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Angiotensin II-Induced Relaxation of Anococcygeus Smooth Muscle via Desensitization of AT_1 Receptor, and AT_2-Receptor-Mediated Relaxation by NOS Pathway

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Running Title: Bimodal Effect of Ang II

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Abbreviations: ASM, anococcygeus smooth muscle; APA, aminopeptidase A; APN, aminopeptidase N; BSA, bovine serum albumin; CRC, concentration response curve; E_{max}, maximal contractile effect; EC_{50}, concentration causing 50% of maximal contractile effect; IC_{50}, concentration causing 50% of maximal inhibitory effect; I_{max}, maximal inhibitory effect; NCM, nitrocellulose membrane; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; NCM, nitrocellulose membrane; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; pD_2, $-logEC_{50}$; $pIC_{50} = -logIC_{50}$; PD123,319, AT_2 receptor antagonist; sGC, soluble guanylate cyclase; SMC, smooth muscle cell; SQ22536, [9-(tetrahydro-2'-furyl)adenine]; TBS-T, Tris-buffered saline Tween; TTX, tetrodotoxin.

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ABSTRACT

We evaluated the role of receptor desensitization, activation of AT_2 receptors, and enzymatic degradation of Ang II by amino/neutral endopeptidases in the rat anococcygeus smooth muscle (ASM) relaxation. Ang II (0.3 nM to 10 μM) produced contractions (E_{max}=21.50 ± 5.73 %) followed by passive relaxations (E_{max} reduced to 9.08 ± 2.55 %). Contractions were inhibited (E_{max}=13.67 ± 2.03 %) by losartan (0.1 μM, AT_1 antagonist) but not by PD123,319 (0.1 μM, AT_2 antagonist). Conversely, the passive relaxation was inhibited (E_{max}=18.00 ± 3.45 %) by PD123,319 (0.1 μM) but not by losartan. Ang II (0.3 μM to 100 μM) produced initial contractions (E_{max}=11.49 ± 9.39 %) followed by active relaxations (I_{max}=47.85 ± 4.23 %) on strips pre-contracted by bethanechol (100 μM). A second administration of Ang II on the background of bethanechol (1 h later) resulted in stronger relaxations (I_{max}=64.03 ± 5.47 %) without the initial contractions. L-NAME (NOS inhibitor), ODQ (guanylate cyclase inhibitor), PD123,319, and TTX (neurotoxin) inhibited the relaxations. The presence of AT_1 and AT_2 receptors was confirmed by Western Blot. Experiments with amastatin (1 μM) and thiorphan (1 μM), aminopeptidase and neutral endopeptidase inhibitors respectively, excluded the involvement of enzymatic degradation in Ang II-induced relaxation of ASM. In conclusion, the rat ASM relaxation by Ang II is because of active and passive relaxations. The passive relaxation depends on desensitization of excitatory AT_1 receptors, and the active relaxation is mediated by stimulation of AT_2 receptors and activation the nNOS/sGC pathway.

Keywords: Ang II, anococcygeus smooth muscle, AT_1 receptor, AT_2 receptor.

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Angiotensin II (Ang II) is the effector peptide of the renin-angiotensin system, which plays a major role in the regulation of smooth muscle tone. In its target cells, Ang II binds to different subtypes of G protein-coupled receptors, named as AT_1 and AT_2 (De Gasparo et al., 2000). Most of the actions of Ang II have been attributed to stimulation of the AT_1 receptor whose effects are frequently subject to tachyphylaxis or desensitisation. Tachyphylaxis is defined as the acute loss of response of certain smooth muscles upon repeated application of the agonist (Motta et al., 2003).

In our preparation, in the repeat experiments, Ang II produces reduced contraction and augmented relaxation. Ang II-induced relaxation in the rat anococcygeus smooth muscle (ASM) may be explained on the basis of the AT_1 receptor desensitisation due to 1) uncoupling from the G protein, 2) sequestration of receptors into endosomal vesicles, and 3) down-regulation of the total receptor number of a cell (Lohse, 1993). Besides all the valuable information on the mechanisms related to desensitisation of the AT_1 subtype, most of the studies regarding tachyphylaxis to Ang II in smooth muscles did not investigate alternative events such as the brake of the AT_1-triggered signal by activation of the AT_2 subtype and the enzymatic degradation of Ang II by local enzymes.

Early studies have suggested that activation of the AT_2 subtype counteracts the effects of AT_1 activation in different tissues (Nouet and Nahmias, 2000; De Godoy and De Oliveira, 2002; Rattan et al., 2002) via the release of inhibitory autacoids such as nitric oxide (NO) (Siragy and Carey, 1999; Israel et al., 2000).

Local enzymatic degradation of Ang II has been described in different organ systems (Ardaillou and Chansel, 1997). Ang II can be hydrolyzed both at its ‘N’ and ‘C’ terminals. The main enzymes responsible for these reactions are the aminopeptidases A (APA) and N (APN),

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and the neutral endopeptidase (NEP). APA acts on Ang II to produce Ang III that also exerts its effects via Ang II receptors (Devynck and Meyer, 1978). APN converts Ang III into the hexapeptide Ang IV (Ang II (3-8)) that is relatively devoid of biological activity (Bennet and Snyder, 1976). NEP converts Ang II to Ang (1-7), which now is believed to display effects distinct from those of Ang II and sometimes even opposite, by activating the Ang (1-7) receptor (Santos et al., 1994).

The rat anococcygeus smooth muscle (ASM) representing different smooth muscles including the gastrointestinal tract has been widely used to investigate the basic mechanisms underlying smooth muscle contraction-relaxation, and the nature of inhibitory neurotransmitters, (Gibson and McFadzean, 2001). In the rat ASM, Ang II has been shown to cause contraction via AT_1 receptor activation while AT_2 receptor leads to inhibition (De Godoy and De Oliveira, 2002).

Present studies showed that ASM displays bimodal effect, contraction in the lower concentrations and relaxation in response to Ang II at supramaximal concentrations (<0.1 μM). Because Ang II plays an important role in smooth muscle tone, the present studies were designed to investigate the mechanisms underlying the bimodal effect of Ang II in the smooth muscle. In that process, we investigated the role of AT_1 receptor desensitisation, AT_2 receptor activation, and local enzymatic degradation, using rat ASM as the model.

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Materials and Methods

Tissue Preparation. Male Sprague-Dawley rats (300-350g) were sacrificed by decapitation and the ASM removed as previously described (Gillespie, 1972)(De Godoy and De Oliveira, 2002). The tissues were then transferred to oxygenated (95% O_2/ 5% CO_2) Krebs' physiological solution of the following composition (in mM): 118.1 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.16 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.1 mM glucose at 37ºC. The studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Measurement of Isometric Tension. The smooth muscle strips (10 mm x 1 mm) were transferred to 2 ml muscle baths containing oxygenated Krebs' solution at 37^{o}C. One end of the muscle strip was anchored at the bottom of the muscle bath while the other end was connected to a force transducer (model FT03; Grass Instruments, Quincy, MA). Isometric tension was measured via the PowerLab/8SP data acquisition system (AD Instruments, Australia) and recorded using Chart 4.1.2 (AD Instruments, Australia). Each smooth muscle strip was initially stretched to a tension of 1 g for optimal force development, followed by an equilibration period of 1 h, in accordance with the previously published work (Gillespie, 1972; Gibson and Pollock, 1973; De Godoy et al., 2003). During this period, the smooth muscles were replenished with Krebs' solution every 20 min.

Drug Responses. Concentration-response curves (CRC) with Ang II and analogues were obtained by adding peptides to the organ bath (0.3 nM to 10 μM) in a cumulative manner as

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explained before (Rathi et al., 2003). Different smooth muscle strips were employed to investigate each peptide to avoid cross interference. To characterize the nature of angiotensin receptors, the tissues were incubated with the selective AT_1 receptor antagonist losartan, the selective AT_2 receptor antagonist PD123,319 (both 0.1 μM), and the selective Ang (1-7) receptor antagonist A-779 (1 μM). Losartan and PD123,319 were added to the organ bath 20 min before administration of the agonists. Shorter incubation of A-779 (5 min) was used to prevent breakdown of the peptide antagonist (Santos et al., 1994). To evaluate the role of local enzymatic degradation on Ang II response, tissues were pre-treated with amastatin (for APA and APN), and thiorphan (for NEP) (both at 1 μM) 20 min before construction of the CRCs. Responses were calculated as percent of maximal contraction by bethanechol (100 μM) added at the end of the experiment.

Active Relaxation. Active relaxation to Ang II was studied by pre-contracting ASM strips with bethanechol (100 μM). When the contraction reached a stable plateau, Ang II (0.3 μM to 100 μM) was administered. Fall in the baseline below the background of bethanechol was considered active relaxation. For this, we first determined the duration of sustained ASM contraction with bethanechol. It was determined to be at least 20 min. Therefore, to examine the inhibitory component of Ang II, studies were completed within this time frame.

To characterize the nature of Ang II receptors in the active relaxation, CRCs were repeated in the presence of losartan (0.1 μM), PD123,319 (0.1 and 0.5 μM), and A-779 (1 μM). Experiments were also repeated in the presence of the neurotoxin, tetrodotoxin (TTX, 1 μM) and the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) to investigate the role of nitrergic inhibitory non-adrenergic, non-cholinergic (NANC) nerve stimulation. The soluble

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guanylate cyclase (sGC) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM), and the selective adenylate cyclase (AC) inhibitor, [9-(tetrahydro-2'-furyl)adenine] (SQ 22536, 1 μM), were also used to examine the role of NOS/sGC pathway. All antagonists/inhibitors were added to the organ bath 20 min before pre-contraction by bethanechol. A-779 was incubated for 5 min before addition of bethanechol to the organ bath to avoid its degradation as explained above. For these experiments, two strips from the same donor rat were used to obtain CRC for Ang II in the absence and presence of the inhibitors. The concentrations of different inhibitors used in this study have been previously shown to be selective in their actions (Fan et al., 2002; Rattan et al., 2002; Sarma et al., 2003; De Godoy and De Oliveira, 2002).

Receptor Desensitization (Passive Relaxation). To determine the contribution of desensitization of the excitatory component (passive relaxation) to relaxation induced by Ang II, a second CRC by Ang II was repeated 1 h following the first CRC in the same tissues. This experiment was repeated in the presence and absence of losartan (0.1 μM), PD123,319 (0.1 and 0.5 μM), L-NAME (100 μM), and TTX (1 μM), all incubated for 20 min before the second pre-contraction by bethanechol (100 μM).

Western Blot Analyses. Western blot studies were performed to determine the relative distribution of AT_1 and AT_2 receptors, and APA, and APN, following the approach previously described in our laboratory (Fan et al., 2002). Iso β-actin expression was used as internal control. The smooth muscle tissues were cut in 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 (16,000g; 4°C) for 15

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min and protein contents in resultant supernatant were determined by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard. The protein samples were immediately aliquoted and stored at -70°C.

The above protein samples were mixed with 2x sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% ß-mercaptoethanol) and placed in boiling water bath for 3 min. Each protein in the sample (20 µl containing 40 µg protein) was separated by 7.5% SDS-polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane (NCM) by electrophoresis at 4°C. To block nonspecific antibody binding, the NCM 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 NCM was then incubated with the specific primary antibodies (rabbit polyclonal IgG, 1:2,000 for AT_1 and AT_2, and β-actin; goat polyclonal IgG, 1:2,000 for APA, and APN) for 1 h at room temperature. After washing with TBS-T, the NCMs were incubated with horseradish peroxidase labeled-secondary antibodies (donkey anti-rabbit and anti-goat IgGs, 1:25,000) for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substrate using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and Hyperfilm MP (Amersham Life Science).

NCMs were then stripped of secondary and primary antibodies by incubating with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) for 15 min at room temperature. NCMs were soaked again overnight at 4°C in TBS-T and immunoblots for β-actin were obtained using the specific primary and secondary antibodies as described above. Bands corresponding to different proteins on X-ray films were scanned (VistaScan32, Astra 1220S, UMAX

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Technologies, Inc., Dallas, TX) and the respective areas and optical densities determined by using Image-Pro Plus 4.0 software (Media Cybernetics; Silver Spring, MD).

Drugs and Chemicals. Ang III, Ang-(1-7), and Ang IV were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Amastatin, Ang II, thiorphan, and PD123,319 were purchased from Sigma-Aldrich (St. Louis, MO). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Losartan was a generous gift from Merck (Rahway, NJ).

Data Analysis. Results are expressed as means ± S.E. Agonist CRCs were fitted using a non-linear interactive fitting program (GraphPad Prism 3.0, Graph Pad Software Incorporated, CA). In contractile experiments, agonist potencies and maximum responses were expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max} (maximum effect elicited by the agonist), respectively, calculated as percent of the maximal contraction induced by bethanechol 100 μM. For relaxation experiments, agonist potencies and maximum responses were expressed as pIC_{50} (negative logarithm of the molar concentration of agonist producing 50% of the maximum inhibitory response) and I_{max} (maximum inhibition elicited by the agonist), respectively, calculated as percent of the maximal fall induced by sodium nitroprusside (10 μM) on the ASM pre-contraction induced by bethanechol (100 μM). In certain experiments where both contraction and relaxation were observed in response to different concentrations of Ang II (e.g. in the background of bethanechol), data in figures was represented as % maximal change.

Statistical significance was tested by using the one-way analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test when three or more different treatments were

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compared. To compare two different treatments obtained on the same tissue, the paired Student t-test was used otherwise the unpaired Student t-test was chosen. A 'p' value less than 0.05 was considered to be statistically significant.

Results

Effects of Ang II and Related Peptides. Ang II and Ang III induced concentration-dependent contraction of the ASM (Fig. 1). There were no significant differences in E_{max} values of the responses of Ang II (21.50 ± 5.73 %) and Ang III (15.50 ± 2.61 %). However, Ang II was more potent (pD_2 = 8.53 ± 0.52) than Ang III (pD_2 = 6.71 ± 0.74) in producing contraction ($p < 0.05$; n = 8). Ang IV and Ang (1-7) on the other hand, did not produce any significant effect. Interestingly, Ang II produced a passive relaxation at the higher concentrations (0.3 μM to 10 μM). With 10 μM Ang II, the contractile response was significantly reduced to 9.08 ± 2.55 % of the maximal contraction ($p < 0.05$; n = 8; Fig. 1). A bimodal effect was also observed with Ang III, but at higher concentration range as compared with Ang II. With 100 μM Ang III, the contractile effect was significantly reduced to 4.48 ± 2.70 % of the maximal contraction caused by bethanechol ($p < 0.05$; n = 8; Fig. 1).

Influence of Amastatin and Thiorphan on Ang II-Induced Contraction. As shown in Fig. 2, amastatin (1 μM), the non-selective inhibitor of APA and APN, caused a significant decrease in Ang II-induced contraction of ASM. In the presence of amastatin, the E_{max} calculated from Ang II-induced CRC was significantly ($p < 0.05$; n = 4 to 6; Fig. 2) lower (6.3 ± 2.45 %) than the control. No significant effect was observed on the pD_2 value. On the other hand, the NEP inhibitor, thiorphan (1 μM), had no significant effect. These results exclude

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participation of local enzymatic degradation by APA, APN, and NEP in the observed smooth muscle relaxation by Ang II.

Influence of Losartan, PD123,319, and A-779 on Ang II-Induced Contraction. In the presence of losartan (0.1 μM), E_{max} (13.67 ± 2.03 %) and pD_2 (6.57 ± 0.09) values of Ang II were significantly ($p < 0.05$) lower than in the control state, suggesting that AT_1 receptor activation provides the excitatory contractile signal in response to Ang II in the ASM. Additionally, these results suggest that losartan behaviors like a non-competitive antagonist. PD123,319 (0.1 μM) produced no significant effect on Ang II-induced contraction. However, it inhibited the passive relaxation (E_{max} = 15.50 ± 0.96 % at 10 μM Ang II) unmasking an inhibitory AT_2-mediated component. Ang (1-7) antagonist A-779 (1 μM) had no significant effect. Data are summarized in Fig. 3.

Influence of Losartan and PD123,319 on Contractions Induced by Ang III. Losartan (0.1 μM) displaced Ang III CRC to the right in a competitive manner (pD_2 = 5.39 ± 0.09; $p < 0.05$; n = 4) with no significant change on E_{max} values (16.27 ± 3.88 %). PD123,319 (0.1 μM), on the other hand, produced no significant effect on Ang III-induced contraction (E_{max} = 20.01 ± 4.88 %; pD_2 = 6.69 ± 0.04) with a tendency to inhibit the passive relaxation (8.73 ± 2.43 % at 100 μM of Ang II). Results are summarized on Fig. 4.

Active Relaxation. To determine the mechanism of ASM relaxation at the higher concentrations of Ang II, the following studies were carried out in the background of bethanechol (100 μM). Once contracted with bethanechol, the contraction of the ASM was

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maintained for at least 20 min (as illustrated on typical tracing in Fig. 5). For this part of the protocol, CRCs to Ang II were therefore constructed in that time frame. In naive strips pre-contracted with bethanechol (100 μM), Ang II (0.3 μM) produced an initial contraction followed by a concentration-dependent active relaxation (I_{max} = 47.85 ± 4.23 %; pIC_{50} = 4.89 ± 0.11) below the level of the contraction induced by bethanechol, which was defined as active relaxation (Fig. 6A). In order to determine the role of angiotensin receptors in the observed responses, ASM strips were incubated with A-779, losartan, or PD123,319 for 20 min before construction of the first CRC to Ang II. A-779 (1 μM) or losartan (0.1 μM) produced no significant effect on the bimodal effect of Ang II. On the other hand, PD123,319 (0.1 μM) augmented the initial contractions elicited by Ang II and preserved the active relaxation by Ang II. Because PD123,319 in contrast to losartan does not loose its selectivity at 0.5 μM (Whitebread et al., 1989), we also evaluated the effect of PD123,319 at 0.5 μM. PD123,319 (0.5 μM) augmented the initial contraction by Ang II significantly (75.94 ± 9.27 %) ($p < 0.05$, $n = 3$). However, it abolished the active relaxation (I_{max} = 2.37 ± 5.25 %) (Fig. 6A). Therefore data suggest that AT_2 receptor activation is primarily involved with the active relaxation caused by Ang II and exclude the involvement of AT_1 and Ang-(1-7) receptors.

Desensitization of the Excitatory Response (Passive Relaxation). In order to evaluate the role of desensitization of the excitatory response in the ASM relaxation by Ang II, a second CRC for Ang II in the background of bethanechol (100 μM) was obtained 1h after the first CRC. The second pre-contraction induced by bethanechol was not significantly different from the first one. As shown in Figure 6B, in the second CRC, the initial contraction by Ang II was nearly abolished, and Ang II became more potent in causing the relaxation (pIC_{50} = 5.59 ± 0.07) (pIC_{50}

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= 4.89 ± 0.11) ($p < 0.05$; n = 6). Significant ($p < 0.05$) differences on I_{max} values were also found. The first CRC produced an I_{max} equivalent to 47.85 ± 4.23 %. On the second CRC, the I_{max} was 64.03 ± 5.47 %. Data suggest that desensitization of the excitatory component may partly contribute to the passive component of the ASM relaxation by Ang II.

In this set of experiments, ASM strips were incubated with losartan or PD123,319 for 20 min before the construction of the second CRC to Ang II. Incubation with losartan caused no significant shift ($p < 0.05$; n = 4) in the second Ang II CRC, suggesting that desensitization of Ang II is selective for AT_1 receptors. PD123,319 on the other hand, caused a significant ($p < 0.05$; n = 4) rightward shift in the second CRC (pIC_{50} = 4.99 ± 0.10). Incubation with 0.5 μM of PD123,319 caused further inhibition (pIC_{50} = 4.65 ± 0.08, n = 3) (Fig. 6B), without any significant change in the I_{max} value. This suggests that PD123,319 is a competitive antagonist. Additionally, the passive component of Ang II-induced relaxation is because of AT_1 receptor desensitization while the active relaxation by Ang II is primarily mediated by AT_2 receptor activation.

Influence of TTX and L-NAME on Active Relaxations. In naive smooth muscle strips, in the background of bethanechol, L-NAME (100 μM) and TTX (1 μM) converted Ang II-induced relaxation to contraction. Additionally, these agents augmented the initial contraction by Ang II to 119.12 ± 19.03 %, and 65.55 ± 27.35 %, respectively ($p < 0.05$; n = 4; Fig. 7A). In repeat experiments, L-NAME and TTX abolished the relaxations ($p < 0.05$; n = 4 -6; Fig. 7B) and no initial contraction was observed. Data suggest that relaxation by Ang II is mediated by desensitization of AT_1 receptors plus AT_2 receptor activation associated with neuronal NOS (nNOS) stimulation.

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Influence of ODQ and SQ 22536 on Active Relaxation. In naive ASM strips, ODQ (1 μM) caused significant ($p < 0.05$; $n = 4$; Fig. 8) antagonism of Ang II-induced relaxation by producing a rightward displacement of the CRC ($pIC_{50} = 4.52 \pm 0.22$) with no significant effect on I_{max} values ($p > 0.05$). SQ 22536 on the other hand, produced no significant effect even at a higher concentration (10 μM) (Fig. 8).

Western Blots Studies to Demonstrate the Presence of AT_1 and AT_2 receptors, APA, and APN in the ASM. Immunoblots using specific antibodies showed the presence of both AT_1 and AT_2 receptors. The studies also demonstrated the presence of APA and APN (Fig. 9). All proteins were identified based on the expected molecular size: AT_1, 40 kDa; AT_2, 40 kDa; APA, 160 kDa; APN, 150 kDa; and β-actin, 43 kDa.

Discussion

The present study reports bimodal effects of Ang II in the rat ASM, a contraction (at lower concentrations) and relaxation (at higher concentrations). Primary focus of the present studies is on the relaxation. We suggest that two events are responsible for this relaxation: passive and active relaxations. We speculate that passive relaxation is partly because of desensitization of the excitatory signal mediated by AT_1 receptor activation. Active relaxation on the other hand occurs primarily by the activation of nNOS/sGC pathway via AT_2 receptor stimulation.

In the present investigation, we also investigated the role of enzymatic degradation (by local peptidases), on ASM effects by Ang II. Degradation of Ang II by local enzymes is well known to play an important role in the termination of Ang II response (Ardaillou and Chansel, 1997).

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The main enzymes for this effect are APA, APN, and NEP (Devynck and Meyer, 1978; Santos et al., 1994). We examined the effects of amastatin (APA and APN inhibitor), and thiorphan (NEP inhibitor). We theorized that if these peptidases play a role in the relaxant effect, this effect should be reversed by the respective inhibitors. The results however show diverse effects. Thiorphan has no effect on the ASM contraction by Ang II. This combined with the lack of effect of Ang-(1-7), and of A-779 (selective antagonist of Ang (1-7)) on Ang II effects, negates the role of NEP in Ang II-induced relaxation of the ASM.

The role of aminopeptidases in Ang II effects in the ASM may be complex. Amastatin attenuates contraction by Ang II in the ASM, but does not reverse the relaxation caused by Ang II. The inhibitory effect of amastatin on the Ang II contraction suggests that APA may have an important role in Ang II-induced contraction, by its conversion into Ang III. This notion is further bolstered by the effects of Ang III in the ASM. Ang III elicits contractions with the efficacy comparable with that of Ang II. Additionally, the effects of Ang III involve the activation of both AT_1 (antagonized by losartan) and AT_2 receptors (inhibited by PD123,319) similar to Ang II in the ASM, as shown in other systems (Devynck and Meyer, 1978; Vatta et al., 1992). The lower potency displayed by Ang III may be related to the absence of the Asp^1 residue on the N terminal, an important residue for the stabilization of the peptide receptor interaction (De Gasparo et al., 2000). APN may be involved with termination of Ang III action by its conversion to Ang IV because incubation with amastatin (1 μM) potentiated ($p < 0.05$) the contraction induced by Ang III ($pD_2 = 7.50 \pm 0.52$; n= 3). Western blot studies further show the presence of APA and APN.

Our studies suggest that the excitatory and inhibitory effects of Ang II in the ASM are mediated via AT_1 and AT_2 subtypes, respectively. Losartan, an AT_1 receptor antagonist,

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competitively inhibits the ASM contraction by Ang II. On the other hand, PD123,319 inhibits the passive relaxation, without modifying the contractile effect. This is noteworthy as one would expect leftward displacement of the whole CRC (including the contractile portion of the curve) because of AT_2 receptor inhibition. We conceptualize that the latter is masked by the dominant AT_1-mediated effect, and that AT_2 receptor activation becomes evident only at higher concentrations of Ang II during the AT_1 receptor desensitization. Additionally, as shown before, AT_1 receptor stimulation may lead to AT_2 receptors inactivation (Hein et al., 1997; De Paolis et al., 1999). To provide additional support for the role of AT_1 in the excitatory and AT_2 receptors in the inhibitory effects, our studies show that losartan inhibits the AT_1-mediated contractions unleashing the inhibitory AT_2-mediated component. Presence of AT_1 and AT_2 receptors is further demonstrated by Western blot studies.

To elucidate the mechanisms of Ang II-induced relaxation of ASM, we carried out studies in bethanechol-pre-contracted smooth muscle strips, and we examined the effect of high concentrations of Ang II (0.3 μM to 100 μM). 0.3 μM Ang II causes a contraction while the higher concentrations cause frank active relaxation. Such experiments also help in explaining the role of desensitization of AT_1 receptor-mediated contraction by Ang II in the relaxation because the repeat curve (second CRC, 1 h apart) shows significantly reduced contraction and augmented relaxation (Fig. 6A vs. 6B; comparing first CRC with second CRC). Initial contraction (by 0.3 μM Ang II) in such experiments in the first CRC (control state; Fig. 6A) is not antagonized by losartan. The simplest explanation for these observations is that these higher concentrations of Ang II competitively displace losartan from AT_1 receptors. In the same set of experiments, PD123,319 on the other hand, enhances the initial contraction and inhibits the active relaxation by Ang II in a concentration-dependent manner, suggesting the role of AT_2

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receptors in the active relaxation.

Above experiments (in concentrations up to 100 μM) (Figs. 6B, and 7B; second CRC after 1 h) reveal the involvement of desensitization (passive relaxation), and an active relaxation via nNOS activation for the observed relaxation with Ang II. PD123,319 concentration-dependently attenuates the relaxations without the initial contractions, and losartan has no significant effect on the relaxations. The lack of effect of losartan is in these results in agreement with earlier suggestion that desensitization to AT_1-mediated contraction may potentiate relaxation of rat mesenteric artery smooth muscle (Widdop et al., 2002).

The data suggest that Ang II-induced active relaxation of the ASM (shown in Figs. 7 and 8) occurs via the activation of AT_2 receptors on the myenteric inhibitory neurons leading to NO/sGC activation. The neurotoxin TTX , and the NOS inhibitor, L-NAME cause similar obliteration of Ang II-induced relaxation. Additionally, sGC inhibitor ODQ inhibits the active relaxation by Ang II. Interestingly, the initial contractions by Ang II were greater with L-NAME as compared to TTX. The involvement of NO/sGC in AT_2-activated relaxation of different smooth muscles by Ang II has been shown before (Siragy and Carey, 1999). But the role of neural NOS in Ang II-induced relaxation of the smooth muscle has not been examined before.

In summary, present studies demonstrate that higher concentrations of Ang II produce ASM relaxation, and that the observed relaxation is the result of two processes, passive and active relaxations. Desensitization of the AT_1 receptor-mediated excitatory signal is responsible for passive relaxations. Stimulation of AT_2 receptors associated with nNOS/sGC pathway on the other hand is responsible for the active relaxation in the smooth muscle. The rat ASM provides a good model for the detailed pharmacological studies to investigate AT_1 and AT_2 receptors in the smooth muscle.

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References

Ardaillou R and Chansel D (1997) Synthesis and effects of active fragments of angiotensin II. *Kidney Int* **52**:1458-1468.

Bennet JPJr and Snyder SH (1976) Angiotensin II binding to mammalian brain membranes. *J Biol Chem* **251**:7423-7430.

De Gasparo M, Catt KJ, Inagami T, Wright JW, and Unger T (2000) International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* **52**:415-472.

De Godoy MAF, Accorsi-Mendonca D, and De Oliveira AM (2003) Inhibitory effect of atropine and hexamethonium on the angiotensin II-induced contractions of rat anycoccygeus smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* **367**:176-182.

De Godoy MAF and De Oliveira AM (2002) Cross-talk between AT_1 and AT_2 angiotensin receptors in rat anococcygeus smooth muscle. *J Pharmacol Exp Ther* **303**:333-339.

De Paolis P, Porcellini A, Gigante B, Gilberti R, Lombardi A, Savoia C, Rubattu S, and Volpe M (1999) Modulation of the AT_2 subtype receptor gene activation and expression by the AT_1 receptor in endothelial cells. *J Hypertens* **17**:1873-1877.

Devynck MA and Meyer P (1978) Angiotensin receptors. *Biochem Pharmacol* **27**:1-5.

Fan Y-P, Puri RN, and Rattan S (2002) 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.

Gibson A and McFadzean I (2001) Biology of the anococcygeus muscle. *Int Rev Cytol* **205**:1-35.

Gibson A and Pollock D (1973) The effects of drugs on the sensitivity of the rat anococcygeus muscle to agonists. *Br J Pharmacol* **49**:506-513.

Gillespie JS (1972) The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br J Pharmacol* **45**:404-416.

Hein L, Meinel L, Pratt MM, Dzau VJ, and Brian KK (1997) Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol* **11**:1266-1277.

Israel A, Cierco M, and Sosa B (2000) Angiotensin AT_2 receptors mediate vasodepressor response to footshock in rats. Role of kinins, nitric oxide and prostaglandins. *Eur J Pharmacol* **394**:103-108.

Lohse MJ (1993) Molecular mechanisms of membrane receptor desensitizaton. *Biochim Biophys Acta* **1179**:171-188.

JPET Fast Forward. Published on June 3, 2004 as DOI: 10.1124/jpet.104.069856
This article has not been copyedited and formatted. The final version may differ from this version.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265-275.

Motta SC, Poletti EF, Souza SE, Correa SA, Jubilut GN, Paiva AC, Shimuta SI, and Narkai CR (2003) Tachyphylactic properties of angiotensin II analogs with bulky and hydrophobic substituents of the N-terminus. *J Pept Res* **62**:227-232.

Nouet S and Nahmias C (2000) Signal transduction from the angiotensin II AT_2 receptor. *Trends Endocrinol Metab* **11**:1-6.

Rathi S, Kazerounian S, Banwait K, Schulz S, Waldman SA, and Rattan S (2003) Functional and Molecular Characterization of Beta Adrenoceptors in the Internal Anal Sphincter. *J Pharmacol Exp Ther* **305**:615-624.

Rattan S, Fan Y-P, and Puri RN (2002) Comparison of angiotensin II (Ang II) effects in the internal anal sphincter (IAS) and lower esophageal sphincter smooth muscles. *Life Sci* **70**:2147-2164.

Santos RAS, Campagnole-Santos MJ, Baracho NCV, Fontes MAP, Silva LCS, Neves LAA, Oliveira DR, Caligiorne SM, Rodrigues ARV, Gropen CJr, Carvalho WS, Silva ACSE, and Khosla MC (1994) Characterization of a new angiotensin antagonist selective for angiontensin (1-7): Evidence that the actions of angiotensin (1-7) are mediated by specific angiotensin receptors. *Brain Res Bull* **35**:293-298.

Sarma D, Banwait K, Basak A, DiMarino AJ, and Rattan S (2003) Inhibitory effect of β_3-adrenoceptor agonist in lower esophageal sphincter smooth muscle: in vitro studies. *J Pharmacol Exp Ther* **304**:48-55.

Siragy HM and Carey RM (1999) Protective role of the angiotensin AT_2 receptor in a renal wrap hypertension model. *Hypertension* **33**:1237-1242.

Vatta MS, Biacontti LG, Locatelli AS, Ponchado ML, and Fernandez BE (1992) Monophasic and biphasic effects of angiotensin II and III on norepinephrine uptake and release in rat adrenal medulla. *Can J Physiol Pharmacol* **70**:821-825.

Whitebread S, Meke M, Kamber B, and De Gasparo M (1989) Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun* **163**:284-291.

Widdop RE, Matrougui K, Levy BI, and Henrion D (2002) AT_2 receptor-mediated relaxation is preserved after long-term AT_1 receptor blockade. *Hypertension* **40**:516-520.

JPET Fast Forward. Published on June 3, 2004 as DOI: 10.1124/jpet.104.069856
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Footnotes

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Figure Legends

Fig. 1. Effect of Ang II, Ang III, Ang IV, and Ang (1-7) on rat ASM. Data represent the mean ± S.E. of 4 to 8 independent determinations. Ang II is significantly more potent than Ang III. Ang IV and Ang (1-7) had no significant effect.

Fig. 2. Effects of amastatin and thiorphan on the contractions induced by Ang II in the ASM. Data represent the mean ± S.E. of 4 to 8 independent determinations. Note a significant ($p < 0.05$) attenuation of Ang II CRC following amastatin.

Fig. 3. Effects of losartan, PD123,319, and A-779 on Ang II-induced contraction of ASM. Note that losartan ($p < 0.05$; $n = 4$ to 8) causes significant attenuation of the contractile effect while PD123,319 attenuates the relaxant effect. A-779 has no significant effect on Ang II-induced contraction of ASM.

Fig. 4. Effects of losartan and PD123,319 on Ang III-induced contraction of ASM. Losartan causes significant rightward ($p < 0.05$; $n = 4$ to 8) parallel shift in the Ang II CRC while PD123,319 tends to inhibit Ang II-induced relaxation of ASM.

Fig. 5. Typical tracings to show the effect of Ang II in the background of bethanechol (100 µM). The tracing in the top panel **A**, shows the sustained nature of bethanechol contraction of ASM. The tracing in the middle panel **B**, shows the initial contraction induced by Ang II followed by active relaxations at higher concentrations of Ang II. The tracing in the bottom panel **C** shows no initial contraction to Ang II but more efficacious and more potent active relaxations with higher concentrations of Ang II than shown in panel **B**. ('w' in the tracings denotes wash)

Fig. 6. Effects of losartan, PD123,319 and A-779 on Ang II-induced response in rat ASM in the background of bethanechol. **A**. First CRC induced by Ang II. Data show that while losartan and A-779 have no significant effects on Ang II-mediated ASM relaxation, PD123,319 (0.5 µM)

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inhibits the response in a concentration-dependent manner. **B**. Second CRC to Ang II, examined 1 hour after the first CRC shows significant ($p < 0.05$) augmentation of Ang II-induced relaxation of the smooth muscle. PD123,319 (0.5 μM) significantly ($p < 0.05$) inhibits the relaxation. Data represent the mean ± SE of 4 to 7 independent determinations.

Fig. 7. Effect of the neurotoxin TTX (1μM), and L-NAME (100 μM) on Ang II-induced relaxations in rat ASM. In the first CRC to Ang II (**A**), both agents cause significant ($p < 0.05$) enhancement of the initial contractions and obliteration of the relaxations. In the second CRC to Ang II (**B**) obtained 1h after the first CRC, active relaxations are abolished by TTX and L-NAME with no initial contraction ($p < 0.05$; $n = 4$ to 6).

Fig. 8. Effect of ODQ and SQ 22536 on Ang II-induced relaxations in ASM. Note that ODQ but not SQ 22536 causes significant antagonism of ASM relaxation by Ang II ($p > 0.05$; $n = 4$ to 6).

Fig. 9. Western blot analyses of AT_1, AT_2, APA, APN, and β-actin expression in the rat ASM demonstrating expected size protein for AT_1 (40 kDa), AT_2 (40 kDa), APA (160 kDa), APN (150 kDa), and β-actin (43 kDa). The protein samples (40 μg/well) were run on a 7.5% SDS-polyacrylamide gel, electrophoresed for 60 min, transferred to NCM, and probed by isoform-specific antibodies for each protein.

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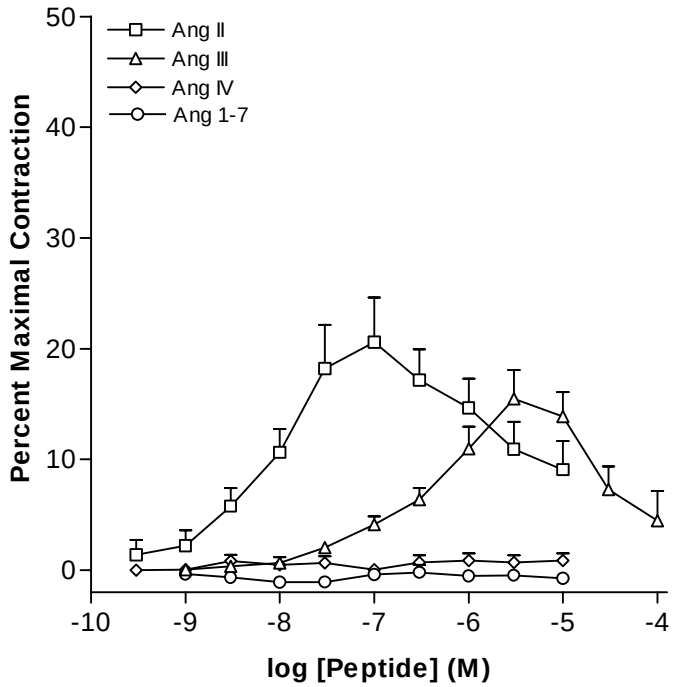

Figure 1

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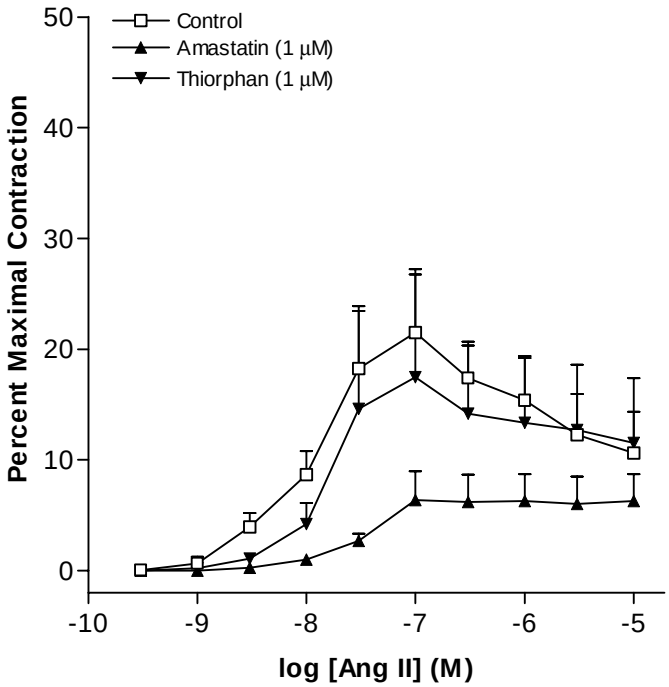

Figure 2

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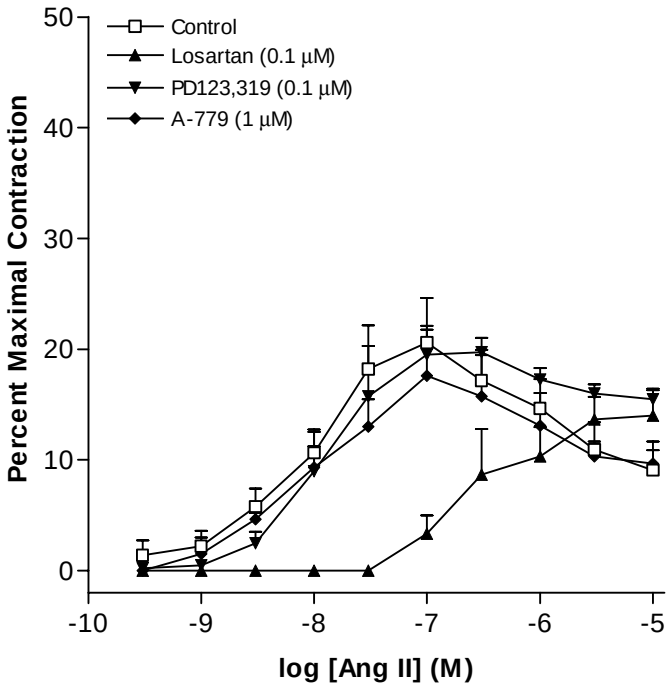

Figure 3

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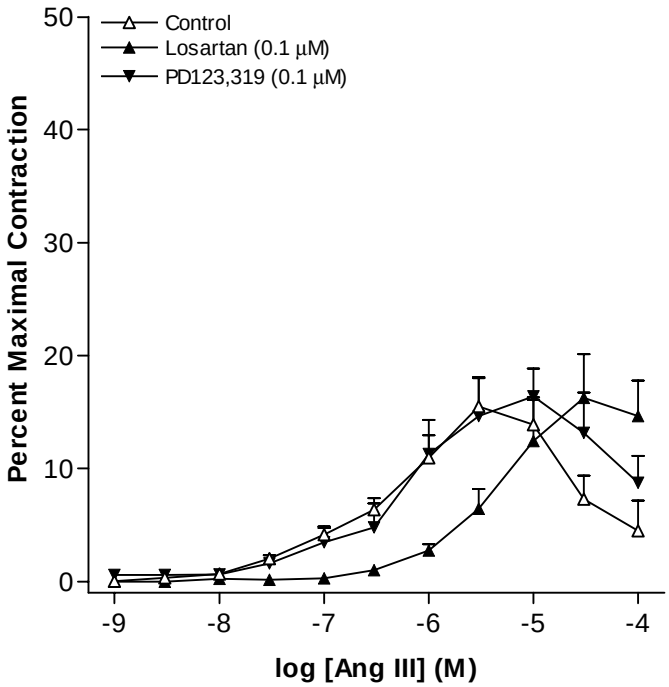

Figure 4

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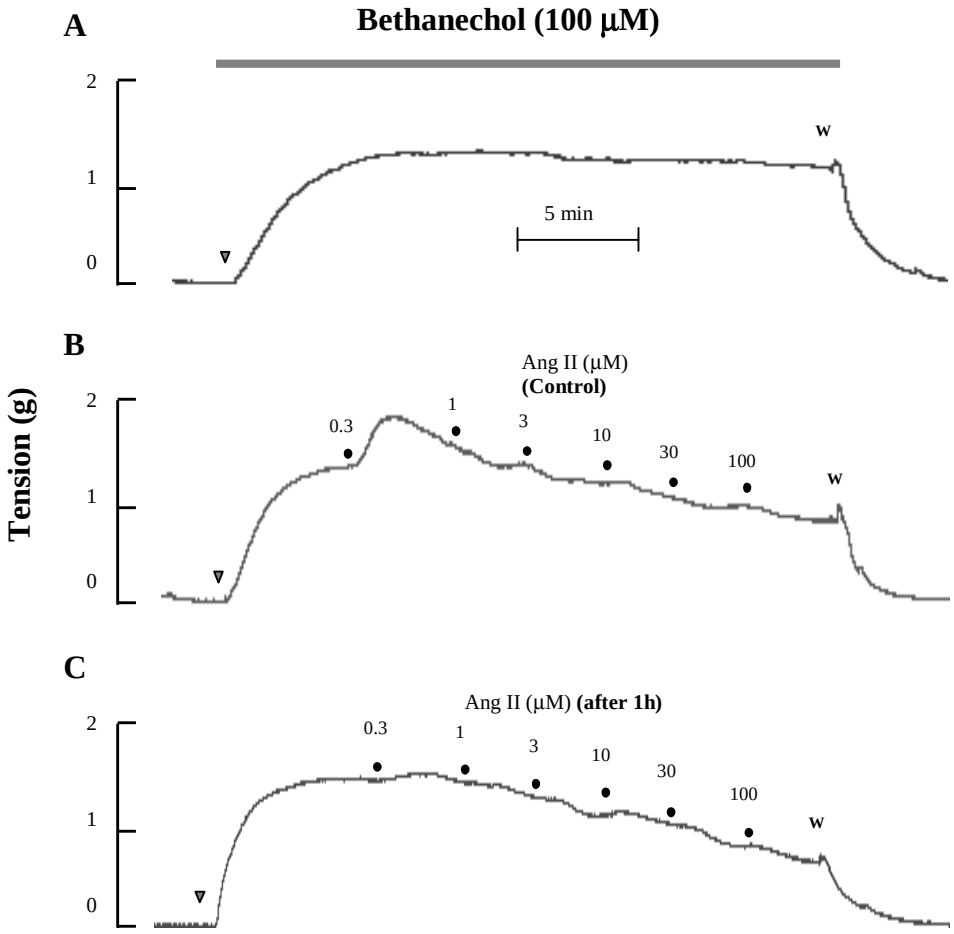

Figure 5

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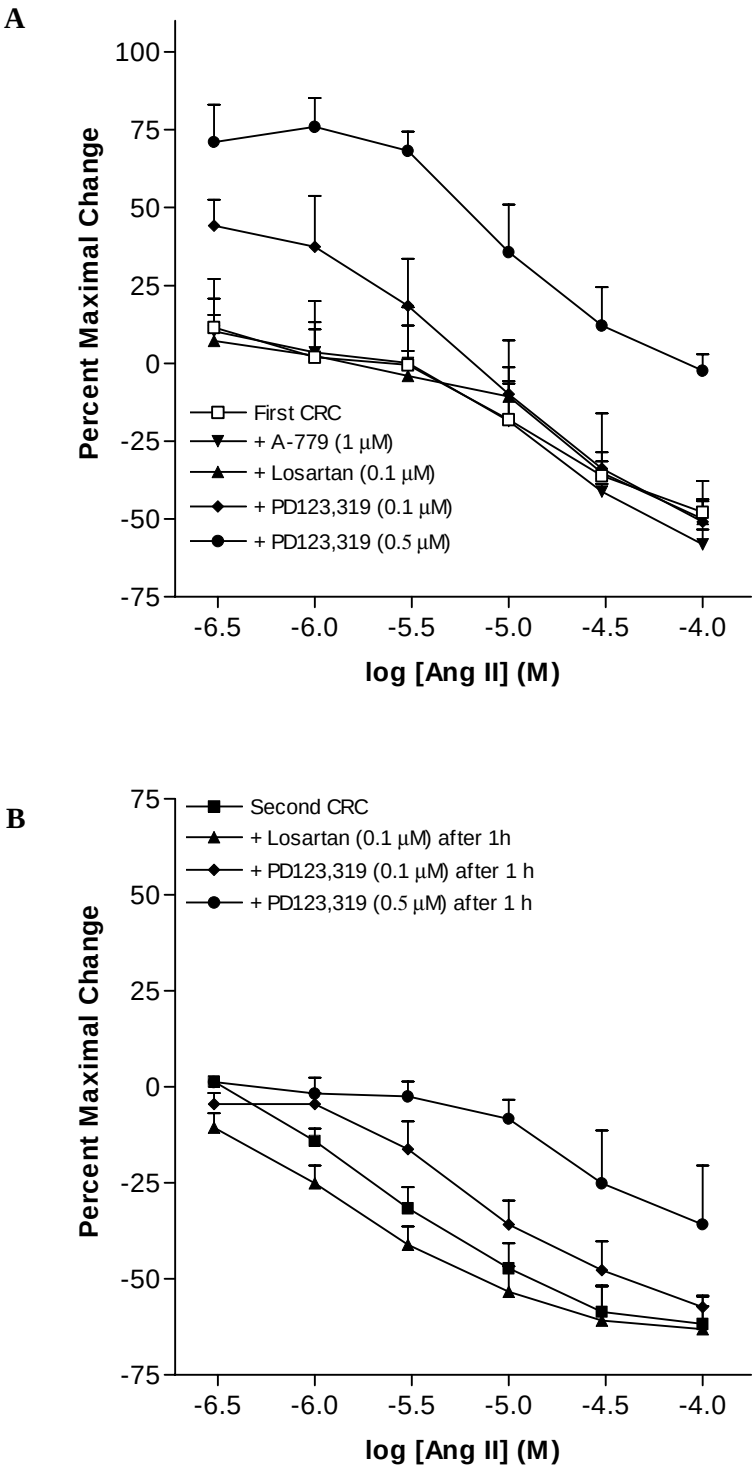

Figure 6

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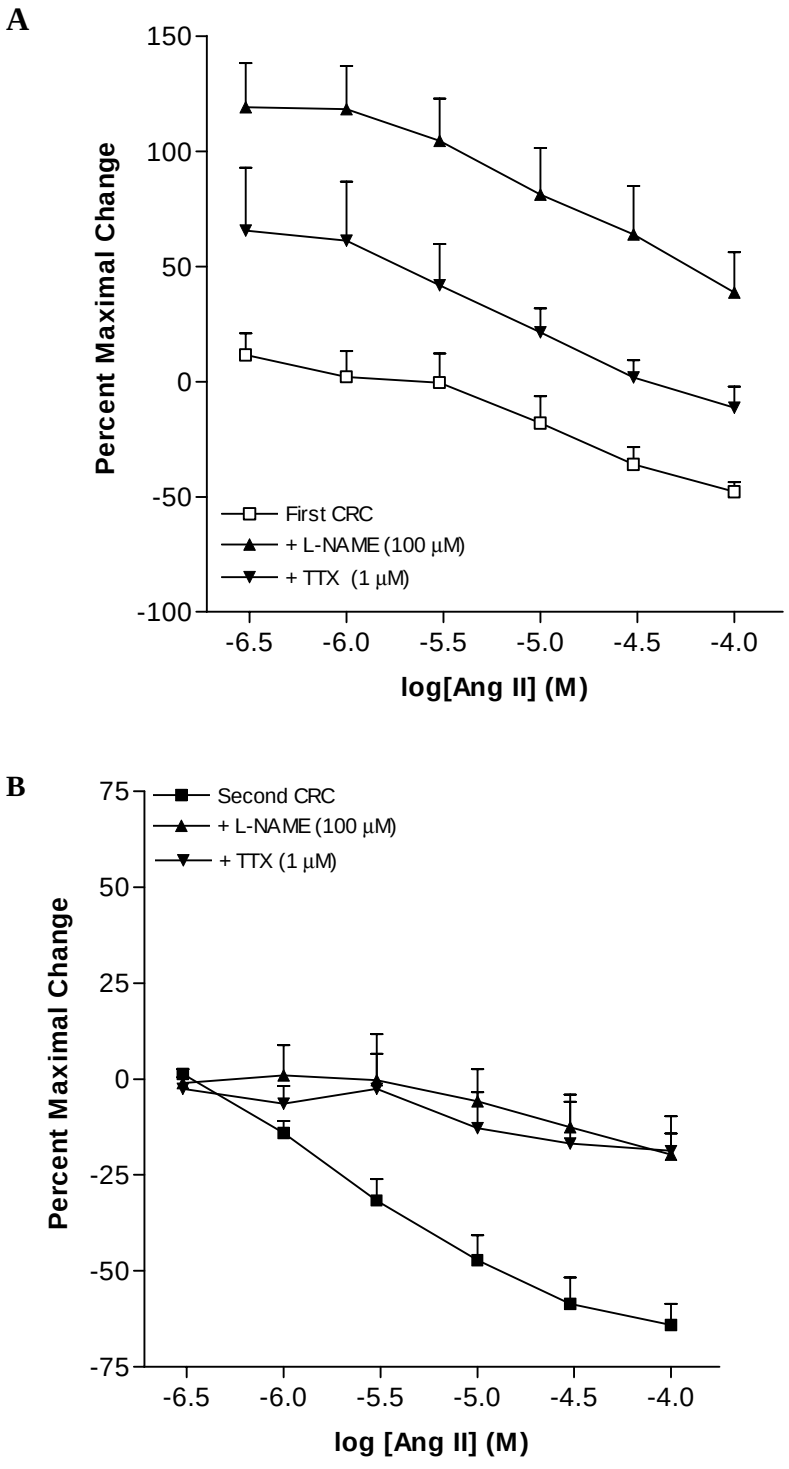

Figure 7

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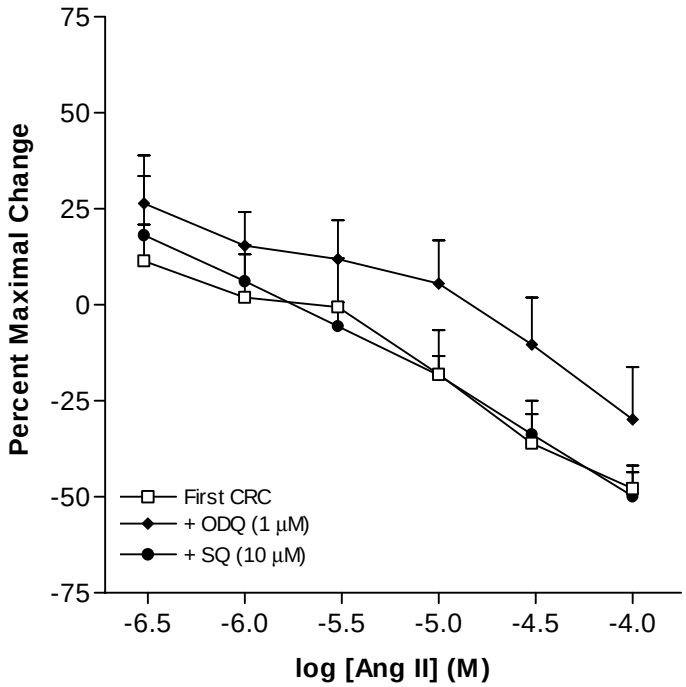

Figure 8

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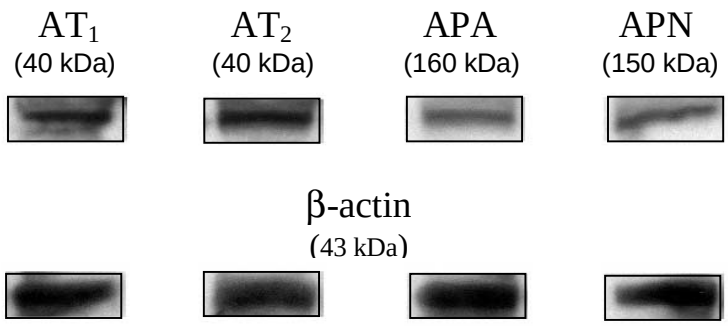

Figure 9