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Effect of cyclosporin A and analogues on cytosolic calcium and vasoconstriction: possible lack of relationship to immunosuppressive activity

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1 The full therapeutic potential of the main immunosuppressive drug, cyclosporin A (CsA), is limited because of its side effects, namely nephrotoxicity and hypertension. Several lines of evidence suggest that the origin of both side effects could be CsA-induced vasoconstriction. However, the underlying molecular mechanisms are not well understood.

2 Diameter measurements of rat isolated mesenteric arteries showed an increase in noradrenaline- and [Arg]^{8}vasopressin-induced vasoconstriction when arteries were pretreated with CsA.

3 Measurements in cultured vascular smooth muscle cells (VSMC) of either cytosolic calcium concentration or of $^{45}Ca^{2+}$ efflux showed that CsA potentiated the calcium influx to several vasoconstrictor hormones: [Arg]^{8}vasopressin, angiotensin II, endothelin-1 and 5-hydroxytryptamine. On the other hand, $^{45}Ca^{2+}$ efflux in response to thapsigargin, which depletes calcium from intracellular pools, was not potentiated by CsA. $^{45}Ca^{2+}$ uptake was not altered by CsA or by any of the analogues tested.

4 Time-course studies in cultured VSMC showed that maximal CsA-induced Ca^{2+} potentiation occurred after ca. 20 h and this effect was reversed over approximately the next 20 h.

5 To investigate the possible role played by the known intracellular targets of CsA, namely cyclophilin and calcineurin, CsA derivatives with variable potencies with respect to their immunosuppressive activity, were tested on the calcium influx to [Arg]^{8}vasopressin. Derivatives devoid of immunosuppressive activity (cyclosporin H, PSC-833) potentiated calcium signalling, while the potent immunosuppressant, FK520, a close derivative of FK506, and MeVal^{4}CsA, an antagonist of the immunosuppressive effect of CsA did not. The latter compound was unable to reverse the calcium potentiating effect of CsA.

6 Our results show that CsA increases the calcium influx to vasoconstrictor hormones in smooth muscle cells, which presumably increases vasoconstriction. Loading of the intracellular calcium pools appears not to be involved. Experiments with derivatives of CsA and FK520 suggest that interactions with cyclophilins and calcineurin are not the mechanism involved. This indicates, for the first time, that the immunosuppressive activity can be dissociated from the calcium potentiating effect of CsA in vascular smooth muscle.

Keywords: Cyclosporin A; cyclosporins; intracellular calcium; hypertensive side effect; smooth muscle cells; vasoconstrictor activity; mesenteric arteries; $^{45}Ca^{2+}$efflux; fura-2

Introduction

Cyclosporin A (CsA) is currently the main immunosuppressive drug used during transplantation and autoimmune disorders. The mechanism of its immunosuppressive effect has recently been elucidated (McKeon, 1991; Schreiber & Crabtree, 1992; Swanson *et al.*, 1992). The formation of a tertiary complex between CsA, the rotamase cyclophilin, and the protein phosphatase, calcineurin, inhibits the activity of the latter. This leads to decreased interleukin-2 (IL-2) production by lymphocyte T cells resulting in inhibition of their activation and proliferation (Cohen *et al.*, 1984; Sigal & Dumont, 1992; Kunz & Hall, 1993).

However, clinical use of CsA is limited due to its nephrotoxicity and ability to induce hypertension (Kahan, 1989; Mason, 1990; Textor *et al.*, 1994). Most studies in animals as well as in CsA-treated patients suggest that CsA-induced vasoconstriction is the underlying cause of both side effects. However, the molecular mechanism of this effect is still an object of some controversy: (1) An increased sympathetic activity has been noted both in experimental models as well as in man (Moss *et al.*, 1985; Scherrer *et al.*, 1990; Morgan *et al.*, 1991; Chiu *et al.*, 1992; Lyson *et al.*, 1993; 1994). (2) Stimulation of the renin-angiotensin system has been described in hypertensive transplant recipients under CsA treatment (Julien *et al.*, 1993) as well as in animals (Müller-Schweinitzer, 1988). (3) In vascular tissue of CsA-treated rats, an increased expression of angiotensin II and endothelin receptors (Iwai *et al.*, 1993; Auch-Schwelk *et al.*, 1994; Nambi *et al.*, 1990) has been measured. (4) Increased levels of vasoconstrictor agents such as thromboxane or endothelin have been detected in plasma of CsA-treated patients (Grieff *et al.*, 1993; Mouquet *et al.*, 1994) as well as in *in vitro* models (Copeland & Yatscoff, 1992; Lanese & Conger, 1993; Conger *et al.*, 1994). (5) In *ex vivo* studies, a decreased production of vasodilator agents such as prostacyclin and nitric oxide has been measured (Auch-Schwelk *et al.*, 1993; Diederich *et al.*, 1994; Roullet *et al.*, 1994; Gallego *et al.*, 1994; Marumo *et al.*, 1995).

We have previously shown that CsA can potentiate the [Arg]^{8}vasopressin-(AVP) or angiotensin II-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in cultured vascular smooth muscle cells (VSMC) (Pfeilschifter & Rüegg, 1987). Similar findings have confirmed these observations (Pfeilschifter, 1988; Kremer *et al.*, 1989; Meyer-Lehnert & Schrier, 1989; Locher *et al.*, 1991; Bokemeyer *et al.*, 1993).

In the present study, we have measured the effect of CsA on

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noradrenaline and AVP-induced contraction on isolated mesenteric arteries. We have also extended our previous findings to other vasoconstrictor hormones using cultured VSMC. In addition, to determine the possible role of cyclophilin or calcineurin inhibition in CsA-induced calcium potentiation, we have investigated the effect of some derivatives of CsA devoid of immunosuppressive activity.

Methods

Diameter measurement of mesenteric arteries

Male Wistar Kyoto rats (200 to 300 g) were anaesthetized by i.p. injection of a solution of pentobarbitone (200 mg kg^{-1} body weight) and were decapitated. Resistance arteries of ca. 200 μM diameter were prepared from the mesenteric bed using segments of the third order branch. They were cleaned of adherent tissue in ice-cold Krebs-Ringer solution (KRS: see below), mounted at both ends onto glass cannulae in a chamber (Living Systems Instrumentation, Burlington, VT, U.S.A.) filled with KRS and placed on a microscope stage. The vessels were continuously superfused with oxygenated KRS at 37°C. A steady pressure of 40 mmHg was created in the vessel with a pump coupled to a feed-back pressure system. Vasoconstrictor drugs were applied via the superfusate in a low [Ca^{2+}] KRS (see Chemicals and buffers) to avoid contractions due to calcium entry through voltage-operated channels. Effects were observed with a video dimension analyser attached to the microscope. The inner diameter and the wall thickness of the vessel were measured continuously by optical density analysis. When indicated, CsA was added to the bath 3 h before the application of the vasoconstrictor hormone.

Culture of smooth muscle cells

In brief, aortae from rats (see above) were treated with collagenase (type II, Worthington) at 70 u/ml^{-1} in Hanks' balanced salt solution for 30 min at 37°C. The adventitia was stripped off mechanically, the endothelium was removed with a fine paint brush and the tissue was thinly cut with a razor blade.

The small pieces of aorta were incubated in an elastase/collagenase (40/70 units ml^{-1} respectively) digestion medium. Cells were dispersed, centrifuged after 90 min at 100 *g* and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with essential amino acids, vitamins, ciproxin and 10% foetal calf serum (FCS). The vascular smooth muscle cells (VSMC) were plated in 25 cm^2 bottles and kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After about 7 days, the cells were confluent. They were detached with 0.25% trypsin solution and seeded at 15 000 cells cm^{-2} into culture bottles (80 cm^2) for further culture and after 5–12 days they were transferred into wells for experiments.

For the measurement of intracellular free calcium using the fura-2 technique, 25 000 cells were seeded on 22 mm diameter glass coverslips pretreated for 1 day with DMEM containing 10% FCS in 6-well culture plates. Confluent VSMC were used after 7 to 8 days.

For $^{45}Ca^{2+}$ flux experiments, 20 000 cells were seeded into 16 mm diameter wells (24-well culture plates) and were used after 7 to 8 days.

Preincubation of VSMC with CsA and related compounds was performed in DMEM without FCS; a 20 h incubation period was used if not indicated otherwise.

As an estimate for toxicity of CsA, total protein of adherent cells was measured by the method of Bradford (1976). At 10^{-6} M CsA no effect was observed.

Determination of $[Ca^{2+}]_c$ in smooth muscle cells

Cytosolic free calcium concentrations ($[Ca^{2+}]_c$) were measured as described previously (Cornwell & Lincoln, 1989; Knot *et al.*, 1991). Seven to eight day-old VSMC grown on glass coverslips were washed 3 times with PSS/1.2 mM Ca^{2+}. Two ml of a solution of fura-2/AM (5 μM) in PSS/1.2 mM Ca^{2+} (containing CsA if indicated) were added to the cells and these were incubated in the dark for 40 min at room temperature. Cells were washed 3 times with PSS/1.2 mM Ca^{2+} and the coverslip was inserted into a thermostated chamber (37°C) on a Nikon Diaphot inverted epifluorescence microscope, being part of a PhoCal single cell fluorescence analyser (Life Science Resources, Cambridge, UK). The cells were illuminated with alternating light of 340 and 380 nm from a rotating filter wheel (6.25Hz). Emission was monitored at 510 nm from a group of about 10 cells (40x objective) and data was analysed using PhoCal software. Calibrations were performed by treating the cells with ionomycin (10^{-5} M) and a higher calcium concentration (6 mM) to obtain the maximal signal, followed by the addition of EGTA (10 mM) to obtain the minimal signal. Background fluorescence, obtained by quenching the Ca^{2+} signal with $MnCl_2$ (1 mM), was subtracted from the signals. Results are given as calculated $[Ca^{2+}]_c$ as described by Grynkiewicz *et al.* (1985).

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

For $^{45}Ca^{2+}$ loading, cells were first washed twice with physiological saline 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 or related analogues as 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 (or more frequently for kinetic experiments) the supernatant was removed and immediately replaced by 0.5 ml of fresh PSS/1.2 mM $CaCl_2$ at 37°C. Agonists were 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 were measured by liquid scintillation counting (Packard Tri Carb 4640).

For kinetic experiments, results are expressed in % c.p.m. min^{-1} per well of total c.p.m. Total c.p.m. is the sum of the c.p.m. in the supernatants plus the cellular content at the end of the experiment.

For dose-response curves, results are expressed as % 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).

Data analysis

Results are expressed as mean ± s.e.mean for $^{45}Ca^{2+}$ efflux experiments (which were done in quadruplicate) and mean ± s.d. for other experiments. *n* refers to the number of experiments. Student's *t* test for paired data was used to test for statistical significance and a value of $P<0.05$ was considered statistically significant.

Chemicals and buffers

Cyclosporin A and analogues were a gift from Sandoz Pharma Ltd (Basel, Switzerland). [Arg]^{8}vasopressin, angiotensin II and endothelin-1 were obtained from Bachem Feinchemikalien AG (Switzerland), thapsigargin and 5-hydroxytryptamine (5-HT) from Sigma Chemie AG (Switzerland), fura-2/AM from Molecular Probes (U.S.A.) and $^{45}Ca^{2+}$ (10–40 mCi mg^{-1} calcium) from Amersham International (UK). All chemicals used were of the purest grade available. Stock solutions of CsA and analogues were prepared at a concentration of 10 mM in ethanol, while peptide hormones and 5-HT were in 0.1 mM acetic acid. At most 0.1% of EtOH was present in the assays

and, as tested, this did not affect contraction or the calcium response. The composition of buffer solutions were: PSS, in mM: NaCl 145, KCl 5, HEPES 5, $MgCl_2$ 1, glucose 10, pH 7.4; containing 0.12 or 1.2 mM $CaCl_2$. KRS, in mM: NaCl 120, KCl 4.7, $NaHCO_3$ 2.4, KH_2PO_4 1.1, $MgSO_4$ 1.2, EDTA 0.033, glucose 9, gassed with 95% O_2, 5% CO_2, pH 7.4; containing 0 or 1.6 mM $CaCl_2$.

Results

Effect of CsA on noradrenaline-induced vasoconstriction in mesenteric arteries

Diameter changes of rat mesenteric resistance arteries were studied in a myograph after preincubation with CsA or vehicle for 3 h. At concentrations of 10^{-6} M and 10^{-5} M, CsA did not influence basal vessel diameter. However, the contractile effect of vasoconstrictor hormones was enhanced by CsA. This was observed with [Arg]^{8}vasopressin (AVP) (not shown) and with noradrenaline (NA) (Figure 1). At a concentration of 10^{-6} M, CsA increased by 40±15% the reduction in vessel diameter induced by NA (3 μM). At 10^{-5} M CsA, the contractile response was more than doubled. With 10^{-5} M NA, a significant increase was still observed with the lower CsA concentration while no further increase of contraction was noted with 10^{-5} M CsA, as the vessel was fully contracted.

CsA increases the ability of vasoconstrictor hormones to elevate $[Ca^{2+}]_c$ in rat VSMC

Addition to rat VSMC of AVP at a concentration of 10^{-8} M induced a sharp (20–30 s) rise in $[Ca^{2+}]_c$, from a baseline of 20–30 nM to 84±30 nM, which decreased and flattened out thereafter (Figure 2a). Pretreatment of the cells with CsA (10^{-6} M) for 20 h did not affect basal $[Ca^{2+}]_c$ (control: 25±6 nM, CsA: 28±7 nM) whereas it increased the $[Ca^{2+}]_c$ peak response to 190±41 nM (Figure 2a and b). The sustained phase of the $[Ca^{2+}]_c$ signal was also potentiated by CsA. Similar results were obtained with 5-HT (10^{-7} M), endothelin (ET, 10^{-8} M) and angiotensin II (AII, 10^{-8} M) although the potentiation was less pronounced for the latter (Figure 2b).

Effect of CsA on $^{45}Ca^{2+}$ efflux in response to four vasoconstrictors and thapsigargin

The potentiating effect of CsA was further investigated in $^{45}Ca^{2+}$ efflux experiments. Pretreatment of VSMC with CsA (10^{-6} M) did not affect basal $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$ preloaded cells (Figure 3, from 3 to 9 min). When hormones (AVP, AII, 5-HT and ET) were added at about their EC_{50}s, a rapid (40–60 s) increase in $^{45}Ca^{2+}$ efflux was noted (Figure 3, from 9 to 11 min 40 s). This increase was potentiated in CsA pretreated cells (Figure 3).

Figure 4a shows concentration-response curves for the four hormones, measured in the absence of CsA or after 20 h of CsA (10^{-6} M) pretreatment. For all four hormones the curves were shifted to the left and their maximal effects were increased. For AII, the effect was less pronounced but still significant. AVP was chosen for further studies.

We next measured the calcium response 10 min after stimulation with the SR-Ca^{2+}-ATPase inhibitor, thapsigargin (TG) (Figure 4b). No potentiation by CsA was observed even at high TG concentration (10^{-5} M), which completely depleted the inositol 1,4,5-trisphosphate-sensitive calcium pools since a subsequent addition of AVP (10^{-7} M) did not further increase $^{45}Ca^{2+}$ efflux (not shown). Very similar results were obtained with cyclopiazonic acid, another SR-Ca^{2+}-ATPase inhibitor (not shown).

Time course of the potentiation and reversal of the effect

When $^{45}Ca^{2+}$ efflux in response to AVP ($10^{-8.5}$ M) was studied after various periods of CsA (10^{-6} M) pretreatment, the time course for the CsA potentiation followed the curve shown in Figure 5. The effect of CsA was already detectable after 9 min but took more than 20 h to reach a maximal effect. When CsA

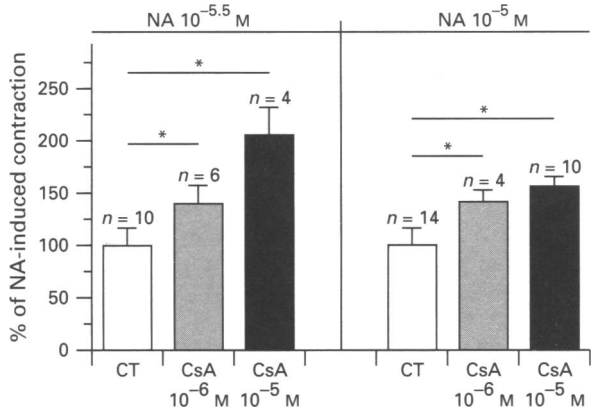

Figure 1 Effect of cyclosporin A (CsA) on noradrenaline (NA)-induced vasoconstriction of rat isolated mesenteric arteries. Vessels were pretreated for 3 h with vehicle (open columns) or CsA (10^{-6} M, stippled columns; 10^{-5} M, solid columns). *n* represents the number of experiments. Values are mean±s.d. A significant difference from control is indicated by $^*P<0.05$.

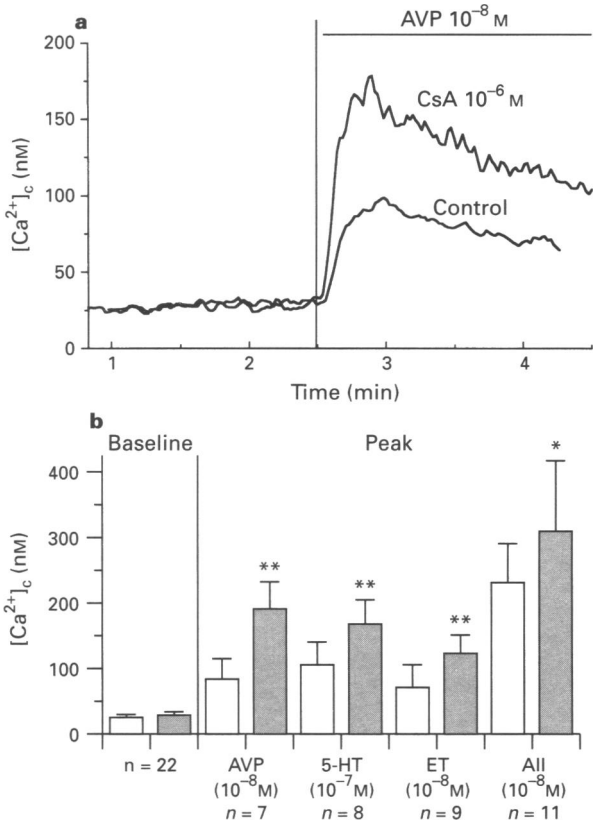

Figure 2 Effect of cyclosporin A (CsA) pretreatment (10^{-6} M for 20 h) on vasoconstrictor hormone-induced rise in $[Ca^{2+}]_c$ in vascular smooth muscle cells (VSMC). (a) Representative result obtained in untreated (control) or CsA-treated cells stimulated with [Arg]^{8}vasopressin (AVP). (b) Mean values of $[Ca^{2+}]_c$ at basal and at peak level obtained in untreated (open columns) or CsA-treated cells (stippled columns) stimulated with AVP, 5-hydroxytryptamine (5-HT), endothelin-1 (ET) and angiotensin II (AII). *n* represents the number of experiments. Values are mean±s.d. A significant difference from control is indicated by $^*P<0.05$; $^{**}P<0.01$.

was removed after 24 h incubation and cells were kept in CsA-free medium, reversal of the Ca^{2+}-potentiation took more than 20 h (Figure 5).

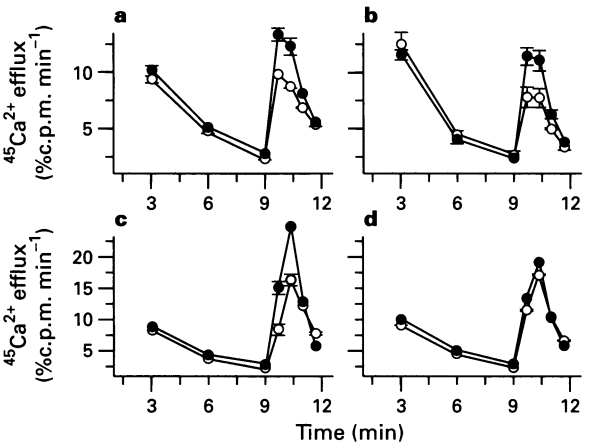

Figure 3 Effect of cyclosporin A (CsA) pretreatment (10^{-6} M for 20 h) on vasoconstrictor hormone-induced rise in $^{45}Ca^{2+}$ efflux in VSMC. Results from control cells are represented by (○) and CsA-treated cells by (●). Hormones were added at the 9th min. (a) 5-Hydroxytryptamine ($10^{-6.5}$ M); (b) [Arg]^{8}vasopressin ($10^{-8.5}$ M), (c) endothelin-1 (10^{-8} M), (d) angiotensin II ($10^{-8.5}$ M). Values are mean ± s.e.mean of 3 experiments in quadruplicate.

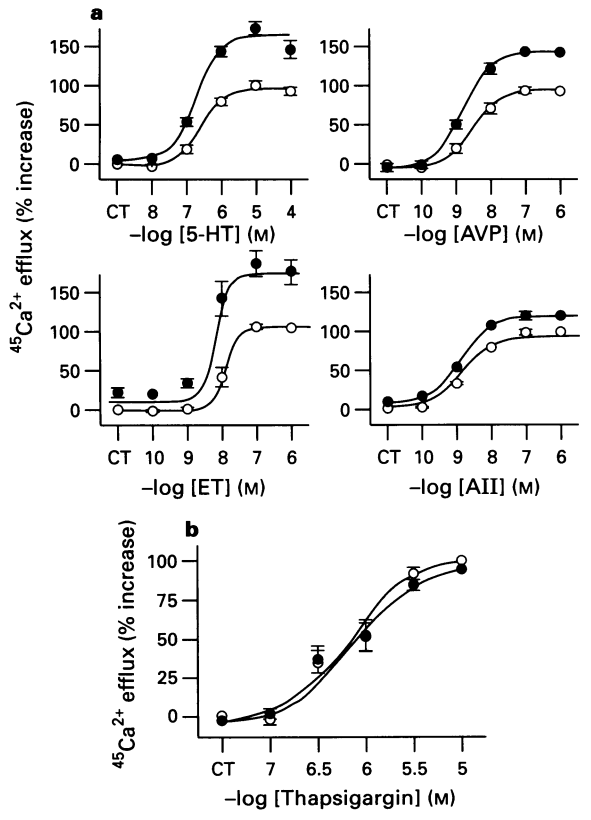

Figure 4 (a) Concentration-response curves for vasoconstrictor hormone-induced rise in $^{45}Ca^{2+}$ efflux in control VSMC (○) or cyclosporin A (CsA)-treated VSMC (10^{-6} M, 20 h) (●). Vasoconstrictor hormones were: 5-hydroxytryptamine (5-HT), [Arg]^{8}vasopressin (AVP), endothelin-1 (ET) and angiotensin II (AII). (b) Results for thapsigargin-induced rise in $^{45}Ca^{2+}$ efflux. Values are mean ± s.e.mean of 3 experiments in quadruplicate.

Test of compounds related to CsA

The $^{45}Ca^{2+}$ efflux in response to AVP ($10^{-8.5}$ M) was measured 20 h after pretreatment with increasing concentrations of various derivatives of CsA (CsG, CsH, PSC-833, MeVal^{4}CsA) and FK520 (ascomycin), the C-21 ethyl derivative of the immunosuppressant FK506 (Liu, 1993) (Figure 6a). FK520 and CsG are immunosuppressive agents with potencies similar to CsA while CsH, PSC-833 and MeVal^{4}CsA are devoid of immunosuppressive activity (Wenger *et al.*, 1986; Boesch *et al.*, 1991; Fliri *et al.*, 1993). CsA and CsG were the most potent compounds to produce a potentiation of the calcium response ($EC_{50} < 10^{-6}$ M, maximal potentiation 157 ± 3% and 158 ± 4%, respectively) but the non-immunosuppressive CsA analogues CsH and PSC-833 also produced an effect (EC_{50} ca 10^{-6} M, max. potentiation 150 ± 6% and 164 ± 8%, respectively). On the other hand, the immunosuppressive agent FK520 did not produce any significant Ca^{2+} potentiation (Figure 6a). Moreover, MeVal^{4}CsA which is known to inhibit the immunosuppressive effect of CsA by binding to cyclophilin (CyP) but not allowing the interaction with calcineurin was ineffective in producing a Ca^{2+} potentiation (Figure 6a). In addition, this derivative did not inhibit the potentiation due to CsA (10^{-6} M) at the concentration tested (10^{-8} M to 10^{-5} M) (not shown).

Measurement of cellular $^{45}Ca^{2+}$ content after pretreatment with the compounds related to CsA showed either no effect or, at higher concentration, a weak inhibition of calcium loading (especially for CsH) but without correlation with the Ca^{2+} potentiating effect (Figure 6b).

Discussion

An increase in vascular resistance due to vasoconstriction may account for the hypertensive and nephrotoxic effects of cyclosporin A (CsA) (Lamb & Webb, 1987; Rego *et al.*, 1990; Garr & Paller, 1990; Roullet *et al.*, 1994; Ressureicao *et al.*, 1995). However, the underlying mechanisms, briefly reviewed in the introduction, are still controversial.

Measurements of diameter changes of rat mesenteric resistance arteries under isotonic conditions showed that CsA did not affect basal diameter but was able to increase contractions induced by vasoconstrictor hormones. It seems therefore that CsA has an action on vasoconstrictor mechanisms induced by noradrenaline or other vasoconstrictor hormones. A CsA-induced decrease of vasodilator factors such as nitric oxide (Auch-Schwelk *et al.*, 1993; Diederich *et al.*, 1994;

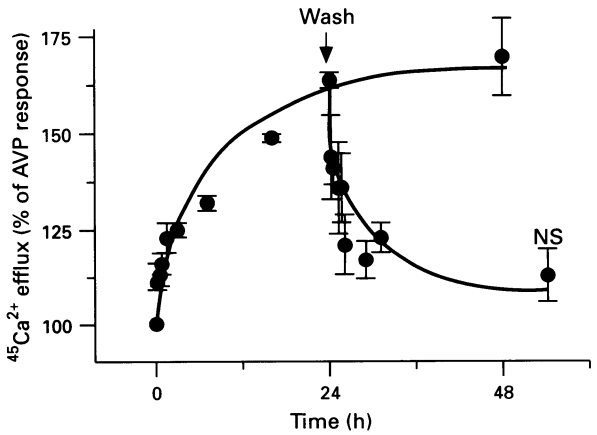

Figure 5 Time course of the establishment and wash-out of the cyclosporin A (CsA) effect on $^{45}Ca^{2+}$ efflux induced by a 2-min stimulation with AVP ($10^{-8.5}$ M). CsA (10^{-6} M) was applied to the VSMC for increasing time periods up to 48 h or washed out after 24 h of incubation. Values are mean ± s.e.mean of 4 experiments in quadruplicate. NS indicates a value not significantly different from control.

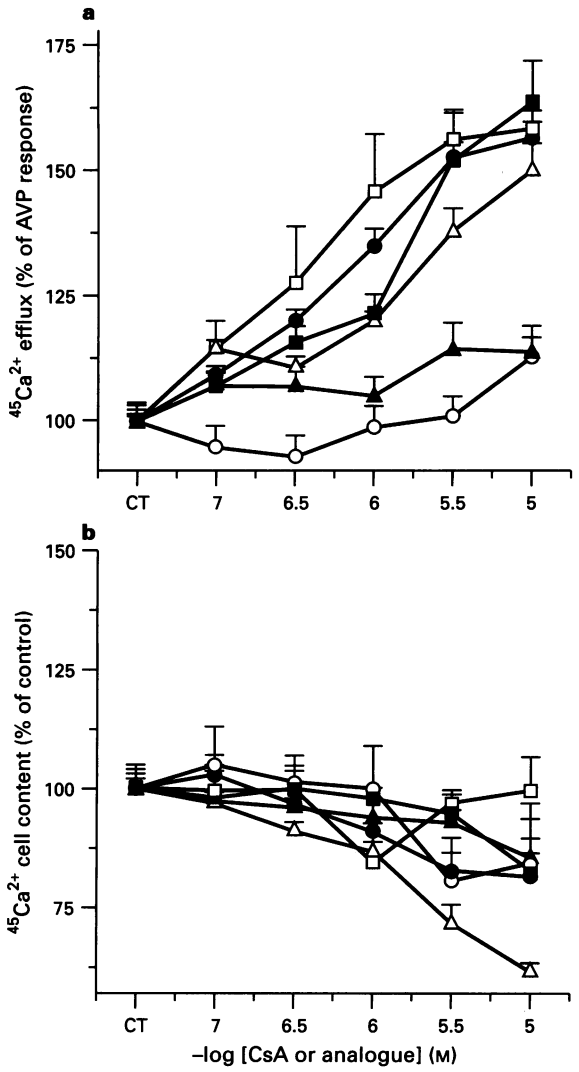

Figure 6 Concentration-dependent effect of cyclosporin A (CsA) (●), CsG (□), CsH (△), PSC-833 (■), MeVal[4]CsA (○) and FK520 (▲) on the potentiation of $^{45}Ca^{2+}$ efflux in response to AVP ($10^{-8.5}$ M) stimulation (a) and on the $^{45}Ca^{2+}$ cell content measured after 15 min of loading with $^{45}Ca^{2+}$ (b). Values are mean ± s.e.mean of 4 experiments in quadruplicate.

Roullet *et al.*, 1994) or the still unidentified endothelium-derived hyperpolarizing factor (Garland *et al.*, 1995) cannot be ruled out, as endothelial cells were still present in our preparation. On the other hand, CsA-mediated release of endothelin from the endothelium, as suggested by Lanese *et al.* (1993), is unlikely to have been a major cause since CsA did not affect the basal level of vasoconstriction.

Experiments with cultured VSMC showed that CsA potentiated the calcium response of different vasoconstrictor hormones without affecting basal calcium levels as demonstrated by $[Ca^{2+}]_c$ measurements (Figure 2a and 2b) or $^{45}Ca^{2+}$ efflux experiments (Figure 3 and 4a). A leftward and upward shift of the concentration-response curve for all vasoconstrictor hormones was observed, indicating an increase in their potency and efficacy in the presence of CsA. As a rise of $[Ca^{2+}]_c$ is the key trigger for vasoconstriction (see e.g. Somlyo & Somlyo, 1994), it is likely that the CsA-induced increase of the calcium response measured in VSMC is responsible for the CsA-induced increase in vasoconstriction detected in isolated mesenteric arteries.

In vivo observations led Scherrer *et al.* (1990) and Lyson *et al.* (1993) to propose that CsA-induced hypertension was due to an action on the central nervous system leading to an increase of sympathetic activity. These results were not confirmed by Kaye *et al.* (1993). Nevertheless, both mechanisms, i.e. activation of the sympathetic system as well as a direct effect on vascular smooth muscle, could be responsible for vasoconstriction *in vivo*. The two effects might even be mechanistically related as $[Ca^{2+}]_c$ regulates functions as diverse as secretion and neurotransmission. Therefore, not only a direct vasoconstrictor effect but also an increase of local vasoconstrictor secretion or an activation of the sympathetic system could be due to the effect of CsA on $[Ca^{2+}]_c$. It is noteworthy that an effect of CsA on calcium signalling has been observed in several cell types, such as smooth muscle cells (Pfeilschifter & Rüegg, 1987), mesangial cells (Pfeilschifter, 1988), T-cells (Hiestand, 1984) and hepatocytes (Nicchita *et al.*, 1985).

Several groups have reported that CsA increased basal cellular calcium influx leading to an increased loading of the inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium pools (Pfeilschifter & Rüegg, 1987; Pfeilschifter, 1988; Meyer-Lehnert & Schrier, 1989; Bokemeyer *et al.*, 1993). As a consequence $[Ca^{2+}]_c$ elevation in response to vasoconstrictor hormones, which act by stimulating the phospholipase C (PLC)-IP_3 pathway, would then be potentiated. Our results do not support these findings. In our VSMC, the $[Ca^{2+}]_c$ elevation induced by a range of concentrations of the SR-Ca^{2+}-ATPase inhibitor thapsigargin (TG), leading at maximal concentration to a complete emptying of the IP_3-sensitive calcium pools, was not potentiated by CsA (Figure 4b). Furthermore, neither CsA nor any of the derivatives tested altered total cellular $^{45}Ca^{2+}$ content (Figure 6b).

The relatively slow time course of both the establishment of the CsA-effect and its reversibility suggests a possible effect on gene expression. CsA could possibly increase the synthesis of hormone receptors as described for angiotensin II or endothelin receptors (Nambie *et al.*, 1990; Iwai *et al.*, 1993; Auch-Schwelk *et al.*, 1994). As in our experiments CsA potentiated the cellular calcium response to four hormones as well as vasoconstriction due to noradrenaline, CsA should increase the synthesis of at least five receptors or activate a common component necessary for the synthesis of these receptors. As an alternative CsA might act downstream of these receptors, on a common target in the signal transduction pathway.

CsA could potentiate the increase in $[Ca^{2+}]_c$ through mechanisms leading to its immunosuppressive effect, i.e. inhibition of the protein phosphatase calcineurin by a complex formed between CsA and the peptidyl-prolyl isomerase cyclophilin (CyP). A role for calcineurin has already been proposed for CsA-induced sympathetic activation (Lyson *et al.*, 1993; Victor *et al.*, 1995) or for the decrease in nitric oxide-mediated vasodilatation (Ressureicao *et al.*, 1995). Even though a correlation between calcineurin inhibition and toxicity has been found for several derivatives of CsA (Lyson *et al.*, 1993) reversal of the CsA effect using a 'CsA-antagonist' such as MeVal[4]CsA has not been tested. Such experiments have been performed with FK506 and its antagonist L-685,818 by Dumont *et al.* (1992) who studied the nephrotoxic effect of these drugs. In fibroblasts, calcineurin can also affect calcium signalling as it has been proposed that it regulates the IP_3-mediated calcium release by dephosphorylation of the IP_3-receptor calcium channel leading to a decrease in its sensitivity (Zhang *et al.*, 1993). Inhibition of calcineurin by CsA should therefore potentiate the calcium response to all hormones activating receptors coupled to the PLC-IP_3 cascade. This would be in line with our observations. The CsA binding protein CyP has not been proposed to regulate calcium signalling but several reports show that another PPIase, FKBP-12, is associated with the ryanodine receptor calcium channel in skeletal muscle (Brillantes *et al.*, 1994; Mack *et al.*, 1994). More recently, the complex calcineurin-FKBP-12 has been shown to modulate IP_3 receptor activity (Cameron *et al.*, 1995).

In order to investigate the role of calcineurin and CyP on the Ca^{2+} response, several derivatives of CsA devoid of immunosuppressive activity (i.e. devoid of the capability to inhibit calcineurin phosphatase activity) were tested. CsA, which

has an inverted chirality at N-methylvaline at position 11 or PSC-833, a derivative of CsA which specifically blocks multidrug resistance (Boesch *et al.*, 1991), are both inactive in inhibiting CyP or calcineurin and are not immunosuppressive (Wenger *et al.*, 1986; Boesch *et al.*, 1991). MeVal[4]CsA is a derivative of CsA with a N-methylvaline instead of N-methylleucine in position 4. This compound is able to bind CyP and inhibits its peptidyl-prolyl *cis-trans* isomerase activity with a higher affinity than CsA but this complex is unable to bind to calcineurin. As a consequence, MeVal[4]CsA can completely reverse the immunosuppressive activity of CsA (Fliri *et al.*, 1993).

Our results show that CsH and PSC-833 potentiated the calcium response to AVP to a similar extent to CsA or CsG. Even though an interaction of these compounds with one of the subtypes of CyPs might occur at the concentrations we have been using, their affinity for CyPs is about one thousandth of that of CsA (M. Zurini, R. Wenger, personal communication) and, therefore, their dose-response curves should be shifted to the right by this factor. Moreover, MeVal[4]CsA did not show any effect on calcium signalling (Figure 6a) and was also unable to reverse the potentiating effect induced by CsA. FK520, a close derivative of FK506 (Liu, 1993), which inhibits calcineurin by binding and inhibiting the PPIase FKBP-12; neither affected the calcium signalling (Figure 6a).

Taken together, these results suggest that in our smooth muscle cell preparation, no correlation between CyP or calcineurin inhibition and calcium response exists and also that FKBP-12 has no role in calcium signalling. To our knowledge this is the first demonstration of a dissociation between the immunosuppressive activity of CsA and analogues, and the effect on the calcium response in VSMC and, therefore, vasoconstriction.

Some comments should be made about the concentrations of CsA used in our experiments. A maximal effect was reached at about 3 μM but a statistically significant effect was already detected at a concentration of 10^{-7} M, which corresponds to plasma concentrations measured during human immunotherapy (see e.g. Fahr, 1993). In most of our experiments we used 10 times higher concentrations. The high lipophilicity of CsA and its repetitive usage in patients might cause its accumulation in cells, making it difficult to assess effective intracellular concentrations. It is noteworthy that for FK506, cytoplasmic concentrations 10 to 400 times higher than extracellularly added concentrations have been measured (Dumont *et al.*, 1994). In most *in vitro* studies, micromolar concentrations of CsA are effective but Lanese & Conger (1993) reported effects on isolated glomeruli at the surprisingly low CsA-concentration of 10^{-11} M. Extracellular concentrations might not necessarily relate to the concentrations obtained at the molecular site of action and intracellular accumulation might vary with cell type or duration of CsA-exposure. As the molecules we have tested have fairly similar structures (with the exception of FK520) we feel confident that their extracellular/intracellular distribution is similar.

In conclusion, our investigations suggest that CsA increases the potency and efficacy of vasoconstrictor hormones in elevating $[Ca^{2+}]_c$ and inducing vasoconstriction. CsA did not modify calcium loading of the cells and of the thapsigargin-sensitive pools but should rather interact with a target of the signal transduction pathway activated by those hormones. An important finding resulting from the investigation of effects of CsA derivatives on cytosolic calcium and vasoconstriction is the lack of relationship to immunosuppression. Further studies are in progress to determine the molecular targets involved in CsA-induced calcium potentiation.

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