

## Original Article

# Age-dependent contribution of Rho kinase in carbachol-induced contraction of human detrusor smooth muscle *in vitro*

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**Aim:** Activation of muscarinic receptors on the detrusor smooth muscle is followed by contraction, which involves both myosin light chain kinase (MLCK) and Rho kinase (ROCK). The aim of this study was to determine the relative contributions of MLCK and ROCK to carbachol-induced contraction of human detrusor smooth muscle *in vitro*.

**Methods:** Detrusor smooth muscle strips were prepared from the macroscopically unaffected bladder wall of patients underwent cystectomy. The strips were fixed in an organ bath, and carbachol or KCl-induced isometric contractions were measured by force transducers.

**Results:** Addition of carbachol (0.4–4 μmol/L) into the bath induced concentration-dependent contractions of detrusor specimens, which was completely abolished by atropine (1 μmol/L). Pre-incubation of detrusor specimens with either the MLCK inhibitor ML-9 or the ROCK inhibitors HA1100 and Y-27632 (each at 10 μmol/L) significantly blocked carbachol-induced contractions as compared to the time-control experiments. Moreover, MLCK and ROCK inhibition were equally effective in reducing carbachol-induced contractions. The residual carbachol-induced contractions in the presence of both MLCK and ROCK inhibitors were significantly smaller than the contractions obtained when only one enzyme (either MLCK or ROCK) was inhibited, suggesting an additive effect of the two kinases. Interestingly, ROCK-mediated carbachol-induced contractions were positively correlated to the age of patients ( $r=0.52$ ,  $P<0.05$ ).

**Conclusion:** Both MLCK and ROCK contribute to carbachol-induced contractions of human detrusor smooth muscle. ROCK inhibitors may be a new pharmacological approach to modulate human bladder hyperactivity.

**Keywords:** bladder hyperactivity; human detrusor; muscarinic receptor; carbachol; atropine; myosin light chain kinase; Rho kinase; Y-27632; HA1100; ML-9

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## Introduction

Acetylcholine is a major excitatory transmitter in urogenital organs leading to a pronounced and sustained contraction of smooth muscle cells. Following the activation of post-junctional muscarinic receptors, the prototypical phospholipase C-dependent signaling pathway<sup>[1]</sup> results in an increase in the intracellular  $Ca^{2+}$  concentration that leads to the calmodulin-mediated activation of myosin light chain kinase (MLCK). Alternatively, there is abundant evidence for muscarinic receptor-dependent activation of voltage-dependent  $Ca^{2+}$  channels with subsequent  $Ca^{2+}$  influx from the extracellular space<sup>[2–4]</sup>. As a consequence, MLCK phosphorylates the regulatory myo-

sin light chain of 20 kDa and thereby initiates contraction<sup>[5]</sup>. However, in addition to this MLCK-dependent mechanism, smooth muscle contraction may also be induced by inhibition of the counteracting enzyme – myosin light chain phosphatase – a mechanism, which is often referred to as  $Ca^{2+}$  sensitization. Inhibition of this enzyme is achieved by phosphorylation of a regulatory targeting subunit, which is a major substrate of Rho kinase (ROCK)<sup>[6,7]</sup>.

In the human urinary bladder, excitatory cholinergic transmission is particularly present in the detrusor smooth muscle which is responsible for the pronounced bladder contraction during micturition<sup>[8]</sup>. Although  $M_2$  receptors are more abundantly expressed than  $M_3$  receptors, the latter subtype seems to be functionally dominant<sup>[9–12]</sup>. The pharmacological differentiation between the MLCK-dependent and the ROCK-dependent components of detrusor contraction is likely to be translationally significant, since alterations of the ROCK path-

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way have been implicated in a number of rodent models of bladder hyperactivity<sup>[13-15]</sup>. In the human detrusor, Ca<sup>2+</sup> sensitization following exposure to carbachol has been observed and was found to involve the ROCK pathway<sup>[3, 16]</sup>. However, the differential contribution of MLCK and ROCK has not been compared directly, and – especially in humans – it is not known which pathway is predominantly involved. Therefore, we recorded isometric contractions from human detrusor muscle strips in an organ bath, and found that both MLCK and ROCK inhibition were equally effective in reducing carbachol-induced contractions, and ROCK-mediated contraction correlates with age.

## Materials and methods

### Preparation of human detrusor samples

Human detrusor samples were obtained from 26 patients (Table 1). All *in vitro* experiments with human material performed in this study were approved by the local ethics committee (University of Rostock), and the informed consent to participate in this study was obtained from each patient.

Immediately following resection of the urinary bladder, a tissue sample of approx 2 cm width was excised from the macroscopically unaffected wall of the detrusor muscle. These samples were submerged into a storage solution containing

(in mmol/L) 120 NaCl, 4.5 KCl, 26 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 0.025 Na<sub>2</sub>-EDTA, 5.5 glucose, 5 HEPES (pH=7.4) and kept at 4 °C for the transfer from the operating room to the laboratory (maximum 1 h). Each detrusor sample was then freed from mucosal tissue and cut into 4–12 muscle strips of 1–2 cm length and 2–3 mm width. Thin nylon threads were sutured to either end of these specimens to enable longitudinal fixation in an organ bath (Panlab ML0146/C, ADInstruments, Spechbach, Germany) filled with a buffer that contained (in mmol/L) 120 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 30 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 0.5 Na<sub>2</sub>-EDTA, 5.5 glucose, 2 sodium pyruvate (pH=7.4) and was gassed with carbogen (95% O<sub>2</sub> and 5% CO<sub>2</sub>).

### Isometric contractions *in vitro*

After fixation in the organ bath, the temperature was slowly raised to 37 °C and the detrusor specimens were slightly stretched and allowed to recover for at least 5 h. During this time, the preparations showed a stable baseline tone with rhythmic activity (see insets in Figure 1A). Isometric contractions of the smooth muscle strips induced by carbachol (CCh) or KCl were measured by force transducers (MLT0201), recorded with a bridge amplifier (ML224) connected to an analog-to-digital converter (Powerlab 4/30, LabChart 7, ADIn-

**Table 1.** Clinical patient data.

Pat #	Age	Sex	Primary disease	Secondary diseases
1	68	M	Urinary bladder cancer	-
2	66	F	Urinary bladder cancer	Diabetes, hypertension
3	61	M	Urinary bladder cancer	Hypothyreosis, multiple transurethral resections
4	80	M	Urinary bladder cancer	-
5	68	M	Urinary bladder cancer	-
6	62	M	Urinary bladder cancer	Hypertension, coronary heart disease and myocardial infarction, stroke
7	73	M	Urinary bladder cancer	Diabetes, hypertension
8	46	M	Urinary bladder cancer	Renal failure with dialysis, hypertension
9	84	F	Urinary bladder cancer	Renal failure, hypertension
10	50	M	Prostate cancer	Miction dysfunction
11	60	M	Urinary bladder cancer	Diabetes
12	80	F	Urinary bladder cancer	Hypothyreosis
13	68	M	Neurogenic micturition dysfunction, shrinking bladder	Alcoholic polyneuropathy
14	74	M	Urinary bladder cancer	Diabetes, hypertension
15	59	M	Urinary bladder cancer	-
16	60	M	Urinary bladder cancer	Hypertension
17	57	M	Urinary bladder cancer	-
18	71	M	Urinary bladder cancer	Hypertension, coronary heart disease and myocardial infarction
19	74	M	Urinary bladder cancer	Prostate cancer, hypertension
20	72	M	Urinary bladder cancer	Hypertension, aortic aneurysm
21	75	M	Urinary bladder cancer	Prostate cancer, hypertension, renal failure, respiratory insufficiency, aortic aneurysm
22	76	M	Urinary bladder cancer	Prostate cancer, hypertension, cardiac failure, respiratory insufficiency
23	82	M	Urinary bladder cancer	Prostate cancer, cardiac failure, aortic aneurysm
24	60	M	Urinary bladder cancer	Prostate cancer
25	62	M	Urinary bladder cancer	Prostate cancer
26	68	M	Neurogenic micturition dysfunction	Multiple sclerosis

struments, Australia).

The major aim of this study was to analyze the effect of different kinase blockers on the carbachol-induced contraction. Since both tachyphylaxis and, to some extent, also tissue deterioration cannot be ruled out in the prolonged course of the experiment, we performed time-control experiments in all parts of this study. Time-control experiments contained only the vehicle of the respective compound (occasionally DMSO). Our organ bath is equipped with four chambers, and we always used one chamber for time-control experiments.

Carbachol, the ROCK blockers HA1100 and Y-27632 as well as the MLCK inhibitor ML-9 were purchased from Tocris Bioscience (Bristol, United Kingdom). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). The application of drugs was performed by adding 100  $\mu\text{L}$  (carbachol, HA1100, Y-27632, ML-9, verapamil) to the organ bath solution to yield the individual final concentration. In the case of KCl, 1000  $\mu\text{L}$  of stock solution (1.5 mol/L KCl) was added (final KCl concentration of 60 mmol/L). All preparations were challenged with KCl at the beginning as well as at the end of the experiment. Experiments were included into statistics when the KCl response at the end of the experiment was at least 50% of the initial contraction (115 out of 165). On average, exposure of smooth muscle preparations to bath solution containing 60 mmol/L KCl caused a mean contraction of  $40 \pm 4$  mN at the beginning, and of  $28 \pm 3$  mN at the end of the experiment (*ie*  $73\% \pm 2\%$ ,  $n=115$ ). The addition of KCl to the organ bath was not corrected for osmolarity, since hyperosmotic control experiments with addition of the same amount of NaCl had no significant effect on smooth muscle tone (contraction of  $1.3 \pm 0.6$  mN compared to  $36.7 \pm 7.1$  mN by KCl application,  $n=19^{[17]}$ ). Drugs were applied for 10–15 min, and the interval between individual drug applications was 45–50 min.

### Statistical analysis

All data are expressed as mean  $\pm$  SEM. Statistical comparison of drug effects was performed using the two-tailed Student's *t* test. The level of significance is indicated (<sup>b</sup> $P < 0.05$ ; <sup>c</sup> $P < 0.01$ ).

## Results

### Carbachol-induced contractions in human detrusor

Smooth muscle contraction is generally induced by membrane depolarization or by pharmacological activation of G protein-coupled receptors such as muscarinic receptors. The dose-response curve for the cholinergic agonist carbachol (CCh, 0.1–100  $\mu\text{mol/L}$ ) obtained with randomly varying concentrations showed dose-dependent contractions of human detrusor smooth muscle preparations with a half-maximal effect between 1–2  $\mu\text{mol/L}$  ( $\text{EC}_{50} = 1.1$   $\mu\text{mol/L}$ , Figure 1A/1C). The detrusor contraction induced by 2  $\mu\text{mol/L}$  carbachol was entirely due to muscarinic receptor activation, since atropine (1  $\mu\text{mol/L}$ ) completely relaxed the pre-contracted detrusor preparation (from  $94 \pm 17$  mN to  $-1 \pm 1$  mN,  $n=7$ ,  $P < 0.05$ ; Figure 1B/1D). Following washout of atropine, CCh-induced contraction partially recovered ( $46 \pm 9$  mN). Hence, for the remainder of the study, 2  $\mu\text{mol/L}$  carbachol was used as the routine

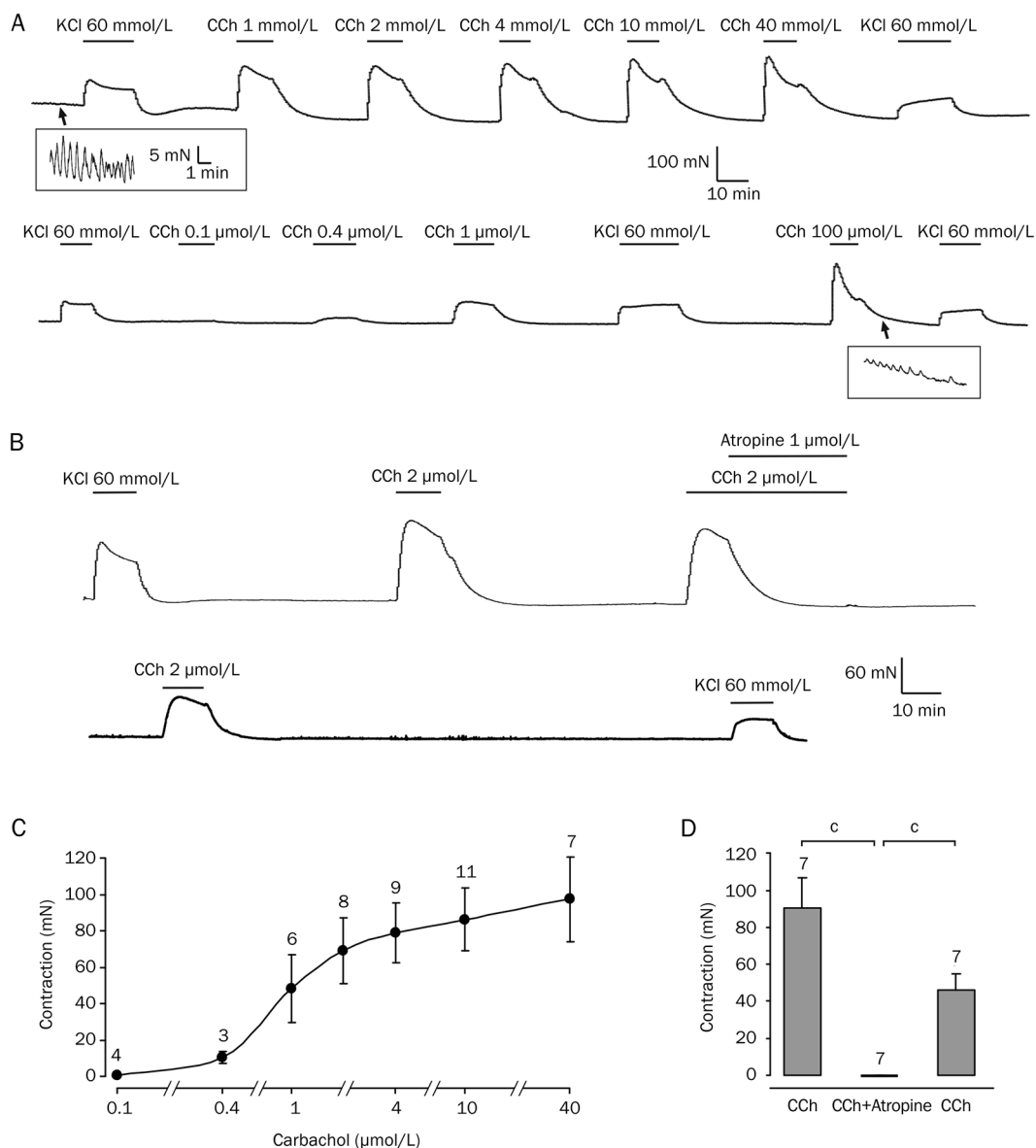
concentration in order to assess the role of the two main routes of smooth muscle contraction: MLCK and ROCK.

### Role of Rho kinase in carbachol-induced contractions

Since we aimed to study the role of these key enzymes in carbachol-induced contractions in the human detrusor muscle, we pre-applied either the MLCK inhibitor ML-9 or the ROCK inhibitor HA1100 (both 10  $\mu\text{mol/L}$ ) before the preparations were challenged with 2  $\mu\text{mol/L}$  carbachol. A typical example of such an experiment is depicted in Figure 2A. In different detrusor specimens taken from the same patient, these compounds were tested alone or in combination (upper two traces). Obviously, both HA1100 and ML-9 were able to depress carbachol-induced contractions suggesting a contribution of both MLCK and ROCK. In addition, these blockers caused a marked relaxation of the baseline tone (arrowheads in Figure 2A) indicating that both enzymes were activated under resting conditions. In another subset of specimens taken from the same patient, no enzyme inhibitor was applied. In these time-control experiments, only little run-down of CCh-induced contractions was observed (lower trace, Figure 2A). Hence, we synchronized the inhibitor experiments (Figure 2B, gray bars) with time-control experiments (Figure 2B, white bars) in order to compare the carbachol response in the presence of an enzyme inhibitor to the control carbachol response at the same time point.

We observed substantial variation of CCh-induced contractions among different patients, but we found a striking correlation between these contractions and those observed by initial KCl-application (correlation coefficient of 0.92,  $n=46$ ,  $P < 0.01$ ). Therefore, we used the initial contraction following KCl to normalize the CCh-induced contractions (Figure 2B). Expressed as the percentage of the KCl effect, the initial contraction induced by carbachol was  $201\% \pm 14\%$  ( $n=34$ ,  $P > 0.7$  versus  $211\% \pm 19\%$ ,  $n=12$ , in time-controls; Figure 2B). The ROCK inhibitor HA1100 significantly reduced this carbachol response to  $150\% \pm 14\%$  ( $n=17$ ,  $P < 0.05$  versus  $207\% \pm 18\%$ ,  $n=12$ , in time-controls). When the MLCK blocker ML-9 was used instead, the contraction by carbachol was reduced to  $136\% \pm 10\%$  ( $n=17$ ,  $P < 0.01$  versus  $207\% \pm 18\%$ ,  $n=12$ , in time-controls). Moreover, the combined application of both inhibitors had an additive effect and further reduced the carbachol response to  $94\% \pm 6\%$  ( $n=34$ ,  $P < 0.001$  versus  $191\% \pm 17\%$ ,  $n=12$ , in time-controls;  $P < 0.01$  versus HA1100 or ML-9 alone). These results suggest that MLCK and ROCK equally and independently contribute to the CCh-induced contraction of human detrusor smooth muscle.

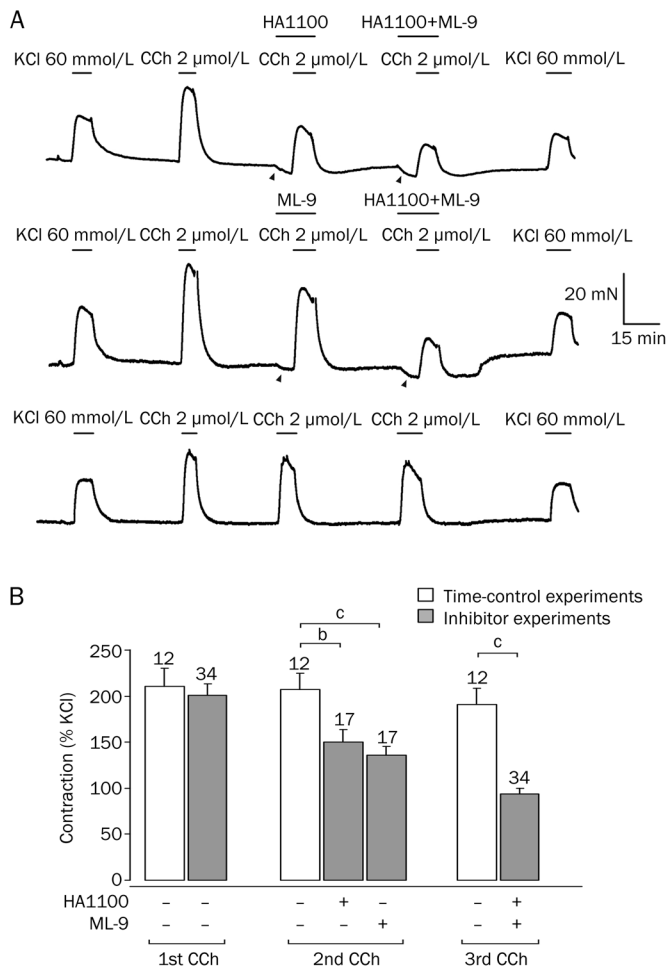
While ROCK preferably acts via inhibition of myosin light chain phosphatase leading to  $\text{Ca}^{2+}$  sensitization in smooth muscle, MLCK-dependent contraction is believed to occur downstream of both  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  release from internal stores. To test the impact of  $\text{Ca}^{2+}$  influx via voltage-dependent L-type  $\text{Ca}^{2+}$  channels, we compared the effects of HA1100 and the L-type  $\text{Ca}^{2+}$  channel blocker verapamil (40  $\mu\text{mol/L}$ ). While HA1100 reduced the carbachol-induced contraction to  $144\% \pm 38\%$  ( $n=6$ ) of the KCl-induced contraction, verapamil



**Figure 1.** Carbachol-induced contractions in human detrusor *in vitro* are atropine-sensitive. (A) Carbachol (CCh, 0.1–100 µmol/L) caused dose-dependent contractions in a human detrusor specimen. The upper trace was started at 6 h following cystectomy. The gap between the upper and the lower trace is 7 h. KCl (60 mmol/L) was used at the beginning and at the end of the experiment to verify stable recording conditions. The insets demonstrate the rhythmic activity of detrusor smooth muscle. Note the different scales within the insets. (B) The upper trace (starting at 5 h following cystectomy) shows contractions induced by carbachol (CCh, 2 µmol/L) in a human detrusor specimen that were abolished by the muscarine receptor antagonist atropine (1 µmol/L). The lower trace shows partial recovery of carbachol-induced contraction following washout of atropine. (C) Dose-response curve of carbachol in human detrusor. The half-maximal contraction was achieved between 1 and 2 µmol/L carbachol ( $EC_{50}=1.1$  µmol/L). Note that a single specimen was tested at multiple concentrations (total number of specimens:  $n=15$  of 4 patients). (D) Carbachol-induced contractions were significantly reduced following atropine (total number of specimens:  $n=7$  of 2 patients). <sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ .

caused a significantly stronger reduction to  $104\pm33\%$  ( $n=6$ ,  $P<0.05$  versus HA1100). However, the combination of both compounds strongly and additively suppressed the carbachol-induced response ( $26\pm9\%$ ,  $n=6$ ,  $P<0.05$  versus HA1100 and verapamil). These data may suggest that the remaining CCh-induced contraction following ROCK inhibition is predominantly mediated by  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels rather than by  $Ca^{2+}$  release from internal stores.

To validate our data showing the involvement of ROCK in carbachol-induced contractions, we chose to also test another ROCK inhibitor, Y-27632 (10 µmol/L), to gauge the effect of this compound on the carbachol response. The effect of carbachol on the detrusor specimens was analyzed in presence of Y-27632 or ML-9 alone (the order of which was randomized), as well as after pre-treatment with both inhibitors in combination (Figure 3A, upper trace). Time-control experiments



**Figure 2.** Carbachol-induced contractions are reduced by HA1100 and ML-9. (A) The upper trace (starting at 9 h following cystectomy) shows contractions induced by carbachol (CCh, 2  $\mu\text{mol/L}$ ) in a human detrusor specimen that were reduced by the ROCK blocker HA1100 (10  $\mu\text{mol/L}$ ) or combined pre-treatment with HA1100 and the MLCK blocker ML-9 (10  $\mu\text{mol/L}$ ). Note the reduction of the basal tone (arrow heads) indicating kinase activity at rest. The middle trace shows a similar experiment with another specimen from the same patient demonstrating that both ML-9 alone and combined application of ML-9 and HA1100 reduced the carbachol-induced contraction. The lower trace shows time-control experiments of another specimen from the same patient demonstrating only little tachyphylaxis upon repetitive carbachol applications. Application of KCl (60 mmol/L) was used at the beginning and at the end of the experiment to verify stable recording conditions. (B) Carbachol-induced contractions (in % of initial response to KCl 60 mmol/L) showed little tachyphylaxis (time-control experiments), but were significantly reduced following HA1100 or ML-9 treatment (total number of specimens:  $n=40$  of 4 patients). <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$ .

(Figure 3A, lower trace) indicated a small run-down of the carbachol response. Pooled data of this experiment are illustrated in Figure 3B. As HA1100, the ROCK inhibitor Y-27632 also significantly reduced the contraction by carbachol. The combined application of both inhibitors Y-27632 and ML-9 had again an additive effect and further reduced the carba-

chol response. These results confirm our previous data and provide further evidence for the finding that ROCK is at least as important as MLCK in carbachol-induced contraction of human detrusor smooth muscle.

#### Age-dependence of Rho kinase-mediated carbachol-induced contraction

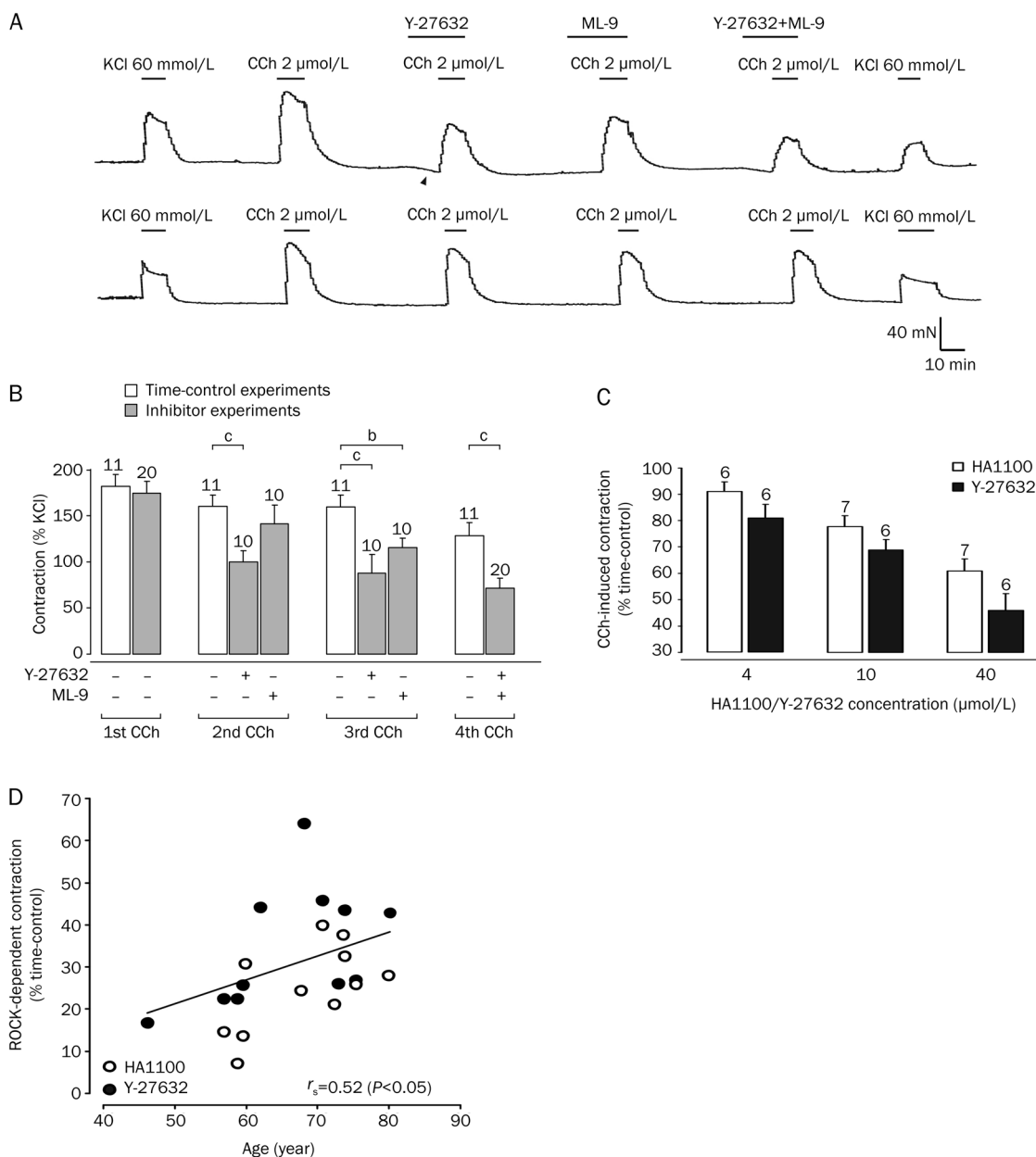
Our data so far demonstrated that ROCK activation is one of the major pathways for human detrusor smooth muscle contraction. Moreover, both blockers HA1100 and Y-27632, showed a significant concentration-dependent inhibitory effect on the CCh-induced contraction (Figure 3C). Another conspicuous observation in our data was the considerable variability of CCh-induced contractions in inhibitor experiments among patients. Since we hypothesized that the patients' age might in part account for this variation, we re-analyzed our data and performed a correlation analysis between age and CCh-induced contraction in the presence of ROCK inhibitor (Figure 3D). Pooling all data with 10  $\mu\text{mol/L}$  HA1100 and 10  $\mu\text{mol/L}$  Y-27632 from 16 patients, we found a significant positive correlation between age and ROCK-mediated CCh-induced contraction (Spearman's rank correlation coefficient of 0.52;  $P<0.05$ ). In contrast to the ROCK-dependent contraction, the MLCK-mediated CCh-induced contraction was not significantly correlated to age (Spearman's rank correlation coefficient of 0.38,  $P=0.31$ ). Hence, ROCK-mediated CCh-induced contractions in human detrusor are strongly age-dependent playing a major role in aging patients.

#### Discussion

The present study evaluated the differential role of MLCK and ROCK in human urinary bladder smooth muscle. We demonstrated that carbachol is capable to activate the ROCK pathway in the human detrusor and thereby confirmed previous reports<sup>[3, 16]</sup>. Moreover, we could show that the combined application of both MLCK and ROCK inhibitors was significantly more potent than one of these compounds alone indicating that these pathways had been activated independently. The clinically relevant finding was the significant correlation between age and ROCK contribution to CCh-induced contraction in human detrusor.

Post-junctional muscarinic receptors mediate the CCh-induced detrusor contraction, predominantly involving  $M_3$  receptor activation<sup>[9-12]</sup>. The potency of carbachol in the present study is consistent with the  $EC_{50}$  values in previous reports on human tissue (0.7–2  $\mu\text{mol/L}$ )<sup>[18-21]</sup>. This is important to note because it indicates that our results obtained from resected human tissue were not largely influenced by the patient population or the surgical procedures involved.

One major finding of our study was that two different ROCK inhibitors significantly reduced the human detrusor contraction induced by carbachol. Involvement of ROCK activation in muscarinic receptor-dependent detrusor contraction has been observed in mice<sup>[22]</sup>, rats<sup>[23-26]</sup>, rabbits<sup>[27]</sup>, and humans<sup>[3, 16]</sup>. Thus, we confirmed that ROCK significantly contributes to the CCh-induced contraction in human tissue. But how does car-



**Figure 3.** Carbachol-induced contractions are reduced by Y-27632 and ML-9, and Rho kinase-mediated contraction is age-dependent. (A) The upper trace (starting at 7 h following cystectomy) shows contractions induced by carbachol (2 μmol/L) in a human detrusor specimen that were reduced by both the ROCK blocker Y-27632 (10 μmol/L) and the MLCK blocker ML-9 (10 μmol/L). Note the reduction of the basal tone (arrow heads) indicating kinase activity at rest. The lower trace shows time-control experiments of another specimen from the same patient demonstrating only little tachyphylaxis upon repetitive carbachol applications. Application of KCl (60 mmol/L) was used at the beginning and at the end of the experiment to verify stable recording conditions. (B) Carbachol-induced contractions (in % of initial response to KCl 60 mmol/L) showed little tachyphylaxis (time-control experiments), but were significantly reduced following Y-27632 or ML-9 (total number of specimens:  $n=31$  of 5 patients). <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$ . (C) Carbachol-induced contractions following ROCK inhibition (in % of the time-matched control response) was dose-dependent for both Y-27632 and HA1100 (total number of specimens:  $n=94$  of 7 patients). (D) The carbachol-induced contraction following ROCK inhibition with either HA1100 (10 μmol/L) or Y-27632 (10 μmol/L) was significantly age-dependent (Spearman's rank correlation coefficient of 0.52,  $P<0.05$ ). In this panel, data from Figures 2B and 3B have been included (total number of patients:  $n=16$ ).

bachol activate this enzyme? The prototypical signal transduction cascade following muscarinic receptor activation includes phospholipase C (PLC) activation,  $Ca^{2+}$  release from internal stores and subsequent activation of myosin light chain kinase.

However, how the cytosolic concentration of  $Ca^{2+}$  ions is raised has already been questioned since L-type  $Ca^{2+}$  channel blockers substantially reduced CCh-induced contractions<sup>[2, 4]</sup>, and PLC inhibitors failed to block CCh-induced contractions<sup>[3]</sup>. We

have performed experiments with verapamil and confirmed that  $\text{Ca}^{2+}$  entry via L-type  $\text{Ca}^{2+}$  channels is a major contributor of the CCh-induced contraction. Moreover, we found that co-application of verapamil and the ROCK inhibitor HA1100 caused a significantly stronger reduction of the CCh-induced contraction than one of these compounds alone, suggesting that ROCK activation appeared independently of  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels. A standard experimental procedure to open these channels is depolarization by high concentration of external  $\text{K}^+$ . It is worth to note that throughout the present study, the CCh-induced contraction was about twice the response than 60 mmol/L KCl within the same detrusor preparation. This is in line with Takahashi *et al*<sup>[16]</sup> who have observed a threefold response to carbachol when compared to 60 mmol/L KCl, and indicates that depolarization per se does not mimic the contraction mediated by muscarinic receptor activation. Hence,  $\text{Ca}^{2+}$  entry via depolarization-activated  $\text{Ca}^{2+}$ -permeable ion channels may only account for a fraction of the CCh-induced contraction in the human detrusor. In general, alternatives to  $\text{Ca}^{2+}$  influx might involve  $\text{Ca}^{2+}$  release from internal stores and/or  $\text{Ca}^{2+}$ -independent cascades of contraction. Actually, both  $\text{Ca}^{2+}$  release from internal stores and protein kinase C activation are potential downstream effects of  $\text{M}_3$  receptor-mediated PLC activation which was, however, identified to be only marginally relevant in CCh-induced contractions<sup>[3]</sup>. Thus, our results support the idea that the muscarinic receptor-mediated ROCK activation may be PLC-independent.

Regardless of whether  $\text{Ca}^{2+}$  influx or  $\text{Ca}^{2+}$  release is more relevant for smooth muscle contraction, the rise in intracellular  $\text{Ca}^{2+}$  concentration is regarded as a key step by binding to calmodulin and activating MLCK<sup>[5]</sup>. We have directly compared the effect of MLCK and ROCK inhibitors, and found that both enzymes are significant and additive contributors to human detrusor contraction. However, this may not imply that both pathways are disjunctive, since Rho-associated kinase may also be activated by L-type  $\text{Ca}^{2+}$  channels<sup>[28]</sup>, and MLC20 phosphorylation has recently been shown to be a target for Rho kinase, at least in myometrial tissue<sup>[29]</sup>. Thus, it is conceivable that both pathways may have cross-links at some certain step within the cascade also in detrusor smooth muscle. With respect to the specificity of the kinase blockers used in this study, the available MLCK inhibitors may also block L-type  $\text{Ca}^{2+}$  channels, while HA1100 and Y-27632 do not have this side effect<sup>[30]</sup>. Nonetheless, co-application of both inhibitors was significantly more effective than using one of them alone.

Interestingly enough, the MLCK inhibitor ML-9 appeared to be less potent in reducing the CCh-induced contraction as compared to the L-type  $\text{Ca}^{2+}$  channel blocker verapamil. This might be due to the metabotropic effect of L-type  $\text{Ca}^{2+}$  channels that has recently been discovered in vascular smooth muscle cells and connects depolarization with ROCK activation<sup>[28, 31]</sup>. In summary, we propose that human detrusor smooth muscle cells have two major mechanisms of muscarinic receptor-mediated contractions: (a) activation of MLCK

predominantly following  $\text{Ca}^{2+}$ -influx via L-type  $\text{Ca}^{2+}$  channels and (b) activation of ROCK that is largely independent of an intracellular  $\text{Ca}^{2+}$  rise, but may in part occur as a consequence of L-type  $\text{Ca}^{2+}$  channel activation.

When analyzing the ROCK contribution with respect to the age of our patients, we discovered a significant and potentially clinically relevant correlation. Thus, ROCK activity appears to be up-regulated with age. Previous reports, mainly in rodent disease models, have indeed suggested an increased ROCK function<sup>[13-15]</sup>. Hence, our results support the hypothesis that ROCK upregulation may underly both detrusor degeneration during aging and those pathologies are often associated with aging such as overactive bladder. In this context, ROCK is becoming a novel pharmacological target to oppose bladder hyperactivity symptoms<sup>[32]</sup>.

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### Author contribution

Timo KIRSCHSTEIN, Chris PROTZEL, Katrin PORATH, and Tina SELLMANN performed the research. Timo KIRSCHSTEIN, Chris PROTZEL, Rüdiger KÖHLING, and Oliver W HAKENBERG designed the research. Timo KIRSCHSTEIN and Katrin PORATH analyzed the data. Timo KIRSCHSTEIN, Chris PROTZEL, Rüdiger KÖHLING, and Oliver W HAKENBERG wrote the paper.

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