Selectivity of ROCK inhibitors in the spontaneously tonic smooth muscle

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Rattan S, Patel CA. Selectivity of ROCK inhibitors in the spontaneously tonic smooth muscle. Am J Physiol Gastrointest Liver Physiol 294: G687–G693, 2008. First published January 10, 2008; doi:10.1152/ajpgi.00501.2007.—The selectivity of different Rho kinase (ROCK) inhibitors in the spontaneously tonic smooth muscle has not been investigated. We examined this issue using Y-27632 [(R)-(+-)trans-N-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxylic acid, HCl], H-1152 [(S)-(+-)2-methyl-5-isoquinolinyl)sulfonfonylhomopiperazine, 2HCl], HA-1077 [(S)-10-(4-pyridyl)-N-[(2,4,6-trichlorophenyl)urea], and ROCK inhibitor II [N-(4-pyridyl)-N'-[(2,4,6-trichlorophenyl)urea]]. We compared these inhibitors in the spontaneously tonic smooth muscle of the internal anal sphincter (IAS). ROCK, protein kinase C (PKC), and myosin light chain kinase (MLCK) activities were determined in the IAS, before and after ROCK inhibition. PKC and MLCK inhibition can be concentration-dependent in the IAS without significant effect in the phasic rectal smooth muscle (10). Conversely, HA-1077 and ROCK inhibitor II were equipotent in the IAS vs. RSM. In the IAS, H-1152 was the most potent whereas ROCK inhibitor II is the least. Y-27632 and H-1152 caused concentration-dependent decrease in the IAS tone that correlates directly with the decreases in ROCK activity, without significant effect in the PKC and MLCK activities. This specifically selective correlation between ROCK activity and decrease in the IAS tone was absent in the case of HA-1077 and ROCK inhibitor II, which also inhibited PKC and MLCK. We conclude that the IAS tone is critically dependent on ROCK activity, and H-1152 and Y-27632 are the most selective and potent ROCK inhibitors in the IAS.

Tonic smooth muscle of the internal anal sphincter (IAS) plays a major role in anorectal continence. Relaxation of the IAS, on the other hand, is important for the expulsion of waste products during the anorectal inhibitory reflex (33). Hypertensive IAS accompanied with an incomplete relaxation of the IAS is associated with Hirschsprung’s disease (30, 33, 47), recurrent anal fissures and hemorrhoids (22, 24). Ser/Thr kinase Rho kinase (ROCK) inhibitors, because of their selective effects, may offer a novel therapeutic approach for such disorders (35).

Increase in intracellular Ca^2+-causes activation of myosin light chain kinase (MLCK) and phosphorylation of 20-kDa myosin regulatory light chain (MLC20) responsible for the initial contraction of the smooth muscle (5, 13). Myosin light chain phosphatase (MLCP) dephosphorylates MLC20 (39), bringing the smooth muscle back to the precontraction or basal level. Sustained contraction or basal tone in the smooth muscle on the other hand may be maintained via ROCK or protein kinase C (PKC) activation.

ROCK and PKC activation may maintain elevated levels of phospho-MLC20 via inhibition of MLCP. ROCK activation accomplishes this via phosphorylation of target/regulatory subunit of MLCP-myosin phosphatase target subunit 1 (MYPT1) and CPI-17 (the endogenous inhibitory protein of the catalytic subunit of MLCP) (16, 18, 20). Conversely, ROCK inhibition may unlease MLCP, leading to relaxation of the IAS (35). PKC inhibits MLCP activity primarily via phosphorylation of CPI-17 (10).

A number of studies have investigated the role of ROCK in different smooth muscle preparations using ROCK inhibitors such as HA-1077 (fasudil), Y-27632 (6, 15, 40, 46), and H-1152 (44). The selectivity of these inhibitors has been reported on the basis of inhibitory potencies for different kinases in in vitro kinase assays only (7, 43). Primarily on the basis of this information, these inhibitors (without actual data) have been assumed to be selective in the smooth muscles as well. Additionally, this ignores the possibility of their nonselective effects on other enzymatic activities such as PKC and MLCK (3, 43).

The objectives of the present investigation were to compare the potency and selectivity of different ROCK inhibitors in the spontaneously tonic IAS smooth muscle while determining their effects on the tone vs. ROCK, PKC, and MLCK activities. In addition, we determined the effect of newly available ROCK inhibitor II [N-(4-pyridyl)-N'-(2,4,6-trichlorophenyl)urea] (42).

MATERIALS AND METHODS

Tissue preparation. Male Sprague-Dawley rats (300–350 g) were killed by decapitation, and the anal canal with an adjacent region of the rectal smooth muscle (RSM) was quickly removed and transferred to oxygenated (95% O2–5% CO2) Krebs physiological solution (KPS) of the following composition (mM): NaCl, 118.07, KCl, 4.69, CaCl2, 1.16, MgSO4, 1.01, NaH2PO4, 25, NaHCO3, and 11.10 glucose (37°C). After the extraneous adventitious tissue was cleaned off, the anal canal was opened in the longitudinal axis and pinned flat with the mucosal side up on a dissecting tray containing oxygenated KPS. The mucosa was removed carefully by sharp dissection, and circular smooth muscle strips (~1 × 7 mm) were prepared as described before (34).

The experimental protocols of the study were approved by the institutional animal care and use committee of Thomas Jefferson University and was in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Measurement of tone and isometric tension. The smooth muscle strips were transferred to 2-ml muscle baths containing oxygenated KPS at 37°C for the recording of isometric tension using force transducers (model FT03; Grass Instruments, Quincy, MA). Isometric...
tension was measured by the powerlab/SP data acquisition system (ADInstruments, Castle Hill, Australia) using Chart recorder 4.1.2 (ADInstruments). Each smooth muscle strip was initially stretched to a tension of 0.7 g. The muscle strips were then given at least 1 h to equilibrate during which they were washed with KPS every 20 min. The smooth muscle strips of the IAS developed spontaneous tone (8, 35, 37), whereas those from the rectum displayed primarily spontaneous phasic activity. The decreases in tension following different drugs were expressed as the percentage of maximal decrease by 50 mM EDTA at the end of the experiment (2).

**Drug responses.** To determine the concentration-response curves with ROCK inhibitors on the basal tone of the IAS smooth muscle, HA-1077, Y-27632, H-1152, and ROCK inhibitor II were added to the muscle bath in a cumulative manner as explained before (36).

**Tissue lysate preparation for kinase assay.** While the isometric tension was monitored, the smooth muscle strips were quick-frozen in the basal state and after treatment with different concentrations of ROCK inhibitors. The tissues were quickly frozen in liquid N2 and stored at −80°C. Later, the respective tissues were cut into 1–mm cubes. The homogenization buffer (50 mM Tris•HCl, pH 7.5; 5 mM EDTA; 10 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 10 mM benzamide; 0.3% wt/vol 2-mercaptoethanol) was added to the tissues in a volume equal to five times the weight. Following homogenization, the homogenates were centrifuged (14,000 rpm) for 5 min and supernatants were collected. Protein concentration in the supernatant was determined using bovine serum albumin as a standard (Pierce, Rockford, IL); 25 μg of proteins in 10 μl of lysates were used for the kinase assay.

**ROCK assay.** ROCK activity was determined by immunokinase assay. The tissue lysates were mixed with 30 μM long S6 kinase substrate peptide (Upstate, Lake Placid, NY). Kinase assays were initiated by the addition of 10 μCi of [γ-32P]ATP (3,000 Ci/mmol) (PerkinElmer, Shelton, CT) and 100 μM ATP, followed by incubation for 10 min at 30°C. [γ-32P]substrate peptide was absorbed onto P81 Whatman phosphocellulose discs (Fisher), and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the discs was measured by liquid scintillation. Changes in the enzymatic activity in the presence of ROCK inhibitors were expressed on percentile bases in reference to the ROCK activity in the basal state (considered as 100) (27, 31, 48).

**PKC assay.** PKC activity was determined using PKC assay kit (Upstate). Briefly, tissue lysates were mixed with 80 μM PKC substrate peptide [QKRPSQRSKYL] (Upstate), 0.1 mg/ml phosphatidylinositol, and 0.01 mg/ml diacylglycerol. Kinase assays were initiated by the addition of 10 μCi of [γ-32P]ATP (3,000 Ci/mmol) and 100 μM ATP, followed by incubation for 10 min at 30°C. [γ-32P]substrate peptide was absorbed onto P81 Whatman phosphocellulose discs (Fisher), and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the discs was measured by liquid scintillation. Changes in PKC activity in the presence of ROCK inhibitors were expressed on percentile bases in reference to the basal activity and basal activity (considered as 100) (28).

**MLCK activity assay.** MLCK activity was assayed with MLC20 as substrate (12, 26). Tissue lysates were mixed with 100 μM CaCl2, 50 mM MOPS, 15 mM dithiothreitol, 10 mM Mg acetate, 0.3 μM CaM, and 18 μM smooth muscle MLC (substrate for MLCK). The assay was initiated by the addition of 10 μCi of [γ-32P]ATP (3,000 Ci/mmol) and 100 μM ATP, followed by incubation for 15 min at 30°C. The aliquots were spotted onto P81 Whatman phosphocellulose discs. The discs were washed three times with 10% trichloroacetic acid with 4% pyrophosphate (15 min each wash), once with 95% ethanol (15 min), and once with ethyl ether for 15 min. The amount of radioactivity on the discs was measured by liquid scintillation, and the changes in MLCK activity were expressed as a percentage of the control. Changes in basal tone of the IAS and in the enzymatic activities (means ± SE of different experiments) following the inhibitors were expressed on percentile basis as ex-

**Drugs and chemicals.** Y-27632 [(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxanecarboxamide, 2HCl] was purchased from Biomol (Plymouth Meeting, PA). HA-1077 [(S)-(+)-(2-methyl-5-isouquinolinyl)sulfonfonylhomopiperazin-2HCl], H-1152 [(S)-(+)-(2-methyl-5-isouquinolinyl)sulfonfonylhomopiperazin-2HCl] and ROCK inhibitor II [N-(4-pyridyl)-N′-(2,4,6-trichlorophenyl)urea] were from Calbiochem (San Diego, CA). MLC20 was purchased from Sigma (St. Louis, MO). PKC substrate peptide, long S6 kinase substrate, and kinase assay reagents were from Upstate. [γ-32P]ATP was from PerkinElmer.

Y-27632, H-1152, and HA-1077 were dissolved and diluted with KPS, ROCK inhibitor II was dissolved initially in dimethyl sulfoxide (DMSO) and further diluted with KPS. Final concentrations of DMSO used in the organ bath had no significant effect on either the basal tone or on any of the enzymatic activities in the IAS.

**Data analysis.** Changes in basal tone of the IAS and in the enzymatic activities (means ± SE of different experiments) following the inhibitors were expressed on percentile basis as ex-

Fig. 1. Concentration response curves of Y-27632 in the internal anal sphincter (IAS; A) vs. rectal smooth muscle (RSM; B). Considering IC50, Y-27632 and H-1152 are ~30-fold more potent in the IAS vs. the RSM (P < 0.05; ANOVA; n = 5–8). IC50 values of H-1152 in causing decrease in the IAS tone are 7.9 × 10−8 M. ROCK, Rho kinase; Inhib, inhibitor.
plained above. The IC₅₀ values were calculated by use of nonlinear regression curve fitting program using GraphPad Prism 5 program. Coefficients of correlation using r² values were also determined via GraphPad Prism 5 that computed individual enzymatic activity vs. the IAS tone values corresponding each concentration of the inhibitors, by linear regression analysis. The statistical significance between different groups was determined by analysis of variance and by unpaired t-test and was considered to be significant with a P value smaller than 0.05.

RESULTS

Effect of Y-27632 on the basal tone and ROCK, PKC, and MLCK activities in the IAS. Y-27632 caused a concentration-dependent decrease in the basal tone of the IAS. In this regard, Y-27632 was ~30-fold more potent in the IAS than in the RSM (n = 5–8; Fig. 1). The concentration of the inhibitor that produces 50% of the maximal effect (IC₅₀) in decreasing the IAS tone and in the phasic activity of the RSM were ~4.36 × 10⁻⁷ and 1.34 × 10⁻⁵ M, respectively (Table 1).

Studies revealed a strong correlation between the decrease in the IAS tone and in the ROCK activity in the presence of different concentrations of Y-27632 (10⁻⁹ to 10⁻⁵ M) (Fig. 2, top) (r² = 0.99). Y-27632 (10⁻⁹ M) caused 50.0 ± 2.4 and 54.2 ± 2.2% decrease in the IAS tone and in the ROCK activity, respectively, without causing a significant change in PKC (Fig. 2, middle) and MLCK activity (Fig. 2, bottom).

Effect of H-1152 on the basal tone and ROCK, PKC, and MLCK activities in the IAS. H-1152 caused a concentration-dependent decrease in the IAS basal tone. H-1152 was ~32-fold more potent in reducing IAS basal tone vs. phasic activity of RSM (n = 5–8; Fig. 1). IC₅₀ values in the IAS vs. RSM were 7.90 × 10⁻⁸ and 2.51 × 10⁻⁶ M, respectively. As determined by the IC₅₀ values, H-1152, among different ROCK inhibitors examined, was most potent in decreasing the IAS basal tone and ROCK activity (Tables 1 and 2).

In agreement with the above functional data, H-1152 was most potent in decreasing ROCK activity in the IAS (Table 2). H-1152 (10⁻⁶ M) caused 90.9 ± 2% decrease in the IAS tone and 87.6 ± 1.2% decrease in the ROCK activity (Fig. 3, top). There was a strong relationship between the decreases in the IAS tone and in the ROCK activity in the presence of 10⁻⁹ to 10⁻⁵ M of H-1152 (r² = 0.99). H-1152 (10⁻⁶ M) that was maximally effective in inhibiting the ROCK activity caused no significant change in either the basal PKC (Fig. 3, middle) or MLCK activity (Fig. 3, bottom).

Effect of HA-1077 on the basal tone and ROCK, PKC, and MLCK activities in the IAS. HA-1077 caused a concentration-dependent decrease in the IAS basal tone and phasic activity of RSM (n = 5–8; Fig. 1). HA-1077 was significantly more potent in the IAS vs. the RSM. IC₅₀ values in the IAS and RSM were 1.82 × 10⁻⁷ and 4.57 × 10⁻⁶ M, respectively (Table 1). EC₅₀ in causing fall in the IAS tone was 3 × 10⁻⁴ M.

Decreases in ROCK activity and IAS tone by HA-1077 were strongly correlated (Fig. 4, top; r² = 0.99). HA-1077 (10⁻⁵ M) caused 71.2 ± 6.9% fall in IAS tone and 47.6 ± 2.1% decrease in ROCK activity. The same concentration of HA-1077 also caused 28.1 ± 2.4 and 41.8 ± 5.6% decrease in PKC (Fig. 4, middle) and MLCK (Fig. 4, bottom) activities, respectively.

Table 1. Comparison of potencies of ROCK inhibitors in inhibiting the basal tone of the IAS tone vs. the phasic activity of RSM

<table>
<thead>
<tr>
<th>ROCK Inhibitor</th>
<th>IC₅₀ (M)</th>
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<tbody>
<tr>
<td>H-1152</td>
<td>7.90 × 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>2.51 × 10⁻⁶</td>
</tr>
<tr>
<td>IAS</td>
<td>4.36 × 10⁻⁷</td>
</tr>
<tr>
<td>RSM</td>
<td>1.34 × 10⁻⁵</td>
</tr>
<tr>
<td>Y-27632</td>
<td>1.82 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>4.57 × 10⁻⁶</td>
</tr>
<tr>
<td>HA-1077</td>
<td>2.95 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>1.54 × 10⁻⁵</td>
</tr>
</tbody>
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ROCK, Rho kinase; IAS, internal anal sphincter; RSM, rectal smooth muscle.
Inhibitory effect of ROCK inhibitors on different smooth muscles (6, 11, 15, 29, 35, 40, 44), there has been a paucity of data determining their selectivity in the spontaneously tonic smooth muscle. To examine this, we focused on the activity of ROCK vs. PKC and MLCK, using a wide range of concentrations of these agents, in the IAS.

Both MLCK and MLCP regulate the levels of phospho-MLC$_{20}$ (39). It is widely accepted that MLCK activation primarily leads to the initial contraction of the smooth muscle whereas inhibition of MLCP either via ROCK or PKC is primarily responsible for the sustained contraction (26, 39). All of the earlier data about the sustained contraction in the smooth muscle have been in response to agonists and not in the spontaneously tonic state.

We speculate that Ser/Thr kinase ROCK maintains IAS basal tone by MLCP inhibition primarily via phosphorylation of target/regulatory subunit of MLCP (MYPT1) at threonine-696 residue and phosphorylation of CPI-17 at threonine-38 residue (31). PKC can also modulate MLCP activity via the phosphorylation of CPI-17 (10).

### DISCUSSION

The studies compare the potency and selectivity of different ROCK inhibitors in the spontaneously tonic smooth muscle of the IAS vs. the phasic RSM. In general, the ROCK inhibitors were significantly more potent in the spontaneously tonic smooth muscle. Additional studies suggest that H-1152 and Y-27632 are more potent and selective than HA-1077 and ROCK inhibitor II in inhibiting the ROCK activity in the IAS.

Among different ROCK inhibitors, H-1152 and Y-27632 are most potent and discriminatory in decreasing the IAS tone vs. the RSM activity. The rank order of potencies of different ROCK inhibitors in the IAS is H-1152 (IC$_{50}$ = 7.90 × 10$^{-8}$ M) > Y-27632 (IC$_{50}$ = 4.36 × 10$^{-7}$ M) > HA-1077 (1.82 × 10$^{-6}$ M) > ROCK inhibitor II (2.95 × 10$^{-6}$ M; H-1152 (1.00 × 10$^{-5}$ M) and Y-27632 (1.86 × 10$^{-5}$ M) almost completely abolish the IAS tone. Data reveal H-1152 to be ~10-fold more potent than Y-27632 in the IAS. H-1152 and Y-27632 are ~30-fold respectively, more potent in the IAS vs. the RSM.

Although there are numerous studies demonstrating the inhibitory effect of ROCK inhibitors on different smooth

### Table 2. Comparison of potencies of ROCK inhibitors in inhibiting ROCK, PKC, and MLCK in the IAS

<table>
<thead>
<tr>
<th>ROCK Inhibitor</th>
<th>IC$_{50}$ M</th>
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<tbody>
<tr>
<td>H-1152</td>
<td>3.90 × 10$^{-8}$</td>
</tr>
<tr>
<td>ROCK</td>
<td>1.00 × 10$^{-5}$</td>
</tr>
<tr>
<td>PKC</td>
<td>2.95 × 10$^{-5}$</td>
</tr>
<tr>
<td>MLCK</td>
<td>1.00 × 10$^{-5}$</td>
</tr>
<tr>
<td>Y-27632</td>
<td>2.00 × 10$^{-5}$</td>
</tr>
<tr>
<td>ROCK</td>
<td>1.07 × 10$^{-7}$</td>
</tr>
<tr>
<td>PKC</td>
<td>1.74 × 10$^{-5}$</td>
</tr>
<tr>
<td>MLCK</td>
<td>1.86 × 10$^{-5}$</td>
</tr>
</tbody>
</table>

MLCK, myosin light chain kinase; NP, not possible to calculate because of the insignificant inhibition of the enzymatic activity.
The studies further demonstrate H-1152 and Y-27632 cause fall in the IAS tone by the selective inhibition of ROCK. In contrast, the inhibitory effects of HA-1077 and ROCK inhibitor II on the IAS tone are relatively less selective and discriminatory because of the narrow range of potency differences between the IAS and RSM. In addition, these agents not only inhibit ROCK but also PKC and MLCK. Our data with HA-1077 are consistent with those of the earlier reports (38, 40, 43).

In vitro studies using inhibition of PKC/h9254 purified from pig aorta smooth muscle (9) suggest that Y-27632 (10^{-5} M) nonspecifically inhibits PKC. However, our studies in the IAS are in agreement with the bulk of the literature (exemplified by two citations) (15, 46), suggesting that H-1152 and Y-27632 (10^{-5} M) have no significant effect on PKC activity.

Our studies identified a strong correlation between decrease in the IAS tone and decrease in ROCK activity following different concentrations of H-1152 and Y-27632. Earlier studies from our laboratory have shown that tonic smooth muscle of the IAS has higher levels and activities of RhoA/ROCK vs. the phasic smooth muscle of the RSM at the cellular level (31, 32). These findings combined with the present data suggest critical dependence of sustained elevated levels of phospho-MLC_{20} and the basal tone in the IAS on RhoA/ROCK-mediated inhibition of MLCP. The transient surge in the levels of phospho-MLC_{20} responsible for the phasic contraction of different smooth muscles on the other hand is considered to be via the higher MLCK activity (4, 41).

In conclusion, ROCK inhibitors like H-1152 and Y-27632 provide selective therapeutic alternatives for anorectal motility disorders characterized with hypertensive IAS (35) over the present options of Ca^{2+} channel blockers (19), nitric oxide donors (1, 17), botulinum toxin (14, 23), and specific phosphodiesterase inhibitors (25, 45). These approaches are often ineffective because of the side effects or desensitization issues. Present data are important in selecting a ROCK inhibitor for the management of hypertensive IAS and in selecting an appropriate tool for defining the molecular role of RhoA/ROCK in the smooth muscle tone.


