Mechanisms of the Endothelial Toxicity of Cyclosporin A
Role of Nitric Oxide, cGMP, and Ca$^{2+}$

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Abstract Cyclosporin A (CyA) is an efficient immunosuppressive agent, which, however, causes functional and structural alterations in endothelial cells. The aim of the present study was to examine the mechanisms of CyA-induced endothelial dysfunction. CyA administration (Wistar rats, 25 mg/kg per day for 15 days) induced a significant inhibition of endothelial-dependent relaxation to acetylcholine on isolated femoral arteries. No changes with CyA were detected in the relaxation response to the endothelium-independent agent (sodium nitroprusside) or the endothelium-dependent receptor-independent agent (Ca$^{2+}$ ionophore). The addition of L-arginine ($10^{-5}$ mol/L) shifted to the left the acetylcholine-mediated vasorelaxing response in CyA-treated segments, an effect that was accompanied by a marked increase of cGMP. $^{45}$Ca$^{2+}$ uptake was higher in CyA-treated segments with respect to control segments but became normalized after incubation with L-arginine or sodium nitroprusside. Deendothelialization or incubation with the L-arginine competitive analogue Nω-nitro-L-arginine (NωNLA) increased $^{45}$Ca$^{2+}$ uptake in control segments but not in CyA-treated segments. In conclusion, in isolated rat arteries, chronic CyA therapy affects endothelial function by uncoupling the acetylcholine-mediated relaxation and interfering with an endothelium-mediated pathway that regulates $^{45}$Ca$^{2+}$ uptake by a mechanism reversed by an L-arginine-dependent cytosolic calcium generation. (Circ Res. 1994;74:477-484.)

Key Words • cyclosporin toxicity • L-arginine • Ca$^{2+}$ • cGMP endothelium-dependent responses • vasodilation • nitric oxide

Cyclosporin A (CyA) is a peptide with a leading role in immunosuppressive treatment, particularly in organ transplantation. In parallel with its outstanding antirejection properties, CyA has considerable toxic effects, with repercussions on both renal and vascular function.

CyA toxicity has been characterized in patients and animal experimental models. As the main effects on kidney and vascular structure and function, CyA has been shown to provoke contraction and hyperplasia of small arteries, with decreased renal cortical flow and glomerular filtration rate.

The principal mechanism of renal damage by CyA appears to be of vascular origin. However, the precise mechanisms of this type of CyA toxicity are still insufficiently clarified. Several studies have been published reporting CyA toxic effects on diverse vascular regulatory systems, including activation of the sympathetic system, loss of the prostacyclin thromboxane A2 equilibrium in the vascular wall, local platelet aggregation and microthrombi formation, and increased Ca$^{2+}$ uptake by vascular smooth muscle and mesangial cells in culture.

New information has been recently provided suggesting that a main site of impaired vascular regulation by CyA is at the endothelial cell level. Studies on endothelial cell cultures have found substantial structural damage when the cells were exposed to CyA. An endothelial effect of CyA was given more support by the evidence of an increased production of the vasoconstricting mediator endothelin 1 in the presence of CyA and the finding of a significant reversal by antibodies against endothelin 1 of the CyA-contracting effects on the renal vasculature. Furthermore, other experiments, done mostly in vitro models of CyA toxicity, have demonstrated that CyA can potentially alter the nitric oxide (NO)-mediated vasodilation.

In vivo evidence for the latter pathophysiological mechanism has been reported by Gerkens and by our laboratory. The appearance of the hemolytic uremic syndrome, an endothelial damage-related illness in patients receiving CyA, is indirect evidence of endothelial damage by the drug. Different therapeutic interventions have been designed to inhibit the CyA-related toxic effects. However, there is no consistent evidence of the usefulness and actual indications of these treatments in clinical practice. Recent data from our laboratory suggested that L-arginine, a substrate for the NO synthase, may contribute to reverse the functional renal and vascular effects of CyA toxicity. Several questions remain, however, about the mechanisms by which L-arginine administration interferes with the toxic effects of CyA.

The present study was therefore designed to characterize the interactions of CyA and L-arginine on the endothelium-dependent relaxation of isolated arteries and to analyze the putative mechanism of these interactions. In particular, the effect of endothelium-related mechanisms...
on $^{47}$Ca$^{2+}$ uptake was compared in CyA-treated, decenedothelized, and No-nitro-l-arginine (NwNLA)-treated vessels.

Materials and Methods

The studies were performed on control and CyA-treated male Wistar rats (250 to 300 g). CyA was administered at a dose of 25 mg/kg IM per day for 15 days, dissolved in 100% ethanol, and prepared in 200 µL olive oil. This CyA administration protocol produced only mild changes in arterial pressure and glomerular filtration rate. Pure CyA was used to avoid the putative effects of the vehicle of CyA used in commercial preparations. The control animals received only the vehicle. At the time of the study, the rats were anesthetized with pentobarbital (100 mg/kg body weight), decapitated, and exsanguinated. Femoral or aortic arteries were isolated and placed in a Petri dish with sterile physiological saline solution (PSS) containing (mmol/L) NaCl 140, KCl 4.6, CaCl$_2$ 2.0, MgCl$_2$ 10, D-glucose 1.0, and HEPES 10, pH 7.4, at 4°C. Vessels obtained by identical procedure were fixed with 10% formol and stained with hematoxylin and eosin or stained with silver nitrate without fixation, for microscopy studies.

Vasorelaxation Studies

Femoral artery segments, 2 mm in length, were cut and cleaned, and the dimensions of the outside diameter were measured using an ocular micrometer included within a Wild M8 Zoom microscope (Kodak). As previously described, each segment was suspended in Krebs-Henseleit solution (mmol/L: NaCl 115, KCl 4.6, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 2.5, NaHCO$_3$ 25, glucose 11.1, and disodium EDTA 0.01) by two fine stainless-steel pins, 50 µm in diameter, introduced into the arterial lumen and connected for isometric recording. The recording system included a Universal transducing cell UC3, a Statham Microscope access bladder U15, and a Beckman type RS recorder. The organ bath contained 6 mL of the solution equilibrated with 95% O$_2$, 5% CO$_2$ to give a pH between 7.3 and 7.4; the temperature was held at 37°C. The segments were stretched to the previously determined optimum resting force of 0.5 g. After equilibration of the vessels for 60 minutes at 37°C, the bath solution was completely replaced. To determine the resting tension for maximal force development, the arteries were subjected to increasing amounts (0.15 to 1.5 g) of resting tension, allowed to equilibrate at each tension, and then challenged with KCl (50 mmol/L) to test the contractile ability of the vessel preparation. The relaxation to acetylcholine and sodium nitroprusside (10$^{-5}$ to 10$^{-4}$ mol/L) was tested on arteries precontracted with prostaglandin F$_{2a}$ (PGF$_{2a}$, 10$^{-5}$ mol/L), both in control conditions and after incubation with l-arginine (10$^{-4}$ mol/L). Additional experiments were done in the presence or absence of superoxide dismutase (30 U/mL) and the l-arginine analogue $N$-benzoyl-$l$-arginine ethyl ester (BAEE, 10$^{-5}$ mol/L), and a concentration-response curve of the contractile effect of PGF$_{2a}$ was constructed. All the drugs were dissolved in PSS containing 0.01% ascorbic acid and added to the organ bath in a volume of 75 µL or less. The dose-response curves for acetylcholine, sodium nitroprusside, and PGF$_{2a}$ were determined in a cumulative manner. l-Arginine, BAEE, and superoxide dismutase were added to the bath 50 minutes before acetylcholine was applied to the vascular rings.

cGMP Measurement in Vascular Segments

Aortic segments were obtained as described above. cGMP was measured in acetylated samples by a kit from Amersham International, Buckinghamshire, England, as described previously. When appropriate, the segments were incubated in the presence of l-arginine (50 minutes, 37°C). After washing, vessels were incubated in Krebs-Henseleit solution (5 minutes) containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 2 mmol/L) with or without acetylcholine (2 minutes). At the end of the experiment, the samples were homogenized with cold 5% trichloroacetic acid and centrifuged (200g, 15 minutes, 4°C). The supernatant was recovered and washed four times with 5 vol diethyl ether. The remaining aqueous phase was dried under an N$_2$ stream. The dried extract was dissolved in a suitable volume of assay buffer before analysis. The sensitivity of the assay, defined as the amount of cGMP needed to reduce zero dose binding to two standard deviations, was 0.5 fmol. The intraassay and interassay variations were <8.9% and <16.0%, respectively.

$^{45}$Ca$^{2+}$ Uptake in Vascular Segments

Vessels were obtained as described in the above sections. The vascular rings were isolated, suspended on stainless-steel pins, and equilibrated in an organ bath containing oxygenated Krebs-Henseleit solution. At the end of the stabilization period, 1 µCi/mL $^{45}$Ca$^{2+}$ was added to the organ bath, and the vascular rings were incubated at different times in the presence or absence of the diverse agents. The experiments with sodium nitroprusside were done in the presence of the phos-

![Fig 1. Graph showing vascular relaxation by acetylcholine (ACh) in control (n=24) and cyclosporin A-treated (n=26) segments. Results are expressed as percent relaxation with respect to the contraction with prostaglandin F$_{2a}$ (10$^{-5}$ mol/L). Values are mean±SEM. In this and the other groups of studies, the experiments with the comparing groups were done in parallel baths, on the same days, and using the same media and reagents to reduce the methodological error. *P<.05 and †P<.01 with respect to control segments.](http://circres.ahajournals.org/)

![Fig 2. Graph showing the effect of incubation with L-arginine (L-Arg, 10$^{-5}$ mol/L, 50 minutes) in vascular relaxation in segments isolated from cyclosporin A (CyA)-treated rats (n=26 for CyA-treated segments and n=15 for CyA-treated segments with L-Arg incubation). Values are mean±SEM. *P<.05 and **P<.001 with respect to CyA-treated segments.](http://circres.ahajournals.org/)
phodiesterase inhibitor IBMX (2 mmol/L) using segments incubated with IBMX but no sodium nitroprusside as controls. In previous calibration experiments, sodium nitroprusside was found to consistently increase cGMP and to provoke more intense and reproducible effects on $^{45}$Ca$^{2+}$ uptake than the cGMP analogue S-bromo-cGMP (authors’ unpublished observations). When the incubation was finished, the uptake was interrupted by washing the vascular rings three times in 4°C PSS with 2 mmol/L EGTA and then stored in liquid N₂. To measure $^{45}$Ca$^{2+}$ uptake, the vascular rings were homogenized with ice-cold 6% trichloroacetic acid and centrifuged (2000g, 15 minutes, 4°C). $^{45}$Ca$^{2+}$ activity was measured using a liquid scintillation counter (model LS 2800, Beckman). The pellets were resuspended in 1 mL PSS and dried at 100°C to determine dry weight. $^{45}$Ca$^{2+}$ uptake was expressed as nanograms $^{45}$Ca$^{2+}$ per milligram dry weight. Most of the experiments were done in segments of aorta, but control experiments with segments of femoral arteries were done to examine whether the characteristics of $^{45}$Ca$^{2+}$ uptake were similar in both types of vessels.

1-Arginine Measurements

Serum and vascular content of L-arginine were measured using arginine kinase. picker. Briefly, the assay was based on the enzymatic transformation of L-arginine and ATP into phosphoarginine and ADP. ADP was measured by two coupled reactions involving pyruvate kinase and lactate dehydrogenase, with measurement of NADH consumption at 340 nm. The samples were preincubated in the reaction medium with arginine kinase to eliminate side reactions, and measurements of absorbance were done at 60 and 180 seconds. The reaction temperature was 30°C.

Drugs

Acetylcholine, L-arginine, PGF₂α, NwNLA, arginine kinase, IBMX, BAEE, and superoxide dismutase were provided by Sigma Chemical Co, St Louis, Mo; sodium nitroprusside was from Merck, Darmstadt, FRG; verapamil was obtained from Knoll, Madrid, Spain; NADH, phosphoenolpyruvate, ATP, lactate dehydrogenase, and pyruvate kinase were from Boehringer Mannheim, Germany; $^{45}$Ca$^{2+}$ and the cGMP kit were purchased from Amersharm International. CyA was a gift of Sandoz, Basel, Switzerland. Meclofenamate was a gift of Dr Vicente Lahera, Facultad de Medicina, Universidad Complutense de Madrid.

Data Analysis

Results are presented as mean±SEM. Unless stated otherwise, results correspond to at least five identical experiments. Changes in variables for the different incubations and dose-response curves were analyzed by one-way and two-way ANOVA for repeated measures and subsequent Scheffe’s test. Comparisons between two groups of data were done by Student’s t test for unpaired observations.

Results

Vasorelaxation Studies: Effects of CyA on Endothelium-Dependent Relaxation in Segments From Femoral Arteries

The response of endothelium-dependent relaxation to acetylcholine was decreased at $10^{-8}$ and $10^{-7}$ mol/L acetylcholine ($P<.05$) in vascular segments obtained from CyA-treated rats compared with control rats (Fig 1). The EC₅₀ for control vascular segments was 5.2×$10^{-8}$ mol/L, and the EC₅₀ for CyA-treated vascular segments was 0.9×$10^{-7}$ mol/L ($P<.01$). However, the dose-response curve of vasorelaxation to the Ca$^{2+}$ ionophore A23187 was not affected in segments of CyA-treated animals (EC₅₀, 6.7×$10^{-7}$ and 7.4×$10^{-7}$ mol/L; n=9 for CyA-treated rats and n=5 for control rats, respectively; $P=NS$).

The endothelium-independent relaxation of vascular segments, as evaluated by the sodium nitroprusside dose-response relation (10⁻¹⁰ to 10⁻⁴ mol/L) was similar in the control and CyA-treated groups (EC₅₀ for control segments, 5.2×$10^{-9}$ mol/L; EC₅₀ for CyA-treated segments, 3.3×$10^{-9}$ mol/L; $P=NS$ between curves). The degree of contraction of arterial segments with PGF₂α was also similar in the segments isolated from CyA-treated rats compared with control rats, as evaluated by a dose-response curve (EC₅₀, 8.6×$10^{-6}$ and 9.2×$10^{-6}$ mol/L; n=16 for CyA-treated rats and n=16 for control rats, respectively).

To assess the role of L-arginine–mediated mechanisms in the observed decrease of acetylcholine-dependent relaxation, experiments were done with L-arginine included in the incubation medium. In the preparations without L-arginine, the levels of L-arginine in the arterial segments decreased spontaneously during the incubation but recovered to normal after incubation with L-arginine (0.5±0.03 mmol/L after incubation in buffer with L-arginine versus 0.175±0.02 mmol/L after incubation in buffer without L-arginine; $P<.05$; n=5 each). To rule out a role for any in vivo changes in L-arginine concentration that may influence the outcome of the experiments, L-arginine levels were measured in plasma.

![Graph showing the effect of incubation of L-arginine (L-Arg, 10⁻⁵ mol/L, 50 minutes) in vascular relaxation in control segments (n=44 for control segments and n=38 for control segments with L-Arg incubation). ACh indicates acetylcholine. Values are mean±SEM ($P>-.05$ between groups).](http://circres.ahajournals.org/Download/Graph)
samples of CyA-treated and control rats after 15 days of treatment with the drug. No differences in l-arginine concentration were found between both groups (1.06±0.06 mmol/L for CyA-treated rats, 1.12±0.04 mmol/L for control rats; P=NS). The addition of l-arginine (10⁻³ mol/L) to the organ bath for 50 minutes shifted to the left the concentration-response curve of the acetylcholine-dependent relaxation in the CyA-treated vascular segments (Fig 2). However, in control vascular segments, the incubation with l-arginine had no significant effect on the acetylcholine-elicited relaxation (Fig 3).

A group of additional experiments was conducted to further examine the putative mechanisms of this different effect of l-arginine on acetylcholine-induced relaxation in arteries from CyA-treated or untreated animals. The hypothesis that the formation of free radicals may account for the lack of potentiation of the acetylcholine-mediated vasorelaxing response was tested by using two types of experiments. First, according to Heim et al²⁴ and Beckman et al,²⁵ the possibility was considered of an interaction of NO with the oxygen-derived free-radical superoxide anion (O²⁻), leading to the formation of the potentially harmful free radical, peroxynitrite, and, subsequently, to the inhibition of a positive interaction. In our experience, some effects of l-arginine may change in the presence of superoxide dismutase.²⁶ The hypothesis was raised, therefore, that O²⁻-dependent peroxynitrite formation could be involved in the absence of a positive effect of l-arginine on the acetylcholine-dependent relaxation of arterial segments from control rats. Accordingly, additional experiments were performed in the presence of the O²⁻-scavenger superoxide dismutase in an attempt to elucidate whether the extracellular superoxide anion and peroxynitrite were responsible for the observed effect of l-arginine on the control segments. The inclusion of superoxide dismutase in the medium did not change the effect of l-arginine on the acetylcholine-dependent relaxation in control or CyA-treated vascular segments (EC₅₀, 4.5x10⁻⁷ mol/L; n=12; P=NS). Further experiments (n=12 segments each) were performed to assess the role of intracellular free-radical generation, which was assessed by adding the putative non–free-radical–generating l-arginine analogue BAEE²⁷ instead of l-arginine. No increase of the acetylcholine-induced vasorelaxation was observed by adding BAEE (10⁻⁵ mol/L) to the incubation medium (EC₅₀, 6.0x10⁻⁶ mol/L in the presence of BAEE; P=NS with respect to acetylcholine alone or acetylcholine+l-arginine).

**Effects of CyA on Arterial ⁴⁵Ca²⁺ Uptake**

⁴⁵Ca²⁺ uptake was higher in aortic segments from CyA-treated animals compared with segments from control animals (Fig 4). In segments of femoral arteries, a similar increase of ⁴⁵Ca²⁺ uptake was observed in arteries of CyA-treated animals (38.2±3.2% with respect to the control rats at 30 minutes; n=4 for CyA-treated rats and n=4 for control rats).

Several maneuvers were done to further characterize the mechanism of increased ⁴⁵Ca²⁺ uptake in arterial segments of CyA-treated rats. First, the role of the substrate for NO synthase, l-arginine, was assessed. After preincubation with l-arginine (10⁻⁵ mol/L) for 50 minutes, ⁴⁵Ca²⁺ uptake returned to normal values in segments from CyA-treated rats (Fig 5), without significant changes in control rats (P=NS, Fig 5). To further examine the role of l-arginine in the regulation of Ca²⁺ uptake, experiments were done in the presence of the l-arginine competitive analogue NwNLA. Fig 6 shows the ⁴⁵Ca²⁺ uptake in the presence of NwNLA (10⁻⁵ mol/L, 30-minute exposure). NwNLA induced a significa

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**Graph 4.** Graph showing ⁴⁵Ca²⁺ uptake: time response in control vascular segments (n=15) and cyclosporin A-treated vascular segments (n=21). Values are mean±SEM. *P<.05 between curves.

**Graph 5.** Bar graph showing the effect of incubation with l-arginine (L-Arg, 10⁻⁵ mol/L, 50 minutes) on ⁴⁵Ca²⁺ uptake at 30 minutes in control segments (n=19), control+L-Arg–treated segments (n=15), cyclosporin A–treated segments (n=16), and cyclosporin A+L-Arg–treated segments (n=18). Values are mean±SEM. *P<.05 with respect to ⁴⁵Ca²⁺ uptake in control segments.
icant increase in Ca\textsuperscript{2+} uptake in control vascular segments, whereas no changes were found in the CyA-treated group.

To assess the putative role of the L-type Ca\textsuperscript{2+} channels in the CyA-induced increase of 45Ca\textsuperscript{2+} uptake, the Ca\textsuperscript{2+} channel blocker verapamil (10\textsuperscript{-6} and 10\textsuperscript{-5} mol/L, 10-minute preincubation) was added to the incubation bath. No inhibition of 45Ca\textsuperscript{2+} uptake was found with verapamil (10\textsuperscript{-5} mol/L) in either control segments (45Ca\textsuperscript{2+} uptake with verapamil was 94±4% of the 45Ca\textsuperscript{2+} uptake without verapamil; P=NS) or CyA-treated segments (45Ca\textsuperscript{2+} uptake with verapamil was 93±2% of the 45Ca\textsuperscript{2+} uptake without verapamil; P=NS).

Based on the above-mentioned results, the role of the endothelium in regulating Ca\textsuperscript{2+} uptake by isolated vessels was tested. As a model of massive endothelial injury, 45Ca\textsuperscript{2+} uptake was measured in segments of deendothelialized arteries from control and CyA-treated rats. As shown in Fig 7, in segments from control rats, the absence of the endothelial layer significantly increased 45Ca\textsuperscript{2+} uptake (P<.05) in a proportion similar to that found with the treatment with NwNLA. Similar results were found in comparative experiments in which the vessels were deendothelialized with an O\textsubscript{2} stream (Table).

In segments from CyA-treated rats, no change of 45Ca\textsuperscript{2+} uptake was observed with deendothelization (deendothelialized CyA-treated artery, 0.78±0.05 ng 45Ca\textsuperscript{2+} per milligram dry weight; nondeendothelialized CyA-treated artery, 0.75±0.03 ng 45Ca\textsuperscript{2+} per milligram dry weight; n=3; P=NS). Of further interest, L-arginine failed to decrease 45Ca\textsuperscript{2+} uptake in arteries from CyA-treated animals when these vessels were deendothelialized (45Ca\textsuperscript{2+} uptake in deendothelialized arteries from rats treated with CyA+10\textsuperscript{-5} mol/L L-arginine, 0.73±0.06 ng Ca\textsuperscript{2+} per milligram dry weight; n=4; P=NS).

The morphology of endothelial cells was optically normal either by the hematoxylin and eosin or silver nitrate stains (n=5 femoral arteries; data not shown). Both types of staining showed a massive endothelial damage in the rubbed vessels (data not shown).

To examine the role of cGMP-mediated mechanisms in the increased 45Ca\textsuperscript{2+} uptake of arteries from CyA-treated animals or of deendothelialized arteries, arterial segments were treated with the endothelium-independent cGMP-producing agent sodium nitroprusside. In the conditions of the present experiments, sodium nitroprusside (10\textsuperscript{-5} mol/L) induces a significant increase of cGMP (authors’ unpublished observations and next section, “cGMP Measurements”). Incubation with sodium nitroprusside (10\textsuperscript{-5} mol/L, 50 minutes) diminished 45Ca\textsuperscript{2+} uptake in control segments, deendothelialized segments, and segments from CyA-treated animals (Fig 7). However, the decrease of 45Ca\textsuperscript{2+} uptake by sodium

### Table 6

<table>
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<th>Time, min</th>
<th>Control</th>
<th>O\textsubscript{2} Flow</th>
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<tbody>
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<td>2</td>
<td>0.23±0.01</td>
<td>0.38±0.02*</td>
</tr>
<tr>
<td>5</td>
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<td>0.50±0.03*</td>
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<tr>
<td>10</td>
<td>0.37±0.01</td>
<td>0.62±0.02*</td>
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<td>30</td>
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<td>0.75±0.02*</td>
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<tr>
<td>60</td>
<td>0.48±0.02</td>
<td>0.78±0.03*</td>
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Values are mean±SEM. *P<.05 between curves.
nitroprusside was more than double on CyA-treated arteries than in control arteries (P<.05). In the presence of IBMX (2 mmol/L) alone, 45Ca2+ uptake was slightly but not significantly decreased in both control and CyA-treated segments (percent decrease of 45Ca2+ uptake, 9.2±5%; P=NS with respect to incubation in the absence of IBMX).

Further experiments were done in the presence of meclofenamate (10-5 mol/L) to examine the putative role of cyclooxygenase derivatives on the 45Ca2+ uptake. No effect of meclofenamate (10-5 mol/L) was detected on 45Ca2+ uptake either in segments of control rats (n=3) or CyA-treated rats (n=4) (93±2% 45Ca2+ uptake in control segments incubated with meclofenamate, 95±6% 45Ca2+ uptake in CyA-treated segments incubated with meclofenamate; P=NS with respect to both groups without meclofenamate).

cGMP Measurements

The levels of cGMP were measured in vessels incubated in the same conditions as in the 45Ca2+ uptake experiments. As shown in Fig 8, left, the increase of cGMP by acetylcholine (10-5 mol/L) was inhibited in vessels from CyA-treated rats; however, the cGMP-increasing effect of sodium nitroprusside (10-5 mol/L) was preserved in vessels from both types of rats (Fig 8, left). In the presence of L-arginine alone (10-5 mol/L), no effect was detected on baseline cGMP levels (Fig 8, right). In segments from CyA-treated rats, acetylcholine elicited a stronger cGMP response after preincubation in the presence of L-arginine (Fig 8, right). However, such an increased effect was not observed in vessels from control rats.

Discussion

The present series of experiments provide new data on the mechanisms of CyA-induced vascular toxicity. These results may also be useful in the understanding of more general vascular regulatory mechanisms, namely, the role of endothelium on Ca2+ uptake. The significant decrease of the acetylcholine-dependent vasorelaxation in segments of CyA-treated rats is consistent with the in vivo findings in rats treated with the same CyA administration protocol13 as well as in vitro findings reported by others.12,28 These results, combined with the preserved vasodilating effect of the nonenzymatic NO donor sodium nitroprusside, indicate that the damaged mechanism is probably located somewhere along the pathway leading from acetylcholine signal transduction to NO and cGMP generation rather than on the guanylate cyclase itself. The absence of significant changes of the vasorelaxing response to the Ca2+ ionophore A23187 and the vasoconstricting response to PGE2, both suggest that, more precisely, the inhibitory effect of CyA may affect the receptor-mediated mechanism that transduces the signal of acetylcholine to the NO-forming system but not the capability of NO synthase to be directly activated by Ca2+-calmodulin. Even though no direct measurements of NO have been done in the present study, the indirect results are clear enough to support the above-mentioned conclusion. In this regard, little is known about the exact mechanisms involved in the triggering of NO formation by agents possessing specific receptors in the endothelial cells, namely acetylcholine, bradykinin, or ATP. The possibility that the treatment with CyA decreases the acetylcholine response by interfering with acetylcholine binding to its receptor is difficult to sustain in light of the intense response to acetylcholine observed in the same vessels in the presence of L-arginine (see following paragraph).

The effect of L-arginine in reversing the inhibition of the acetylcholine-mediated vasodilation in CyA-treated animals was also in accordance with our in vivo data14 and with preliminary results reported by Solagni et al.29 Balligand and Godfraind30 reported a vasorelaxing effect of L-arginine on rat aortic rings, which was independent of the presence or absence of CyA, therefore suggesting that the NO-forming system was well preserved. These authors postulate that, because of its lipophilic characteristics, CyA might disorder the cell membrane bilayer and dis regulate transducing mechanisms. However, their finding that not only the acetylcholine-mediated but also the ionophore-mediated response was altered suggests that, in their model, the CyA-induced alterations might involve an interaction between CyA and calmodulin. It is necessary to remark,
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however, that the experiments were done in conditions that differ substantially from those used in the present series of studies, i.e., after acute incubation with CyA of previously untreated vessels, ≈8 hours after the vessel extraction and without simultaneous challenging with L-arginine and acetylcholine; therefore, their conclusions cannot be directly extrapolated to the model reported in the present study.

Our results with L-arginine suggest that the CyA-induced defect involves a decreased efficiency of the acetylcholine-induced NO synthesis, which could be normalized in the presence of excess L-arginine. The fact that the vessels of CyA-treated animals had a significantly higher peak of cGMP formation in the combined presence of acetylcholine and L-arginine, as well as the preserved cGMP formation induced by sodium nitroprusside, illustrated that the soluble guanylate cyclase activity was not impaired.

The results obtained with L-arginine on the vasorelaxing response of the arteries of CyA-treated rats differed from those in control rats. The absence of improvement of the acetylcholine-induced vasorelaxation with L-arginine in control animals indicates that, in this type of preparation, the vasorelaxing response to acetylcholine does not need the supply of exogenous L-arginine to reach its normal magnitude; in other words, in normal vessels, L-arginine appears to add no additional vasorelaxing potency to acetylcholine. The alternative hypothesis of an interfering role of freeradical formation can be ruled out by the experiments with superoxide dismutase and BAEE.

The results on 45Ca2+ uptake provided information about an insufficiently known aspect of vascular regulation, i.e., the putative control of Ca2+ turnover by endothelium-mediated mechanisms. Meyer-Lehnert and Schrier et al. and Schierecht and Ruege31 raised the possibility of an increased Ca2+ uptake and Ca2+ release as a mechanism of CyA-induced increased contractility of vascular smooth muscle cells. In the present study, 45Ca2+ uptake experiments in whole arteries examined the possibility that differences in cellular Ca2+ were involved in the CyA effects. The results showed that pretreatment of the animals with CyA favors 45Ca2+ uptake by isolated vessels, by a mechanism not inhibited by verapamil. The possible relations of this finding with the presence of endothelial dysfunction were examined by deendothelialization or selective inhibition of the metabolism of L-arginine to NO. Both maneuvers favored an increased 45Ca2+ uptake by the control arterial segments. The absence of an additive effect of NwNLA and deendothelialization on the increased 45Ca2+ uptake in segments from CyA-treated rats gave further support to the hypothesis that a similar mechanism involving NO synthesis may be operative in all these three cases, namely, CyA and NwNLA treatment or deendothelialization. However, these findings cannot be automatically reconciled with the apparent absence of baseline inhibition of NO-dependent mechanisms in the vasorelaxation studies (see above paragraphs).

Taken as a whole, the present vasorelaxation and 45Ca2+ uptake experiments suggest that CyA damages the endothelium-dependent mechanisms necessary to increase agonist-mediated NO production and to block the progressive accumulation of Ca2+ without affecting the baseline status. No data are available, at the present time, to clarify the possible relations of these two affected functions of the endothelium, and in this regard, CyA toxicity may constitute a useful tool for exploring several specific properties of the vessel wall.

The above-mentioned results raised the possibility that an endothelium-mediated mechanism regulates the Ca2+ content of the vascular tissue. A principal role of cGMP, the final intracellular mediator of the NO-dependent mechanism, has been proposed in regulating Ca2+ turnover in vascular smooth muscle cells.32 The role of cGMP in the present conditions was substantiated by the experiments in the presence of sodium nitroprusside, which markedly increases intracellular cGMP levels. The fact that L-arginine also inhibited the increase of 45Ca2+ uptake in CyA-treated vessels in the absence of any detectable increase of cGMP content cannot be readily explained from the present data; however, the possibility exists that L-arginine alone may increase cGMP in a localized cellular domain that affects 45Ca2+ uptake but is below threshold for the detection of cGMP changes by radioimmunoassay. However, the possibility cannot be ruled out that L-arginine acted in this case by a mechanism different from the NO/cGMP generation, but this subject is out of the scope of the present study. In any case, our results with deendothelialized vessels clearly disclosed that the presence of endothelium was necessary for the observed effect of L-arginine on 45Ca2+ uptake in the arteries of CyA-treated animals.

In view of the present results, experiments are needed to assess the precise intracellular mechanisms by which CyA affects 45Ca2+ uptake and its possible role in decreased vasorelaxation. As a more general consequence, the role of NO synthase-dependent mechanisms in the regulation of vascular Ca2+ content in other pathological or physiological situations deserves further attention.

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