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FK506 attenuates 1-methyl-4-phenylpyridinium- and 3-nitropropionic acid-evoked inhibition of kynurenic acid synthesis in rat cortical slices

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Abstract. Kynurenic acid (KYNA), the only known endogenous glutamate antagonist, is produced in the brain by kynurenine aminotransferases (KATs) I and II. Mitochondrial toxins, 1-methyl-4-phenylpyridinium (MPP^+) and 3-nitropropionic acid (3-NPA), were previously shown to reduce KYNA synthesis *via* interference with KAT I and II. Data presented here demonstrate that immunophilin ligand, FK506 (10-130 μM), but not CsA (1-50 μM), or ryanodine receptor blocker, dantrolene (1-100 μM), enhances the formation of KYNA in cortical slices. FK506, but not CsA or dantrolene, abolished the inhibition of KYNA synthesis evoked by MPP^+ and 3-NPA. None of studied compounds influenced the activity of KAT I and KAT II. FK506 is the first among currently used drugs that might stimulate KYNA synthesis. This effect does not seem to arise from the interference with KATs or calcineurin activity.

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INTRODUCTION

Pathological sequel culminating in the development of neuronal loss and the occurrence of seizures might involve different genetically determined and/or acquired alterations in cellular metabolism. Research data accumulated within last decade indicate that disturbed mitochondrial function may contribute to the pathogenesis of human neurodegenerative diseases and epilepsy (Bloem et al. 1990, Borlongan et al. 1995, Urbanska et al. 1998). Mitochondrial toxins, such as 3-nitropropionic acid (3-NPA) or 1-methyl-4-phenylpyridinium (MPP^+), diminish oxidative phosphorylation *via* interference with mitochondrial respiratory chain, reduce the level of available ATP, and thus cause metabolic inhibition similar to the one evoked by ischemia/anoxia (Brouillet et al. 1998, Ramsay et al. 1986). Various patterns of experimental application of 3-NPA, an irreversible inhibitor of succinate dehydrogenase found in the environment, may evoke either Huntington's chorea-like symptoms or initiate the occurrence of seizures, both susceptible to the protection with glutamate antagonists (Beal et al. 1993, Borlongan et al. 1995, Urbanska et al. 1998, Urbanska et al. 1999). Administration of MPP^+, inhibiting the activity of mitochondrial complex I, serves as a model of parkinsonism in animals (Burns et al. 1983). Moreover, MPP^+ evokes seizures in rodents, which can be prevented with the use of glutamate receptor antagonists (Turski and Stephens 1992). These data are in line with hypothesis linking a defect in mitochondrial energy metabolism with increased vulnerability of neurons to endogenous glutamate (Beal et al. 1993, Novelli et al. 1988). It is broadly accepted now that an excessive activation of glutamate inhibiting receptors induces abnormal intracellular accumulation of Ca^{2+} followed by a variety of changes culminating in the cell death (Budd and Nicholls 1996).

Cyclosporin A (CsA), FK506 and dantrolene are three drugs able to modulate intracellular Ca^{2+} levels. The first two compounds are immunosuppressants, potently inhibiting Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, in the presence of their respective cytoplasmic immunophilin proteins, cyclophilin and FK506-binding protein (FKBP) (Gold 2000). Calcineurin modulates a number of key Ca^{2+} signaling pathways in neurons, and has been implicated in Ca^{2+}-dependent negative feedback inactivation of N-methyl-D-aspartate receptors and voltage-sensitive Ca^{2+} channels (Rusnak and Mertz 2000). Experimentally used CsA and FK 506 are effective neuroprotectants in global cerebral ischemia, traumatic brain injury and glutamate-induced neurotoxicity (Kikuchi et al. 1998, Singleton et al. 2001, Uchino et al. 2002). Both compounds may also ameliorate toxicity evoked by MPP^+ and 3-NPA (Guo et al. 2001, Leventhal et al. 2000). Dantrolene, an inhibitor of Ca^{2+} release from endoplasmic reticulum *via* blockade of ryanodine receptors, is used to treat malignant hyperthermia and neuroleptic malignant syndrome (Heiman-Patterson 1993). Abnormalities of endoplasmic reticulum-mediated Ca^{2+} signaling were linked to the excitotoxic processes (Mattson et al. 2000). Accordingly, dantrolene may prevent delayed neuronal loss associated with the experimental transient ischemia and can reduce glutamate-evoked toxicity (Gepdiremen et al. 2001, Nakayma et al. 2002).

Kynurenic acid (KYNA) is the only known endogenous antagonist of glutamate receptors within the mammalian brain (Turski et al. 1989, Stone 2000). Recently it was shown that KYNA also blocks cholinergic α7-nicotinic receptor (Hilmas et al. 2001). KYNA is synthesized *via* an irreversible transamination of its bioprecursor, L-kynurenine, along so-called kynurenine pathway, by kynurenine aminotransferases (KATs) I and II, enzymes found predominantly in glial cells (Guidetti et al. 1997). KYNA was implicated as a modulator of physiological glutamate neurotransmission, and its altered metabolism has been suggested as one of the factors contributing to the development of seizures and neurodegenerative disorders (Stone 2000). Recently we have shown that MPP^+ and 3-NPA inhibit cortical synthesis of KYNA *via* interference with its synthesizing enzymes (Luchowski et al. 2002). In this study we have aimed to evaluate the effect of CsA, FK506 and dantrolene on the brain production of KYNA and their influence on MPP^+- and 3-NPA-induced reduction of KYNA synthesis.

METHODS

Male Wistar rats (220-250 g) housed under standard laboratory conditions were used. Experimental procedures have been approved by the Local Ethical Committee in Lublin and are in agreement with the European Communities Council Directive on the use of animals in experimental studies. L-kynurenine (sulphate salt), kynurenic acid, CsA, dantrolene (sodium salt), 3-NPA, MPP^+, pyruvate, pyridoxal-5'-phosphate, 2-mercaptoethanol, and cellulose membrane dialysis tubing were obtained from Sigma (St.

Louis, USA), FK506 was purchased from Fujisawa Healthcare (USA), whereas all HPLC reagents were obtained from Baker. KYNA production *in vitro* was investigated as previously described (Luchowski et al. 2002). Briefly, animals were killed by decapitation and their brains rapidly removed from the skull. Cortical slices (1 x 1 mm base) were placed at culture wells (8 per each well) containing oxygenated Krebs-Ringer buffer, pH 7.4, and incubated in the presence of 10 μM L-kynurenine and solutions of tested compounds (2 h, 37°C), in a final volume of 1 ml. At the end of incubation period, the incubation media were rapidly separated from the tissue, acidified, centrifuged and supernatant was applied to the columns containing cation-exchange resin (Dowex 50 W^+). Water eluate containing KYNA was subjected to HPLC and KYNA was detected fluorimetrically (Varian HPLC system; ESA catecholamine HR-80, 3 μm, C_{18} reverse-phase column). The mean control production of KYNA in the presence of 10 μM L-kynurenine was 6.1 ± 0.7 pmol/1 h/well.

The activities of KAT I and KAT II were assayed according to the method of Guidetti et al. (1997) with modification. Briefly, cortical brain tissue was homogenized (1:10; wt:vol) in 5 mM Tris-acetate buffer, pH 8.0, containing 50 μM pyridoxal-phosphate and 10 mM 2-mercaptoethanol. The resulting homogenate was dialyzed overnight at 8°C, using cellulose membrane dialysis tubing, against 4 l of the buffer composed as above. The enzyme preparation was incubated in the reaction mixture containing 2 μM L-kynurenine, 1 mM pyruvate, 70 μM pyridoxal-5'-phosphate, 150 mM Tris-acetate buffer, at pH of 7.0 or 9.5, for KAT II or KAT I, respectively (all given concentrations are the final ones) and solution of tested drug in varying concentrations. Glutamine (final concentration 2 mM), the inhibitor of KAT I, was added to samples assaying KAT II activity. Blanks contained the enzyme preparation that was heat-deactivated at 100°C for 10 min. The incubation (37°C, 2 h) was ended by a rapid transfer of samples to an ice-bath and the addition of 50% trichloroacetic acid (14 μl) and 0.1 N HCl (1 ml) to each sample. Denaturated protein was removed by centrifugation and the supernatant was applied to a Dowex 50W^+ column. Further procedures were performed as described above. The mean control activity of KAT I and KAT II in the presence of 2 μM kynurenine was 1.35 ± 0.12 and 0.94 ± 0.09 pmol/mg tissue/h, respectively.

The statistical comparisons of results were performed using an ANOVA test, with the adjustment of *P* value by the method of Bonferroni. Statistical significance of differences from control values is indicated as $P<0.05$.

RESULTS

FK506 used in the concentration of 10, 30, 60, 90, 110 and 130 μM significantly increased the formation of KYNA in cortical slices to 125% ($P<0.05$), 130% ($P<0.01$), 135% ($P<0.001$), 136% ($P<0.001$), 122% ($P<0.05$) and 119% ($P<0.05$), respectively, but applied in the concentration ≤ 5 μM and ≥ 150 μM did not change KYNA production (Fig. 1). Neither CsA (1-50 μM) nor dantrolene (1-100 μM) altered KYNA formation (Fig. 1). In the presence of FK506, used in the concentration of 30 μM (increasing KYNA formation *per se*), but not of 3 μM, 3-NPA-induced impairment of KYNA production evoked has been abolished (Fig. 2A). CsA used in the concentration of 50 μM and dantrolene (100 μM) did not alter the inhibition of KYNA production evoked by 3-NPA (Fig. 2B,C). Similarly, FK506 used in the concentration of 30 μM, but not of 3 μM, prevented the decrease of KYNA synthesis evoked by MPP^+ (Fig. 3A). Neither CsA (50 μM) nor dantrolene (100 μM) influenced the impairment of KYNA production evoked by MPP^+ (Fig. 3B,C). FK506 (0.1-100 μM), CsA (1-50 μM) and dantrolene (1-100 μM) did not influence the activity of KAT I and KAT II (Table I).

DISCUSSION

The above data show that FK506 increases *de novo* synthesis of KYNA in rat brain cortical slices, while CsA and dantrolene are ineffective. Moreover, in the presence of FK506, the KYNA production inhibited by 3-NPA and MPP^+ is restored. In contrast, neither CsA nor dantrolene influenced KYNA synthesis reduced by 3-NPA or MPP^+ administration. The observed effect does not seem to be mediated *via* changes in the activity of KYNA biosynthetic enzymes, KAT I or KAT II, which was unaffected by the studied drugs.

According to the available literature there are surprisingly few means to enhance the production of KYNA. These include lowering the content of extracellular sodium, use of co-factors of KATs, such as 2-oxoacids, or substances increasing the level of KYNA bioprecursor, L-kynurenine, due to the blockade of its metabolic enzyme, kynurenine 3-hydroxylase (Hodgkins et al. 1999, Stone 2000, Turski et al. 1989). FK506 is the first, until

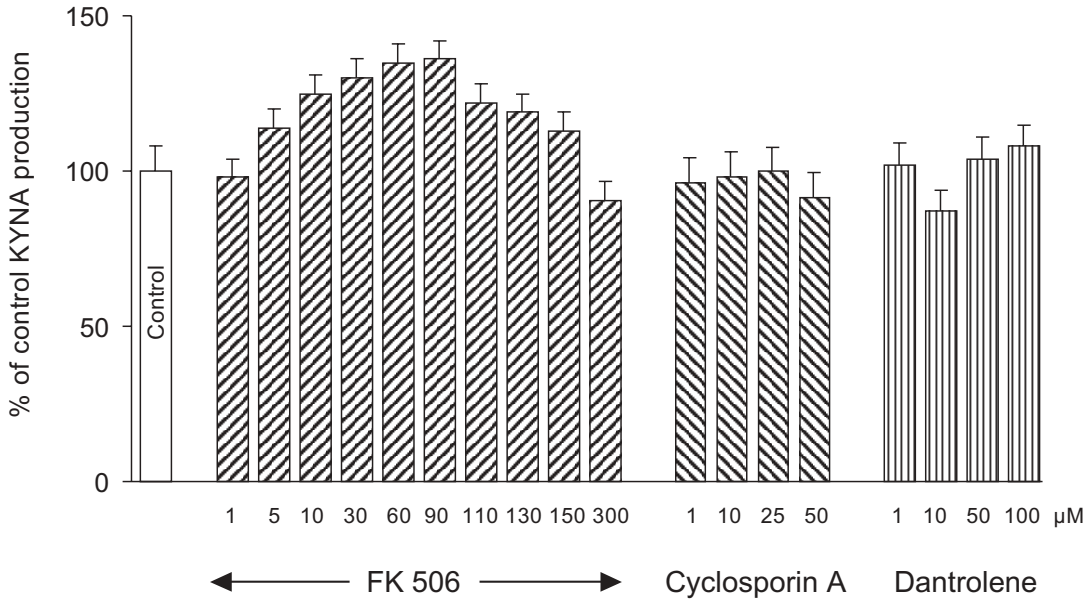

Fig. 1. The effect of FK506, cyclosporin A (CsA) and dantrolene on the production of kynurenic acid (KYNA) in cerebral cortical slices. Slices (1 x 1 mm base) were placed at culture wells (8 per well) containing 1 ml of oxygenated Krebs-Ringer buffer, pH 7.4. The tissue was incubated in the presence of 10 µM L-kynurenine and solutions of tested drugs, for 2 h at 37°C. Newly synthesized KYNA was quantified fluorimetrically, with HPLC. Data are mean values ± SD of six determinations. *, $P<0.05$ (ANOVA) vs. control (100%).

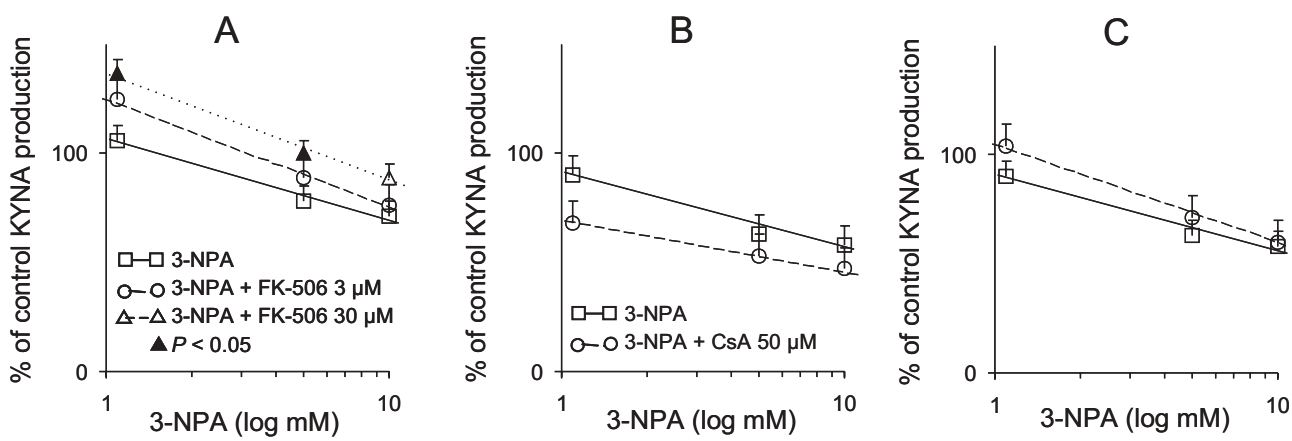

Fig. 2. The effect of FK506 (**A**), cyclosporin A (CsA) (**B**) and dantrolene (**C**) on the 3-NPA-evoked impairment of kynurenic acid (KYNA) production in cerebral cortical slices. Slices (1 x 1 mm base) were placed at culture wells (8 per well) containing 1 ml of oxygenated Krebs-Ringer buffer, pH 7.4. The tissue was incubated in the presence of 10 µM L-kynurenine and solutions of tested drugs, for 2 h at 37°C. Newly synthesized KYNA was quantified fluorimetrically, with HPLC. Data are mean values ± SD of six determinations. Filled symbols represent data points that have reached statistical significance ($P<0.05$; ANOVA) vs. 3-NPA. Dose-regression lines were calculated using GraphPad software. **A**: 3-NPA: y = -16.285Ln(x) + 106.75, r = -0.99; 3-NPA + FK506 3 µM: y = -22.456Ln(x) + 126.66, r = -1.0; 3-NPA + FK506 30 µM: y = -22.184Ln(x) + 138.3, r = -1.0; **B**: 3-NPA: y = -15.04Ln(x) + 90.423, r = -0.99; 3-NPA + CsA: y = -9.5778Ln(x) + 68.794, r = -1.0; **C**: 3-NPA: y = -15.04Ln(x) + 90.423, r = -0.99; 3-NPA + dantrolene: y = -20.237Ln(x) + 105.36, r = -1.0.

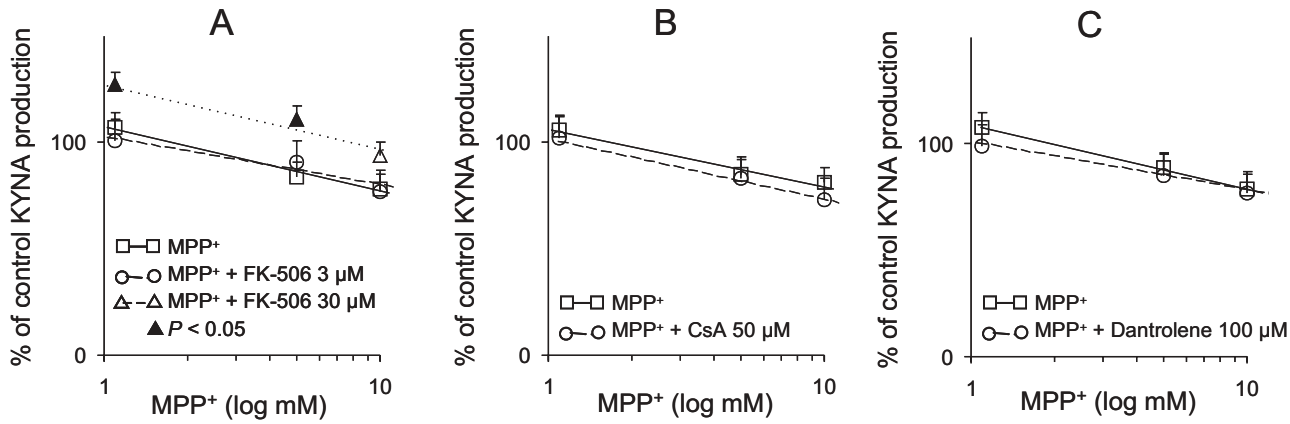

Fig. 3. The effect of FK506 **(A)**, cyclosporin A (CsA) **(B)** and dantrolene **(C)** on the MPP^+-evoked impairment of kynurenic acid (KYNA) production in cerebral cortical slices. Slices (1 x 1 mm base) were placed at culture wells (8 per well) containing 1 ml of oxygenated Krebs-Ringer buffer, pH 7.4. The tissue was incubated in the presence of 10 μM L-kynurenine and solutions of tested drugs, for 2 h at 37°C. Newly synthesized KYNA was quantified fluorimetrically, with HPLC. Data are mean values ± SD of six determinations. Filled symbols represent data points that have reached statistical significance ($P<0.05$; ANOVA) vs. MPP^+. Dose-regression lines were calculated using GraphPad software. **A**: MPP^+: y = -13.472Ln(x) + 107.66, r = -0.99; MPP^+ + FK506 3 μM: y = -10.179Ln(x) + 103.26, r = -0.95; MPP^+ + FK506 30 μM: y = -14.238Ln(x) + 129.69, r = -0.97; **B**: MPP^+: y = -11.74Ln(x) + 106.35, r = -0.99; MPP^+ + CsA: y = -13.042Ln(x) + 103.42, r = -1.0; **C**: MPP^+: y = -13.042Ln(x) + 109.42, r = -1.0; MPP^+ + dantrolene: y = -9.8498Ln(x) + 100.16, r = -1.0.

Table I

The influence of FK506, cyclosporin A (CsA) and dantrolene on the activity of kynurenine aminotransferases I and II (KAT I and II) in rat brain

Substance	Concentration (μM)	KAT I	KAT II
FK506	0.1	97.4 ± 4.4	102.1 ± 6.7
	0.5	105.1 ± 6.0	102.9 ± 5.8
	1.0	100.1 ± 7.5	98.8 ± 6.1
	5.0	106.2 ± 7.3	109.6 ± 8.1
	10.0	105.5 ± 4.9	96.5 ± 4.6
	30.0	104.7 ± 9.2	99.2 ± 4.3
	100.0	96.6 ± 6.0	101.7 ± 6.7
CsA	1.0	107.4 ± 6.2	98.4 ± 5.5
	10.0	99.9 ± 7.5	101.3 ± 5.1
	50.0	95.2 ± 4.7	96.6 ± 7.8
Dantrolene	1.0	94.9 ± 5.8	102.4 ± 5.6
	10.0	102.4 ± 4.7	100.1 ± 4.0
	100.0	96.0 ± 7.5	97.2 ± 6.2

Data are expressed as % of control values ± SD of six determinations. Tissue preparation was incubated (2 h, 37°C) in the presence of tested compounds, 2 μM L-kynurenine, 1 mM pyruvic acid and 70 μM pyridoxal 5'-phosphate in 150 mM Tris-acetate buffer, pH 7.0 for KAT II and pH 9.5 for KAT I analysis. Glutamine (final concentration 2 mM), the inhibitor of KAT I, was added to samples assaying KAT II activity. Blanks contained the enzyme preparation that was heat-deactivated at 100°C for 10 min. Newly synthesized KYNA was quantified fluorimetrically, with HPLC. The statistical comparisons of results were performed using ANOVA test, with the adjustment of P value by the method of Bonferroni.

now, currently used drug which is able to increase KYNA formation. Surprisingly, the evaluation of concentration dependency revealed that FK506 action follows the bell-shape curve what indicates the presence of saturation mechanism or feedback inhibition.

The nature of the observed in this study stimulatory action of FK506 is not clear. FK506, as well as CsA and dantrolene, display neuroprotective action in various experimental models including cerebral ischemia, traumatic brain injury, application of glutamate, MPP^+ or 3-NPA (Guo et al. 2001, Leventhal et al. 2000, Singleton et al. 2001, Uchino et al. 2002, Zhang et al. 1993). The suggested mechanisms underlying their beneficial effects include calcineurin (serine/threonine protein

phosphatase 2B) inhibition, interference with mitochondrial permeability transition (MPT), and blockade or ryanodine receptors (Bochelen et al. 1999, Uchino et al. 2002, Xu et al. 1998). Both CsA and FK 506 are immunophilin ligands reducing the activity of calcineurin, which modulates various Ca^{2+} signaling pathways and may contribute to the inactivation of N-methyl-D-aspartate receptors (Rusnak and Mertz 2000). Nonetheless, the enhancement of KYNA production does not seem to result from the inhibition of calcineurin activity, since in our paradigm only FK506, and not CsA, was active.

Recent evidence suggests that MPT may be involved in Ca^{2+} signaling as well as necrotic and apoptotic cell death (Lemasters et al. 1998). CsA binds to cyclophilin D, believed to be a component of mitochondrial megachannel, what favours the closed state of the pore and blocks onset of the MPT (He and Lemasters 2002, Uchino et al. 2002). However, the data presented here exclude the possibility that modulation of MPT may stimulate KYNA synthesis, because CsA turned out ineffective.

Furthermore, the alterations in the level of Ca^{2+} released from endoplasmic reticulum do not appear to influence KYNA formation. Dantrolene, which prevents abnormal Ca^{2+} release from the endoplasmic reticulum by blocking ryanodine receptors (Xu et al. 1998), did not change KYNA synthesis in cortical slices.

What could be the mechanism behind the enhancement of KYNA synthesis by FK506? Intracellular effects of FK506 are mediated by group of immunophilins including: FKBP-13 (FKBP-15), which is present in endoplasmic reticulum; FKBP-25, which is largely uncharacterized; FKBP-65, which is also present in endoplasmic reticulum, where it serves as a chaperone protein for tropoelastin; and human FKBP-52 (rabbit FKBP-59), or heat shock protein 56 (hsp-56), which (together with hsp-90) is a component of a subclass of steroid receptor complexes (Gold 2000). Accordingly, only FK506, and not CsA or dantrolene, has been clearly shown to exhibit significant neuroregenerative activity, arising from the binding of FKBP-52 (Gold 2000, Guo et al. 2001). However, short incubation time used in the present experimental approach (2 h) seems to rule out the prominent role of neuroregenerative action of FK506 in the synthesis of KYNA. Nevertheless, it cannot be excluded that other(s) immunophilin participates in the FK506-induced effects. Apparently, the hypothetical action of immunophilins would not be related to the interference with KATs activity, however, it could be speculatively linked with the modulation of kynurenine 3-hydroxylase, an enzyme involved in the metabolism of KYNA precursor, L-kynurenine (Stone 2000), or with the altered sodium balance. Indeed, FK506, but not CsA, was shown to inhibit aldosterone-induced sodium transport in A6 cells (Rokaw et al. 1996). Further studies exploiting more selective pharmacological tools are needed to clarify this issue.

In the presence of FK506, used in the concentrations raising KYNA synthesis *per se*, the inhibitory effects of mitochondrial toxins, 3-NPA and MPP^+, were abolished. These substances, interfering with mitochondrial respiratory chain, were recently shown to decrease KYNA formation, at least partially *via* inhibition of its biosynthetic enzymes, KATs (Luchowski et al. 2002). Interestingly, others have shown that FK506, but not CsA, protects against the hypoxic injury in part by preventing the mitochondrial dysfunction in concert with the enhancement of heat shock response in hepatocytes (Kaibori 2001). It seems possible that protective effect of FK506 against mitochondrial toxins-related inhibition of KYNA synthesis may, at least partially, result from the restoration of ATP levels.

CONCLUSIONS

Presented data suggest that action of FK506, as opposed to CsA or dantrolene, might include the stimulation of KYNA synthesis, the effect possibly not related to the interference with KATs or calcineurin activity. FK506 is the first of already used in the therapy drugs showing among other actions the ability to enhance formation of endogenous antagonist of glutamate receptors, KYNA.

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