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S. Rattan, R. A. Haj and M. A. F. De Godoy
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Involvement of Rho and Rho-Associated Kinase in Sphincteric Smooth Muscle Contraction by Angiotensin II

S. Rattan, R. N. Puri and Y.-P. Fan
Experimental Biology and Medicine, September 1, 2003; 228 (8): 972-981.

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Role of pp60c-src and p44/42 MAPK in ANG II-induced contraction of rat tonic gastrointestinal smooth muscles

R. N. Puri, Y.-P. Fan and S. Rattan
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Animal model for angiotensin II effects in the internal anal sphincter smooth muscle: mechanism of action

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Animal model for angiotensin II effects in the internal anal sphincter smooth muscle: mechanism of action. *Am J Physiol Gastrointest Liver Physiol* 282: G461–G469, 2002; 10.1152/ajpgi.00207.2001.—Effect of ANG II was investigated in *in vitro* smooth muscle strips and in isolated smooth muscle cells (SMC). Among different species, rat internal and sphincter (IAS) smooth muscle showed significant and reproducible contraction that remained unmodified by different neurohumoral inhibitors. The AT₁ antagonist losartan but not AT₂ antagonist PD-123319 antagonized ANG II-induced contraction of the IAS smooth muscle and SMC. ANG II-induced contraction of rat IAS smooth muscle and SMC was attenuated by tyrosine kinase inhibitors genistein and typhostin, protein kinase C (PKC) inhibitor H-7, Ca²⁺ channel blocker nifedipine, Rho kinase inhibitor Y-27632 or p^{44/42} mitogen-activating protein kinase (MAPK^{44/42}) inhibitor PD-98059. Combinations of nifedipine and H-7, Y-27632, and PD-98059 caused further attenuation of the ANG II effects. Western blot analyses revealed the presence of both AT₁ and AT₂ receptors. We conclude that ANG II causes contraction of rat IAS smooth muscle by the activation of AT₁ receptors at the SMC and involves multiple intracellular pathways, influx of Ca²⁺, and activation of PKC, Rho kinase, and MAPK^{44/42}.

internal anal sphincter; smooth muscle tone; tyrosine kinase; Rho kinase; mitogen-activating protein kinase; protein kinase C; calcium influx

INTERNAL ANAL SPHINCTER (IAS) smooth muscle tone plays a pivotal role in anorectal continence. IAS smooth muscle tone is primarily myogenic in nature (3, 24), but a number of neurohumoral factors may exert modulatory effects on the tone. However, the knowledge of neurohumoral factors causing a rise in the basal tone of the IAS is very limited.

ANG II is a potent smooth muscle contractile neurohumoral agonist (9, 28). Three main types of ANG II receptors have been described AT₁, AT₂, and AT₄. A majority of ANG II actions is known to be mediated by the specific activation of AT₁. AT₁ receptor is a member of a superfamily of peptide hormone receptors with seven membrane-spanning regions linked to G proteins. Most of the actions of AT₁ receptor are mediated via the activation of phospholipase C that hydrolyzes

phosphatidyl inositol 4,5-bisphosphate to produce inositol 1,4, 5-trisphosphate (IP₃) and diacylglycerol. IP₃ and diacylglycerol in turn lead to increase in the levels of free intracellular Ca²⁺ concentration and activation of protein kinase C (PKC), respectively. In addition, AT₁ receptor activation may lead to influx of Ca²⁺ and activation of tyrosine kinase pathway (9, 26, 27, 29). AT₂ receptors on the other hand have been suggested to attenuate the responses mediated by the AT₁ receptors (8), in different systems. Besides its direct action on the smooth muscle, ANG II may cause smooth muscle contraction by indirect effects by the release of autacoids such as prostaglandins and histamines, activation of sympathetic nerve terminals, nitric oxide synthase pathway, and via the release of endothelins and growth factors (9, 13, 28).

Despite the abundant literature in different smooth muscles, there are limited data on the effects of ANG II in the gastrointestinal smooth muscle that characterizes the mechanism and site(s) of excitatory effect, type of ANG II receptors, and receptor distribution (1, 9). As it relates to the tonic smooth muscle of the gastrointestinal tract, studies (19) have been reported primarily in the lower esophageal sphincter (LES). Those studies were primarily limited to the ANG II effects (19) rather than specific information on ANG II receptors and signal transduction mechanisms. Effects and mechanism of action of ANG II in the IAS have not been reported before.

Because ANG II has been shown to exert potent contractile effects in different smooth muscles, it was considered important to investigate ANG II actions in the IAS. The IAS smooth muscle tone, like that in the LES, is primarily myogenic in nature but can be modulated by the neurohumoral factors (3, 10–12). The IAS has significant importance in the pathophysiology of a number of motility disorders such as anorectal incontinence and severe constipation (15, 23). Anorectal incontinent patients could benefit from agents that cause selective increase in the IAS tone. The studies were therefore designed to compare the effects of ANG II in the IAS of different species and then to pursue, in depth, the site and mechanism of action of ANG II and

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relative distribution of AT₁ versus AT₂ receptors in the species that showed pronounced and reproducible effects.

MATERIALS AND METHODS

Preparation of smooth muscle strips. Adult Sprague-Dawley rats (weighing ~250 g) of either sex were used for the study. Some studies were also carried out in opossums (*Didelphis virginiana*) (weighing from 2 to 3.5 kg) and New Zealand rabbits (weighing from 2.5 to 3.5 kg). All experimental procedures in animals were carried out in accordance with the approved standards described in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, 86-23, 1985. Animals were anaesthetized with pentobarbital sodium (50 mg/kg ip) followed by laparotomy. Studies focused primarily on the IAS, the lower-most smooth muscle of the gastrointestinal tract. Some studies were also carried out in the smooth muscles of the distal colon adjacent to the IAS (rectum), the LES, and the adjacent esophageal body. The isolated organs were transferred immediately to oxygenated (95% O₂-5% CO₂) Krebs solution of the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose. The respective smooth muscle tissues were carefully freed of the adjoining nonsmooth muscle tissues and other extraneous structures, opened, and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs solution. Mucosal and submucosal layers were removed by sharp dissection, and circular smooth muscle strips (~1 × 10 mm) of esophageal body, LES, IAS, and rectal circular smooth muscle were prepared for the recording of isometric tension as described previously (5).

Measurement of isometric tension. Different smooth muscle strips prepared above were secured at both ends with silk sutures and transferred to 2-ml muscle baths containing oxygenated Krebs solution at 37°C. One end of the muscle strip was anchored at the bottom of the muscle bath and the other attached to the force transducer (model FT03; Grass Instruments, Quincy, MA). Isometric tension was measured using computerized PowerLab recorder (CB Sciences, Milford, MA). The muscle strips were initially stretched with 10 mN of force and then allowed to equilibrate for at least 1 h with regular washing at 20-min intervals. Only the strips that developed a spontaneous and steady tone and relaxed in response to electrical field stimulation were considered for the studies on the IAS and LES. Optimal length of each smooth muscle was determined in the beginning of the experiment, and baseline to measure the active tone of the smooth muscle strips was determined at the end of each experiment, as described previously (5, 18).

Effects of different concentrations of agonists on the isometric tension of each smooth muscle strip were examined in a cumulative manner before and after the addition of different antagonists. Each smooth muscle served as its own control. The antagonists were added 15 min before repeating the concentration-response curve. Concentrations of different antagonists used have been previously determined to be selective in blocking their respective actions or pathways. At the conclusion of each experiment, the smooth muscle strips were carefully freed from the suture material, blotted dry, and weighed accurately. Isometric tension was expressed either on absolute basis in gram or as millinewtons, and smooth muscle contractions were expressed as percent maximal contraction obtained with 1 × 10⁻⁴ M bethanechol (5).

Isolation of the smooth muscle cells. IAS smooth muscle cells (SMCs) were isolated as described before (6). Briefly, the smooth muscle tissues were cut into small pieces (1-mm cubes) and incubated in oxygenated Krebs solution containing 0.1% collagenase (CLS II, 217 U/mg dry weight), 0.01% soybean trypsin inhibitor, and mixtures of amino acids and multivitamins at 31°C for two successive 60-min periods. Partially digested tissue pieces were then filtered through a 500-μm Nitex mesh. The tissues trapped on the mesh were rinsed with 5-ml collagenase-free Krebs solution and incubated for another 30 min in oxygenated Krebs solution to disperse the SMCs. The SMCs were harvested by filtration through a 500-μm Nitex mesh, centrifuged at 350 g for 10 min, and resuspended to obtain about 3,000 to 4,000 cells/mm³.

Measurement of changes in SMC lengths by scanning micrometry. Lengths of the isolated SMC were measured by scanning micrometry as described previously (6). Aliquots (30 μl) of the rat IAS-SMC were treated either with bethanechol (1 × 10⁻⁴ M) or ANG II (1 × 10⁻⁷ M) for 5 min followed by their fixation with 0.1% acrolein. The mean length of 30 SMC at random was determined by micrometry using phase contrast microscopy, and the results were calculated as percentage of control cell length or shortening. To examine the effects of the specific antagonists on the ANG II-induced contraction of SMC, the inhibitors were added 15 min before the addition of different concentrations of ANG II.

Western blot analyses. Western blot analyses to determine the relative distribution of AT₁ and AT₂ receptors were performed following the approach previously described in our laboratory (5). Respective tissues were cut into small pieces, rapidly homogenized in five volumes of boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4), and then microwaved for 10 s. The homogenates were centrifuged (16,000 g, 4°C) for 15 min, and the protein contents in resultant supernatants were determined by the method of Lowry et al. (17) using BSA as the standard. All of the samples were mixed with 2× sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol) and placed in a boiling water bath for 3 min. An aliquot (of 20 μl containing 40 μg protein) of each sample was separated by 7.5% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane at 4°C. To block nonspecific antibody binding, the nitrocellulose membrane was soaked overnight at 4°C in Tris-buffered saline Tween (TBS-T; composed of: 20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 1% BSA. The nitrocellulose membranes were then incubated with the specific primary antibodies (1:1,000 for AT₁) or anti-sera to AT₂ receptor (raised in rabbit, 1:2,000), for 1 h at room temperature. After washing with TBS-T, the membranes were incubated with the horseradish peroxidase labeled-secondary antibody (donkey anti-rabbit IgG, 1:1,500 dilution) for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substrate using a Western blot detection kit and Hyperfilm MP (Amersham Life Science). Bands corresponding to different proteins on X-ray films were scanned (SnapScan.310; Agfa, Ridgefield Park, NJ) and their relative densities determined by using Image-Pro Plus 4.0 software (Media Cybernetics; Silver Spring, MD).

Drugs and chemicals. The following chemicals were used in the study: ANG II, H-7, tyrphostin B46, and Rho kinase inhibitor HA-1077 (20) (Calbiochem, San Diego, CA); losartan (AT₁ antagonist) (gift from Merck, Rahway, NJ); PD-123319 ditrifluoroacetate (AT₂ antagonist), prazosin, atropine, hexamethonium, tetrodotoxin (TTX), ω-conotoxin GVIA, N^G-

nitro-L-arginine methyl ester, indomethacin, nicardipine, collagenase, soybean trypsin inhibitor (Sigma, St. Louis, MO); cimetidine (Smith Kline, King of Prussia, PA); methysergide maleate (Sandoz Research Institute, East Hanover, NJ); genistein (RBI, Natick, MA); PD-98059, (BioMol, Plymouth Meeting, PA); Y-27632 (another potent and specific inhibitor of Rho kinase; was a gift from Yoshitomi Pharmaceutical Industries, Osaka, Japan); polyclonal antibody to ANG II receptor 1 (AT₁) (Santa Cruz Biotechnology, Santa Cruz, CA); and antiserum for AT₂ receptor was kindly provided by Dr. Andrew S. Greene (Department of Physiology, Medical College of Wisconsin, Milwaukee, WI).

Data analysis. Data were expressed as means \pm SE of different experiments. The rise in the basal tension was computed in reference to percent maximal (100%) withbethanechol (1×10^{-4} M). All the absolute values (expressed as either g or mN) and percentile increases in basal IAS tension were in reference to zero line determined with EGTA at the conclusion of each experiment. The statistical significance between different groups was determined by two-way ANOVA or by Student's paired and unpaired *t*-test where appropriate. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of ANG II on rat basal tone of IAS smooth muscle. ANG II caused a concentration-dependent rise in the basal tone of rat IAS (Fig. 1). Maximal increase

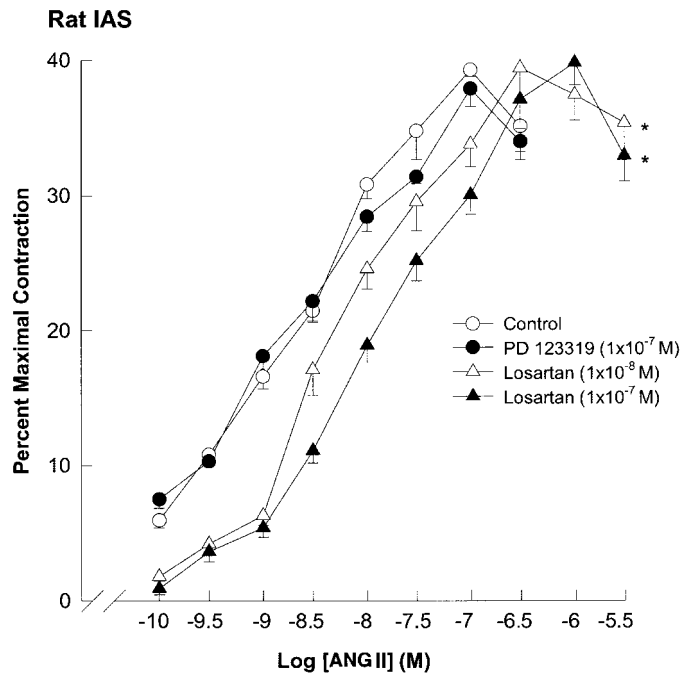


Fig. 1. Concentration-response curves showing the contractile effect of ANG II in the rat internal and sphincter (IAS) smooth muscle before and after treatment with the AT₂ receptor antagonist PD-123319 and AT₁ receptor antagonist losartan. Effects of ANG II in the basal IAS tone were calculated on the basis of %maximal contraction with 1×10^{-4} M bethanechol. The AT₁ receptor antagonist caused significant and concentration-dependent rightward shifts in the control concentration-response curve obtained with ANG II alone ($*P < 0.05$; $n = 5-8$). PD-123319 on the other hand had no significant effect on the increase in the IAS smooth muscle tone by ANG II ($P > 0.05$; $n = 5-8$). All values in this and the subsequent graphs represent mean \pm SE of different observations.

in rat IAS tone of $39.3 \pm 0.5\%$ was observed with 1×10^{-7} M. The threshold concentration of ANG II was 1×10^{-10} M that caused an increase in the basal IAS tone of $5.9 \pm 0.6\%$. A typical tracing to exemplify the effect of ANG II in the rat versus the rabbit and opossum IAS has been shown in Fig. 2. The IAS smooth muscle was found to be distinctly sensitive to ANG II because the smooth muscle strips prepared from the distal colon adjacent to the IAS (rectal smooth muscle) elicited either no or a relatively poor contraction. Likewise, in the case of LES, the smooth muscles of opossum displayed maximal contraction with ANG II followed by that in the rat. To the contrary, the rabbit LES showed no significant response to ANG II. The esophageal body of different species examined including opossum produced limited or no contraction with ANG II (data not included).

Effect of selective antagonists of AT₁ and AT₂ receptors on ANG II-induced contraction of rat IAS smooth muscle. The AT₂ receptor antagonist PD-123319 caused no significant modification of concentration-response curve showing rise in the basal tone of the IAS smooth muscle by ANG II ($P > 0.05$; $n = 5$ to 7; Fig. 1). On the other hand, AT₁ receptor antagonist losartan caused a concentration-dependent (1×10^{-8} and 1×10^{-7} M) rightward and significant shifts in the ANG II concentration-response curve ($P < 0.05$; $n = 5-8$).

Influence of cholinergic and adrenergic antagonists, TTX, and ω -conotoxin on ANG II-induced rise in the IAS smooth muscle basal tone. ANG II-induced contraction of IAS smooth muscle was not significantly modified by the neurotoxins TTX and ω -conotoxin, and cholinergic antagonist atropine (1×10^{-6} M) ($P > 0.05$; Fig. 3; $n = 5-8$). The contraction, however, was partially inhibited by α_1 -adrenoceptor antagonist prazosin (1×10^{-5} M) ($n = 5-8$). We also examined the influence of antagonists of other neurohumoral substances such as histamine (combination of pyrilamine and cimetidine), 5-hydroxytryptamine (methysergide), and prostaglandins (indomethacin) on ANG II-induced contraction of the IAS smooth muscle. None of those neurohumoral antagonists modified significantly the effect of ANG II. In these series of experiments, ANG II-induced contraction of rat IAS smooth muscle in control was 35.6 ± 3.2 and after the addition of these inhibitors was 29.5 ± 2.8 , 32.5 ± 2.6 , $33.9 \pm 3.9\%$, respectively ($P > 0.05$; $n = 5-8$). Data suggest that the contractile action of ANG II in the IAS smooth muscle is largely via its direct action on the SMCs.

Influence of Ca²⁺ influx, PKC, tyrosine kinase, and Rho kinase inhibitors on the contractile responses of ANG II. Contractile action of ANG II in the IAS smooth muscle was significantly attenuated by the inhibitors of L-type Ca²⁺-channels (nicardipine), PKC (H-7), tyrosine kinase (genistein), and Rho kinase (and Y-27632) ($P < 0.05$; $n = 5-8$; Fig. 4). ANG II-induced contraction of the IAS smooth muscle was also significantly attenuated by other inhibitors of PKC, tyrosine kinase, Rho kinase, calphostin C (1×10^{-6} M), tyrphostin B46 (1×10^{-5} M), and HA-1077

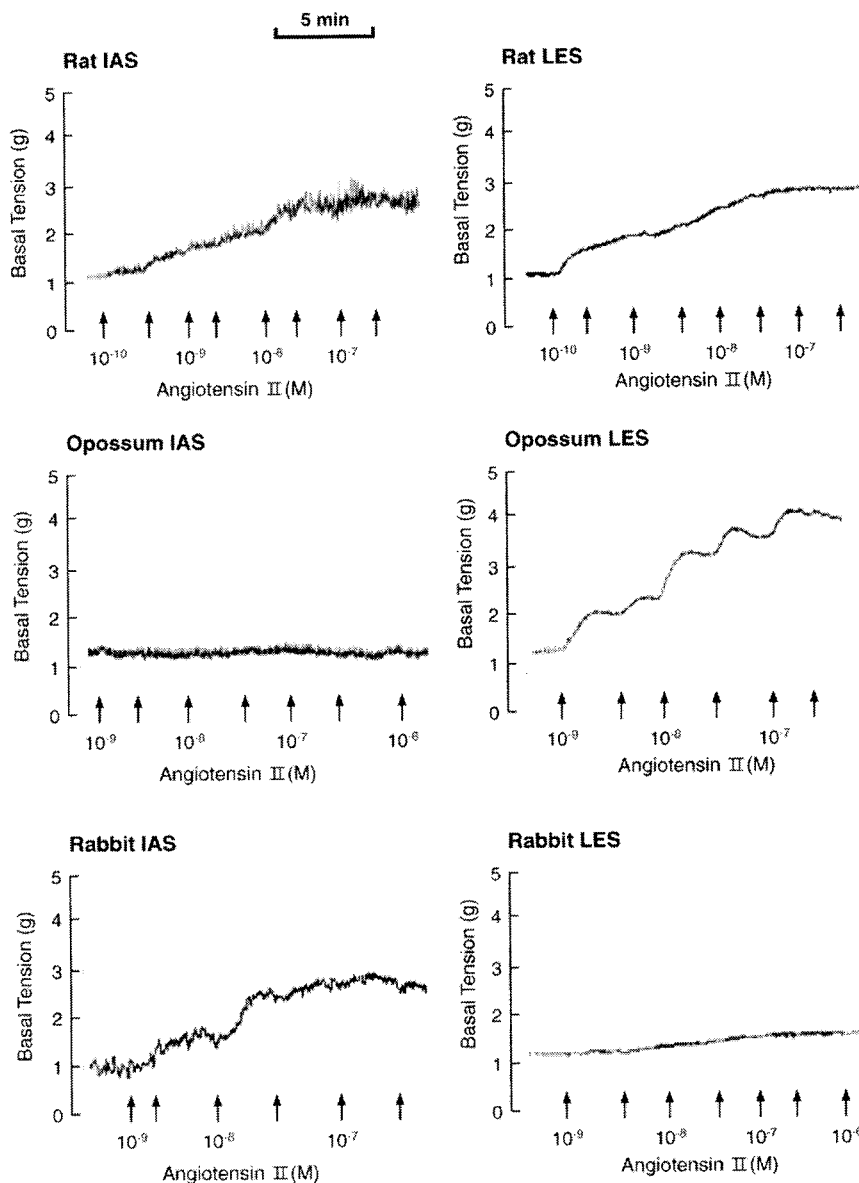


Fig. 2. Typical tracings showing the effects of ANG II in the rat vs. opossum and rabbit IAS and lower esophageal sphincter (LES) smooth muscles. Note a pronounced effect of ANG II in the rat IAS compared with the other species. ANG II in the opossum IAS caused no significant increase, whereas it had a modest effect in the basal tone of the rabbit IAS smooth muscle. *Right*, effects of ANG II in the LES in different species. Note that the opossum showed the maximal contraction, followed by that in the rat, and contraction was negligible in the rabbit.

(1×10^{-6} M), respectively ($P < 0.05$; $n = 5-8$) (data not shown). Tyrosine kinase inhibitors genistein and tyrphostin caused the maximum attenuation of the ANG II-induced contraction of the IAS smooth muscle.

Effect of ANG II on the SMC isolated from IAS. ANG II caused concentration-dependent contraction of the IAS-SMC. This effect was antagonized significantly by the AT_1 antagonist losartan in a concentration-dependent manner ($P < 0.05$; $n = 4$; Fig. 5). The effect of ANG II in the IAS-SMC was not significantly modified by the AT_2 receptor antagonist PD-123319 (data not shown).

Effect of Ca^{2+} channel blocker, PKC, and tyrosine kinase, Rho kinase and $p^{44/42}$ mitogen-activating kinase inhibitors on SMC contraction by ANG II. Maximal shortening of SMC by ANG II was caused by 1×10^{-7} M and was comparable to that induced bybethanechol 1×10^{-4} M (Fig. 5). The shortening of the

IAS-SMC by ANG II was significantly attenuated by the inhibitors of different pathways, namely, Ca^{2+} influx (nicardipine), PKC (H-7), tyrosine kinase (genistein), Rho kinase (HA-1077), and $p^{44/42}$ mitogen-activating kinase (MAPK^{44/42}; PD-98059). ($P < 0.05$; $n = 4$; Fig. 6).

Influence of combined inhibition of different pathways on ANG II-induced contraction of the IAS-SMC. In a separate series of experiments, we also examined the effect of combined antagonism of Ca^{2+} channel, PKC, tyrosine kinase, Ca^{2+} channels, Rho kinase, and MAPK^{44/42}. The combination of H-7 and HA-1077 caused significantly more attenuation of ANG II-induced contraction of IAS smooth muscle, compared with their individual use ($P < 0.05$; $n = 5-8$; Fig. 7). The combination of Rho kinase and MAPK^{44/42} inhibitors also produced significantly higher inhibition of ANG II effects than their individual effect ($P < 0.05$; $n = 5-8$; Fig. 8). However, inhibition of tyrosine kinase

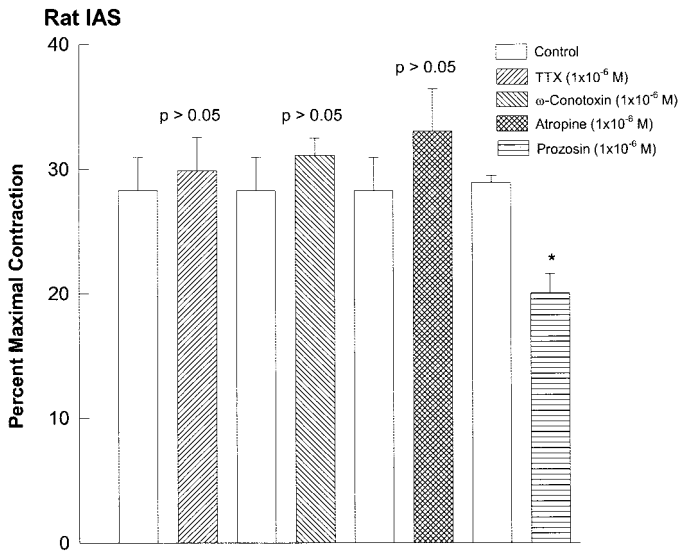


Fig. 3. Influence of neuronal blockade with tetrodotoxin (TTX) and ω-conotoxin, and cholinergic and α₁-adrenoceptor blockade on ANG II-induced contraction in rat IAS smooth muscle. These inhibitors with the exception of α₁-adrenoceptor antagonist prazosin caused no significant attenuation of ANG II-induced contraction of the IAS smooth muscle (*P* > 0.05; *n* = 5–8). Prazosin caused a partial but significant attenuation of ANG II-induced contraction of the IAS smooth muscle (**P* < 0.05; *n* = 5–8).

pathway by genistein in rat IAS caused significantly greater attenuation of ANG II-induced contraction than that of any other pathway. Data suggest a possible link between tyrosine kinase and other pathways.

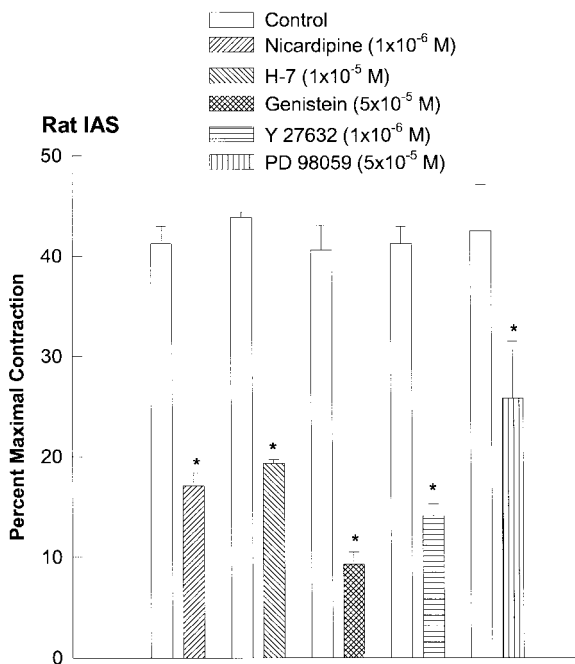


Fig. 4. Influence of L-type Ca²⁺ channel blocker nicardipine, PKC inhibitor H-7, tyrosine kinase inhibitor genistein, Rho kinase inhibitor Y-27632, and p^{44/42} MAP kinase (MAPK^{44/42}) inhibitor PD-98059 on ANG II-induced contraction in rat IAS smooth muscle. These antagonists caused a significant attenuation of increase in the basal IAS tone by ANG II (1 × 10⁻⁷ M) (**P* < 0.05; *n* = 5–8).

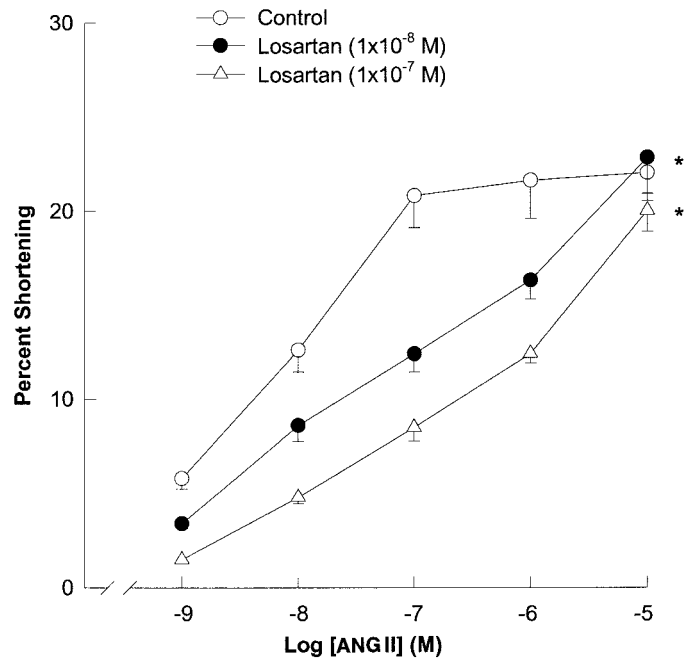


Fig. 5. Concentration-dependent contraction of the rat IAS smooth muscle cells (SMC) by ANG II and antagonism by the specific inhibitor of the AT₁ receptor losartan. Note that ANG II caused concentration-dependent contraction of the SMCs that was antagonized by losartan in a concentration-dependent manner (**P* < 0.05; *n* = 4).

Relative distribution of AT₁ and AT₂ receptors in the anorectal versus esophageal smooth muscles of rat. Immunoblots using specific antibodies show the presence of both AT₁ and AT₂ receptors in different tissues examined (Fig. 9). However, the relative distribution of AT₂ in relation to AT₁ (AT₂/AT₁) was found to be higher in esophageal body (EB) and rectal smooth muscles. The order of calculated relative values of AT₂/AT₁ was as follows: EB > rectum > LES > IAS (being highest in EB). Western blots are shown in Fig. 9A and densitometric analysis in Fig. 9B. Data suggest that the lack of ANG II response in the nontonic tissues of EB and rectal smooth muscles compared with IAS and LES may not be due to the lack of AT₁ receptors.

DISCUSSION

Present studies for the first time report the actions of ANG II in the IAS smooth muscle. Studies show that rat is an appropriate animal model to investigate the effects and mechanism of action of ANG II and antagonists in the IAS smooth muscle. ANG II causes contraction of rat IAS mostly by its action directly at the smooth muscle. Data suggest that the excitatory effect of ANG II in the IAS involves multiple signal transduction pathways.

Effects of ANG II in the tonic gastrointestinal smooth muscle are not only species specific but also tissue specific. Rat IAS was found to be maximally responsive to the contractile actions of ANG II; rabbit IAS showed a modest contraction, whereas opossum IAS showed no response. Furthermore, the distal co-

lonic smooth muscle of rat (rectum) adjacent to the IAS either produces no significant contraction or only a limited contraction by ANG II. Thus rat IAS offers an important model to investigate specific agents (ANG II agonists and antagonists) in the basal tone of the IAS smooth muscle. Agents that cause selective increase or decrease in the basal tone of the IAS smooth muscle are of significance in anorectal incontinence associated with the IAS smooth muscle dysfunction and in the pathophysiology of spastic IAS responsible for severe constipation.

The contractile action of ANG II was determined to be primarily via its action directly at the smooth muscle of the IAS. The excitatory effect of ANG II in the IAS smooth muscle remained intact following pretreatment of the tissues with the neurotoxins TTX and ω -conotoxin and different neurohumoral antagonists. The only neurohumoral antagonist that caused partial attenuation of ANG II effects was the α_1 adrenoceptor-antagonist prazosin. This suggests the involvement of adrenergic nerve terminals activation for a part of the contractile actions of ANG II. Such a partial action of ANG II in the smooth muscle that involves adrenergic nerve terminals has been shown before (4, 13). Lack of effect of neurotoxins and neurohumoral antagonists on ANG II-induced contraction of IAS smooth muscle com-

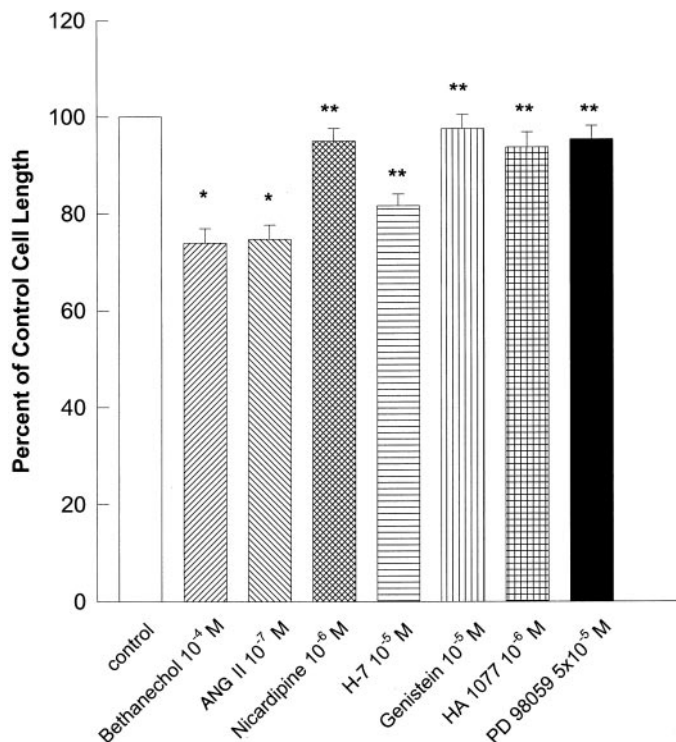


Fig. 6. Influence of different inhibitors on ANG II-induced contraction of rat IAS SMC. ANG II (1×10^{-7} M) caused a significant shortening of the SMC ($*P < 0.05$; $n = 4$) comparable to that by bethanechol (1×10^{-4} M). ANG II-induced shortening of the IAS SMC was attenuated significantly by different agents, nicardipine (inhibitor of L-type Ca^{2+} -channels), H-7 (PKC inhibitor), genistein (tyrosine kinase inhibitor), HA-1077 (Rho kinase inhibitor), and PD-98059 (MAPK^{44/42} inhibitor) ($**P < 0.05$; $n = 4$). However, genistein caused the maximal inhibition of the SMC contraction.

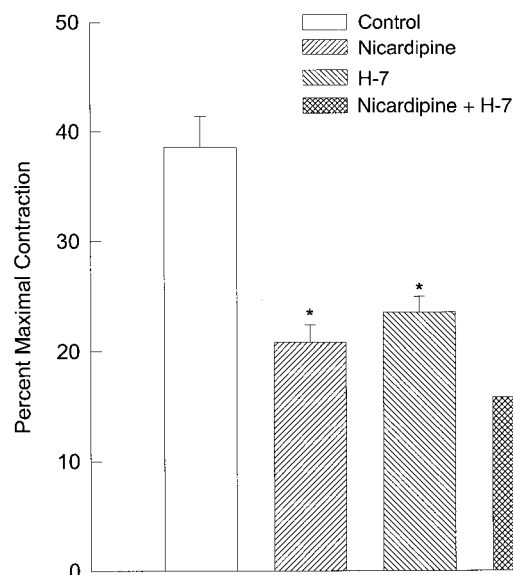


Fig. 7. Influence of Ca^{2+} channel blocker nicardipine and PKC inhibitor H-7 alone, and their combination on ANG II-induced contraction of the IAS smooth muscle. These agents caused significant ($*P < 0.05$; $n = 5-8$) attenuation of ANG II effect. A combination of nicardipine and H-7, however, caused even greater attenuation ($**P < 0.05$; $n = 5-8$) of ANG II-induced contraction of IAS smooth muscle. In these experiments, the IAS smooth muscle contraction caused by ANG II alone, after nicardipine, H-7, and nicardipine plus H-7 was 38.6 ± 2.8 , 20.8 ± 1.6 , 23.5 ± 1.4 , and $15.8 \pm 2.3\%$, respectively.

bined with contraction of the isolated IAS-SMC following ANG II suggests a majority of ANG II to be via its direct action at the smooth muscle.

Contractile action of ANG II in the IAS smooth muscle is due to the activation of AT_1 receptors. This was evident by the selective antagonism of ANG II effects by the AT_1 receptor antagonist losartan in a concentration-dependent manner. The AT_2 receptor antagonist PD-123319 on the other hand had no significant effect on ANG II-induced contraction of the smooth muscle. This was found to be the case both in the studies with smooth muscle strips and the isolated SMC. Interestingly, the sensitivity of AT_1 receptors to losartan in rat IAS was found to be severalfold more than the opossum LES (7). Whether the AT_1 receptors in the rat IAS smooth muscle being highly sensitive to AT_1 antagonists belong to a specific subclass of AT_1 receptors AT_{1A} or AT_{1B} (9, 16) remains to be determined. In addition, the relative density of AT_1 receptors was found to be higher in the rat IAS than the adjoining nonsphincteric smooth muscle of rectum.

It is possible that the net effect of ANG II in a given tissue may not depend entirely on the presence and activation of AT_1 receptors but also on the AT_2 receptors that may otherwise be silent in exerting their independent action. The AT_2 receptors have been shown to suppress the action of the AT_1 receptors (8). It is of interest that in the present study, the smooth muscle tissues examined not only showed the presence of AT_1 but also AT_2 receptors. Actually, the smooth

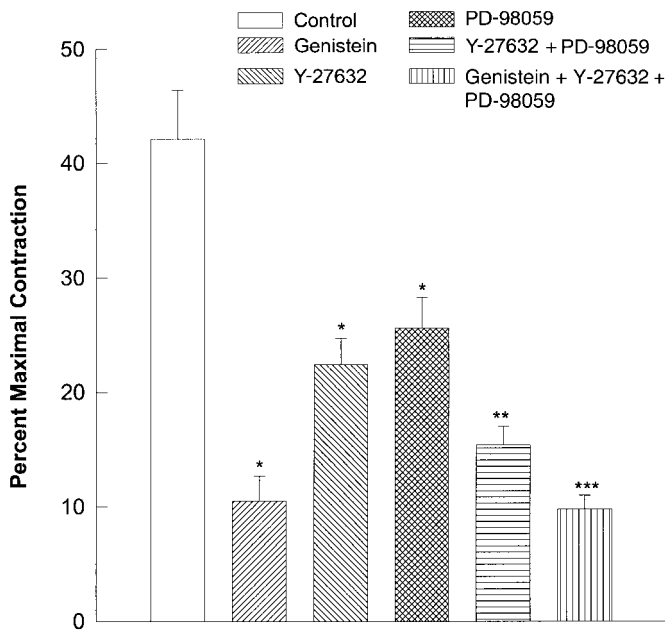


Fig. 8. Influence of tyrosine kinase inhibitor genistein, Rho kinase inhibitor Y-27632, MAPK^{44/42} inhibitor PD-98059 used alone, and their combinations on ANG II-induced contraction of the IAS smooth muscle. These agents caused significant (**P* < 0.05; *n* = 5) attenuation of ANG II effect. The combination of Y-27632 and PD-98059 caused further attenuation (***P* < 0.05; *n* = 5–8). The attenuation was even higher when ANG II effects on IAS smooth muscle were examined in the presence of these agents in combination with genistein (***)*P* < 0.05; *n* = 5–8).

of the AT₂ receptors and their interaction with the AT₁ in the signal transduction mechanisms by ANG II in these smooth muscles remain to be determined.

ANG II-induced contraction of rat IAS smooth muscle involves multiple pathways, i.e., influx of Ca²⁺, and activation of PKC, MAPK^{44/42}, and Rho kinase. Antagonism of these pathways by their selective and respective inhibitors caused a partial attenuation of the IAS smooth muscle contraction by ANG II. An increase in the concentration of any of the inhibition caused no further attenuation of ANG II-induced smooth muscle contraction. The combination of the Ca²⁺-channel blockade with PKC inhibition and MAPK^{44/42} with Rho kinase inhibition by PD-98059 and Y-27632, respectively, caused a small but significant increase in the attenuation of ANG II effects compared with the individual use of the inhibitors. Presently, the exact significance of these findings is not known.

There is an abundance of literature in different systems to suggest that an early upstream activation of tyrosine kinase is involved in ANG II-induced activation of MAPK^{44/42} and Rho kinase (2, 14, 21, 22, 25, 28). Whether such an upstream regulation of tyrosine kinase is involved in the IAS smooth muscle is difficult to ascertain from the present studies. However, in present studies, inhibition of tyrosine kinase produced a greater attenuation of ANG II effects than the individual or combined inhibition of different pathways. Actually, tyrosine kinase inhibition nearly obliterated the effect of ANG II in the IAS smooth muscle. Furthermore, this association between the pathways leading to tyrosine kinase activation and other pathways in the rat IAS smooth muscle is in agreement with the

muscles that respond poorly had a higher ratio of AT₂/AT₁ receptors, suggesting relatively higher levels of AT₂ receptors in such tissues. The exact significance

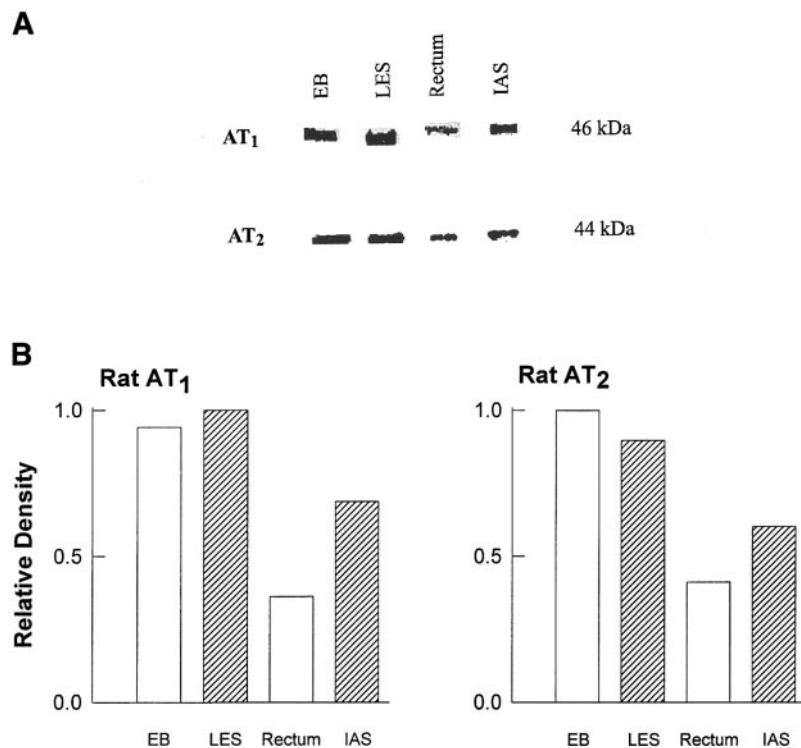


Fig. 9. A: Western blots showing the relative distribution of AT₁ and AT₂ receptors in the rat IAS and the rectal smooth muscle vs. that in the esophageal body (EB) and LES. B: densitometric analyses showing quantitative and relative distribution of AT₁ and AT₂ receptors in the esophageal and anorectal tissues. Not only AT₁ but also AT₂ receptors were present in the tissues examined. The order of calculated ratios of AT₂/AT₁ in different tissues were as follows: EB > rectum > LES > IAS (being highest in the EB).

literature cited above but was not found to be the case in the opossum LES (7). In the opossum LES, tyrosine kinase inhibitor genistein had no significant effect on ANG II-induced contraction. Whether there is an upstream regulation of Ca^{2+} influx, PKC, MAPK^{44/42}, and Rho kinase activation by tyrosine kinase, these pathways are completely independent, or there is any cross talk between different pathways for ANG II-induced contraction of the IAS smooth muscle, is not currently known.

The multiplicity of intracellular pathways by agonists like ANG II suggests alternative cellular mechanisms for the maintenance of the tonic smooth muscle contraction (in the basal as well as stimulated state). This may render the tonic smooth muscle adaptable under certain pathophysiological conditions. It is possible that in the acute setting (similar to that presented in studies here), each pathway plays a significant role in the mediation of ANG II effects, and the absence of one may be compensated by other pathway(s).

A lack of reproducible effect of ANG II in the IAS of species other than rat is of significant interest. This may not be simply because of the lack of AT₁ receptors in these species because such receptors were shown to be present there. This, however, may be related to relatively higher levels of AT₂ (reflected in the AT₂/AT₁ ratios) compared with AT₁ receptors. It is well known that AT₂ receptors, otherwise silent in causing the IAS smooth muscle contraction, may suppress the functional expression of AT₁ receptors. Further studies are needed to resolve this issue. Another explanation for the species differences in the actions of ANG II may be the differences in signal transduction machinery linked to AT₁ receptor of IAS SMCs of different species.

In summary, we conclude that rat IAS provides an excellent model to pursue the investigation of ANG II for the following aspects: characterization of ANG II receptor types, role of ANG II in the pathophysiology and therapeutic potentials of anorectal motility disorders, signal transduction cascade for the IAS smooth muscle contraction; and determination of the significance of AT₂ receptors in gastrointestinal smooth muscle.

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