Angiotensin neuromodulation of adrenergic and purinergic co-transmission in the guinea-pig vas deferens

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1 The effects of angiotensin II (AII) and angiotensin III (AIII) on the isolated vas deferens of the guinea-pig were studied via three parameters: the overflow of adenosine 5'-triphosphate (ATP) and tritiated noradrenaline (NA), the mechanical response to field stimulation and the mechanical response to exogenous NA and α,β -methylene ATP (α,β -mATP).

2 At 2 Hz, AII enhanced the overflow of ATP and NA, whereas at 20 Hz, AII enhanced the overflow of NA but was without significant effect on ATP overflow. AIII, at 2 Hz, inhibited the overflow of ATP, but enhanced NA overflow, whereas at 20 Hz ATP overflow was unaffected, but NA overflow was still enhanced.

3 At 2 Hz, AII enhanced both phases of the response to field stimulation and at 20 Hz the overall response. AIII at 2 Hz enhanced the adrenergic response, but was without effect on the purinergic response to field stimulation. At 20 Hz, AIII was without effect on the overall response.

4 AII enhanced responses to exogenous NA and α,β -mATP, whereas AIII was without effect.

5 These results provide evidence that both ATP and NA release are capable of being modulated by angiotensins. Furthermore, modulation of ATP release is frequency-dependent, whereas [³H]-NA release is not. These results raise questions about the mechanisms of storage and release of the sympathetic co-transmitters NA and ATP; they also show that angiotensin receptors in the guinea-pig vas deferens are not a homogeneous population.

Introduction

The guinea-pig vas deferens has been widely used for the study of the action of co-transmitters. The neurogenic response of the vas deferens is biphasic, consisting of an initial twitch response and a slow second component. This second phase is adrenergic in nature (Swedin, 1971), whereas the twitch response has been shown to be non-adrenergic, non-cholinergic in nature and is due to the release of adenosine 5'-triphosphate (ATP) (Levitt *et al.*, 1984; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1985; Kirkpatrick & Burnstock, 1987; Kasakov *et al.*, 1988). ATP and noradrenaline (NA) are stored together in storage vesicles of sympathetic nerve terminals (Lagercrantz & Stjärne, 1974; Lagercrantz, 1976; Fried *et al.*, 1978). ATP release in response to neurogenic stimulation of the guinea-pig vas deferens has been shown in a number of studies (Kirkpatrick & Burnstock,

1987; Lew & White, 1987; Kasakov *et al.*, 1988). ATP which is released originates from sympathetic nerve terminals and its release is blocked by tetrodotoxin (Kirkpatrick & Burnstock, 1987). ATP release from guinea-pig vas deferens has been shown to be of neuronal origin as release is not affected with the nerves intact and with the mechanical response blocked by α,β -methylene ATP (α,β -mATP) and prazosin (Kasakov *et al.*, 1988), nor is ATP release affected following depletion of NA by reserpine pretreatment, suggesting that ATP release is not secondary to NA release (Kirkpatrick & Burnstock, 1987).

In the early 1960s, angiotensin II (AII) was shown to facilitate adrenergic neurotransmission (McCubbin & Page, 1963; Benelli *et al.*, 1964). This was shown in the guinea-pig vas deferens to be a direct effect, only facilitating NA release when the nerves were actively firing (Bell, 1972). More recently, the pharmacological effects of AII, and its active

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metabolite, angiotensin III (des-Asp¹-angiotensin II) (AIII), have been studied on purinergic and adrenergic neurotransmission in the rabbit vas deferens (Trachte *et al.*, 1984; Trachte, 1988). The latter study drew conclusions on ATP and NA release by analysis of the mechanical response of the vas deferens to field stimulation.

The purpose of this study was to examine the action of AII and AIII on neurotransmission in the guinea-pig vas deferens by directly measuring the release of endogenous ATP and the release of radio-labelled NA. The actions of these substances on the mechanical response to field stimulation and the pharmacological response to exogenously added α , β -mATP and NA were also studied.

Methods

General

Male guinea-pigs (250–400 g) were stunned and exsanguinated. The prostatic portions of the vasa deferentia were excised and stripped of connective tissue. Following incubation (60 min) with [³H]-NA, the isolated preparations (10–20 mg wet weight) were placed between pairs of platinum ring electrodes (15 mm apart) and were allowed to equilibrate, with one end tied to the holder and the other attached to a Grass force-displacement transducer (FTO3 C). Contractions were measured as changes in isometric tension by a Washington data recording system. The preparations, at an initial tension of 0.5–1 g, were superfused at a flow rate of 2.5 ml min⁻¹ by use of a Watson-Marlow peristaltic pump with a modified Krebs solution (MKS) (mm: NaCl 133, KCl 4.7, CaCl₂ 2.5, MgSO₄ 2.5, NaH₂PO₄ 1.4, NaHCO₃ 16.3, glucose 7.7 and ascorbic acid 0.1 and gassed continuously with 5% CO₂ in O₂ at 36°C). After a 90 min equilibration period the superfusate was collected, before, during and after stimulation, in polypropylene vials for subsequent determination of [³H]-NA and ATP levels. The preparations were stimulated electrically twice, at 9 and 45 min for 240 pulses with trains of rectangular pulses of 0.1 ms duration, 50 V, and either at 2 or 20 Hz delivered by Grass SD9 stimulators. Perfusate was collected for periods corresponding to the length of the stimulus train. Therefore at 2 Hz the collection period was 120 s, whereas at 20 Hz the collection period was 12 s. Results are expressed as the ratio of responses to the second stimulation (S₂) versus responses to the first stimulation (S₁), that is the S₂/S₁ ratio. Control values for S₂/S₁ ratios were obtained when the second stimulation was carried out in the absence of any drug. Experimental data were obtained by the

superfusion of the preparation with MKS containing the angiotensins 15 min before the second stimulation. This superfusion continued until the end of the experiment. Tissues from different animals were used to assess the effects of the angiotensins.

ATP overflow

ATP overflow from the sympathetic nerves supplying the vas deferens was assayed with the luciferin-luciferase technique described by Stanley & Williams (1969). Briefly, 50 mg of luciferin-luciferase was dissolved in 12.5 ml of sterile water and left in the dark for 2 h to equilibrate. Under these conditions the assay was capable of measuring ATP levels as low as 30 fmol. Prior to the experiment, a calibration curve for ATP was prepared to be used as a standard. Automatic injection of 200 μ l of luciferin-luciferase solution to 100 μ l of superfusate or standards produced bioluminescence which was measured by a Packard Picolite Luminometer. The MKS was assayed for ATP content. This was used as a blank and subtracted from each sample.

[³H]-noradrenaline overflow

After preparation (described above), the tissues were incubated for 60 min in continuously gassed MKS containing 0.1 nM tritium-labelled noradrenaline ([³H]-noradrenaline, New England Nuclear; specific activity 1624.3 GBq mmol⁻¹; two preparations in 5 ml medium) at 36°C. After rinsing five times with MKS, the preparations were placed between the stimulating electrodes and superfused to wash out the excess [³H]-NA during the 90 min equilibration period. Samples (2.5 ml for 2 Hz and 0.25 ml for 20 Hz) were added in a ratio of 1:3 to Optiphase mp (LKB) scintillation cocktail and ³H-radioactivity in the samples was counted in a Beckman 7500 liquid scintillation system. The superfusion was stopped immediately after the end of the experiment and the preparations were weighed and dissolved in 0.5 ml Optisolv (LKB) tissue solubilizer. The total tissue [³H]-NA radioactivity was counted as described above. The results were calculated as the percentage fractional rate (FR%; mean $\pm$ s.e.mean) of the basal release and stimulation-evoked release of [³H]-NA which were calculated according to Alberts *et al.* (1981). Previous investigations in rodent vas deferens have shown that [³H]-NA is the major constituent of the total radioactivity released upon stimulation of the tissue (Beattie *et al.*, 1986; Kapocsi *et al.*, 1987). Hence the evoked efflux of [³H]-NA reflected a quasi-physiological release of NA (Reichenbacher *et al.*, 1982).

Pharmacological study

Prostatic portions of the vas deferens were tied to tissue holders and placed in 5 ml organ baths containing MKS at 36°C and equilibrated for 90 min. After the equilibration period, either NA or α,β -mATP at bath concentrations of 30 μ M and 1 μ M respectively were added for short periods every 15 min. The tissues were washed as soon as a maximum response was reached to prevent desensitization. α,β -mATP was used as it is a stable analogue of ATP. After establishing control responses to agonists, the angiotensins were added 10 min before a further addition of NA or α,β -mATP. Results were calculated as the percentage response to each agonist relative to the previous response when no angiotensins were present.

Analysis of results

The effects of the angiotensins were assessed by the comparison of S_2/S_1 ratios for the experimental data versus S_2/S_1 ratios for the control data. The results for ATP overflow, [3 H]-NA overflow and mechanical responses were evaluated by Student's *t* test for unpaired observations. Results from the pharmacological study were evaluated by Student's *t* test for paired observations. In both cases, probability less than 0.05 was considered statistically significant.

Drugs

Angiotensin II, angiotensin III, noradrenaline, α,β -methylene ATP and luciferin-luciferase were obtained from Sigma. AII, AIII and α,β -mATP were first prepared as stock solutions in distilled water. NA was prepared in a 0.4% ascorbic acid solution to prevent oxidation. Luciferin-luciferase was prepared in sterile water.

Results

Field stimulation

Control responses The contractile response of the vas deferens to electrical field stimulation (EFS) at 2 Hz (stimulation period lasting 2 min) exhibited two distinct phases: an initial twitch response which reached its maximum in 2–3 s and then faded rapidly and a second slow phase which reached its maximum in 10–15 s and whose amplitude slowly decreased, but which lasted for the duration of the stimulation period. The second stimulation period, 30 min later, exhibited similar contractions but both phases were slightly reduced in amplitude (S_2/S_1 ratio for 1st phase was 0.81 ± 0.09 , 2nd phase was

Figure 1 Effects of angiotensin II (AII) and angiotensin III (AIII) on the overflow of ATP from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of ATP are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) Overflow at 2 Hz in the presence of AII (100 nM) compared to control (C, stippled columns). (b) Overflow at 20 Hz in the presence of AII (100 nM) compared to control. (c) Overflow at 2 Hz in the presence of AIII (100 nM) compared to control. (d) Overflow at 20 Hz in the presence of AIII (100 nM) compared to control. Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by using Student's *t* test for unpaired observations (NS = not significant, ***P* < 0.01).

0.84 ± 0.06 , $n = 6$) (Figure 3). During both stimulation periods there was a marked rise in [3 H]-NA and ATP overflow over basal levels. [3 H]-NA overflow was little affected during the second stimulation (S_2/S_1 ratio was 1.05 ± 0.06 , $n = 6$) (Figure 2). However, ATP overflow during the second phase was reduced (S_2/S_1 ratio was 0.50 ± 0.06 , $n = 6$) (Figure 1).

At 20 Hz (stimulation period lasting 12 s) the distinction between the two phases was not clear. Therefore mechanical responses are given as the overall magnitude of the response and not divided into two distinct phases. The magnitude of the response elicited by the second stimulation was unchanged (S_2/S_1 ratio was 1.02 ± 0.03 , $n = 6$) (Figure 3). Again during both stimulation periods there was a marked rise in [3 H]-NA and ATP overflow over basal levels. [3 H]-NA release elicited by stimulation was unchanged during the second stimulation (S_2/S_1 ratio was 0.95 ± 0.06 , $n = 6$) (Figure 2). ATP overflow was only slightly diminished during the second stimulation (S_2/S_1 ratio was 0.85 ± 0.11 , $n = 6$) (Figure 1).

Figure 2 Effects of angiotensin II (AII) and angiotensin III (AIII) on the overflow of [³H]-noradrenaline ([³H]-NA) from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of [³H]-NA are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) Overflow at 2 Hz in the presence of AII (100 nM) compared to control (C, stippled columns). (b) Overflow at 20 Hz in the presence of AII (100 nM) compared to control. (c) Overflow at 2 Hz in the presence of AIII (100 nM) compared to control. (d) Overflow at 20 Hz in the presence of AIII (100 nM) compared to control. Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by using Student's *t* test for unpaired observations (***P* < 0.01, ****P* < 0.001).

Angiotensin II The addition of angiotensin II (AII) (100 nM) to the superfusate 15 min before the second stimulation had no effect on the baseline tension of the preparations. At 2 Hz, when compared to the control values, AII enhanced both phases of the response. The S_2/S_1 ratio for the purinergic phase of the response was 1.06 ± 0.07 ($n = 8$) (Figure 3a) and for the adrenergic phase it was 1.09 ± 0.09 ($n = 6$) (Figure 3b). AII at this frequency also enhanced, relative to the second control stimulation, the stimulus-evoked overflow of both [³H]-NA (S_2/S_1 ratio was 1.72 ± 0.19 , $n = 6$) (Figure 2a) and of ATP (S_2/S_1 ratio was 0.80 ± 0.06 , $n = 6$) (Figure 1a). At 20 Hz, AII enhanced the magnitude of the response (S_2/S_1 ratio was 1.15 ± 0.04 , $n = 6$) (Figure 3c). At this frequency, AII enhanced the overflow of [³H]-NA (S_2/S_1 ratio was 1.54 ± 0.16 , $n = 6$) (Figure 2b); however, compared to controls it was without significant effect on ATP overflow (S_2/S_1 ratio was 0.99 ± 0.13 , $n = 6$) (Figure 1b).

Angiotensin III Angiotensin III (AIII) (100 nM) did not affect the baseline tension of the preparations. At 2 Hz, compared to controls, AIII significantly enhanced the adrenergic phase of the response (S_2/S_1 ratio was 1.00 ± 0.04 , $n = 6$) (Figure 3b); however, it

Figure 3 Effects of angiotensin II (AII) and angiotensin III (AIII) on the mechanical response of the guinea-pig vas deferens to electrical field stimulation. Alterations in the mechanical responses are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) The purinergic response at 2 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control (C, stippled columns). (b) The adrenergic response at 2 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control. (c) The total response at 20 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control. Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by Student's *t* test for unpaired observations (NS = not significant, **P* < 0.05).

was without significant effect on the fast initial purinergic phase (S_2/S_1 ratio was 0.90 ± 0.03 , $n = 6$) (Figure 3a). At this frequency, AIII enhanced the stimulus-evoked overflow of [³H]-NA (S_2/S_1 ratio was 1.58 ± 0.09 , $n = 6$) (Figure 2c), whereas it decreased the overflow of ATP in response to EFS (S_2/S_1 ratio was 0.16 ± 0.06 , $n = 6$) (Figure 1c). At 20 Hz, relative to control values AIII did not alter the mechanical response of the tissue to EFS (S_2/S_1 ratio was 1.02 ± 0.06 , $n = 6$) (Figure 3c). At this frequency AIII enhanced the stimulus-evoked overflow of [³H]-NA to EFS (S_2/S_1 ratio was 1.72 ± 0.19 , $n = 6$) (Figure 2d). It was however, compared to controls, without significant effect on ATP overflow (S_2/S_1 ratio was 0.75 ± 0.07 , $n = 6$) (Figure 1d).

Pharmacological responses

Responses to exogenously added NA (30 μ M) and α, β -mATP (1 μ M) did not show tachyphylaxis provided the tissues were washed immediately the

Figure 4 Effects of angiotensin II (AII) and angiotensin III (AIII) on the response of guinea-pig isolated vas deferens to noradrenaline (NA) and exogenous α,β -methylene ATP (α,β -mATP). Responses to drugs are compared to the control value (C, stippled columns). (a) Responses to NA in the presence of AII (100 nM) and AIII (100 nM). (b) Responses to α,β -mATP in the presence of AII (100 nM) and AIII (100 nM). Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by Student's *t* test for paired observations (NS = not significant, **P* < 0.05, ****P* < 0.001).

responses reached their maximum. AII (100 nM) added 10 min before the addition of NA produced a slight increase in the response to a value $109 \pm 3\%$ of the control level. It produced a large increase in the response to α,β -mATP ($141 \pm 7\%$ of the control value). AIII (100 nM) did not significantly affect responses to either NA or α,β -mATP, respective values being 102 ± 4 and $98 \pm 6\%$ of the control responses (Figure 4).

Discussion

Interpretation of these results should take into account pre- and postjunctional interactions of NA, ATP and adenosine, resulting from ecto-enzymatic breakdown of ATP. There is also the possibility of an interaction with the neuromodulator neuropeptide Y (NPY). NPY is known to coexist with NA and ATP (Fried *et al.*, 1985; Stjärne *et al.*, 1986) and to be released along with NA and ATP upon stimulation of sympathetic nerves of the guinea-pig vas deferens (Kasakov *et al.*, 1988). NPY has a dual function; postjunctionally at low concentrations

potentiating NA- and ATP-mediated responses and at higher concentrations producing prejunctional inhibition of release of NA and ATP (Stjärne & Lundberg, 1986). It is therefore possible that the angiotensins, by affecting the release of NPY, may have subsequent effects on NA and ATP release and their postjunctional effects. Confirmation of any involvement of NPY will have to await a study on the effect of angiotensins on NPY release. There is also a possible interaction with the autoinhibition by adenosine and NA itself. Adenosine is known to inhibit the release of both ATP and NA (Sneddon *et al.*, 1984), whereas NA inhibits its own release (see Starke, 1987).

The biphasic nature of the response and the concomitant release of both [3 H]-NA and endogenous ATP confirms previous studies that the response of this tissue to field stimulation is due to NA and ATP acting as co-transmitters (see Burnstock, 1982; Burnstock, 1985). Many investigations into the action of angiotensins on sympathetic nerve stimulation in the vas deferens (Hughes & Roth, 1971; Bell, 1972; Johnson *et al.*, 1974) were carried out before the concept of co-transmission was raised (Burnstock, 1976). These investigations therefore were only concerned with the enhancement/inhibition of adrenergic transmission. More recently some experiments have been carried out on the effects of angiotensins on both purinergic and adrenergic components of the rabbit vas deferens to sympathetic nerve stimulation (Trachte *et al.*, 1984). However, these studies deduced angiotensin effects on release by the indirect method of analysing pharmacological studies which have the inherent problems of pre- and postjunctional interactions. Neither in this paper nor in a later study (Trachte, 1988) was ATP release measured, either by direct measurement of endogenous ATP by the luciferin-luciferase technique (Stanley & Williams, 1969) or by the indirect method of labelling with [3 H]-adenosine. This latter method however, cannot exclude uptake and release from non-neuronal as well as neuronal sources. By directly measuring endogenous ATP release in the present study, this problem has been overcome.

Purinergic transmission

Against low-frequency stimulation (2 Hz), angiotensins differed in their effects on ATP overflow. They also differed in that AII enhanced responses of the agonists postjunctionally, whereas AIII was without effect. However, at high frequency (20 Hz) the angiotensins were without significant effect on ATP overflow. The purinergic component of the mechanical response was enhanced by AII but unaffected by AIII at 2 Hz. This enhancement of the

mechanical response by AII is to be expected, as AII enhances both the overflow of ATP and the action of ATP on its postjunctional receptors. Surprisingly, AIII does not reduce the mechanical response, although it inhibits the overflow of ATP and is without postjunctional effects. At present this observation is difficult to explain. Our results show some similarity to recent studies carried out in the rabbit vas deferens (Saye *et al.*, 1986; Trachte, 1988). The latter study, as in the present study, showed that at 2 Hz the angiotensins enhanced the adrenergic phase of the response. However the reported inhibition of the purinergic phase by both of the angiotensins was not repeated in this study, although AIII has been shown to inhibit the overflow of ATP. Saye *et al.* (1986) reported that at low frequencies AII potentiated both phases of the response as is seen in the present study, whereas AIII inhibited both phases of the response. Interestingly, Saye *et al.* (1986) found that the inhibition by AIII was turned into a potentiation in the presence of indomethacin. As both studies mentioned used the same tissue and stimulation parameters it is difficult to explain the discrepancy. We conclude that AII and AIII have opposing actions on ATP release at low frequency. This is probably due to an action at different angiotensin receptors situated prejunctionally (Trachte *et al.*, 1984; Trachte, 1988). Moreover, angiotensin neuro-modulation is frequency-dependent, as at high frequencies the angiotensins do not affect ATP release. These results also provide evidence for receptors located postjunctionally for the angiotensins. At these receptors AII markedly enhances the responses to α, β -mATP and slightly to NA, whereas AIII would seem to have no affinity for these receptors.

Adrenergic transmission

Both angiotensins enhance [3 H]-NA overflow at both low and high frequencies. AII enhances slightly the response to exogenous NA, whereas AIII has no effect. Mechanical responses to field stimulation at 2 Hz bear out this enhancement. However, the response at 20 Hz, which is due to both purinergic and adrenergic components, shows that AII enhances the mechanical response to field stimulation, whereas AIII has no effect. As neither angiotensin affects ATP overflow, at this frequency, the mechanical response will be due to the actions of the angiotensins on NA overflow and their postjunctional effects if any. At 20 Hz, it is likely that more NA is being released than is required to produce the maximal response. Therefore the enhancement seen by AII is probably due to the postjunctional enhancement of ATP and NA action, whereas AIII does not have any effects postjunctionally. The angiotensins have been shown to facilitate adrenergic

transmission in a variety of tissues and species (Magnan & Regoli, 1979; Boke & Malik, 1983; Trachte, 1988) and at low and high frequencies (Zimmerman *et al.*, 1972). By measuring [3 H]-NA release, our study shows that facilitation of adrenergic transmission by angiotensins can be attributed to an increase in the amount of NA released and is not due to a postjunctional action.

The lack of effect of the angiotensins on the resting tension suggests that the angiotensins, at least at the concentrations used, are without direct effect on the smooth muscle of the vas deferens.

The reason for the disparate effects of angiotensins on ATP and NA release is unclear. It is possible that in the vas deferens there are nerves that are predominantly purinergic and those that are predominantly adrenergic. This hypothesis could also be used to explain the observation that adrenergic transmission is predominant at the epidymal end and purinergic transmission is predominant at the prostatic end of the rodent vas deferens (McGrath, 1978; Rohde *et al.*, 1986). On the other hand, NA and ATP are known to be stored in small dense-cored vesicles and in large vesicles (Lagercrantz & Stjärne, 1974; Lagercrantz, 1976; Fried *et al.*, 1978), and so a neuromodulator may alter release from one type of vesicle in one way and the release from another type in another. Also the ratio of small vesicles to large vesicles may vary, as may the ratio of catecholamine to ATP in the vesicles within different nerves or even within the same nerve terminal. Large vesicles may contain both ATP and NA or there may be vesicles containing only NA and vesicles containing only ATP, as has been suggested for NPY and NA storage (Fried *et al.*, 1985).

In summary, angiotensins were found to have differing actions at low frequency and no effect at high frequency on purinergic transmission in the guinea-pig vas deferens. In contrast, both angiotensins enhance adrenergic transmission at low and high frequency. This action is predominantly prejunctional as shown by the direct method of measurement of release of transmitters, although AII also facilitates transmission postjunctionally. The different actions of the angiotensins suggests that they are acting prejunctionally on different receptor subtypes, with different affinities for AII and AIII. The opposing effects on purinergic and adrenergic transmission by the angiotensins suggest that ATP and NA storage and/or release are not similar in the guinea-pig vas deferens. This is borne out by the finding that ATP neuromodulation by angiotensins is frequency-dependent, whereas NA modulation is not. Our results provide the first direct evidence of the effects of angiotensins on both ATP and NA release and the role of frequency of stimulation on neuromodulation.

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