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Role of cGMP as second messenger of adenosine in the inhibition of renin release

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Role of cGMP as second messenger of adenosine in the inhibition of renin release. Adenosine is known to be a potent inhibitor of renin release from the kidneys. The aim of this study was to investigate the transmembrane signalling avenue that the second messenger of adenosine causes inhibition of renal renin release. Using short term cultures of juxtaglomerular cells isolated from rat kidneys, we found that adenosine inhibited spontaneous renin release from these cells up to 40% of control, in a dose dependent fashion between 10^{-10} M to 10^{-6} M. Half maximal inhibition was observed at 2×10^{-8} M adenosine. The inhibitory effect of adenosine on renin release could be mimicked by the A_1-receptor agonist N^6-cyclohexyladenosine (CHA) and could be attenuated by the A-receptor antagonist theophylline (5×10^{-5} M). The A_2-receptor agonist 5'-N-ethylcarboxamideadenosine (NECA) had no inhibitory effect on renin release. These findings indicate that the inhibitory effect of adenosine is mediated by A_1-receptors on juxtaglomerular cells. Adenosine had no effect on either transmembrane calcium influx or the cytosolic free calcium concentration in the isolated juxtaglomerular cells. Adenosine also did not alter the cellular level of cyclic AMP in the concentration range employed. However, adenosine led to a dose dependent increase of the cellular level of cyclic GMP. Half maximal increase of cGMP was observed at 10^{-8} M adenosine. The effect of adenosine on cyclic GMP could be mimicked by the A_1-receptor agonist CHA and could be attenuated by the A-receptor antagonist, theophylline. We infer from our results that inhibition of renin release by adenosine could be mediated by an A_1-receptor linked rise of cGMP in renal juxtaglomerular cells. This conclusion is supported by our previous finding that cGMP is an inhibitory signal for renin release from renal juxtaglomerular cells.

Intrarenal infusion of adenosine (ado) has been found to strongly inhibit renin release from the kidneys [1–3]. There is good evidence obtained from whole kidney experiments that this effect of ado on renin release is not mediated by either the baroreceptor or the macula densa receptor [4], suggesting a direct interaction between ado and juxtaglomerular cells. This conclusion is confirmed by the finding that ado also inhibits renin release from renal cortical slices [5, 6], isolated afferent arterioles [7], and isolated glomeruli [6]. The demonstration of a direct interaction between ado and juxtaglomerular cells is, however, lacking so far. Furthermore, there is experimental evidence that the inhibitory effect of ado on renin release is mediated by cell surface receptors of the A_1-subclass [5, 7, 8]. However, the mechanism by which occupancy of adenosine receptors leads to an inhibition of renin release from juxtaglomerular cells has not been clarified so far.

There is good evidence to indicate that renin release from juxtaglomerular cells is affected by the intracellular concentrations of calcium and of cyclic nucleotides in a way that calcium [9, 10] and cGMP [11] act as inhibitory signals, while cAMP is a stimulatory signal [9, 10]. A substance that inhibits renin release, such as ado, could therefore do so by either raising the concentrations of calcium or cGMP, or by lowering the concentration of cAMP within juxtaglomerular cells. We have recently developed a method that permits an establishment of reproducible, short-term cell cultures containing around 90% juxtaglomerular cells [11]. Moreover, we have shown that this cell culture model is suitable for the study of renin release on a cellular level [11–13].

Using such juxtaglomerular cells isolated from rat kidneys we investigated the effects of ado on renin release, transmembrane calcium influx, cytosolic free calcium concentration and intracellular levels of cAMP and cGMP.

We found that ado inhibits renin release from juxtaglomerular cells most likely by an A_1-receptor mediated increase in intracellular cGMP.

Methods

Cell culture

Short-term cultures of rat renal juxtaglomerular cells were made as described previously. In brief, a single cell suspension was prepared by kidney perfusion with citrate, enzymatical dissociation with trypsin and collagenase, and sieving over a 22 μm screen exactly as described [13]. This single cell suspension was then further separated on a 25% isoosmotic percoll gradient and cells with a density of 1.06 g/ml were used for culture. For a typical isolation procedure the kidneys of a single rat were used. They gave a yield of around 40 million single cells and were mixed with 4×30 ml 25% isoosmotic percoll. From the four gradients a total of around 3 million cells were harvested from the bands with a density of 1.06 g/ml. For experiments to determine renin secretion, 10^5 cells were seeded per 7 cm^2 dish; for experiments to determine cyclic nucleotides 3×10^5 cells were seeded per dish. On the second day of culture around between 20 to 30% of the seeded cells were attached, and around 90% of the attached cells were juxtaglomerular cells as judged from the specific immunostaining for rat renin [11]. Experiments with the cells were done two days after seeding as

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a rule. Distribution of renin containing cells was determined in every third cell preparation with indirect immunofluorescence staining as described in [13].

Renin release

Determination of renin release from the cultured cells was done exactly as described in [12] by measuring the linear increase of renin activity of the cell supernatant 10, 20 and 30 minutes after onset of the experiments. In brief, the culture medium was replaced with prewarmed, Hepes buffered saline (132 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM NaOAc, 2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes, pH 7.2). The culture dishes were placed on a heating block at 37°C, and the time dependent increase of the renin activity of the buffer was monitored. Renin activity was determined by its ability to generate angiotensin I from the plasma of bilaterally nephrectomized rats as described in [14]. Angiotensin I was measured with a commercially available radioimmunoassay (Isotopen Dienst West, Teufen, Switzerland).

Intracellular contents of cAMP and cGMP

Intracellular levels of cyclic nucleotides were examined under the same experimental conditions as the renin release. In brief, five minutes after onset of the experiments the buffer was removed from the cells, and the dishes were placed on an ice block. After the addition of 0.4 ml of ice-cold buffer (5 mM potassium phosphate, 0.2 mM EDTA, 0.5 mM 3-isobutyl-methylxanthine and 150 mM KCl, pH 6.8), the cells were scraped off with a teflon policeman. The cell suspension so obtained was sonicated, boiled for five minutes, and centrifuged. An aliquot was removed from the sonicated cell suspension for protein determination. The supernatants were assayed for cAMP and cGMP using radioimmunoassays (New England Nuclear, Boston, Massachusetts, USA).

^{45}Calcium-influx

^{45}Ca-uptake into the cultured cells was determined as outlined in [12]. In brief, the culture medium was substituted by a Hepes-buffered saline containing 2 μCi/ml ^{45}Ca. Simultaneously agents were added. After either 2, 5, 10 or 20 minutes the buffer was withdrawn and the cells were quickly washed with 10 × 1 ml ice-cold Krebs solution containing 10 mM $CaCl_2$. The cells were lysed by the addition of 1 ml 1 N NaOH and the radioactivity was counted with a β-scintillation counter.

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+}, $[Ca]_i$, was measured using quin-2. About 2 × 10^7 cells were incubated with 25 μM quin-2 AM [tetrakis (acetoxymethyl)ester of quin-2] in RPMI 1640 for 20 minutes, followed by another 40 minute incubation with 4 volumes of medium. After the incubation period, aliquots of 1 × 10^6 cells were washed twice and resuspended in Hepes-buffered saline. Fluorescence of quin-2 loaded cells was measured at 37°C in a Perkin-Elmer fluorescence spectrophotometer L93 using excitation wavelength of 340 nm and emission wavelength of 490 nm. Each trace was monitored for at least five minutes. The fluorescence signal was calibrated at the end of each individual trace essentially as described by Tsien, Pozzan and Rink [15]. Cell numbers were determined with a Coulter counter.

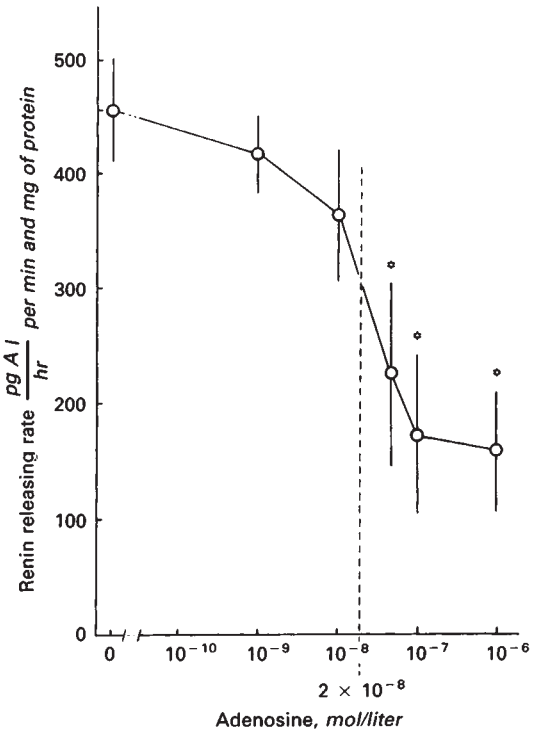

Fig. 1. *Dependency of spontaneous renin release from the isolated juxtaglomerular cells on the extracellular concentration of adenosine.* Data are mean ± SE of 8 experiments. The vertical dashed line indicates the concentration of adenosine required for half maximal effect. Asterisk indicates $P < 0.05$ vs. control.

Protein determination

Protein was determined according to the method of Lowry et al [16] using bovine serum albumin as a standard. One mg of cellular protein corresponds to around two million cells.

Statistics

Levels of significance were calculated utilizing Students unpaired t-test. $P < 0.05$ was considered significant.

Reagents

All reagents for the culture medium were obtained from Boehringer (Mannheim, FRG). Percoll was from Pharmacia Diagnostics, Uppsala, Sweden). Adenosine, N^6-cyclohexyladenosine (CHA) and theophylline were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. 5'-N-ethylcarboxamideadenosine (NECA) was from Byk Gulden Company (Konstanz, FRG). ^{45}Ca was from Amersham (U.K.) and quin-2 AM was obtained from Calbiochem.

Results

The effect of adenosine(ado) on the spontaneous renin release from the isolated juxtaglomerular cells is shown in Figure 1. Ado led to a dose dependent inhibition of renin release to around 40% of control in a concentration range between 10^{-9} to 10^{-6} M. Half maximal inhibition was observed at 2 × 10^{-8} M. To test whether or not the inhibitory effect of ado on renin release was mediated by cell surface receptors, the effect of both

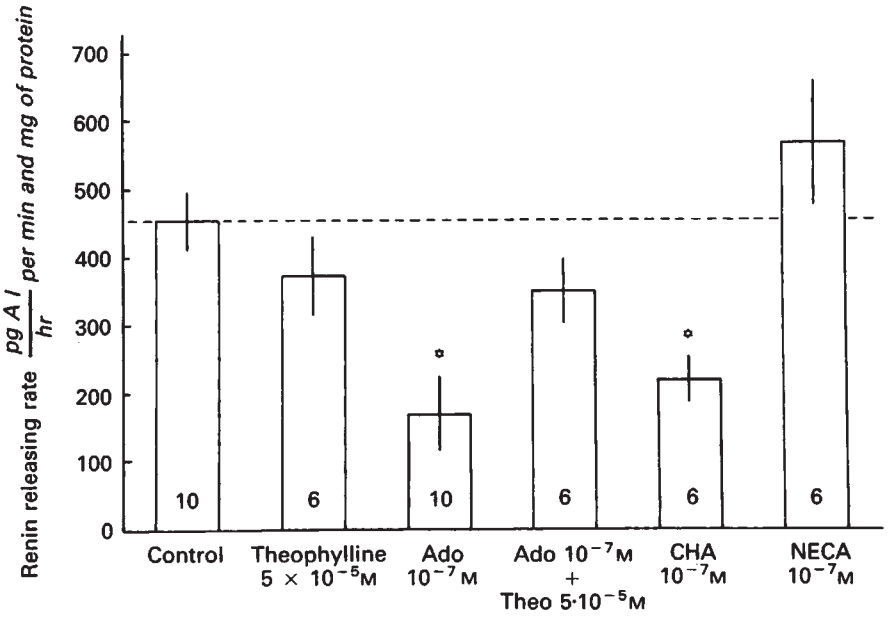

Fig. 2. *Renin releasing rate from the isolated juxtaglomerular cells in presence of adenosine (10^{-7} M), theophylline (5×10^{-5} M), CHA (10^{-7} M) and NECA (10^{-7} M).* Data are mean ± SE. Numbers at the bottom of the columns indicate numbers of experiments. The dashed line indicates the mean of control. Asterisk indicates $P < 0.05$ vs. control.

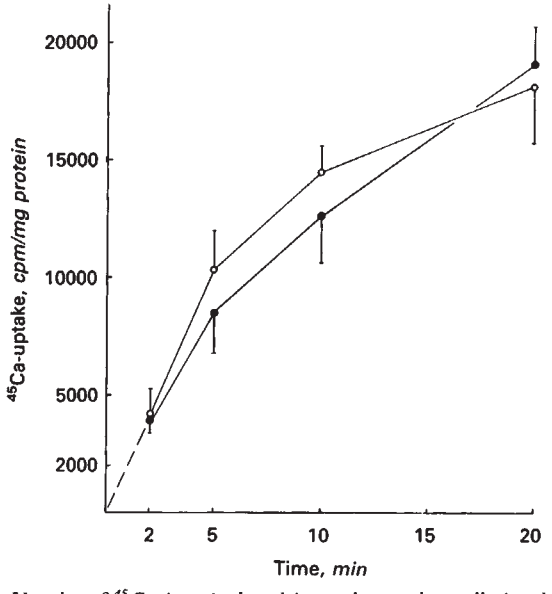

Fig. 3. *Uptake of ^{45}Ca into isolated juxtaglomerular cells in absence and presence of adenosine (10^{-7} M) as a function of time.* Data are mean ± SEM of four experiments.

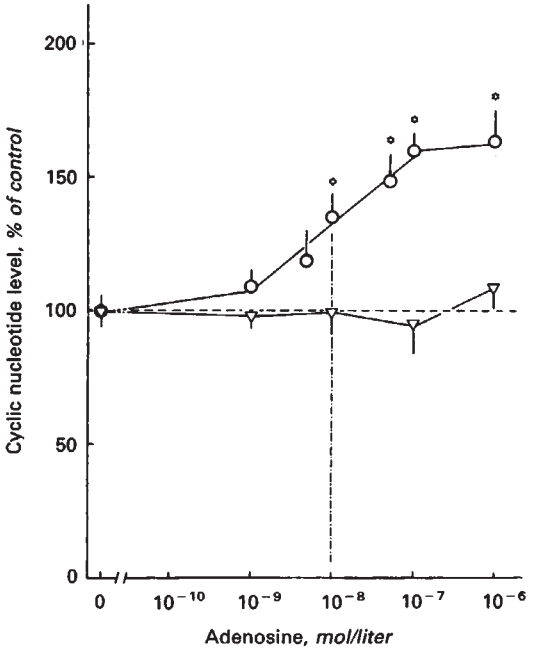

Fig. 4. *Dependency of intracellular levels of cAMP and cGMP in isolated juxtaglomerular cells on the extracellular concentration of adenosine.* Data are mean ± SE of ten experiments and are given as percentage of the mean of the control value (= absence of adenosine). Mean of controls were 460 fmol/mg protein for cAMP and 60 fmol/mg of protein for cGMP. The horizontal dashed line indicates the mean of control, the vertical dashed line indicates the concentration of adenosine required for half maximal effect on cGMP. Asterisk indicates $P < 0.05$ vs. control.

A-receptor antagonists and A-receptor agonists on renin release was investigated. As shown in Figure 2, theophylline at a low concentration (5×10^{-5} M), considered to antagonize adenosine A-receptor effects [17], alone did not significantly alter the spontaneous renin release from the cells. However, it obviously abolished the inhibitory effect of 10^{-7} M ado. We further investigated the effects of the A-receptor agonists [17] N^6-cyclohexyl adenosine(CHA) and 5'-ethylcarboxamide adenosine(NECA) on renin release from the isolated juxtaglomerular cells. As it can be seen in Figure 2, CHA (10^{-7} M) but not NECA (10^{-7} M) mimicked the inhibitory effect of 10^{-7} M ado on renin release.

Since the results obtained so far indicated that the inhibitory effect of ado on renin release was mediated by cell surface receptors of ado, the next set of experiments were designed to identify possible intracellular messengers that might mediate the effect of ado in juxtaglomerular cells.

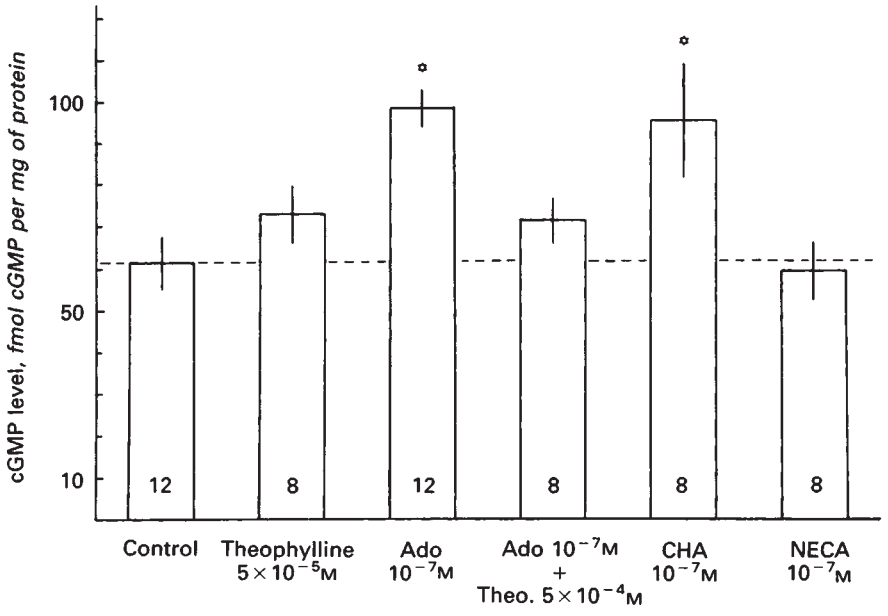

Fig. 5. *Intracellular levels of cGMP in isolated juxtaglomerular cells in presence of adenosine (10^{-7} M), theophylline (5×10^{-5} M), CHA (10^{-7} M) and NECA (10^{-7} M).* Data are mean ± SE. Numbers at the bottom of the columns indicate the numbers of experiments. The dashed line indicates the control value. Asterisk indicates $P < 0.05$ vs. control.

There is good experimental evidence that renin release is inversely related to the intracellular concentration of calcium [9, 10]. We therefore tested the effect of ado (10^{-7} M) on both transmembrane calcium influx and cytosolic free calcium in the isolated juxtaglomerular cells. Using the ^{45}Ca-tracer method, ^{45}calcium uptake is shown in Figure 3. It is obvious that adenosine did not enhance ^{45}Ca-uptake by the cells. Cytosolic-free calcium concentration as monitored with the quin-2 method was 233 ± 28 nM (mean ± SE; $N = 7$) in the absence of ado and 231 ± 9 nM (mean ± SE; $N = 7$) in the presence of ado. For comparison angiotensin II (10^{-7} M) caused an increase of Ca^{2+}_i to 529 ± 7 nM ($N = 5$). Both results indicate that ado at a concentration at which it maximally inhibited renin release did not alter calcium influx or cytosolic free calcium. Cyclic nucleotides such as cAMP [9, 10] and cGMP [11] are also considered as second messengers that are involved in the intracellular control of renin release. We therefore investigated the effects of ado on the intracellular levels of cAMP and cGMP in the juxtaglomerular cells. Figure 4 shows the cellular contents of cAMP and cGMP five minutes after addition of adenosine. In pilot experiments for this study, in which we determined cyclic nucleotide levels after 2, 5 and 15 minutes, as well as in recent studies [11, 12], a five minute interval was found as the best time point for optimal agonist-induced changes of both cAMP and cGMP. As it can be seen from Figure 4, ado did not affect the intracellular level of cAMP in the concentration range between 10^{-9} M to 10^{-6} M. On the other hand a dose dependent increase of the level of cGMP was observed in this concentration range of ado. Half maximal increase of cGMP; was achieved with 10^{-8} M ado.

Since we have recently shown that cGMP is an inhibitory signal for renin release from isolated juxtaglomerular cells [11] the question arises whether or not the inhibitory effect of ado on renin release could be explained by the rise of $cGMP_i$. As documented in Figure 2, the inhibitory effect of ado on renin release could be attenuated by theophylline and could be mimicked by CHA but not by NECA. Figure 5 shows the intracellular cGMP levels under these experimental conditions. It can be seen that theophylline itself did not significantly alter $cGMP_i$ but it abolished the rise in $cGMP_i$ induced by ado. It is furthermore evident that the effect of ado on $cGMP_i$ could be mimicked by CHA but not by NECA. We want to emphasize here that none of the experimental conditions demonstrated in Figure 5 led to any change in $cAMP_i$ in the isolated juxtaglomerular cells. For comparison isoproterenol (10^{-5} M) led to an increase of $cAMP_i$ to 346 ± 65% of the control ($N = 5$).

To see whether the rise in $cGMP_i$ induced by ado would be sufficient to explain its inhibitory effect of renin release we further compared the effects of ado on both renin release and $cGMP_i$ with a correlation between renin release and $cGMP_i$ that was obtained in a recent study [11]. This correlation is depicted as a dotted line in Figure 6. Together with this line experimental data obtained with ado (closed circles) and CHA (open circle) are shown in this figure. It is evident from Figure 6 that the experimental points obtained with ado and CHA fit very well to the correlation between $cGMP_i$ and renin release which was obtained from independent experiments.

Discussion

This study was designed to investigate by which way of transmembrane signalling adenosine(ado) inhibits renal renin release. Using a cell preparation highly enriched in juxtaglomerular cells we found that ado caused a dose dependent inhibition of renin release from the cells (Fig. 1). This result indicates that ado inhibits renin release by a direct action on renal juxtaglomerular cells, and this result thus confirms the conclusions drawn from experiments done with whole kidneys, renal cortical slices [5, 6], isolated afferent arterioles [7], and isolated glomeruli [6], which predicted a direct interaction between ado and juxtaglomerular cells. We further found that inhibition of renin release from juxtaglomerular cells by ado was dose dependent in a concentration range between 10^{-9} M to 10^{-6} M, and half-maximal inhibition was observed at 2×10^{-8} M. These findings are in harmony with the results Churchill and

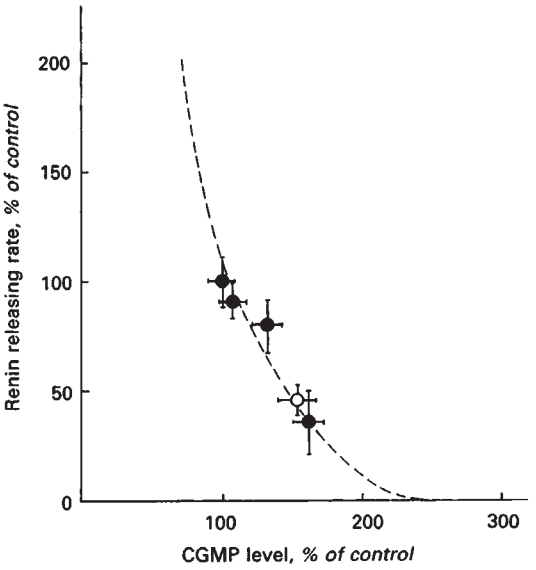

Fig. 6. *Relation between cellular levels of cGMP and renin release from isolated juxtaglomerular cells in presence of ado (0 – 10^{-6} M) (closed circles) and CHA (open circles).* Renin releasing rate values are taken from Figs. 1 and 2, and cGMP levels are taken from Figs. 3 and 4. Data are given as the percentage of the control value, which was obtained in absence of ado and CHA. The dashed line is the last square regression curve for a correlation between cGMP and renin releasing rate obtained in a recent study in which atrial natriuretic peptide, sodium nitroprusside, M&B 22948 and methylene blue were used in order to vary cGMP [11].

Churchill obtained from experiments with renal cortical slices [5]. Moreover our results indicate that the inhibitory effect of ado on renin release is mediated by A_1-cell surface receptors on juxtaglomerular cells, because the effect of ado could be blocked by the A-receptor antagonist theophylline [17] and could be mimicked by the A_1-receptor agonist CHA [17], but not by the A_2-receptor agonist NECA [17] (Fig. 2). Again these findings fully confirm the results of experiments performed with whole kidneys [8, 18], renal cortical slices [5] and isolated arteriols [7]. Under the assumption that the effect of ado is mediated by A_1-cell surface receptors on juxtaglomerular cells the question arises about the intracellular second messenger that mediates the effect of ado.

One candidate for such a second messenger role is calcium, because calcium is known as an inhibitory intracellular signal for renin release [9, 10]. We therefore determined the effect of ado at a concentration (10^{-7} M) that almost maximally inhibits renin release (Fig. 1) on transmembrane calcium influx and on the cytosolic-free calcium concentration. However, we failed to detect any effect of ado on each of these parameters. The result that ado did not enhance transmembrane calcium influx into the juxtaglomerular cells complements reports on the lack of effect of calcium channel blockers on the inhibition of renin release caused by ado [4, 5, 19] in whole kidneys [4, 19] and renal cortical slices [5]. The entirety of these results indicate that inhibition of renin release is not caused by an enhanced calcium influx. The finding that ado does not increase cytosolic free calcium is a new finding and suggests that ado does not lead to a release of calcium from intracellular stores. Taken together experimental evidence clearly argues against a role of calcium as a second messenger of ado in inhibition of renin release from renal juxtaglomerular cells.

Further candidates for second messengers in the control of renin release are cyclic nucleotides such as cGMP and cAMP. In a recent study we have obtained evidence that cGMP is also involved in the intracellular control of renin release, in a way that cGMP is a potent inhibitory signal [11]. It was obvious therefore to test whether or not cGMP could be an intracellular messenger that mediates inhibition of renin release by ado. And indeed we found that ado led to an increase in $cGMP_i$ (Fig. 4). Three pieces of evidence were obtained to suggest that cGMP could be the second messenger of ado to inhibit renin release from juxtaglomerular cells. First, both the inhibition of renin release and increase of $cGMP_i$ caused by ado are likely to be mediated by A_1-receptors, because both effects could be attenuated by theophylline and could be mimicked by CHA but not by NECA (Figs. 2, 5). Second, the dose response curves of ado for the increase in $cGMP_i$ and for the decrease of renin release were very similar (Figs. 1, 4). Third, the experimental data obtained with ado matched with a correlation between $cGMP_i$ and renin release that was obtained from independent experiments (Fig. 6).

Finally a possible role of cAMP as the second messenger of ado in the inhibition of renin release should be discussed. Cyclic AMP is known as a stimulatory signal for renin release [9, 10]. Since A_1-receptors have been found to be linked to the adenylate cyclase in an inhibitory fashion in a variety of cells [20] one could speculate that ado impairs renin release by lowering the intracellular concentration of cAMP due to an inhibition of the adenylate cyclase. We determined the effect of ado on the cellular level of cAMP in juxtaglomerular cells in the same concentration range of ado in which inhibition of renin release was observed, but we failed to detect any effect of ado on $cAMP_i$ (Fig. 4). Assuming that A_1-receptors are inhibitorily linked to the adenylate cyclase also in juxtaglomerular cells, it might be remarkable that no decrease of $cAMP_i$ in presence of ado occurred. However, it has been shown for other cells that A_i-receptor occupancy does not affect basal $cAMP_i$, but attenuates the rise in $cAMP_i$ induced by activators of the adenylate cyclase [21]. At first sight the lack of effect of ado on $cAMP_i$ seems to argue against a second messenger role of cAMP for ado in the inhibition of renin release. But from our measurements we cannot rule out the possibility that ado causes locally restricted decreases of cAMP that are crucial for the intracellular control of renin release. Rossi, Churchill and Churchill have recently reported that A_1-receptor mediated inhibition of renin release from renal cortical slices could be prevented by pretreatment of the donor animals with pertussis toxin [22]. Since pertussis toxin is known to inactivate the inhibitory guanine nucleotide binding regulatory protein of the adenylate cyclase [23], they inferred from their finding that ado inhibits renin release by the inhibition of the adenylate cyclase [22]. We have also performed experiments with isolated juxtaglomerular cells that were pretreated with pertussis toxin (islet activating protein, IAP; 100 ng/ml) for 16 hours. We found that pretreatment with IAP raised renin release two- to threefold from control cells (that is, in the absence of ado) but did not alter either $cAMP_i$ or $cGMP_i$. On the other hand, pretreatment with IAP prevented both the inhibition of renin release and the rise in $cGMP_i$ but did not affect $cAMP_i$ in presence of ado (10^{-7} M).

Thus, although these findings fully confirm the results reported by Rossi et al [22], they can be more easily explained if one assumes that cGMP is the physiological antagonist of cAMP in that it inhibits renin release from juxtaglomerular cells. In summary we have presented evidence in this study that inhibition of renal renin release by adenosine is effected by a direct interaction between ado and A_1-receptors on juxtaglomerular cells. Concerning the second messenger that mediates this effect of ado, the experimental evidence points towards cGMP. By which mechanism occupancy of A_1-receptors leads to a rise in $cGMP_i$ in renal juxtaglomerular cells remains to be clarified. In a recent study clear evidence was obtained that occupancy of A_1-receptors stimulates guanylate cyclase activity in vascular smooth muscle cells [24]. In view of the fact that renal juxtaglomerular cells are modified vascular smooth muscle cells [25] one could speculate that stimulation of guanylate cyclase activity is also the mechanism by which ado raises $cGMP_i$ in renal juxtaglomerular cells.

Acknowledgments

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