Pre- and postsynaptic studies concerning the interaction between the renin angiotensin system and the sympathetic nervous system
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No involvement of the AT₂-receptor in angiotensin II-enhanced sympathetic transmission \textit{in vitro}
Introduction

Angiotensin II has been shown to interact with the sympathetic nervous system (SNS) at several levels. These interactions involve the enhancement of noradrenergic neurotransmission at central nervous structures, the sympathetic ganglia, the adrenals as well as peripheral sympathetic nerve terminals\(^{1,2}\).

The receptors through which angiotensin II exerts its effects are subclassified into AT\(^{-}\)-receptors and AT\(^{2}\)-receptors, respectively. The AT\(^{-}\)-subtype is sensitive to the reference compound losartan whereas the AT\(^{2}\)-subtype is sensitive to PD123177 and to low concentrations of the related agent PD123319\(^{3,4}\).

Although it has been claimed that the majority of effects elicited by angiotensin II are mediated through the AT\(^{-}\)-receptor subtype, the physiological role of the AT\(^{2}\) receptor is recently drawing a great deal of attention. Cardiovascular remodelling, vasodilatation, apoptosis, foetal development and anti-thrombotic activity are some of the effects presumed to be mediated through the AT\(^{2}\)-receptor\(^{5,6}\).

The AT\(^{-}\)-receptor is known to mediate the facilitatory actions of angiotensin II on noradrenergic neurotransmission\(^{7,8}\). Recently, however, several studies suggested that besides AT\(^{-}\)-involvement the AT\(^{2}\)-receptor may be associated with angiotensin II-enhanced sympathetic nerve traffic. In conscious rats, administration of angiotensin II into the cerebral ventricles resulted in significant pressor effects, which were associated with a marked vasoconstriction at the level of the mesenteric and hindquarter vascular beds. Both PD 123319 and EXP-3174, administered into the cerebral ventricles, abolished the cardiovascular response to central angiotensin II infusion, thus indicating the involvement of the AT\(^{2}\)-receptor\(^{9,10}\). In addition, PD123319 (0.01 and 0.1 µM) could abolish the synergistic interaction between losartan (10 nM) and angiotensin II concerning stimulation-induced noradrenaline efflux as well as nerve stimulated vasoconstriction in the rat caudal artery\(^{11}\). This phenomenon might be explained by an unmasking of a latent population of AT\(^{2}\)-receptors that subserve further facilitation.

In accordance with these observations we recently demonstrated that inhibition of angiotensin II-mediated facilitation in the pithed rat by irbesartan (1 - 60 mg/kg) resulted in a U-shaped dose response curve\(^{12}\). The highest dose of irbesartan caused less than maximal sympatho-inhibition. This U-shaped dose-response relationship was not observed when PD123319 (5 mg/kg + 50 µg/kg/min) was co-administered. Hence, the irbesartan-mediated “upstroke” may be explained by the involvement of the AT\(^{2}\)-receptor after AT\(^{-}\)-blockade with high-dose irbesartan.
It was the objective of the present study to further investigate the possible role of the AT₁-receptor in angiotensin II-mediated facilitation *in vitro*. We applied the noradrenaline spillover technique to study the influence of selective AT₁- and AT₂-blockade on the exocytotic release of neurotransmitter. This straightforward model was shown to be suitable to investigate the interactions between the renin angiotensin system and the peripheral sympathetic nervous system.

Accordingly, we studied the effects of the selective AT₂-receptor antagonist PD123319 on angiotensin II-enhanced sympathetic outflow evoked by electrical field stimulation (EFS) in the isolated vena cava inferior of the rat. Additionally, we investigated the inhibitory effect of irbesartan on the sequelae of angiotensin II-enhanced, EFS-evoked sympathetic nerve traffic in the presence or absence of PD123319. By selectively excluding the AT₁-receptor (irbesartan) we intended to address or unmask a possible latent population of AT₂-receptors, as we previously described for the pithed rat preparation. Subsequently, we antagonized this receptor population by applying PD123319. Furthermore, we determined the sympatholytic potency of irbesartan in the present model.

**Material, methods & animals**

The experimental protocol was approved by the committee on Animal Experiments of the Academic Medical Center Amsterdam. Male Wistar rats, weighing 240-260g were used. The rats were stunned and decapitated. The thoracic cavity was opened and the vena cava inferior was dissected free from its connective tissue and transferred to a petri plate containing physiological salt solution (PSS), gassed with a mixture of 95% O₂ and 5% CO₂ at room temperature.

**Rat isolated vena cava inferior preparations**

The proximal and distal ends of the vena cava were ligated with fine silk threads, subsequently the preparation was transferred to an organ bath. The PSS was composed as follows (mmol/l): NaCl 118, Na₂HPO₄ 1.2, NaHCO₃ 25, KCl 4.7, CaCl₂ 1.6, MgSO₄ 1.2, and glucose 11.0. Ascorbic acid (0.3) and Na₂EDTA (0.03) were added to prevent the oxidation of noradrenaline.
Radiolabelling of noradrenergic transmitter stores

In order to radioactively label their noradrenergic vesicles, the isolated veins were incubated for 45 min in 2.0 ml of PSS containing 0.1 μmol/l [7,8-3H]-noradrenaline (specific activity 28.8 to 52.0 Ci/mmol) in a 5 ml glass-jacketed organ bath. The medium was continuously bubbled with carbogen and maintained at a temperature of 37°C.

After the incubation period the isolated veins were washed with [3H]-noradrenaline-free PSS (10× 2 ml and 4× 5 ml) to remove superficially bound, non-neuronal radioactivity before the experimental procedures were started. After the wash-procedure the veins were mounted vertically between platinum wire electrodes (2 cm) placed along either side of the preparations in a 25 ml organ bath and subjected to a tension of 0.5 g.

The organ bath contained 20.0 ml PSS. Desipramine (0.6 μmol/l) and corticosterone (40 μmol/l) were added in order to rule out uptake-1 and uptake-2 of [3H]-noradrenaline, respectively. Yohimbine (1 μmol/l) was added to the PSS to rule out any α2-adrenergic auto-inhibitory effects on [3H]-noradrenaline release. The veins were equilibrated for a total period of 48 min. After an initial period of 18 min the preparations were subjected to a 2 min period of electrical field stimulation (EFS) with a train of 3 ms rectangular bipolar wave pulses of 150 mA, at a frequency of 2 Hz. (S1) (Danish Myo Technology Current Stimulator, model CS 200). This 'priming' stimulation has been proven to increase the reliability and stability of the subsequent basal and EFS-induced [3H]-noradrenaline spillover.

Stimulation of intrinsic sympathetic nerves

After the equilibration period veins were subjected to two additional periods of EFS (trains of 2 min, 3 ms, 150 mA, 2Hz). The second period of stimulation (S2) was applied directly after the equilibration period of 48 min and the tritium outflow thus evoked was taken as control value. Subsequently, a third period (S3) was applied 24 min after S2. The ratio between S3 and S2 was calculated to quantify the influence exerted by the drugs to be investigated.

Measurement of tritium outflow

Samples of 0.5 ml each were repeatedly taken from the organ bath starting at 36 min after washout. The actual tritium outflow could be obtained by calculating the incremental accumulation in each sample. We corrected for the reduced volume, which decreased stepwise by the repeated drawing of samples.

The mean basal tritium efflux/min preceding the stimulation periods S2 and S1 was determined as the mean outflow/min of tritium in two 6 min samples prior to each period of stimulation. For
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$S_2$, we subtracted the radioactivity (DPM) measured in the sample taken at $t=36$ min from that of $t=42$ min and the radioactivity measured in the sample taken at $t=42$ min from that of the sample at $t=48$ min. Accordingly, we could determine the outflow/min of radioactivity during two 6 min time intervals prior to $S_2$. Basal outflow was determined by averaging these values. An equivalent procedure was applied for $S_3$.

The release/min evoked by EFS ($S_2$ and $S_3$, 2 min samples) was calculated by subtracting the corresponding mean basal efflux/min from the apparent EFS-evoked efflux/min.

At the end of the experiment the residual radioactivity of the tissue was measured. By adding the total released tritium to this value, the initial content of tritium was calculated. The effect of EFS on the release could then be expressed as a fraction of the total tissue content present at the moment of stimulation, the ‘fractional release’ of radioactivity (FR$_2$ and FR$_3$). Accordingly, the effects of pharmacological interventions are expressed as the ratio FR$_3$/FR$_2$.

**Detection of tritium in the samples and tissue**

After the experiment the tissues were kept overnight in 2 ml of 0.5 M quarternary ammonium hydroxide solved in toluene (Soluene, Packard). Radioactivity was measured by liquid scintillation counting (Tri Carb 2900TR, Packard) in 20-ml aliquots (with either samples or tissue) after addition of 5 ml of the scintillation fluid (Ultima Gold, Packard). Corrections for counting efficiency were made by external automatic standardization.

Three different experiments were performed:

**Experiment 1: Angiotensin II and EFS-evoked tritium-label outflow**

To investigate the influence of angiotensin II on EFS-evoked noradrenaline release it was added to the medium 150 seconds prior to $S_2$. Three different concentrations were studied; 1, 10 and 100 nM, respectively. To express the effect of angiotensin II the ratio FR$_3$/FR$_2$ was used.

**Experiment 2: Irbesartan, PD123319 and angiotensin II-facilitated $[^3H]$noradrenaline outflow**

In another series of experiments we studied the influence of the selective $\text{AT}_1$-antagonist irbesartan (1 nM - 1μM) and the selective $\text{AT}_2$-blocker PD123319 (10 nM) on angiotensin II-enhanced EFS-evoked sympathetic outflow. Either irbesartan, in one particular concentration, PD123319 (10 nM) or vehicle was added to the medium 20 minutes before $S_2$. Angiotensin II (10 nM) was added 150 seconds prior to $S_3$. To characterize the sympahto-inhibitory effects of
irsestaran and PD123319 the ratio FR₂/FR₁ was used. PD123319 10 nM is known to be effective in blocking the AT₁-receptor.

**Experiment 3: Irbesartan combined with PD123319 and angiotensin II-facilitated tritium-label outflow**

In a third series of experiments we investigated whether AT₁-inhibition by irbesartan (1 and 10 nM) combined with AT₂-blockade by PD 123319 (10 nM) could influence angiotensin II mediated facilitation differently when compared to inhibition by irbesartan alone, thus demonstrating the presence and function of the AT₂ receptor. Irbesartan (1 or 10 nM) and PD123319 (10 nM), or vehicle was added to the medium 20 minutes before S₁. Angiotensin II (10 nM) was added 150 seconds prior to S₂. To characterize the effects of irbesartan and PD123319 the ratio FR₂/FR₁ was used.

**Drugs and chemicals**

Irbesartan (Sanofi, Amilly, France) was dissolved in NaOH 1M. The pH of the solution was adjusted to 7.5 using 1M HCl. Angiotensin II (Bachem, Bubendorf, Switzerland, synthetic human sequence) and PD123319 (Parke Davis, Ann Arbor, USA) were dissolved in distilled water. Stock solutions of angiotensin II (10⁻³, 10⁻⁴) were stored in 50-µl aliquots at -20°C. Desipramine HCl (Sigma, St. Louis, USA) and yohimbine HCl (Sigma, St. Louis, USA) were dissolved in distilled water. Hydrocortisone Hydrogenosuccinates (Bufa, Uitgeest, Holland) was dissolved in DMSO. Stock solutions of desipramine (6.10⁻⁴), yohimbine (1.10⁻³) and corticosterone (4.10⁻²) were further diluted with PSS. Tritiated levo-[7,8-³H]-noradrenaline (Amersham Pharmacia Biotech, Little Chalfont, England) had a specific radioactivity of 28.8 - 52.0 Ci/mmol and a radioactive concentration of 1.0 mCi/ml. Soluene and Ultima Gold solutions were obtained from Packard Bioscience (Groningen, Netherlands).

**Statistical analysis**

All data are expressed as means ± S.E.M. Student’s t - test (two-tailed, unpaired) was used to evaluate statistical significance of differences between means of control and treatment groups. ANOVA followed by Dunnett’s test was performed for multiple comparisons with a control group. Differences at p<0.05 were considered to indicate statistical significance.

In order to determine the sympatho-inhibitory potency of irbesartan we used a computer program (GraphPad Prism, GraphPad, San Diego, USA). The curve was fitted to log concentration-effect data. The underlying equation is E=Eₘᵟₓ*ₐᵣ*(Aᵣ+IC₅₀ᵣ)⁻¹. In this equation
E is the response obtained at a given concentration $A$, $E_{\text{max}}$ is the maximally attainable response, $IC_{50}$ the concentration antagonist for the half maximal effect, and the exponent $p$ describes the slope of the relationship (Hill-coefficient). The curve was fitted to averaged concentration-effect data.

**Results**

**Basal parameters**

In table 1 the data concerning the basal outflow samples (DPM), the absolute fractional release $FR_2$ (DPM), the total tritium amount contained by the organ at the start of the experiments (DPM), the fractional release $FR_2$ (as % total tritium) and the relative response to $S_3$ compared to $S_2$ ($FR_3/FR_2$) are summarized for the control experiments.

<table>
<thead>
<tr>
<th>Basal outflow (DPM)</th>
<th>Total tritium content (DPM)</th>
<th>Absolute value $FR_2$ (DPM)</th>
<th>$FR_2$ (% of total at $S_2$)</th>
<th>$FR_3/FR_2$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.29 ± 0.66 x 10³</td>
<td>4.55 ± 0.59 x 10³</td>
<td>1.15 ± 0.14 x 10⁴</td>
<td>2.61 ± 0.17</td>
<td>0.70 ± 0.02</td>
<td>7</td>
</tr>
</tbody>
</table>

**Experiment 1: Angiotensin II and EFS-evoked tritium-label outflow**

Angiotensin II (1 nM – 0.1 μM) did not influence the basal tritium efflux (data not shown). In contrast, angiotensin II caused a concentration-dependent increase of EFS-evoked noradrenaline spillover. All concentrations applied caused a significant enhancement of the spillover. The maximal facilitation, by angiotensin II 10 nM, amounted to 66.9 % ($FR_3/FR_2$ 1.18 ± 0.04, n=9). Angiotensin II 0.1 μM evoked less than maximal enhancement of EFS-evoked sympathetic outflow, resulting in a ‘bell-shaped’ curve (figure 1).
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Experiment 2: Irbesartan, PD123319 and angiotensin II-facilitated [\(^3\)H]noradrenaline release

The highest concentrations of irbesartan (1 μM) and PD123319 (10 nM) neither influenced the basal efflux of tritium (data not shown) nor the EFS-evoked tritium spillover (FR\(_3\)/FR\(_2\) 0.71 ± 0.12 and 0.71 ± 0.14, respectively) (n=4, p>0.05). Moreover, PD123319 (10 nM) did not influence the angiotensin II-facilitated (10 nM) EFS-evoked noradrenaline spillover (FR\(_3\)/FR\(_2\) 1.16 ± 0.07) (figure 2). Irbesartan, however, concentration-dependently attenuated the subsequent angiotensin II-mediated (10 nM) enhancement of EFS-evoked sympathetic outflow. After exposure to the two lowest concentrations of irbesartan (0.1 and 1 nM) the noradrenaline spillover did not significantly differ from the EFS-evoked spillover in the presence of angiotensin II (10 nM) alone. Conversely, at the higher three concentrations used (10 nM - 1 μM) the AT\(_1\)-antagonist significantly inhibited the angiotensin II-mediated responses (p<0.05). The IC\(_{50}\) value, which is the concentration of irbesartan that causes 50% reduction of the enhancement of EFS-evoked spillover by angiotensin II 10 nM, amounted to -7.99 ± 0.03 (expressed as log M).

![Figure 1](image-url)  

**Figure 1.** Enhancing effect of angiotensin II (1 nM – 0.1 μM) on the EFS-evoked [H]-noradrenaline outflow from isolated rat vena cava inferior preparations. The preparations were stimulated at 24-min intervals. Angiotensin II was added to the organ bath 150 seconds before S\(_3\). The ratio between fractional releases evoked by S\(_3\) (FR\(_3\)) and S\(_2\) (FR\(_2\)) is shown on the ordinate, for controls and angiotensin II, respectively. Columns represent means ± SEM. □, control; ■, angiotensin II. Asterisk indicates p<0.05 compared with control (ANOVA followed by Dunnett’s test, n=6-9 per group).
**Experiment 3: Irbesartan combined with PD123319 and angiotensin II-facilitated tritium-label outflow**

AT₁-inhibition by irbesartan (1 and 10 nM) combined with AT₂-blockade by PD 123319 (10 nM) could influence angiotensin II-mediated facilitation. As in the experiment with irbesartan alone, irbesartan 10 nM (in combination with PD123319 10 nM) significantly reduced the angiotensin II-enhanced noradrenaline spillover. However, when compared to the inhibitory effect of irbesartan alone, the combination of PD123319 10 nM and either concentration of irbesartan (1 and 10 nM) inhibited angiotensin II-mediated facilitation to the same extent (figure 3).

**Figure 2.** Inhibitory effect of irbesartan (0.1 nM – 1 μM) and PD123319 (10 nM) on the facilitation by angiotensin II (10 nM) of EFS-evoked [3H]-noradrenaline outflow from rat isolated vena cava inferior. The veins were stimulated at 24-min intervals. Angiotensin II (10 nM) in the presence or absence of irbesartan or PD123319 was added to the organ bath 150 seconds prior to S3. The antagonists were added 20 min before S3. The ratio between fractional releases evoked by S1 (FR₁) and S2 (FR₂) is shown on the ordinate, the concentrations of the receptor antagonists on the abscissa. □ angiotensin II (10 nM); □ angiotensin II + PD123319; ■ angiotensin II + irbesartan. Columns represent the mean ± SEM. Asterisk indicates p<0.05 compared with the angiotensin II group (ANOVA followed by Dunnett’s test, n=5-9 per group).
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Figure 3. Inhibitory effect of irbesartan (1 nM and 10 nM) combined with PD123319 (10 nM) □ , compared to irbesartan alone ■ , on the facilitation by angiotensin II (10 nM) of EFS-evoked [3H]-noradrenaline outflow from rat vena cava inferior. The veins were stimulated at 24-min intervals. Angiotensin II (10 nM) in the presence of irbesartan or the combination irbesartan/PD123319 was added to the organ bath 150 seconds prior to S3. The antagonists were added 20 min before S3. The ratio between fractional releases evoked by S3 (FR3) and S2 (FR2) is shown on the ordinate, the concentrations of the receptor antagonists (log M) on the abscissa. Data are presented as means ± SEM. Asterisk indicates p<0.05 compared with the angiotensin II/irbesartan 0.1 nM group (ANOVA followed by Dunnnett’s test, n=5-9 per group). To evaluate statistical significance between irbesartan and the combination irbesartan/PD123319 groups Student’s t-test (two-tailed, unpaired) was used.

Discussion

We deliberately compared the sympatho-inhibitory effects of AT1-blockade by irbesartan alone on the one hand, and combined AT1/AT2-blockade by irbesartan plus PD123319 on angiotensin II-augmented sympathetic nerve traffic on the other hand. We observed no difference between the two approaches. Accordingly, we conclude that the AT2-receptor is not involved in prejunctural facilitation in the present in vitro investigation. The inability to demonstrate a U-shaped concentration-effect relationship for irbesartan supports this conclusion.

Previously, we demonstrated in the pithed rat model that the increase in diastolic blood pressure (DBP) to electrical stimulation of the thoraco-lumbar spinal cord could be dose-dependently inhibited by selective AT1-receptor blockade16,18. However, the highest dose of irbesartan (60
mg/kg) caused less than maximal attenuation of the rise in DBP. We hypothesized that unmasking of a latent population of AT₂-receptors, that mediates further facilitation, might explain our observations. Indeed, the selective AT₂-blocker PD123319 (5 mg/kg + 50 µg/kg/min) could abolish the upstroke when co-administered with irbesartan.

In the present study, as to be expected, angiotensin II (1 nM – 0.1 μM) caused a concentration-dependent enhancement of EFS-evoked sympathetic nerve traffic, with a maximum of approximately 67% (at 10 nM) (figure 1). Previous studies reported similar magnitudes. In numerous vascular and cardiac tissues angiotensin II–enhanced sympathetic transmission varied from approximately 60% to 90%\(^{10,21}\). Additionally, we observed a ‘bell-shaped’ concentration-effect relationship, as was shown previously\(^{13,19,21}\). This rather curious phenomenon might be explained by the concomitant release of anti-facilitatory prostaglandins\(^{33}\). PD123319, in a concentration known to block the AT₁-receptor (10 nM)\(^{5}\), did not influence the augmentation induced by angiotensin II (figure 2). Therefore, it appears that the AT₁-receptor does not play a significant role in angiotensin II-mediated facilitation, as was previously reported\(^{13,24}\). Conversely, irbesartan concentration-dependently attenuated angiotensin II-enhanced, EFS-evoked sympathetic transmission to baseline levels (figure 2), thus confirming the general view that angiotensin II-facilitated sympathetic nerve traffic is mediated through the AT₁-receptor subtype\(^{11,15,25}\).

The sympatholytic potency (pIC\(_{50}\)) of irbesartan amounted to 7.99 ± 0.03. We previously reported a pIC\(_{50}\) value of irbesartan of 9.20 ± 0.03 in the same model using rabbit thoracic aortic ring preparations \(^{11}\). This discrepancy might be explained by species differences (rat versus rabbit). However, the angiotensin II concentration used in the present study (10 nM) differed a tenfold (1 nM) compared to concentration used previously, which is more likely to be responsible. Interestingly, we did not observe a U-shaped concentration-response relationship for irbesartan in contrast to our previous findings with high-dose irbesartan in the pithed rat model \(^{16,18}\). However, several in vitro studies, by us and others, investigating the pharmacological properties of AT₁-receptor antagonists concerning angiotensin II-enhanced transmission neither showed an U-shaped concentration-response relationship\(^{12,13,20}\).

Several explanations can be thought of for these contrasting findings. First, the prejunctional AT₁-receptor might be (functionally) absent in vitro, possibly by down-regulation, as has been described for murine neuronal cells\(^{20}\). Similarly, in the pithed rat, postjunctional \(\alpha_2\)-adrenoceptor-mediated vasoconstriction can be demonstrated to occur\(^{27}\). By contrast, functional \(\alpha_2\)-
adrenoceptors cannot be demonstrated \textit{in vitro} in various models\textsuperscript{28,29}. Similarly, the same concept of \textit{in vivo} / \textit{in vitro} differences may explain the lack of "upstroke" observed in the current study. Conversely, at the postjunctional site the AT\(_2\)-receptor appears to remain present, since angiotensin II (with AT\(_1\)-blockade) induced a concentration-dependent relaxation in rat isolated mesenteric arteries, which could be inhibited by PD123319\textsuperscript{27}. Second, the maximal concentration of irbesartan (1 \(\mu\)M) used in the present study may not be sufficient to unmask a latent population of AT\(_2\)-receptors and hence no upstroke can be observed. This explanation, however, is very unlikely regarding the plateau that was reached using the two highest concentrations of irbesartan.

As suggested by several authors the beneficial effects of AT\(_1\)-receptor blocker (ARB) therapy in hypertension and heart failure may, at least partly, be explained by angiotensin II-mediated stimulation of the unopposed AT\(_2\)-receptor\textsuperscript{31,33}. We therefore attempted to demonstrate a role for the putative AT\(_2\)-receptor in angiotensin II-mediated facilitation by (partly) blocking the prejunctional AT\(_1\)-receptor. When irbesartan (1 and 10 nM) was administered together with PD123319 (10 nM) (combined AT\(_1\) and AT\(_2\) blockade), the sympatholytic effects of irbesartan were not different compared to irbesartan-mediated inhibition alone (figure 3). Consequently, as suggested earlier, it appears that the AT\(_2\)-receptor is not present at the level of the sympathetic nerve terminals \textit{in vitro}.

In conclusion, the facilitating effect of angiotensin II on EFS-evoked sympathetic nerve traffic is mediated exclusively by prejunctionally located AT\(_1\)-receptors. The angiotensin II-mediated enhancement could be concentration-dependently antagonized (pIC\(_{50}\) 7.99 ± 0.03) by the selective AT\(_1\)-receptor antagonist irbesartan. The selective AT\(_2\)-receptor antagonist PD123319, either alone or in combination with (partial) AT\(_1\)-blockade (irbesartan), proved unable to influence angiotensin II-mediated facilitation. Therefore, we conclude that the AT\(_2\)-receptor is not involved in angiotensin II-mediated enhancement of sympathetic nerve traffic \textit{in vitro}. 

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References


