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FK506 promotes adenosine release from endothelial cells via inhibition of adenosine kinase

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Abstract

The immunosuppressants, cyclosporin A and tacrolimus (FK506) induce an increase in plasma levels of adenosine and mimic ischemic preconditioning. However, the mechanism of action of the two drugs on adenosine metabolism is not clear. Since inhibition of adenosine kinase promotes an increase in endogenous adenosine release, we tested a hypothesis that FK506 induces adenosine release via inhibition of adenosine kinase activity. In cultured endothelial cells, FK506 enhanced release of tracer adenosine and inhibited uptake of tracer adenosine. It also reduced adenosine kinase activity of the cell membrane fraction. In addition, FK506 does not inhibit membrane transport of tracer adenosine. These observations indicate that FK506 inhibits in situ adenosine kinase activity in endothelial cells. Other cell signaling inhibitors were found to inhibit adenosine uptake via inhibition of adenosine transport. In conclusion, FK506 promotes adenosine release from endothelial cells by a novel mechanism involving inhibition of adenosine kinase activity associated with the membrane. © 2001 Published by Elsevier Science B.V.

Keywords: FK506; Adenosine; Adenosine kinase; Endothelial cell

1. Introduction

Recent studies have suggested that the immunosuppressants, cyclosporin A and tacrolimus (FK506) mimic cardioprotective effects of ischemic preconditioning. Cyclosporin A has been shown to preserve post-ischemic left ventricular function in isolated rat hearts, with inhibition of mitochondrial ion pore opening in cardiomyocytes implicated as a mechanism of the action (Duchen et al., 1993). Cyclosporin A treatment also preserved myocardial function and nitric oxide production in isolated guinea pig hearts during ischemia–reperfusion (Massoudy et al., 1997). In addition, both cyclosporin A and its analog FK506 have been shown to reduce post-ischemic myocardial infarct size in isolated rabbit hearts (Weinbrenner et al., 1998). FK506 also improved post-ischemic cardiac function in isolated rat hearts (Haines et al., 2000). This finding raised the possibility of a role for calcineurin, a known target of both cyclosporin A and FK506. In brain and liver where both cyclosporin A and FK506 induce ischemic preconditioning, these drugs reduced infarct size in animal models of ischemia–reperfusion injury (Bochelen et al., 1999; Garcia-Criado et al., 1997; Toung et al., 1999). However, the mechanisms underlying the protective effect remain unclear.

Both cyclosporin A and FK506 can induce an increase in adenosine plasma levels in kidney transplant recipients, and the plasma levels of cyclosporin A and adenosine are closely correlated. The mechanism of action of the two drugs is not clear but a reduction in adenosine uptake by red blood cells has been observed (Guieu et al., 1998). Because adenosine also has a potent immunosuppressive effect, it has been proposed that endogenous adenosine action participates in cyclosporin A- and FK506-mediated immunosuppression (Guieu et al., 1998). In addition, adenosine is a mediator of ischemic preconditioning (Millar et al., 1996), and it also has anti-inflammatory and anti-thrombotic effects (Bouma et al., 1997b; Kitakaze et al., 1991). Therefore, it is possible that cyclosporin A- and FK506-induced organ protection is mediated by endogenous adenosine.

Among the enzymes involved in adenosine metabolism, adenosine kinase plays an important role in regulating formation and release of endogenous adenosine in cardiomyocytes as well as vascular endothelial cells (Decking et al., 1997; Kowaluk et al., 1998; Smolenski et al., 1994).

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Inhibition of adenosine kinase increases the extracellular adenosine level by increasing intracellular adenosine concentration and thereby increasing net release of adenosine from cells (Decking et al., 1997; Gorman et al., 1997). In fact, several studies have shown that adenosine kinase inhibitors have potent anti-inflammatory effects in animal models of inflammation (Bouma et al., 1997b; Cronstein et al., 1995). In rat models of septic shock, in vivo administration of an adenosine kinase inhibitor, GP-1-515 significantly reduced mortality, and this beneficial effect was attributed to A_2 adenosine receptor-mediated endogenous adenosine actions, such as decreased pulmonary neutrophil accumulation and decreased plasma levels of tumor necrosis factor-α (Firestein et al., 1994). Other studies also demonstrated that GP-1-515 reduced neutrophil degranulation (Bouma et al., 1997a), and neutrophil transmigration and vascular leakage in a skin model of inflammation in vivo (Rosengren et al., 1995). Accordingly, adenosine kinase is thought to play an important role in regulating endogenous adenosine release, which in turn regulates a vast array of cellular functions (Bouma et al., 1997b; Mullane and Bullough, 1995). Hence, understanding the biochemical mechanisms involved in the regulation of adenosine kinase will be important in the development of therapeutic drugs targeting the adenosine system.

We tested the hypothesis that FK506 induces adenosine release from cultured endothelial cells via inhibition of adenosine kinase activity. In the present study, we chose endothelial cells as a model system because recent studies suggest that the vascular endothelium is a potentially important source and target of endogenous adenosine (Mullane and Bullough, 1995).

2. Materials and methods

2.1. Materials

Rat prostate endothelial cells (YPEN-1, ATCC CRL-2222) were purchased from American Type Culture Collection (Rockville, MD). We chose YPEN-1 for this study because we obtained relatively high and easily extractable adenosine kinase activity from this cell line in our preliminary experiments. However, these results may be true for different endothelial cells because similar results were obtained from cow pulmonary artery endothelial cells (CPAE, ATCC CCL-209) as indicated in the results. Cells were serum-starved for 24 h at confluency before experiments unless stated otherwise.

All the cell culture media and reagents were purchased from GIBCO BRL (Rockville, MD). The immunosuppressants, cyclosporin A, FK506 and rapamycin, and kinase inhibitors, genistein (4′,5,7-trihydroxyisoflavone), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole), SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole), PD98059 (2′-amino-3′-methoxyflavone) and wortmannin were purchased from Calbiochem (San Diego, CA). The inhibitors of adenosine kinase, 5′-amino-5′-deoxyadenosine (NdADO) and 5′-iodotubercidin (4-amino-5-iodo-7-(*b*-D-ribofuranosyl)pyrrolo[2,3-D]-pyrimidine) were purchased from Sigma (St. Louis, MO) and RBI (Natick, MA), respectively. The inhibitors of adenosine transport, dipyridamole, and of adenosine deaminase, *erythro*-9-(2-hydroxy-3-nonyl) adenine (EHNA) were purchased from Sigma.

2.2. Preparation of cytosol and crude membrane fractions

Cytosol and membrane fractions were prepared using slight modifications of previously described methods (Andres and Fox, 1979; Stokoe et al., 1994). All procedures were carried out at 4 °C unless stated otherwise. Cells were grown in P-100 cell culture plates. Confluent cells were treated with drugs for 30 min in Hank's balanced salt solution (HBSS) at 37 °C. After treatment, cells were rinsed twice with cold phosphate buffered saline (PBS) and homogenized in cold 10 mM Tris–HC1 solution using a Polytron homogenizer. The Tris–HC1 solution contained 30 nM okadaic acid to preserve phosphorylation of adenosine kinase (Sparks et al., 1998). The homogenate was centrifuged for 10 min at $1500 \times g$ to remove cell debris and nuclei. The supernatant was recentrifuged for 1 h at $100{,}000 \times g$, and the resulting supernatant was used as a cytosolic fraction. The pellet was rinsed twice and resuspended with a hypotonic buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.4), and homogenized with 30 strokes in a Dounce homogenizer, resulting in the appearance of a particulate solution, which we call the crude membrane fraction. Adenosine kinase was purified from the above cytosolic fraction using size exclusion, CM and DEAE–sepharose ion exchange and AMP affinity chromatography according to the method of Andres and Fox (1979). After purification, the purity of the enzyme preparation was $> 85\%$ on SDS-PAGE (data not shown). Protein concentration of the enzyme solution was measured with a Bio-Rad kit making use of the Bradford method.

2.3. Assay of adenosine kinase activity

Activity of the purified adenosine kinase was measured as generation of [^{14}C] AMP from [U-^{14}C] adenosine (Amersham, Piscataway, NJ) using a previously described method (Gorman et al., 1997). The reaction mixture contained an appropriate amount of [U-^{14}C] adenosine (513 mCi/mol) and unlabeled adenosine in a sample buffer (50 mM Tris–HCl, 100 mM KC1, 5 mM ATP, 5.4 mM $MgCl_2$, 0.4 mg/ml bovine serum albumin, pH 7.4), resulting in a final adenosine concentration of 20 μM. The purified adenosine kinase prepared in the sample buffer was added to the mixture, and the reaction was run for 5

min at 37 °C. The reaction was stopped by heating at 90 °C for 3 min followed by chilling on ice for 3 min. AMP generation was linear (< 20% of the substrate added) under these conditions. The sample was centrifuged and assayed by high performance liquid chromatography (HPLC). HPLC conditions were designed to isolate adenine nucleotides and adenosine. A Waters C18 Nova-pak column was perfused isocratically at 1 ml/min with 65% solution A (0.5% of 1 M tetrabutylammonium dihydrogen phosphate (Pic-A), an ion-pairing reagent) and 35% solution B (70:30 methanol–water). Fractions of the eluate were collected every 1–2 min and counted on a liquid scintillation counter. The adenosine peak on the chromatogram was integrated and used to calculate total adenosine concentration, the specific activity and AMP concentration. Adenosine kinase activity in the cytosol or membrane fraction was measured using the same method for the purified enzyme preparation except 20 μM EHNA and 10 μM α, β-methyleneadenosine 5′-diphosphate (AOPCP) were added in the reaction mixture. This is to inhibit activities of adenosine deaminase (adenosine degradation) and 5′-nucleotidase (adenosine formation), respectively.

2.4. Adenosine release assay

Adenosine release was measured using a previously described method with slight modifications for a cell culture system (Deussen et al., 1986). Cells were grown in 24-well cell culture plates. Adenine nucleotide pools of confluent cells were prelabeled by exposure to [2, 8-^{3}H] adenosine (50 nM, 41 Ci/mmol; New England Nuclear, Boston, MA) for 35 min at 37 °C in 250 μl of HBSS containing 5 μM EHNA to inhibit adenosine deamination. Each well was then washed three times with HBSS, treated in triplicate with drugs in 500 μl of HBSS containing 5 μM of EHNA and incubated at 37 °C for 30 min. Immediately before the incubation period, medium from the vehicle-treated samples was collected from each plate and counted as background. After incubation, 400 μl of the medium in each well were collected and total radioactivity was measured using a liquid scintillation counter.

2.5. Adenosine uptake assay

Adenosine uptake was measured using slight modifications of a previously described method (Deussen et al., 1993). Cells were grown in 24-well cell culture plates. After 48 h of serum-starvation, confluent cells were pretreated in triplicate with drugs for 10 min in 450 μl of HBSS in each well. We then added 50 μl of HBSS containing [2, 8-^{3}H] adenosine (50–100 nM, 2–4 Ci/mmol), EHNA and unlabeled adenosine into the incubation medium, resulting in final EHNA and adenosine concentrations of 5 μM and 100 nM, respectively, and the cells were pulsed for 30 min at 37 °C. Each well was then washed twice with cold HBSS containing 100 μM unlabeled adenosine to terminate further tracer adenosine uptake. Cells were dissolved in 1.4 N perchloric acid and subsequently neutralized with 1.4 N KOH/1.4 N $NaHCO_3$. Total radioactivity in the solubilized cells was measured using a liquid scintillation counter.

2.6. Adenosine transport assay

Membrane transport of adenosine was measured using slight modifications of a previously described method (Sobrevia et al., 1994). Cells were grown in 24-well cell culture plates. Confluent cells were pretreated in triplicate with the drugs for 15 min at 22 °C in 250 μl of HBSS in each well. We then added 250 μl of HBSS containing the respective drug and [2, 8-^{3}H] adenosine (200 nM, 41 Ci/mmol) into the incubation medium and pulsed for 15 s in the presence of the adenosine kinase inhibitor, NdADO (10 μM), in order to measure only the membrane transport component of adenosine uptake (Gu et al., 1996). The adenosine transport was terminated by washing each well twice with cold HBSS containing an adenosine transport inhibitor, dipyridamole (10 μM) to prevent loss of tracer adenosine from cells. Cells were dissolved in 1.4 N perchloric acid and subsequently neutralized with 1.4 N KOH/1.4 N $NaHCO_3$. Total radioactivity in the solubilized cells was measured using a liquid scintillation counter.

2.7. Statistical analysis

Data are presented as means ± S.E. Statistical analysis was performed with *t*-test for comparison of two groups. Analysis of variance (ANOVA) was used to compare three or more groups, followed by the Bonferroni/Dunn's multiple comparison procedure. When data were normalized to percent changes from the control, the Kruskal–Wallis test was used for comparison of more than two groups. A P value of less than 0.05 was considered significant.

3. Results

3.1. Adenosine release

In order to evaluate the effect of FK506 on adenosine metabolism in endothelial cells, we prelabeled adenine nucleotide pools of YPEN-1 with [^{3}H] adenosine and then measured the tracer release into the fresh medium with and without drug treatment. FK506 significantly increases tracer release from the cells in a concentration-dependent manner (Fig. 1A). This increased release (70%) is significantly blocked by dipyridamole (10 μM), an adenosine transport inhibitor (Fig. 1B). We used the specific adenosine kinase inhibitor NdADO as a positive control for adenosine release from the cells. As expected, NdADO (10 μM) substantially increases tracer release (224%), which is also blocked by dipyridamole. Dipyridamole itself does

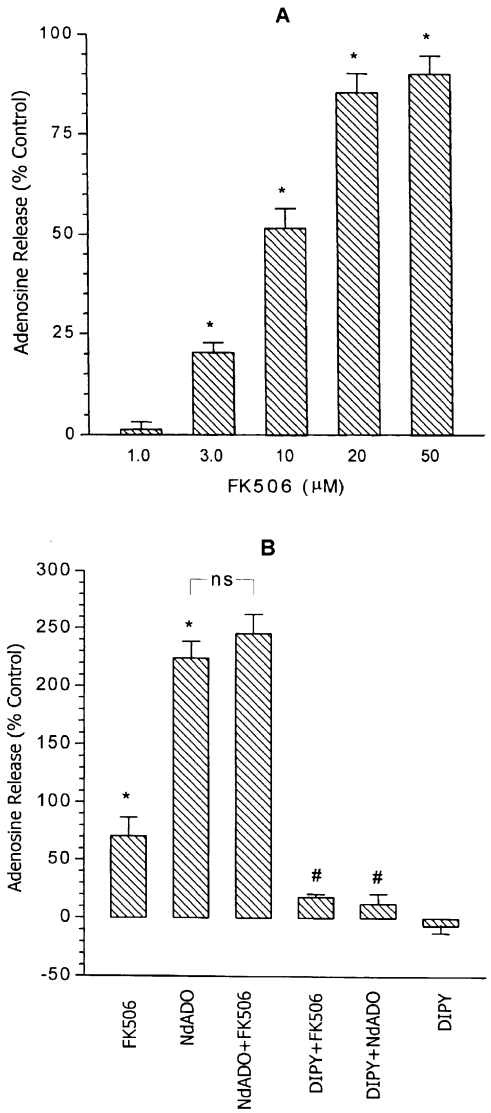

Fig. 1. FK506 increases adenosine release from YPEN-1. (A) Concentration-dependent effect of FK506 on adenosine release. (B) Effects of different drugs on adenosine release. Adenine nucleotide pools of confluent cells were prelabeled by exposure to [^{3}H] adenosine for 35 min. The cells were treated with the indicated drugs, and the tracer release from the cells was measured for 30 min. Dipyridamole (DIPY) and 5′-amino-5′-deoxyadenosine (NdADO) were used at 10 μM. Data are means ± S.E. ($n = 4$ for panel A and $n \geq 4$ for panel B). $^{*}P < 0.05$ versus control; $\#P < 0.05$ versus the respective treatment; ns, not significant.

not change tracer release from the cells. In order to determine if the FK506-induced increase in adenosine release is due to adenosine kinase inhibition, FK506 (10 μM) was added in the presence of NdADO (10 μM). As shown in Fig. 1B, the FK506 had minimal effect on adenosine release when adenosine kinase is completely inhibited by NdADO. These results indicate that FK506 induces an increase in adenosine formation by adenosine kinase inhibition in endothelial cells. Furthermore, the increase in adenosine formation is intracellular, and adenosine leaves the cells via the dipyridamole-sensitive purine transporter.

3.2. Adenosine uptake

We used [^{3}H] adenosine uptake (adenosine transport plus metabolism) into endothelial cells as an indirect measure of adenosine kinase activity in the cells. In preliminary experiments, adenosine and adenine nucleotides in the solubilized cells were separated using a Sep-pak cartridge, and the radioactivity associated with each fraction was counted. In control cells, 92% of radioactivity in the solubilized cells was associated with adenine nucleotides after 30 min of incubation at 37 °C in the absence of EHNA, an adenosine deaminase inhibitor. This shows the predominant role of adenosine kinase in adenosine uptake even when the degradation of intracellular adenosine to inosine is a possibility. When testing the effect of FK506 on adenosine uptake, we measured [^{3}H] adenosine uptake in the presence of EHNA. This assured that $> 92\%$ of the observed uptake of the tracer is due to adenosine kinase activity.

Cells were treated with FK506 following the protocol described in Section 2. After the cells were pulsed with [^{3}H] adenosine for 30 min under FK506 treatment, we measured the tracer level in the solubilized cells. FK506 decreases adenosine uptake into the cells in a concentration-dependent manner (Fig. 2A). In control cells, adenosine uptake rate was 0.5 ± 0.04 pmol/min/10^6 cells. FK506 at concentrations of 3 μM and higher is effective. Dipyridamole (10 μM) was used to measure background counts and inhibited the adenosine uptake into the cells by 90%.

In order to evaluate a potential mechanism of action of FK506 on adenosine uptake in endothelial cells, we used another immunosuppressant rapamycin, which is known to be a competitive inhibitor of the FK506 action on calcineurin (Bierer et al., 1990). However, we found that rapamycin also significantly decreases adenosine uptake into the cells in a concentration-dependent manner (Fig. 2B). The adenosine kinase inhibitor, 5′-iodotubercidin was used as a positive control, which inhibited the adenosine uptake by 77%. Another inhibitor of calcineurin, cyclosporin A, induced significant but only 25% inhibition at 100 μM (Fig. 2C), indicating that it is much less potent than FK506. Concentrations of cyclosporin A up to 10 μM have been previously tested in a similar preparation and reported to be without induction of endothelial damage (Bierer et al., 1990). Similar results on the concentration-dependent effect of these drugs on adenosine uptake were obtained from another endothelial cell line, CPAE (data not shown). Combined, these results suggest that calcineurin may not be the only mediator of actions of FK506, and that other mechanisms are involved in the FK506-induced inhibition of adenosine uptake.

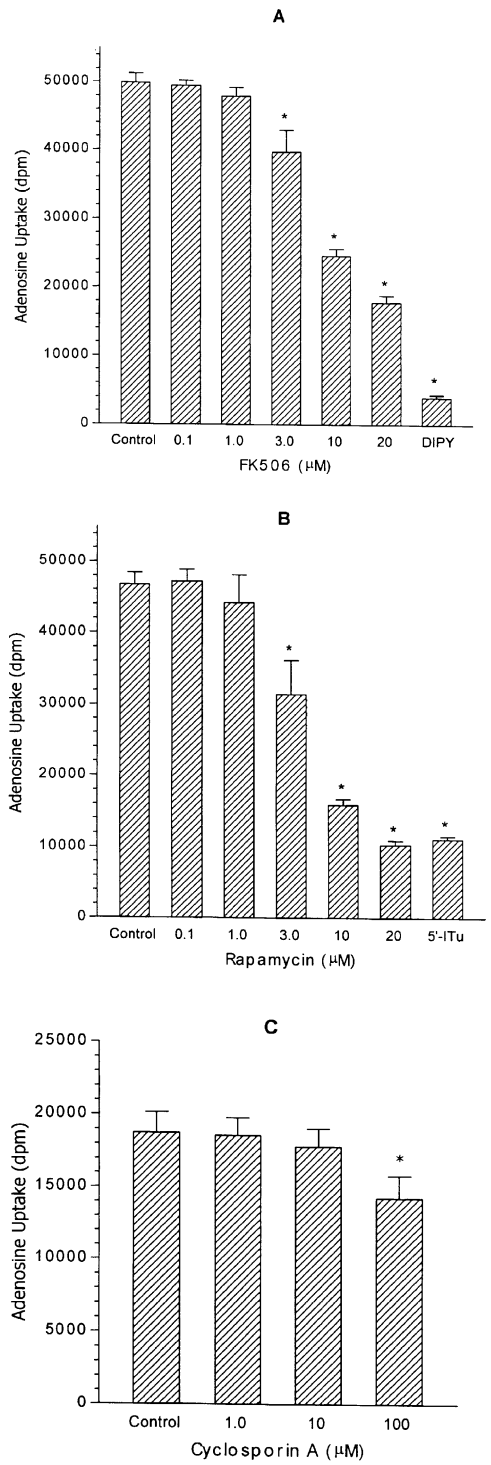

Fig. 2. Concentration-dependent effect of FK506, rapamycin or cyclosporin A on adenosine uptake. Confluent cells were pretreated with the indicated concentrations of FK506 (A), rapamycin (B) or cyclosporin A (C) for 10 min, and then exposed to [^{3}H] adenosine for 30 min. The uptake of adenosine into the cells was determined as described in Section 2.5. The adenosine transport inhibitor, dipyridamole (DIPY, 10 μM) or the adenosine kinase inhibitor, 5′-iodotubercidin (5′-ITu, 10 μM) was used as a positive control. Data are means ± S.E. ($n = 3$). $^*P < 0.05$ versus control.

3.3. Adenosine kinase activity

The uptake data strongly suggest that FK506 inhibits adenosine kinase activity in intact endothelial cells. If adenosine kinase activity is inhibited by a covalent modification of the enzyme, adenosine kinase activity in a cytosolic fraction should be decreased by previous treatment of the intact cells with FK506. However, neither FK506 nor rapamycin pretreatment changes the activity of adenosine kinase in the cytosolic fraction of the cells (Fig. 3A). In addition, FK506 pretreatment did not change the activity of adenosine kinase purified from the cell cytosol (data not shown).

It is possible that a cellular fraction of adenosine kinase, which modulates uptake of adenosine, is associated with the plasma membrane. In this case, FK506 might have no effect on total adenosine kinase or cytosolic adenosine kinase, while inhibiting the activity of membrane-associated adenosine kinase. Therefore, we tested the hypothesis

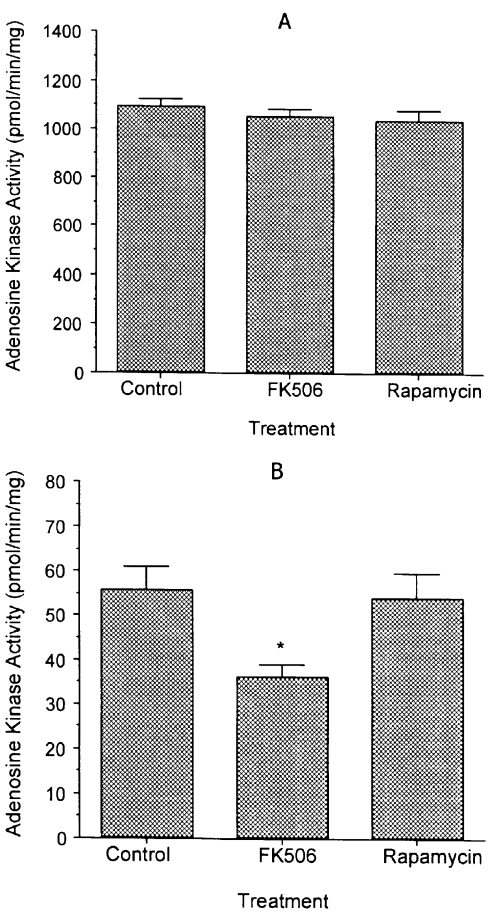

Fig. 3. Activities of adenosine kinase in cytosol (A) and membrane (B) fractions of YPEN-1 previously exposed to FK506 or rapamycin. Intact cells were pretreated with FK506 (10 μM) or rapamycin (10 μM) for 30 min. Cytosol and membrane fractions were prepared as described in Section 2. Data are expressed as the rate of AMP generation in pmol/min/mg protein in the sample (means ± S.E.). Control, $n = 8$ or 9; FK506, $n = 6$; rapamycin, $n = 5$. $^*P < 0.05$ versus control.

that the activity of adenosine kinase in cultured endothelial cells depends on the activity of the enzyme associated with the cell membrane fraction. Intact cells were treated with FK506, using the same protocol in previous experiments. Following treatment using the maximal concentration (10 μM), which has been previously demonstrated not to induce endothelial dysfunction in a similar preparation (Benigni et al., 1992), a crude membrane fraction was prepared, and adenosine kinase activity was measured as described in Section 2. Indeed, FK506 significantly decreases the activity of adenosine kinase by 35% in the crude membrane fraction (Fig. 3B). However, when cells were treated with rapamycin (10 μM) using the same protocol, no effect on the enzyme activity of the crude membrane fraction was observed.

3.4. Effects of mitogen-activated protein kinase (MAPK) inhibitors on adenosine metabolism

We further evaluated a potential signaling mechanism involved in adenosine kinase inhibition induced by FK506. Because FK506 as well as rapamycin and cyclosporin A have been shown to modulate MAPK pathways (Matsuda and Koyasu, 2000), we tested effects of the specific inhibitors for the MAPK pathways on adenosine uptake in endothelial cells. In each case, cells were treated with a MAPK inhibitor using a concentration, which has been previously demonstrated to be effective in a similar preparation (Laird et al., 1998). A tyrosine kinase inhibitor, genistein (10–100 μM) as well as specific p38 MAPK inhibitors, SB203580 (0.3–10 μM) and SB202190 (0.3–10 μM) decrease adenosine uptake in a concentration-dependent manner (data not shown). However, the inhibitors for MAPK/ERK kinase (MEK), PD98059 (0.1–10 μM), and for phosphatidyl inositol 3-kinase, wortmannin (0.1–10 μM) do not change adenosine uptake (data not shown). Similar results were also obtained with CPAE (data not shown).

We found that genistein and SB203580 have no effect on the adenosine kinase activity in either cytosolic or crude membrane fraction (data not shown) but inhibit adenosine transport (Fig. 4). Of note, the membrane transport of adenosine (the extent to which [^{3}H] adenosine is metabolized) was determined by pulsing the cells with [^{3}H] adenosine for 15 s in the presence of NdADO (10 μM) to measure only the transport component of adenosine uptake (Gu et al., 1996). Dipyridamole at a concentration, which completely inhibits the dipyridamole-sensitive purine transporters, was used as a positive control in adenosine transport assay (Fig. 4). Genistein (100 μM) is almost as effective as dipyridamole in inhibiting adenosine transport. Rapamycin and SB203580 are also effective in reducing adenosine transport. These drugs as well as dipyridamole, at a concentration known to block adenosine transport (Newby, 1986), had no effect on adenosine release (data not shown). By contrast, the adenosine kinase inhibitor, NdADO, and FK506, which inhibits the membrane fraction adenosine kinase activity but not adenosine transport (Fig. 4), both increase adenosine release (Fig. 1).

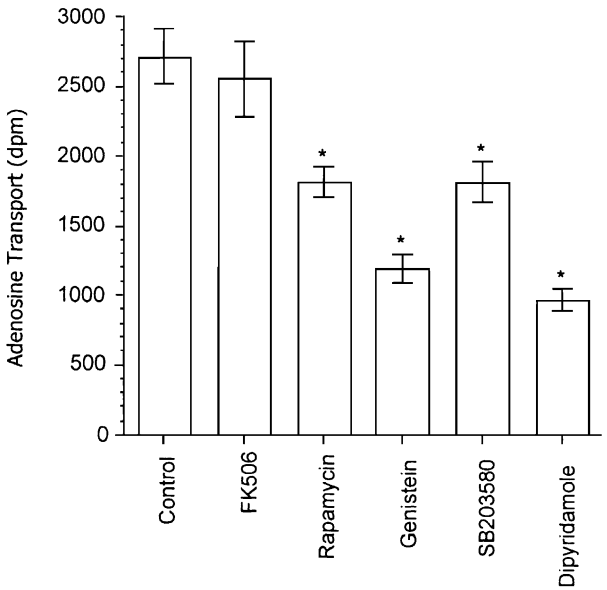

Fig. 4. Adenosine transport into YPEN-1 under the different drug treatments. Confluent cells were pretreated with the indicated drugs for 15 min. Then, the cells were exposed to [^{3}H] adenosine and pulsed for 15 s. The membrane transport of adenosine into the cells was determined as described in Section 2. The adenosine transport inhibitor, dipyridamole (10 μM) was used as a positive control. FK506 (10 μM); rapamycin (10 μM); genistein (100 μM); SB203580 (10 μM). Data are means ± S.E. ($n = 4$). $^*P < 0.05$ versus control.

4. Discussion

4.1. FK506 inhibits adenosine kinase activity in cultured endothelial cells

Three lines of evidence indicate that FK506 inhibits in situ adenosine kinase activity in cultured endothelial cells. FK506: (a) inhibits uptake of tracer adenosine (Fig. 2A), (b) enhances release of tracer adenosine (Fig. 1) and (c) reduces adenosine kinase activity of a crude membrane fraction of endothelial cells (Fig. 3B). In addition, FK506 does not inhibit membrane transport of tracer adenosine (Fig. 4). Each of these lines of evidence is evaluated below.

We used adenosine uptake as a screening tool to establish the possibility that FK506 inhibits in situ adenosine kinase activity. We did this because our own preliminary experiments, as well as the work from other laboratories, indicated that it is extremely unlikely that adenosine kinase could be inhibited without an inhibition of adenosine uptake (Decking et al., 1994; Spychala et al., 1997). In preliminary experiments, we found that when cultured endothelial cells are exposed to extracellular [^{3}H] adeno-

sine, more than 90% of the tracer taken up into the cells is in the form of adenine nucleotides. This means that adenosine kinase predominates over adenosine deaminase in providing the major pathway for adenosine metabolism, once adenosine enters the cell. This result is comparable to previous observations in human and porcine endothelial cells in culture (Shryock et al., 1988; Smolenski et al., 1994; Sobrevia et al., 1994). In fact, the role of adenosine deamination in cultured endothelial cells was small even at high concentrations of adenosine (10 μM) in the incubation medium (Sobrevia et al., 1994). Nevertheless, we included an inhibitor of adenosine deaminase, EHNA, in all uptake and release experiments so that the intracellular sink for adenosine would be via adenosine kinase.

FK506 induces an increase in adenosine release after the adenine nucleotide pools of cultured endothelial cells are prelabeled with [^{3}H] adenosine (Fig. 1). Because an adenosine deaminase inhibitor, EHNA, was added during both prelabeling and release periods, the elevated tracer in the medium cannot be inosine or its metabolites. Other investigators have shown that endothelial cells can also release adenine nucleotides and cAMP and can form extracellular adenosine via extracellular dephosphorylation of AMP by ecto-5′-nucleotidase (Borst and Schrader, 1991; Deussen et al., 1993; Kroll et al., 1987). However, in our experiment, the purine transporter inhibitor dipyridamole blocked the increase in tracer release in response to FK506. Adenine nucleotides and cAMP do not leave cells via the dipyridamole-sensitive purine transporter (Thorn and Jarvis, 1996). Moreover, addition of FK506 in the presence of the maximal concentration of the adenosine kinase inhibitor, NdADO, did not cause further increase in adenosine release. Therefore, intracellular adenosine is the only source for tracer found in the incubation medium in our release experiments, and the increase in adenosine release by FK506 is due to adenosine kinase inhibition.

Intracellular adenosine has three possible fates: (a) phosphorylation to AMP, (b) deamination to inosine and (c) exit from the cell by a purine transporter. When adenosine kinase is inhibited, the importance of the other pathways will be increased. Thus, adenosine kinase inhibition is expected to result in increased adenosine release (Decking et al., 1994). Indeed, the adenosine kinase inhibitors used in our experiments uniformly increase adenosine release, as does FK506 (Fig. 1).

Pretreatment of cultured endothelial cells with FK506 did not cause inhibition of purified adenosine kinase or adenosine kinase activity of the cytosolic fraction. However, FK506 causes a decrease in activity of adenosine kinase in membrane fraction isolated from cultured endothelial cells previously exposed to the drug (Fig. 3B). This finding raises the possibility that FK506 acts either by decreasing the activity of adenosine kinase associated with the plasma membrane (e.g. dephosphorylation) (Sparks et al., 1998), or by translocating the membrane adenosine kinase to the cytosol. If the latter is the case, one might expect to see a reciprocal change in cytosolic adenosine kinase activity when membrane adenosine kinase activity changes. However, cytosolic adenosine kinase activity is so much greater than membrane adenosine kinase activity that changes in the cytosolic enzyme activity due to transfer to or from the membrane would be impossible to detect (Fig. 3A). In a recent abstract, Spychala and Mitchell (1998) reported similar results in T lymphocytes although a decrease in adenosine kinase activity in response to cyclosporin A or FK506 was observed in the cell lysate preparation.

In order to determine activity of adenosine kinase associated with the plasma membrane of cultured endothelial cells, we used a crude membrane fraction according to the method previously described. This method has been used to measure activity of a protein kinase Raf translocated to plasma membrane (Stokoe et al., 1994). This fraction also contains mitochondria and microsomes. However, we believe that it is likely that adenosine kinase activity is associated with plasma membrane. Spychala recently found a detectable amount of adenosine kinase activity in a plasma membrane fraction isolated from lymphocytes (personal communication). The K_m of adenosine kinase in the membrane fraction was two to six times lower than that of adenosine kinase in the cytosolic fraction, suggesting a modification of the enzyme associated with plasma membrane. It is possible that adenosine kinase associated with plasma membrane would be easily accessible to both adenine nucleotide pools and adenosine transporters on the cell membrane. There is no evidence that mitochondria or microsomes contain adenosine kinase.

4.2. Effects of MAPK inhibitors on adenosine uptake and adenosine transport in cultured endothelial cells

We planned to use rapamycin, genistein and SB203580 as tools to explore the pathway by which FK506 exerts its effect on adenosine kinase. Initially, we were encouraged by the fact that all three of these agents inhibit adenosine uptake. However, further experiments indicated that these agents do not act on adenosine uptake by inhibiting adenosine kinase activity. They (a) have no effect on cytosolic or membrane fraction adenosine kinase activity, (b) have no effect on adenosine release and (c) inhibit adenosine transport into endothelial cells (Fig. 4).

The membrane transport of adenosine was measured by observing the first seconds of uptake of tracer adenosine in the presence of the specific inhibitor of adenosine kinase activity, NdADO. This means that radioactivity associated with the cells gives a measure of adenosine entering the cells. The portion of this accumulation blocked by dipyridamole gives an indication of the activity of the dipyridamole-sensitive purine transporters. Genistein was almost as effective in blocking adenosine transport as dipyridamole, and both rapamycin and SB203580 had substantial activity (Fig. 4).

In the present study, the mechanism of actions of these drugs on adenosine transporters were not explored. It could be either nonspecific effects of the drugs on adenosine transporters or a cell signaling-mediated effect. It has been shown in cultured chromaffin cells that secretagogues, such as acetylcholine and nicotine, and protein kinase C activators inhibited adenosine uptake into the cells via downregulation of high affinity adenosine transporters (Delicado et al., 1991). In fact, the effect of these drugs on adenosine transport is important in the in vivo context because adenosine transport inhibition has been shown to induce an increase in the extracellular adenosine level and to potentiate adenosine actions (Thorn and Jarvis, 1996).

In summary, it appears that immunosuppressive drugs like FK506 and cyclosporin A may act in novel ways to elevate extracellular adenosine levels. Our results indicate that FK506 promotes adenosine release by a novel mechanism involving inhibition of the adenosine kinase activity associated with membrane. Therefore, it is possible that endogenous adenosine action is involved in FK506- and cyclosporin A-induced ischemic organ protection. Interestingly, it has been recently found that the anti-inflammatory effect of aspirin is also in part mediated by endogenous adenosine action. (Cronstein et al., 1999a,b). Further studies are required to evaluate biochemical mechanisms involved in the FK506-induced inhibition of adenosine kinase activity associated with cell membrane.

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