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Cyclosporin A inhibits PGE_2 release from vascular smooth muscle cells

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Summary: The influence of the fungoid undecapeptide cyclosporin A (CyA) on PGE_2 release from cultured rat aortic smooth muscle cells was investigated in this study. We found that CyA time and concentration dependently (ED_{50}: 500 ng/ml) inhibited PGE_2 release from the cells. CyA attenuated both basal and PGE_2 release evoked by angiotensin II (10^{-10}-10^{-6}M), arginine vasopressine (10^{-10}-10^{-6}M)and ionomycin (10^{-9}-10^{-6}M). CyA (1µg/ml) did not affect the conversion of exogenous arachidonic acid (1µM) into PGE_2. The inhibitory effect of CyA was neutralized by high concentrations of the calcium ionophore ionomycin (> $3x10^{-6}$M). Taken together our results indicate that CyA inhibits both basal and vasoconstrictor evoked PGE_2 release from vascular smooth muscle by impairing the availability of free arachidonic acid rather than by inhibiting the conversion of arachidonic acid into PGE_2.

The fungoid undecapeptide cyclosporin A (CyA) (1) is a potent inhibitor of interleukin-2 production in T-lymphocytes(2). Due to this action CyA has become a powerful tool in the management of allograft rejection. However, its usefulness is limited by severe adverse reactions. Thus CyA has detrimental effects on blood vessels causing arteriolopathy and enhanced arteriolar constriction resulting in systemic hypertension and reduction of renal blood flow(cf 3). The mechanism by which CyA alters arteriolar function is not yet understood. It has been observed that CyA impairs vascular prostaglandin formation in vivo(4) and

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endothelial prostacyclin formation in vitro(5) and that exogenous prostaglandins are capable to protect from the detrimental vascular effects of CyA(6,7). Vasodilatory prostaglandins, in particular PGE_2, are produced by vascular smooth muscle cells(VSMC) in response to vasoconstrictive hormones. Since this modulatory reaction is important for the regulation of VSMC function (8) the present study was done to find out whether CyA alters PGE_2 formation in response to vasoconstrictors in cultured VSMC from rat aorta.

Materials and Methods

cell culture: Cultures of VSMC from rat aorta were prepared and handled as described previously (9). The cells were grown on 24-well plates in RPMI 1640 medium (supplemented with 10% FBS, 25 mM Hepes, 1 U/ml insulin and penicillin/streptomycin). Subconfluent cultures between the second and fourth passage were used for the experiments.

experiments: Cells were preincubated with normal cell culture medium containing CyA for different times (0.15 - 24 hours) at a single concentration (1µg/ml) or at different concentrations (0.01 - 10 µg/ml) for a single time interval (3 hours). After preincubation the multiwell plates were placed on a heater maintaining them at 37°C. The medium was removed and the cells were quickly washed twice with prewarmed L-15 medium (without supplementation). The cells were then incubated with 0.2ml of this medium with or without agents. After five minutes the medium was removed and immediately centrifuged at 4000g for five minutes. The supernatant was frozen in liquid nitrogen and stored at -80°C until assay of PGE_2. Concentrations of PGE_2 were determined with a commercially available radioimmunoassay (New England Nuclear). The cells were lysed in 1N NaOH and cellular protein was determined according to the method of Lowry (10) using bovine serum albumin as a standard.

Determination of fractional arachidonic acid release: Cells were prelabelled with 0.2µCi/ml of ^{14}C-arachidonic acid (New England Nuclear) in DMEM medium supplemented with fatty acid free bovine serum albumin for 24 hours. CyA (10µg/ml) was added during the last three hours of the labelling period. Afterwards the cells were thoroughly washed with L-15 medium and incubated with this medium (with and without angiotensin II, 10^{-7}M) for 15 minutes. The supernatant then was removed and the cells were lysed by the addition of 1N NaOH. Fractional AA-release was calculated from the ratio of radioactivity in the supernatant over the sum of radioactivities present in cells and supernatant.

chemicals: CyA as a pure powder was a generous gift from the Sandoz Company (Basle,Switzerland). Arginine vasopressin (AVP),

angiotensin II (AII)and arachidonic acid (AA) were purchased from Sigma, ionomycin was obtained from Calbiochem. Stocks of CyA (20mg/ml), ionomycin (10 mM) and arachidonic acid (3mM) were prepared in pure ethanol. Further dilutions were made in L-15 medium.

Results

In vivo, the effects of CyA occur in a time and concentration dependent fashion. In order to find out whether CyA affects PGE_2 from VSMC at all, the cells were preincubated with a single concentration of CyA (1µg/ml) for different times. PGE_2 was stimulated with a submaximal concentration of the calcium ionophore ionomycin (10^{-6}M). The results shown in table I indicate that CyA time dependently inhibited ionomycin evoked PGE_2 release. Already after one hour of preincubation the inhibitory effect of CyA on PGE_2 release was maximal and this effect of CyA was not altered by longer incubation.
Further experiments were therefore done after a preincubation period of three hours with CyA as a rule. In figs. 1 and 2 the effect of CyA on PGE_2 release evoked by the vasoconstrictive hormones AVP and AII are shown. It is evident that CyA already diminished basal PGE_2 release. Taking all experiments together the ED_{50} value for the inhibitory action of CyA on basal PGE_2 release was estimated to around 500 ng/ml. The figures further show that CyA also attenuated PGE_2 release evoked by AVP and AII. Obviously CyA did not shift the dose-response curves for both hormones.

Table 1

PGE_2 release from VSMC in response to ionomycin (1µM) after various times of preincubation with CyA (1µg/ml)

	PGE_2 (ng/min per mg cellular protein)					
time (h)	0.15	0.5	1	3	5	24
control	5.2±0.4	5.0±0.2	5.5±0.7	4.9±0.6	4.5±0.2	4.2±0.4
CyA (1µg/ml)	4.9±0.5	3.9±0.6	2.4±0.5	2.5±0.3	2.3±0.6	2.0±0.3
CyA/co	0.95	0.78	0.44	0.52	0.50	0.48

Data are mean + SEM of quadruplicates. Basal PGE_2 release was 1.2+0.2 ng/min per mg cellular protein.

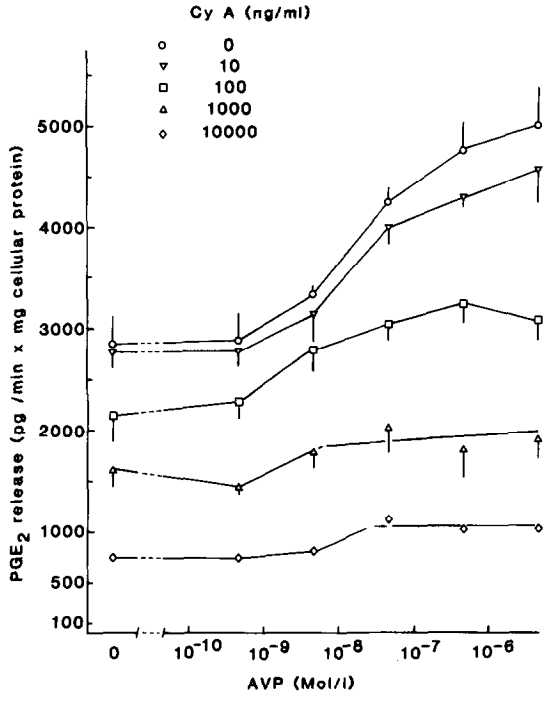

Figure 1. Effect of CyA on PGE_2 release from VSMC in response to arginine vasopressin (AVP). Preincubation time with CyA was three hours. Data are mean + SEM of five experiments.

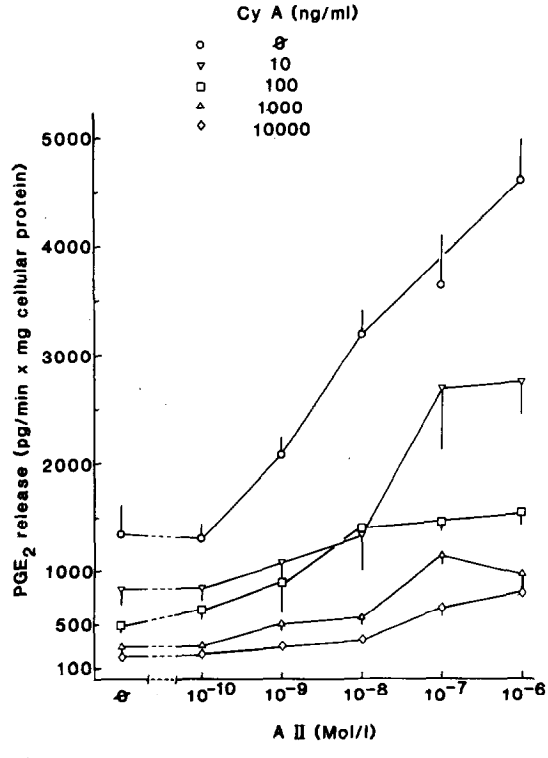

Figure 2. Effect of CyA on PGE_2 release from VSMC in response to angiotensin II (AII). Data are mean + SEM of five experiments.

Table 2

Effect of CyA on PGE_2 release from VSMC in presence of exogenous arachidonic acid (1µM)

CyA (µg/ml)	0	0.01	0.1	1.0	10.0
PGE_2 (ng/min x mg)	10.4±2.1	14.4±1.0	10.6±2.5	13.5±1.4	12.9±0.9

Data are mean + SEM of five experiments. Basal PGE_2 release was 2.0+0.3 ng/min x mg).

Cellular prostaglandin formation in general is determined by the activity of phospholipases which liberate the substrate arachidonic acid (AA) one the one hand and the conversion rate of AA into prostaglandins by cyclooxygenase activity on the other (11). To examine whether CyA could affect the conversion of AA by interference with cyclooxygenase activity, PGE_2 formation in presence of exogenous AA (1µM) was determined. The results (table II) clearly show that the conversion of AA into PGE_2 was not altered by CyA.

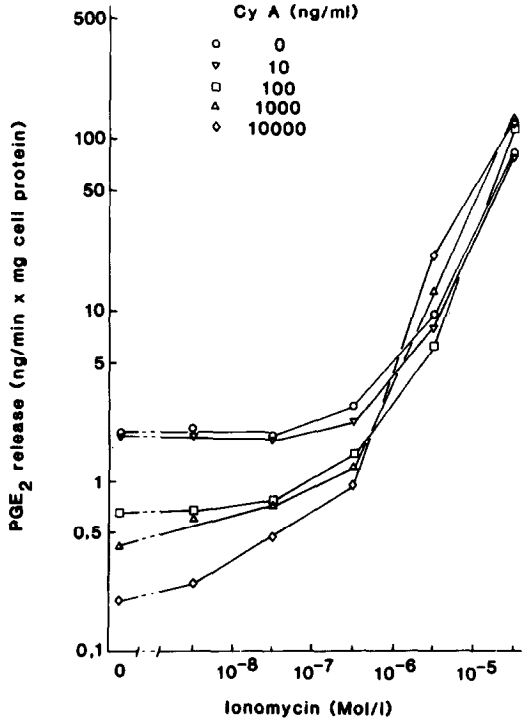

Figure 3. Effect of CyA on PGE_2 release from VSMC in response to ionomycin. Data are mean of five experiments.

The activity of phospholipases, in particular of phospholipase A_2 is calcium dependent (12,13). To find out out whether or not CyA could affect phospholipase activity, PGE_2 formation in presence of the calcium ionophore ionomycin was measured. The results (fig.3) show that CyA inhibited ionomycin evoked PGE_2 release up to a concentration of around 10^{-6}M. However, at high concentrations of ionomycin (>$3x10^{-6}$M) no inhibitory effect of CyA on prostaglandin formation could be observed. Phospholipase activity was further assessed by determination of the fractional ^{14}C-arachidonic acid release from prelabelled VSMC. Fractional AA-release within 15 minutes from control cells was 1.6 +/- 0.2 % (mean+/-SEM;n=5). In presence of angiotensin II (10^{-7}M) this figure was raised to 8.2 +/- 0.4%. After 3 hours of preincubation with CyA (10µg/ml) fractional AA-release in presence of A II was 2.9 +/- 0.7 %.

Discussion

This study was undertaken to determine if CyA alters PGE_2 production by rat aortic smooth muscle cells. We found that CyA inhibited both basal and vasoconstrictor evoked PGE_2 formation by a factor of 1.5 - 2 in a concentration dependent fashion. The ED_{50} value was estimated to be around 500ng/ml and this figure is in the range of conventional therapeutic levels attained in serum in vivo in man and laboratory animals (3). Concerning the mechanism by which CyA could inhibit PGE_2 formation our results indicate that CyA does not inhibit cyclooxygenase activity, because the conversion of exogenous arachidonic into PGE_2 was not altered by CyA (table II). Thus it seems likely that CyA reduced the availability of free arachidonic acid (AA). The level of free AA is determined by the rate of reacyclation by the activity of acyl-transferases on the one hand and by the rate of deacylation by the activity of phospholipases (PLA) on the other (11). The observations that PGE_2 release in response to specific, receptor-mediated (in the case of AVP and AII) and unspecific (in the case of ionomycin) stimulation of PLA activity was blunted by CyA

argue in favour of an interference of CyA with the liberation of AA. Impaired deacylation of AA from phospholipids could result from a reduction of AA stores or from a functional inhibition of PLA activity. From the findings that impaired angiotensin II induced AA release and that high concentrations of ionomycin were able to overcome the inhibitory effect of CyA on PGE_2 release (fig.3) we may infer that CyA acts by inhibiting PLA activity rather than by depleting pools for AA. This conclusion fits with recent findings demonstrating that CyA inhibits phospholipase activity in macrophages (14) and in renal mesangial cells (15). Our observation that the inhibitory action of CyA on PGE_2 formation was modulated by the calcium ionophore could indicate that CyA affects the interaction between calcium and PLA. It might be of relevance in this context that the activation of PLA_2 by calcium appears to be mediated by calmodulin (16,17). For T-lymphocytes evidence was recently provided that CyA binds to calmodulin and antagonizes calmodulin activity (18). Under the assumption that CyA binds to calmodulin also in vascular smooth muscle cells a calmodulin antagonism could be the mechanism by which CyA inhibits phospholipase activtity and in consequence PGE_2 formation. To prove this hypothesis remains a task for future research.

In summary our results indicate that attenuation of PGE_2 release could be one pathophysiologic mechanism by which CyA alters vascular smooth muscle cell function.

Acknowledgements

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