Effect of Adenosine on Membrane Potential and Ca^{2+} in Juxtaglomerular Cells

Comparison with Angiotensin II

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Key Words

Adenosine • Afferent arteriole • Angiotensin II •
Cytoplasmic free calcium • Membrane potential •
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Abstract

Background: Renin is mainly secreted from the juxtaglomerular cells (JGC) in the kidney situated in the afferent arteriole close to the vessel pole. Angiotensin II (ANG II) and adenosine inhibit renin secretion and synergistically constrict the afferent arteriole. ANG II depolarises JGC and increases the cytoplasmic free Ca^{2+} concentration $[\text{Ca}^{2+}]_i$. The responses of JGC to adenosine are less known. **Methods:** Effects of adenosine on membrane potential and $[\text{Ca}^{2+}]_i$ were studied in afferent arterioles from NaCl-depleted rats and mice. **Result:** Stimulation of A_1 adenosine receptors (A_1AR) by adenosine (10 μM) or cyclohexyladenosine (1 μM) increased the spiking frequency of JGC, slightly depolarised the cells and, in $\leq 50\%$ of the cases, increased $[\text{Ca}^{2+}]_i$. These effects were much smaller than those of ANG II (3 nM). Simultaneous application of cyclohexyladenosine and ANG II gave only additive effects on $[\text{Ca}^{2+}]_i$; in addition, responses to ANG II in JGC from A_1AR knockout mice were similar to those from control mice. **Conclusion:** The small changes in membrane potential and $[\text{Ca}^{2+}]_i$ in response to A_1AR stimulation as com-

pared to those of ANG II may suggest that these 2 tissue hormones use different signal transduction mechanisms to affect JGC function, including the inhibition of renin release.

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Introduction

Adenosine exerts various important functions in the kidney. It alters the tone of the afferent arteriole in response to changes in NaCl delivery at the macula densa, i.e. it is the mediator of tubuloglomerular feedback [1; for a review, see 2]. An increase in luminal NaCl concentration at the macula densa leads to a constriction of the afferent arteriole, thereby reducing the glomerular filtration rate. Adenosine acts via the A_1 adenosine receptor (A_1AR) which, by liberating the G protein $\text{G}_{i,\beta\gamma}$, activates phospholipase C (PLC) [3]. PLC, in turn, most probably generates inositol 1,4,5-trisphosphate (IP_3) leading to a mobilisation of Ca^{2+} from intracellular stores. In experiments using pressurised isolated afferent arterioles, adenosine (maximal effect typically at 1–10 μM) induces vasoconstriction [3, 4], and Gutiérrez et al. [5] have shown that adenosine (100 μM) induces a modest increase in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in this preparation. Pharmacological evidence [6] and the use of

genetically engineered mice lacking either the angiotensin II (ANG II) receptor type 1A [7] or the A₁AR [8, 9] have shown that the afferent arteriolar vasoconstrictor response to adenosine is potentiated by ANG II and vice versa, i.e. a synergistic interaction exists between the 2 tissue hormones in this respect [for reviews, see 2, 10, 11]. ANG II acts via the ANG II receptor type 1 coupled to G_q, leading to a final increase in [Ca²⁺]_i by the G_{qα}/PLC/IP₃ signalling pathway [12] and to membrane depolarisation.

Another important effect of adenosine in the kidney is the inhibition of renin secretion from the juxtaglomerular cells (JGC) [for reviews, see 2, 13, 14]. These cells are located in the media of the afferent arteriole near the entrance into the glomerulus [reviews: 15, 16] and they can transform into smooth muscle cells and vice versa according to the needs of the body [17, 18].

Renin secretion from JGC is under multiple control mechanisms. A striking feature is the inverse dependence of renin secretion on [Ca²⁺]_i, the so-called 'Ca²⁺ paradox' of renin secretion, i.e. an increase in [Ca²⁺]_i decreases the rate of renin secretion from JGC and vice versa [13, 15, 19].

The mechanism by which adenosine inhibits renin secretion is not entirely clear. In isolated JGC in culture, a partial inhibition of spontaneous renin release was obtained by adenosine (60% at 0.1 μM [20]) and by the selective A₁AR agonist cyclohexyladenosine (CHA; ~40% at 0.01 μM [21]). In the isolated perfused juxtaglomerular apparatus, CHA at 0.1 μM depressed the rise in renin secretion in response to low NaCl at the macula densa by 90% [22]. Hence, adenosine inhibits renin secretion via A₁AR activation. However, the subsequent steps in the signalling pathway remain to be defined. Since cAMP is a strong stimulator of renin release [for a review, see 19], inhibition of adenylyl cyclase by G_{iα} is a plausible possibility. On the other hand, adenosine (10 μM) has been shown to slightly depolarise JGC and to increase spontaneous spiking of the membrane potential [23], which may promote Ca²⁺ entry that in turn may inhibit renin secretion.

In this study, we investigated the effects of adenosine and CHA on membrane potential and [Ca²⁺]_i in isolated afferent arterioles near the glomerular vessel pole, where JGC are concentrated. In addition, we tested for a potential synergy between adenosine and ANG II. In order to increase the number of renin-positive cells, kidneys from NaCl-depleted rats were used, in which ~95% of the vessels are renin positive [24].

Methods

Animals

Animal experimentation was conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the German Law on the Protection of Animals. Sprague-Dawley rats (males, 250–500 g), CD-1 and C57 mice (males, 20–35 g) were used (Charles River, Germany). Additionally, mice lacking A₁AR and the respective control animals (A₁AR^{-/-}, A₁AR^{+/+} [9]) were used. These mice were bred in our laboratory and genotyped as previously described [25].

To induce NaCl depletion, rats and C57 mice were treated once with furosemide (rats: 10 mg/kg, i.p.; mice: 25 mg/kg, i.p.) and kept on an NaCl-depleted diet (0.015% Na⁺, 0.015% Cl⁻, C1036, Altromin, Germany) for at least 2 weeks after furosemide application. Control animals were not treated with furosemide and were kept on a standard diet (0.2% Na⁺, C1324, Altromin).

Preparation of Glomeruli

Animals were killed by cervical dislocation, exsanguinated and the kidneys removed. A freshly isolated kidney was transferred in a HEPES-buffered physiological salt solution (PSS) containing (in mM) NaCl 142, KCl 2.8, MgCl₂ 1, CaCl₂ 1, D(+)-glucose 11, buffered with HEPES (10 mM) and titrated to pH 7.4 with NaOH at 37°C. The kidney was decapsulated, cut longitudinally into 2 halves and the isolated cortex was minced with a razor blade. Glomeruli were prepared by modifications of published methods [26–28]. The minced tissue was incubated for 50 min in 20 ml PSS with 20 mg collagenase A (Roche Diagnostics, Germany) at 37°C under gentle shaking. The suspension was then passed through stainless-steel sieves with meshes of 200 and 125 μm. The final material was collected on a 63-μm (rats) or 43-μm (mice) sieve. Microscopic inspection showed that the preparations consisted mainly of glomeruli and some contamination with tubular fragments; in about 10% of the cases, a remnant of the afferent arteriole up to 100 μm in length remained attached to the glomerulus. Figure 1 shows the regions where measurements were performed.

Electrophysiology

Glomeruli were transferred to the recording chamber equipped with a poly-D-lysine-coated cover slide. The patch-clamp technique was used in the whole-cell configuration as described by Hamill et al. [29] and at 37°C. The bath solution was PSS. Patch pipettes were drawn from borosilicate glass capillaries (GC 150, Harvard Apparatus, UK) and heat polished using a horizontal micro-electrode puller (Zeitz, Germany). Pipettes were filled with (in mM) K-glutamate 135, NaCl 10, MgCl₂ 1, HEPES 10, EGTA 1, ATP 1, GTP 0.3, titrated to pH 7.2 with NaOH and had a resistance of 3–5 MΩ. Data were recorded with an EPC9 amplifier using the Pulse software package (HEKA, Germany). Potentials given in these experiments were corrected for a liquid junction potential of 10 mV [30].

Calcium Measurements

For measurement of [Ca²⁺]_i, fura-2 dye was used. Epifluorescence was measured with an inverted microscope (Diaphot 300, Nikon, Japan), equipped with a ×100 fluor oil immersion objective (numerical aperture, 1.3; Nikon) and an ImageMaster fluorescence imaging system (PTI, N.J., USA). Excitation wavelengths

were set to 340 and 380 nm, and the microscope was equipped with a dichroic mirror (430DCLPO2, Omega Optical, Vt., USA) and an interference barrier filter (510WB40, Omega Optical). Cells were loaded with the indicator by incubation of glomeruli in PSS with 16 μM fura-2 acetoxymethyl (Invitrogen, Germany), the membrane-permeable acetoxymethyl ester of fura-2 (stock solution 1 mM in DMSO), and 0.04% (v/v) pluronic F127 (stock solution 2% in DMSO) for 60 min at room temperature. After washing with PSS for 30 min, fluorescence measurements were performed by selecting an area of about 10–20 cells from the afferent arteriole near the entrance into the glomerulus (fig. 1). These cells are clearly distinguishable from smooth muscle cells which have a spindle-like appearance and a spiral arrangement around the vessel [15, 31]. About 38 pictures resulting in 19 ratios/min were recorded and stored. During washout periods, sampling frequency was reduced to avoid dye bleaching. The fura-2 fluorescence ratio (R) was calibrated following Gryniewicz et al. [32]. In accordance with other groups [33, 34], calibration was performed in the absence of glomeruli using 5 μM fura-2 pentapotassium salt. The following parameters were obtained: $R_{\min} = 0.52$, $R_{\max} = 15.41$, $Sf2/Sb2 = 10.32$, and the K_D value of Ca^{2+} binding to fura-2 was determined to be $192 \pm 18 \text{ nM}$ ($n = 3$). Due to the cell-free calibration procedure, the absolute values for $[\text{Ca}^{2+}]_i$ have to be taken with care.

In general, functional integrity of the cells was tested with an application of 3 nM ANG II for 1 min, and the size of the response was used as the internal standard for a second (test) stimulation. Response parameters were the response frequency, the maximal Ca^{2+} increase and the area under the curve of $[\text{Ca}^{2+}]_i$ versus time.

Calculations and Statistics

Data are shown as means $\pm$ SEM. Concentration-response relationships were evaluated by fitting the logarithmic form of the Hill equation

$$y = A / (1 + 10^{n(p_x - pEC_{50})})$$

to the data according to the method of least squares using the programme SigmaPlot 9 (Systat Software, Germany). Here A denotes the extent (amplitude) of $[\text{Ca}^{2+}]_i$ increase, n denotes the Hill coefficient and EC_{50} is the midpoint of the curve with $pEC_{50} = -\log EC_{50}$, x is the concentration of ANG II with $p_x = -\log x$. Due to the lognormal distribution of the EC_{50} values [35], the error is given by the 95% confidence interval in parentheses.

Statistical differences of the data were examined with t tests, rank-sum tests, ANOVA or χ^2 tests depending on the nature of the data. A difference was assumed to be significant if $p < 5\%$.

Results

Responses to $A_1\text{AR}$ and ANG II Receptor Stimulation

Animals on a sodium-depleted diet were used to increase the number of JGC in afferent arterioles. For patch-clamp recordings, cells with a roundish shape and strong granulation were selected at the vessel pole (fig. 1). In a first series ($n = 6$; fig. 2a) combined current and voltage clamp measurements were performed. CHA (1 μM) elic-

Fig. 1. Afferent arteriole with an enclosed erythrocyte. Note the roundish shape and the granulated appearance of the cells. The pipette approaching the distal part of the arteriole is seen on the right side. The region marked by the white line represents the area typically used for measurement of fura-2 fluorescence.

ited a very small inward current as compared to the large inward currents evoked by ANG II (10 nM). Inward current responses were mirrored by corresponding depolarisations and are a complex superposition of an increase in Cl^- and a decrease in K^+ efflux [36]. For a detailed documentation of the cellular response to adenosine or CHA, we focused on continuous current clamp measurements at high frequency. JGC had a resting membrane potential of $-57 \pm 2 \text{ mV}$ ($n = 17$). Figure 2b and c shows the response of JGC to stimulation by adenosine (10 μM), the $A_1\text{AR}$ -selective agonist CHA (1 μM) and ANG II (3 nM). All agonists induced a depolarisation in combination with increased spiking activity; however, the response to adenosine or CHA was much smaller than that to ANG II. Adenosine (10 μM) depolarised JGC by $5 \pm 1 \text{ mV}$ ($n = 10$) and CHA (1 μM) by $4 \pm 1 \text{ mV}$ ($n = 6$). The mean depolarisation induced by subsequent application of ANG II (3 nM) was $39 \pm 1 \text{ mV}$ ($n = 9$), independent of whether adenosine or CHA had been given previously.

Next, it was examined whether adenosine or CHA affected $[\text{Ca}^{2+}]_i$. Figure 1 shows the region of the vessel from which fluorescence was evaluated, and it is estimated that typically 10–20 cells contributed to the signal. Cells were first stimulated with ANG II (3 nM) which led to a reliable increase in $[\text{Ca}^{2+}]_i$; subsequent superfusion with adenosine (10 μM) gave a much smaller effect (fig. 3). The Ca^{2+}

Fig. 2. Patch-clamp recordings from JGC in afferent arterioles. **a** Experiment with simultaneous current (I) clamp and voltage (E) clamp recording. The upper graph shows the current needed to clamp the cell to -60 mV. The lower graph shows the alternatively recorded membrane potential. **b, c** Changes in membrane potential only. The preparations were treated with $10 \mu\text{M}$ adenosine, 3 or 10 nM ANG II and $1 \mu\text{M}$ CHA as indicated. The lower graphs in **b** and **c** show a section of the respective experiment in an expanded time scale. The preparations had a resting membrane potential slightly negative to -60 mV. Adenosine (**b**) and CHA (**c**) produced small depolarisations (<5 mV) and strong spiking activity. After washout of the drugs, spiking decreased slowly and the baseline almost returned to control levels. ANG II induced a large depolarisation of ~ 40 mV initially superimposed with strong spiking. Washout led to a slow repolarisation, which was not yet complete after 10 min and was accompanied by strong spiking activity. Subsequent stimulations of adenosine receptors with adenosine and CHA produced more pronounced responses than the first stimulation preceding the application of ANG II.

Fig. 3. A_1 AR stimulation and $[\text{Ca}^{2+}]_i$ in rat JGC. Original recording from a JGC, stimulated first with ANG II (3 nM for 1 min) and, after washout, with adenosine ($10 \mu\text{M}$ for 1 min).

response to adenosine was, however, inconsistent and only 1 out of 6 preparations responded to this stimulus. Decreasing the adenosine concentration (1 , 0.1 and $0.01 \mu\text{M}$) did not lead to significant changes in the responder rate as judged by a χ^2 test ($n \geq 6$; data not shown). Similar observations were made with CHA (0.01 – $10 \mu\text{M}$, responder rates were between 20 and 50%), with the best result obtained at $1 \mu\text{M}$ CHA, at which concentration 11 out of 22 preparations responded. The magnitude of the responses to adenosine and CHA was always much smaller than the response to ANG II (3 nM). In the series with

$1 \mu\text{M}$ CHA the area under the curve was only 22% of the corresponding response to 3 nM ANG II.

Several manoeuvres were tried to increase the response rate of JGC to the Ca^{2+} -mobilising effect of A_1 AR stimulation. First, the order of stimulation was changed to CHA first, ANG II second. To reduce damage of the preparation by collagenase, the exposure time to the enzyme was shortened from 50 to 20 min; to avoid inadvertent activation of $A_{2(A)}$ receptors, the antagonist ZM241385 (100 nM) was added, and the HEPES-buffered solution was replaced by a bicarbonate-buffered solution. None of these mea-

tures showed any effect. It was also examined whether the response to A₁AR stimulation depended on the region of the vessel, going from the vessel pole with predominantly JGC to a more proximal region with mostly smooth muscle cells. In 14 experiments and using 1 μ M CHA as the stimulus, the region between 50 and 100 μ m from the glomerulus was evaluated: the results were similar to those obtained in the JGC region (data not shown).

In addition to preparations from rat kidney, preparations from C57 mice on a sodium-depleted diet and CD-1 mice fed with standard chow were also used. The response of mouse JGC to stimulation with 3 nM ANG II was very similar to that found in rat JGC. However, none of the arterioles of either mouse strain showed any response to CHA (100 nM or 1 μ M, $n = 3$ –10).

Is There an Endogenous Tone of Adenosine in the Preparation?

One reason for the unreliable response to A₁AR stimulation might be the presence of endogenous adenosine, secreted from the tissue during preparation and experiment, and which might desensitise the receptor. First it was investigated whether such endogenous adenosine might affect basal $[Ca^{2+}]_i$. To this end, preparations were treated with adenosine deaminase type II to inactivate endogenous adenosine; however, no significant difference with time-matched controls was observed (fig. 4a). Similarly, superfusion with an A₁AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine, did not affect basal $[Ca^{2+}]_i$ either (fig. 4b).

Additionally, it was investigated whether pretreatment with adenosine deaminase type II (4 U/ml) affected the response to CHA (1 μ M). Though the response rate seemingly increased from 50% (11 of 22) to 67% (6 out of 9), this was not significant (χ^2 test, $p = 0.46$).

Is There a Synergy between Adenosine and ANG II?

A synergy between adenosine and ANG II in the constriction of the afferent arteriole has been demonstrated [6, 7, 37, 38]. Therefore, we examined whether or not a synergy can also be seen in the $[Ca^{2+}]_i$ response of rat JGC. One experimental approach is illustrated in figure 5a.

Following the reference ANG II application and wash-out, CHA (100 nM) was applied and, in the continued presence of CHA, ANG II (3 nM) was added again. In agreement with the data shown above, 6 out of 13 arterioles responded to CHA with a slight increase in $[Ca^{2+}]_i$. Figure 5a describes a sample of a responding arteriole, and figure 5b shows the mean from all 13 experiments.

The total increase in $[Ca^{2+}]_i$ induced by ANG II together with CHA was significantly greater than that by ANG II alone in time-matched controls ($p < 0.05$). However, if $[Ca^{2+}]_i$ in the presence of CHA – just before ANG II application – was used as the baseline, significance was lost (fig. 5b).

In additional experiments, this protocol was modified by using higher CHA concentrations or by using 10 nM adenosine instead of CHA, by simultaneous application of the agonists, by inverting the application protocol (i.e. adenosine + ANG II first) and by using a bicarbonate-buffered medium supplemented with albumin (1 mg/ml). In all these experiments, only additive effects were seen.

In another series of experiments the preparation was conditioned by a low concentration of ANG II (30 or 100 pM), and the test stimulation by CHA was applied in the presence of ANG II (fig. 5c). Superfusion with low concentrations of ANG II induced a slight increase in basal $[Ca^{2+}]_i$ (30 pM: $9 \pm 3\%$, $n = 7$, 100 pM: $17 \pm 3\%$, $n = 9$) and did not affect the response to 3 nM ANG II. Application of CHA (1 μ M in the presence of 30 pM ANG II, and 10 μ M in the presence of 100 pM ANG II; $n = 5$) elicited a response in each of 3 experiments. The effects of CHA were again much smaller than the responses to 3 nM ANG II and not different from those obtained in the absence of ANG II. Therefore, low concentrations of ANG II do not increase the reactivity of JGC to A₁AR stimulation.

ANG II and $[Ca^{2+}]_i$ in A₁AR Knockout and Control Mice

The generation of A₁AR knockout (A₁AR^{-/-}) mice [9] allowed us to test the hypothesis that a putative release of endogenous adenosine via activation of A₁AR might affect the $[Ca^{2+}]_i$ response of mouse JGC to stimulation by ANG II. To this end, concentration-response curves for the $[Ca^{2+}]_i$ response to ANG II in preparations from A₁AR^{-/-} mice were compared with those from the corresponding control (A₁AR^{+/+}) mice. Figure 6a shows that JGC from A₁AR^{-/-} mice responded to ANG II (3 nM) in a manner comparable to JGC from rats (fig. 3, 5a). Figure 6b compares the dependence of the peak $[Ca^{2+}]_i$ response on ANG II concentration from knockout and control mice. At no concentration was there any significant difference between the 2 preparations. The fit of the Hill equation to the data from control mice gave a maximal increase of 60 ± 11 nM with an EC₅₀ value of 6 (1, 36) nM and a Hill coefficient of 0.6 ± 0.2 . Assuming the same maximum response, the resulting parameters for JGC from A₁AR^{-/-} mice were similar [EC₅₀ = 9 (4, 20) nM, Hill coefficient = 0.6 ± 0.1].

Fig. 4. Effect of adenosine deaminase type II (ADA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) application (indicated by horizontal bars) on basal $[Ca^{2+}]_i$. The curves show the mean of normalised experimental results. **a** Application of ADA (4 U/ml for 5 min): in the control experiments, the corresponding concentrations of albumin (0.02 mg/ml) [21] and solvent (0.2% glycerol) were present. Traces were averaged over min 3–5 and this was set to 100%; then ADA or control was superfused. Averaging $[Ca^{2+}]_i$ over min 6–8 gave values of $95 \pm 2\%$ for ADA ($n = 18$) and $99 \pm 2\%$ ($n = 16$) for control; the difference was not significant. **b** Application of the A_1AR antagonist DPCPX (100 nM for 6 min). Setting the values from min 4–6 to 100%, $[Ca^{2+}]_i$ in min 8–12 was $108 \pm 2\%$ after DPCPX ($n = 11$); time-matched controls gave $[Ca^{2+}]_i = 103 \pm 2\%$ ($n = 10$); the difference was not significant.

To exclude an insufficient stimulation of the A_1AR by endogenous adenosine we measured the responses to 3 and 30 nM ANG II in the continuous presence of 0.1 μM CHA (fig. 5a). As one would expect, there was no response of JGC from ($A_1AR^{-/-}$) mice to CHA ($n = 42$ from 4 animals). JGC from ($A_1AR^{+/+}$) mice showed a slight, but not significant increase in $[Ca^{2+}]_i$ ($n = 47$ from 4 animals). The response to 3 nM ($n = 21$ $A_1AR^{-/-}$; $n = 23$ $A_1AR^{+/+}$) and 30 nM ($n = 21$ $A_1AR^{-/-}$; $n = 24$ $A_1AR^{+/+}$) ANG II did not reveal any significant differences between wild-type and A_1AR knockout mice.

Fig. 5. Tests for synergy between CHA and ANG II in increasing $[Ca^{2+}]_i$. **a** Experiment demonstrating the protocol: after standard stimulation with ANG II (3 nM) and washout, the preparation was superfused with CHA (100 nM) for 4 min and then with ANG II (3 nM) in the continued presence of CHA for a further 1 min. **b** Area under the curve (AUC) of the second (i.e. test) stimulation normalised with respect to the preceding standard stimulus of ANG II (3 nM). In control experiments applying ANG II as the test stimulus, the second application reached $94 \pm 7\%$ ($n = 10$) of the 1st response. In 13 experiments, CHA alone increased $[Ca^{2+}]_i$ to $11 \pm 5\%$, and CHA + ANG II to $118 \pm 7\%$. The response to the combined agonists is significantly higher than the response to ANG II alone (* $p < 0.05$); however, if the response to CHA alone is subtracted, the difference compared with control is no longer significant ($107 \pm 7\%$; $n = 13$). **c** Experiment with 1 μM CHA in the presence of a small conditioning concentration of ANG II (30 pM).

Fig. 6. $[Ca^{2+}]_i$ increase in response to ANG II in JGC from A_1AR knockout ($A_1AR^{-/-}$) and control ($A_1AR^{+/+}$) mice. **a** Response of JGC from an $A_1AR^{-/-}$ mouse to ANG II (3 nM). **b** Dependence of the maximum increase in $[Ca^{2+}]_i$ (peak increase) in response to ANG II in JGC from $A_1AR^{+/+}$ and $A_1AR^{-/-}$ mice ($n = 5$ –22 per point). Fitting the equation outlined in the Methods section to the original (individual) values from control mice gave a maximal increase of 60 ± 11 nM with an EC_{50} value of 6 (1, 36) nM and a Hill coefficient of 0.6 ± 0.2 . The data from $A_1AR^{-/-}$ mice could not be analysed successfully using 3 variable parameters; however, setting the maximal effect to 60 nM gave the fit shown with $EC_{50} = 9$ (4, 20) nM and Hill coefficient = 0.6 ± 0.1 .

Discussion

In this study, we measured the effect of A_1AR stimulation on membrane potential and $[Ca^{2+}]_i$ in JGC in isolated non-perfused afferent arterioles from NaCl-depleted rats and compared the effects to ANG II.

Electrophysiological Effects

A_1AR stimulation induced spiking of the membrane potential of JGC but only minor depolarisations; ANG II,

in contrast, induced massive depolarisations initially superimposed by spiking. Similar observations were made by Bührle et al. [23] in JGC in the isolated hydronephrotic mouse kidney. The difference between the stimulation of the A_1AR and the ANG II receptor is of interest since the concentrations of adenosine and CHA used here are saturating at the A_1AR [39–41] and much higher than those required for the inhibition of renin secretion [20–22], whereas the concentration of ANG II (3 nM) is far below half-saturation (fig. 6).

The inwardly rectifying K^+ channel (most probably Kir2.1) determines the membrane potential of JGC, and the ANG-II-induced depolarisation has been mainly attributed to a decrease in the open probability of this channel [24, 26, 36]. The pathway by which ANG II induces inhibition of Kir2.x may be related to lipid metabolism. The activity of Kir2.x channels strongly depends on the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the membrane [42]. ANG II receptor stimulation induces G_q -mediated activation of PLC and breakdown of PIP_2 . This may be sufficient to reduce the activity of the Kir2.x channel, thereby depolarising the cell [43, 44].

Furthermore, PKC-mediated Kir2.1 inhibition has been shown [45, 46]. Activation of protein kinase C by PLC might therefore, alone or together with PIP_2 depletion, induce cell depolarisation. Regarding A_1AR stimulation, it is conceivable that activation of PLC (which has been shown to be mediated by G_i [3]) is weaker than the stimulation elicited by ANG II. Hence, A_1AR stimulation may not sufficiently reduce PIP_2 to greatly affect Kir2.x activity, thus resulting only in minor depolarisations.

The mechanism leading to A_1AR stimulation-induced spiking activity has not been investigated. It seems plausible, however, that by activation of PLC and formation of IP_3 [3], Ca^{2+} sparks may have been generated which, in turn, activate Ca^{2+} -activated Cl^- channels leading to depolarising spikes [47, 48]. A Ca^{2+} -activated Cl^- current has been shown in JGC [26, 36] and is activated by 5 μM adenosine [49]. The locally restricted calcium sparks [47, 48] are, however, too small and too short lasting to be detected by our $[Ca^{2+}]_i$ detection system.

Ca^{2+} Responses

Adenosine and CHA induced small and inconsistent increases in $[Ca^{2+}]_i$ (maximum responder rate, 67%). A stimulation of A_2AR reducing or masking the response to A_1AR stimulation can be excluded as lower, more A_1AR -specific concentrations of adenosine or CHA [50]

did not increase, but decrease, changes in $[Ca^{2+}]_i$. Activation of A_2AR actually may have contributed to the observed increase in $[Ca^{2+}]_i$ in response to adenosine, as increases in cAMP can increase $[Ca^{2+}]_i$ in this preparation [51]. This, however, should be irrelevant in response to CHA.

Potential explanations for the observed inconsistency of the $[Ca^{2+}]_i$ response induced by A_1AR activation include the heterogeneity of cells contributing to the fluorescence signal (JGC vs. smooth muscle cells). Indeed, adenosine constricts afferent arterioles of mice via the PLC pathway [3], which typically leads to an increase in $[Ca^{2+}]_i$. A very modest increase in $[Ca^{2+}]_i$ in response to adenosine (100 μM) has been shown in afferent arterioles from rabbits [5]. This might suggest that smooth muscle cells are responders and JGC are non-responders. However, the results of this study did not support such a notion as no difference in the signal was found close to or 50 μm proximal to the vessel pole. In addition, smooth muscle cells and JGC are coupled by gap junctions [16], limiting independent responses of the 2 cell types. In a recent publication, Hansen et al. [49] detected a reproducible increase of 25% of $[Ca^{2+}]_i$ in mouse afferent arterioles in response to 0.5 μM adenosine; however, this was a small effect compared with that on luminal diameter and the increase in $[Ca^{2+}]_i$ in response to ANG II (fig. 6). Another possibility is that an endogenous tone of adenosine, due to release from the preparation, desensitises A_1AR . However, no evidence to support this notion was found.

Several manoeuvres were tried to increase the responder rate to adenosine and CHA, in particular the exploitation of the known synergy between A_1AR and ANG II receptor stimulation with regard to afferent arteriolar constriction [6, 7, 37, 38]. However, no enhancement of the Ca^{2+} response to CHA by ANG II and vice versa was found and, in addition, the $A_1AR^{-/-}$ mice showed the same responsiveness to ANG II as control mice. How can the present findings be explained considering the well-documented synergy between the 2 tissue hormones for afferent arteriolar constriction? Lai et al. [52] confirmed the synergy of adenosine and ANG II receptor stimulation with respect to arteriolar contraction; however, the increase in $[Ca^{2+}]_i$ was almost independent from pretreatment with adenosine (similar to the present findings), and the authors claimed a sensitisation of the contractile fibres to $[Ca^{2+}]_i$ by adenosine instead. Another potential explanation may include the fact that in the functional studies cited above, the preparation was perfused. Luminal pressure is known to depolarise smooth

muscle cells [53] and, at a perfusion pressure of 80 mm Hg, the resting membrane potential of smooth muscle cells in rat afferent arterioles was reported to be -40 mV [54] as compared to the value of -57 mV determined here in the non-perfused preparation. The signalling pathway leading from G protein activation to the release of Ca^{2+} from the sarcoplasmic reticulum contains voltage-dependent steps and is enhanced by depolarisation [55, 56]. In addition, the value of -40 mV is near the potential where L-type Ca^{2+} channels open massively [57], such that small depolarisations may lead to substantial increases in $[Ca^{2+}]_i$. Hence, in smooth muscle cells, both Ca^{2+} mobilisation from stores and Ca^{2+} entry are favoured by the depolarised potentials at physiological perfusion pressure, and both processes may contribute to the constriction of the afferent arteriole induced by physiological concentrations of adenosine ($\sim 0.1 \mu M$ [3]). In turn, hyperpolarisation by K_{ATP} channel openers inhibits IP_3 production in vascular smooth muscle cells [58], suggesting that the more polarised potential in the non-persurised preparation may be the reason for the small and inconsistent $[Ca^{2+}]_i$ response to A_1AR stimulation. In comparison, the stimulation of the PLC pathway by ANG II could be so robust that the modulatory role of membrane potential does not play a major role.

The inconsistent response of the Ca^{2+} increase after adenosine and CHA has effectively rendered any investigation impossible into the mechanism by which A_1AR stimulation is translated into the observed small $[Ca^{2+}]_i$ increase. Therefore, we could not determine whether the small depolarisations activated Ca^{2+} entry via low-threshold voltage-dependent Ca^{2+} channels (e.g. T channels [59, 60]) or whether or not Ca^{2+} mobilisation from intracellular stores was involved [3]. The fact that the electrical response to A_1AR stimulation was observed regularly, whereas the Ca^{2+} response was inconsistent, may speak against a causal link between them. However, it is also possible that the depolarisation just reaches the threshold for the Ca^{2+} response.

Despite these limitations, the study shows clear differences in the responses of JGC to ANG II receptor type 1 and A_1AR stimulation with respect to membrane potential and $[Ca^{2+}]_i$. Moderate concentrations of ANG II show strong effects, whereas even saturating concentrations of adenosine or CHA show only small, if any, responses. Considering a similar efficacy of adenosine and ANG II to inhibit renin release [20, 61], the present finding of much smaller changes in membrane potential and $[Ca^{2+}]_i$ in response to adenosine suggests that, in contrast to ANG II, the main pathway used by adenosine does not

involve these parameters. Effects of adenosine and ANG II were additive and the responses to either one did not depend on co-stimulation of the other system. The present studies suggest that the synergy between the 2 tissue hormones, which is well documented for afferent arteriolar constriction, is not reflected in JGC at the level of $[Ca^{2+}]_i$.

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