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Cyclosporine A enhances renin secretion and production in isolated juxtaglomerular cells

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Cyclosporine A enhances renin secretion and production in isolated juxtaglomerular cells. Stimulation of the renin-angiotensin system is a major side effect of the fungoid immunosuppressant cyclosporine A (CyA). The aim of this study was to find out whether or not this effect of CyA results from a direct interaction with renal juxtaglomerular (JG) cells, which are the site of renal renin synthesis and release. Using primary cell cultures from rat renal cortex containing more than 80% JG cells, we found that CyA (0.01 to 10 μg/ml) stimulated renin secretion threefold. This stimulation was paralleled by a dose-dependent twofold increase of inactive renin within the cells, while the active intracellular renin remained the same. In order to identify a possible second messenger which could mediate the effects of CyA on JG cells, we examined the simultaneous effects of a single concentration of CyA (1 μg/ml) on renin secretion, prostaglandin formation and intracellular cAMP concentration. However, prostaglandin formation and cAMP were not detectably altered by CyA in experiments where renin secretion was significantly enhanced. Our results indicate that cyclosporine A stimulates renin secretion and renin synthesis by a direct effect on renal juxtaglomerular cells. This action of CyA is not mediated by changes in cellular prostaglandin or intracellular cAMP.

Cyclosporine A (CyA) has become a powerful tool in the management of allograft rejection. However, its usefulness is limited by side effects, in particular, by nephrotoxicity [1, 2]. Experience has shown that reduced doses of CyA result in fewer side effects. Nevertheless, development of hypertension has also been seen under these reduced doses [3]. Recent attention has been focused on a possible link between CyA and an elevation of plasma renin activity [4–11]. Thus an increase in renal renin content [8] and hypertrophy of the juxtaglomerular apparatus [9] have been observed after CyA administration. The mode of action by which CyA stimulates the renin-angiotensin system (RAS) is not well understood, and in particular it is not clear whether or not CyA affects the RAS by a direct effect of renal JG cells or indirectly by its well documented detrimental effects on renal blood flow [10], glomerular filtration [1, 12], and tubular function [4].

It was the aim of this study, therefore, to examine the effect of CyA on isolated renal juxtaglomerular cells and to obtain some indications as to how CyA could alter JG cell function. For our study we used primary cell cultures from rat renal cortex enriched in JG cells (80 to 90%). This cell preparation has been shown to allow the study of renin secretion on a cellular level [13]. We found that CyA stimulated renin secretion from isolated juxtaglomerular cells in a dose dependent manner. Moreover, evidence for a dose dependent increase of intracellular storage of renin was obtained. Both findings together indicate a stimulation of renin production in isolated JG cells by CyA.

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Methods

Cell culture

Short-term cultures of rat renal juxtaglomerular cells were made as described previously [14]. Briefly, a single cell suspension was prepared by kidney perfusion with citrate, enzymatic dissociation with trypsin and collagenase, and sieving over a 22 μm screen [15]. This single cell suspension was 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 male rat (120 g body wt) were used. They yielded around 40 million cells which were mixed with 4 × 30 ml 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. Three × 10^5 cells were seeded per 7 cm^2 dish. On the second day of culture between 20 and 30% of the seeded cells were attached, and around 90% of the attached cells contained renin as judged from the specific immunostaining for rat renin [14]. As a rule, experiments with the cells were performed two days after seeding. Distribution of renin containing cells was determined in every third cell preparation with indirect immunofluorescence staining [15].

Renin release

Long term incubation. The culture medium was withdrawn and replaced by prewarmed fresh culture medium containing 0.5% instead of 2% fetal bovine serum. In different series of experiments the medium was withdrawn after either 1.5, 4, or 24 hours, centrifuged, and stored at −80° until assay of renin activity. The cells were scraped off the bottom of the culture dish in ice cold buffer (0.2 M maleate buffer, pH 6.0) plus 5 mM phenylmethylsulfonylfluoride (PMSF), 10 mM EDTA and 0.1% gentamycin) sonicated (50 watts for 30 seconds), frozen in liquid nitrogen, and stored at −80° until assaying renin activity and protein content.

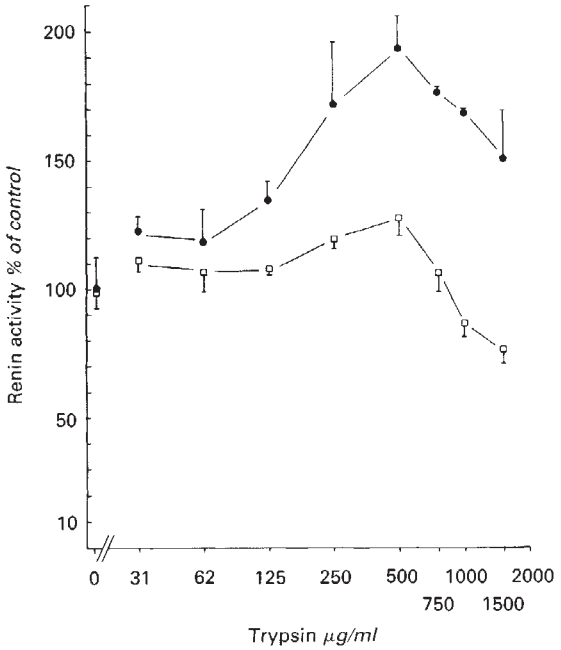

Fig. 1. *Dependence of renin activity in cells and supernatant on the trypsin concentration used for activation.* Data (mean ± SEM; $N = 5$) are presented as percentage of control (absence of trypsin). Mean control renin activity was 4.6 ng AI/hr and 10.6 ng AI/hr for cells (●) and supernatant (□), respectively.

Short term incubations. The culture medium was replaced with 1 ml prewarmed, Hepes buffered saline (132 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM Na acetate, 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 allowed to incubate for 10 minutes. Then cyclosporine A (1 μg) was added to the dishes in a volume of 100 μl. After different times of incubation the buffer was removed from the cells, centrifuged, frozen in liquid nitrogen, and stored at −80°C until assaying renin activity and PGE_2. 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-methyl-xanthine 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. The supernatant was frozen in liquid nitrogen and stored at −80°C until assaying cAMP; the pellet was lysed in 1 N NaOH and used for protein determination. In the present study assays were carried out immediately prior to the addition of cyclosporine and 10, 30 and 60 minutes after the addition.

Activation of inactive renin

Activation of inactive renin was achieved by trypsin treatment. In order to find out the optimal trypsin concentration, the dependence of renin activity on trypsin concentration was first examined. As shown in Figure 1, 500 μg/ml trypsin turned out to be optimal for activation of both the cellular and extracellular renin; therefore this concentration was used throughout the activation experiments. Activation of samples was carried out in the following way: To 10 μl of cell homogenate or to 100 μl medium, 0.1 M phosphate buffer (pH 7.0) was added to a final volume of 400 μl. The activation reaction was started by adding 2 μl of a trypsin solution (stock: 100 mg/ml in 0.1 M phosphate buffer, pH 7.0). After an incubation at 4°C for 60 minutes the reaction was stopped by the addition of 2 μl soybean trypsin inhibitor (stock: 100 mg/ml in water). After addition of trypsin inhibitor the reaction vessels were allowed to stand at room temperature for 15 minutes. The samples thus obtained were subsequently assayed for renin activity.

Renin activity determination

Renin activity was determined by its ability to generate angiotensin I from the plasma of bilaterally nephrectomized rats as described elsewhere [16]. Angiotensin I was measured with a commercially available radioimmunoassay (Isotopendienst West, Teufen, Switzerland).

Determination of PGE_2

Concentrations of PGE_2 in the buffer were determined by radioimmunoassay (New England Nuclear, Boston, Massachusetts, USA) after appropriate dilution.

Determination of cAMP

The supernatants were assayed for cAMP using a cAMP binding assay for cAMP exactly as described [17].

Determination of LDH activity

LDH activities in cellular homogenates or buffers were assayed with a commercially available kit (Sigma Chemical Company, St. Louis, Missouri, USA). The samples for LDH determination were not frozen but subsequently were assayed after generation.

Determination of protein

Protein was determined according to the method of Lowry [18] using bovine serum albumin as a standard.

Statistics

Levels of significance were calculated utilizing Student's unpaired t-test. $P < 0.05$ was considered significant.

Reagents

All reagents for cell preparation and cell culture were obtained from Boehringer (Mannheim, FRG). Percoll was obtained from Pharmacia (Uppsala, Sweden). Trypsin, TPA and 8-bromo cGMP were purchased from Sigma. CyA and CyH as pure powders were from the Sandoz Company (Basle, Switzerland). As a rule, stock solutions of CyA and CyH were made as 20 mg/ml in pure ethanol. Dilutions were made in water or buffer. The final concentration of ethanol in all experiments was less than 0.1%.

Results

The effect of CyA on renin release was assessed by incubating isolated juxtaglomerular cells for either 1.5, 4 or 24 hours with different concentrations of CyA (0.01 to 10 μg/ml). The effect of CyA on renin secretion is shown in Figure 2. It is obvious from this figure that CyA enhanced renin release at each time examined in a dose dependent manner. To discover whether the increase of renin activity by CyA was the result of an enhanced conversion of inactive to active renin the media

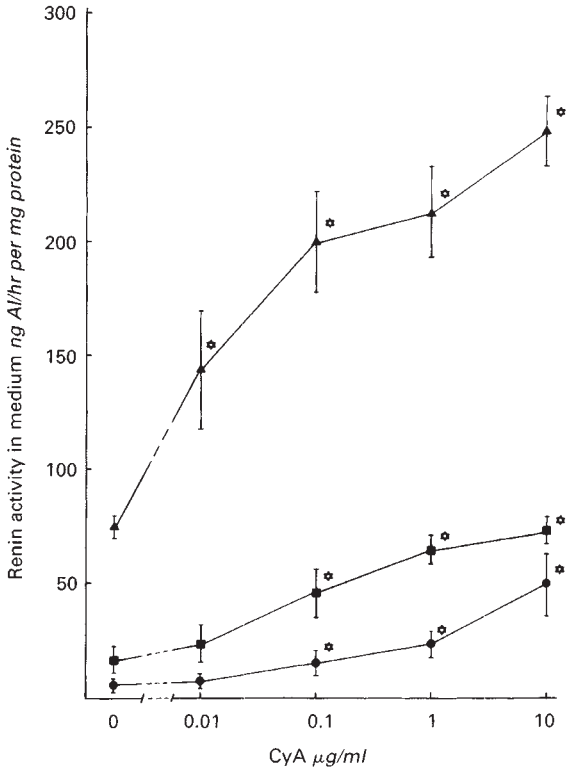

Fig. 2. *Effect of cyclosporine A (CyA) on renin release from isolated rat juxtaglomerular cells.* Cells were incubated for either 1.5 (●), 4 (■) or 24 (▲) hours with CyA. Data are mean ± SEM of five experiments. Asterisk indicates $P < 0.05$ vs. control.

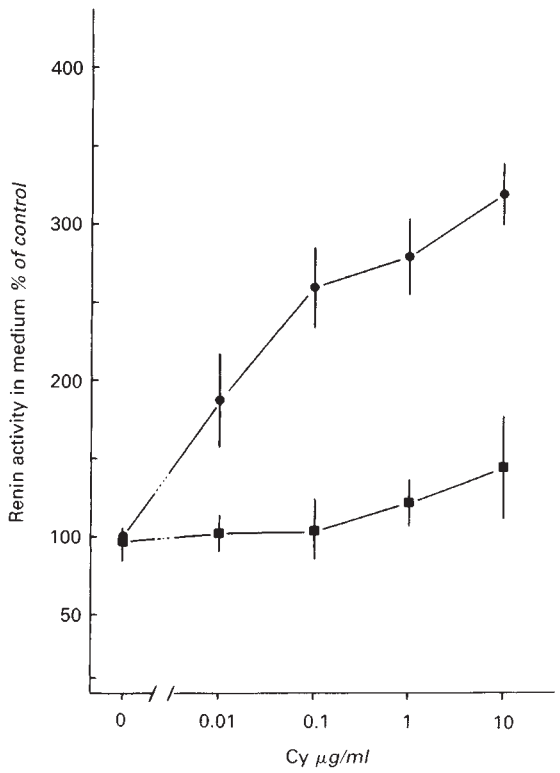

Fig. 3. *Effects of cyclosporine A and H on 24 hours renin release from isolated rat juxtaglomerular cells.* Data (mean ± SEM; $N = 5$) are presented as percentage of control (absence of cyclosporine). Mean control values were 72 ng AI/hr per mg cellular protein and 24 hours, and 58 ng AI/hr per mg cellular protein and 24 hours for CyA (●) and CyH (■), respectively.

were also subjected to trypsin activation. Trypsin treatment increased renin activity by 45 ± 9% (mean ± SEM; $N = 15$) in the absence of CyA, and this figure was not significantly altered by CyA. For instance, renin activities after trypsin activation after 24 hour incubation were 94 ± 8.4, and 312 ± 69 ng AI/hr per mg protein (mean ± SEM; $N = 5$) in the absence and presence of 10 μg/ml of CyA, respectively.

To assess a possible damage of the cells by CyA causing an unspecific passive release of renin from the cells, cellular morphology, cellular protein, intra- and extracellular lactate dehydrogenase (LDH) activities and intracellular renin activities were recorded during the incubation experiments. Cellular morphology as examined by phase contrast microscopy showed no obvious alteration in presence of CyA. Furthermore, CyA did not cause an obvious detachment of cells from the surface of the culture dishes. This observation was supported by the results of protein determinations of attached cells after 1.5, 4 and 24 hours of incubation. Cellular protein per 7 cm^2 petri dish ranged from 80 to 145 μg, and this figure was not significantly altered in presence of CyA.

LDH activity was determined in cells and supernatants after 1.5 and 24 hours of incubation. LDH activity of cells incubated in absence of CyA was not significantly different between the two times. Cellular LDH activity was to 4213 ± 303 mU/mg cell protein (mean ± SEM; $N = 10$), and this figure was not significantly affected by CyA in the concentration range of 0.01 to 10 μg/ml. LDH activities in the supernatants were always below the detection limit (10 mU) of the assay used. Since 200 μl of supernatant were assayed, the total amount of extracellular LDH was less than 50 mU per dish. The cellular LDH activities averaged 450 mU per dish, indicating that extracellular LDH activity was less than 10% of intracellular activity both in the absence and presence of CyA.

To gain some information about the specificity of the observed effects of CyA on renin release we also examined the effect of another cyclosporine, CyH, on the 24-hour renin release (Fig. 3). It can be seen from Figure 3 that CyH had almost no effect on renin release from the isolated JG cells.

Intracellular renin activities were determined after 1.5, 4 and 24 hour incubations in the same experiments shown in Figure 2. In order to measure both active and inactive renin, cellular renin activity was determined with and without trypsin activation. For cells incubated for 1.5 hours in the absence of CyA, renin activity was 1122 ± 84 ng AI/hr per mg of protein (mean ± SEM; $N = 5$) without trypsin activation, and 2356 ± 302 ng AI/h-4 per mg protein with trypsin activation. For the series of experiments in which the cells were incubated for four hours the respective values were 1458 ± 76 and 3645 ± 160. Neither afer 1.5 nor after 4 hours of incubation were cellular renin activities with and without trypsin activation significantly altered by CyA. After 24 hours of incubation, however, CyA caused a concentration dependent increase in trypsin activatable renin, while initially active renin was not altered by CyA (Fig. 4).

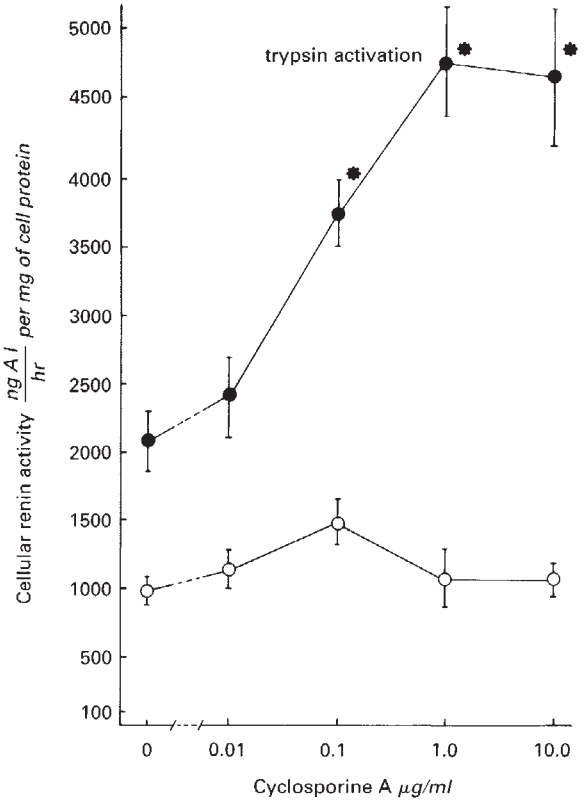

Fig. 4. *Effect of cyclosporine A (CyA) on active (○) and trypsin activatable (●) renin within isolated rat juxtaglomerular cells after 24 hours of incubation with CyA.* Data are mean ± SEM of five experiments. Asterisk indicates $P < 0.05$ vs. control.

Table 1. Effect of 12-O-tetradecanoylphorbol 13-acetate (TPA) and 8-bromo cyclic GMP on 24-hr renin release from isolated rat juxtaglomerular cells in the absence and the presence of CyA (10 μg/ml)

	Renin activity released *ng AI/hr per mg cellular protein and 24 hrs*	
	without CyA	with CyA
TPA 0	45.4 ± 6.2	94.4 ± 8.3
TPA 100 nM	31.8 ± 2.8[a]	45.4 ± 10.6[a]
8-bromo cGMP 0	32.6 ± 3.6	72.0 ± 5.3
8-bromo cGMP 10 μM	20.8 ± 2.4[a]	43.5 ± 3.1[a]

Data are mean ± SEM of five experiments.
[a] $P < 0.05$ vs. control

The sum of the results obtained from the experiments described above suggests that CyA enhances renin release from isolated juxtaglomerular cells by a specific effect rather than by causing cell damage. The next series of experiments was therefore done to gain some insight into the intracellular mechanism by which CyA stimulates renin secretion.

First, we determined the lag time required for CyA to stimulate renin secretion. Isolated juxtaglomerular cells were incubated with or without a single concentration of CyA (1 μg/ml) and the amount of renin released after 10, 30 and 60 minutes was determined (Fig. 5A). The amount of renin released was significantly higher in presence of CyA after only 10 minutes, indicating a fast onset of action for CyA on juxtaglomerular cells.

It has been reported that CyA is capable of enhancing prostaglandin release from tissues [19–21]. Since prostaglandins, in particular PGE_2, are stimulators of renin secretion [22], we measured the rate of PGE_2 release and renin secretion in the same experiments (Fig. 5B). It is obvious in this figure that CyA (1 μg/ml) did not alter PGE_2 release.

Another second messenger that has been established as a stimulatory signal for renin secretion is cAMP [23]. Therefore, in some experiments we measured the intracellular cAMP levels in addition to renin secretion and PGE_2 release (Fig. 5C). It can be seen in this figure that CyA (1 μg/ml) did not affect intracellular cAMP levels.

A final set of experiments was done to examine whether or not renin release evoked by CyA could be inhibited by manipulations that usually inhibit renin release from isolated JG cells. There is evidence that addition of phorbol esters and an increase of intracellular cGMP inhibits renin release [13]. We therefore examined the effects of the phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA, 100 nM) and the cyclic GMP derivative 8-bromo cGMP (10 μM) on 24-hour renin release in the absence and presence of CyA (10 μg/ml). The results of these experiments are documented in Table 1. It is apparent that both the basal and CyA stimulated renin release were attenuated by TPA and 8-bromo cGMP.

Discussion

Under cyclosporine A (CyA) treatment an increase of plasma renin activity (PRA) has often been observed in humans and in laboratory animals [4], in particular, during acute administration of the drug. Since PRA is primarily determined by the rate of renin secretion from the kidneys, the question arises as to how CyA stimulates renal renin release. CyA has been found to cause a variety of changes in renal function including a decrease of renal blood flow [10], a decrease of glomerular filtration [1, 12] and tubular damage [4]. Renin secretion on the other hand is known to be dependent on the renal perfusion pressure, on glomerular filtration, and in particular, on the load of sodium chloride in the region of the macula densa [22]. At the moment it is now known whether the enhancement of renin secretion is a consequence of the alterations of renal functional parameters by CyA or is due to a direct effect of CyA on juxtaglomerular cells, which are the site of renal renin production and secretion. Our experiments as documented in Figure 1 clearly show that CyA is capable of stimulating renin release from isolated juxtaglomerular cells in a dose dependent fashion. The increase of extracellular renin activity was not due to an increased conversion of prorenin to renin, because the proportion of active to inactive renin was not affected by CyA. Since CyA did not alter apparent cellular morphology and cellular protein content, nor did it cause the release of unspecific cytosolic proteins such as LDH, we may infer that CyA stimulates renin release from isolated juxtaglomerular cells by a specific effect rather than by causing cell damage. Experiments with CyH provide a further indication that this effect of CyA is a specific cyclosporine effect. CyH differs from CyA only at amino acid position 11, and only weakly binds to cyclosphilin. CyH is orders of magnitude less effective in immunosuppression than CyA, and the same holds for adverse renal side effects (Dr.

Ryffel, personal communication). Thus the observation that CyH did not enhance renin secretion argues against an unspecific cyclosporine effect on the one hand and fits well with the in vivo observations on the other. Concerning the direct effect of CyA on renin secretion, our results are in harmony with the more single finding obtained by Baxter et al [24] that CyA in a concentration of 8 μM stimulates renin release from renal cortical slices in a time interval between 30 to 240 minutes.

We obtained evidence that CyA led to an increase of trypsin activatable renin activity within the cells in a dose dependent fashion in an incubation interval between 4 and 24 hours (Fig. 4). Since trypsin activatable renin is considered prorenin [25], our results as documented in Figure 4 indicate that CyA increased the amount of intracellular prorenin without changing the amount of primarily active renin.

Together with the observation that extracellular renin was also increased by CyA, these findings suggest that CyA not only stimulates renin secretion but also renin production in isolated juxtaglomerular cells, at least after 24 hours.

This conclusion fits well with the observations that CyA in vivo not only increases PRA but also renal renin content [8]. Taken together we infer from our results that CyA is capable of stimulating renin secretion and renin production in isolated juxtaglomerular cells by a direct effect in these cells.

We tried to gain some insight as to how CyA could affect juxtaglomerular cell function. The intracellular mediation of the effects of CyA on target cells is generally not well understood. There is evidence for a cytosolic binding protein for CyA named cyclosphilin [26]. The dissociation constant for CyA to cyclosphilin is 2×10^{-7} M. Interestingly this value is very close to the concentrations for CyA required for half maximal effects on renin secretion and intracellular accumulation of prorenin, which we estimated to be around 10^{-7} M from the data presented in Figures 2 and 4. Thus a mediation of the effects of CyA on juxtaglomerular cells by binding to cyclosphilin is consistent with these data.

Our results as documented in Figure 5A indicate that the effect of CyA on renin secretion begins within the first ten minutes, suggesting a rapid onset of action on juxtaglomerular cells. An almost immediate onset of action of CyA was also observed by Nicchita, Kamoun and Williamson [27] who studied the influence of CyA on calcium handling in isolated hepatocytes.

There are some reports in the literature that CyA is capable of enhancing renal PGE_2 release [19–21]. Since PGE_2 is a stimulator of renin secretion [22] we examined whether or not enhancement of renin secretion by CyA could be due to an enhancement of PGE_2 formation in the isolated cells. However, as shown in Figure 5B we failed to detect a stimulation of PGE_2 release by CyA in the same experiments in which renin secretion was stimulated (Fig. 5A). This in vitro finding parallels the in vivo results of Baxter et al [28] who observed a stimulation of renin secretion without an enhancement of renal prostaglandin release by CyA. Thus we may infer that stimulation of renin secretion from juxtaglomerular cells by CyA is not due to an enhanced prostaglandin synthesis.

The best characterized, stimulatory intracellular signal for renin secretion in juxtaglomerular cells is cAMP [23]. It was therefore obviously important to examine the influence of CyA on intracellular cAMP levels in isolated juxtaglomerular cells.

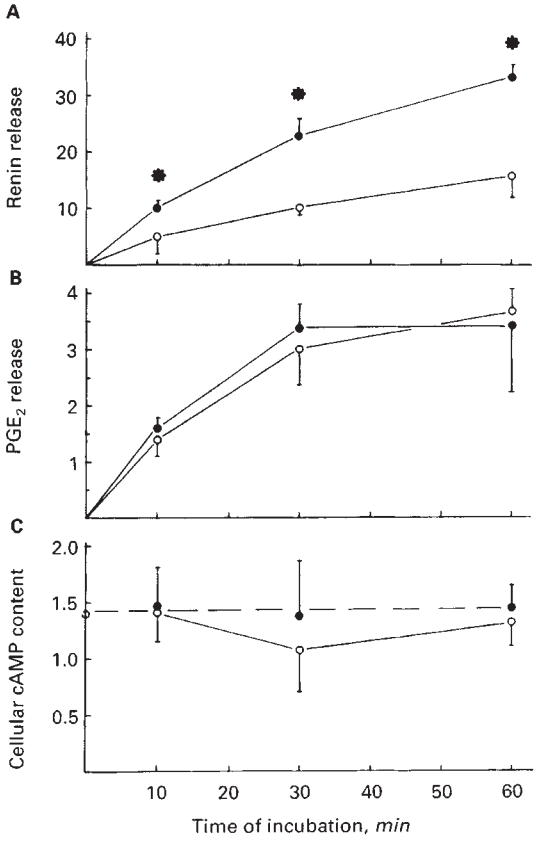

Fig. 5. A *Time course of extracellular renin activity (ng A I/hr per mg of prot.) after addition of cyclosporine (CyA) 0 (○) or 1 μg/ml (●) to isolated rat renal juxtaglomerular cells.* Data are mean ± SEM of five experiments. Asterisk indicates $P < 0.05$ vs. control. **B** Time course of extracellular prostaglandin E_2 (PGE_2) (ng PGE_2/per mg of protein) after addition of CyA 0 (○) or 1 μg/ml (●) to isolated rat renal juxtaglomerular cells. Data are mean ± SEM of five experiments. **C** Time course of intracellular cAMP levels (pmol cAMP/mg prot.) after addition of CyA 0 (○) or 1 μg/ml (●) to isolated rat renal juxtaglomerular cells. Data are mean ± SEM of five experiments.

As documented in Figure 5C, CyA (1 μg/ml) did not affect intracellular cAMP in the same experimental conditions under which a stimulation of renin secretion was observed. Thus we may infer that cAMP is not likely to be the second messenger mediating the effect of CyA on juxtaglomerular cells. Also Duggin et al [29] did not observe a stimulation of cAMP release from renal tissues by CyA.

Recently it has been shown that CyA also binds to calmodulin [30]. In this context it has been demonstrated that CyA inhibits calmodulin dependent reactions. Moreover, binding of CyA to calmodulin was found to be competitively inhibited by calmodulin antagonists. Since calmodulin antagonists are well documented stimulators of renin secretion [23], calmodulin antagonism could be a possible explanation for the stimulatory effect of CyA on renin secretion. Half maximal inhibition of calmodulin mediated reactions were observed at around 100 nM CyA [30].

As already mentioned above, this is the same concentration of CyA at which half maximal effects on renin secretion and on intracellular renin content were observed.

Although our results do not permit us to infer a mechanism by which CyA enhances renin secretion, some findings indicate that the mechanism of renin secretion is not totally different in presence of CyA. This conclusion is made from the observation that phorbol ester and cyclic GMP inhibit renin secretion in the absence and the presence of CyA (Table 1).

In summary, the results obtained in this study suggest that CyA has a direct effect on rat renal juxtaglomerular cells. It enhances renin secretion from these cells with a fast onset of action. With some delay it also leads to an increase in the intracellular amount of prorenin. Since CyA causes both an enhanced release of renin and, simultaneously an increase in renin stores, we may infer that CyA also stimulates renin production. The mode of action by which CyA could affect juxtaglomerular cell function is less clear. From our experiments neither prostaglandins nor cAMP appear to be mediators. If CyA acts as a calmodulin antagonist in juxtaglomerular cells, as it has been demonstrated so far only for T-lymphocytes [30], then a calmodulin antagonism would be a likely explanation for the effects of CyA on juxtaglomerular cells.

From a more clinical point of view it is of importance that the effects of CyA on isolated juxtaglomerular cells occurred at concentrations that are typical extracellular concentrations during CyA treatment [4]. Thus a direct effect of CyA on juxtaglomerular cells in vivo is not unlikely. In vivo CyA causes an increase of plasma renin activity during acute or short term administration. Long-term administration has been reported to lower plasma renin activity and to depress the RAS. The cell culture system of JG cells only permits short-term studies [15]. Our results can therefore only be compared with the situation of acute administration of CyA in vivo, and these results could help to understand how CyA elevates plasma renin activity.

Acknowledgments

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