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Upregulation of vasopressin V_{1A} receptor mRNA and protein in vascular smooth muscle cells following cyclosporin A treatment

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1 The major side effects of the immunosuppressive drug cyclosporin A (CsA) are hypertension and nephrotoxicity. It is likely that both are caused by local vasoconstriction.

2 We have shown previously that 20 h treatment of rat vascular smooth muscle cells (VSMC) with therapeutically relevant CsA concentrations increased the cellular response to [Arg^8]vasopressin (AVP) by increasing about 2 fold the number of vasopressin receptors.

3 Displacement experiments using a specific antagonist of the vasopressin V_{1A} receptor ($V_{1A}R$) showed that the vasopressin binding sites present in VSMC were exclusively receptors of the V_{1A} subtype.

4 Receptor internalization studies revealed that CsA (10^{-6} M) did not significantly alter AVP receptor trafficking.

5 $V_{1A}R$ mRNA was increased by CsA, as measured by quantitative polymerase chain reaction. Time-course studies indicated that the increase in mRNA preceded cell surface expression of the receptor, as measured by hormone binding.

6 A direct effect of CsA on the $V_{1A}R$ promoter was investigated using VSMC transfected with a $V_{1A}R$ promoter-luciferase reporter construct. Surprisingly, CsA did not increase, but rather slightly reduced $V_{1A}R$ promoter activity. This effect was independent of the cyclophilin-calcineurin pathway.

7 Measurement of $V_{1A}R$ mRNA decay in the presence of the transcription inhibitor actinomycin D revealed that CsA increased the half-life of $V_{1A}R$ mRNA about 2 fold.

8 In conclusion, CsA increased the response of VSMC to AVP by upregulating $V_{1A}R$ expression through stabilization of its mRNA. This could be a key mechanism in enhanced vascular responsiveness induced by CsA, causing both hypertension and, *via* renal vasoconstriction, reduced glomerular filtration.

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Abbreviations: AURE, AU-rich elements; AVP, [Arg^8]vasopressin; CsA, cyclosporin A; CsH, cyclosporin H; PKA, protein kinase A; $V_{1A}R$, V_{1A} subtype of vasopressin receptors; VSMC, vascular smooth muscle cells

Introduction

Cyclosporin A (CsA) is widely used as an immunosuppressive drug to prevent graft rejection and treat autoimmune diseases (Borel *et al*., 1996). Unfortunately, CsA treatment is accompanied by significant side effects, mainly hypertension and nephrotoxicity, which limit its utilization (Kahan, 1989; Textor *et al*., 1994). Both side effects have been proposed to be due to increased local vasoconstriction (Lamb & Webb, 1987; Radermacher *et al*., 1998). Several causative mechanisms have been postulated for the CsA-induced increase in vasoconstriction, including decreased production of vasodilator substances (Auch-Schwelk *et al*., 1993; Albillos *et al*., 1998; Bartholomeusz *et al*., 1998), increased production of vasoconstrictor hormones, such as endothelin-1, thromboxane A_2 or angiotensin II (Conger *et al*., 1994; Abassi *et al*., 1996; Bartholomeusz *et al*., 1998), and increased vascular sensitivity to vasoconstrictor hormones (Garr & Paller, 1990; Mikkelsen *et al*., 1992; Gotze *et al*., 1994). We have previously shown in cellular models of rat and human vascular smooth muscle cells (VSMC) that treatment with CsA increased the response to vasoconstrictor hormones by upregulating membrane expression of angiotensin II and vasopressin receptors (Pfeilschifter & Ruegg, 1987; Lo Russo *et al*., 1996; 1997; Avdonin *et al*., 1999). Subsequent increases in inositol phosphate production and cytosolic calcium responses were also observed, which lead to enhanced vasoconstriction e.g. of isolated resistance vessels (Lo Russo *et al*., 1996).

Vasopressin receptors of the V_{1A} subtype are expressed in rat VSMC (Stassen *et al*., 1987; Yazawa *et al*., 1996) and play an important physiological role in vasoconstriction

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(Mohring *et al*., 1979; Stam *et al*., 1998). The V_{1A} receptor belongs to the family of G protein coupled receptors (Morel *et al*., 1993) and acts through activation of phospholipase C_β causing release of inositol 1,4,5-trisphosphate, which leads to an elevation of cytosolic calcium concentrations (Doyle & Ruegg, 1985; Thibonnier, 1992). The V_{1A} receptor and its promoter have been cloned (Morel *et al*., 1992; Murasawa *et al*., 1995a) but little is known about the regulation of its expression by xenobiotics.

As our previous results strongly suggest that CsA increased expression of V_{1A} receptors in rat VSMC *via de novo* protein synthesis (Lo Russo *et al*., 1997), we have studied V_{1A} receptor internalization, mRNA levels and stability as well as V_{1A} receptor promoter activity.

Methods

Chemicals and buffers

CsA and CsH were gifts from Novartis Pharma (Basel, Switzerland). Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS), *Bam*HI, Trizol® reagent, oligo(dT) 12–18 primer, M-MLV reverse transcriptase and first strand buffer were from Life Technologies (Basel, Switzerland). Ciproxin was from Bayer AG Pharma (Zürich, Switzerland) and actinomycin D from Alexis Corporation (Läufelingen, Switzerland). [^{3}H][Arg^8]vasopressin was purchased from NEN Life Science Products (Geneva, Switzerland), [Arg^8]vasopressin and [d(CH$_2$)$_5^1$, Tyr(Me)^2, Arg^8]vasopressin from Bachem (Bubendorf, Switzerland). Beetle D-luciferin potassium salt, pGL3-Basic vector, RQ1 RNase-Free DNase and dNTP were from Promega Corporation (Zürich, Switzerland). Chlorophenolred-β-D-galactopyranoside (CPRG) and Asp718 were from Roche Diagnostics (Rotkreuz, Switzerland). TaqMan probes and 2 × TaqMan Universal PCR Master Mix were from PE Applied Biosystems (Rotkreuz, Switzerland). Primers were custom synthesized by Microsynth (Balgach, Switzerland). Stock solutions of CsA and CsH were prepared in ethanol at concentrations of 10^{-2} M, the stock solutions of [Arg^8]vasopressin and [d(CH$_2$)$_5^1$, Tyr(Me)^2, Arg^8]vasopressin (10^{-3} M) were in 10 mM acetic acid. The physiological salt solution (PSS) contained (in mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) 5 and glucose 10, adjusted to pH 7.4 with 5 N NaOH.

Cell culture

Vascular smooth muscle cells (VSMC) were isolated from aortae of male Wistar Kyoto rats (200–300 g) as described (Lo Russo *et al*., 1996). VSMC were seeded at a density of 10,000 cells/cm^2 in culture flasks (80 cm^2) or multi-well plates in Dulbecco's modified Eagle medium (DMEM) supplemented with essential amino acids, vitamins, ciproxin (0.001%, w v^{-1} and 10% foetal calf serum (FCS), and kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For studies with CsA, cells were used at confluence (after 7–9 days of culture) between passages 6 and 11.

The serum was removed 20 h before experiments by incubation for 15 min at 37°C in FCS-free DMEM followed by replacement with FCS-free DMEM containing vehicle (ethanol 0.01–0.1%) or 10^{-6}–10^{-5} M CsA.

Construction of pGL3-V1 vector

A 2 kb *Bam*HI/Asp718 fragment of the 5′-flanking region of the rat V_{1A} receptor gene (a gift from S. Murasawa and H. Matsubara, Osaka, Japan, GenBank™ accession number D83546) was cloned into the multiple cloning site (Asp718/*Bgl*II) of the pGL3-Basic vector containing a luciferase reporter gene. Partial sequencing of the resulting construct (pGL3-V1) was performed using the Thermo Sequenase sequencing kit (Amersham Pharmacia Biotech, Dübendorf, Switzerland) to check the correct structure of the vector (data not shown).

Transfections

VSMC were seeded at a density of 21,250 cells/cm^2 in 12-well plates in DMEM containing 10% FCS. Medium was replaced after 20 h and, after 30 min, cells were transfected with 5 μg well^{-1} of pGL3-Basic or pGL3-V1 using the calcium phosphate precipitation technique. Briefly, plasmids were mixed with $CaCl_2$ (250 mM) in transparent tubes. An equal volume of 2 × HeBS (in mM): HEPES 40, NaCl 270, KCl 10, Na_2HPO_4 1.5, glucose 10, (pH 7.05), was added and samples were incubated for 2–3 min at 37°C to allow the precipitate to form. This suspension was dispersed evenly onto the cells and this was followed by incubation overnight. Fresh medium (DMEM with 10% FCS) was added the next day to allow cells to recover for 8–10 h before the CsA treatment was performed.

As transfection efficiency may vary from one well to another, cells were co-transfected with 5 μg well^{-1} of a CMV βGal construct in order to allow for normalization. When necessary, pBS-SK+ plasmid was added as carrier.

Cell lysis and enzymatic assays

Cells were lysed in 200 μl of a lysis buffer containing 25 mM Tris, 2 mM DTT, 2 mM *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA), 10% glycerol and 0.5% Triton X-100. Aliquots were used for measurement of luciferase and β-galactosidase activities. Luciferase measurements were performed in 96-well plates using 20 μl of cell lysate and 100 μl of luciferase assay reagent: 20 mM tricine, 0.1 mM EDTA, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 33.3 mM DTT, 270 μM coenzyme A, 470 μM beetle D-luciferin and 530 μM ATP. Luminescence was measured with a MicroLumat Plus luminometer (EG&G Berthold, Regensdorf, Switzerland).

β-Galactosidase assays were performed by using 30 μl of cell lysate and 20 μl of chlorophenolred-β-D-galactopyranoside (CPRG, 4 mg ml^{-1} in H_2O) plus 150 μl of buffer (in mM): Na_2HPO_4 60, NaH_2PO_4 40, KCl 10, $MgSO_4$ 1, 2-mercaptoethanol 50 (pH 7.3). After 2–2.5 h incubation at 37°C, the reaction was stopped by the addition of 100 μl of 1 M Na_2CO_3. Absorbance was read at 575 nm in a microtiter plate reader with 30 μl of lysis buffer plus 20 μl of an aqueous CPRG solution (4 mg ml^{-1}) and 150 μl of buffer as blank.

Polymerase chain reaction

Isolation of total RNA Total RNA was isolated from VSMC cultured in 10 cm diameter petri-dishes or 6-well plates using the Trizol® reagent (Life Technologies, Basel, Switzerland). Isolation was performed according to the manufacturer's protocol. Values in parenthesis are for 6-well plates. Briefly, cells were washed twice with PBS and 3 ml (1 ml) of Trizol® reagent were added. Cell lysate was homogenized by passing it several times through a pipette tip and transferred to polypropylene tubes. After 5 min of incubation at room temperature, 0.6 ml (0.2 ml) of chloroform was added. Tubes were vigorously shaken and incubated for 2–3 min. After centrifugation for 15 min at 4°C at $12{,}000 \times g$ the upper aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 1.5 ml (0.5 ml) of 2-propanol. The precipitate was collected by centrifugation at 4°C at $12{,}000 \times g$ for 10 min. The RNA pellet was washed with 70% ethanol and air-dried before resuspending in 60 μl (20 μl) of RNase-free water. Typically, 160–200 μg of total RNA were obtained from about 10 million cells.

DNase treatment and reverse transcription Total RNA (5 μg) was treated with RQ1 RNase-Free DNase (1 unit μg^{-1} of RNA) for 30 min at 37°C in 10 μl of 1 × first strand buffer. Reaction was terminated by addition of 1 μl of 20 mM EGTA (pH 8.0) and DNase was inactivated by incubation for 10 min at 65°C.

The entire DNase treated RNA (5 μg) was reverse-transcribed in a 20 μl reaction mix (0.5 mM dNTP, 250 ng oligo(dT)12–18 primer, 200 U M-MLV reverse transcriptase, 10 mM DTT, 1 × first strand buffer, pH 8.3). Samples were incubated at 37°C for 60 min followed by 15 min at 70°C for enzyme inactivation.

Real-time quantitative PCR analysis

Real-time quantitative PCR analysis was performed using a PE Applied Biosystems 7700 Sequence Detector (Rotkreuz, Switzerland) and the primers and probes shown in Table 1.

Primers and probes

Complementary DNA was amplified in a 25 μl volume containing 12.5 μl of the 2 × TaqMan Universal PCR Master Mix, 100 or 200 nM of $V_{1A}R$ or GAPDH probes, respectively, 300 nM of corresponding reverse and forward primers and 1/100 of the RT reaction. Thermal cycling conditions were: one initial step of 2 min at 50°C, 10 min at 95°C followed by 45 amplification cycles of 15 s at 95°C, 1 min at 60°C. During elongation, the 5′–3′ exonuclease activity of the Taq DNA polymerase cleaves the probe, thus releasing the reporter (carboxyfluorescein, Fam) from the quencher (carboxytetramethylrhodamine, Tamra).

Two negative controls were included: one PCR reaction mixture with water in place of the DNA sample and one with a non reverse-transcribed RNA sample. Amplification product never appeared in these controls.

For each experiment, calibration curves were established for both probes ($V_{1A}R$ and GAPDH) using dilutions of cDNA obtained from untreated cells. Values were then transformed according to line parameters obtained from the calibration. Results were normalized by dividing $V_{1A}R$ values by GAPDH values for each sample.

mRNA decay experiments

CsA effect on $V_{1A}R$ mRNA stability was examined by inhibiting mRNA transcription with actinomycin D. Before adding actinomycin D the cells were incubated for 10 h with or without 10^{-5} M CsA in DMEM without serum. Then medium was substituted by medium containing 5×10^{-6} M of actinomycin D and CsA or vehicle. Total RNA was extracted at different time points and the receptor mRNA was measured by real-time quantitative PCR as described above. $V_{1A}R$ mRNA levels were normalized with respect to GAPDH mRNA. During the observation period of 6 h, GAPDH mRNA decreased only marginally and, more importantly, CsA did not alter GAPDH mRNA stability (data not shown). The results are expressed in per cent of the mRNA quantity determined at time zero (before incubation of actinomycin D).

[^{3}H]-AVP binding

Confluent VSMC in 24-well plates treated with CsA (10^{-6} M) or vehicle were washed twice with 0.5 ml PSS containing 1.2 mM $CaCl_2$ and 1% (w v^{-1}) of bovine serum albumin (PSS 1% BSA). Solutions of [^{3}H]-AVP for the determination of total and non-specific binding were prepared in PSS 1% BSA containing protease inhibitors (aprotinin 0.1 μg/ml, pepstatin 0.2 μg/ml, leupeptin 1 μM and phenylmethanesulfonylfluoride 5 μM). Non-specific binding was measured in the presence of 3×10^{-7} M of unlabelled AVP (a 200 fold excess). Typically, binding was performed for 10 min at room temperature, using 0.2 ml of 1.5×10^{-9} M [^{3}H]-AVP. Each well was washed four times with 0.5 ml of ice-cold PSS 1% BSA. Cells were detached with 50 μl of trypsin/EDTA (0.25/1%; w v^{-1} respectively) followed by the addition of 250 μl of a sodium dodecyl-sulphate solution (1%; w v^{-1}). Radioactivity was measured by liquid scintillation counting after addition of scintillation liquid (Ultima Gold, Packard, Zürich, Switzerland). Specific binding was calculated as the difference between total and non-specific binding. Results are expressed in per cent of control.

Table 1 PCR primers and TacMan probes used for real-time quantitative RT–PCR analysis

	$V_{1A}R$	*GAPDH*
Forward primer	5′-ACC ACA TCT GGC GCA ACA-3′	5′-CTG CCA AGT ATG ATG ACA TCA AGA A-3′
Reverse primer	5′-GTA ACC AAA AGC CCC TTA TGA AAG-3′	5′-AGC CCA GGA TGC CCT TTA GT-3′
TaqMan probe	5′-FAM-CAG CGT CCT CGC GAC ACA GCA-TAMRA-3′	5′-FAM-TCG GCC GCC TGC TTC ACC A-TAMRA-3′
Length of amplicon	109 bp	72 bp

[^{3}H]-AVP displacement studies

Confluent rat VSMC in 24-well plates were incubated for 20 h in FCS-free DMEM and washed twice with 0.5 ml of PSS 1% BSA. Solutions of [^{3}H]-AVP were prepared in PSS 1% BSA containing protease inhibitors as described above. Solution for the determination of total [^{3}H]-AVP binding contained 1.5×10^{-9} M of ligand. Displacement of the [^{3}H]-AVP from its binding sites was measured in the presence of unlabelled AVP or [d(CH$_2$)$_5^1$, Tyr(Me)^2, Arg^8]vasopressin (V_{1A}R antagonist) at concentrations ranging from 10^{-11}–10^{-7} M. Binding and cells harvesting were performed as described above.

AVP receptor internalization studies

Binding was performed as described above with incubations at 4°C or at room temperature for 10 or 60 min. After rinsing the cells with 0.5 ml of ice-cold PSS 1% BSA to remove unbound ligand, cells were incubated for 10 min at room temperature with 0.5 ml of acid wash buffer (glycine 50 mM, NaCl 150 mM, pH 3.0) to dissociate the ligand from its surface receptor (Briner *et al*., 1992). After incubation, the radioactivity released into the supernatant was determined representing detached radioligand. Cells were rinsed four times with 0.5 ml of ice-cold PSS 1% BSA and lysed as described above. The internalized radioligand (acid wash resistant radioactivity) was measured by counting the cellular lysate. The internalized [^{3}H]-AVP is expressed as percentage of total specific [^{3}H]-AVP binding (intracellular [^{3}H]-AVP plus acid wash released [^{3}H]-AVP). Specific binding was calculated as the difference between total and non-specific binding determined in the presence of 200 fold excess of unlabelled AVP.

Data analysis

Results are presented as means of at least three independent experiments with standard errors of the mean (s.e.m.). Unpaired Student's *t*-test was applied to evaluate significance between groups.

Results

Displacement experiments with V_{1A} receptor antagonist

As found previously (Lo Russo *et al*., 1997), CsA treatment of VSMC for 20 h increased [^{3}H]-AVP binding (Figure 1A). It is very likely that the [^{3}H]-AVP binding sites present in VSMC are of the V_{1A} subtype (Stassen *et al*., 1987; Yazawa *et al*., 1996). In order to ensure that no other receptor subtype, such as V_2 or oxytocin receptors are present in VSMC, we performed displacement experiments using [d(CH$_2$)$_5^1$, Tyr(Me)^2, Arg^8]vasopressin, a specific V_{1A} receptor antagonist (Manning *et al*., 1992). Although this V_{1A} antagonist had a slightly higher affinity for the receptor than vasopressin, both compounds displaced bound [^{3}H]-AVP with a similar efficacy (Figure 1B and Table 2). This suggests that only a single class of AVP receptors are present in VSMC and that they are of the V_{1A} subtype.

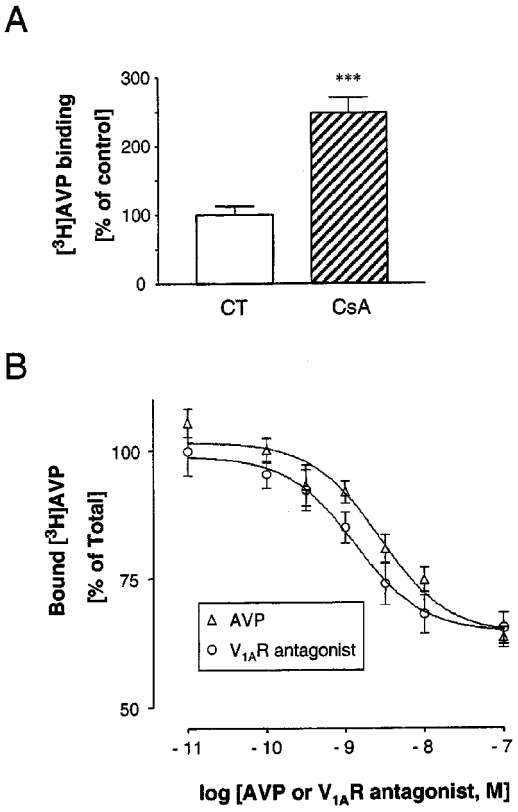

Figure 1 Displacement experiments with V_{1A}R antagonist. [^{3}H]-AVP binding to VSMC was measured as described in methods. (A) Cells were treated with CsA (10^{-6} M) or vehicle for 20 h. Results are expressed in per cent of control and are the mean ± s.e.mean of 16 independent experiments done in triplicate. ***indicates significant difference ($P<0.001$) from control (CT). (B) [^{3}H]-AVP concentration was 1.5×10^{-9} M and increasing concentrations (10^{-11}–10^{-7} M) of AVP or V_{1A} receptor antagonist ([d(CH$_2$)$_5^1$, Tyr(Me)^2, Arg^8]vasopressin) were added to establish the displacement curves. Each value is the mean of 3–4 independent experiments done in quadruplicate.

Effect of CsA on internalization of AVP receptors

The increase in [^{3}H]-AVP binding could be due either to an increase in receptor synthesis or to a decrease in ligand-receptor internalization. Therefore, we studied the effect of CsA on AVP receptor processing. Figure 2 shows that CsA had no significant effect on receptor internalization regardless of temperature or incubation time. In fact, in the presence of AVP, receptors were only significantly internalised at room temperature ($P<0.05$), which is in agreement with previous findings showing that internalization of AVP receptors is temperature dependent (Briner *et al*., 1992).

This suggests that decreased internalization of the receptor is not responsible for the increase in [^{3}H]-AVP binding observed after CsA treatment. Therefore, the CsA-induced increase in AVP receptors is likely due to an increase in cell surface expression of the receptor caused by *de novo* protein synthesis.

Time-course of CsA effect on [^{3}H]-AVP binding and on V_{1A} receptor mRNA levels

We next examined the time dependent effect of CsA on cell surface AVP receptor expression. Figure 3A shows that the

Table 2 Parameters of the regression lines for [^{3}H]-AVP displacement experiments

	pEC$_{50}$ −log (M)	*Confidence interval*	*Top* (per cent of total)	*Confidence interval*	*Bottom* (per cent of total)	*Confidence interval*	R^2
AVP	8.6±0.14	8.3–8.9	102±1.9	98–106	64±2.6	59–69	0.89
Antag.	8.9±0.18	8.5–9.3	99±2.6	94–104	64±2.6	59–70	0.83

Non-linear regression analyses for sigmoidal dose-response curves were performed using GraphPad Prism® (GraphPad Software, San Diego, U.S.A.). Confidence intervals are for 95% probability levels. Antag, $V_{1A}R$ antagonist.

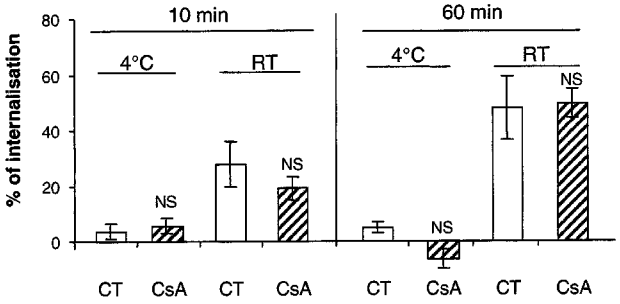

Figure 2 Receptor internalization. The effect of 20 h CsA treatment (10^{-6} M) on receptor internalization was determined after 10 or 60 min incubation of VSMC with [^{3}H]-AVP at 4°C or room temperature (RT). Results represent the mean±s.e.mean of 3–5 independent experiments done in triplicate. NS: not significantly different ($P>0.05$) from control (CT). Using one-sample *t*-test, none of the per cent of internalization values at 4°C were significantly different from a theoretical mean of 0.0 ($P>0.05$).

CsA-induced increase over control in [^{3}H]-AVP binding was significant after 8 h and was 2.6±0.3 fold the control levels after 20 h.

In order to investigate if this effect was due to either increased protein synthesis or increased protein stability, we measured $V_{1A}R$ mRNA levels. Primers and the probe were chosen to be specific for the V_{1A} receptor subtype, known to be present in brain, hepatic and vascular tissues (Penit *et al*., 1983; Aiyar *et al*., 1986; Stassen *et al*., 1987; Yazawa *et al*., 1996; Stam *et al*., 1998). Figure 3B shows that $V_{1A}R$ mRNA was increased by CsA treatment while it remained stable in cells treated with vehicle (0.01% ethanol). The maximal increase was reached already after 3–6 h of CsA treatment and was maintained at least up to 20 h. Thus, the increase in $V_{1A}R$ mRNA preceded the increase in [^{3}H]-AVP binding by about 4 h (Figure 3), the average time necessary for protein synthesis.

Effect of CsA on $V_{1A}R$ promoter activity

The increase in $V_{1A}R$ mRNA could be either due to increased rate of transcription or to increased mRNA stability. To address this question, we investigated the effect of CsA on the activity of the $V_{1A}R$ promoter using a construct (pGL3-V1) in which the luciferase reporter gene is driven by about 2 kb of the $V_{1A}R$ promoter (Murasawa *et al*., 1995a).

As ratios pGL3-V1/pGL3-Basic were typically around 3 to 4 (data not shown), which are close to the basal transcriptional activity observed by Murasawa *et al*. (1995a), we concluded that the $V_{1A}R$ promoter had basal transcriptional activity in our cells. However, CsA did not increase the $V_{1A}R$ promoter activity but slightly decreased it after 6 and 20 h of treatment (Figure 4A). This strongly suggests that the $V_{1A}R$ mRNA increase was not due to a direct stimulation of the $V_{1A}R$ promoter but rather that CsA acted by an alternative pathway in VSMC, such as stabilization of $V_{1A}R$ mRNA. However, because we used a 2 kb fragment of the $V_{1A}R$ promoter, we cannot exclude that other regions of the $V_{1A}R$ gene may be involved in transcriptional regulation by CsA.

Effect of cyclosporin H on $V_{1A}R$ promoter activity

To assess if the inhibitory effect of CsA on $V_{1A}R$ promoter activity involves the calcineurin pathway, the effect of the CsA analogue cyclosporin H (CsH) was investigated. CsH is not immunosuppressive because its K_i for calcineurin inhibition is about 1000 times higher than that of CsA (Fliri *et al*., 1993). At an identical concentration (10^{-6} M), CsH showed a weaker but still significant effect compared to CsA (Figure 4B), thus implying that calcineurin phosphatase activity is unlikely to be involved in decreasing $V_{1A}R$ basal promoter activity.

Effect of cyclosporin A on $V_{1A}R$ mRNA stability

The effect of CsA on $V_{1A}R$ mRNA stability was examined by measuring mRNA decay after inhibition of new mRNA transcription with actinomycin D. A 10 h treatment of VSMC with CsA (10^{-5} M) increased the amount of mRNA of $V_{1A}R$ 3.1±0.5 fold over control (data not shown). When untreated cells were incubated for 6 h with actinomycin D the level of $V_{1A}R$ mRNA decreased to 33±10% of the initial value while it decreased to 61±17% in CsA-treated cells (Figure 5). These data indicate that CsA increased $V_{1A}R$ mRNA stability. The half-life for the decay of $V_{1A}R$ mRNA without CsA was 3.6 h while CsA increased the half-life to 8.1 h (2.3 fold). This correlates well with the 3 fold elevation in $V_{1A}R$ mRNA steady-state levels by CsA.

Discussion

Understanding the mechanisms involved in CsA-mediated vasoconstriction is of primary importance, as CsA currently is the most widely used immunosuppressive drug in the prophylaxis and treatment of transplant rejection and in the treatment of immunoregulatory disorders (Faulds *et al*., 1993; Borel *et al*., 1996).

CsA is known to act on the immune system by inhibiting the synthesis of the T-cell growth factor interleukin-2 in activated T-cells (Borel *et al*., 1996). At the molecular level, CsA binds to cyclophilins and this binary complex interacts with calcineurin thereby inhibiting calcineurin phosphatase

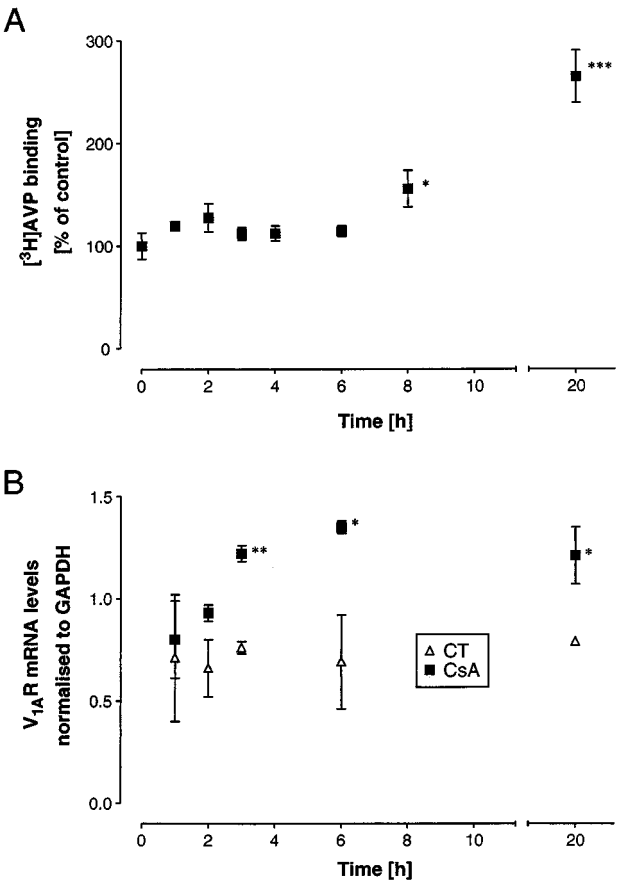

Figure 3 Time course of CsA effect on [^{3}H]-AVP binding and $V_{1A}R$ mRNA levels. (A) [^{3}H]-AVP binding was measured after treatment of VSMC with CsA (10^{-6} M). Results are expressed in per cent of control and are the mean ± s.e.mean of 3–11 independent experiments done in triplicate. Asterisks indicate significant differences from 0 h control (* $0.01 < P < 0.05$, *** $P < 0.001$). (B) mRNA levels were measured using quantitative PCR. VSMC were treated with CsA (10^{-6} M) or vehicle. Results represent the mean ± s.e.mean of three independent experiments done in triplicate after normalization to GAPDH mRNA levels. Asterisks indicate significant differences from control (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$).

activity (Klee *et al*., 1998). As a result the nuclear factor of activated T-cells (NF-AT) remains in its inactive, phosphorylated state and the genes for several cytokines, including interleukin-2, remain silent (Rao *et al*., 1997).

We have previously shown that CsA increased contractile responses of isolated arteries to stimulation with noradrenalin (Lo Russo *et al*., 1996). This effect correlated well with a potentiation of the cytosolic calcium responses to various vasoconstrictor hormones in vascular smooth muscle cells. This was further related to an increase in the number of vasoconstrictor hormone receptors, in particular those of vasopressin and angiotensin II (Lo Russo *et al*., 1997; Avdonin *et al*., 1999). In this study, we investigated the mechanism of CsA-mediated potentiation of the vasopressin-induced calcium response using rat aortic smooth muscle cells.

Besides their role in immunosuppression, cyclophilins are known to play important roles in protein folding and trafficking in and through particular cellular compartments (Walsh *et al*., 1992; Helekar *et al*., 1994; Helekar & Patrick, 1997; Göthel & Marahiel, 1999). By inhibiting cyclophilin function, CsA may impair these processes and the CsA effect on [^{3}H]-AVP binding may, thus, be due to alterations in receptor trafficking. However, our results (Figure 2) show that CsA does not change vasopressin receptor internalization and hence suggest that cyclophilins are not directly involved in the internalization process. Since CsA did not change the internalization of vasopressin receptors, we investigated whether CsA altered $V_{1A}R$ promoter activation and mRNA expression.

The steady-state levels of $V_{1A}R$ mRNA were augmented after CsA treatment (Figure 3B). As changes in mRNA levels may be attributable either to increased rates of gene transcription or to decreased rates of degradation of the transcripts, the role of CsA in the regulation of the $V_{1A}R$ promoter was studied. Murasawa *et al*. (1995a) showed that basal promoter activity was cell-type specific and identified potential binding sites for the transcription factors AP-1, AP-2, SP-1, NF-κB and PEA3 in the 5′-flanking region of the gene. However, the regulation of the $V_{1A}R$ expression by these factors has not been reported.

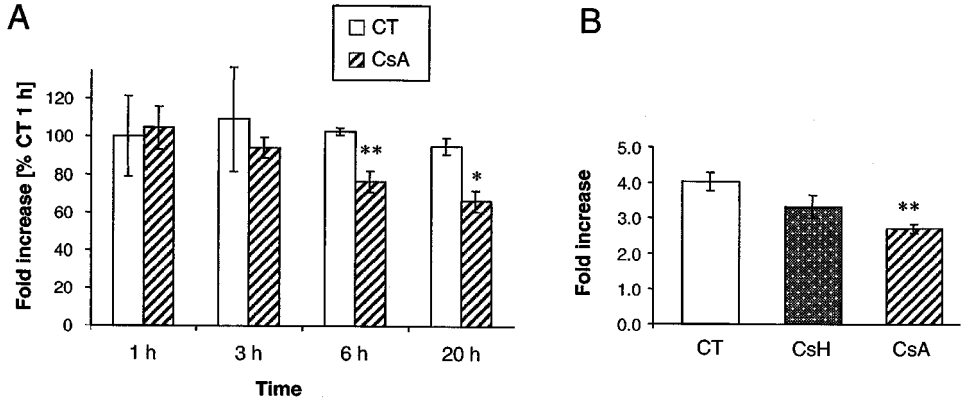

Figure 4 Effect of CsA and CsH on $V_{1A}R$ promoter activity. VSMC were transfected with a $V_{1A}R$ promoter-luciferase reporter construct and a β-galactosidase construct. Normalized luciferase activities were determined relative to cells transfected with a empty plasmid (pGL3-Basic). Results are the mean ± s.e.mean of three experiments done in triplicate. Asterisks indicate significant differences from control (*$0.01 < P < 0.05$, **$0.001 < P < 0.01$). (A) Time-course study of the effect of CsA (10^{-6} M) on $V_{1A}R$ promoter activity. Results are expressed as per cent of control after a 1 h treatment. (B) Cells were treated with CsA or CsH (10^{-6} M each) or control buffer for 20 h.

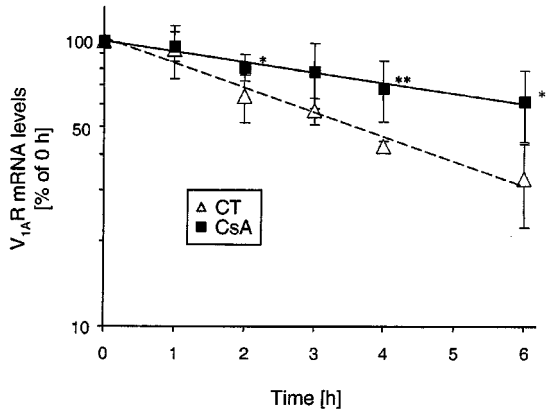

Figure 5 Effect of CsA on $V_{1A}R$ mRNA stability. VSMC were incubated for 10 h with CsA (10^{-5} M) and the CsA effect on $V_{1A}R$ mRNA stability was examined by measuring mRNA decay after inhibition of mRNA transcription with actinomycin D (5×10^{-6} M). The mRNA levels were determined by quantitative RT–PCR as described in methods. $V_{1A}R$ mRNA levels were normalized with respect to GAPDH mRNA. The results are expressed as per cent of the mRNA quantity determined at time zero of actinomycin D treatment and are the mean ± s.e.mean of 5–9 independent experiments done in triplicate. Asterisks indicate significant differences from control (*$0.01 < P < 0.05$, **$0.001 < P < 0.01$).

Surprisingly, CsA decreased the activity of the promoter after transfection of the pGL3-V1 reporter construct into VSMC. The significance of the inhibition of the $V_{1A}R$ promoter by CsA still remains unclear but Murasawa *et al.* (1995a) suggested the presence of a negative regulatory element in the promoter region, which might be involved in the observed repression by CsA.

The fact that CsA did not stimulate $V_{1A}R$ promoter activity but increased the corresponding mRNA levels suggested that CsA may increase $V_{1A}R$ mRNA levels by mRNA stabilization. Such a mechanism has previously been described for vasopressin and angiotensin receptors in vascular smooth muscle cells exposed to dexamethasone (Murasawa *et al.*, 1995b) and insulin (Nickenig *et al.*, 1998), respectively. It is interesting to note that both the glucocorticoid and the insulin pathways have been linked to the induction of hypertension (Prichard *et al.*, 1992; Whitworth, 1994). In addition, a role for CsA in mRNA stabilization has recently been described (Luo *et al.*, 1999). Therefore, we have measured the effect of CsA on $V_{1A}R$ mRNA decay. These experiments showed indeed that CsA increased $V_{1A}R$ mRNA stability (Figure 5).

It has previously been described that an increase in the length of the poly-A tail of mRNAs results in their stabilization (Atwater *et al.*, 1990). Activation of a protein kinase A (PKA) dependent pathway was proposed to increase the average length of some mRNAs (Emanuel *et al.*, 1998). Therefore, an indirect action of CsA on PKA could explain our results. On the other hand, it has been shown that AU-rich elements (AURE) present in the 3′ untranslated region of mRNAs can confer mRNA instability (Ross, 1996) and a relationship between RNA-binding proteins and the modulation of the stability of mRNAs has been demonstrated (Gillis & Malter, 1991; Pende *et al.*, 1996). Recently, expression of an AURE-binding protein, AUF1, was reported in human vascular smooth muscle cells, which specifically bound to the human AT_1 receptor mRNA (Pende *et al.*, 1999). This suggests that AUF1 plays a role in the modulation of receptor mRNA stability in smooth muscle cells. Therefore, an effect of CsA on this pathway may be an alternative mechanism for the observed CsA-induced mRNA increase.

We have previously shown that the transcriptional inhibitor actinomycin D blocked the CsA-induced increase in [^{3}H]-AVP binding (Lo Russo *et al.*, 1997). This supports the notion that CsA acts at the transcriptional level, either directly on the transcription of the $V_{1A}R$ gene or by altering the expression of an unknown protein involved in mRNA turnover. However, our observations that CsA (i) decreased $V_{1A}R$ promoter activity, (ii) increased $V_{1A}R$ mRNA already after 3 h, a rather short time for an indirect mechanism involving new protein synthesis and (iii) attenuated $V_{1A}R$ mRNA decay, suggest that transcriptional activation of the $V_{1A}R$ gene is not required. Therefore, the stabilization of $V_{1A}R$ mRNA represents a very likely mechanism for the action of CsA on $V_{1A}R$.

In conclusion, we propose that CsA increases the cellular response to AVP by augmenting $V_{1A}R$ mRNA levels *via* the stabilization of its mRNA. Further studies are needed to elucidate how CsA causes mRNA stabilization, in particular with respect to the roles of PKA and AURE-binding proteins.

The increase in $V_{1A}R$ by CsA could be a key mechanism in the exaggerated vascular responsiveness to AVP as well as to other vasoconstrictor hormones. A detailed knowledge of the underlying mechanisms would be very useful to understand the mechanistic basis of side effects, in particular hypertension, which occur upon CsA treatment in humans.

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