Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans

Satish Rattan, Jagmohan Singh, Sumit Kumar, and Benjamin Phillips

1Department of Medicine, Division of Gastroenterology and Hepatology, Thomas Jefferson University, Philadelphia, Pennsylvania; and 2Department of Surgery, Division of Colorectal Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania

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Rattan S, Singh J, Kumar S, Phillips B. Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans. Am J Physiol Gastrointest Liver Physiol 308: G924–G933, 2015. First published April 16, 2015; doi:10.1152/ajpgi.00017.2015.—The extracellular signal that triggers activation of rho-associated kinase (RhoA/ROCK), the major molecular determinant of basal internal anal sphincter (IAS) smooth muscle tone, is not known. Using human IAS tissues, we identified the presence of the biosynthetic machineries for angiotensin II (ANG II), thromboxane A$_2$ (TXA$_2$), and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). These end products of the renin-angiotensin system (RAS) (ANG II) and arachidonic acid (TXA$_2$ and PGF$_{2\alpha}$) pathways and their effects in human IAS vs. rectal smooth muscle (RSM) were studied. A multipronged approach utilizing immunocytochemistry, Western blot analyses, and force measurements was implemented. Additionally, in a systematic analysis of the effects of respective inhibitors along different steps of biosynthesis and those of antagonists, their end products were evaluated either individually or in combination. To further describe the molecular mechanism for the IAS tone via these pathways, we monitored RhoA/ROCK activation and its signal transduction cascade. Data showed characterized higher expression of biosynthetic machineries of RAS and AA pathways in the IAS compared with the RSM. Additionally, specific inhibition of the arachidonic acid (AA) pathway caused ~80% decrease in the IAS tone, whereas that of RAS led to ~20% decrease. Signal transduction studies revealed that the end products of both AA and RAS pathways cause increase in the IAS tone via activation of RhoA/ROCK. Both AA and RAS (via the release of their end products TXA$_2$, PGF$_{2\alpha}$, and ANG II, respectively), provide extracellular signals which activate RhoA/ROCK for the maintenance of the basal tone in human IAS.

arachidonic acid pathway; renin-angiotensin system; Rho kinase; basal smooth muscle tone
MATIERS AND METHODS

Tissue preparation. Tissue samples of internal anal sphincter (IAS) and rectal (RSM) smooth muscles from ten human subjects (from adult men as well as women) who underwent surgical resection of anorectum, were provided by the departments of surgery and pathology. None the subjects were on any of the medications that could directly or indirectly affect AA or RAS pathway. All tissue specimens were assessed to be normal and free of any inflammatory conditions. The studies were approved by the Institutional Review Board of Thomas Jefferson University. Tissue samples were obtained in the operating room and immediately placed in oxygenated, 0.22 µM filtered Krebs physiological solution (KPS) at 37°C. The composition of KPS was (in mM) 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.10 glucose. The IAS and rectal tissues were identified and cleaned of the extraneous serosal and mucosal tissues by an experienced colorectal surgeon using sharp dissection, and tissue identification was confirmed by functional studies.

Measurement of isometric tension. The smooth muscle strips were transferred to 2-ml muscle baths containing oxygenated KPS at 37°C. Isometric tension was measured with force transducers (FORT10, WPI, Sarasota, FL), and a PowerLab/8SP data-acquisition system (AD Instruments, Colorado Springs, CO) was employed, as described before (43).

Effects of different agents were examined as cumulative concentration (1 nM to 10 µM)-response curves (CRC) as previously explained (15, 46), before and after different antagonists. In certain experiments, we also examined the effects of the inhibitors given in different combinations as outlined in RESULTS.

Isolation of SMCs and cell culture. Smooth muscle cells (SMCs) from the IAS and RSM were isolated as described previously (38, 43, 45). Briefly, the circular smooth muscle tissues were minced into ~1-mm cubes with sharp scissors and kept for incubation in oxygenated KPS (containing 0.1% collagenase type I and 0.01% soybean trypsin inhibitor) at 37°C. The cell suspension was filtered through a 500-µm Nitex mesh. The filtrate (in collagenase-free KPS) containing the cells was centrifuged at 350-500 g for 3 min. The cells in the pellet were resuspended on collagen-coated plates in DMEM growth medium with 5% fetal bovine serum, 5% penicillin-streptomycin, 50 µg/ml gentamicin, 2 µg/ml amphotericin B in 100 mm tissue culture dishes (Corning, CA) at 37°C in an incubator with regulated humidity and 5% CO2.

Immunocytochemistry analysis of isolated SMCs from IAS and RSM. The SMCs were grown overnight in chambered slides and treated with 100 nM of ANG II, U46619, and PGF2a for 10 min and fixed with 4% paraformaldehyde and then washed three times with PBS. These cells were then stained with secondary antibodies (FITC and Texas red-conjugated secondary antibody from LI-COR Biosciences) by 10.220.33.1 on September 23, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/ by 10.220.33.1 on September 23, 2017. The tissue samples were rinsed with PBS and suspended in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM EDTA, 250 mM sucrose, and 1 mM dithiothreitol). The homogenates were centrifuged at 100,000 g for 30 min at 4°C (Beckman L8-70 M Ultracentrifuge; Beckman Coulter, Fullerton, CA). The supernatants were then transferred to a fresh tube and used as the cytosolic fractions. The pellets were resuspended and homogenized in buffer containing 1% Triton X-100. The pellet extract was centrifuged at 800 g for 10 min, and the supernatant was collected as the particulate fraction (43).

Total protein lysates of IAS and RSM tissue samples for Western blot studies. The tissue samples were rinsed with PBS and suspended in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM EDTA, 250 mM sucrose, and 1 mM dithiothreitol, 1% Triton X-100) and homogenized by using tissue homogenizer (IKA ultra, Turrax, Wilmington, DE). The tissue extracts were centrifuged at 800 g for 10 min, and protein concentrations in the resultant supernatants were determined by use of a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) (45).

Western blot studies. Protein (30 µg) was mixed in 30 µl of lysates with Laemmli sample buffer (with final concentrations of 62.5 mM Tris, 1% SDS, 15% glycerol, 0.005% bromphenol blue, and 2% mercaptoethanol) and placed in boiling water bath for 5 min. Proteins in the samples were separated by SDS-PAGE gel (7.5% gel for ACE, COX-1, COX-2, ROCK II, and phosphorylated form of myosin-binding subunit-i at threonine residue 696 (pThr696-MYPT1) vs. nonphosphorylated form of MYPT1; 10% gel for renin, AT1-R, TPR, FPR, and RhoA; 15% gel for myosin light chain (MLC20) and phosphorylated form of MLC20 (pThr18/Myc-19-MLC20) and electrophoretically transferred onto a polyvinylidene difluoride membrane by use of the iBlot Dry Blotting System (Invitrogen, Carlsbad, CA) at RT.

The membrane was soaked for 1 h at RT in LI-COR buffer, following which it was incubated overnight with specific primary antibodies (1:1,000 for ACE, COX-1, COX-2, renin, AT1-R, TPR, FPR, FPR, RhoA, ROCK II, p-MYPT1, MYP1, MLC20, and p-MLC20, and 1:2,000 for α-actin) diluted in LI-COR buffer containing 0.1% Tween 20. After washing three times for 10 min each with Tris-buffered saline with Tween 20 (TBS-T) the membranes were incubated in dark for 1 h, respectively with IRdye680 and IRdye800 conjugated secondary antibody from LI-COR Biosciences (Lincoln, NE). The membranes were washed three times for 10 min each with TBS-T and finally kept in PBS buffer on shaker for 10 min at RT and the dark and scanned by LI-COR Infrared scanner (LI-COR Biosciences).

Chemicals and reagents. ANG II, captopril, indomethacin, Sc-560, BBP, PGF2α, α-actin, and MLC20 primary antibody were obtained from Sigma (St. Louis, MO). Rofecoxib, AL8810, U46619, and SQ29548 were from Cayman Chemical (Ann Arbor, MI). Y27632 was purchased from Biomol (Plymouth Meeting, PA). H77 was from Bachem (Bubendorf, Switzerland). Losartan was a gift from Merck (Rahway, NJ). Primary antibodies of renin, ACE, COX-1, COX-2, ROCK II, pThr696-MYPT1, MYP1, AT1-R, TPR, FPR, and secondary antibodies conjugated with Texas red and FITC against mouse, goat, and rabbit were from Santa Cruz Biotechnology (Santa Cruz, CA). IRdye680- and IRdye800-conjugated secondary antibodies against mouse, goat, and rabbit were from LI-COR Biosciences.

Data analyses. Results are expressed as means ± SE of four to eight independent determinants. ANOVA and Student’s t test were used to determine the statistical significance at the level of p < 0.05.

ACKNOWLEDGMENTS

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REFERENCES

A *-test was used to compare two different groups. A $P$ value $< 0.05$ was considered statistically significant.

**RESULTS**

**Effects of ANG II on basal tone in human IAS.** ANG II (1 nM to 1 μM) produced concentration-dependent increase in the IAS tone, with maximal increase of $19.1 \pm 1.6\%$ (Fig. 1A). Cross-reactivity protocols revealed a selective and significant antagonism ($P < 0.05; n = 8$) of ANG II CRC by the AT1-R antagonist losartan and not by the TPR antagonist SQ29548.

**Effects of U46619 and PGF2α on basal tone in human IAS.** Stable analog of TXA2 and TPR agonist U46619 also produced decrease in human IAS tone following pretreatment with different renin-angiotensin system (RAS) and arachidonic acid (AA) inhibitors. Data in A show concentration-dependent decrease in the IAS tone with of H77 (renin inhibitor), captopril [angiotensin-converting enzyme (ACE) inhibitor], and losartan (AT1-R antagonist). Data in B show inhibitory effect of PLA2, dual cyclooxygenase (COX), COX-1, and COX-2 inhibitors, 4-bromophenacylbromide (BPB), indomethacin, Sc-560, and rofecoxib, respectively, in the IAS tone. C: data show comparative inhibitory effects of TXA2 receptor (TPR) and FPR antagonists SQ2958 and AL1180, respectively, in the IAS tone. In general, the AA pathway inhibitors produce more pronounced decrease in the IAS tone than the RAS inhibitors.
concentration-dependent increase in the IAS tone, with significantly greater efficacy and potency than ANG II. Normalized at 100% increase in the IAS tone with U46619, maximal effect with ANG II was 20% increase in the IAS tone whereas that with U46619 and with PGF$_{2\alpha}$ was 100 and 65%, respectively (Fig. 1, A–C). Comparison of EC$_{50}$ values (for ANG II $3 \times 10^{-8}$ M, and for U46619 and PGF$_{2\alpha}$ $1 \times 10^{-8}$ M) revealed that U46619 and PGF$_{2\alpha}$ were 30 times more potent than ANG II. Cross-reactivity experiments showed selective and significant rightward shift in the CRC with SQ29548 ($^*P < 0.05; n = 8$; Fig. 1B) and not with losartan ($P > 0.05; n = 8$; Fig. 1B). These data suggest selective activation of TPR by U46619 and PGF$_{2\alpha}$.

These inhibitors produced concentration-dependent decrease in the IAS tone. These inhibitors (with the exception of renin inhibitor H-77) produced a limited decrease with captopril and losartan, respectively. These data as plotted in Fig. 2A, on the basis of % of residual absolute basal IAS tone following captopril and losartan, respectively, were 82.7 ± 2.0 and 75.6 ± 3%. Percent of basal IAS tone following the maximally effective concentration of renin inhibitor H-77 on the other hand was 52.9 ± 2.0% (or 48.1 ± 2.0% decrease).

**Effects of AA pathway inhibitors (nonselective PLA$_2$ inhibitor BPB, dual COX inhibitor indomethacin, COX-1 inhibitor SC-560, COX-2 inhibitor rofecoxib, TPR antagonist SQ29548, and FPR antagonist AL8810), on human IAS tone.** These inhibitors of prostanoid production (Fig. 2B) and respective antagonists of TXA$_2$ and PGF$_{2\alpha}$ (Fig. 2C) produced concentration (1 nM to 10 μM)-dependent and significant decrease in the IAS tone ($^*P < 0.05; n = 8$).

BPB and indomethacin produced 67.0 ± 4.0 and 83 ± 6.0% decreases in the IAS tone. These values plotted in Fig. 2B were 33.0 ± 4.0 and 17.0 ± 6.0% of basal IAS tone, respectively. Although Sc-560 (COX-1 inhibitor) and rofecoxib (COX-2 inhibitor) individually caused only 18.0 ± 3 and 28.5 ± 5% decrease in IAS tone, respectively, their combination produced ~80% decrease in the IAS tone (data not shown).

SQ29548 and AL8810 in the maximally effective concentrations induced 49.0 ± 3 and 65 ± 8.60% decrease in IAS tone.

**Fig. 3. Data in A show the effect of losartan and SQ29548 (TPR antagonist), given alone and in combination, on the basal tone in human IAS.** The cumulative effect of these antagonists is significantly ($^*P < 0.05; n = 6–8$) greater than their individual use, suggesting the role of both AA and RAS pathways.

Effects of RAS pathway inhibitors (renin inhibitor H-77, ACE inhibitor captopril, and AT$_1$-R antagonist losartan) on human IAS tone. These inhibitors produced concentration (1 nM to 10 μM)-dependent and significant ($^*P < 0.05; n = 8$; Fig. 2A) decrease in the IAS tone. These inhibitors (with the exception of renin inhibitor H-77) produced a limited decrease in the basal IAS tone: 17.0 ± 1.8 and 24.4 ± 3.5% decrease with captopril and losartan, respectively. These data as plotted in Fig. 2A, on the basis of % of residual absolute basal IAS tone following captopril and losartan, respectively, were 82.7 ± 2.0 and 75.6 ± 3%. Percent of basal IAS tone following the maximally effective concentration of renin inhibitor H-77 on the other hand was 52.9 ± 2.0% (or 48.1 ± 2.0% decrease).
Combined effects of AT₁-R and TPR antagonists (losartan + SQ29548), and TPR and FPR antagonists (SQ29548 + AL8810), on the basal tone. In the maximally effective concentrations (10 μM), the combined effect of losartan and SQ29548 was 65.0 ± 5% decrease in the IAS tone (or as shown in Fig. 3A, % of basal IAS tone following the combination was 37.0 ± 4.0). Individually, these antagonists produced 24.4 ± 3.5 and 42.0 ± 5% decrease in the IAS tone (or resulted in 75.6 ± 5.0 and 58.9 ± 5.9% basal tone as shown in Fig. 3A), respectively.

Similarly, in fresh series of experiments, SQ29548 and AL8810, when given individually and in combination produced 39.0 ± 3, 57.0 ± 8.6, and 75.0 ± 7.27% decrease in the IAS tone (leading to 60.9 ± 4.3, 42.7 ± 7.6, and 25.2 ± 7.2% basal tone; n = 6–8; Fig. 3B), respectively.

Values with the residual basal IAS tone (on percentile basis) following the maximal effect of RAS and AA pathway inhibitors administered either individually or in different combinations are given in Table 1.

On the basis of the above data, the AA pathway appears to provide a more potent extracellular signal for the basal tone in human IAS compared with the RAS pathway (Fig. 3, A and B).

Effects of ANG II and U46619 in the IAS vs. RSM. To determine whether stimulation of the RAS and AA pathways yields increased tonicity uniquely in the IAS, a comparison was made between the circular muscle layer of the rectum and the circular muscle layer of the IAS when exposed to agonists of these pathways. This comparison of the AT₁-R agonist (ANG II) and TPR agonist (U46619) revealed significantly

Table 1. Effects of different inhibitors on basal tone in human IAS

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mechanism of Action</th>
<th>% of Basal Tone</th>
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<tr>
<td>H-77</td>
<td>renin inhibitor</td>
<td>52.9 ± 2.0</td>
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<tr>
<td>Captopril</td>
<td>ACE inhibitor</td>
<td>82.7 ± 2.0</td>
</tr>
<tr>
<td>Losartan</td>
<td>AT₁-R antagonist</td>
<td>75.6 ± 3.0</td>
</tr>
<tr>
<td>BPB</td>
<td>PLA₂ inhibitor</td>
<td>33.0 ± 4.0</td>
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<tr>
<td>Indomethacin</td>
<td>nonselective COX inhibitor</td>
<td>17.0 ± 6.0</td>
</tr>
<tr>
<td>Sc-560 + Rofecoxib</td>
<td>COX-1 + COX-2 inhibitors</td>
<td>20.0 ± 1.7</td>
</tr>
<tr>
<td>SQ29548</td>
<td>TPR antagonist</td>
<td>50.9 ± 4.0</td>
</tr>
<tr>
<td>AL8810</td>
<td>FPR antagonist</td>
<td>35.2 ± 5.7</td>
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<tr>
<td>Losartan + SQ29548</td>
<td>AT₁-R antagonist</td>
<td>37.0 ± 4.0</td>
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<td>SQ29548 + AL8810</td>
<td>TPR antagonist</td>
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Values represent the residual % basal internal anal sphincter (IAS) tone (means ± SE) following the maximal effect of different inhibitors given either individually or in different combinations. Data given in RESULTS represent the absolute decrease in the IAS tone following these treatments. ACE, angiotensin-converting enzyme; BPB, 4-bromophenacylbromide; COX, cyclooxygenase; FPR, prostaglandin F₂α receptor; TPR, TXA₂ receptor.
higher responses in the IAS vs. RSM to either agonist (*P < 0.05; n = 6–8; Fig. 3, C and D). Once again, a more significant increase in IAS tone is seen with stimulation of the AA pathway (U46619).

Relative distribution of renin, ACE, AT₁-R, COX-1, COX-2, TPR, and FPR in human IAS vs. RSM SMCs. Immunocytochemistry analysis demonstrated significantly higher IFI/unit area of the IAS vs. RSM SMCs for renin, ACE, and AT₁-R (*P < 0.05; n = 4; Fig. 4, top). Similar data were obtained with the IIF for COX-1, COX-2, TPR, and FPR in the IAS vs. RSM SMCs (*P < 0.05; n = 4; Fig. 4, bottom).

Likewise, Western blot data revealed significantly higher expression (*P < 0.05; n = 4) of renin, ACE, and AT₁-R (Fig. 5A) and of COX-1, COX-2, TPR, and FPR (Fig. 5B) in the IAS vs. RSM.

These data insinuate a more significant role of the RAS and AA pathways in the mechanics of IAS compared with RSM. Influence of rho kinase inhibition on the contractile effect of ANG II, U46619, and PGF₂α. Y27632 (ROCK inhibitor) (0.1 μM) significantly attenuated increase in the IAS tone caused by ANG II, U46619, and PGF₂α (*P < 0.05; n = 8; Fig. 6, A–C). These data suggest the role of RhoA/ROCK acting as a common mediator in the increase in IAS tone by these agonists.

Protein expression of RhoA/ROCK II in response to ANG II, U46619, and PGF₂α stimulation in human IAS. Western blot data revealed that ANG II, U46619, and PGF₂α significantly increased the expression of both RhoA (Fig. 7A) and ROCK II (Fig. 7B) in the particulate compared with the cytosolic fractions. This increase was attenuated by pretreatment of the cells with Y27632 (*P < 0.05; n = 5).

These data provide further support for the role of RhoA/ROCK in the mediation of basal tone via both the RAS and AA pathways. Translocations of RhoA/ROCK II to the membrane (captured in particulate fractions) are known markers for the activation of RhoA/ROCK pathway (25, 38).

Data further revealed that the increase in p-MYPT1 and p-MLC₂₀ levels were significantly attenuated by Y27632 (*P < 0.05; n = 4). [MYPT1 is the direct downstream target of activated ROCK causing MYPT1 phosphorylation (42)]. Together, these data support the role of RhoA/ROCK in the mediation of basal tone via RAS and AA pathways.

**DISCUSSION**

For the first time (regardless of the species), our study has been able to demonstrate that the end products of AA and RAS pathways, TXA₂, PGF₂α, and ANG II, provide extracellular signals for the activation RhoA/ROCK for the maintenance of the basal tone in the IAS. The studies have direct relevance in the human pathophysiology of the IAS because they were conducted in human tissues. Data demonstrate that myogenic tone in intact human IAS is regulated via the autocrine control of AA (~80%; via thromboxane A₂ and PGF₂α) and RAS (~20%; via ANG II) pathways. TXA₂, PGF₂α, and ANG II are synthesized within the IAS SMCs and subsequently released, resulting in the activation of their respective GPCRs TPR, FPR, and AT₁-R. Such activation leads to the stimulation of RhoA/ROCK, causing increase in p-MYPT1 and p-MLC₂₀ that is ultimately responsible for the basal IAS tone.

It is noteworthy that, although the overall control mechanism for the human IAS tone is similar to that in rats (primarily via AA vs. RAS) (15, 17), certain differences exist. According to the previously published studies, rat IAS tone is primarily mediated via TXA₂. In those studies, SQ29548 (TPR antagonist) was more efficacious than AL-8810 (FPR antagonist) in reducing the basal IAS tone (15). Conversely, in humans, the
IAS tone is regulated predominantly via PGF\textsubscript{2\alpha} generation and FPR activation. It is evident from the present data that the FPR antagonist AL-8810 is more efficacious than the TPR antagonist SQ29548 in producing decrease in the IAS tone. Additionally, in contrast with rat IAS studies (17, 18), there are significant differences in the nature of PLA\textsubscript{2} and COX in the relative contribution of basal tone in human IAS.

Although the exact nature of PLA\textsubscript{2} and COX in the human IAS tone is not known, it appears that rather than a single isoform [unlike the secreted isoform of PLA\textsubscript{2} (sPLA\textsubscript{2}) and COX-1 in the case of rat], in human IAS multiple subtypes of PLA\textsubscript{2} and COX may be involved. Not shown, in human IAS sPLA\textsubscript{2}-specific inhibitor MJ33 in contrast with the rat studies (18) produces only \sim 15% whereas nonselective PLA\textsubscript{2} inhibitor BPB produces 67% decrease in the tone. Likewise, in contrast with the limited effect of the individual use of COX-1 and COX-2 inhibitors, their combined use and the dual COX inhibitor indomethacin produces a profound decrease in IAS tone in humans.

The conclusion for the AA and RAS regulation of the basal tone in human IAS is based on the presence of biosynthetic machinery for these pathways, the effects of their end products (prostanoids TXA\textsubscript{2} and PGF\textsubscript{2\alpha} and ANG II, respectively, and their mechanistic convergence on the common RhoA/ROCK signal transduction (43). Finally, selective inhibition of the respective pathways causes corresponding decrease in the basal tone in intact human IAS.

Immunocytochemistry and Western blot studies reveal discrete presence of the biosynthetic machinery for both RAS (renin, ACE, and AT\textsubscript{1}-R) and AA (COX-1,2, TPR, and FPR) in the human IAS. Functional data reveal that natural ligands of RAS (ANG II) and AA (TXA\textsubscript{2} and PGF\textsubscript{2\alpha}) produce concentration-dependent increase in the IAS tone, selectively antagonized by their respective antagonists. These data, combined

**Fig. 6.** Data show that ROCK inhibitor Y27632 causes concentration-dependent and significant inhibition (*\textit{P} < 0.05; \textit{n} = 8) of increases in the IAS tone produced by ANG II (A), U46619 (B), and PGF\textsubscript{2\alpha} (C).

**Fig. 7.** Western blots and corresponding quantitative data show that ROCK inhibitor Y27632 (Y2) significantly inhibits (*\textit{P} < 0.05; \textit{n} = 5) increases in expressions of RhoA (A) and ROCK II (B), in the particulate fractions caused by ANG II, U46619 (U4), and PGF\textsubscript{2\alpha}. P, particulate; c, cytosolic; Rel., relative.
with the specific decrease in the IAS tone following the selective inhibition and higher expression of AT1-R, TPR, and FPR and severalfold more potent excitatory effects of their agonists in the IAS vs. RSM, suggest the role of RAS and AA pathways in the basal tone in human IAS. The present data show that PGF2\(_\alpha\)/H9251 and TXA2 contribute to the majority (~80%) of the IAS tone, whereas the contribution by ANG II may be limited to ~20%. This notion is supported by the limited decrease (~20%) in the IAS tone by the RAS inhibitors, compared with ~80% decrease in the IAS tone following AA pathways inhibitors.

The unexpected higher efficacy of H-77 (in contrast with the smaller effect by the inhibition of other components of RAS) on the basal IAS tone may be because of nonselective wide spectrum effect with renin blockade (23). Likewise, limited effects of COX-1 and COX-2 inhibitors given alone in the human IAS tone may be explained on the basis of the cross talk between the two, and possibly by the compensatory upregulation.

**Nature of Extracellular Signal for the Basal Tone in Human IAS**

![Diagram of Extracellular Signal](http:// FontAwesome.com)

Fig. 8. Western blots and corresponding quantitative data show that ANG II, U46619, and PGF2\(_\alpha\), cause significant (*P < 0.05; n = 4) increase in the expressions of p-MYPT1 (A) and p-MLC\(_{20}\) (B) in the IAS, which are significantly (*P < 0.05; n = 4) attenuated by Y27632.

Fig. 9. Model showing that the basal tone in human IAS is mediated via the autocrine control of AA (via thromboxane A\(_2\) and PGF2\(_\alpha\)) and RAS (via ANG II) pathways. ANG II is synthesized from angiotensinogen with the involvement of renin and ACE, respectively, inhibited by H77 and captopril. TXA2 and PGF2\(_\alpha\) are synthesized via PLA2 and COX-1 and COX-2. As listed, the inhibitors of the AA pathways along different steps are BPB, Sc-560, rofecoxib, indomethacin, SQ2958, and AL1180. Following their release, TXA2 and PGF2\(_\alpha\) activate their respective G protein-coupled receptors (GPCR) TPR, FPR, and AT1-R and recruit their specific G proteins (which in the IAS per se are not presently known). This leads to the activation of Rhoa/ROCK, causing increase in the p-MYPT1 and inhibition of myosin light chain phosphatase. These events lead to increase in p-MLC\(_{20}\), responsible for the basal tone in the IAS, Y27632 as indicated is a well-known inhibitor of ROCK.
tion of one subtype of COX following inhibition of the other. This notion is supported by the profound decrease in the IAS tone following dual COX inhibitor indomethacin and by the combined use of rofecoxib and Sc-560. In further support of this, as stated above, other AA pathway inhibitors (e.g., non-selective PLA2 inhibition) and combined use of TPR and FPR antagonists also produce ~80% decrease in the IAS tone. Together, these data support the concept of a predominant role of AA pathway in the IAS tone. Further decreases in the IAS tone with the combined use of both RAS and AA pathway inhibition (by losartan and SQ29548) suggest that these pathways may lie in parallel.

Functional and Western blot data show that the ROCK-selective inhibitor Y27632 significantly blocks increase in the IAS tone caused by ANG II, U46619, and PGF2α. Evidence for the predominant role of RhoA/ROCK is also supported by the data wherein ANG II, U46619, and PGF2α produce significant displacement of RhoA/ROCK from the cytosol to the membrane. Such translocations of RhoA/ROCK serve as important markers for RhoA/ROCK activation (37, 38). Activation of RhoA/ROCK increases the levels of pThr18/Ser19-MLC20. Since basal IAS tone in humans is predominantly mediated via RhoA/ROCK (43), we surmise that these agents utilize RhoA/ROCK for the basal IAS tone.

In support of the present data, recent studies have reported pathophysiological and therapeutic significance of RAS and AA pathways in the esophageal achalasia (7, 8) and in decreased colonic motility in patients with slow-transit constipation on the basis of lower levels of TXA2, PGF2α, and COX-1 in the colon smooth muscle (11). The data that we have presented here in humans have direct implications in the SMC-targeted, novel, and safe therapy of hyper- and hypotonic IAS. Current treatment for hypotonic IAS diseases is limited to available approaches such as Ca2+-channel blockers, NO donors, and phosphodiesterase inhibitors (35, 49). Similarly, in the case of FI associated with the hypotonic anal sphincter, stimulation of AT1-R, TPR, and FPR provides potential novel therapeutic avenues. Moreover, in case of anorectal motility disorders the distinct advantage of topical anal application of pharmacological agents targeting AA and RAS pathways may provide attractive therapeutic options (30).

In summary, as illustrated in Fig. 9, present data demonstrate that TXA2, PGF2α, and ANG II provide extracellular signals that trigger the activation of RhoA/ROCK for the basal IAS tone in humans. These autocinines in turn activate TPR, FPR, and AT1-R GPCRs followed by recruitment of receptor-specific G proteins. RhoA/ROCK activation via increase in p-MYPT1 leads to the inhibition of myosin light chain phosphatase. This sequence of events causes increase in p-MLC20, responsible for the basal tone in the human IAS. These studies have direct implications on the pathophysiology and smooth muscle-specific therapeutic targeting in a number of rectoanal disorders, which are currently difficult to manage. In support of this concept, a recent study has reported a decrease in mean resting anal pressure (free of cardiovascular side effects) in half of healthy volunteers of African American origin, using topical anal application of 5% captopril (29). It is our expectation that this study’s breakdown and delineation of the pathways leading to basal tone of the internal anal sphincter via the RAS and AA pathways in humans will have real-world implications in the treatment of patients with both hypertonic and hypotonic anorectal diseases.

**REFERENCES**

13. Daniel EE, Crankshaw J, Sarna S. Evidence for the role of angio-
21. Daniel EE, Crankshaw J, Sarna S. Evidence for the role of angio-


Patel CA, Rattan S. Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle. Am J Physiol Gastrointest Liver Physiol 291: G830–G837, 2006.


