The effect of cyclosporine A to enhance vasoconstrictor-induced calcium (Ca2+) mobilization in vascular smooth muscle cells may contribute to important side effects in cyclosporine therapy such as hypertension and nephrotoxicity. On the other hand, atrial natriuretic peptide (ANP) is known to diminish vasoconstrictor-stimulated Ca2+ mobilization. The present study, therefore, examined the interaction of cyclosporine and ANP on Ca2+ kinetics in cultured rat vascular smooth muscle cells. Intracellular free calcium concentrations ([Ca2+]i) were measured using fura-2. 45Ca2+ was used to estimate Ca2+ efflux and cellular Ca2+ influx. Preincubation of the cells with cyclosporine (10 μg/ml) for 12 minutes lowered basal [Ca2+]i from 48±4 to 28±3 nM (p<0.01). However, in the presence of cyclosporine, the angiotensin II (10−8 M)–stimulated rise of [Ca2+]i was increased from 296±22 to 460±47 nM (p<0.001). ANP (5×10−9 M) blocked the Ca2+ mobilization by angiotensin II (71±7 versus 69±7 nM, NS) and also completely inhibited the effect of angiotensin II in the presence of cyclosporine (77±5 versus 78±5 nM, NS). Basal efflux as well as angiotensin II–stimulated 45Ca2+ efflux were not altered by preincubation with cyclosporine, indicating that the effect of cyclosporine on [Ca2+]i was not due to an inhibition of 45Ca2+ efflux. Cyclosporine increased cellular 45Ca2+ influx within 5 minutes (2,375±76 versus 3,268±183 cpm/mg protein per 5 minutes, p<0.001); this effect was diminished in the presence of 5×10−9 M ANP (3,268±183 versus 2,680±118 cpm/mg protein per 5 minutes, p<0.05). These results suggest that cyclosporine stimulates transmembrane Ca2+ influx, thereby increasing angiotensin II–sensitive intracellular Ca2+ stores and thus augmenting angiotensin II–induced Ca2+ mobilization in the vascular smooth muscle cells. This cellular effect of cyclosporine in vitro was markedly diminished by ANP. ANP may thus prove to be beneficial in attenuating potentially severe side effects of cyclosporine in vivo. (Hypertension 1993;21:166–172)

Key Words • cyclosporine • natriuretic peptides, atrial • calcium • muscle, smooth, vascular

Cyclosporine A (CyA), a fungal cyclic polypeptide, is an immunosuppressive drug that acts primarily on T-lymphocytes. This immunosuppression seems to be mediated by inhibition of production of T-lymphocyte–derived interleukin-2.1–3 CyA has little in vivo effect against other lymphocytes and is much less myelotoxic than other drugs.4 The clinical use of CyA has provided new perspectives in organ transplantation and the treatment of autoimmune diseases.

The extensive use of CyA, however, has been accompanied by potentially severe side effects. Nephrotoxicity is the most common adverse effect,5,6 and arterial hypertension has also been reported frequently.6–8 Functional hemodynamic alterations seem to play a major role in CyA nephrotoxicity,9 and increased vascular resistance has been reported in heart transplant patients with CyA–associated hypertension.10 The implication that these side effects may reflect an increase of contractility of vascular smooth muscle cells is supported by observations made in vitro in cultured cells. The normal transitory increase in intracellular calcium, after stimulation with angiotensin II (Ang II) or arginine vasopressin,11 that activates the contractile mechanism in vascular smooth muscle cells12,13 was found to be enhanced in the presence of CyA.14,15

Atrial natriuretic peptide (ANP) is known to be a potent vasodilator.16,17 An important component of this effect is the interaction of ANP with vasopressor substances on the cellular level of vascular smooth muscle cells. It has been shown that ANP decreases the Ca2+ mobilization induced by Ang II or vasopressin.18–20 Because of these contrary effects of CyA and ANP it is conceivable that the vasoconstrictor effects of CyA may be antagonized by ANP on the cellular level. Antagonistic effects of ANP on CyA–associated side effects may prove to be of potential therapeutical relevance in vivo.

Therefore, the present study was designed to investigate the potential interaction of these substances on the vasopressor-induced Ca2+ mobilization in vitro. We examined the effects of Ang II, CyA, and ANP on intracellular free calcium concentrations ([Ca2+]i) and transmembrane Ca2+ fluxes in vascular smooth muscle cells in culture.

Methods

CyA dissolved in cremophore was obtained from Sandoz, Basel, Switzerland. The vehicle cremophore...
EL, Ang II, TMB8, fura-2-acetoxy methylester (fura 2-AM), and fura-2-free acid were purchased from Sigma Chemical Co., St. Louis, Mo. 4Ca2+ was purchased from Amersham, Braunschweig, Germany. ANP [ANP-(1-28)] was obtained from Peninsula, Maidenhead, England. The rats were purchased from the Charles River Wiga GmbH, Sulzfeld, Germany.

Preparation of Aortic Smooth Muscle Cells and Cell Culture

Rat aortic smooth muscle cells were isolated by using a modified method originally described by Chamley et al.21 Male Sprague-Dawley rats were decapitated under ether anesthesia. Then the thoracic aortas were rapidly dissected out under sterile conditions. The aortas of four rats were pooled each time. The surrounding adipose and connective tissues were then eliminated, and the aortas were incubated at 37°C for 30 minutes in Eagle’s minimum essential medium (MEM) that contained collagenase (385 units/ml), elastase (90 units/ml), and trypsin-inhibitor (inhibits 0.85 mg trypsin per milliliter). Then the adventitia and small fragments of outer medium to be stripped off with forceps under sterile conditions. Afterward, the aortas were minced with sterile scalpels and incubated a continuous stirring. After this incubation, the resulting single-cell suspension was centrifuged for 5 minutes at 1,000 rpm. The cell pellet was resuspended in fresh MEM. This procedure was repeated twice. The cells were plated onto 35-mm culture dishes and grown at 37°C in a humidified atmosphere of 95% air–5% CO2.

Confluent cultures were passaged by incubation with 1 ml phosphate-buffered saline (PBS) (mM: NaCl 137, KCl 2.7, Na2HPO4 8.1, KH2PO4 1.5, and pH 7.4) that contained 0.05% trypsin and 0.02% EDTA for 10 minutes at 37°C. The passages were performed when the cells had reached confluence. Hormone-stimulated Ca2+ kinetics were conducted with cells between the third and tenth passage. For each set of experiments the number of measurements equals the number of cell preparations. A total of 20 different cell preparations were used in the present study.

Measurements of Intracellular Free Ca2+

For measurements of [Ca2+]i, using a modified method described by Hassid et al,22 cells were grown on round coverslips in culture medium. The coverslips were washed twice with MEM without fetal bovine serum. Cells were then incubated for 60 minutes at 37°C with 1 ml serum-free MEM that contained 4 μM fura-2-AM. At the end of the loading period, the coverslips were washed twice with physiological saline solution (PSS) (mM: NaCl 140, KCl 4.6, MgCl2 1, CaCl2 2, glucose 10, HEPES 10, pH 7.4). The coverslips were then inserted into cuvettes and preincubated with various effectors for 10 minutes at 37°C in PSS. Then the cuvettes were placed in the thermostable holder of a Hitachi 4000F fluorescence spectrophotometer that faced both the oncoming beam and the detector at an angle of 45°. Fluorescence of the fura 2-loaded monolayers was measured with the excitation wavelengths of 340 nm and 380 nm that changed every 4 seconds and an emission wavelength of 500 nm. In Ca2+-saturated medium, fura-2 reaches the maximal fluorescence at an excitation wavelength of 340 nm and in Ca2+-free medium at 380 nm.22 Autofluorescence was measured in cells of the same passage that had not been loaded with fura-2. [Ca2+]i was calculated as described by Grynkiewicz et al22 using the following equation:

\[
[Ca^{2+}]_i = K_d \cdot \frac{(R - R_{min})}{(R_{max} - R)} \cdot \frac{S_{f2}}{S_{b2}}
\]

A value of 224 nM was used as the dissociation constant (Kd) of the fura 2–Ca2+ complex, as measured by Grynkiewicz et al.22 R is the experimentally determined fluorescence emission ratio of the cytoplasmic dye within the cells at the two excitation wavelengths of 340 nm and 380 nm. Rmax is the maximal ratio measured in the presence of saturating Ca2+ (2 mM), whereas Rmin is the ratio given by Ca2+-free dye. Sf2/Sb2 is the ratio of fura-2 fluorescence values at 380 nm in Ca2+-free and Ca2+–replete solution.

As described by Hassid et al,22 we used medium simulating the intracellular milieu to measure Rmin (mM): KCl 95, NaCl 20, K-2-(N-morpholino)propane sulfonic acid (MOPS) 10, MgSO4 2, K2H2EGTA 10. For the measurement of Rmax, we substituted 10 mM CaK2EGTA plus 2 mM CaCl2 for K2H2EGTA. As discussed by Tsien et al,24 the actual values of the calibrating parameters depend on the microviscosity of the medium. Therefore the aforementioned media were supplemented with 50% glycerol to give an estimated microviscosity equal to that found in the cytosol of cultured mesangial cells.22 Rmax, Rmin, and Sf2/Sb2 values were found to be 13.82, 1.35, and 6.24, respectively. Neither CyA nor ANP affected the fluorescence of fura-2 at the concentrations we used.

4Ca2+ Efflux

The culture medium was removed by aspiration, and cell monolayers were rinsed twice with PSS and loaded with 8 μCi of 4Ca2+ in 1 ml PSS at 37°C for 3 hours. Then the cultures were rapidly rinsed 10 times (30 seconds total) with 1 ml PSS, and another 1 ml PSS was added. The medium was removed and replaced with 1 ml PSS at 1, 2, 3, and 4 minutes, and at 30-second intervals thereafter. Ang II was added as indicated during the time course of 4Ca2+ efflux. Cells were preincubated with CyA, cromephore, or ANP for 10 minutes before the start of the experiment. The 4Ca2+ extruded from the cells in each time interval was measured by liquid scintillation counting. Cells were collected from the culture dish using sodium dodecyl sulfate (SDS) that contained alkaline solution (0.1% SDS, 2% Na2CO3, 0.1 N NaOH). Protein was determined by the method of Lowry et al.25

4Ca2+ Influx

The culture medium was removed by aspiration, and the cells were washed twice with PSS. Then the cells were incubated for 5 minutes at 30°C with 2 μCi of 4Ca2+ in the presence or absence of any effector in 1 ml PSS. To terminate influx, external 4Ca2+ was removed by rinsing the dish eight times with PSS at 4°C.
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**Figure 1.** Bar graph shows effect of pretreatment with cyclosporine A (CyA) or cremophore (cremo) on basal and angiotensin II (All)–induced increase in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in vascular smooth muscle cells. Fura-2–loaded cells were preincubated with CyA or an equivalent volume of cremophore for 12 minutes at 37°C.

Intracellular Free Ca\(^{2+}\)

Changes in [Ca\(^{2+}\)]\(_i\) in smooth muscle cells in response to a vasoconstrictor hormone are of particular interest since the amount of free cytosolic Ca\(^{2+}\) is an important determinant of the force of contraction. Within seconds, 10\(^{-8}\) M Ang II caused an increase of [Ca\(^{2+}\)]\(_i\) from basal levels of 48±4 nM (n=73) to 296±22 nM (n=38) (p<0.001). [Ca\(^{2+}\)]\(_i\) returned to a lower level still significantly above baseline within approximately 4 minutes.

After preincubation with CyA (10 μg/ml) for 12 minutes at 37°C, basal [Ca\(^{2+}\)]\(_i\) declined to 29±3 nM (p<0.01 versus control). Cremophore (130 μg/ml) in an equivalent volume did not affect basal [Ca\(^{2+}\)]\(_i\) (Figure 1). Ang II–stimulated [Ca\(^{2+}\)]\(_i\), rose from 296±22 to 460±47 nM after preincubation with CyA (p<0.001). Cremophore alone had no significant effect on the Ang II–stimulated increase of [Ca\(^{2+}\)]\(_i\) (Figure 1).

To examine the role of intracellular Ca\(^{2+}\) release for the mechanism of action of CyA, [Ca\(^{2+}\)]\(_i\) was measured in the presence of TMB8, an inhibitor of sarcoplasmic Ca\(^{2+}\) release.\(^{26}\) Under these conditions, Ang II–stimulated Ca\(^{2+}\) mobilization did not differ between CyA-treated and control cells (Figure 2). This indicates that enhanced Ca\(^{2+}\) release from intracellular stores may contribute to the potentiating effect of CyA on the Ca\(^{2+}\) mobilization by Ang II.

Preincubation with ANP for 5 minutes decreased the Ca\(^{2+}\) mobilization by Ang II (10\(^{-8}\) M) in a dose-dependent manner (Figure 3). ANP (5×10\(^{-9}\) M) increased basal [Ca\(^{2+}\)]\(_i\) from 48±4 to 71±7 nM after preincubation for 12 minutes (p<0.05). The effect of

**Figure 2.** Bar graph shows effect of TMB8 on the potentiating effect of cyclosporine A (CyA) on angiotensin II (All)–induced increase in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in vascular smooth muscle cells. Fura-2–loaded cells were preincubated with CyA plus TMB8 for 12 minutes at 37°C.

**Figure 3.** Bar graph shows dose response for atrial natriuretic peptide (ANP)–induced inhibition on angiotensin II (All)–induced (10\(^{-8}\) M) increase in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in vascular smooth muscle cells.
the subsequent stimulation by 10^{-8} M Ang II was completely blocked (69±7 nM) (Figure 4). This complete inhibition of the effect of Ang II was not affected by preincubation with CyA (77±5 nM versus 78±5 nM, NS) (Figure 4). At a lower concentration of 2x10^{-9} M ANP neither basal [Ca^{2+}]_i nor Ang II-stimulated Ca^{2+} mobilization were altered (Figure 5). However, with 2.10^{-9} M ANP the augmentory effect of CyA on Ang II-induced increase in [Ca^{2+}]_i was significantly reduced from 460±47 to 270±27 nM (p<0.05) (Figure 5).

45Ca^{2+} Efflux
To examine whether extrusion of Ca^{2+} from the cell may be attenuated by CyA, thus contributing to the enhancement of Ang II-stimulated [Ca^{2+}]_i, we measured 45Ca^{2+} efflux from the cells by measuring 45Ca^{2+} efflux. The spontaneous efflux rate decreased rapidly during the first few minutes, and the further decline was considerably slower. The addition of CyA or ANP did not affect the pattern of spontaneous efflux (data not shown).

Ang II increased 45Ca^{2+} efflux in a dose-dependent manner. Within 4 minutes, efflux returned to prestimulated levels (Figure 6). Preincubation with CyA for 10 minutes at 37°C did not alter the maximal Ang II (10^{-9} M)-stimulated efflux at 90 seconds (2,312±768 versus 2,568±586 cpm/mg protein per 30 seconds, NS) (Figure 7). Neither did CyA affect the efflux rates after addition of lower concentrations of Ang II (10^{-9} M, 10^{-10} M) (data not shown). Preincubation with ANP (5x10^{-9} M) blocked the effect of Ang II in the absence (2,312±343 versus 658±155 cpm/mg protein per 30 seconds, p<0.01) as well as in the presence of CyA (2,568±586 versus 765±168 cpm/mg protein per 30 seconds, p<0.01) (Figure 7).

These results of the efflux studies argued against an effect of CyA to inhibit Ca^{2+} extrusion from the cells, a mechanism that might have contributed to enhanced [Ca^{2+}]_i levels.

45Ca^{2+} Influx
The results of the 45Ca^{2+} efflux studies and the measurements of [Ca^{2+}]_i indicated that CyA may en-
Figure 7. Line graph shows effect of cyclosporine A (CyA) and atrial natriuretic peptide (ANP) (5 x 10^{-9} M) on the angiotensin II (Ang II)-induced 45Ca^{2+} efflux. Cells were preincubated at 37°C with CyA, ANP, or both for 10 minutes before the start of the experiment. Incubation medium contained CyA and ANP throughout the entire experiment.

In patients treated with CyA, hypertension is a severe side effect. CyA-induced hypertension appears to be related to an increase in peripheral arterial and venous vascular resistance. In heart transplant patients, this side effect was found in the absence of increase in circulating concentrations of vasoconstrictors such as norepinephrine and Ang II. These findings are compatible with the effect of CyA enhancing the vasoconstrictor response to such endogenous pressor agents rather than increasing their release.

In contrast to CyA, ANP causes a vasodilation that seems to be most readily demonstrable in the presence of vasoconstrictors. Also in kidneys, CyA and ANP have opposite effects on vascular resistance. In micropuncture studies in rats, Barros et al. showed CyA to increase the afferent and efferent glomerular vascular resistance and to decrease the glomerular filtration rate (GFR). On the other hand, ANP dilates the afferent arterioles and increases the GFR.

After preincubation with CyA for 12 minutes, we found a significant decrease in basal [Ca^{2+}]. At present there appears to be no ready explanation for this finding. It would be tempting to speculate that a decrease in basal [Ca^{2+}], might reflect increased intracellular binding of Ca^{2+} to proteins such as calmodulin. This could contribute to enhanced contractility in the presence of CyA. Effects of CyA on Ca^{2+} transport across intracellular membranes might also be involved.
In studies using quin2 for measurement of \([Ca^{2+}]_j\), we\textsuperscript{14} as well as other authors\textsuperscript{15} did not find ANP to affect basal \([Ca^{2+}]_j\). This difference may be due to the higher sensitivity of fura-2 compared with quin2.\textsuperscript{2,5} In accordance with other studies, CyA did not alter basal \([Ca^{2+}]_j\) efflux.\textsuperscript{14,15}

Ang II is known to induce a rapid rise in \([Ca^{2+}]_j\), mainly by mobilization of \([Ca^{2+}]_j\) from the sarcoplasmic reticulum. As has been shown previously, in the present study we could demonstrate that in the presence of CyA, the Ang II-stimulated rise of \([Ca^{2+}]_j\) was enhanced. These findings indicate that CyA may enhance the \([Ca^{2+}]_j\) content in intracellular stores such as the sarcoplasmic reticulum. This is supported by our finding that in the presence of TMB8, an intracellular \([Ca^{2+}]_j\) antagonist, the activity of CyA to enhance Ang II-induced \([Ca^{2+}]_j\) mobilization was attenuated. Zhao et al\textsuperscript{20} reported that cremophore, the vehicle of CyA, modulates the activity of protein kinase C, an enzyme affecting \([Ca^{2+}]_j\) mobilization. Therefore studies were performed with cremophore in the presence and absence of Ang II. However, cremophore neither affected \([Ca^{2+}]_j\) nor \([45Ca]_i\) transmembrane fluxes. Preincubation with ANP (5 x 10\(^{-9}\) M) for 12 minutes increased basal \([Ca^{2+}]_j\). Again this effect on basal \([Ca^{2+}]_j\), was not demonstrated in studies using quin2.\textsuperscript{14,19} The Ang II stimulation of \([Ca^{2+}]_j\), was inhibited by preincubation with ANP in a dose-dependent manner. Simultaneous preincubation with ANP (5 x 10\(^{-9}\) M) and CyA blocked the \([Ca^{2+}]_j\) mobilization by Ang II as well as the potentiating effect of CyA on Ang II-induced rise in \([Ca^{2+}]_j\). ANP at a concentration of 2 x 10\(^{-8}\) M did not affect Ang II-stimulated \([Ca^{2+}]_j\) mobilization but blocked the potentiating effect of CyA. Therefore, ANP in low concentrations may attenuate CyA-associated side effects, but the normal vascular response may be maintained.

Compatible with our in vitro study is the study of Capasso et al,\textsuperscript{37} who examined the effect of ANP on CyA-treated rats. After infusion of CyA for 7 days, GFR decreased significantly. This effect was completely reversed by infusion of ANP. Other authors\textsuperscript{38} have also demonstrated a beneficial effect of ANP on CyA nephrotoxicity in rats. These findings also stress the point that CyA-associated side effects are at least in part functional and reversible for a certain period of time (for review, see Reference 39).

To investigate the mechanism of CyA to increase the Ang II-stimulated \([Ca^{2+}]_j\) mobilization, the effect of CyA on \([45Ca]_i\) transmembrane fluxes was examined. The studies of \([45Ca]_i\) efflux did not disclose an inhibiting effect of CyA on \([Ca^{2+}]_j\) extrusion mechanisms that might have contributed to enhanced \([Ca^{2+}]_j\) levels. We found that \([45Ca]_i\) influx, which can be considered to be unidirectional under the present experimental conditions,\textsuperscript{40} was increased by CyA, thus confirming studies of our own\textsuperscript{2} as well as of other authors.\textsuperscript{15} Our findings are consistent with those of Haller et al\textsuperscript{41} who recently described that CyA induced changes of protein kinase C activity in vascular smooth muscle cells. Phosphorylation of proteins by protein kinase C and a concomitant cellular \([Ca^{2+}]_j\) influx are known to be associated with the sustained phase of vascular smooth muscle cell contraction and transmembrane fluxes.\textsuperscript{42-44}

The experiments with TMB8 already indicated that in the presence of CyA, the amount of \([Ca^{2+}]_j\) releasable from intracellular stores may be enhanced. This interpretation gains further support by the finding that CyA stimulates unidirectional \([Ca^{2+}]_j\) influx. Thereby, it may enhance the content of intracellular \([Ca^{2+}]_j\) stores.\textsuperscript{45} Consequently, increased amounts of \([Ca^{2+}]_j\) may be available for mobilization by Ang II. Stimulation of \([Ca^{2+}]_j\) influx appears to be the primary cellular effect of CyA. The data of the present study showed ANP to inhibit the CyA-induced \([Ca^{2+}]_j\) influx. This effect of ANP may cause the inhibition of the augmentary effect of CyA on Ang II-induced increase of \([Ca^{2+}]_j\). Interpreting the present results with regard to the in vivo conditions must be made with caution. It is of note, however, that Ang II and ANP concentrations in the nanomolar range and CyA in a concentration of 10 \(\mu g/ml\) are frequently used in cell culture systems.\textsuperscript{14,19,45}

This concentration of CyA is approximately 100 times higher than in vivo. In spite of the acknowledged limitations, such studies of cell cultures may provide insights into basic pathophysiological mechanisms underlying the adverse effects of CyA and its interaction with ANP. As mentioned above, there is experimental evidence that the proposed cellular mechanisms may indeed be active in vivo.

Although the present in vitro study focused on the potential effects of CyA on the \([Ca^{2+}]_j\) mobilization in vascular smooth muscle cells, data from other studies have suggested further mechanisms such as the prostaglandin system,\textsuperscript{46,47} endothelin,\textsuperscript{48,49} and sympathetic nerve activity\textsuperscript{50} to be implicated in CyA-induced side effects.

In conclusion, data from the present study suggest that direct cellular effects of CyA in vascular smooth muscle cells may play a pivotal role in the pathogenesis of CyA-induced side effects such as arterial hypertension. CyA was shown to induce transmembrane \([Ca^{2+}]_j\) uptake, thus leading to an increase in Ang II-sensitive intracellular \([Ca^{2+}]_j\) stores. ANP inhibits this primary cellular event and consequently the augmentary effect of CyA on Ang II-induced \([Ca^{2+}]_j\) mobilization. The present findings may be of significant clinical relevance since ANP may represent a potential therapeutic tool in the treatment or prophylaxis of CyA-associated side effects. Further investigations will no doubt be necessary to examine this potential therapeutic value of ANP in patients treated with CyA.

Acknowledgment

We thank Karin Kappes-Horn for her excellent technical assistance.

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Atrial natriuretic peptide blunts the cellular effects of cyclosporine in smooth muscle.
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Hypertension. 1993;21:166-172
doi: 10.1161/01.HYP.21.2.166

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X, Online ISSN: 1524-4563

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