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Biphasic effects of cyclosporin A on formyl-methionyl-leucyl-phenylalanine stimulated responses in HL-60 cells differentiated into neutrophils

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1 The immunosuppressive drug cyclosporin A (CsA) depresses neutrophil oxidative burst which may lead to an increased susceptibility to infection in transplant patients. Using specific CsA analogues we investigated the mechanism of inhibition of the oxidative burst and evaluated short and long-term effects of CsA on dimethylsulphoxide-differentiated HL-60 neutrophils.

2 A biphasic pattern was observed: a 4 h pre-treatment with CsA (1 μM) diminished the fMLP induced $[Ca^{2+}]_c$ rise, reactive oxygen species (ROS) production, and β-glucuronidase release by about 40%, whereas a 20 h pre-treatment increased these responses by about 1.5 fold.

3 [MeVal[4]]CsA, which binds with high affinity to cyclophilin but inhibits the interaction of the CsA-cyclophilin complex with calcineurin, blocked the stimulation observed with CsA after a 20 h incubation but did not alter the CsA effects after a 4 h pre-treatment.

4 PSC 833 (1 μM), a potent multi drug resistance transporter (MDR) inhibitor, diminished ROS production to the same extent as a 4 h CsA incubation but was ineffective after a 20 h pre-treatment. An involvement of MDR as a basis for CsA or PSC 833 action was ruled out based on the results of the calcein retention assay.

5 [^{3}H]CsA uptake showed that CsA and [MeVal[4]]CsA, but not CsH or PSC 833 were strongly taken up and retained by the cells.

6 In conclusion, the reduction of the responses after 4 h appear to be due to a primary reduction of calcium signalling, while the enhanced responses after 20 h may be due to calcineurin inhibition.

Keywords: Calcium; calcineurin; cyclophilin; cyclosporin; degranulation; HL-60 cells; multi-drug resistance transporter; neutrophil; reactive oxygen species

Introduction

Cyclosporin A (CsA), a fungal cyclic undecapeptide produced by *Tolypocladium inflatum Gams*, is a potent immunosuppressive drug used in transplant rejection therapy (Borel *et al*., 1996). It acts by inhibiting interleukin-2 production which constitutes the early cellular response of T-helper cells to antigenic stimulation (Schreiber & Crabtree, 1992; Sigal & Dumont, 1992, Kunz & Hall, 1993; Borel *et al*., 1996). The primary target for CsA in T-cells is the cytosolic protein cyclophilin, which catalyses the *cis-trans* isomerization of peptide bonds at prolyl residues (Schreiber & Crabtree, 1992; Sigal & Dumont, 1992; Kunz & Hall, 1993). The formation of a tertiary complex between CsA, cyclophilin and the calcium-dependent phosphatase, calcineurin (McKeon, 1991), leads to inhibition of the enzymatic function of the latter. This inhibition prevents nuclear translocation of the transcription factor NF-AT (nuclear factor of activated T-cells) required for cytokine gene expression during T-cell activation (Sigal & Dumont, 1992; Kunz & Hall, 1993; Borel *et al*., 1996). CsA is also known to bind directly to the multi-drug resistance transporter (MDR) resulting in its blockade (Foxwell *et al*., 1989; Boesch *et al*., 1991; Borel *et al*., 1996).

Allografted patients treated with CsA show an increased susceptibility to infections. As short-term (1–2 h) *in vitro* incubation of neutrophils with CsA has been shown to depress phagocytosis and respiratory burst (Kurokawa *et al*., 1992; Thorat *et al*., 1994) it has been proposed that these effects may contribute to this increased susceptibility (Kurokawa *et al*., 1992; Thorat *et al*., 1994).

The human HL-60 promyelocytic cell line can be differentiated into neutrophils by incubation with dimethylsulphoxide (DMSO) (Klinker *et al*., 1996). In such treated cells, functional multimeric NADPH oxidase can be activated at the membrane by the chemoattractant peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Tauber, 1987; Levy *et al*., 1990; Morel *et al*., 1991; Seifert & Schultz, 1991; Segal & Abo, 1993). fMLP elicits a respiratory burst in neutrophils by binding to its receptor and stimulating a G-protein which activates phospholipase C. Signalling proceeds *via* the classical pathway, yielding inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of calcium from the endoplasmic reticulum through interaction with the IP_3 receptor. DAG in conjunction with high levels of cytoplasmic calcium ($[Ca^{2+}]_c$) activates protein kinase C. These events trigger the assembly of NADPH oxidase producing superoxide anion radicals ($O_2^{\cdot-}$) and the release of granules containing β-glucuronidase. $O_2^{\cdot-}$ is further transformed to hydrogen peroxide and hypochlorite, collectively termed reactive oxygen species (ROS) during this oxidative burst (Tauber, 1987; Levy *et al*., 1990; Morel *et al*., 1991; Seifert & Schultz, 1991; Segal & Abo, 1993).

The mechanisms by which CsA decreases ROS production in neutrophils, which constitute the primary defence against invading micro-organisms, are not well understood and its long-term effects on neutrophils have not, to our knowledge, been reported. HL-60 cells can be differentiated into

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neutrophils and thus constitute an useful model to study the mechanisms triggering the oxidative burst (Seifert & Schultz 1991; Klinker *et al*., 1996). We have used these cells to investigate the effects of CsA on the fMLP-induced ROS formation. In addition, degranulation was studied by measuring β-glucuronidase release. Both oxidative burst and degranulation are calcium-dependent processes. We have therefore examined changes in cytosolic calcium concentrations ($[Ca^{2+}]_c$). Analogues of CsA, selective for certain intracellular targets, were used to determine the possible contribution of cyclophilin and calcineurin or of the MDR on the biphasic CsA effects observed.

Methods

Drugs

CsA and its analogues (CsH, PSC 833 and [MeVal^4]CsA) were a gift from Novartis Pharma (Basel, Switzerland). [^{3}H]CsA was provided by Amersham (Zurich, Switzerland). RPMI 1640 medium, fetal calf serum (FCS), penicillin and streptomycin were from Life Technologies (Basel, Switzerland), luminol and fura-2 acetoxymethyl ester (fura-2/AM) from Molecular Probes (Oregon, U.S.A.), horseradish peroxydase (type II), fMLP, and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide and cytochalasin B from Sigma (Buchs, Switzerland). Stock solutions of luminol (10 mM), fura/2AM (10 mM) and fMLP (1 mM), respectively, were made in DMSO and those of CsA and its analogues in ethanol at a concentration of 10 mM. The HEPES-buffered salt solution (HBSS) contained 130 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, and 5 mM glucose, adjusted to pH 7.4.

Cell culture

The human promyelocytic cell line HL-60 was kindly provided by Drs. D. Lew and K.H. Krause (Division of Infectious Diseases, University Hospital of Geneva, Switzerland). Cells at an initial density of 2.0×10^6 cells ml^{-1} were grown in 75 ml culture flasks in RPMI 1640 medium containing 10% heat-inactivated FCS, penicillin (5 U ml^{-1}) and streptomycin (50 μg ml^{-1}) under a 95% air/5% CO_2 atmosphere. HL-60 cells were differentiated by culturing them for 3 days in 1.3% DMSO and another 3 days in 0.65% DMSO in the above medium. Cells were used 2 days after the second differentiation stage.

ROS measurements

Differentiated HL-60 cells (2×10^6 cells ml^{-1}) were treated with CsA or its analogues in the above medium containing 0.65% DMSO for the times indicated, centrifuged at 900 r.p.m. for 5 min at room temperature and resuspended at a concentration of 2.5×10^6 cells ml^{-1} in HBSS. Aliquots (0.2 ml) were preincubated at 37°C with sodium azide (90 μM), horseradish peroxydase (1 U ml^{-1}), and luminol (50 μM) for 5 min and stimulated with fMLP as described (Wymann *et al*., 1987). Luminescence measurements were performed using a microtiter-plate luminometer (Dynatech ML 3000, Embrach, Switzerland).

$[Ca^{2+}]_c$ measurements

HL-60 cells pre-treated with CsA or analogues under the above conditions were centrifuged (900 r.p.m. for 5 min), suspended at 2×10^7 cells ml^{-1} in HBSS supplemented with 1% bovine serum albumin (BSA), and preincubated at 37°C for 5 min. After 45 min of incubation with 5 μM of fura-2/AM, the suspension was diluted 2 fold, and centrifuged again. Cells were resuspended at 2.5×10^6 cells ml^{-1} in HBSS without BSA, and stimulated at 37°C with 1 μM fMLP. Fura-2 fluorescence was detected with a Perkin-Elmer LS 50 B spectrofluorimeter thermostated at 37°C. Excitation and emission measurements were at 340 nm and 505 nm, respectively. Calibration was performed as previously described (Leung *et al*., 1996) by sequential addition of 10 μM ionomycin to measure F_{max}, followed by 20 mM EGTA and 3 mM Tris (pH 9) to measure F_{min}. $[Ca^{2+}]_c$ was calculated according to the equation $[Ca^{2+}]_c = K_d\ (F\text{-}F_{min})/(F_{max}\text{-}F)$ using a K_d of 224 nM (Grynkiewicz *et al*., 1985). CsA alone was not fluorescent and did not alter the fluorescence excitation spectrum of fura-2 free acid. There was also no change in autofluorescence of HL-60 cells exposed to CsA for times up to 24 h.

β-Glucuronidase release

Degranulation was assessed using the marker enzyme β-glucuronidase as described (Absolom, 1986). Briefly, DMSO-differentiated HL-60 cells (2.5×10^6 cells ml^{-1}) with or without CsA pretreatment were incubated in HBSS for 5 min in the presence of cytochalasin B (5 μg ml^{-1}). fMLP (1 μM) was added, and after 10 min the tubes were put on ice, and centrifuged at 1600 r.p.m. in the cold for 15 min. β-glucuronidase activity in the supernatant was measured spectrophotometrically (Absolom, 1986).

Influence of DMSO and ethanol on cellular function and of CsA on cell viability

The solvents used for preparing stock solutions of fMLP, luminol, and of cyclosporins, namely DMSO and ethanol may scavenge $^{\cdot}OH$ (Oya *et al*., 1986) or decrease agonist induced calcium responses (Yasui *et al*., 1994). Therefore they were tested at the maximal concentrations used (0.3%) with respect to their ability to alter fMLP-induced responses of HL-60 cells. No ROS scavenging or $[Ca^{2+}]_c$ modulating effects were observed at these concentrations. Cell viability was evaluated by trypan-blue exclusion, yielding more than 95% viable cells after 1, 4 or 20 h incubation with 1 to 10 μM of CsA.

[^{3}H]-CsA uptake assay

Drug uptake by cells was monitored as previously described (Charuk *et al*., 1995). Briefly, DMSO-differentiated HL-60 cells were centrifuged and resuspended at 2.5×10^6 cells ml in HBSS containing 50 nM [^{3}H]CsA and CsA or analogues at various concentrations. After 30 min at 37°C the tubes were chilled on ice and centrifuged at 900 r.p.m. for 5 min at 0°C. Supernatants were removed and replaced by 250 μl of a 1% sodium dodecyl sulphate solution. The radioactivity of the resulting cellular lysate was determined by scintillation counting (Packard Tri Carb 4640). Background radioactivity, corresponding to the c.p.m. found after addition of [^{3}H]CsA to cells followed by immediate cooling and centrifugation, was subtracted.

MDR assay by calcein uptake

A method previously described for other cell lines (Tiberghien & Loor, 1996) was adapted and used. Briefly, DMSO-

differentiated HL-60 cells were centrifuged at 900 r.p.m. for 5 min, resuspended at 10^6 cells ml^{-1} in HBSS containing a cyclosporin derivative at the concentration indicated or vehicle and incubated for 30 min at 37°C. Calcein acetoxy-methylester (calcein-AM, final concentration 0.25 μM) was added and the cells were further incubated for 15 min at 37°C. To stop the reaction, tubes were dipped into ice, centrifuged in the cold and resuspended at a final concentration of 2.5×10^6 cells ml^{-1} in HBSS. The calcein retention in the cell suspension was measured as calcein-specific fluorescence with an excitation at 490 nm, and emission at 530 nm using a Perkin-Elmer 5λ fluorimeter (Tiberghien & Loor, 1996).

Data analysis

Results are means of at least three independent experiments. Vertical bars indicate standard errors of the mean (s.e.m.). results on luminol-dependent chemiluminescence are shown as relative light units (RLU) and were calculated as percentages of control versus values from CsA-treated cells. Calculation of IC_{50} and EC_{50} values was performed by non-linear regression using the software lnPlotPrism (GraphPad Software, San Diego, CA, U.S.A.). Unpaired Student's *t*-test was applied to evaluate a significant variation between treatment groups.

Results

Time-course of the effects of CsA on fMLP-stimulated ROS formation

Addition of 1 μM fMLP to differentiated HL-60 cells increased within about half a minute the ROS formation by over 1000-fold as measured by luminol dependent chemiluminescence (Figure 1). CsA (1 and 10 μM) had no immediate effects on this signal. Cells exposed to CsA for 4 h displayed a reduced peak intensity while those exposed for 20 h had an increased peak intensity (Figure 1). When superoxide dismutase (50 U ml^{-1}) together with catalase (200 U ml^{-1}) were added, the chemiluminescence was less than 15% of control level (Figure 1) indicating that extracellular superoxide anion radical was principally detected under these conditions.

Results after 2, 4, 6, and 20 h pre-treatment with CsA are shown in Figure 2. A decrease in chemiluminescence was noted after incubating the cells for 2 or 4 h with CsA while at 6 h the inhibitory effect disappeared. CsA (1 μM and 10 μM) diminished ROS production after 4 h pre-treatment from $100 \pm 12\%$ to $62 \pm 3\%$ and to $40 \pm 7\%$, respectively ($n=7$, $P<0.05$ for both; Figure 2A). After 20 h of pre-treatment, CsA increased ROS production (from $100 \pm 1\%$ to $128 \pm 10\%$ for 1 μM and to $147 \pm 16\%$, for 10 μM, $n=6$, $P<0.05$ for both concentrations; Figure 2A). The same responses were noted if cells exposed to CsA were washed by centrifugation and resuspension, indicating that the effects could not be abolished by removal of extracellular CsA.

It is possible that CsA was metabolised by the cells during these prolonged incubation times. Therefore, a fresh solution of CsA (1 μM) was added to cells every 2 h and the fMLP-elicited ROS production was measured after 2, 4, 6 and 8 h together with the control (Figure 2B). The results of these time-course studies did not significantly differ from those obtained with 1 μM or 4 μM CsA (Figure 2B) or of the biphasic pattern shown in Figure 2A.

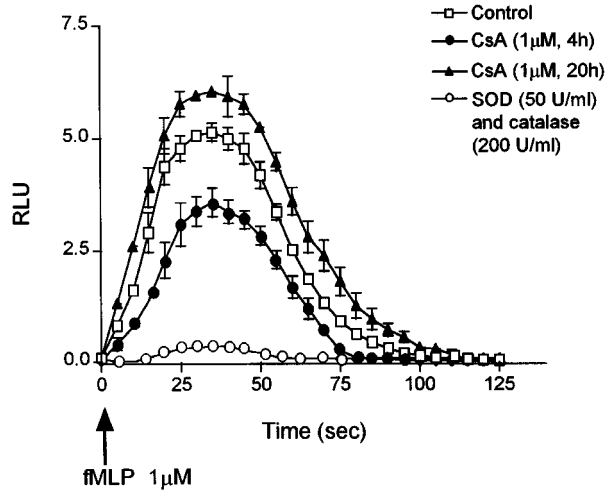

Figure 1 Representative results of ROS formation by differentiated HL-60 cells as measured by luminol-enhanced chemiluminescence. Cells were left untreated or pretreated with CsA (1 μM) for 4 or 20 h and stimulated by fMLP. In one set of experiments, superoxide dismutase and catalase were added 2 min before fMLP stimulation of cells. Data represent means ± s.e.m. of three independent experiments. RLU (relative light units) are arbitrary units of the luminometer.

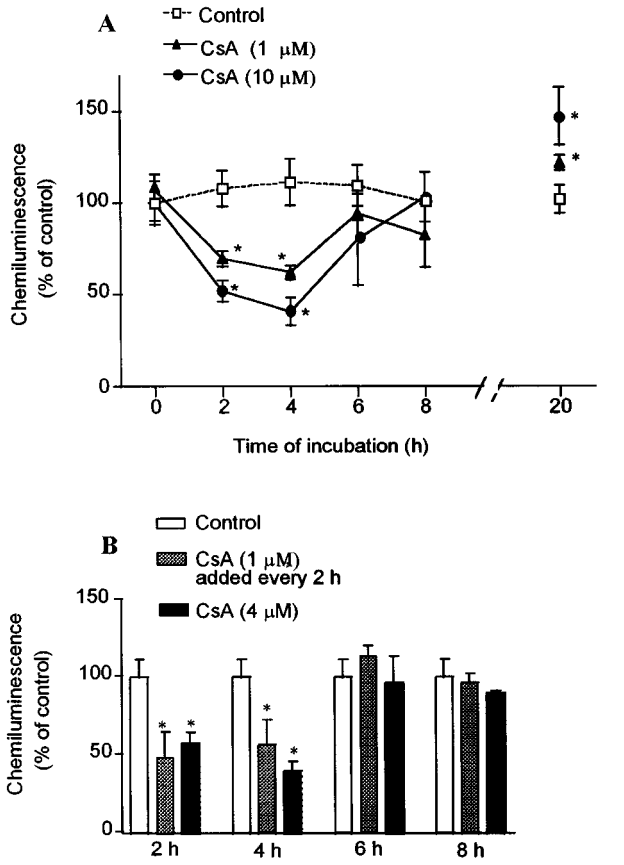

Figure 2 Time course of CsA effect on the fMLP-induced ROS production in differentiated HL-60 cells. (A) After various incubation times in absence or in presence of 1 or 10 μM CsA, cells were stimulated by 1 μM fMLP and luminol-enhanced chemiluminescence was measured. (B) In order to investigate the possibility that CsA was metabolized, 1 μM of CsA was added every 2 h and fMLP-elicited chemiluminescence production was measured. In an additional set of experiments 4 μM CsA was added to the cells; this corresponds to the final concentration reached after 8 h. Values are means ± s.e.m. ($n=6-7$, each in quadruplicate). Significant differences in results between control and treated cells are marked by *(Student's *t*-test, $P<0.05$).

Concentration-dependence of the effects of CsA on fMLP-stimulated ROS formation

When cells were exposed to CsA (1 μM) for 4 or 20 h respectively, and stimulated by various concentrations of fMLP, the response was altered in a concentration-dependent manner (Figure 3A). After a 4 h incubation period with CsA, the fMLP concentration-response curve was shifted to the right (from an EC_{50} value of 48 ± 5 nM for control to 140 ± 15 nM for CsA 4 h-treated cells) and the maximal effect was decreased by $40\pm3\%$. On the other hand, after a 20 h CsA pre-treatment, the maximal effect of fMLP was enhanced by $25\pm4\%$, but the potency was unaltered (EC_{50} value of 48 ± 5 nM for control versus 53 ± 7 for CsA 20 h, not significant; Figure 3A).

The dose-response curve for CsA with respect to the fMLP-elicited ROS production is shown in Figure 3B. The chemiluminescence peak intensities of cells pre-treated with CsA and stimulated with fMLP were reduced (to $62\pm18\%$) in a concentration-dependent manner after 4 h of pre-treatment and increased (to $45\pm3\%$) after a 20 h CsA pre-treatment period. For solubility reasons, CsA concentrations larger than 10 μM could not be investigated.

Effects of CsA analogues on the fMLP-induced ROS production

In order to investigate the possible mediation of these effects by intracellular targets of CsA such as cyclophilin, calcineurin and the MDR, we tested CsA analogues specific for these targets. PSC 833, a specific MDR blocker (Tiberghien & Loor, 1996) showed similar inhibitory profiles as CsA after 4 h of pre-incubation. In contrast to CsA, PSC 833 had no effect on fMLP-elicited ROS production after the 20 h incubation period (Figure 4).

CsH blocked the fMLP-elicited chemiluminescence by $80\pm7\%$ at a concentration of 1 μM ($n=5$, $P<0.05$) at all incubation times tested (Figure 4). [MeVal^4]CsA, a CsA antagonist which binds to cyclophilin with a higher affinity than CsA, thereby preventing the interaction of the CsA-cyclophilin complex with calcineurin (Fliri *et al*., 1993), reversed the 20 h CsA pre-treatment effect on fMLP-mediated ROS production (Table 1).

Effect of CsA and its antagonist on the fMLP-induced $[Ca^{2+}]_c$ rise

Pre-treatment of DMSO-differentiated HL-60 cells with CsA for up to 20 h did not significantly affect basal $[Ca^{2+}]_c$ values as detected by fura-2 fluorescence. Moreover, if CsA was added together with fMLP there was no effect on the fMLP stimulated $[Ca^{2+}]_c$ rise. However, if cells were pre-treated for 4 h with CsA (1 μM), the fMLP-induced $[Ca^{2+}]_c$ rise was significantly decreased (from $100\pm15\%$ to $56\pm12\%$; $n=8$, $P<0.05$; Figure 5). Conversely, a 20 h CsA pre-treatment significantly increased the fMLP-induced $[Ca^{2+}]_c$ rise (from $100\pm10\%$ to $197\pm7\%$; $n=8$, $P<0.05$). [MeVal^4]CsA alone, or together with CsA, was ineffective after 4 h but fully antagonised the effect on $[Ca^{2+}]_c$ after a 20 h CsA pre-treatment (Table 1). The latter effect was also observed when CsA or [MeVal^4]CsA pre-treated cells were centrifuged,

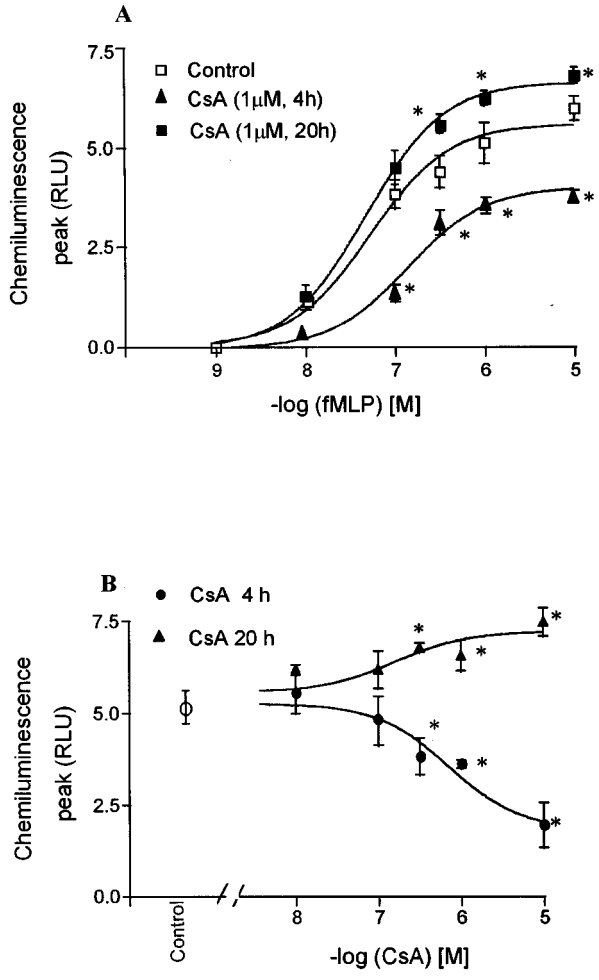

Figure 3 Concentration-dependency of fMLP-induced ROS production under CsA pretreatment. (A) After 4 or 20 h of incubation in the absence or presence of 1 μM CsA, differentiated HL-60 cells were stimulated by various fMLP concentrations. CsA or the vehicle added at the same time as fMLP did not affect chemiluminescence. (B) After 4 or 20 h of incubation with various CsA concentrations, cells were stimulated by fMLP (1 μM). Values are means$\pm$s.e.m. ($n=3$, each in quadriplicate). Significant differences in results between control and treated cells are marked by *(Student's *t*-test, $P<0.05$).

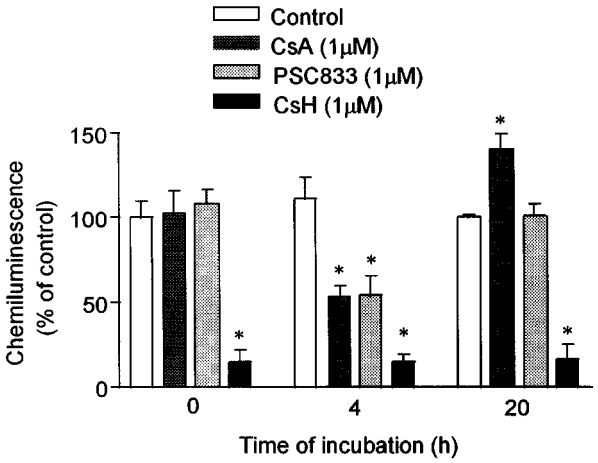

Figure 4 Effect of various incubation times with CsA or CsA analogues on the ROS production stimulated by fMLP (1 μM). HL-60 cells were left untreated (control) or pre-treated with 1 μM of CsA, PSC 833 or CsH for 0, 4 or 20 h, and stimulated by fMLP (1 μM). Values are means$\pm$s.e.m. ($n=3-5$, each in quadruplicate). Significant differences in results between control and treated cells are marked by *(Student's *t*-test, $P<0.05$).

Table 1 Effects of CsA or of [MeVa[4]]CsA on the fMLP responses after 4 or 20 h incubation periods. [MeVa[4]]CsA is a CsA antagonist which binds to cyclophilin with a higher affinity than CsA and prevents the interaction of the CsA-cyclophilin complex with calcineurin

	$[Ca^{2+}]_c$		ROS		βglucuronidase	
Assay	*(% of control)*		*(% of control)*		*(% of control)*	
Incubation time	*4 h*	*20 h*	*4 h*	*20 h*	*4 h*	*20 h*
Control	100±15	100±22	100±15	100±8	100±9	100±10
CsA	56±12*	157±6*	62±9*	128±10*	52±12*	152±5*
[MeVal[4]]CsA	90±5	104±13	99±17	82±15	95±10	92±9
CsA + [MeVal[4]]CsA	57±5*	110±15	56±14*	83±12	52±10*	92±12

Cells were left untreated (control) or were pre-treated with 1 μM of CsA or 1 μM of [MeVal[4]]CsA, respectively, or co-incubated with CsA and with [MeVal[4]]CsA (1 μM) for 4 or 20 h and stimulated by fMLP (1 μM). ROS formation, $[Ca^{2+}]_c$ increase and β-glucuronidase release were assayed as described in Methods. Values are means±s.e.m. ($n=3-8$). Significant differences between control and treated cells are marked by *(Student's *t*-test, $P<0.05$).

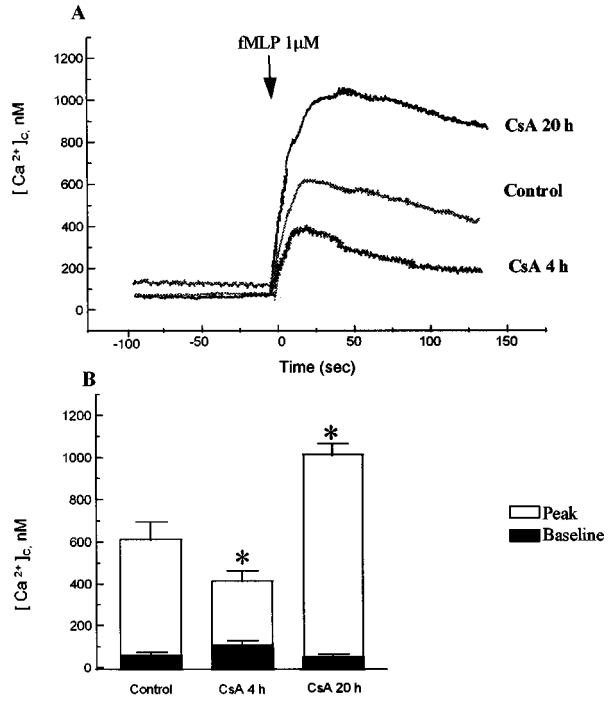

Figure 5 Effect of 4 or 20 h incubation periods with CsA (1 μM) on the $[Ca^{2+}]_c$ rise stimulated by fMLP (1 μM) in fura-2 loaded differentiated HL-60 cells. (A) Typical recording. (B) $[Ca^{2+}]_c$ values at baseline and at fMLP-peak. Values are means±s.e.m. of eight independent experiments performed in duplicate. Significant differences in results between control and treated cells are marked by *(Student's *t*-test, $P<0.05$).

resuspended in buffer and then tested for their sensitivity to fMLP.

Effect of CsA and its antagonist on the fMLP-induced β-glucuronidase release

The effect of CsA on degranulation as tested by β-glucuronidase release is shown in Table 1. CsA (1 μM) pre-treatment for 4 h significantly decreased (from 100±9% to 52±12%; $n=8$, $P<0.05$) the fMLP-induced β-glucuronidase release whereas a 20 h CsA incubation period significantly increased (from 100±10% to 152±5%; $n=7$, $P<0.05$). The 20 h effect, but not the 4 h effect, was abolished by [MeVal[4]]CsA. These time-dependent effects were also observed after removal of CsA by centrifugation and resuspending the cells in CsA-free buffer. Moreover, CsA added at time zero had no effect on the fMLP-stimulated enzyme release. Under basal conditions, neither CsA nor [MeVal[4]]CsA affected enzyme release.

Effect of CsA and its analogues on [^{3}H]CsA uptake

[^{3}H]CsA uptake was studied to determine the effect of CsA and its analogues on their net cellular accumulation. Initial experiments showed that after an incubation period of 30 min with 50 nM [^{3}H]CsA a steady state was reached. PSC 833 and CsH had no effect on the cellular accumulation of [^{3}H]CsA whereas CsA and [MeVal[4]]CsA (both at 1 μM) decreased the cellular level of [^{3}H]CsA by 60±5% indicating competition (Figure 6).

Effect of CsA and its analogues on the MDR activity

Calcein has been shown to be a substrate for the MDR in various cells expressing this protein (Tiberghien & Loor, 1996). Measurement of calcein accumulation was thus used to test the functional presence of the MDR. PSC 833 and CsA, each tested at a concentration of 10 μM, had no effect on calcein accumulation implying the absence of a functional MDR in these cells.

Discussion

The effect of CsA on the neutrophil function are not clearly understood, although it has been shown that CsA is able to suppress the fMLP-induced ROS production in human neutrophils (Kurokawa *et al*., 1992; Thorat *et al*., 1994). We here report that long-term (20 h) incubation of DMSO-differentiated HL-60 cells with CsA caused an upregulation of their $[Ca^{2+}]_c$ response, ROS formation and β-glucuronidase release after stimulation with fMLP. However, after short-term incubation (2–4 h) with CsA, a decrease in these fMLP-stimulated responses was observed suggesting a biphasic effect of CsA on neutrophil function.

The 4 h as well as the 20 h effect occurred in a similar CsA concentration range (0.1–10 μM) which falls within the range of plasma levels found in CsA treated patients (Fahr, 1993). No effect was observed upon incubation of the cells with CsA for less than 30 min excluding the possibility of ROS scavenging or blockage of the fMLP receptor as has been previously reported with CsH (Wenzel-Seifert & Seifert, 1993). Therefore the inhibition of ROS production by CsH is taken to be due to fMLP receptor antagonism. The extended period of time required for inhibiting and stimulating effects, suggest that CsA affects signalling

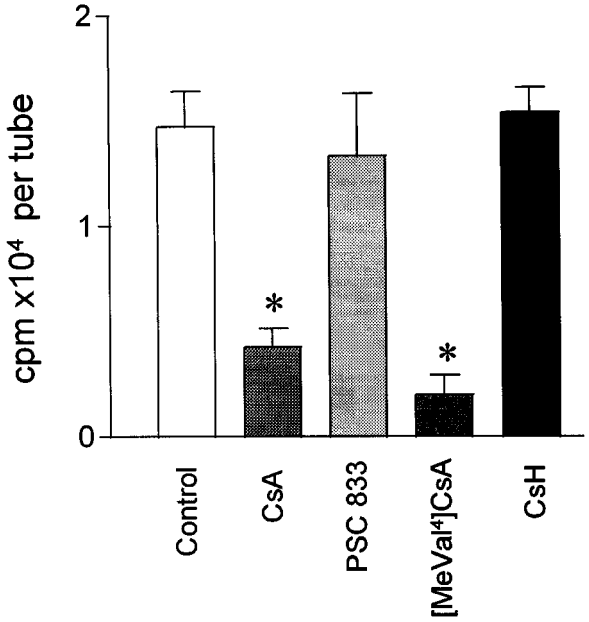

Figure 6 Effect of CsA and analogues on [^{3}H]CsA accumulation in differentiated HL-60 cells. The four cyclosporins were added for 30 min at 1 μM together with [^{3}H]CsA as described in Methods. Values are means$\pm$s.e.m. ($n=3-6$, each done in duplicate). Significant differences in results between control and treated cells are marked by *(Student's t-test, $P<0.05$).

components *via* complex interactions possibly by regulating transcription or protein synthesis. Consistent with our observations, it has been demonstrated that addition of CsA several minutes before fMLP stimulation had no effect on $[Ca^{2+}]_c$ or ROS production in neutrophils (Wenzel-Seifert *et al*., 1991; Wenzel-Seifert & Seifert, 1993).

Since the three parameters studied in response to fMLP were affected to a similar extent and with similar kinetics, the molecular mechanisms involved are likely to be linked and located upstream of calcium signalling. The decrease after 4 h pre-treatment may be due to an accumulation of CsA which interferes with a transduction step between the fMLP receptor and the calcium messenger. Downstream, the reduction of $[Ca^{2+}]_c$ subsequently leads to decreased effects on ROS formation and β-glucuronidase release. Similarly, effects after a 20 h CsA pre-treatment may also be caused by augmentation of a step taking place before $[Ca^{2+}]_c$ rise as subsequent responses were increased too. Therefore, it is possible that CsA stimulated an increase in the calcium response, possibly by upregulation of the fMLP receptor, as has been observed for vasopressin receptors in smooth muscle cells (Lo Russo *et al*., 1997).

The known intracellular target of CsA, the cyclophilins, inhibit the protein phosphatase calcineurin in the presence of CsA (McKeon, 1991; Schreiber & Crabtree, 1992; Sigal & Dumont, 1992; Kunz & Hall, 1993). The formation of this ternary complex is necessary to obtain an immunosuppressive effect. CsA derivatives devoid of immunosuppressive activity (CsH, [MeVal^4]CsA and PSC 833) were used in order to investigate potential intracellular targets involved in ROS production specifically affected by CsA. [MeVal^4]CsA is a CsA derivative known to bind to cyclophilins with an approximately 2–3 fold higher affinity than CsA (M. Zurini, R. Wenger, personal communication), thereby preventing the interaction of the CsA-cyclophilin complex with calcineurin and reversing the immunosuppressive activity of CsA (Fliri *et al*., 1993). [MeVal^4]CsA alone showed no effect on the fMLP-induced responses at all times studied. Moreover, this CsA antagonist was unable to reverse the inhibitory effect of CsA after 4 h incubation indicating that inhibition of calcineurin is not involved in these early events. In contrast, a 20 h co-incubation of CsA with [MeVal^4]CsA abolished the stimulation of CsA on the fMLP-induced responses, suggesting that calcineurin inhibition by CsA was causing the increased response at prolonged incubation times. A related finding has been made in adrenal glomerulosa cells (Baukal *et al*., 1994).

The affinity of PSC 833, a potent blocking agent of MDR (Archinal-Mattheis *et al*., 1995; Borel *et al*., 1996), for cyclophilin is approximately 1/1000 of that of CsA (M. Zurini, R. Wenger, personal communication). PSC 833 did not mimic the CsA effects at 20 h but suppressed the fMLP-induced ROS production, similar to CsA after 4 h pre-treatment. This would be in line with an involvement of the MDR in the CsA effect at 4 h. Tumour cell lines, such as HL-60 cells, have indeed been shown to express MDR under specific conditions (Broxterman *et al*., 1995; Zhou *et al*., 1996). This transporter is associated with decreased intracellular drug accumulation resulting from enhanced drug efflux. The fluorescent MDR substrate calcein was used to test for the presence of a MDR transport system. None of the various cyclosporins used affected the accumulation of calcein suggesting that HL-60 neutrophils used in this study did not express a functional MDR. Thus, the effects of CsA and PSC 833 are unlikely to be a result of MDR inhibition.

To see whether the effects observed were due to changes observed in effective intracellular concentration of CsA, we determined the effects of various cyclosporin analogues on the cellular accumulation of [^{3}H]CsA. Despite their similar chemical structure and hydrophobicity, the cyclosporins tested differed greatly in their ability to modulate [^{3}H]CsA accumulation. CsA and [MeVal^4]CsA diminished the accumulation of [^{3}H]CsA, while CsH, devoid of interaction with cyclophilin (Fliri *et al*., 1993) did not. These data strongly indicate that an association with cyclophilin is responsible for [^{3}H]CsA accumulation. As for CsH, PSC 833 was unable to compete with [^{3}H]CsA for cellular accumulation. This, in conjunction with the calcein results, implies that it is unlikely that the MDR is responsible for the decreased effects at 4 h. To determine which of the cellular targets causes the CsA effect at 4 h further studies are needed.

In summary, CsA showed biphasic effects on the fMLP-induced cellular responses which were concentration and time dependent. While the exact mechanisms of these biphasic effects are not yet fully understood, our findings, using DMSO-differentiated HL-60 cells as a model for neutrophils, suggest a modulation of an early step in calcium triggering. Pharmacological evidence points to an implication of the phosphatase calcineurin in the effect observed after 20 h incubation. The results of [^{3}H]CsA accumulation experiments demonstrate that cyclophilins are present in DMSO-differentiated HL-60 cells, and mediate the long-term potentiation of the fMLP responses by inhibiting calcineurin.

In addition to its well known action on the signal transduction system of T cells involved in inhibiting the cellular immune response, CsA and some of its more specific analogues could be useful for the investigation of pathways involved in defence cells such as neutrophils. Our findings support the notion that although short term incubation of these cells leads to a decrease, a long term incubation increases their primary defence reaction.

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