

Differential Rho-kinase dependency of full and partial muscarinic receptor agonists in airway smooth muscle contraction

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1 In airway smooth muscle (ASM), full and partial muscarinic receptor agonists have been described to have large differences in their ability to induce signal transduction, including Ca^{2+} -mobilization. Despite these differences, partial agonists are capable of inducing a submaximal to maximal ASM contraction.

2 To further elucidate transductional differences between full and partial muscarinic receptor agonists, we investigated the contribution of Rho-kinase (an important regulator of Ca^{2+} -sensitization) to methacholine-, pilocarpine- and McN-A-343-induced bovine tracheal smooth muscle (BTSM) contraction, using the selective Rho-kinase inhibitor Y-27632. In addition, we measured Ca^{2+} -mobilization and -influx in BTSM cells in response to these agonists in the absence and presence of Y-27632.

3 Whereas treatment with Y-27632 (1 μM) significantly decreased potency (pEC_{50}) for all agonists, maximal contraction (E_{max}) was reduced by 23.4 ± 2.8 and $50.4 \pm 7.9\%$ for the partial agonists pilocarpine and McN-A-343, respectively, but was unaffected for the full agonist methacholine. However, E_{max} of methacholine became Rho-kinase dependent after taking away its receptor reserve using the irreversible muscarinic receptor antagonist propylbenzylcholine mustard.

4 Pilocarpine and McN-A-343 induced a very small Ca^{2+} -mobilization and -influx as compared to methacholine. In addition, an inverse relationship of these two parameters with the Rho-kinase dependency was observed. Interestingly, no inhibitory effects of Y-27632 were observed on Ca^{2+} -mobilization and -influx for all three agonists, indicating that the effects of Y-27632 on contraction are most likely on the level of Ca^{2+} -sensitization.

5 In conclusion, in contrast to the full agonist methacholine, the partial muscarinic receptor agonists pilocarpine and McN-A-343 are dependent on Rho-kinase for their maximal contractile effects, presumably as a consequence of differences in transductional reserve, indicating an agonist-dependent role for Rho-kinase in ASM contraction. Moreover, an inverse relationship exists between Rho-kinase dependency and both Ca^{2+} -mobilization and Ca^{2+} -influx for these agonists.

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Abbreviations: ASM, airway smooth muscle; BTSM, bovine tracheal smooth muscle; CCRC, cumulative concentration–response curve; KH, Krebs–Henseleit; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; pEC_{50} , $-\log_{10}$ of the concentration causing 50% of the effect

Introduction

Muscarinic receptor stimulation in airway smooth muscle (ASM) results in the activation of phospholipase C, subsequently followed by the production of sn-1,2-diacylglycerol and inositol1,4,5-trisphosphate (IP_3) (Takuwa *et al.*, 1986; Meurs *et al.*, 1989; Yang *et al.*, 1991). In response to IP_3 , Ca^{2+} is being mobilized from intracellular stores (Hashimoto *et al.*, 1985), causing a rapid, transient rise in intracellular Ca^{2+} -concentration [Ca^{2+}]_i (Yang *et al.*, 1993), which is followed by a sustained influx of extracellular Ca^{2+} . Smooth muscle contraction is then initiated through the formation of Ca^{2+} -calmodulin and subsequent activation of myosin light chain kinase (MLCK), resulting in the phosphorylation of the

20 kDa regulatory myosin light chain (MLC_{20}) (de Lanerolle & Paul, 1991; Pfitzer, 2001). Recently, it has been established that contractile stimuli do not exert their effects only by increasing [Ca^{2+}]_i, but also by increasing Ca^{2+} -sensitivity of the smooth muscle. One of the main regulators involved in this so-called Ca^{2+} -sensitization is Rho-kinase, which acts through inhibition of myosin light chain phosphatase, resulting in an enhanced MLC_{20} phosphorylation and thus an increased level of contraction at a certain [Ca^{2+}]_i (Fukata *et al.*, 2001; Somlyo & Somlyo, 2003).

Contraction of ASM preparations by muscarinic receptor agonists is mediated primarily through M_3 -receptor stimulation. Several studies have demonstrated that the involvement of the M_2 -receptor in ASM contraction is minor or negligible (Roffel *et al.*, 1988; 1990; Watson *et al.*, 1995). Meurs *et al.*

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(1988) compared concentration–response curves (CRCs) for contraction and inositol phosphate accumulation in response to partial and full muscarinic receptor agonists, and demonstrated that a strong linear correlation between these two parameters exists. In addition, they showed a considerable reserve of inositol phosphate production for the full agonists methacholine and oxotremorine, but not for the partial agonist McN-A-343 (Meurs *et al.*, 1988). This transduction reserve is in accordance with studies showing that acetylcholine induces maximal force development by only occupying 4% of the available muscarinic receptors (large receptor reserve), whereas McN-A-343 has to occupy 80% of the receptors (low receptor reserve) to achieve the same degree of force (Gunst *et al.*, 1987; 1989). Furthermore, it has been demonstrated that canine tracheal smooth muscle shortens at a significantly faster rate when contracted with acetylcholine than with McN-A-343 (Gunst *et al.*, 1992). Large differences between full and partial M_3 -agonists regarding Ca^{2+} -mobilizing capacity have been described, whereas no differences in dependency on Ca^{2+} -influx through voltage-dependent channels appear to be present (al-Hassani *et al.*, 1993). To further elucidate mechanisms underlying the differences between full and partial M_3 -receptor agonists, we investigated the contribution of Rho-kinase to ASM-contraction, Ca^{2+} -mobilization and Ca^{2+} -influx in response to the full muscarinic agonist methacholine and the partial muscarinic agonists pilocarpine and McN-A-343 (Meurs *et al.*, 1988; Offer *et al.*, 1991). We demonstrate that these agonists are differentially dependent on Ca^{2+} -mobilization/-influx and Rho-kinase for their contractile effects, and that the functional dependency on Ca^{2+} -mobilization and -influx is inversely correlated with Rho-kinase dependency.

Methods

Tissue preparation and organ-culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs–Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5% CO_2 and 95% O_2 , pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (10 mM), HEPES (20 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin ($45 \mu g ml^{-1}$), penicillin (100 U ml^{-1}), streptomycin (100 $\mu g ml^{-1}$) and amphotericin B (1.5 $\mu g ml^{-1}$). Next, tissue strips were transferred into suspension culture flasks (containing 2.5 ml medium per tissue strip) and maintained overnight.

Isometric tension measurements

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5%

CO_2 and 95% O_2 , pH 7.4, at 37°C. Subsequently, strips were mounted for isometric recording (Grass force–displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37°C, continuously gassed with 5% CO_2 and 95% O_2 , pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two wash-outs, maximal relaxation was established by the addition of 0.1 μM (–)-isoprenaline. In over 95% of the experiments, no basal myogenic tone was detected. Tension was now re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min, cumulative CRCs were constructed to stepwise increasing concentrations of methacholine, pilocarpine or McN-A-343 (1 nM–100 μM). When maximal agonist-induced tension was obtained, the strips were washed several times and maximal relaxation was established using (–)-isoprenaline (10 μM). When used, the Rho-kinase inhibitor Y-27632 (1 μM) was applied to the organ bath 30 min before agonist addition. This concentration has been shown to be effective and selective in smooth muscle (Uehata *et al.*, 1997; Gosens *et al.*, 2004b; Schaafsma *et al.*, 2004). In a separate set of experiments, 100 μM of the alkylating muscarinic receptor antagonist propylbenzilylcholine mustard (PrBCm) was preincubated for 15 min, followed by several washouts, prior to the construction of a cumulative CRC of methacholine.

Isolation of bovine tracheal smooth muscle (BTSM) cells

After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, two times at the size of 300 μm and three times at 100 μm . Tissue fragments were washed under sterile conditions (three times) and maintained overnight in DMEM supplemented with $NaHCO_3$ (10 mM), HEPES (20 mM), penicillin (100 U ml^{-1}), streptomycin (100 $\mu g ml^{-1}$) and 10% fetal bovine serum (FBS) in an incubator shaker (Innova 4000) at 37°C, 55 r.p.m.

Tissue fragments were washed three times in Krebs–Ringer–Henseleit (KRH) buffer containing (mM): NaCl 125.0, KCl 6.0, $MgCl_2$ 2.5, $CaCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.0, HEPES 25.0, pH 7.4, supplemented with 2.0 mM (\pm)-dithiothreitol (DTT) and resuspended in a digestion mixture of collagenase P (0.75 $mg ml^{-1}$), papain (1 $mg ml^{-1}$) and trypsin inhibitor (1 $mg ml^{-1}$) in KHR buffer. The suspension was incubated for 20 min at 37°C (55 r.p.m.) and then gently dispersed with a wide-bored pipette. After another 10 min of incubation at 37°C (70 r.p.m.), the suspension was gently dispersed again and filtered over a 50 μm gauze. The cells were collected by centrifugation (1000 $\times g$, 10 min), washed three times with KHR, pH 7.4, supplemented with 2 $mg ml^{-1}$ FBS (KRH/FBS), and were allowed to regenerate for 1 h at 37°C (55 r.p.m.). Subsequently, the cells were incubated with the fluorescent dye Fura-2/AM (3 μM) for 30 min at 37°C (55 r.p.m.). The loaded cells were washed three times in KRH/FBS and resuspended to a density of 1×10^6 cells ml^{-1} . The cells were kept at room temperature on a Rock-N-Roller (Breda Scientific, The Netherlands) and used within 2–4 h, during which they remained viable and responsive.

Intracellular Ca^{2+} -measurements

Fura-2 fluorescence of the cells (excitation wavelengths: 340 and 380 nm; emission wavelength: 510 nm) was measured at 37°C with a Perkin-Elmer spectrometer (LS-50B). Each cuvet contained 2 ml of magnetically stirred cell suspension. Ca^{2+} -mobilization and -influx induced by methacholine (30 nM–300 μ M), pilocarpine (100 nM–300 μ M) or McN-A-343 (100 nM–300 μ M), added at $t=60$ s, were measured. When used, Y-27632 (1 μ M) was added 30 min prior to agonist addition. The intracellular Ca^{2+} -concentration ($[Ca^{2+}]_i$) was calculated every 0.2 s according to Grynkiewicz *et al.* (1985). At the end of the experiment, the maximal fluorescence ratio (R_{max}) was determined after adding 0.01% of Triton-X-100 as a permeabilizing agent. The minimal fluorescence ratio (R_{min}) was determined by addition of 5 mM EGTA to the permeabilized cells.

Data analysis

All data represent means \pm s.e.m. from n separate experiments. CRCs of contractile responses were analyzed by measuring myogenic tension. No corrections were made for basal tone. Maximal tension (E_{max}) and pEC_{50} were calculated from the CRCs. Curves were fitted using the logistic four-parameter model (Sigmaplot 9.0, SPSS Inc.).

Agonist-induced transient rise of $[Ca^{2+}]_i$, representing Ca^{2+} -mobilization from internal stores, was expressed as the maximal increase above basal after addition of the agonist. The plateau level of $[Ca^{2+}]_i$, representing Ca^{2+} -influx, was measured 2 min after agonist addition and expressed as $[Ca^{2+}]_i$ above basal. CRCs for Ca^{2+} -mobilization and -influx were expressed as a percentage of the maximal methacholine-induced Ca^{2+} -mobilization.

Statistical significance of differences between data was determined using the two-tailed Student's t -test for paired observations or one-way analysis of variance, where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Dulbecco's modification of Eagle's Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Foetal bovine serum, $NaHCO_3$ solution (7.5%), HEPES solution (1 M), sodium pyruvate solution (100 mM), nonessential amino acid mixture, gentamycin solution (10 mg ml^{-1}), penicillin/streptomycin solution (5000 U ml^{-1} ; 5000 μ g ml^{-1}) and amphotericin B solution (250 μ g ml^{-1}) (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Dithiothreitol (DTT), soybean trypsin inhibitor, Fura-2/AM, pilocarpine hydrochloride and (–)-isoprenaline hydrochloride were from Sigma Chemical Co. (St Louis, MO, U.S.A.). McN-A-343 was purchased from RBI, Natick, MA, U.S.A.

(+)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632) was obtained from Tocris Cookson Ltd (Bristol, U.K.). L(+)-ascorbic acid was from Merck (Darmstadt, Germany). Propylbenzylcholine mustard was from NEN products (Boston, MA, U.S.A.). Papain and Collagenase P were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Effects of Rho-kinase inhibition on methacholine-, pilocarpine- and McN-A-343-induced ASM contraction

In BTSM strip preparations, pilocarpine and McN-A-343 reached up to 87 and 84%, respectively, of the maximal contraction (E_{max}) induced by methacholine, indicating that both pilocarpine and McN-A-343 are partial agonists (Figure 1, Table 1).

To determine the contribution of Rho-kinase, we used the selective Rho-kinase inhibitor Y-27632 (1 μ M). With methacholine, no effect of Rho-kinase inhibition was observed on E_{max} , but a significant decrease in potency (pEC_{50}) was observed. Interestingly, treatment with Y-27632 significantly decreased E_{max} of pilocarpine and McN-A-343 by 23.4 ± 2.8

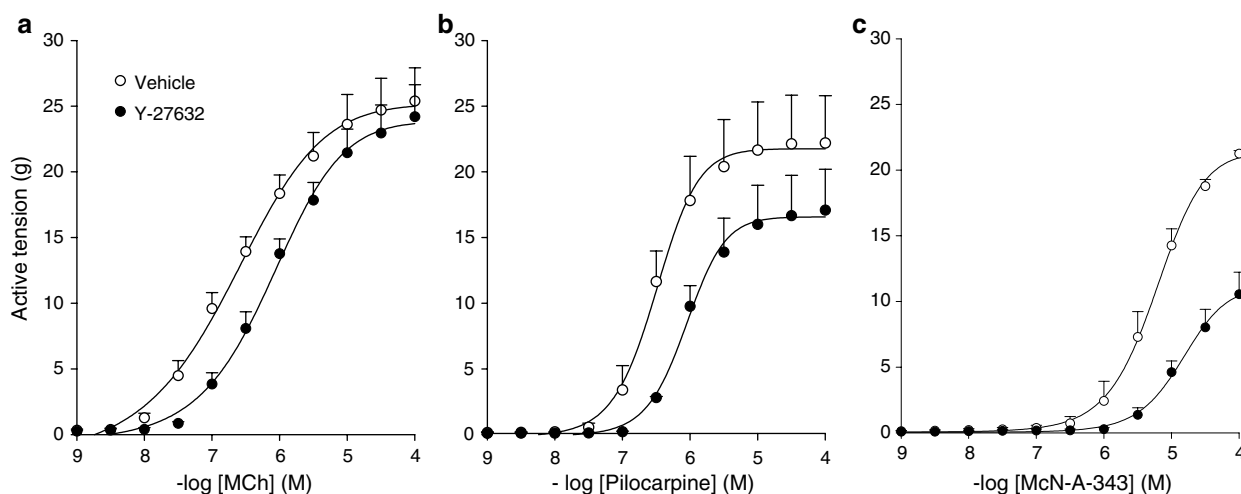


Figure 1 Effects of Rho-kinase inhibition on contraction induced by full and partial M_3 -receptor agonists. Methacholine (MCh, a)-, pilocarpine (b)- and McN-A-343 (c)-induced contraction in the absence (open circles) and presence (closed circles) of 1 μ M Y-27632 of BTSM strips. Data represent means \pm s.e.m. of 3–7 experiments, each performed in duplicate.

Table 1 Effects of Rho-kinase inhibition on contractile properties of bovine tracheal smooth muscle preparations following administration of muscarinic receptor agonists

	Vehicle		Y-27632	
	pEC_{50} ($-\log M$)	E_{max} (g)	pEC_{50} ($-\log M$)	E_{max} (g)
Methacholine	6.73 ± 0.15	25.4 ± 2.5	6.19 ± 0.15**	24.2 ± 2.4
after PrBCm	3.59 ± 0.14	21.8 ± 1.2	2.91 ± 0.17*	16.3 ± 1.6*
Pilocarpine	6.52 ± 0.08	22.2 ± 3.6	6.09 ± 0.02*	17.1 ± 3.1*
McN-A-343	5.26 ± 0.09	21.3 ± 0.2	4.93 ± 0.04*	10.5 ± 1.7*

Data represent means ± s.e.m. of 3–7 experiments, each performed in duplicate. * $P < 0.05$, ** $P < 0.01$ vs vehicle-treated.

and $50.4 \pm 7.9\%$, respectively, and also significantly decreased potency (Figure 1, Table 1). Remarkably, the effect of Y-27632 in decreasing pEC_{50} values diminished in the order methacholine > pilocarpine > McN-A-343.

Effects of Rho-kinase inhibition on intracellular Ca^{2+} homeostasis in response to methacholine, pilocarpine and McN-A-343

Ca^{2+} -mobilization and -influx were determined in response to several concentrations of methacholine, pilocarpine and McN-A-343 (Figure 2). Both mobilization and influx were calculated as a percentage of the maximal methacholine-induced Ca^{2+} -mobilization, which amounted to 349.7 ± 53.9 nM Ca^{2+} . Mobilization induced by pilocarpine and McN-A-343 (Figure 2a) was significantly lower as compared to that induced by methacholine, and reached up to only 10.0 ± 0.8 and $5.8 \pm 1.6\%$, respectively ($P < 0.001$, both). Ca^{2+} -influx in response to these agonists was also significantly lower (8.9 ± 1.1 and $7.1 \pm 1.7\%$, respectively; Figure 2b) as compared to methacholine-induced influx ($13.9 \pm 0.5\%$; $P < 0.01$, both).

Next, to determine whether the effects of Rho-kinase inhibition on contraction (Figure 1) could be linked to the effects on Ca^{2+} -mobilization or -influx, we measured Ca^{2+} -responses induced by $100 \mu M$ methacholine, pilocarpine and McN-A-343 in the presence and absence of $1 \mu M$ Y-27632. As shown in Figure 3, Y-27632 had no inhibitory effect on Ca^{2+} -mobilization or -influx induced by either agonist, indicating that the inhibitory effects of Y-27632 on maximal pilocarpine- and McN-A-343-induced contractions are not caused by changes in Ca^{2+} -mobilization or -influx.

Effects of muscarinic receptor alkylation on the Rho-kinase dependency of the methacholine-induced contraction

To determine whether differences in receptor reserve could be responsible for the difference in Rho-kinase dependency of the contraction induced by full and partial agonists, we removed receptor reserve of the full agonist methacholine by using the alkylating agent propylbenzilylcholine mustard (PrBCm). In contrast to methacholine-induced contraction of untreated strips (Figure 1a), maximal methacholine-induced contraction was Rho-kinase dependent after treatment with PrBCm ($24.8 \pm 9.2\%$ inhibition of E_{max} in the presence of Y-27632;

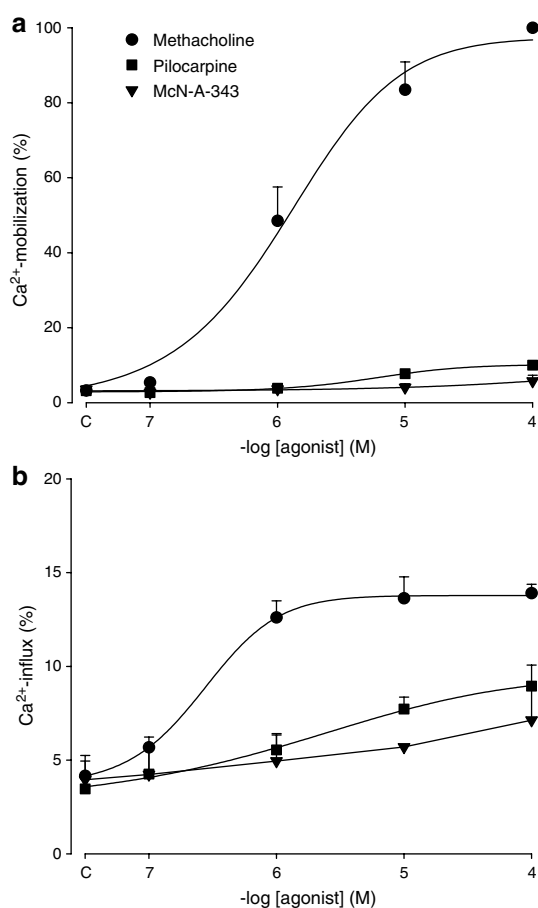


Figure 2 Ca^{2+} -mobilization and -influx in response to full and partial M_3 -receptor agonists. Methacholine (circles)-, pilocarpine (squares)- and McN-A-343 (triangles)-induced Ca^{2+} -mobilization (a) and -influx (b) in BTSM cells. Data are expressed as percentage of maximal Ca^{2+} -mobilization induced by methacholine and represent means ± s.e.m. of three experiments, each performed in duplicate.

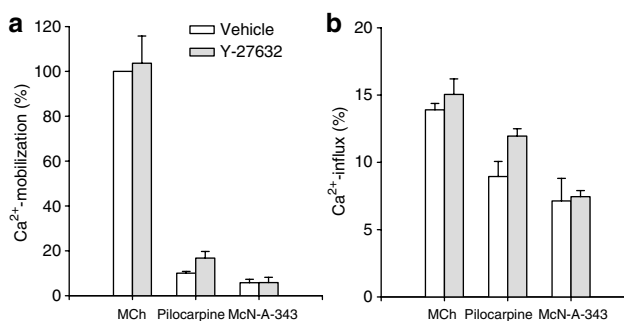


Figure 3 Effects of Rho-kinase inhibition on Ca^{2+} -responses induced by full and partial M_3 -receptor agonists. Ca^{2+} -mobilization (a) and -influx (b) in response to methacholine (MCh), pilocarpine and McN-A-343 in the absence (white bars) and presence (gray bars) of $1 \mu M$ Y-27632 in BTSM cells. Data are expressed as percentage of maximal Ca^{2+} -mobilization induced by methacholine and represent means ± s.e.m. of three experiments, each performed in duplicate.

$P < 0.05$; Figure 4). These results suggest that the contribution of Rho-kinase to muscarinic agonist-induced contraction is dependent on receptor/transduction reserve. Interestingly, after PrBCm pretreatment, the reduction of the pEC_{50} value

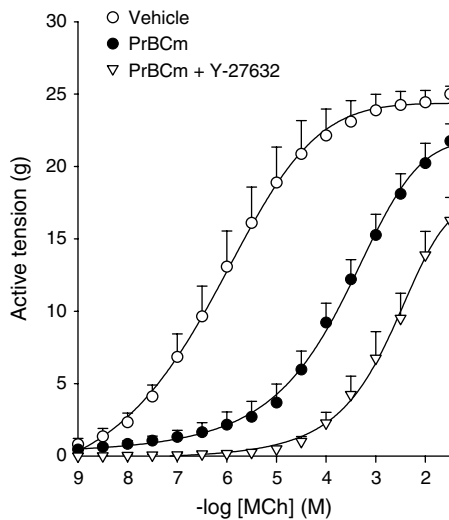


Figure 4 Effects of Rho-kinase inhibition on methacholine-induced contraction after pretreatment with propylbenzylcholine mustard (PrBCm). Methacholine (MCh)-induced contraction in the absence (closed circles) and presence (triangles) of $1 \mu\text{M}$ Y-27632 of PrBCm-treated BTSM strips. Data represent means \pm s.e.m. of 3–4 experiments, each performed in duplicate.

of methacholine by Y-27632 was of similar magnitude as in nonalkylated preparations (Table 1).

Relationship between Rho-kinase dependency and Ca^{2+} -mobilization and -influx

For several agonist concentrations, we have plotted the effectiveness of Y-27632 in decreasing contraction (% inhibition) against the induced Ca^{2+} -mobilization and the Ca^{2+} -influx (Figure 5). An inverse relationship between Rho-kinase dependency and Ca^{2+} -mobilization (Figure 5a) as well as Ca^{2+} -influx (Figure 5b) was found.

Discussion

In the present study, we demonstrate that full and partial muscarinic agonists are differentially dependent on Rho-kinase and Ca^{2+} -mobilization for their contractile effects. Moreover, we show that there is an inverse (exponential) relationship between the Rho-kinase dependency of the contraction and the Ca^{2+} -mobilization as well as the Ca^{2+} -influx induced by different concentrations of the agonists. These findings correspond to observations in canine tracheal smooth muscle using acetylcholine and McN-A-343, indicating that differences in Ca^{2+} -kinetics are possibly a consequence of differences in potency of muscarinic agonists to activate the same subcellular pathways, rather than from the activation of distinct subcellular mechanisms (al-Hassani *et al.*, 1993).

A receptor-dependent role of Rho-kinase in agonist-induced ASM contraction appears to exist. Both in guinea-pig (Schaafsma *et al.*, 2005) and human (Gosens *et al.*, 2004a) ASM, it was found that growth factors, which are coupled to receptor tyrosine kinases, induce contractions which are fully Rho-kinase dependent, whereas contractions elicited by histamine, which are mediated through $\text{G}_{q/11}$ -coupled H_1 receptors,

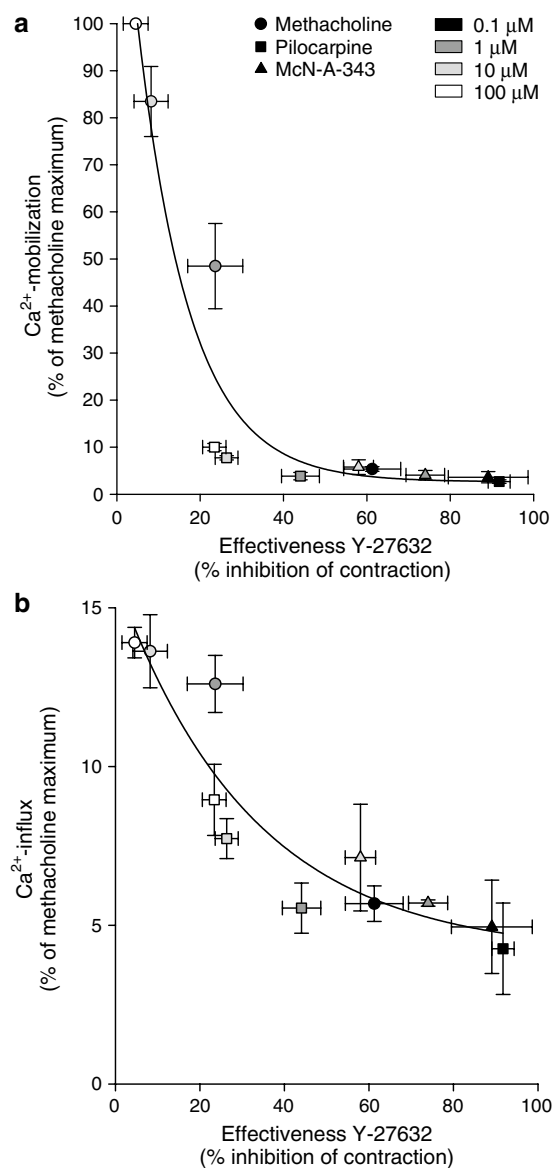


Figure 5 Relationship between methacholine (circles)-, pilocarpine (squares)- and McN-A-343 (triangles)-induced Ca^{2+} -mobilization (a) and -influx (b) in BTSM cells and Rho-kinase dependency of methacholine-, pilocarpine- and McN-A-343-induced contraction of BTSM strips. Data represent means \pm s.e.m. of 3–7 experiments, each performed in duplicate.

are not (Gosens *et al.*, 2004a; Schaafsma *et al.*, 2004). In addition, potency and maximal contraction induced by $\text{PGF}_{2\alpha}$ are governed by Rho-kinase activity in guinea-pig tracheal smooth muscle to an important extent, whereas histamine-induced contractions are not (Schaafsma *et al.*, 2004). The agonists used in the present study all function through activation of the M_3 -receptor in order to induce ASM contraction. The observed differential involvement of Rho-kinase by partial and full agonists therefore implies that there is not only a receptor-dependent, but also an agonist-dependent role for the participation of Rho-kinase in ASM contraction.

Rho-kinase inhibition resulted in a decrease in potency of methacholine, pilocarpine and McN-A-343, whereas E_{max} was

affected only for pilocarpine and McN-A-343. Remarkably, the strongest decrease in potency was observed for methacholine and the smallest for McN-A-343; in contrast to the effects on E_{\max} , which were absent for methacholine and most pronounced for McN-A-343. This indicates that methacholine is only dependent on Rho-kinase for its contractile effects in the lower concentration range, without requiring Rho-kinase to achieve its maximal contractile response. In BTSM, there is a considerable reserve of inositol phosphate production (transduction reserve) for methacholine, but not for McN-A-343 (Meurs *et al.*, 1988). If the maximally stimulated M_3 -receptor generates more Rho-kinase activity than 'required' for maximal contractile effect, inhibition of this signal transduction will affect the potency, not the efficacy, of the agonist. In other words, methacholine induces the largest IP_3 -dependent Ca^{2+} -mobilization and -influx, which is probably sufficient to achieve maximal contraction independent of Rho-kinase-mediated Ca^{2+} -sensitization. In contrast, very low levels of Ca^{2+} -mobilization are observed in response to pilocarpine and McN-A-343, presumably as a consequence of a small and neglectable transduction reserve, respectively (Meurs *et al.*, 1988), and therefore these agonists do rely on Rho-kinase for their maximal contractile response. Also, fully in line with this interpretation, maximal methacholine-induced contraction becomes Rho-kinase dependent after pretreatment with the irreversible muscarinic receptor antagonist PrBCM, demonstrating that the contribution of Rho-kinase to muscarinic agonist-induced contraction is dependent on transductional reserve. Remarkably, in the presence of Y-27632, a similar decrease of potency is observed in PrBCM-treated strips as in untreated strips. An additional explanation for the differences between full and partial agonists in the modulation of the maximal contraction might therefore be that partial

agonists have different relative activation profiles for G_q - and $G_{i/o}$ -proteins as compared to full agonists, as demonstrated in Chinese hamster ovary cells (Akam *et al.*, 2001).

The inverse relationship between Rho-kinase dependency and both Ca^{2+} -mobilization and Ca^{2+} -influx, as shown in Figure 5, might suggest that Y-27632 is capable of reducing these Ca^{2+} -responses. However, we demonstrate that Y-27632 does not inhibit Ca^{2+} -mobilization or Ca^{2+} -influx for all three agonists at their maximal concentration. In addition, no effect of Rho-kinase inhibition was observed when a submaximal concentration of methacholine ($1 \mu M$) was applied (data not shown), corresponding to findings in guinea-pig trachealis, which showed no effects of Y-27632 (1 – $10 \mu M$) on methacholine ($1 \mu M$)-induced Ca^{2+} -mobilization (Ito *et al.*, 2001). This strongly suggests that the effects of Rho-kinase inhibition are on the level of Ca^{2+} -sensitization rather than on Ca^{2+} -mobilization or -influx.

In conclusion, this study shows that full and partial muscarinic receptor agonists are differentially dependent on Rho-kinase for their contractile effects, indicating an agonist-dependent role for Rho-kinase in ASM contraction. Furthermore, we demonstrate that there is an inverse relationship between both Ca^{2+} -mobilization and -influx and the functional Rho-kinase dependency, presumably as a consequence of differences in transduction reserve between full and partial M_3 -receptor agonists. Moreover, Ca^{2+} -mobilization and -influx in response to M_3 -receptor stimulation seem to be independent of Rho-kinase, suggesting that differences in functional Rho-kinase dependency are on the level of Ca^{2+} -sensitization rather than on Ca^{2+} -mobilization or -influx.

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References

- AKAM, E.C., CHALLISS, R.A. & NAHORSKI, S.R. (2001). $G(q/11)$ and $G(i/o)$ activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype. *Br. J. Pharmacol.*, **132**, 950–958.
- AL-HASSANI, M.H., GARCIA, J.G. & GUNST, S.J. (1993). Differences in Ca^{2+} mobilization by muscarinic agonists in tracheal smooth muscle. *Am. J. Physiol.*, **264**, L53–L59.
- DE LANEROLLE, P. & PAUL, R.J. (1991). Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. *Am. J. Physiol.*, **261**, L1–L14.
- FUKATA, Y., AMANO, M. & KAIBUCHI, K. (2001). Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol. Sci.*, **22**, 32–39.
- GOSENS, R., SCHAAFSMA, D., GROOTTE BROMHAAR, M.M., VRUGT, B., ZAAGSMA, J., MEURS, H. & NELEMANS, S.A. (2004a). Growth factor-induced contraction of human bronchial smooth muscle is Rho-kinase-dependent. *Eur. J. Pharmacol.*, **494**, 73–76.
- GOSENS, R., SCHAAFSMA, D., MEURS, H., ZAAGSMA, J. & NELEMANS, S.A. (2004b). Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype. *Eur. J. Pharmacol.*, **483**, 71–78.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.
- GUNST, S.J., GERTHOFFER, W.T. & AL-HASSANI, M.H. (1992). Ca^{2+} sensitivity of contractile activation during muscarinic stimulation of tracheal muscle. *Am. J. Physiol.*, **263**, C1258–C1265.
- GUNST, S.J., STROPP, J.Q. & FLAVAHAN, N.A. (1987). Analysis of receptor reserves in canine tracheal smooth muscle. *J. Appl. Physiol.*, **62**, 1755–1758.
- GUNST, S.J., STROPP, J.Q. & FLAVAHAN, N.A. (1989). Muscarinic receptor reserve and beta-adrenergic sensitivity in tracheal smooth muscle. *J. Appl. Physiol.*, **67**, 1294–1298.
- HASHIMOTO, T., HIRATA, M. & ITO, Y. (1985). A role for inositol 1,4,5-trisphosphate in the initiation of agonist-induced contractions of dog tracheal smooth muscle. *Br. J. Pharmacol.*, **86**, 191–199.
- ITO, S., KUME, H., HONJO, H., KATOH, H., KODAMA, I., YAMAKI, K. & HAYASHI, H. (2001). Possible involvement of Rho kinase in Ca^{2+} sensitization and mobilization by MCh in tracheal smooth muscle. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **280**, L1218–L1224.
- MEURS, H., ROFFEL, A.F., POSTEMA, J.B., TIMMERMANS, A., ELZINGA, C.R., KAUFFMAN, H.F. & ZAAGSMA, J. (1988). Evidence for a direct relationship between phosphoinositide metabolism and airway smooth muscle contraction induced by muscarinic agonists. *Eur. J. Pharmacol.*, **156**, 271–274.
- MEURS, H., TIMMERMANS, A., VAN AMSTERDAM, R.G., BROUWER, F., KAUFFMAN, H.F. & ZAAGSMA, J. (1989). Muscarinic receptors in human airway smooth muscle are coupled to phosphoinositide metabolism. *Eur. J. Pharmacol.*, **164**, 369–371.
- OFFER, G.J., CHILVERS, E.R. & NAHORSKI, S.R. (1991). Beta-adrenoceptor induced inhibition of muscarinic receptor-stimulated phosphoinositide metabolism is agonist specific in bovine tracheal smooth muscle. *Eur. J. Pharmacol.*, **207**, 243–248.
- PFITZER, G. (2001). Invited review: regulation of myosin phosphorylation in smooth muscle. *J. Appl. Physiol.*, **91**, 497–503.

- ROFFEL, A.F., ELZINGA, C.R., VAN AMSTERDAM, R.G., DE ZEEUW, R.A. & ZAAGSMA, J. (1988). Muscarinic M2 receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur. J. Pharmacol.*, **153**, 73–82.
- ROFFEL, A.F., ELZINGA, C.R. & ZAAGSMA, J. (1990). Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm. Pharmacol.*, **3**, 47–51.
- SCHAAFSMA, D., GOSENS, R., BOS, I.S., MEURS, H., ZAAGSMA, J. & NELEMANS, S.A. (2004). Allergic sensitization enhances the contribution of Rho-kinase to airway smooth muscle contraction. *Br. J. Pharmacol.*, **143**, 477–484.
- SCHAAFSMA, D., GOSENS, R., BOS, S., MEURS, H., ZAAGSMA, J. & NELEMANS, A. (2005). Role of contractile prostaglandins and Rho-kinase in growth factor-induced airway smooth muscle contraction. *Respir. Res.*, **6**, 85.
- SOMLYO, A.P. & SOMLYO, A.V. (2003). Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.*, **83**, 1325–1358.
- TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1986). Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositide in bovine tracheal smooth muscle measurements of the mass of polyphosphoinositides, 1,2-diacylglycerol, and phosphatidic acid. *J. Biol. Chem.*, **261**, 14670–14675.
- UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, K., INUI, J., MAEKAWA, M. & NARUMIYA, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*, **389**, 990–994.
- WATSON, N., MAGNUSSEN, H. & RABE, K.F. (1995). Pharmacological characterization of the muscarinic receptor subtype mediating contraction of human peripheral airways. *J. Pharmacol. Exp. Ther.*, **274**, 1293–1297.
- YANG, C.M., CHOU, S.P. & SUNG, T.C. (1991). Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells. *Br. J. Pharmacol.*, **104**, 613–618.
- YANG, C.M., YO, Y.L. & WANG, Y.Y. (1993). Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M3 muscarinic receptors. *Br. J. Pharmacol.*, **110**, 983–988.

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