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Effects of the novel cyclosporine derivative PSC833 on glucose metabolism in rat primary cultures of neuronal and glial cells

Fatima Cruz, Armin Wolf*

Novartis Pharma AG, Experimental Toxicology, CH-4002 Basel, Switzerland

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Abstract

Cyclosporine A (CsA) and the cyclosporine D congener PSC833 are known to cause transient CNS symptoms at high dosages in animal and man. Since impaired glucose metabolism plays a fundamental role in many heriditary and drug-induced neurological disorders, it was the purpose of the present study to evaluate whether this mechanism of pathogenesis might apply to PSC833 and CsA, using neural cells from rats. PSC833 and CsA were investigated in primary cultures of rat neuronal and glial cells at the concentration of 0.1, 1, 10, and 20 μM for 24 and 48 hr. Lactate dehydrogenase was determined as a marker of cytotoxicity. Cell proliferation was determined in astrocytes. Cellular glucose metabolism was investigated by ^{13}C-NMR using [1-^{13}C]glucose as a substrate. Glucose and lactate concentrations in the cell culture supernatants were determined spectrophotometrically. PSC833 at 10 μM was not cytotoxic in neuronal or glial cells nor did it inhibit proliferation in astrocytes 24 hr after incubation. Under the same conditions, the determination of [1-^{13}C]glucose and [3-^{13}C]lactate revealed significantly increased glucose consumption and lactate production in both cell types, as well as decreased levels of Krebs cycle intermediates. In the cell culture medium of both cell types after treatment with 10 μM PSC833, the rates of glucose consumption and lactate formation increased in comparision to controls, between 60–83% and 54–78%, respectively. PSC833 (10 μM) and CsA (20 μM) resulted in nearly similar increased glucose consumption and lactate production. The major PSC833 metabolite in rats, M9, which was devoid of CNS effects, did not cause significant changes in glucose metabolism. The present data suggest that PSC833-impaired tricarboxylic acid cycle activity, resulting in decreased Krebs cycle metabolites, can cause energy depletion and acidosis, which might contribute to the transient neurological symptoms of PSC833 and CsA.

Keywords: PSC833; Cyclosporine A; Neurotoxicity; Neuronal primary culture; Astrocyte primary culture; NMR; Glucose metabolism

1. Introduction

PSC833 [(3'-keto-Bmt1)-(Val2)-cyclosporine] represents a novel class of multidrug resistance modifier successfully used to reverse MDR of anticancer drugs by Pgp-170 kDa protein, preventing the efflux of anticancer drugs out of cancer cells. The compound is remarkably free of side effects, and although being a member of the cyclosporine family, it is devoid of immunosuppressive activity. Concerning its *in vitro* pharmacological activities to reverse MDR, PSC833 is about 3–10 times more potent than CsA, which is itself about one order of magnitude more potent than other known chemosensitizers, including verapamil, quinidine, or amiodarone, which have already entered clincial trials in MDR reversal [1,2]. PSC833 has been formulated for oral and intravenous co-administration with anticancer drugs. Preliminary clincial results obtained so far in Phase I/II trials suggest very promising results of PSC833 co-therapy during acute leukemia [3].

Neurotoxicity is a well-known adverse side effect of CsA seen in clinical as well as preclinical studies [4–6]. Among cyclosporines, the cyclosporine D (CsD) derivatives can induce CNS effects after oral treatment in rats, in the form of reversible impairment of locomotion and excitatory behavior at concentrations less than 100 mg/kg/day. In order to demonstrate similar CNS effects with CsA in rats, doses greater than 100 mg/kg/day are necessary [7]. Chemically, PSC833 is a CsD derivative. A single intravenous bolus injection of PSC833 at a dose level of 30 mg/kg caused transient neurological symptoms, such as ataxia, impaired

* Corresponding author. Tel.: +41-61-324-1735; fax: +41-61-324-1027.

E-mail address: armin.wolf@pharma.novartis.com (A. Wolf).

Abbreviations: CsA, cyclosporine A; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LDH, L-lactate dehydrogenase (EC 1.1.1.27); MDR, multidrug resistance, PC, pyruvate carboxylase (EC 1.2.4.1); PDH, pyruvate dehydrogenase complex (EC 1.2.4.1, 1.8.1.4. 2.3.1.12, 3.1.3.43); and TCA, tricarboxylic acid.

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locomotion, and hunched posture [8]. CsA at the same dose levels was devoid of such effects. The PSC833 metabolite M9, which is C-hydroxylated in position λ of amino acid number 9, L-methyl-leucine, is the major metabolite of PSC833 in rats and did not cause any signs of neurotoxicity. Both compounds, when administered by the same route and at the same dose level, exposed the brain to a nearly similar extent [8].

The mechanisms underlying the adverse side effects of PSC833 and CsA are not yet understood. One mechanism reported to be common in many heriditary neurological disorders and various drug-induced cases of neurotoxicity is the impairment of brain glucose metabolism [9–13,15,16]. Glucose is the energy-generating substrate of the normal adult brain which serves as the major carbon source, lead via Krebs cycle activity to the biosynthesis of important metabolites such as neurotransmitters [17]. In contrast to other organs, brain energy metabolism depends almost exclusively on the oxidation of glucose [17]. Since glucose is the basic substrate for maintaining many neurological functions and impairment of its metabolism has been reported to contribute to several heriditary and drug-induced neurological disorders [15,16], it was the aim of the present study to evaluate whether this mechanism also underlies the neurological effects of PSC833. In the present study, we investigated the effects of PSC833 on cerebral glucose metabolism in rat primary cultures of cortical neurons and astrocytes, using ^{13}C-NMR techniques and spectrophotometric methods. CsA and the PSC833 metabolite M9 were used as reference compounds.

2. Materials and methods

2.1. Chemicals

[1-^{13}C]glucose (99% ^{13}C-enriched) and deuterated solvents were obtained from Dr. Glaser AG. Auxiliary enzymes and co-factors were obtained from Boehringer Mannheim. FBS and DMEM were obtained from GIBCO BRL. The rest of the products were from Sigma Chemical Co. CsA and PSC833 were from Novartis Pharma AG. The PSC833 metabolite M9 was synthesized by Dr. Roland Wenger, Novartis Pharma AG.

2.2. Animals

Permission for the animal studies was obtained from the Veterinäramt Basel-Landschaft, CH-4410 Liestal, Switzerland, and all study protocols were in compliance with the institutional guidelines. Female pregnant Wistar rats were obtained from Biological Research Laboratories. They were kept in MacrolonR cages with wood shavings as bedding, under optimal hygienic conditions, at a temperature of 22–23°, a relative humidity of 50–70°, and fluorescent light on a 12-hr day/12-hr night cycle. They were given water and rodent food (pellets) *ad lib*. Two days after acclimatization, the animals were dissected.

2.3. Primary cultures of cortical neurons

Neuronal cultures were prepared from the cerebral cortex of 17-day-old rat embryos as described by Gomeza *et al*. [23]. Tissue was cleaned of meninges and dissociated with papain (7 min, 37°, 0.4 mg/mL in a 10-mM phosphate buffer solution, pH 7.5, with 6 mM glucose) and subsequently disaggregated by passing through a Pasteur pipette. After centrifugation (800 *g*, 5 min), pelleted cells were resuspended in DMEM containing 10% FBS, penicillin (50 IU/mL), and streptomycin (0.5 mg/mL) and plated on poly-L-lysine (10 mg/mL)-coated plates at a density of 10^6 cells/mL. Medium was replaced after 3 hr by a serum-free medium prepared with DMEM supplemented as described by Brewer *et al*. [24]. This medium was changed every two days up to the sixth day after seeding, when the experiment was performed. Cells were maintained at 37°, 95% air and 5% CO_2. Neuronal cultures were characterized immunohistochemically using a monoclonal antibody antineurofilament 160 kD (anti-NF160) [25]. The monoclonal antibodies antiglial fibrillary acidic protein (specific for astrocytes) [26], antivimentin (specific for astrocytes and fibroblast) [27], and antifibronectin (specific for fibroblast) [28] were used to verify the purity of the cultures. Neuron enrichment was higher than 90%. Experiments were performed after cells were maintained for 6 days in culture.

2.4. Primary cultures of cortical astrocytes

Primary cultures of astrocytes were prepared from 1-day-old Wistar rats, essentially as described by Clarke *et al*. [29]. Cerebral cortices were removed and cleaned of meninges and blood vessels, dissociated mechanically by passing sequentially through 20 GI and 23 GI sterile needles, and collected by centrifugation at 800 *g* for 5 min. Cells were resuspended in DMEM containing 10% (v/v) FBS and non-essential amino acid mixture. Cells were used at confluence. Astrocyte preparations were characterized immunohistochemically using a monoclonal antibody antiglial fibrillary acidic protein (anti-GFAP) [26]. The monoclonal antibodies anti-NF160 and antifibronectin were used to determine the purity of the cultures, which was higher than 95%.

2.5. Compound incubation conditions

In order to achieve solubility, PSC833 or CsA was first dissolved in DMSO. The stock solution was further diluted so that the final DMSO concentration reached 0.1% in each cell culture plate. The same amount of DMSO was used in control plates.

2.6. ^{13}C-NMR investigations

Neurons and astrocytes were grown and maintained with their respective media until the adequate state of confluence was reached. Afterwards, cells were incubated with [1-^{13}C]glucose in a medium containing the tested compounds for 24 hr. To this end, the medium used during the culture was substituted by a defined medium without glucose, pyruvate, and glutamine but supplemented with 5 mM [1-^{13}C]glucose. Incubations were maintained at 37°, 95% air and 5% CO_2 for 24 hr, taking aliquots at different times to obtain the metabolic balance. After the incubation, cells and media were extracted with $HClO_4$ at 5% v/v, neutralized with KOH, and lyophilized. Freeze-dried extracts were reconstituted with D_2O (99%) prior to NMR acquisition.

^{13}C-NMR spectra (22°, pH 7.5) were obtained on a Bruker DMX-500 spectrometer operating at 125.7 MHz for ^{13}C, using a 5-mm ^{13}C-NMR dual probe. Acquisition conditions were: 80° pulses, spectral width 28 kHz, 1.17 sec acquisition time, 5.67 sec total cycle time, 65 k data points. Proton decoupling was obtained using composite ^{1}H pulses applied only during acquisition. Chemical shifts were calibrated with an external reference of dioxane (67.4 ppm). Peak assignments were based on literature values and on the addition of appropriate compounds [30,31].

2.7. Determination of lactate dehydrogenase activity

The leakage of the cytosolic enzyme LDH, expressed as the percentage of LDH activity in the cell culture medium as part of the LDH activity on the plate, was used as marker of cytotoxicity. The test was performed as described by Wolf *et al*. [32].

2.8. Determination of glial cell proliferation

Proliferation rates were determined by measuring the amount of [^{3}H]thymidine incorporated by the cells according to the procedure described by Mirsalis *et al*. [33]. Astrocytes were seeded on 12-well plates and maintained with DMEM, containing 10% FBS, until 60–70% confluence was reached approximately 10 days after starting culture. Afterwards, cells were incubated with the test compounds for 24 or 48 hr. Four hours before the treatment was finalized, 1 μCi/mL of [^{3}H]thymidine was added to each well. When the treatment was stopped, the supernatants were discarded and the cells were washed twice with 1 mL PBS (4°). After the last washing procedure, 1 mL of ice-cold trichloroacetic acid (10%) was added to the cell layer of each well, all of which were also put on ice. The trichloroacetic acid precipitates were dissolved with 0.5 mL of 1 N NaOH and transferred to the scintillation vials. Ten milliliters of scintillation liquid was added and radioactivity was measured in a β-counter. Results were expressed as percentage of controls.

2.9. Determination of glucose

Glucose was determined in the cell culture supernatant spectrophotometrically at the wavelength of 340 nm, using enzymatic end point reactions coupled to the increase in NADPH [34]. The test kit was purchased from Boehringer Mannheim (Cat. No. 716251).

2.10. Determination of lactate

The determination is based on the LDH reaction, which catalyzes the oxidation of L-lactate to pyruvate by NAD^{+}, with the simultaneous formation of NADH. The amount of NADH formed is stoichiometric to the amount of L-lactate and was measured in the cell culture medium at a wavelength of 340 nm [35].

2.11. Determination of protein contents

Protein concentration was measured using the method described by Bradford [36].

2.12. Statistical analysis

Data presented are the means of measurements in cells derived from 4–6 animals, with each measurement performed in triplicate. The results are given as the mean value ± standard error.

Data were analyzed by two statistic data sets, A and B. For data set A, a two-way analysis of variance was performed (both group and data numbers were regarded as qualitative). If the effect of the data number was not significant, it was omitted. A quantile plot was used to visually judge the normality of the residuals. Residuals were considered normal although there were only relatively few data points.

For data set B, a one-way analysis of variance was performed for the group effect. A quantile plot was used to visually judge the normality of the residuals. If the residuals were not normally distributed, we tried to achieve (approximate) normality by omission of outliners or by transforming the data. In a few cases, normality could not be achieved due to unequal variance of the groups; however, the differences in variance were not considered too great.

For both data sets, a multiple comparison method was further applied using the methods of Turkey [43], Sidak [44], and Dunnett [42]. The Dunnett test compares every treated group with the control group, while the other two methods can be used to compare each group with each other group. The multiple comparison method delivers an estimation of the difference of the expected response between the two compared groups, the standard error of the response, and a lower and upper confidence limit for the difference. If the two limits do not include zero, the difference is significantly different from zero at the level of 5%. By repeating the method for 1% and 0.1%, we could find out how strong

Table 1
Effect of PSC833 on LDH leakage in neurons and astrocytes, as well as on astrocyte cell proliferation, after 24 and 48 hr of incubation

PSC833 Concentration [μM]	Neurons				Astrocytes							
	24 hr		48 hr		24 hr				48 hr			
	LDH leakage [% of total]	$P <$	LDH leakage [% of total]	$P <$	LDH leakage [% of total]	$P <$	Proliferation [% of control]	$P <$	LDH leakage [% of total]	$P <$	Proliferation [% of control]	$P <$
0	6.8 ± 1.8		6.8 ± 0.5		8.5 ± 1.5		100.0 ± 3.0		12.5 ± 0.5		100.0 ± 8.3	
0.1	6.5 ± 2.3		8.4 ± 0.4		8.5 ± 1.0		94.5 ± 2.1		12.5 ± 0.8		111.2 ± 10.4	
1	7.4 ± 2.0		6.3 ± 0.5		8.5 ± 1.0		95.2 ± 5.2		13.0 ± 0.5		108.1 ± 10.4	
10	11.3 ± 3.2		15.4 ± 2.1	0.05	11.0 ± 1.2		90.4 ± 6.0		18.0 ± 2.3	0.001	24.1 ± 2.6	0.001
20	10.9 ± 0.5	0.001	19.0 ± 1.1	0.001	12.5 ± 1.5	0.05	83.6 ± 2.6		18.0 ± 2.5	0.001	18.0 ± 1.8	0.001

Data are expressed as means ± SE (N = 3). Statistically significant differences compared with the respective controls are expressed by the *P* values.

the significance was. The S-Plus software (version 5) was used for the computation [45]. Thereafter, the three methods of Turkey [43,44] and Dunnett [42] were used adaptively, i.e. in every case the most sensitive of the methods were used.

3. Results

3.1. Cytotoxicity

PSC833 and CsA were investigated in rat primary cultures of neuronal and glial cells at the concentration of 0.1, 1, 10, and 20 μM for 24 and 48 hr. LDH leakage was determined as a marker of cytotoxicity. PSC833 and CsA induced neuronal cell death in a dose- and time-dependent manner (Tables 1 and 2). While PSC833 and CsA were not clearly cytotoxic after 24 hr at the concentration of 10 μM, it became very obvious that both compounds caused increased LDH leakages at concentrations of 20 μM. After 48 hr of incubation, both compounds became cytotoxic at concentrations of 10 μM, whereby the CsA effects were always more pronounced compared to those of PSC833.

In control astrocytes, the basal LDH leakage rates were in general higher than in control neurons. Increasing concentrations of CsA and PSC833 resulted in nearly similar small increases in the LDH leakages in comparison to that of controls (Tables 1 and 2). After 24 hr of incubation with CsA or PSC833, LDH values were statistically significantly increased at a concentration of 20 μM. After 48 hr, LDH leakages were increased at 10 and 20 μM.

PSC833 and CsA were evaluated concerning their effects on glial cell proliferation. While CsA caused a dose-dependent inhibitory effect on proliferation at 10 and 20 μM 24 hr after incubation, PSC833 under the same conditions did not change cell proliferation to the same extent (Tables 1 and 2). After 48 hr, PSC833 and CsA both inhibited astrocyte proliferation at 10 μM, the effects of CsA always being more pronounced than those of PSC833.

Summarizing the results from both cytotoxicity tests, the determinations of LDH leakages, and proliferation rates, it appears that CsA in comparision to PSC833 at concentrations of 10 μM continued the trend to be cytotoxic, although statistically significant differences to controls were not reached in all test systems.

3.2. ^{13}C-NMR investigations

PSC833 and CsA were incubated at concentrations of 0.1, 1, and 10 μM with neurons for 24 hr, and [1-^{13}C]glucose metabolism was determined after incubating the cells with [1-^{13}C]glucose. NMR investigations revealed PSC833-increased [1-^{13}C]glucose consumption by the formation of [3-^{13}C]lactate, as well as decreased TCA cycle flux as

Table 2
Effect of CsA on LDH leakage in neurons and astrocytes, as well as on astrocyte cell proliferation, after 24 and 48 hr of incubation

CsA Concentration [μM]	Neurons				Astrocytes							
	24 hr		48 hr		24 hr				48 hr			
	LDH-leakage [% of total]	$P <$	LDH-leakage [% of total]	$P <$	LDH-leakage [% of total]	$P <$	Proliferation [% of control]	$P <$	LDH-leakage [% of total]	$P <$	Proliferation [% of control]	$P <$
0	7.4 ± 0.4		10.5 ± 1.2		8.5 ± 1.5		100.0 ± 12.0		12.5 ± 0.5		100.0 ± 6.7	
0.1	6.7 ± 0.4		15.6 ± 0.1		11.5 ± 2.8		100.0 ± 10.0		14.5 ± 0.1		108.4 ± 10.4	
1	8.4 ± 0.4		10.5 ± 2.0		11.6 ± 2.5		99.5 ± 8.0		14.5 ± 0.3		70.5 ± 12.8	0.01
10	11.9 ± 0.5		32.2 ± 4.8	0.001	10.0 ± 1.0		47.2 ± 12.0	0.01	23.0 ± 2.3	0.001	15.4 ± 1.5	0.001
20	16.2 ± 2.0	0.001	29.5 ± 0.6	0.001	11.0 ± 0.2	0.001	10.2 ± 5.0	0.01	26.5 ± 2.5	0.001	1.7 ± 0.2	0.001

Data are expressed as means ± SE (N = 3). Statistically signficant differences compared with the respective controls are expressed by the *P* values.

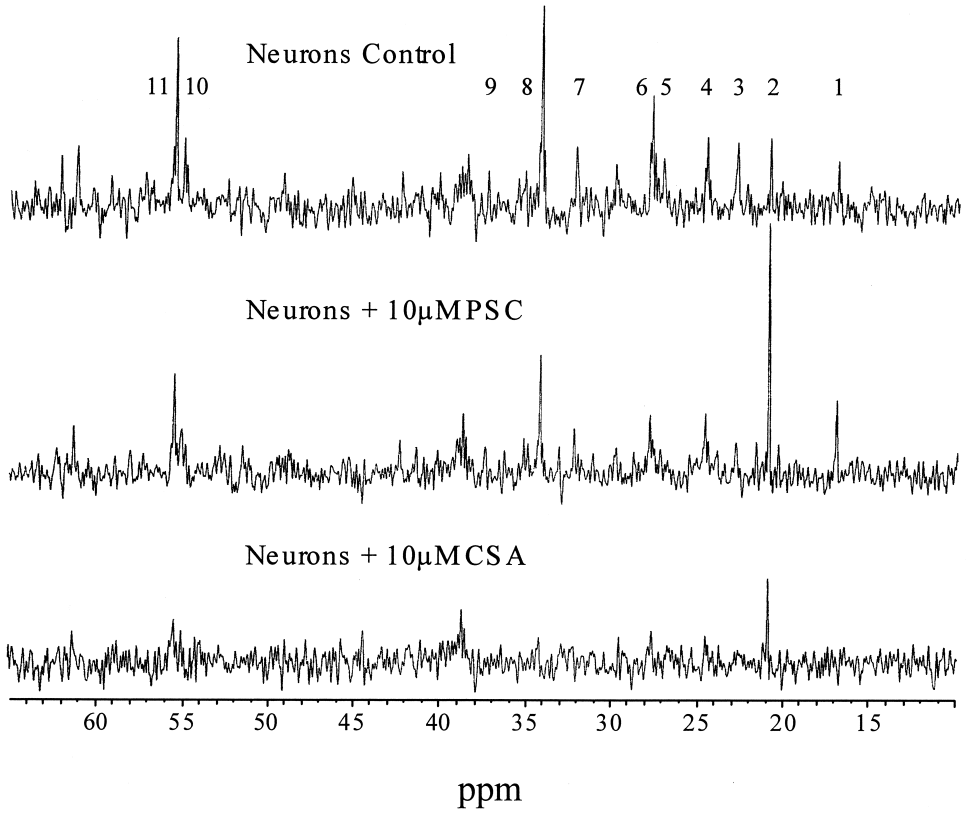

Fig. 1. ^{13}C-NMR spectra (125.7 MHz, 22°, pH 7.5) from the aliphatic region (10–65 ppm) of the $HClO_4$ extracts of neurons incubated for 24 hr with 0.1% DMSO (upper panel), 10 μM PSC833 (middle panel), and 10 μM CsA (lower panel). Assignments: 1. C3 alanine; 2. C3 lactate; 3. C6 *N*-acetyl aspartic acid; 4. C2 acetate; 5. C3 glutamine (or glutathione); 6. C3 glutamate; 7. C4 glutamine (or glutathione); 8. C4 glutamate; 9. unassigned; 10. C2 glutamine (or glutathione); 11. C2 glutamate.

compared to the controls. Fig. 1 shows representative ^{13}C-NMR spectra (region 10–65 ppm) obtained from extracts of neurons incubated with 5 mM [1-^{13}C]glucose and 0.1% DMSO, 10 μM PSC833, or 10 μM CsA. In the spectra, glutamate, glutamine, *N*-acetyl-aspartate, alanine, and lactate were clearly identified by their characteristic ^{13}C resonances. In neurons, incubations with the non-cytotoxic concentration of 10 μM PSC833 for 24 hr caused a clear decrease in the Krebs cycle intermediates, reflected by decreased intensities of ^{13}C resonances from the carbons of glutamate and increased ^{13}C resonances of intracellular lactate and alanine. CsA at a concentration of 10 μM resulted in an even stronger decrease in all resonances compared to PSC833, except for the [3-^{13}C]lactate peak. Incubations of neurons with 0.1 and 1 μM PSC833 or CsA, respectively, did not result in differences compared to the controls (data not shown).

The same incubations performed with glial cells also resulted in increased glucose consumption as measured by [3-^{13}C]lactate production in response to PSC833, but not CsA. Fig. 2 depicts representative ^{13}C-NMR spectra (region 10–65 ppm) obtained from extracts of astrocytes incubated with 5 mM [1-^{13}C]glucose and 0.1% DMSO, 10 μM PSC833, or 10 μM CsA. Incubations of astrocytes with 10 μM PSC833 showed a clear decrease in the intermediates of the TCA cycle and associated metabolites (glutamate, glutamine), as well as an increase in [3-^{13}C]lactate formation. The decrease in the intensity of the C4 resonance of glutamine or glutamate was not the same as in the C2 carbon. Incubations of astrocytes with 10 μM CsA resulted in significant decreases in all resonances of the spectrum. Astrocytes incubated with 0.1 and 1 μM PSC833 or CsA, respectively, did not cause significant changes in the spectra as compared to the controls (data not shown).

3.3. Glucose utilization and lactate formation

Cell culture supernatants of neurons and astrocytes were investigated for glucose consumption and lactate production by means of spectrophotometric methods. Fig. 3 shows the time-course of glucose consumption and lactate production during 24-hr incubation of neurons and astrocytes with 5 mM glucose in the presence of 0.1, 1, and 10 μM PSC833 or CsA. In neuronal cell culture supernatants, PSC833 resulted in a linear glucose consumption rate of 0.28 (μmol.mg protein^{-1}.hr^{-1}), significantly higher than that found in control cells (0.17 μmol.mg protein^{-1}.hr^{-1}) or cells incubated with 10 μM CsA (0.15 μmol.mg protein^{-1}.hr^{-1}) (Fig. 3A). At the same time, the rate of lactate production of neurons significantly increased by 0.48

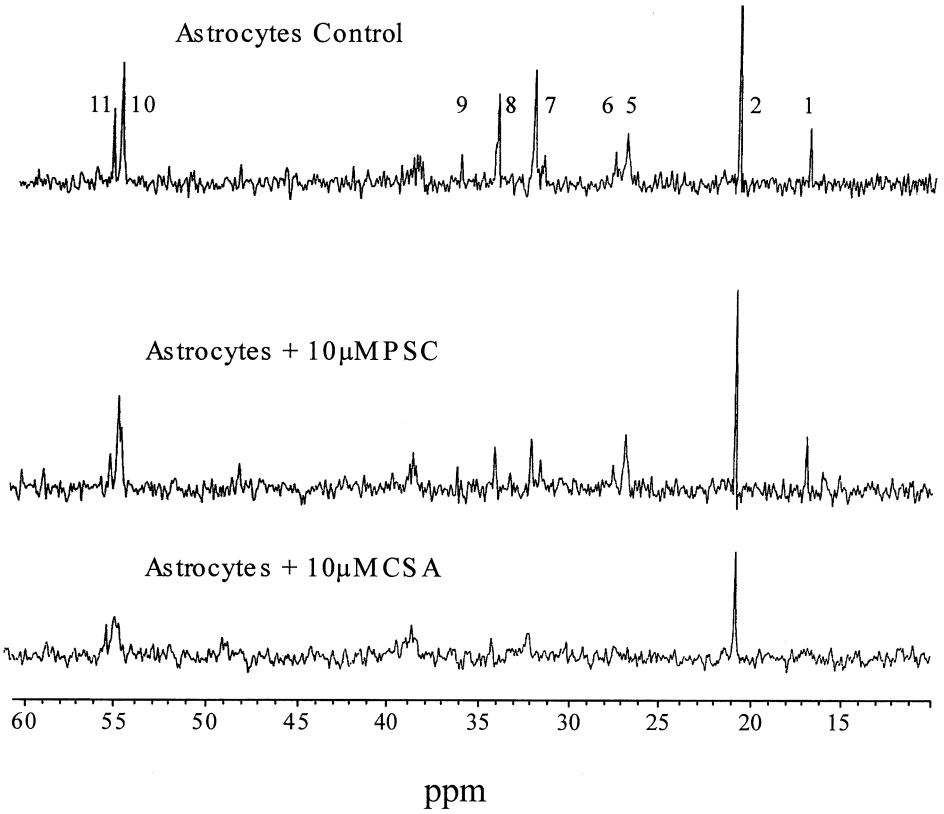

Fig. 2. ^{13}C-NMR spectra (125.7 MHz, 22°, pH 7.5) from the aliphatic region (10–65 ppm) of the $HClO_4$ extracts of astrocytes incubated for 24 hr with 0.1% DMSO (upper panel), 10 μM PSC833 (middle panel), and 10 μM CsA (lower panel). Assignments: 1. C3 alanine; 2. C3 lactate; 3. C6 *N*-acetyl aspartic acid; 4. C2 acetate; 5. C3 glutamine (or glutathione); 6. C3 glutamate; 7. C4 glutamine (or glutathione); 8. C4 glutamate; 9. unassigned; 10. C2 glutamine (or glutathione); 11. C2 glutamate.

μmol.mg protein^{-1}.hr^{-1} in comparison to CsA (0.23 μmol.mg protein^{-1}.hr^{-1}) or controls (0.27 μmol.mg protein^{-1}.hr^{-1}). This means that after PSC833 treatment, the metabolic rates of glucose oxidation in comparison to controls were statistically significantly greater by 60% than that of controls, and the rate of lactate formation was 78% greater. Under the same conditions, CsA decreased glucose oxidation and lactate formation rates between 12 and 15%, without reaching statistically significant differences compared to controls. No significant differences in the time-courses of glucose consumption and lactate production were found in neurons under the same conditions in the presence of 0.1 and 1 μM PSC833 or CsA in comparison to controls (data not shown).

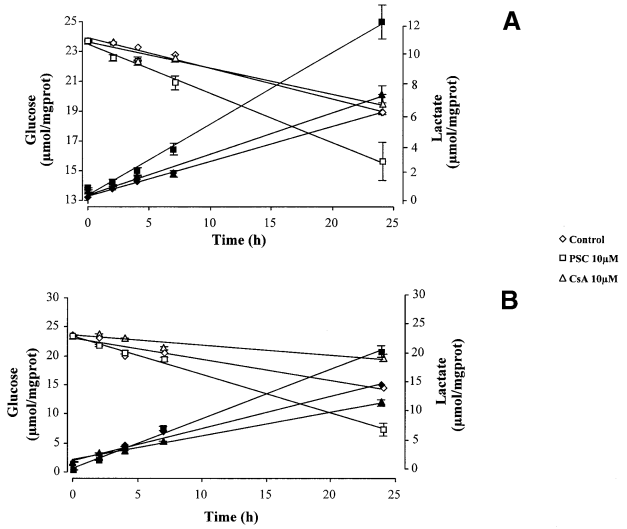

Fig. 3. Effects of PSC833 and CsA on the kinetics of glucose utilization (open symbols) and lactate formation (filled symbols) after incubating neurons (A) and astrocytes (B) with 0.1% DMSO (control), 10 μM PSC833, or 10 μM CsA.

Glucose metabolism in astrocytes and neurons appeared to be similar in response to PSC833 and CsA (Fig. 3B). In the presence of 10 μM PSC833 and 5 mM glucose, astrocytes showed higher glucose consumption rates (0.66 μmol.mg protein^{-1}.hr^{-1}) and lactate production (0.94 μmol.mg protein^{-1}.hr^{-1}) compared to the controls, where glucose consumption was 0.36 (μmol.mg protein^{-1}.hr^{-1}) and lactate production was 0.61 (μmol.mg protein^{-1}.hr^{-1}). Incubations with 10 μM CsA resulted in a decrease in both glucose and lactate metabolic rates of 0.17 and 0.45 (μmol.mg protein^{-1}.hr^{-1}, respectively). This means that after PSC833 treatment the metabolic rates of glucose oxidation in comparison to controls were statistically significantly greater by 83%, and the rate of lactate formation was

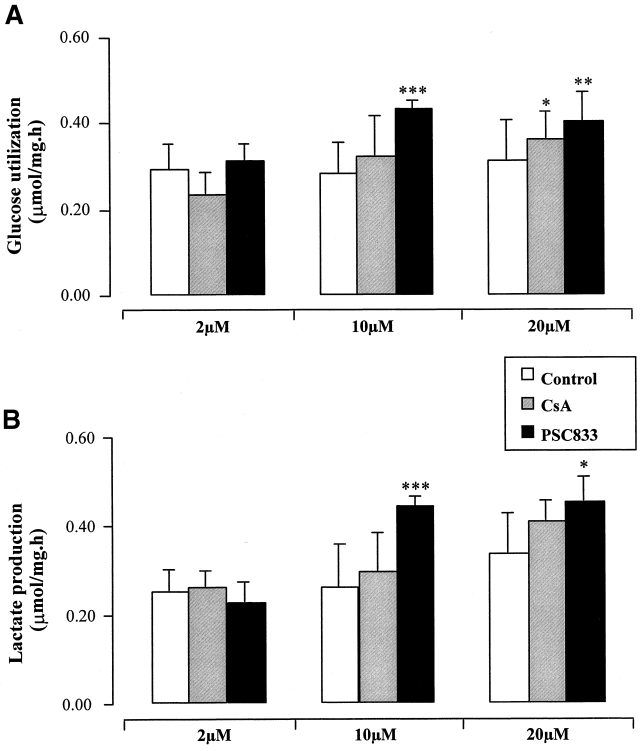

Fig. 4. Glucose utilization (A) and lactate formation (B) in neurons after a 20-hr treatment with PSC833 or CsA. Data are expressed as means ± SE (N = 3). Statistically significant differences compared with the respective controls are expressed as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

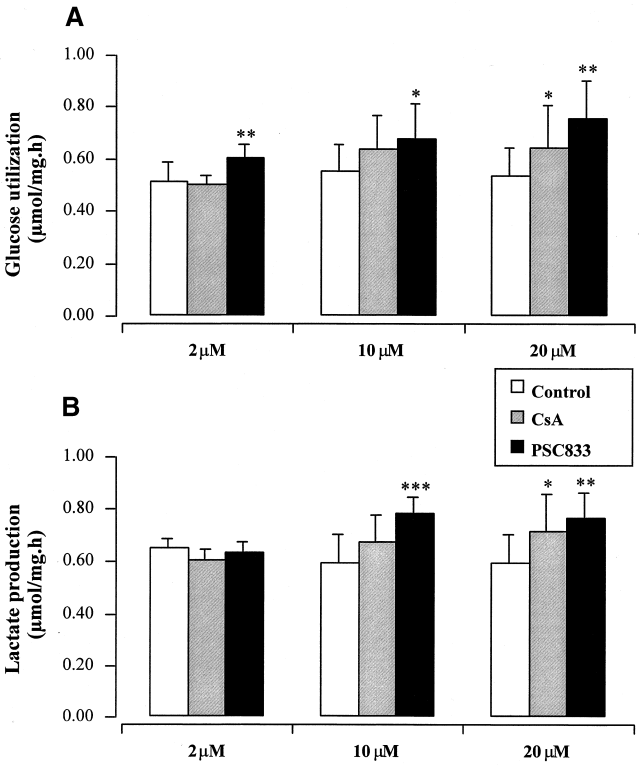

Fig. 5. Glucose utilization (A) and lactate formation (B) in astrocytes after a 20-hr treatment with PSC833 or CsA. Data are expressed as means ± SE (N = 3). Statistically significant differences compared with the respective controls are expressed as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

54% greater. Under the same conditions, CsA decreased glucose oxidation and lactate formation rates between 53 and 26%, respectively, although without reaching statistically significant differences from controls. As in neurons, no statistically significant differences compared to controls were found in the metabolic rates of astrocytes incubated with 5 mM glucose in the presence of 0.1 and 1 of both PSC833 and CsA (data not shown).

In another series of independent experiments, neuronal and glial cells were incubated with CsA and PSC833 at the concentrations of 2, 10, and 20 μM for 24 hr. Lactate and glucose were determined in the cell culture supernatant. Each concentration group had its respective solvent control group. Results are depicted in Figs. 4 and 5. As seen in the previous experiment, PSC833, already at 10 μM, increased glucose utilization (Fig. 4A) and lactate production (Fig. 4B) in a statistically significantly different manner compared to respective controls, while CsA under the same conditions did not. These effects were also found at 20 μM PSC833. At a concentration of 20 μM, CsA treatment also resulted in statistically significantly increased glucose utilization and lactate formation. For both compounds, these effects were similar in astrocytes (Fig. 5), except that PSC833 caused a statistically significant increase in glucose consumption in comparison to its respective control already at a concentration of 2 μM (Fig. 5A).

Figs. 6 and 7 summarize glucose consumption and lactate production in neuronal and glial cell culture supernatants in another set of experiments after treatment with PSC833 and its metabolite M9. Whereas PSC833 caused a significant dose-dependent increase in glucose utilization and lactate formation in both cell types, there were no statistically significant differences in M9-treated cultures compared with controls, not even at the highest M9 concentration. However, there seemed to be a trend toward slightly increased glucose utilization and lactate formation in both cell types with increasing M9 concentrations. After PSC833 treatment of neurons, glucose consumption and lactate formation were statistically significantly different from that found after M9 treatment (Fig. 6). In astrocytes, both parameters were lower after M9 treatment than after PSC833 treatment, which reached statistical significance compared to controls at 10 and 20 μM (Fig. 7).

4. Discussion

4.1. ^{13}C-NMR investigations

Fig. 8 illustrates the general metabolism of [1-^{13}C]glucose in neural cells. Glucose enters the intracellular space through a specific glucose transporter. Once inside the cell, [1-^{13}C]glucose is degraded through the glycolytic pathway

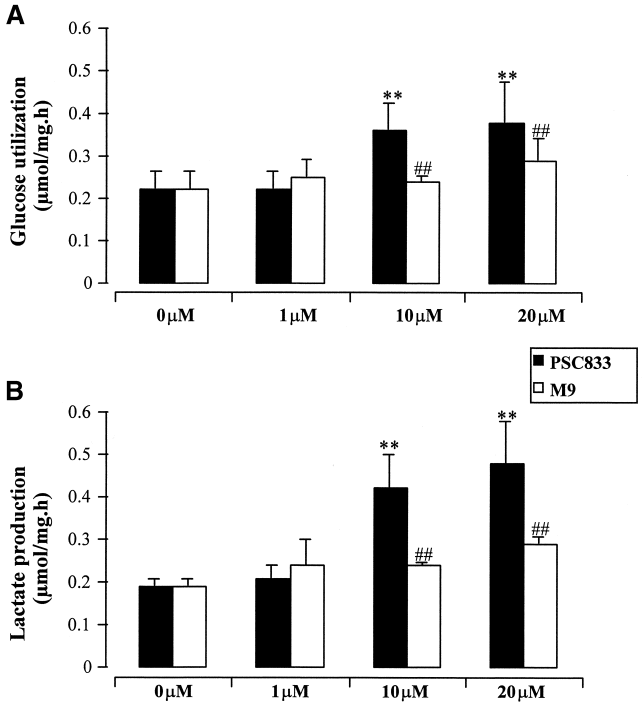

Fig. 6. Glucose utilization (A) and lactate formation (B) in neurons after a 20-hr treatment with PSC833 or M9. Data are expressed as means ± SE (N = 3). Statistically significant differences compared with the respective controls are expressed as $^{**}P < 0.01$; differences compared with respective PSC833 group are indicated by $^{\#\#}P < 0.01$.

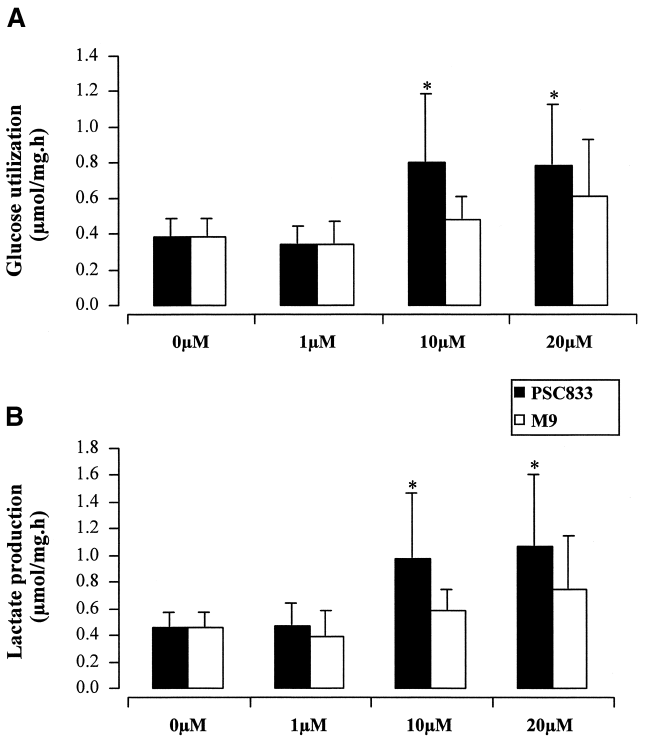

Fig. 7. Glucose utilization (A) and lactate formation (B) in astrocytes after 20-hr treatment with PSC833 or M9. Data are expressed as means ± SE (N = 3). Statistically significant differences compared with the respective controls are expressed as $^{*}P < 0.05$.

to an equimolar mixture of [3-^{13}C]-labeled and unlabeled pyruvate. In neurons, [3-^{13}C]pyruvate could be transaminated to [3-^{13}C]alanine, reduced to [3-^{13}C]lactate, or enter the TCA cycle through the activity of the PDH, yielding [2-^{13}C]acetyl-CoA. Condensation of [2-^{13}C]acetyl-CoA with unlabeled oxaloacetate produces [4-^{13}C]α-ketoglutarate and then [4-^{13}C]glutamate. The exchange between α-ketoglutarate and glutamate is much faster than the TCA cycle rate [19]. Thus, labeling in glutamate accurately reveals labeling in α-ketoglutarate at every turn of the cycle. These metabolites are easily detected in the ^{13}C-NMR spectra of extracts and provide a well-established method to study flux through the TCA cycle and associated anaplerotic reactions [20,21,37].

In the incubations of astrocytes with [1-^{13}C]glucose, the labeling pattern of metabolites is the same as in neurons, with the exception of an additional entrance of [3-^{13}C]pyruvate to the TCA cycle catalyzed by the glial PC enzyme which is not present in neurons [38]. In this case, PC flux would label glutamate C2 in the first turn of the cycle. Then, during the first turn of the cycle, PC originates [2-^{13}C]glutamate while PDH yields [4-^{13}C]glutamate.

4.2. Mechanism of PSC833-impaired glucose metabolism

By means of NMR investigations, monitoring of the metabolism of [1-^{13}C]glucose, and quantitative lactate and glucose determination, we were able to demonstrate for the first time that PSC833 impairs the neural glucose metabolism. At non-cytotoxic concentrations, PSC833-increased lactate formation, as well as increased glucose oxidation, might be regarded as the result of increased anaerobic glycolysis, induced by an impaired TCA cycle. CsA induced the same changes and was equipotent to PSC833 at concentrations twice as high as that of PSC833.

Most of the brain's energy consumption is used for active transport of ions to sustain and restore membrane potentials discharged during the process of excitation and conduction. Only aerobic utilitzation of glucose is capable of providing the brain with sufficient energy to maintain normal function. It is well known that the brain is submitted to the Pasteur effect when the cellular steady state of ATP is disturbed [15]. Phosphofructokinase is the key enzyme in controlling glycolytic flux. At steady state, ATP levels in brain are sufficient to keep phosphofructokinase inhibited. When the ATP levels drop, phosphofructokinase is activated and produces an increase in the glycolytic flux [17]. From our results, we can only make indirect conclusions based on the reduced TCA activity on an impaired energy supply, since no direct ATP measurements were performed. A direct impairment of the cellular energy state by CsA in rat cortical slices was reported by Serkova *et al*. [40,41], who found decreased high-energy phosphate by means of ^{31}P-NMR analysis.

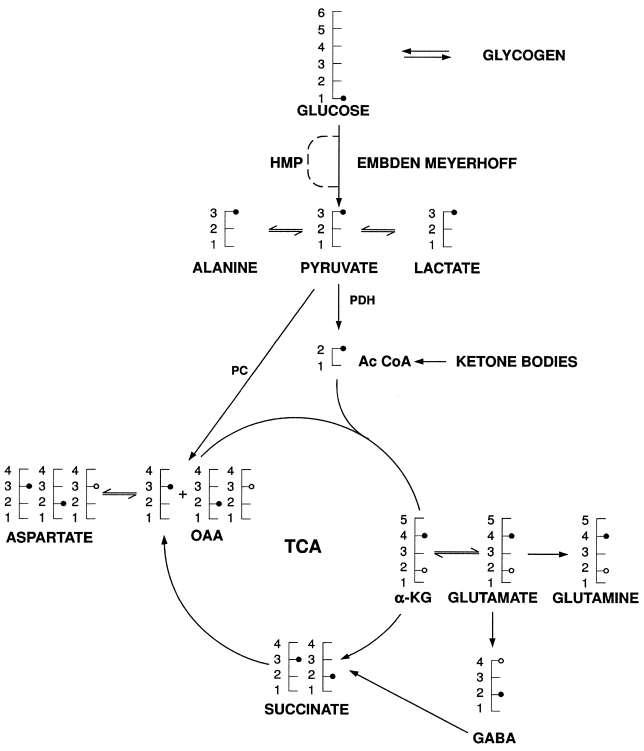

Fig. 8. ^{13}C-labeling patterns of different cerebral metabolites using [1-^{13}C]glucose as a substrate. In the TCA cycle, labeling derived from PDH entrance is indicated as a filled symbol, while labeling derived from PC entrance is indicated as an open symbol. Abbreviations used: OAA: oxaloacetate, GABA: γ-aminobutyric acid, α-KG: α-ketoglutarate.

The PSC833- as well as the CsA-mediated disturbances in the energy metabolism in astrocytes could be the result of an altered entrance of acetyl-CoA into the TCA, catalyzed by PC or the PDH complex. This can be concluded from the spectra of astrocytes after cyclosporine treatment, in which C4 intensities of glutamate and glutamine were found to be more decreased in comparison to the C2 resonances. As described above, labeling in C2 of glutamate derives from the activity of pyruvate carboxylase on [3-^{13}C]pyruvate and the subsequent entrance of the TCA cycle, while C4 glutamate derives from the entering of [2-^{13}C]acetyl-CoA into the TCA cycle, catalyzed by the PDH complex. In the case of neurons, where no PC activity is present [38], we can speculate on an impairment of the TCA cycle at the level of the PDH.

In the literature, it has also been reported that hereditary PDH deficiencies could be related to ataxia [39]. A partial deficit in PDH complex in terms of high-energy demand would have to be compensated for by excessive glycolysis and lactic acid production.

Our data suggest that PSC833 as well as CsA may inhibit entrance of pyruvate into the PCA cycle at the level of PDH and PC. Such a mechanism could explain the neurological effects that were observed in rats at high PSC833 dosages.

The specific inhibitory effects of PSC833 and CsA on the formation of TCA intermediates could also directly lead to deficiency in the production of putative excitatory neurotransmitters and thus to impaired excitatory and conductory functions. As a consequence of the reduced pyruvate oxidation, tissue concentrations of glutamic acid and aspartic acid are decreased. The tissue deficiency in putative excitatory neurotransmitters is thought to be responsible for the mechanism in Friedreich's ataxia [46]. The decreased TCA cycle activity might also have an effect on the intracellular redox state of the NAD+/NADH and NADP+/NADPH couples, which could be important modulators of neuronal cellular functions [47].

4.3. Relevance of the in vitro data

The incidence of CsA neurotoxicity was estimated between 25% and 60% of organ-transplanted patients. Preclinical studies with rats did not show neurological symptoms at doses up to 100 mg/kg/day after oral administration. Among the cyclosporines, the D-derivatives [(Val)^2 dihydrocyclosporine] seemed to be most potent, causing a CNS effect already at lower dose levels than 100 mg/kg/day in the form of reversible impairment and excitatory behavior [7]. PSC833 also belongs to the class of cyclosporine D-derivatives. Previous studies carried out in rats showed that 2 hr after a single intravenous bolus application of 30 mg/kg PSC833, transient neurological symptoms such as ataxia, impaired locomotion, and hunched posture were observed [8]. The application of the PSC833 metabolite M9 under the same conditions resulted in a nearly similar passage through the blood-brain barrier as PSC833 and in the similar brain exposure, but was not neurotoxic. Two hours after a single i.v. bolus injection of 30 mg/kg PSC833 or M9, brain concentrations were 10.7 ± 1.9 and 9.6 ± 1.1 μg/g tissue (or mL), respectively. The brain-blood partition coefficients (K_p) for both compounds were about 2.0 [8].

Since both compounds, PSC833 as well as M9, reach *in vivo* the same brain exposure, and M9 was not neurotoxic, this correlates with the ineffectiveness concerning its effect on glucose metabolism. This helps to validate the present *in vitro* model and supports the proposed mechanism of action.

In summary, we have shown that PSC833 exerts a specific effect on the glucose metabolism of neural cells at a non-cytotoxic concentration, inducing an impairment of glycolysis and TCA cycle, which results in lactic acidosis and a decrease in TCA cycle intermediates. The resulting impaired energy metabolism, as well as the decrease of Krebs cycle intermediates, could have an impact on neurophysiological functions. The relevance of the current findings is supported by the good correlation between the *in vitro* effects of CsA, PSC833, and M9 on glucose metabolism in neurons and astrocytes and their ability to induce neurotoxicity *in vivo*. The current mechanism suggests the possibility of antagonizing the CsA or PSC833 neurological findings by restoring normal brain glucose levels.

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