

Calcium blockers inhibit cyclosporine A-induced hyperreactivity of vascular smooth muscle cells

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Abstract. Cyclosporine belongs to the group of the most commonly used immunosuppressants. Hypertension occurs in approximately 30% of patients treated with this drug. However, the pathogenesis of this occurrence has not been explained to date. The purpose of our study was to clarify the mechanisms leading to the evolution of hypertension induced by cyclosporine A (CsA). We examined the changes in transmission within receptors and around the receptors. We also aimed to elucidate the mechanisms responsible for averting arterial hyperresponsiveness induced by the drug. Experiments were performed on isolated and perfused tail arteries of Wistar rats. Tissues surrounding the artery were removed and the proximal segment (length of 2-3 cm) was used for cannulation. Cannulated arteries were placed in a 20-ml glass chamber (vertical position). The contraction force in our model was measured by an increased degree of perfusion pressure with a constant flow rate (approximately 1 ml/min). The results showed that in the presence of CsA, the concentration-response curves/phenylephrine (PHE) curve shifted to the left. Cyclosporine increased the reactivity of the arteries to PHE. This effect was directly linked to the

increase in the receptor reserve. The analysis of the reactivity of vascular smooth muscle showed that CsA increased the influx of calcium ions from the extracellular to the intracellular area. No difference was found between the contraction triggered by Bay-K8644 in the presence of CsA and the control probe. The increase in perfusion pressure induced by CsA was blocked by L-type calcium channel blockers (nifedipine and diltiazem). The results from our experiments show that CsA increases the reactivity of vessels to the effect of catecholamines. CsA also enhances signal transmission between G-protein coupled receptors (GPCRs) and calcium channels. The activation of protein kinase C also plays a significant role in this process. Our results suggest that the best choice for the pharmacotherapy of hypertension induced by CsA would be calcium channel antagonists.

Introduction

Cyclosporine A (CsA) is the most commonly used drug in immunosuppressive therapy after kidneys, liver, heart or bone marrow transplantation (1). The first polypeptide was isolated from the fungus *Tolypocladium inflatum*. CsA was introduced into clinical practice as a new generation of immunosuppressants in the 1980's. It is widely used to prevent the rejection of transplanted organs. However, the drug also has toxic effects. The spectrum of the usage of CsA is not limited only to transplantology; it has also been used to treat patients with uveitis - the first effective therapy was documented in 1979 (2,3). CsA has also been used in other cases where conventional treatment has not been effective (4,5).

CsA inhibits the activity of lymphocytes, especially Th and Tc, decreasing the production of pro-inflammatory interleukin (IL)-2. This effect is based on the binding of CsA to the cytosolic protein, cyclophilin of lymphocytes. The complex form of CsA and cyclophilin binds to the calcium ions, calmodulin and calcineurin (CN). The inhibition of CN phosphatase activity, which leads to the dephosphorylation of the nuclear factor of activated T lymphocytes (NF-AT), prevents its activation. In this case, the promoter for IL-2 is blocked (6,7). The application of CsA is associated with many side-effects, such as hypertension, renal failure, hepatotoxicity, neurological disorders or cancers, which cannot be eliminated

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Abbreviations: AVP, arginine vasopressin; CRCs, concentration response curves; CsA, cyclosporine A; DAG, diacylglycerole; ER, endoplasmic reticulum; GPCRs, G-protein coupled receptors; IP₃, inositol 1,4,5-trisphosphate; IP₃R, inositol 1,4,5-trisphosphate receptor; NF-AT, nuclear factor of activated T lymphocytes; PHE, phenylephrine; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; ROC, receptor-operated channel; RyR, ryanodine receptor; SMOG, second messenger-operated channel; VOC, voltage-operated channel; VSMCs, vascular smooth muscle cells

Key words: cyclosporine A, vascular smooth muscle cells, receptors reserve, calcium signals, protein kinase C, BayK-8644

to date (8). One of the main reasons for restricting the usage of CsA in certain situations is nephrotoxicity and hypertension in approximately 30% of patients. However, the mechanism of this action remains unknown (9).

Hypertension caused by immunosuppressants is one of the main risk factors of cardiovascular diseases. High blood pressure persisting for long periods of time may lead to organ complications, atherogenesis, renal failure, heart failure or stroke. Decisions regarding the treatment of hypertension should be based on clinical investigations that include classification of hypertension, presence of organ complication, additional risk factors (intercurrent diseases) and age (9). Due to the similar effectiveness of drugs from five basic groups, the European Society of Hypertension, European Society of Cardiology and Polish Society of Hypertension do not recommend a specific class of hypertension drugs. The effectiveness of treatment depends on the choice of appropriate therapy (mono- or polytherapy), drug dose, interactions between drugs and the tolerance of drug by the patient (10,11).

Role of calcium ions in vascular smooth muscle tone. The contraction of vascular smooth muscle is upregulated by membrane G-protein coupled receptors (GPCRs), which increase phospholipase C (PLC) activity. Vascular receptors which demonstrate pressor effect are as follows: α_1 , V_1 , ET_A and AT_1 . The complex agonist/receptor leads to G_q protein activation (first order relay). Through the subunit of G_q protein, PLC is activated. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerole (DAG) (12,13).

The activation of protein kinase C (PKC) is significant in off-receptor signal regulation induced by DAG. There are many isolated isoenzymes of PKC which demonstrate tissue specificity. PKC enhances the contraction of vascular smooth muscle by L-type calcium channel phosphorylation. The importance of PKC activation is proven by the fact that phorbol esters, a group of synthetic PKC activators, induce the contraction of vascular smooth muscle (14-18).

Calcium ion homeostasis is upregulated by their passive transport to the intracellular space of the myocyte and through active transport to the endoplasmic reticulum (ER) and extracellular area in cooperation with ion exchangers.

Ca^{2+} ion concentration inside the myocyte is $\sim 10^{-7}$ M, which contrasts with their high concentration in extracellular fluid (2×10^{-3} M) (20). The influx of calcium ions from the extracellular area goes by channels activated via second messenger-operated channel (SMOC), receptor-operated channel (ROC), voltage-operated channel (VOC) (types L, N and P), Q, R, T and channels dependent on ER (20,21). IP_3 receptors (IP_3 Rs) activate the influx of Ca^{2+} ions induced by the increase in the concentration of IP_3 . The second channel in the ER area is the ryanodine receptor (RyR). At concentrations from 10^{-9} to 10^{-6} M/l, ryanodine shows agonistic actions, but at concentrations higher than 10^{-5} M/l, it blocks the receptors (22,23).

Calcium signal modulation depends on proteins which function as the intracellular detectors for these ions. Calmodulin, activated by calcium ions, belongs to this group of proteins. Complex Ca^{2+} /calmodulin regulates enzyme activity, such as phospholipases or kinases (19,24-26).

The aim of the study was to investigate whether calcium blockers inhibit CsA-induced hyperactivity of vascular smooth muscle cells (VSMCs).

Materials and methods

Animals. Experiments were performed on isolated, perfused Wistar rat tail arteries. Animals were housed under a 12/12-h light/dark cycle and had unlimited access to food and water. Male rats weighing 250-270 g were narcotized by intraperitoneal injection of 120 mg urethane per 1 kg of body mass. Rats were sacrificed by stunning and cervical dislocation. CsA was applied intraperitoneally. The study protocol was approved by the local ethics committee. All experiments were carried out in accordance with the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication No. (NIH) 8523: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, USA].

Drugs and solutions. CsA (1 and 10 nM, or accordingly, 121.6 and 1,216 ng/ml) dissolved in ethanol solution (70%). Krebs solution contained NaCl (71.8 mM/l), KCl (4.7 mM/l), $CaCl_2$ (1.7 mM/l), $NaHCO_3$ (28.4 mM/l), $MgSO_4$ (2.4 mM/l), KH_2PO_4 (1.2 mM/l) and glucose (11.1 mM/l). All reagents were purchased from the Sigma-Aldrich Chemical Company (Poznan, Poland).

Study design and performance. After dissection from surrounding tissues, a 2-3 cm long segment of rat tail artery was cannulated and connected to a perfusion device. The distal part was weighed with a 500-mg weight and the tail was placed in a 20-ml container filled with oxygenated Krebs solution at 37°C. The perfusion pressure was continuously measured. We gradually increased the perfusion solution flow using a peristaltic pump up to 0.25-1 ml/min. Vessel contractions induced by phenylephrine (PHE) [an adrenergic α_1 receptor, arginine vasopressin (AVP)] and vasopressin (non-selective agonist α_1 receptor, V_1 , V_2) were measured as an increase in perfusion pressure.

Data analysis and statistical procedures. Perfusion pressure was measured on Nyketron 1675 and TZ-21S devices. Concentration response curves (CRCs) were calculated according to the method described by van Rossum (27,28). Maximal response of tissue (E_{max}) was calculated as a percentage of maximal response of PHE or AVP administered in KCl solution. Half maximal effective concentration (EC_{50}) was estimated using classical pharmacological methods with pD_2 , the negative logarithm of the EC_{50} . The number of the CRC and E_{max} was used in all calculations estimating the statistical significance.

Results

PHE, agonist of the α_1 adrenergic receptor, increased the perfusion pressure, depending on the concentration. The mean perfusate pressure for PHE (10^{-5} M/l) was 92 (± 6.2) mmHg in perfusate with Ca^{2+} and 60 (± 4.3) mmHg in perfusate without Ca^{2+} . CRCs had sinusoidal shape (Fig. 1). CRCs for PHE were compared in the absence and presence of CsA (1 nM/l). In the

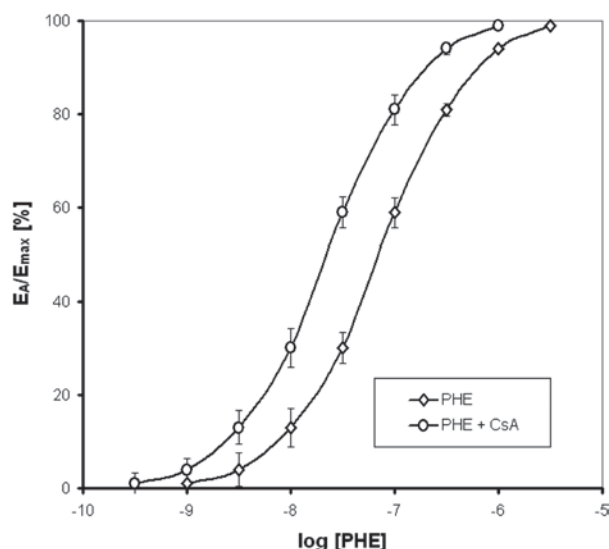


Figure 1. Concentration response curves for phenylephrine (PHE) prior to and after incubation in the presence of cyclosporine-A (CsA) (1 nM/l; n=12, $p<0.05$).

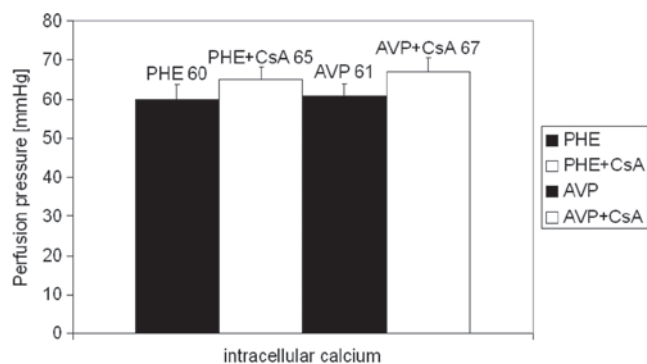


Figure 2. Changes in intracellular calcium levels in the absence or presence of cyclosporine A (CsA) (1 nM/l; n=16 phenylephrine (PHE) vs. PHE + CsA, $p=ns$; arginine vasopressin (AVP) vs. AVP + CsA, $p=ns$).

presence of CsA, curves were shifted to the left. A similar shift of CRC and E/RT curves in the presence of CsA was shown in our earlier investigation (stimulated by AVP).

Mobilization of Ca^{2+} ions from ER of arteries in the absence or presence of CsA (1 nM/l). In the performed experiment, the contraction of arteries was triggered by PHE (10^{-5} M/l) and AVP (10^{-5} M/l). Values of pressure changes were presented as the means for $n=16 \pm SD$. The experiment was carried out in a fluid without Ca^{2+} . The mean value of perfusion pressure, induced by PHE and AVP was $60 (\pm 4.3)$ mmHg and $61 (\pm 4.0)$ mmHg, respectively. During the incubation in the presence of CsA (1 nM/l), no significant changes in contraction effect were found for PHE and AVP. Perfusion pressure induced by the action of the drug in both cases was not significantly different compared to the control. Perfusion pressure, after incubation in the presence of CsA, was $65 (\pm 3.9)$ mmHg for PHE and $67 (\pm 3.9)$ mmHg for AVP. Reactivity of the arteries during the incubation in the presence of CsA was not changed. The absence of significant differences in the perfusion pressure

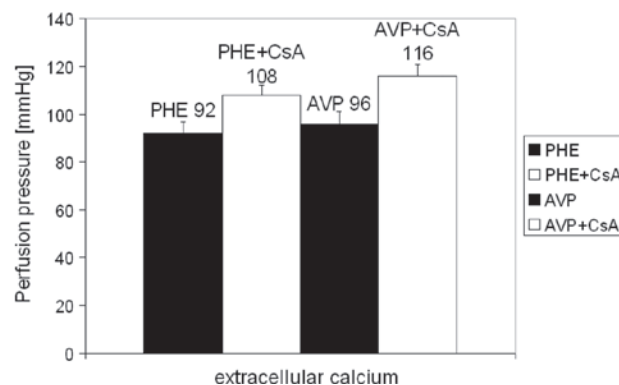


Figure 3. Changes in extracellular calcium levels in the absence or presence of cyclosporine A (CsA) (1 nM/l; n=16, phenylephrine (PHE) vs. PHE + CsA, $p=0.0001$; arginine vasopressin (AVP) vs. AVP + CsA, $p<0.0001$).

proves that CsA does not change the mobilization of intracellular fraction of Ca^{2+} ions (Fig. 2).

Mobilization of Ca^{2+} ions from extracellular fraction in the absence or presence of CsA (1 nM/l). In the performed experiment, the contraction of arteries was triggered by PHE (10^{-5} M/l) and AVP (10^{-5} M/l). Values of pressure changes were presented as the means for $n=16 \pm SD$. The experiment was carried out in Ca^{2+} fluid. The mean value of perfusion pressure induced by PHE and AVP was $92 (\pm 6.2)$ mmHg and $96 (\pm 5.2)$ mmHg, respectively. During the incubation in the presence of CsA (1 nM/l), an increase in the contraction effect of PHE was observed. Perfusion pressure induced by the action of the drug increased to $108 (\pm 4.2)$ mmHg and was different compared to the control ($p<0.0001$). Increased reactivity of arteries to the pressor action of AVP and PHE demonstrates the mobilization of Ca^{2+} ions from the extracellular fraction (Fig. 3).

Changes in perfusion pressure induced by PHE before and after application of CsA (1 nM/l) in the presence of nifedipine, diltiazem and losartan. In the performed experiment, the contraction of arteries was triggered by PHE (10^{-5} M/l). The control values for PHE and PHE and CsA (1 nM/l) were $92 (\pm 6.2)$ mmHg and $108 (\pm 4.2)$ mmHg, respectively. The derivative of 1,4-dihydropyridine, nifedipine (3×10^{-5}), successfully decreased the perfusion pressure to $87 (\pm 5.3)$ mmHg during incubation in the presence of CsA ($p<0.0001$). A similar result was found for the benzothiazepine derivative, diltiazem. The mean pressure value after incubation in the presence of CsA was $93 (\pm 5.3)$ mmHg and was 15 mmHg lower than the control ($p<0.0001$). Losartan, an angiotensin receptor antagonist, did not reduce artery reactivity in the presence of CsA. The mean pressure value for losartan was $106 (\pm 4.3)$ mmHg and was 2 mmHg lower than the control ($p=0.2614$) (Fig. 4).

Changes in perfusion pressure induced by AVP before and after application of CsA (1 nM/l) in the presence of nifedipine, diltiazem and losartan. In the performed experiment, the contraction of arteries was triggered by AVP (10^{-5} M/l). The control values for AVP alone and AVP plus CsA (1 nM/l) were $96 (\pm 5.2)$ mmHg and $116 (\pm 4.8)$ mmHg, respectively. The derivative of 1,4-dihydropyridine, nifedipine, successfully decreased the perfusion pressure to $91 (\pm 4.5)$ mmHg during

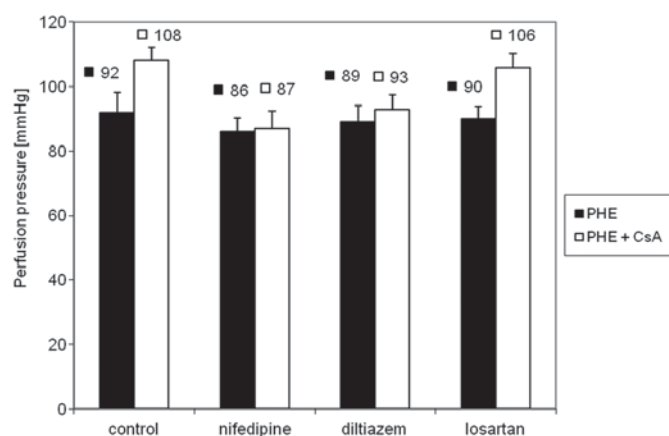


Figure 4. Changes in perfusion pressure induced by phenylephrine (PHE) in the absence or presence of cyclosporine A (1 nM/l) for nifedipine, diltiazem and losartan (n=16). Control vs. nifedipine, $p<0.0001$; control vs. diltiazem, $p<0.0001$; control vs. losartan, $p=0.2614$.

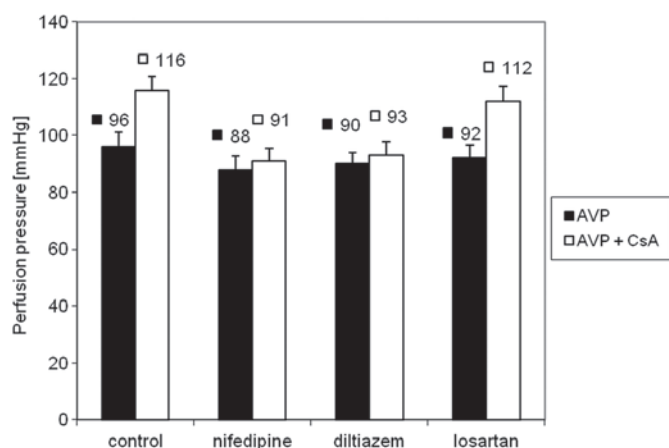


Figure 5. Changes in perfusion pressure induced by arginine vasopressin (AVP) in the absence or presence of cyclosporine A (1 nM/l) for nifedipine, diltiazem and losartan (n=16). Control vs. nifedipine, $p<0.0001$; control vs. diltiazem, $p<0.0001$; control vs. losartan, $p=0.1245$.

incubation in the presence of CsA ($p<0.0001$). A similar result was found for the benzothiazepine derivative, diltiazem. The mean pressure value after incubation in the presence of CsA was $93 (\pm 5)$ mmHg and was 23 mmHg lower than the control ($p<0.0001$). Losartan did not reduce artery reactivity in the presence of CsA. The mean pressure value for losartan was $112 (\pm 5.4)$ mmHg and was 4 mmHg lower than the control ($p=0.1245$) (Fig. 5).

Effect of CsA on vasoconstriction induced by BayK-8644. In the performed experiment, vasoconstriction was triggered by the calcium channels agonist, BayK-8644. Arteries were incubated in the presence of CsA (1 nM/l).

To determine how CsA induces hyperreactivity of arteries, two different patterns were made. The first pattern was based on a contraction triggered by BayK-8644 in fluid without Ca^{2+} . The mean perfusion pressure value for BayK 8644 in the presence of CsA was $15 (\pm 4.1)$ mmHg and was not significantly different compared to the control [$12 (\pm 3.3)$ mmHg] ($p=0.1198$).

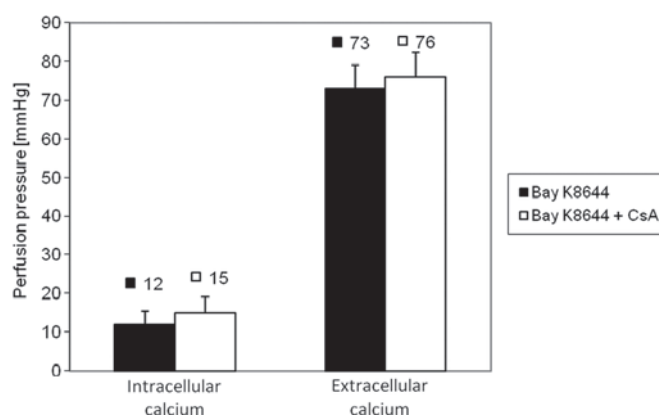


Figure 6. Influence of cyclosporine A (CsA) (1 nM/l) on vasoconstriction induced by BayK-8644. Model 1, BayK-8644 vs. BayK-8644 + CsA, $p=ns$; model 2, BayK-8644 vs. BayK-8644 + CsA, $p=ns$.

In the second pattern, the arteries were incubated in a fluid with Ca^{2+} . Perfusion pressure induced by BayK-8644 was $73 (\pm 5.9)$ mmHg. The incubation of arteries in the presence of CsA did not change their reactivity on contraction triggered by BayK-8644. The mean perfusion pressure value in this pattern was $76 (\pm 3.9)$ mmHg and was not significantly different compared to the control ($p=0.2375$) (Fig. 6).

Discussion

The reactivity of VSMCs is upregulated through the autonomic nervous system. The tension depends on the hormonal activity and local chemical signals. Abnormal vasoreactivity caused by endothelial dysfunction or impaired signal transmission, induces the development of hypertension in the group of patients treated with CsA (29).

Hyperreactivity is the result of complex mechanisms, which lead to the increased concentration of Ca^{2+} ions in a myocyte. However, vasopressin, norepinephrine and endothelin-1 are ligands for a number of transmembrane metabotropic receptors. The mechanisms of signal transmission in their area are similar and lead to the activation of intracellular calcium influx (30). Moreover, there is a possibility that all receptors bound with the G-protein and IP_3/DAG cascade are involved in enhancing vasopressin effect via CsA.

No effect of CsA on the mobilization of the intracellular calcium pool suggests that there was a significant increase of transmission within the IP_3R and ryanodin receptors during the exposure of the arteries to the drug (Fig. 2).

Artery incubation in the presence of CsA increased the influx of calcium ions from the intracellular fraction (Fig. 3). Therefore, the increased intracellular influx from the extracellular compartment may be considered as an important element of hypertension pathogenesis.

In our experiments estimating the vessel contractions induced with the L-type calcium channel agonist, BayK-8644, no significant differences were found in perfusion pressure in both fluids (with and without Ca^{2+}) in the presence of CsA (Fig. 6).

Contraction induced by BayK-8644 initiates calcium ion influx from the extracellular area. Apart from vasoconstriction action, BayK-8644 may exert inotropic effects (31). CsA did

not increase the reactivity of arteries on the pressor action of BayK-8644. The obtained results demonstrate that the drug does not modulate the agonist binding site, and does not change the mobilization of the calcium pool from the ER fraction. In our study, we observed that, in the presence of CsA, the vaso-sensitivity of the pressor action of PHE and AVP increased. The reason for this hyperreactivity is the enlargement of the receptor reserve pool (α_1 , V_1). This was confirmed by the higher value of K_A/ED_{50} in both cases. Higher receptor reserve in consequence leads to the higher sensitivity of pressure factors (32,33). The data available in the literature show the impact of CsA on the concentration of GPCR receptors.

It was also proven that CsA at a dose of 25 mg/kg body mass/day applied for 4 weeks induced the expression of mRNA, which encodes the receptor for endothelin-1 (subtype ET_A) in the aorta and mesenteric artery by 170 and 176%, respectively. This fact shows an important role of endothelin-1 and its receptor in the pathogenesis of hypertension in a group of patients treated with immunosuppressants. The selective ET_A -FR 139317 receptor antagonist may protect from this action (34). We should also emphasize the role of angiotensin II, whose concentration increases with long-term use (35). Based on our experiments, we conclude that CsA directly increases the activity of PKC, dependent of Ca^{2+} . Shin *et al* confirmed that the hyperreactivity of PKC (β -isoform) plays an important role in the pathogenesis induction, which leads to renal fibrosis in patients treated with CsA (36).

Based on the results in the following study, we may consider a direct influence of CsA on calcium channels. This is due to the reduction or even complete inhibition of vasoconstriction effect of the drug in the presence of the calcium antagonist.

Analyzing the costs of hypertension drugs used after exposure on CsA, it can be concluded that the effective variant in pharmacotherapy of hypertension induced by CsA may be calcium channel antagonists (37). Carrier *et al* showed that the application of diltiazem by the infusion effectively decreases renal perfusion pressure and may protect from the nephrotoxic effects of the drug. However, even after application of diltiazem, a significant elevation in the concentration of endothelin-1 from 0.5 ± 0.4 to 3.8 ± 0.6 pg/ml in the presence of CsA was observed. Although the calcium channel blockade by diltiazem effectively reduced the vasoconstrictive action of the immunosuppressant, they did not prevent the expression of endothelin-1 (38).

The use of the angiotensin receptor antagonist was not effective in the hypertension therapy of patients treated with CsA (Figs. 4 and 5). Hypertension in transplant recipients almost always requires matching three drugs from different therapeutic groups. As a diuretic, patients should receive loop diuretics. If there are no arbitrary indications (coronary artery disease and myocardial infarction), β -blocker application may be suspended. Observations from past years have shown that β -blockers foster the growth of body mass in patients, which increases the risk of T2 diabetes. Calcium antagonists belong to a preferable group of drugs in hypertension therapy after transplantation. It has been proven that those medications reduce the renal failure triggered by infarction and nephrotoxicity caused by calcineurin inhibitors (39). Certain drugs from this group inhibit CYP3A4, which causes the retention of CsA in the blood and necessity of frequent monitoring of the drug concentration

(40). The greatest inhibition of CsA metabolism was demonstrated for verapamil, and lower inhibition was shown for drugs, such as diltiazem, nicardipine and amlodipine. Nifedipine, nitrendipine and isradipine did not affect the metabolism of CsA. Calcium channel antagonists belong to the preferable group of drugs used in patients with hyperlipidemia, since contrary to diuretics and β -blockers, they do not affect the lipid homeostasis (41). Unlike many other drugs used chronically, omission of a single dose of the third-generation calcium antagonist does not cause an increase of blood pressure. Contrary to ACE inhibitors and β -blockers, amlodipine does not react with non-steroidal anti-inflammatory drugs, and can be used with antibiotics and anti-diabetic medications. Van der Schaaf *et al* performed experiments in patients with hypertension and kidney transplantation treated with CsA. The particularly beneficial effect of amlodipine at improving renal function was demonstrated. Amlodipine was a better controller of blood pressure (ABPM 111 ± 9 mmHg) than lisinopril (ABPM 115 ± 9 mmHg). Amlodipine enlarged glomerular filtration rate, effective renal plasma flow and decreased renal vascular resistance. These parameters did not differ significantly during therapy with lisinopril (42). Chanard *et al* proved its superiority over the β -blocker teratolol. Amlodipine decreased the concentration of uric acid in the plasma from 483 ± 99 to 431 ± 110 μ mol/l, whereas teratolol increased it from 450 ± 98 to 476 ± 84 μ mol/l (43).

In conclusion, CsA increases vascular reactivity by enhancing signal transmission between GPCR and calcium channels. The effect of CsA is also significantly inhibited by the calcium blockers, nifedipine and diltiazem.

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