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Mechanism of enhanced vasoconstrictor hormone action in vascular smooth muscle cells by cyclosporin A

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1 The use of the immunosuppressive drug cyclosporin A (CsA) is limited by two major side effects, nephrotoxicity and hypertension, which are caused by drug-induced local vasoconstriction. We have recently shown that CsA potentiates the contraction of isolated resistance arteries to vasoconstrictor hormones and increases the calcium response to these agents in vascular smooth muscle cells (VSMC). The goal of the present study was to investigate further the molecular mechanism(s) involved in these effects.

2 Stimulation of VSMC with [Arg]^{8}vasopressin (AVP) induced a concentration-dependent increase in total inositol phosphates (InsP) and cellular calcium response (as measured by $^{45}Ca^{2+}$ efflux). Preincubation of VSMC with CsA increased both InsP formation and $^{45}Ca^{2+}$ efflux.

3 The potentiating effect of CsA on AVP-elicited InsP formation and $^{45}Ca^{2+}$ efflux was inhibited by co-incubation with the protein synthesis inhibitors actinomycin D and cycloheximide, indicating that CsA acted on gene expression.

4 Binding experiments with [^{3}H]-AVP on VSMC showed that CsA increased the number of AVP receptors by about two fold without affecting receptor affinity. Actinomycin D completely blocked this increase.

5 These results demonstrate for the first time that incubation of VSMC with CsA increases the expression of AVP receptors, resulting in a potentiation of InsP formation and calcium response upon stimulation with AVP. This effect of CsA is likely to occur with other vasoconstrictor hormone receptors as well and could be a key mechanism in the induction of vasoconstriction, and subsequent drug-induced nephrotoxicity and hypertension.

Keywords: Cyclosporin A; smooth muscle; inositol phosphates; calcium; vasopressin; vasoconstrictor hormones; receptors; hypertension

Introduction

Cyclosporin A (CsA) is the most widely used immunosuppressive drug for preventing graft rejection and autoimmune diseases. However, drug-induced nephrotoxicity and hypertension limit its utilization (Kahan, 1989). It has been shown that an increase in vasoconstriction by CsA is responsible for both side-effects (Lamb & Webb, 1987; Rego *et al*., 1990; Sturrock *et al*., 1993). We have recently demonstrated that CsA potentiates the cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) increase in response to vasoconstrictor hormones in vascular smooth muscle cells (VSMC) (Lo Russo *et al*., 1996; 1997). The known pharmacological targets of CsA, i.e. cyclophilin and calcineurin (Liu *et al*., 1991), were not involved in mediating the $[Ca^{2+}]_c$ increase (Lo Russo *et al*., 1996).

Stimulation of vasoconstrictor hormone receptors in VSMC activates phospholipase C which leads to an increase in the production of inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ binds to its intracellular receptor, induces extrusion of calcium from the calcium pools in the endoplasmic reticulum and subsequently, causes an elevation of $[Ca^{2+}]_c$ (Berridge, 1993).

As CsA has been shown to potentiate the calcium response to at least four vasoconstrictor hormones (Pfeilschifter & Rüegg, 1987; Meyer-Lehnert & Schrier, 1989; Kremer *et al*., 1989; Lo Russo *et al*., 1996; 1997), we have investigated its effect on the formation of inositol phosphates (InsP) in response to stimulation of VSMC with one of them, namely [Arg]^8 vasopressin (AVP). The effect of CsA upstream of InsP formation, i.e. with respect to density of AVP receptors, was also investigated and inhibitors of transcription or translation (actinomycin D and cycloheximide, respectively) were tested with respect to receptor levels, InsP formation and Ca^{2+} response.

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Methods

Culture of smooth muscle cells

Vascular smooth muscle cells (VSMC) were isolated from aortae of male Wistar Kyoto rats (200–300 g) as described by Lo Russo *et al*. (1996). VSMC were seeded at 15 000 cells cm^{-2} into culture flasks (80 cm^2) in Dulbecco's modified Eagle medium (DMEM) supplemented with essential amino acids, vitamins, ciproxin and 10% foetal calf serum (FCS), and kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were used between passage 5 and 9. After 6–7 days they were transferred into culture wells for $^{45}Ca^{2+}$ flux and inositol phosphate measurements: 20 000 cells were seeded into 16 mm diameter wells of 24-well culture plates and were used at confluence after 7 to 8 days.

Preincubation of VSMC with CsA or protein synthesis inhibitors was performed in DMEM without FCS for 20 h.

As an estimate for toxicity of CsA or protein synthesis inhibitors, total protein was measured after solubilization of adherent cells with 0.1% sodium dodecyl-sulphate by use of the method of Bradford (1976).

$^{45}Ca^{2+}$ efflux experiments

For $^{45}Ca^{2+}$ loading, cells were washed twice with physiological salt solution (PSS, see below) at 37°C and incubated for 15 min at 37°C in PSS/0.12 mM $CaCl_2$ containing 1 μCi of $^{45}Ca^{2+}$/well (and CsA if indicated). The cells were rapidly washed 4 times with ice-cold PSS/1.2 mM $CaCl_2$. $^{45}Ca^{2+}$ efflux was initiated by incubating the cells at 37°C in PSS/1.2 mM $CaCl_2$ (0.5 ml/well). After 3, 6, 9 and 11 min supernatants were collected and immediately replaced by 0.5 ml of fresh PSS/1.2 mM $CaCl_2$ at 37°C. AVP was added in the buffer at the 9th min. Cellular $^{45}Ca^{2+}$ content was determined by detaching the cells with 50 μl of a solution of trypsin/EDTA (0.25/1%; w/v,

respectively) followed by addition of 250 μl of a sodium dodecyl-sulphate (1%; w/v) solution.

The radioactivity of the supernatants and of the lysate was measured by liquid scintillation counting (Packard Tri Carb 4640).

For dose-response curves, results are expressed as % of increase of $^{45}Ca^{2+}$ efflux (0% corresponds to the basal $^{45}Ca^{2+}$ efflux and 100% corresponds to the maximum agonist-induced $^{45}Ca^{2+}$ efflux in control cells).

InsP measurements

Confluent VSMC in 24 well plates were kept for 24 h in DMEM without FCS and inositol. After this preincubation, 2 μCi ml^{-1} of myo-[2-^{3}H]-inositol and CsA (if indicated) were added for 20 h. VSMC were washed two times with PSS/1.2 mM $CaCl_2$ and incubated at 37°C for 15 min in PSS containing 50 mM LiCl and 95 mM NaCl. After AVP was added at the concentration indicated, incubation was continued for another 15 min. Reactions were terminated by the addition of ice-cold perchloric acid (0.4 M). Samples were neutralized by adding KOH (1 M) and tris(hydroxymethyl)aminomethane chloride (final concentration: 0.1 M). The precipitate (protein and $KClO_4$) was discarded after centrifugation at 3000 r.p.m.

Supernatants were applied to columns of Dowex AG1-X4 (formate form, 50–100 mesh). The resin was washed twice with 5 ml of water to eliminate free [^{3}H]-inositol. Total [^{3}H]-inositol phosphates (InsP) were eluted twice with 2 ml of HCl (1 M) and radioactivity was determined by liquid scintillation counting (Packard Tri Carb 4640).

For dose-response curves, results are expressed as % of increase of InsP formation (0% corresponds to the basal InsP and 100% corresponds to the maximum of agonist-induced InsP formation in control cells).

[^{3}H]-AVP binding

Confluent monolayers of VSMC grown in 24-well plates were washed twice with binding buffer (PSS/1.2 mM $CaCl_2$ containing 1% bovine serum albumin) at 20°C. Binding was initiated by adding 200 μl of binding buffer containing 0.25 to 10 nM [^{3}H]-AVP (64.2 Ci mmol^{-1}) with or without 300 nM AVP at 20°C. After 10 min, the binding buffer was aspirated. In order to remove the unbound radioligand, cells were washed four times with ice-cold binding buffer. Cells were lysed by adding 50 μl of a solution of trypsin/EDTA (0.25%/1%; w/v, respectively) followed by addition of 250 μl of a sodium dodecyl-sulphate (1%; w/v) solution. Radioactivity of the lysate was measured by liquid scintillation counting (Packard Tri Carb 4640). Specific binding was determined by subtracting from total binding the radioactivity bound in the presence of 300 nM unlabelled AVP.

Data analysis

Results are expressed as mean $\pm$ s.e.mean for $^{45}Ca^{2+}$ efflux and InsP measurement. Student's t test for paired data was used to test for statistical significance of differences; a value of $P<0.05$ was considered significant. Receptor densities (B_{max}) and dissociation constants (K_D) were calculated from [^{3}H]-AVP binding experiments by the programme STEP (Pliska *et al.*, 1986; Pliska, 1995).

Chemicals and buffers

Cyclosporin A was a gift from Sandoz Pharma Ltd (Basle, Switzerland). [Arg]^{8}vasopressin (AVP) was obtained from Bachem Feinchemikalien AG (Switzerland), [Arg]^{8}vasopressin[phenylalanyl-3,4,5-^{3}H(N)] (64.2 Ci mmol^{-1}) from Dupont (U.S.A.), $^{45}Ca^{2+}$ (10–40 mCi mg^{-1} calcium) and myo-[2-^{3}H]-inositol from Amersham International (U.K.). All chemicals used were of the purest grade available. Stock solutions of CsA were prepared at a concentration of 10 mM in ethanol, while the 1 mM stock solution of AVP was in 0.1 mM acetic acid. At most, 0.1% of ethanol was present in the assays and, as tested, this did not affect calcium or InsP responses. The composition of the physiological salt solution (PSS) was, in mM: NaCl 145, KCl 5, HEPES 5, $MgCl_2$ 1, glucose 10, pH 7.4; containing 0.12 or 1.2 mM $CaCl_2$.

Results

Effect of CsA on InsP formation and $^{45}Ca^{2+}$ efflux in response to AVP

Stimulation of VSMC with increasing concentrations of [Arg]^{8}vasopressin (AVP) induced a rapid and concentration-dependent increase in $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$ pre-loaded cells. Measurement of InsP formation gave a similar dose-response curve (Figure 1). When cells were pretreated with CsA (20 h, 10^{-6} M) no change in basal InsP formation or $^{45}Ca^{2+}$ efflux was observed. However, the effects of AVP on these two second messengers were potentiated resulting in an increased efficacy as seen by an upward shift of the dose-response curves (Figure 1). Conversely, no or only a slightly significant change of potency was noted (compare pEC_{50} values in Table 1).

The CsA-induced potentiation of InsP formation or $^{45}Ca^{2+}$ efflux were concentration-dependent with a threshold at 10^{-7} M CsA (114$\pm$4% for InsP formation and 109$\pm$2% for $^{45}Ca^{2+}$ efflux as compared to control, $P<0.05$) (Figure 2). Maximal effects were obtained at $10^{-5.5}$ M CsA (155$\pm$5% and 152$\pm$3% for InsP formation and $^{45}Ca^{2+}$ efflux, respectively). The slight decrease of InsP formation observed with 10^{-5} M CsA is probably due to an enhanced sensitivity of VSMC to CsA toxicity as InsP were measured after prolonged exposure to foetal calf serum-free medium (48 versus 24 h for $^{45}Ca^{2+}$ efflux measurement). An increase in [^{3}H]-inositol incorporation into phosphoinositides can be ruled out as basal [^{3}H]-InsP associated radioactivity was unaltered after CsA treatment of VSMC (see also Figures 1 and 2).

In order to test whether the potentiation of CsA on InsP formation could be caused by its stimulating effect on the cytosolic calcium concentration ($[Ca^{2+}]_c$) we measured InsP formation under conditions that prevented a rise in $[Ca^{2+}]_c$. We found that incubation with the calcium chelator BAPTA/AM (50 μM for 45 min), which completely prevented the AVP-induced rise in $[Ca^{2+}]_c$ as well as $^{45}Ca^{2+}$ efflux, did not affect

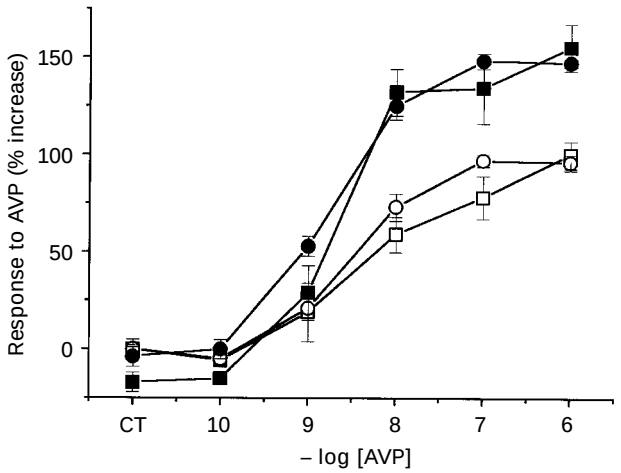

Figure 1 Effects of cyclosporin A (CsA) pretreatment on [Arg]^{8}vasopressin (AVP)-induced rise in inositol phosphate (InsP) formation (■, □) and $^{45}Ca^{2+}$ efflux (●, ○) in VSMC. Results from cells treated with vehicle alone are represented by open symbols and those treated with CsA (10^{-6} M for 20 h) by solid symbols. Values are mean of three experiments performed in quadruplicate; vertical lines show s.e.mean.

Table 1 Effect of preincubations of VSMC with vehicle alone (CT) or cyclosporin A (CsA, 10^{-6} M, 20 h) on InsP formation and $^{45}Ca^{2+}$ efflux in response to AVP

	pEC_{50} (−log M)		*Maximal effect* (%)	
	CT	*CsA*	*CT*	*CsA*
InsP formation	8.21 ± 0.28	8.72 ± 0.12 (NS)	100 ± 2	155 ± 12**
$^{45}Ca^{2+}$ efflux	8.49 ± 0.09	8.78 ± 0.04*	98 ± 4	148 ± 2***

NS: not significantly different from control ($P > 0.1$); *$P \leqslant 0.025$, **$P \leqslant 0.02$, ***$P < 0.01$, significantly different from control.

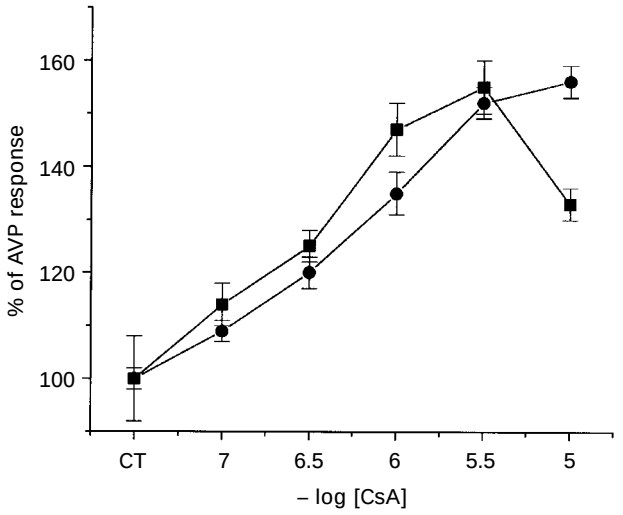

Figure 2 Concentration-dependent effects of cyclosporin A (CsA) on inositol phosphate (InsP) formation (■) and $^{45}Ca^{2+}$ efflux (●) stimulated by AVP. VSMC were pretreated with increasing concentrations of CsA or vehicle alone, 20 h before stimulation with AVP ($10^{-8.5}$ M). InsP formation or $^{45}Ca^{2+}$ efflux were measured. Values are mean of three experiments performed in quadruplicate; vertical lines show s.e.mean.

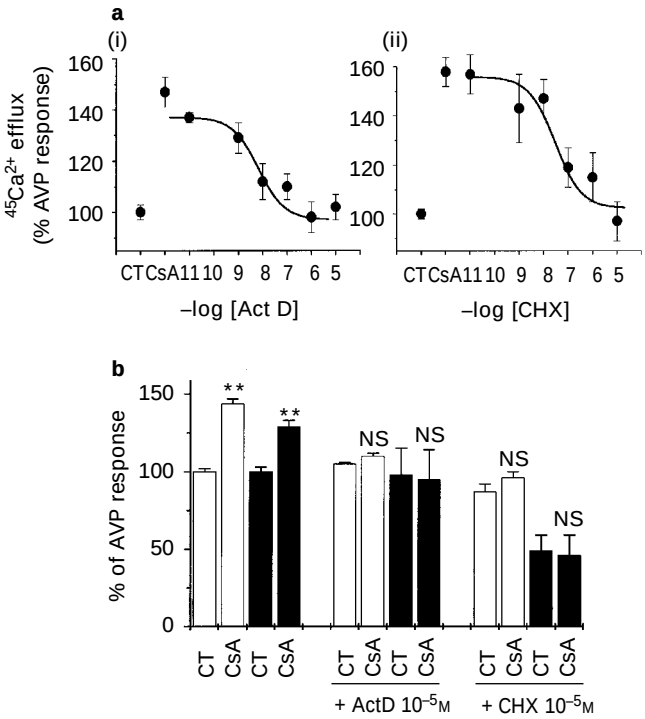

Figure 3 Effect of protein synthesis inhibitors on the potentiating effect of cyclosporin A (CsA). (a) VSMC were pretreated with vehicle alone (CT) or with CsA (10^{-6} M, 20 h) alone or with increasing concentrations of (i) actinomycin D (ActD) or (ii) cycloheximide (CHX). Cells were stimulated with AVP ($10^{-8.5}$ M) and $^{45}Ca^{2+}$ efflux was measured. (b) ActD or CHX (10^{-5} M) was tested on InsP formation (solid columns) or $^{45}Ca^{2+}$ efflux (open columns) in VSMC pretreated with vehicle alone (CT) or CsA (10^{-6} M, 20 h) and stimulated with AVP ($10^{-8.5}$ M). Values are mean ± s.e.mean of three experiments performed in quadruplicate. Paired data, **$P < 0.01$; NS, not significantly different from CsA-free control, paired t test.

either AVP-stimulated InsP formation or the potentiating effect of CsA on these metabolites (data not shown).

Effect of actinomycin D and cycloheximide on the potentiation by CsA of InsP formation and $^{45}Ca^{2+}$ efflux in response to AVP

We have recently shown that the time of incubation necessary for CsA to reach maximal potentiation of the calcium response to AVP was about 20 h (Lo Russo *et al*., 1996). This suggests that protein synthesis may be required for the establishment of this effect. In order to examine this, an inhibitor of transcription, actinomycin D (ActD), and an inhibitor of translation, cycloheximide (CHX), were tested on CsA-mediated increases in both InsP formation and $^{45}Ca^{2+}$ efflux induced by AVP.

VSMC were pretreated with vehicle alone or with CsA (10^{-6} M, 20 h) in the presence or absence of ActD or CHX. As can be seen, these agents inhibited the effect of CsA on AVP-induced $^{45}Ca^{2+}$ efflux concentration-dependently; the IC_{50} value was $10^{-8.5}$ M for ActD and $10^{-7.5}$ M for CHX (Figure 3a). The effects of these inhibitors at their maximally blocking concentration (10^{-5} M) on CsA-potentiated AVP-induced InsP formation and $^{45}Ca^{2+}$ efflux were measured (Figure 3b). ActD did not significantly affect the $^{45}Ca^{2+}$ efflux in response to AVP (105 ± 1%) but completely inhibited the potentiating effect of CsA (110 ± 2% for ActD + CsA versus 144 ± 3% for CsA). Similar results were obtained with CHX (87 ± 5% for CHX and 96 ± 4% for CHX + CsA). Addition of ActD did not affect InsP formation in response to AVP (98 ± 17%) but fully inhibited the CsA-mediated potentiation (95 ± 19% for ActD + CsA versus 129 ± 4%). However, CHX partially inhibited the response to AVP (presumably due to a toxic effect of CHX) but no potentiating effect of CsA was observed in the presence of CHX (49 ± 10% for CHX versus 46 ± 13% for CHX + CsA).

Effect of CsA on AVP receptors

In further experiments we studied if the expression of AVP receptors was affected by CsA. Maximal specific equilibrium [^{3}H]-AVP binding was obtained at 20°C after 10 min incubation. Coincubation with cytochalasin B (50 μM for 15 min), known to inhibit ligand internalization, resulted in unaltered [^{3}H]-AVP binding. VSMC were pretreated with CsA (10^{-6} M, 20 h) or vehicle alone and binding of [^{3}H]-AVP was performed as described in Methods. Incubation of VSMC with CsA increased the number of AVP receptors about 1.8 fold as analysed by a Scatchard plot and calculated by the STEP programme (Figure 4 and Table 2). On the other hand, the K_D value was not significantly affected by CsA (3.1 nmol l^{-1} for vehicle alone versus 2.1 nmol l^{-1} for CsA-treated cells, Table 2). These values are very close to the EC_{50} values obtained for InsP formation and $^{45}Ca^{2+}$ efflux measurements (see Figure 1 and Table 1).

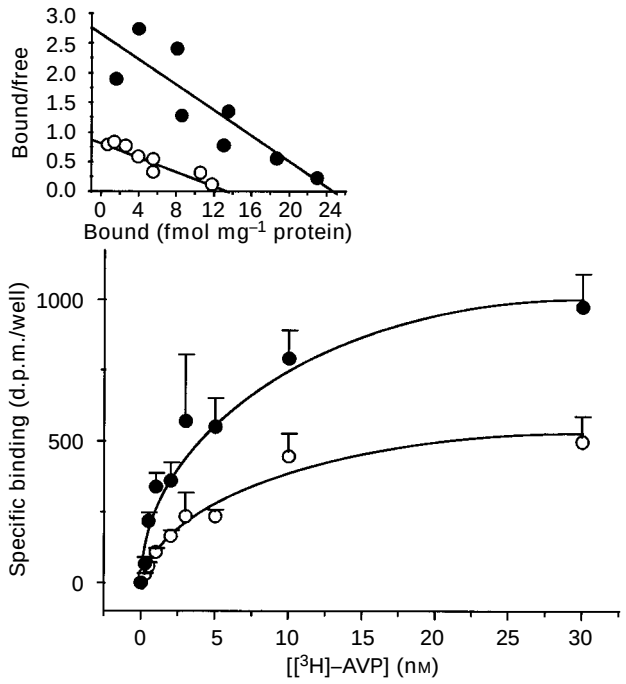

Figure 4 Specific binding isotherms and Scatchard plot (inset) for [^{3}H]-AVP binding to VSMC treated with vehicle alone (○) or cyclosporin A (CsA, 10^{-6} M, 20 h; ●). Values are mean of four to six experiments performed in duplicate; vertical lines show s.e.mean.

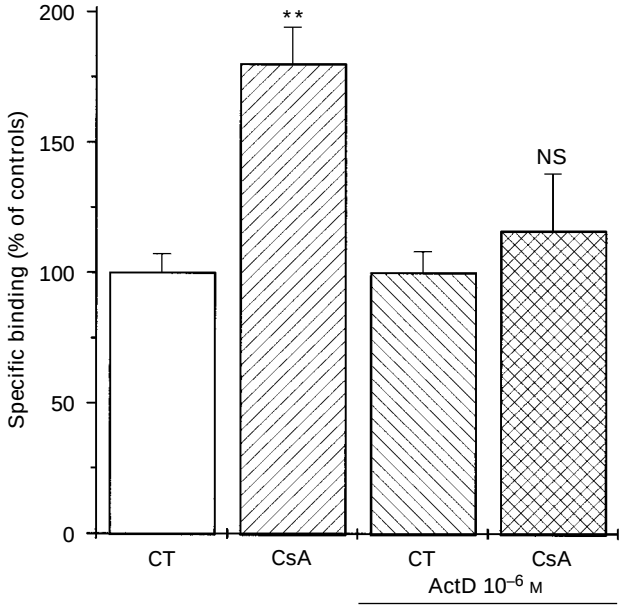

Figure 5 Effect of actinomycin D (ActD, 10^{-6} M, 20 h) on the increase of AVP receptors in VSMC induced by cyclosporin A (CsA) treatment (10^{-6} M, 20 h) measured as specific [^{3}H]-AVP binding. [^{3}H]-AVP concentration was 1.5 nM. Controls (CT) were determined as the specifically bound radioactivity obtained with cells treated with vehicle alone or with ActD. Values are mean ± s.e.mean of 6–8 experiments performed in duplicate. Paired data, $^{**}P<0.01$; NS, not significantly different from CsA-free control, paired *t* test.

Table 2 Results of [^{3}H]-AVP binding experiments

	pK_d (–log M)	B_{max} (fmol/well)	*Confidence limit*[1]
Control	8.51 ± 0.10	3.3	2.9–3.7
CsA (10^{-6} M)	8.68 ± 0.09 (NS)	6.2*	5.8–6.8

VSMC were preincubated with vehicle alone (control) or cyclosporin (CsA, 10^{-6} M) for 20 h and binding assay was performed on the cellular monolayer. Results are mean ± s.e.mean of four to six experiments performed in duplicate. Values for pK_d and B_{max} were determined by the STEP programme. Significance was calculated by non-parametric *t* test.
[1]Confidence limits are for 95% probability level.
NS: not significantly different from control; *significantly different from control ($P<0.01$).

Effect of ActD on [^{3}H]-AVP binding

Since protein synthesis inhibitors blocked the CsA-mediated potentiation of $^{45}Ca^{2+}$ efflux and InsP formation in response to AVP, the effect of ActD on the CsA-induced increase of the number of AVP receptors was investigated.

VSMC were pretreated with CsA (10^{-6} M) or vehicle alone (CT) and ActD (10^{-6} M) where indicated. [^{3}H]-AVP binding measurements were performed as described (see above). As shown in Figure 5 the CsA-mediated increase in the AVP receptor number (178 ± 17%) was completely abolished when cells were co-incubated with ActD (112 ± 23%, not significant from control without CsA).

Discussion

Cyclosporin A (CsA) is currently the most widely prescribed immunosuppressive drug used in transplant rejection therapy and is also used in some autoimmune diseases. Side effects which occur in most patients are nephrotoxicity and drug-induced hypertension (Kahan, 1989; Textor *et al.*, 1994). Drug-induced vasoconstriction is likely to be at the origin of both side effects (Lamb & Webb, 1987; Rego *et al.*, 1990; Sturrock *et al.*, 1994). We have recently shown that CsA increased contractile responses of isolated arteries in response to vasoconstrictive hormones; this effect was correlated with a potentiation of the elevation of $[Ca^{2+}]_c$ in vascular smooth muscle cells (VSMC). With the use of specific analogues, we have also shown that the potentiating effect of CsA was unrelated to inhibition of cyclophilin or calcineurin by CsA (Lo Russo *et al.*, 1996).

Elevation of $[Ca^{2+}]_c$ after hormone stimulation is due to the formation of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) which induces calcium release from the endoplasmic reticulum (Berridge, 1993). A potentiation of the calcium response by CsA could be due to increased $InsP_3$ formation, decreased $InsP_3$ metabolism, increased $InsP_3$ action (e.g. due to an increased sensitivity of the $InsP_3$ receptor), or to decreased Ca^{2+} extrusion (e.g. by lowered activity of the Ca^{2+} ATPases).

Measurement of total InsP formation after stimulation of VSMC with AVP showed that CsA did not affect basal levels of InsP but potentiated accumulation of InsP after hormone stimulation. This potentiating effect of CsA was very well correlated with the CsA-mediated increase in calcium response (Figures 1 and 2), suggesting an effect of CsA upstream of $InsP_3$ formation. Inhibition of InsP degradation by CsA is unlikely to occur as the InsP1-phosphatase inhibitor lithium (Berridge, 1993), was present during the experiments.

Locher *et al.* (1991) have also observed elevated InsP formation in VSMC at a single and relatively high CsA concentration (25 μM) but, as opposed to our results, basal InsP was also increased. On the other hand, McNally *et al.* (1991) did not detect an effect of CsA on InsP formation in response to noradrenaline in vessels from spontaneously hypertensive rats (SHR). The discrepancy of their results with respect to ours might be due to the fact that their incubation time with CsA was much shorter (2 h versus 20 h) and that the InsP-response in vessels from SHR might already be upregulated making a further increase difficult.

The potential target of CsA, upstream of InsP formation, was investigated. Measurements of [^{3}H]-AVP binding to its receptors, presumably of the V_1 subtype in VSMC (Doyle &

Rüegg, 1985), indicated that CsA increased the number of AVP receptors without significantly affecting their affinity (Table 2). These results strongly suggest that the increased Ca^{2+} response to AVP is linked to an increased expression of AVP receptors in VSMC. This is consistent with the fact that the effect of CsA on both cellular $[Ca^{2+}]_c$ and arteriolar contraction was detectable only in the presence of agonist (Lo Russo *et al*., 1996). We have found now that the formation of InsP paralleled the effects on $^{45}Ca^{2+}$ efflux (Figures 1 and 2) and that the pEC_{50} values of AVP for InsP formation and $^{45}Ca^{2+}$ efflux (Table 1) were essentially the same as the pK_d value of AVP in binding experiments (Table 2), indicating that there is no gain between receptor activation and second messenger; a role for spare receptors can therefore be ruled out.

The CsA-induced increase of receptors (Figure 4, Table 2) could be due to either inhibition of receptor down-regulation or increase in receptor synthesis at the translational or transcriptional level. Experiments with the transcription inhibitors actinomycin D (ActD) (Reich & Goldberg, 1964) and cycloheximide (CHX), an inhibitor of translation (Kay & Korner, 1966), suggest that gene expression was required, as both inhibitors attenuated the potentiating effects of CsA on InsP formation and $^{45}Ca^{2+}$ efflux, leaving the Ca^{2+} and InsP response to AVP unaffected (Figure 3). In addition, ActD inhibited the CsA-induced gain in AVP receptors (Figure 5), suggesting the CsA increases the synthesis of receptors at the level of transcription. The requirement for protein synthesis is in agreement with the relatively long incubation times of VSMC with CsA needed for a full effect (more than 20 h) (Lo Russo *et al*., 1996).

Our earlier studies have shown that CsA potentiates the calcium and the contractile responses to at least five vasoconstrictive hormones: noradrenaline, endothelin, 5-hydroxytryptamine (5-HT), angiotensin II and AVP (Lo Russo *et al*., 1996; 1997). It is therefore very likely that CsA also upregulates the expression of other vasoconstrictor hormone receptors in cells and tissues. Indeed, increases in endothelin and angiotensin II receptor levels have been demonstrated in vascular smooth muscle tissues of rats treated with CsA (Nambi *et al*., 1990; Iwai *et al*., 1993, 1995; Takeda *et al*., 1995).

In conclusion, our results show clearly that AVP receptors are upregulated in VSMC. This finding together with the observation of increased cellular calcium responses to angiotensin II, 5-HT and endothelin (Lo Russo *et al*., 1996; 1997) strongly support the hypothesis that CsA increases the expression of several vasoconstrictor hormone receptors in VSMC. This would have major consequences on vasoconstriction, leading to such manifestations as hypertension, decreased glomerular filtration rate and, finally, nephrotoxicity.

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