This is to certify that the

dissertation entitled

REGULATION OF ADENOSINE KINASE AND ADENOSINE RELEASE FROM ENDOTHELIAL CELLS

presented by

Kwan-Ki Hwang

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

Major professor

Date Dec 15, 1999

REGULATION OF ADENOSINE KINASE AND ADENOSINE RELEASE FROM ENDOTHELIAL CELLS

By

Kwan-Ki Hwang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1999

ABSTRACT

REGULATION OF ADENOSINE KINASE AND ADENOSINE RELEASE FROM ENDOTHELIAL CELLS

By

Kwan-Ki Hwang

Adenosine (ADO) has widespread effects on mammalian systems. Adenosine kinase (AK) is an important regulator of extracellular levels of ADO, which exerts its effects via stimulation of ADO receptors on the cell membrane. In the present study, we investigated the biochemical mechanisms involved in regulation of AK and ADO release. We used cultured endothelial cells (EC) as a model system since EC on the vascular wall are a potentially important source and target of endogenous ADO.

AK was purified from cow pulmonary artery endothelial cells (CPAE) and rat prostate endothelial cells (YPEN-1), and the Vmax and Km values for both cell lines were determined. We evaluated a potential for modification of AK activity by a specific phosphorylation of the enzyme. Purified AK from EC is immunoreactive with both phosphoserine and phosphothreonine antibodies, and the serine/threonine phosphatase, PPλ decreases the activity of

AK purified from EC. We also evaluated effects of different signaling effectors and inhibitors as pharmacological probes in order to identify the signaling mechanisms involved in regulation of AK activity in EC. Three lines of evidence indicate that the immunosuppressant, FK506 inhibits *in situ* AK activity in EC. FK506 inhibits uptake of tracer ADO, enhances release of tracer ADO and reduces AK activity of a crude membrane fraction of EC. In addition, FK506 does not inhibit transport of tracer ADO. Another immunosuppressant (rapamycin), a tyrosine kinase inhibitor (genistein) and p38 MAPK inhibitor (SB203580) decreased ADO uptake via inhibition of ADO transport. Hormones, or the signaling effectors and inhibitors involved in PKA and PKC pathways or other protein kinases (MEK and PI_3K) and phosphatases (PP1 and PP2A) did not have a significant effect on ADO uptake, measured as an index of AK activity. In conclusion, phosphorylation is a potential mechanism for regulation of AK activity in EC. The mechanism of action of FK-506 in ADO metabolism is inhibition of AK, and AK activity associated with the plasma membrane could be responsible for changes in the extracellular ADO levels. Therefore, FK506 promotes ADO release from EC by a novel mechanism involving inhibition of the AK activity associated with the plasma membrane.

To God and to my family

ACKNOWLEDGMENTS

I want to express my gratitude to my mentor, Dr. Harvey V. Sparks, for his advice and support throughout my graduate career. I would like to extend my thanks to the members of my dissertation committee, Drs. Gregory D. Fink, Kathleen A. Gallo, L. Karl Olson and William S. Spielman, for their advice and guidance. I would also like to thank my colleagues, Carolyn S. Hall, Wojciech Nowak and Dr. Narayanan Parameswaran. Valuable advice from Dr. Jozef Spychala in University of North Carolina at Chapel Hill is also gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

LIST OF ABBREVIATIONS

5′-ITu	5′-Iodotubercidin
5′-NDADO	5′-Amino-5′-deoxyadenosine
5′-NT	5′-Nucleotidase
8-SPT	8-(*p*-Sulfophenyl)theophylline
ADA	Adenosine deaminase
ADO	Adenosine
AK	Adenosine kinase
AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
CAMP	Adenosine 3′,5′-cyclic monophosphate
CsA	Cyclosporin A
DAG	1,2-Diacyl-sn-glycerol
EC	Endothelial cells
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
ERK	Extracellular signal-regulated kinase
FKBP	FK506 binding protein
GTP	Guanosine 5′-triphosphate
I-1	Inhibitor-1
IL	Interleukin
IMP	Inosine 5′-monophosphate
IP_3	Inositol 1,4,5-triphosphate
IκB	Inhibitor κB
JNK	*jun*-amino terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NE	Norepinephrine
NF-AT	Nuclear factor of activated transcription
NFκB	Nuclear factor κB
NMR	Nuclear magnetic resonance
PI_3K	Phosphatidyl inositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPλ	Protein phosphatase lambda
RTK	Receptor tyrosine kinase
TNF-α	Tumor necrosis factor-α

1. INTRODUCTION

Adenosine is well known as an important regulator of vascular tone and cardiac function (Berne, 1980; Shryock and Belardinelli, 1997). In addition, ADO has immunosuppressive, anti-inflammatory and anti-thrombotic effects (Bouma et al., 1997b; Kitakaze et al., 1991). These effects of ADO are mediated by stimulation of ADO receptors on the cell membrane, resulting in G protein-coupled signaling cascades (Olsson and Pearson, 1990). It has been suggested that AK is an important regulator of extracellular ADO levels. AK phosphorylates and recycles ADO to AMP, and inhibition of AK causes a substantial release of ADO from EC as well as from cardiac myocytes (Decking et al., 1997; Ely et al., 1992; Kroll et al., 1993). Although AK has been purified and cloned from several sources, the biochemical mechanisms that regulate the enzyme activity are still unknown. Recently, it has been found that inhibition of cardiac AK can be achieved by decreasing the phosphorylation level of the enzyme (Sparks et al., 1998), suggesting that a signaling pathway is involved in the regulation of AK and ADO release. In the present study, we used cultured EC as a model system to study regulation of AK and ADO release. EC play active roles in vascular tone, inflammation and thrombosis (Liao, 1998; Luscinskas and Gimbrone, 1996). It is also known to

be a potentially important source and target of endogenous ADO (Mullane and Bullough, 1995). Therefore, the purpose of the study was to purify and to characterize AK from cultured EC. We also evaluated the potential for modification of AK activity by biochemical mechanisms including second messenger systems, a specific phosphorylation of the enzyme, and association of the enzyme with plasma membrane in cultured EC.

1.1. Physiological activities of ADO

ADO is a purine nucleoside, which participates in the regulation of physiological activity in many different mammalian cells and tissues (Fig. 1). It has widespread effects on the cardiovascular, nervous, renal and immune systems (Olsson and Pearson, 1990; Shryock and Belardinelli, 1997). First, ADO has anti-adrenergic effects on the heart. It has been shown to have negative inotropic, chronotropic, dromotropic and anti-arrhythmic effects on the heart, and inhibits sympathetic nerve discharge. These effects reduce myocardial energy demand and protect the heart under excessive catecholamine stimulation or oxygen deficiency (Ely and Berne, 1992; Shryock and Belardinelli, 1997). ADO also regulates vascular tone in the coronary, cerebral, splanchnic and renal circulations (Olsson and Pearson, 1990). The adenosine hypothesis of coronary vasodilation

was based on the findings that ADO release is increased during periods of decreased cellular oxygen supply/demand ratio, such as hypoxia and ischemia. It suggests that ADO increases coronary blood flow during periods of the decreased oxygen supply/demand ratio, returning the ratio toward normal in the heart (Berne, 1980; Sparks and Bardenheuer, 1986). The main source of ADO in the heart was found to be cardiac myocytes (Deussen et al., 1986).

Second, ADO has anti-infarct effect of ischemic preconditioning in several organs including heart, brain and kidney (Millar et al., 1996; Sweeney, 1997). Ischemic preconditioning (a sublethal episode of ischemia) renders the organ resistant to infarction caused by a subsequent ischemic insult, and this protective effect is mediated by ADO (Millar et al., 1996; Thornton et al., 1990). It is thought that activation of A_1 and A_3 ADO receptors, PKC and ATP-sensitive K^+ channels confers the protection (Downey et al., 1993; Wang et al., 1997).

There are four different types of ADO receptors, namely A_1, A_{2A}, A_{2B} and A_3 ADO receptors classified on the basis of both pharmacological analyses and receptor cloning studies (Shryock and Belardinelli, 1997). Stimulation of different ADO receptors modulates the activity of various second messenger systems via G protein-coupled signaling cascades. The anti-adrenergic effects of ADO are mediated by A_1 ADO

receptors (G_i) on cardiac myocytes, which decrease cAMP formation, stimulate K^+ efflux and inhibit Ca^{2+} influx among their effects. The vasodilatory effect of ADO is thought to be mediated by stimulation of A_2 ADO receptors (G_s) on vascular smooth muscles cells, resulting in an increase of cAMP formation and a decrease of Ca^{2+} influx (Olsson and Pearson, 1990). It has been also reported that ADO increases release of an endothelium-derived relaxing factor, nitric oxide (NO), by an ADO receptor-mediated mechanism in EC (Li et al., 1995).

Recently, the anti-inflammatory and anti-thrombotic effects of ADO have been emphasized (Bouma et al., 1997b; Mullane and Bullough, 1995). Many investigators have identified ADO as an important modulator of neutrophil functions both *in vivo* and *in vitro* studies. ADO inhibited adherence of neutrophils to endothelium and generation of superoxide anions from activated neutrophils, preventing endothelial injury and preserving the endothelium-mediated coronary artery relaxation. A_2 ADO receptor stimulation in neutrophils has been implicated in the mediation of these anti-inflammatory actions of ADO (Cronstein et al., 1992; Cronstein et al., 1985; Zhao et al., 1996). It also inhibited neutrophil degranulation, particularly the release of elastase, a serine protease via A_2 and A_3 ADO receptor stimulation (Bouma et al., 1997a).

In the study of a canine ischemia-reperfusion model *in vivo*, A_2 ADO receptor stimulation reduced cardiac infarct size. This protective effect was associated with inhibition of neutrophil accumulation, superoxide generation and adherence to the coronary endothelium (Jordan et al., 1997). Other investigators also found that ADO inhibited neutrophil function at multiple stages of an adhesion cascade including the selectin-mediated initial interaction with endothelium (rolling), the integrin-mediated adhesion (arrest) and leukocyte transmigration (Grisham et al., 1989; Bouma et al., 1997b; Bullough et al., 1996; Firestein et al., 1995). Moreover, ADO has been found to reduce release of several monocyte pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8 in both *in vivo* and *in vitro* studies (Bouma et al., 1997b).

ADO inhibits platelet aggregation and thrombus formation (Kitakaze et al., 1991) via A_2 ADO receptor-mediated mechanism (Hourani and Cusack, 1991). It also prevents platelet adhesion to coronary endothelium via both A_1 and A_2 ADO receptors. In isolated guinea pig hearts under ischemia and reperfusion, endogenous ADO released from the hearts prevented platelet adhesion even in the presence of thrombin. Both A_1 and A_2 ADO receptor antagonism blunted the anti-thrombotic effect of endogenous ADO under these conditions (Seligmann et al., 1998). Consequently, ADO

exerts beneficial cardiovascular, anti-inflammatory and anti-thrombotic activities that may provide protection during ischemia-reperfusion and inflammation.

1.2. Role of AK in ADO metabolism

The major pathways and enzymes involved in ADO metabolism are illustrated in Fig. 1 (Kochan et al., 1994). One of the major pathways for the formation of ADO is the dephosphorylation of AMP to ADO catalyzed by 5'-NT. There are different isoforms isolated from cardiac tissue. One of these is a membrane-bound ecto-5'-NT, which catalyzes dephosphorylation of AMP derived from extracellular ATP or cAMP. In addition, there are two soluble, cytosolic isoforms, one with a relatively high affinity for AMP and another with a high affinity for IMP (Kroll et al., 1987; Minamino et al., 1995; Newby, 1991).

The extracellular level (interstitial or plasma) of ADO is not only dependent upon its rate of formation but also its rate of removal by cellular uptake and deamination. The cellular uptake of ADO is an effective and rapid process in EC, red blood cells and cardiac myocytes (Nees et al., 1985; Sparks et al., 1985). ADO is transported across the plasma

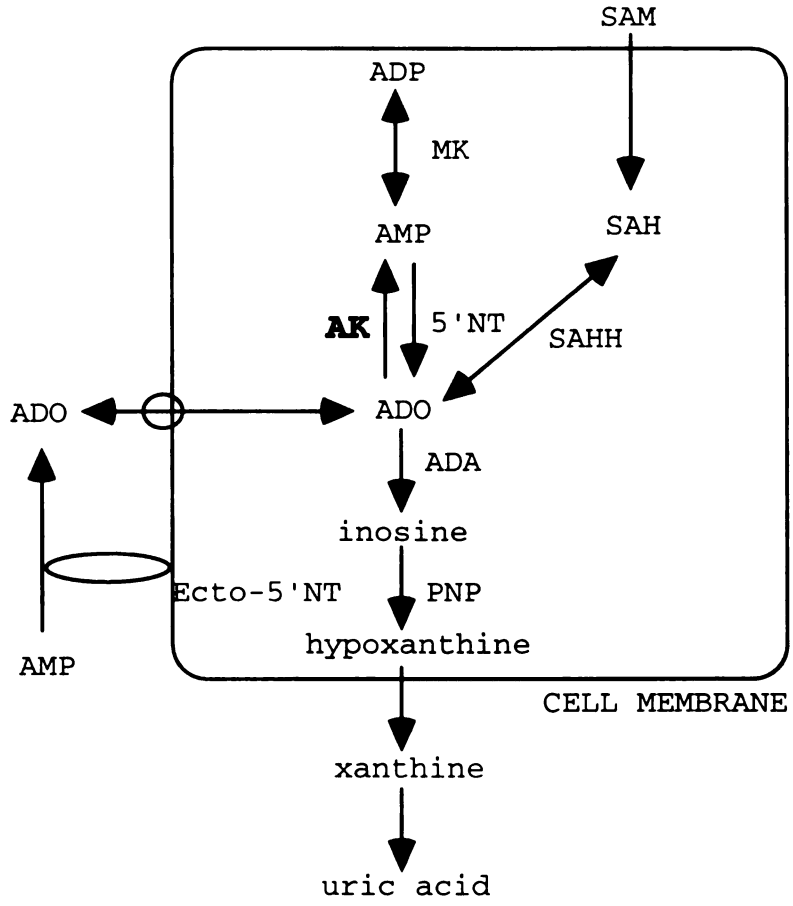

Figure 1. The major pathways and enzymes involved in ADO metabolism

AK, adenosine kinase; 5'-NT, 5'-nucleotidase; ADA, adenosine deaminase; MK, myokinase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; SAHH, SAH hydrolase; PNP, purine nucleoside phosphorylase.

membrane through ADO transporters by facilitated diffusion, and pharmacological inhibition of the transporters leads to an increase in the extracellular ADO levels (Thorn and Jarvis, 1996). The major pathways for the removal of ADO are the rephosphorylation of ADO to AMP by AK (a salvage pathway) and the deamination of ADO to inosine by cytosolic and ectoenzyme forms of ADA (a catabolic pathway). Inhibition of these enzymes also promotes an increase in ADO levels and potentiates various ADO actions (Olsson and Pearson, 1990; Shryock and Belardinelli, 1997). AK is an abundant enzyme present in many mammalian cell types including cardiac myocytes and EC (Kochan et al., 1994). It catalyzes the phosphorylation of ADO to AMP as well as several purine nucleosides and analogs to nucleoside monophosphates, using either ATP or GTP as the phosphoryl group donor (Lin et al., 1988).

Among the enzymes involved in ADO metabolism, AK is thought to play an important role in regulating formation and release of endogenous ADO. Endogenous adenosine formation can be increased by (a) increased AMP concentration, (b) activation of 5'-NT or (c) inhibition of AK. Until recently, an increase in ADO formation was thought to be mediated via an increase in AMP concentration and/or activation of 5'-NT (Olsson and Pearson, 1990; Sparks and Bardenheuer, 1986). Deking *et al.* (1997), however,

provided evidence on the importance of AK in isolated perfused guinea pig hearts. They found that hypoxia provoked a 15-20 fold increase in ADO release. Because only a 3-4 fold increase could be accounted for by ADO formation directly from AMP, as measured by NMR, they concluded that most of the increase in ADO release occurred because of AK inhibition. Furthermore, they have also shown that in the well-oxygenated heart, more than 90% of ADO formed in the heart is recycled to AMP via AK. In addition, pharmacological inhibition of AK by 5'-ITu caused an increase (15-20 fold) in ADO release in the heart comparable to hypoxia without a change in cytosolic AMP concentration. These results provided evidence that there is an active cycling between AMP and ADO (Kroll et al., 1993). Whether ADO is formed intracellularly (by cytosolic 5'-NT) or extracellularly (by ecto-5'-NT), it moves across the plasma membrane via facilitated diffusion (by ADO transporter) and is rephosphorylated to AMP by AK. Therefore, inhibition of AK increases the extracellular ADO level either by increasing intracellular ADO concentration or by reducing extracellular ADO uptake (Gorman et al., 1997).

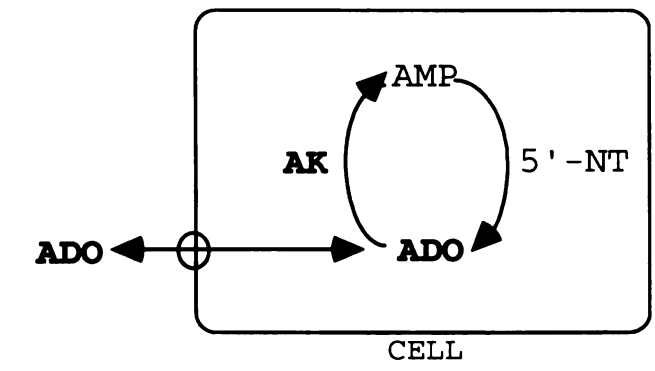

Figure 2. The active cycling between AMP and ADO

Recently, many investigators have evaluated the therapeutic potential of endogenous ADO produced by AK inhibition in *in vivo* and/or *in vitro* animal models of ischemia, epilepsy, nociception and inflammation (Kowaluk et al., 1998). For example, in the brain, where ADO has neuroprotective effects, pharmacological inhibition of AK reduced the infarct size in a rat model of acute brain ischemia (a temporary middle cerebral artery occlusion). This protection was observed without a significant change in other physiological parameters including body weight and brain temperatures (Jiang et al., 1997; Miller et al., 1996; Tatlisumak et al., 1998). Several studies have shown that AK inhibitors also have potent anti-inflammatory effects in both *in vivo* and *in vitro* models. In rat models of septic shock *in vivo*, administration of an AK inhibitor, GP-1-515

significantly reduced mortality, and this beneficial effect was attributed to A_2 ADO receptor-mediated endogenous ADO actions, such as decreased pulmonary neutrophil accumulation and decreased plasma level of TNF-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 *in vivo* skin model of inflammation (Rosengren et al., 1995). Accordingly, AK is thought to play an important role in regulating endogenous ADO release, which in turn regulates a vast array of cellular functions (see Section 1.1). Hence understanding the biochemical mechanisms involved in regulation of AK will be important in the development of therapeutic drugs targeting the adenosine system.

1.3. Regulation of AK

Although AK has been purified and cloned from several sources (Singh et al., 1996; Spychala et al., 1996), biochemical signaling mechanisms which regulate the enzyme activity are still unknown. The long-term goal of this study is to identify signal transduction systems and biochemical mechanisms responsible for the regulation of AK activity.

A survey of human tissues isolated from autopsy specimens indicated that AK activity is high in brain but is

relatively low in skeletal muscle. The level of AK mRNA, however, is much lower in the brain than that of the skeletal muscles, and hence other factors regulating the protein synthesis or enzymatic activity could be postulated (Snyder and Lukey, 1982). Other investigators have shown that stimulation of cultured EC with a Ca^{2+} ionophore concentration dependently increases ADO release (Deussen et al., 1993), suggesting a role of Ca^{2+} signaling in ADO metabolism. Induction of HL-60 myeloid and lymphoid cell differentiation by PMA results in a decrease in AK activity and mRNA levels (Spychala et al., 1997). In addition, it has been recently demonstrated that the immunosuppressants CsA and FK506, which inhibit PP2B (calcineurin), decrease both ADO uptake and lysate AK activity in T-lymphocytes, and increase ADO release, further indicating that AK activity can be regulated by non-energetics-dependent biochemical mechanisms (Spychala and Mitchell, 1998).

Studies from our laboratory have shown that elevated inorganic phosphate (Pi) concentration causes independent allosteric inhibition of AK purified from isolated retrograde perfused guinea pig hearts (Gorman et al., 1997). Preliminary results, however, indicate that other mechanisms of AK inhibition exist. When guinea pig hearts were infused with NE and with NE in the presence of ADO receptor antagonists 8-SPT or caffeine, or when hearts were made

hypoxic, ADO release was increased in excess of what can be explained by increased cytosolic Pi concentration, as measured by NMR. In addition, the activities of AK purified from hypoxic hearts and hearts infused with NE and with NE in the presence of 8-SPT or caffeine were significantly lower than that of the control hearts, suggesting other mechanisms of AK regulation (Gorman et al., 1997).

Because reversible phosphorylation is a common mechanism of enzyme regulation, the authors postulated that phosphorylation of AK regulates its activity. In line with that, treatment of purified AK with the serine/threonine phosphatase (PPλ) decreased its activity as well as the threonine phosphorylation level of the enzyme. The same result was also obtained with hypoxic guinea pig hearts, where a decreased level in phosphorylation, a decrease in AK activity and an increase in ADO release compared to the normal controls were observed (Sparks et al., 1998). To our knowledge this is the first demonstration of AK being modified by a phosphorylation, and it is a significant determinant of AK activity and contributes to the regulation of myocardial ADO release. To further elucidate the signaling pathways involved in the modification of AK, we have used cultured EC as a model system.

1.4. Vascular endothelium and ADO

Recent studies suggest that the vascular endothelium is a potentially important site of both ADO formation and actions. The endothelial cell has all the key enzymes involved in ADO metabolism (Kochan et al., 1994). It has been demonstrated that endothelium makes a significant contribution (14%) to myocardial ADO release from Langendoff-perfused guinea pig hearts in normoxia (Kroll et al., 1987). In fact, removal of endothelium significantly reduced ADO release from a canine coronary artery and from an isolated rabbit aorta (Minamino et al., 1995; Sedaa et al., 1990). The ADO release from EC is also increased under stress conditions, such as ischemia and hypoxia. For example, hypoxia and/or ischemia increased ADO release from EC of isolated perfused guinea pig hearts (Borst and Schrader, 1991; Deussen et al., 1986). In addition, in cultured EC, AK inhibition by 5'-ITu also induces a substantial increase in ADO release, indicating that the active cycling between AMP and ADO is operating in this cell type as well as cardiac myocytes (Fig. 2) (Smolenski et al., 1994).

It has been well known that EC also play an active role in vascular tone, inflammation and thrombosis (Liao, 1998; Luscinskas and Gimbrone, 1996). The vascular endothelium mediates vasodilation by releasing endothelium-derived

relaxing factor NO, endothelium-derived hyperpolerizing factor and prostacyclin via G protein-coupled receptor stimulation in EC. It can also produce endothelium-derived contracting factors including endothelin, angiotensin II, thromboxane A_2 and superoxide anions (Liao, 1998) (Vanhoutte, 1997).

In particular, vascular endothelium participates in localized accumulation of activated leukocytes, which is the cellular hallmark of inflammation. Vascular endothelium can be activated by inflammatory cytokines, such as IL-1 and TNF-α, oxidized low-density lipoproteins, and bacterial endotoxins, such as LPS. The activated endothelium facilitates leukocyte-endothelial adhesive interactions via the cell-surface expression of various adhesion molecules: endothelial-leukocyte adhesion molecules (e.g. E and P-selectin), intercellular adhesion molecules (e.g. ICAM-1 and 2) and a vascular cell adhesion molecule (e.g. VCAM-1) among them (Luscinskas and Gimbrone, 1996). Furthermore, activated endothelium also produces various leukocyte chemoattractants, such as monocyte chemotactic protein-1, IL-8 and platelet-activating factor (Springer, 1994). Therefore, EC can play an active role in an adhesion cascade by mediating the initial interactions with leukocytes (rolling), leukocyte adhesion (arrest) and the subsequent movement across the endothelial junctions (transmigration)

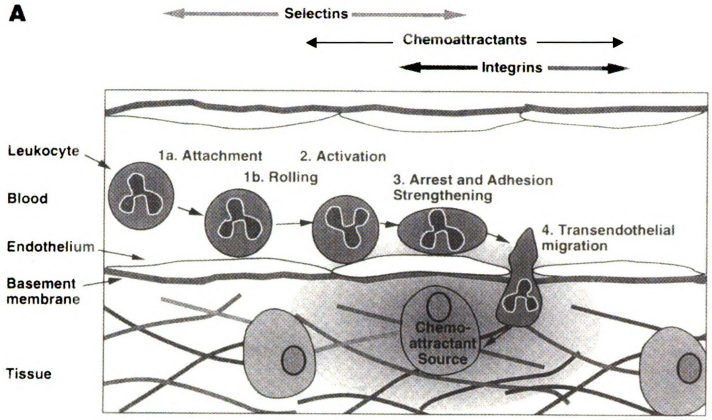

Figure 3. The adhesion cascade during inflammation (Springer 1994). ADO inhibits leukocyte functions at multiple stages of an adhesion cascade including the selectin-mediated initial interaction with endothelium (rolling), the integrin-mediated adhesion (arrest) and leukocyte transmigration.

(Luscinskas and Gimbrone, 1996).

In addition to its role in inflammation, activation of vascular endothelium is an important feature of the artherogenic process. In fact, it has been proposed that artherogenesis is a result of chronic inflammatory processes (Liao, 1998). Both leukocytes and blood platelets contribute to myocardial ischemia-reperfusion injury by interacting with activated endothelium and with one another (Becker et al., 1998; Becker et al., 1996; Gawaz et al., 1996).

ADO has been known to inhibit neutrophil functions at multiple stages of the adhesion cascade and to inhibit platelet functions (see Section 1.1). Since the neutrophil-mediated endothelial dysfunction and thrombogenesis are an important feature of ischemia-reperfusion injury, the beneficial effects of endogenous ADO from EC has been implicated in organ protection during such conditions (Mullane and Bullough, 1995). A recent study has shown that an increase in local endogenous ADO release from EC by AK inhibition attenuated the adhesion of activated neutrophils to the EC monolayer in a selectin-dependent manner (Firestein et al., 1995). ADO has been also shown to reduce endothelial release of pro-inflammatory cytokines, such as IL-6 and IL-8 (Bouma et al., 1996). Moreover, endogenous ADO activates antioxidant defense systems in EC, providing

cytoprotective effects in a pathophysiological condition, such as oxygen free radical-mediated reperfusion injury. In this study, the authors provided evidence that endogenous ADO increases activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in bovine and human endothelial cells via A_3 ADO receptor-dependent mechanism (Maggirwar et al., 1994).

1.5. Therapeutic potential of endogenous ADO from EC

The amounts of ADO formed by EC are less than those derived from parenchymal cells, such as cardiac myocytes in the heart (Borst and Schrader, 1991). However, its release at a critical site could be more important than relative amount because of the strategic location of EC between the circulation and the vascular wall (Mullane and Bullough, 1995). Intravascular infusion of ADO has been shown to preserve endothelial function, for example, by interfering with leukocyte functions during ischemia-reperfusion (Grisham et al., 1989). Moreover, large size ADO analogs with their movement restricted within the vascular compartment also exhibited many of the beneficial effects of ADO in the isolated perfused heart including vasodilation, and negative inotropic and chronotropic effects. And these effects can be blocked by an ADO receptor antagonist, 8-SPT,

suggesting an ADO receptor-mediated mechanism in vascular endothelium (Balcells et al., 1992).

The therapeutic potential of exogenous ADO or its analog, however, is limited because of the severe cardiovascular side effects, such as hypotension and bradycardia (Belardinelli et al., 1989), and the very short half-life of ADO (1.5 second at 1 μM exogenous ADO) in the plasma (Moser et al., 1989). Therefore, drug development has been focused on an alternative drug, which could increase endogenous ADO locally at the target site of its actions (Bouma et al., 1997b). Endogenous ADO formed by EC may directly participate in local regulation of blood flow, anti-inflammation and anti-thrombosis in an autocrine and paracrine manner (Mullane and Bullough, 1995). In fact, the capacity of ADO uptake and rephosphorylation by EC is so effective that it serves as a metabolic barrier for both interstitial and intravascular ADO at concentrations below 1 μM (Nees et al., 1985). Hence the development of an endothelium-specific AK-regulating agent that controls endogenous ADO formation may have therapeutic benefits.

1.6. FK506 and endogenous ADO release

Recent studies have suggested that the immunosuppressants, CsA and FK506 mimic cardioprotective effects of ischemic preconditioning. CsA has been shown to

preserve post-ischemic left ventricular function in isolated rat hearts. Inhibition of the mitochondrial ion pore opening in the cardiomyocyte has been implicated as a mechanism of the protection (Duchen et al., 1993). CsA treatment also preserved myocardial function and NO production in isolated guinea pig hearts during ischemia-reperfusion (Massoudy et al., 1997). In addition, both CsA and its analog FK506 have been shown to reduce post-ischemic myocardial infarct size in isolated rabbit hearts, and hence the role of PP2B (calcineurin) is implicated, which is one of the known targets of both CsA and FK506.

Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase, which plays an important role in intracellular Ca^{2+} signaling and in cytokine-mediated T-lymphocyte activation. Calcineurin binds to FKBP12 and inhibits IP_3 receptors. Upon dephosphorylation of its substrates, calcineurin unmasks the nuclear localization signal of cytoplasmic NF-AT transcription factors and activates NF-κB via inhibition of IκB, allowing the transcription factors to translocate into the nucleus. Calcineurin also activates PP1 via inhibition of inhibitor-1 and DARP-32, which are specific inhibitors of PP1 (Guerini, 1997; Lee and Burckart, 1998). CsA and FK506 bind to the immunophilins cyclophilin and FKBP, respectively. The CsA/cyclophilin or FK506/FKBP complex binds to calcineurin and inhibits its activity.

Inhibition of calcineurin leads to intracellular Ca^{2+} mobilization, inhibition of nuclear translocation of transcription factors, such as NF-AT and NF-κB, and inhibition of PP1 (Burkhardt and Kalden, 1997).

It has been recently found that both CsA and FK506 can induce an increase in ADO plasma levels in kidney transplant recipients, and the plasma levels of CsA and ADO were closely correlated. The mechanism of action of the two drugs is not clear but a reduction in ADO uptake by red blood cells was observed.

ADO also has a potent immunosuppressive effect. It was found that people with inherited ADA deficiency manifest severe combined immunodeficiency due to accumulation of endogenous ADO leading to lymphotoxicity (Hirschhorn, 1995). In addition, the immunosuppressive effect of ADO can be mediated by A_{2A} ADO receptors in T-lymphocytes where ADO inhibits T-lymphocyte proliferation and functions (Huang et al., 1997; Koshiba et al., 1997). Therefore, it has been proposed that endogenous ADO action is implicated in CsA and FK506-mediated immunosuppression (Guieu et al., 1998).

Since ADO has been well known as 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), it is possible that CsA and FK506-induced cardioprotection was mediated by endogenous

ADO. In fact, it has been demonstrated that both CsA and FK506 inhibit ADO uptake and increase ADO release from T-lymphocytes (Spychala et al., 1996). In the present study, we tested a hypothesis that FK506 induces ADO release from cultured EC via inhibition of AK activity.

1.7. Cell signaling mechanisms

Hormones, growth factors and cytokines through their respective receptors, activate a series of signal transduction cascades within the cell, leading to biological responses, such as proliferation, apoptosis and inflammation. In order to identify signaling mechanisms involved in AK regulation in cultured EC, we evaluated the effect of two different hormones that interact through the G protein-coupled receptors, norepinephrine and thrombin, and a factor that interacts through a receptor tyrosine kinase, insulin. We also used several signaling effectors and inhibitors for different kinases and phosphatases as pharmacological probes to identify the signaling pathways (Fig. 4).

The most well-known family of cell surface receptors are the seven transmembrane spanning receptors coupled to heterotrimeric G proteins, which act on different effectors, such as ion channels and enzymes generating second messengers. For example, norepinephrine can modulate

endothelial function via stimulation of α and β-adrenoceptors. β_2-adrenoceptors (Gs) are coupled to adenylate cyclase, which forms cAMP upon stimulation. The subsequent activation of PKA by cAMP reduces the production of inflammatory mediators from EC (Vanhoutte, 1997). In addition, stimulation of α_1-adrenoceptor (Gq) induces an increase in release of ADO and adenine nucleotides preferentially from vascular endothelium in isolated rabbit aorta (Sedaa et al., 1990).

Thrombin is a coagulation protease, which activates platelets via G protein-coupled (Gq/Gi) protease-activated receptors (PARs). PAR1 is activated by proteolytic cleavage of its amino-terminus exodomain by thrombin, and it has been demonstrated in EC that the activated PAR1 can be downregulated by internalization. Thrombin and the activated platelets have been shown to play an important role in myocardial infarction and thrombogenesis (Coughlin, 1999).

The biological actions of insulin via its receptor tyrosine kinase involve PI_3K and MAPK pathways. Activation of the PI_3K pathway via insulin receptor substrates 1 and 2 has been shown to increase the endothelial NO production, which induces vasodilation and reduces various inflammatory and thrombotic reactions. An insulin-stimulated MAPK has recently been described (Hsueh and Law, 1999).

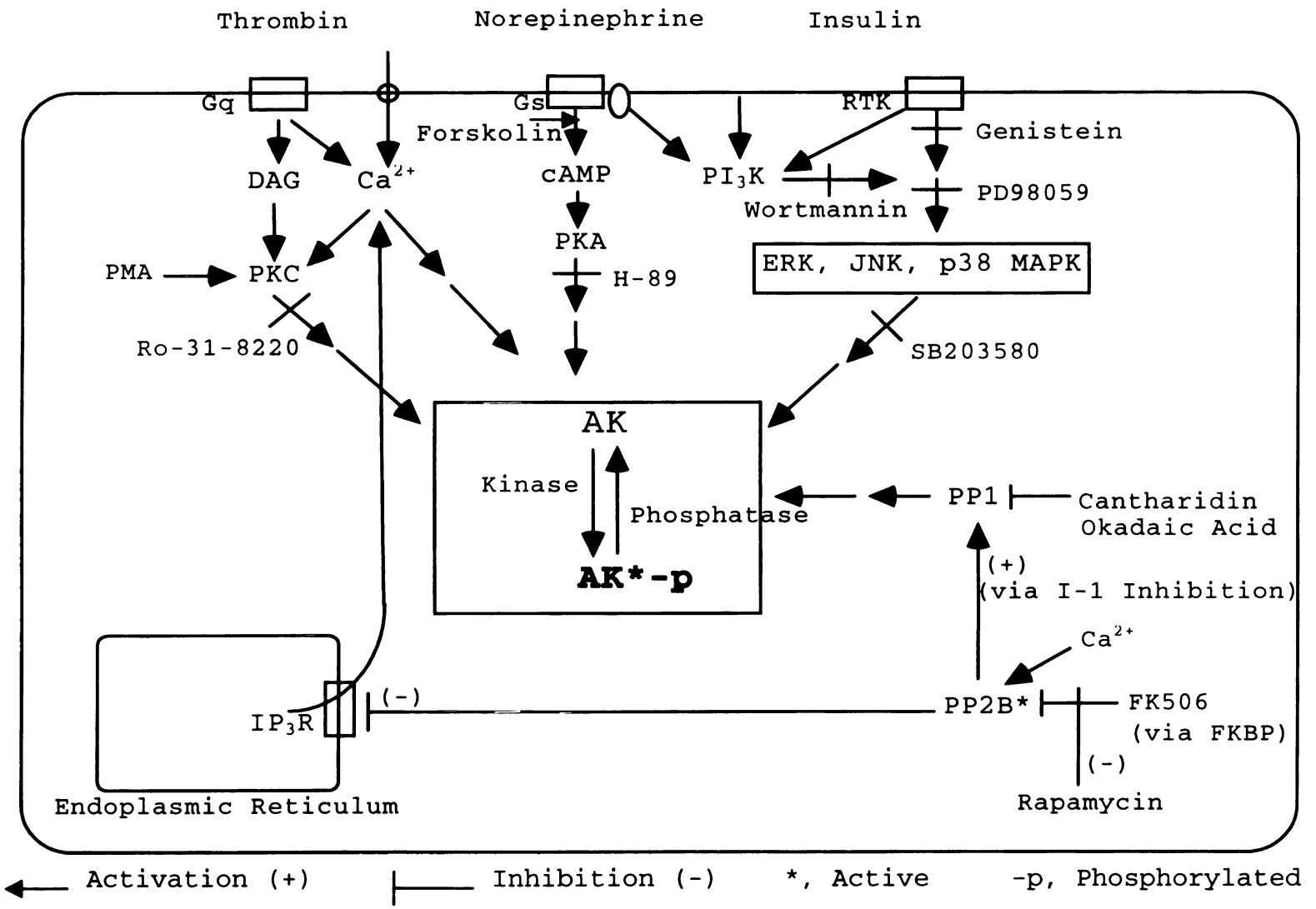

Figure 4. A hypothetical signaling mechanism involved in AK regulation.

Three parallel MAPK pathways are currently well known, namely the ERK, JNK and P38 MAPK pathways. Activation of the MAPK cascade, especially that of the ERK pathway, by various growth factors is associated with proliferation and differentiation in a number of cell systems. Cellular stress, endotoxin, and inflammatory cytokines, such as IL-1 and TNF-α can activate JNK and p38 MAPK. Activation of JNK and P38 MAPK pathways is associated with apoptosis and inflammation (Brunet and Pouyssegur, 1997). Also, inhibitors of P38 MAPK pathway inhibit the pro-inflammatory effects of various cytokines on EC as well as leukocytes (Detmers et al., 1998). Because ADO has a potent anti-inflammatory effect in several systems, we hypothesized that the inhibition of pro-inflammatory effects by P38 MAPK inhibitors may be through a decrease in AK activity and an increase in ADO release.

In summary, ADO has a number of anti-inflammatory and anti-thrombotic activities. Understanding the mechanisms involved in the regulation of ADO formation and release might be helpful for drug development. In that regard, and also because of the location of EC in the microvasculature, ADO formed by EC may directly participate in anti-inflammation and anti-thrombosis. Therefore, the development of agents that control endogenous ADO release from EC may have therapeutic benefits with reduced systemic

side effects of excess ADO or its analogs. Results from our own laboratory and others have strongly indicated that this could be achieved by inhibiting AK. Given the fact that AK can be regulated by phosphorylation, understanding the signaling mechanisms of AK regulation in EC will prove beneficial in the areas of molecular drug targeting.

1.7. Overall and specific hypotheses

Overall Hypothesis:

Regulation of AK by extracellular signals, second messenger systems and reversible phosphorylation of the enzyme is a physiologically significant mechanism controlling ADO formation by cultured EC.

Specific hypotheses:

1. The activity of AK in cultured EC can be modulated by exposing the intact cells to extracellular signals.
2. The activity of AK in cultured EC can be modulated by exposing the intact cells to agents, which modulate second messenger systems.
3. The activity of AK purified from cultured EC depends on a specific phosphorylation of the enzyme.
   a. Dephosphorylation of purified AK by a specific protein phosphatase decreases its activity.

b. The activity of purified AK is correlated with the degree of a specific phosphorylation when the cells are exposed to the specific effectors and inhibitors (Specific hypotheses 1 and 2).

4. The regulation of AK activity is responsible for changes in ADO release from cultured EC.

2. MATERIALS AND METHODS

2.1. Cell culture

Cow pulmonary artery endothelial cells (CPAE, ATCC CCL-209) and rat prostate endothelial cells (YPEN-1, ATCC CRL-2222) were purchased from American Type Culture Collection (Rockville, MD). CPAE is a normal cell line with endothelial cell morphology (Del Vecchio and Smith, 1981). Cells were grown in Dulbecco's modified Eagles medium (DMEM) with 20% fetal bovine serum (FBS) and 1% non-essential amino acids, and passages between 18 and 25 were used for experiments. YPEN-1 is a transformed cell line infected with adenovirus 12-SV40 hybrid virus (Yamazaki and Pienta, 1995). Cells were grown in minimum essential medium (MEM) with 5% FBS and 1% non-essential amino acids. Both lines of cells were serum-starved for 24 hours at confluency before experiments unless stated otherwise. All the cell culture media and reagents were purchased from GIBCO BRL (Rockville, MD).

2.2. Preparation of cytosol and crude membrane fractions

Cytosol and membrane fractions were prepared using slight modifications of the methods previously described (Andres, 1979; Stokoe, 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(hysdoxymethyl)aminomethane (Tris)-HCl using a Polytron homogenizer. The Tris-HCl solution contained 30 nM okadaic acid in order to preserve phosphorylation of AK. The homogenate was centrifuged for 10 min at 1,500 *g* in order to remove cell debris and nuclei. The supernatant was re-centrifuged for 1 h at 100,000 *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.

2.3. Purification of AK

We isolated AK using slight modifications of the method previously described (Andres and Fox, 1979). The cytosolic fraction was adjusted to pH 6.0 with 0.2 N HCl, then mixed for 30 min with an equal volume of carboxymethyl-cellulose resin (Pharmacia) previously equilibrated to pH 6.0 in 10 mM sodium acetate (NaAc). This suspension was centrifuged, and the supernatant was mixed with an equal volume of

diethylaminoethyl cellulose (pH 6.0 in 10 mM NaAc) for 30 min. After centrifugation, the supernatant was applied to a 1 ml 5'-AMP-Sepharose 4B affinity column equilibrated to pH 6.0 with 10 mM NaAc. The column was washed sequentially with 3 ml 10 mM NaAc, pH 6.0; 1 ml 10 mM NaAc/1 M KCl, pH 6.0; 1 ml 10 mM Tris-HCl/1 M KCl, pH 7.4; 1 ml 0.1 M Tris-HCl/5 mM ATP, pH 7.4; and 1 ml 0.1 mM Tris-HCl/5 mM ATP/5 mM $MgCl_2$/5 mM ADO, pH 7.4. The final eluate was filtered through a Millipore ultrafree 15 filter (10K molecular weight size exclusion). The residue was resuspended in 10 mM Tris-HCl/5 mM ATP/5.4 mM $MgCl_2$ (pH 7.4), re-filtered and frozen at -80 °C until use. Protein concentration of the enzyme solution was measured with a Bio-Rad kit making use of the Bradford method.

2.4. Assay of AK activity

Purified AK activity was measured as generation of [^{14}C]AMP from [U-^{14}C]adenosine (Amersham, Piscataway, NJ) (Gorman, 1997). The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM ATP, 5.4 mM $MgCl_2$, 0.4 mg/ml bovine serum albumin and appropriate amount of [U-^{14}C]adenosine (513 mCi/mmol) and unlabelled ADO. AK sample 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 and chilled on ice for 3 min. AMP generation was

linear (10-20% 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 ADO. A Waters C18 Nova-pak column was perfused isocratically at 1 ml/min with 65% solution A (0.5% 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 ADO peak on the chromatogram was integrated and used to calculate total ADO concentration, the specific activity and AMP concentration. AK 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 AOPCP were added in the reaction mixture.

2.5. Western analysis

The purified AK samples were run on a 12% sodium dodesylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred to a nitrocellulose membrane. The membrane was incubated with a blocking buffer containing 0.1% gelatin in NET buffer (1.5 M NaCl, 0.05 M EDTA, pH 8.0, 0.5 M Tris, pH 7.5, 0.001% Triton-X100) and immunoblotted using a primary antiserum to phosphoserine, phosphothreonine or to AK (Kaufmann et al., 1987). The

membrane was washed with NET buffer and incubated with a horseradish peroxidase-conjugated secondary antibody (GIBCO). The membrane was washed again and incubated with 3 µl hydrogen peroxide, 90 mM p-coumaric acid (Sigma) and 250 mM 3-aminophthalhydrazide (Fluka) in 0.1 N Tris (pH 8.5, 10 ml) for 1 min. The immunoreactive bands were visualized by enhanced chemiluminescence.

2.6. ADO uptake assay

ADO uptake was measured using slight modifications of the method previously described (Deussen, 1993). Cells were grown in 24-well cell culture plates. After 48-hours 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 (New England Nuclear, Boston, MA; 5-10 nM, 2-4 Ci/mmol), 5 µM EHNA and unlabeled ADO into the incubation medium, resulting in a final ADO concentration of 100 nM, and the cells were pulsed for 30 min at 37 °C. Each well was then washed twice with cold HBSS containing 100 µM unlabeled ADO in order to terminate ADO 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.

2.7. ADO release assay

ADO release was measured using the method previously described with slight modifications for a cell culture system (Deussen, 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) for 35 min at 37 °C in 250 µl of HBSS containing 5 µM EHNA in order to inhibit ADO deamination. Each well was then washed three times with HBSS, treated in triplicate with drugs in 500 µl HBSS containing 5 µM 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 was collected and total radioactivity was measured using a liquid scintillation counter.

2.8. ADO transport assay

ADO transport was measured using slight modifications of the method previously described (Sobrevia, 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 [2,8-^{3}H]adenosine (100 nM, 41 Ci/mmol) into the incubation medium and pulsed for 15 s in the presence of the AK inhibitor, 5'-NDADO (10 µM) in order to measure only the transport component of ADO uptake (Gu, 1996). ADO uptake was terminated by washing each well twice with cold HBSS containing an ADO transporter inhibitor, dipyridamole (10 µM) to prevent loss of tracer ADO 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.

2.9. Statistical analysis

Data are presented as means ± SE. 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. Purification and characterization of AK in cultured EC

AK was purified from both cow pulmonary artery endothelial cells (CPAE) and rat prostate endothelial cells (YPEN-1). Purification of AK was accomplished by size selection, ion exchange and affinity chromatography. The purification was confirmed by detection of a dominant band approximately at 40 kDa on a SDS-PAGE gel (Fig. 5A) and a single band on a Western blot, which was immunoreactive with a polyclonal AK antibody raised against an antigenic sequence conserved in both human and rat AK (Fig. 5B). On the SDS-PAGE gel, the AK band intensity of the purified sample was greater than 85% of the total band intensity on the lane, indicating that more than 85% of the proteins in the purified sample were AK. In terms of the specific activity (Vmax), AK from both cell lines was purified 1900-fold.

Figure 6 and 7 are double reciprocal plots for the purified AK activity with ADO as a substrate. The Vmax and Km for ADO of AK purified from cow EC are 236.3±19.7 mol/min/mg and 2.8±0.6 μM, respectively. The Vmax and Km for ADO of AK purified from rat EC are 608.1±22.3 nmol/min/mg and <0.5 μM, respectively. The Vmax and Km

values are considerably different between cow and rat AK even though they were purified by the same method.

Studies from our laboratory indicated that guinea pig heart AK activity is modulated by threonine phosphorylation (Sparks et al., 1998). To evaluate the potential for modification of AK activity in EC by a specific phosphorylation of the enzyme, we labeled the purified AK on a Western blot with a phosphoserine (data not shown) or a phosphothreonine antibody (Fig. 8). The AK is immunoreactive with both phosphoserine and phosphothreonine antibodies, indicating that AK from EC contains phosphoryl amino acid residues as does guinea pig heart AK.

Finally, purified AK from both cow and rat EC was treated with the serine/threonine phosphatase, PPλ in order to determine a role of the specific phosphorylation in AK activity. The treatment decreased the activity of AK purified from EC by 73±1.9% and 67±1.3% for cow and rat AK, respectively (Fig. 9). The activity after treatment with PPλ was compared to activity after treatment with a concentration of 5'-Itu (8 μM) capable of >95% inhibition of AK (Gupta, 1996). The degree of inhibition by PPλ treatment was 89% for CPAE and 68% for YPEN-1 of the inhibition by 5'-ITu.

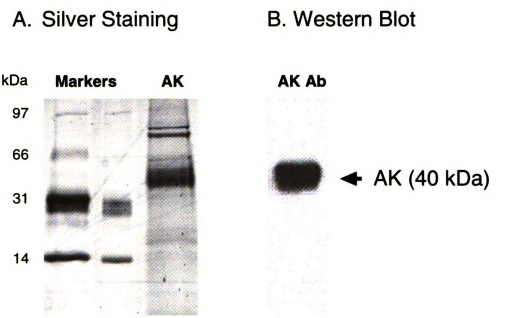

Figure 5. Adenosine kinase purified from CPAE.

A. Silver staining of the purified AK sample on a SDS-PAGE gel.

B. Western blot labeled with an AK antibody.

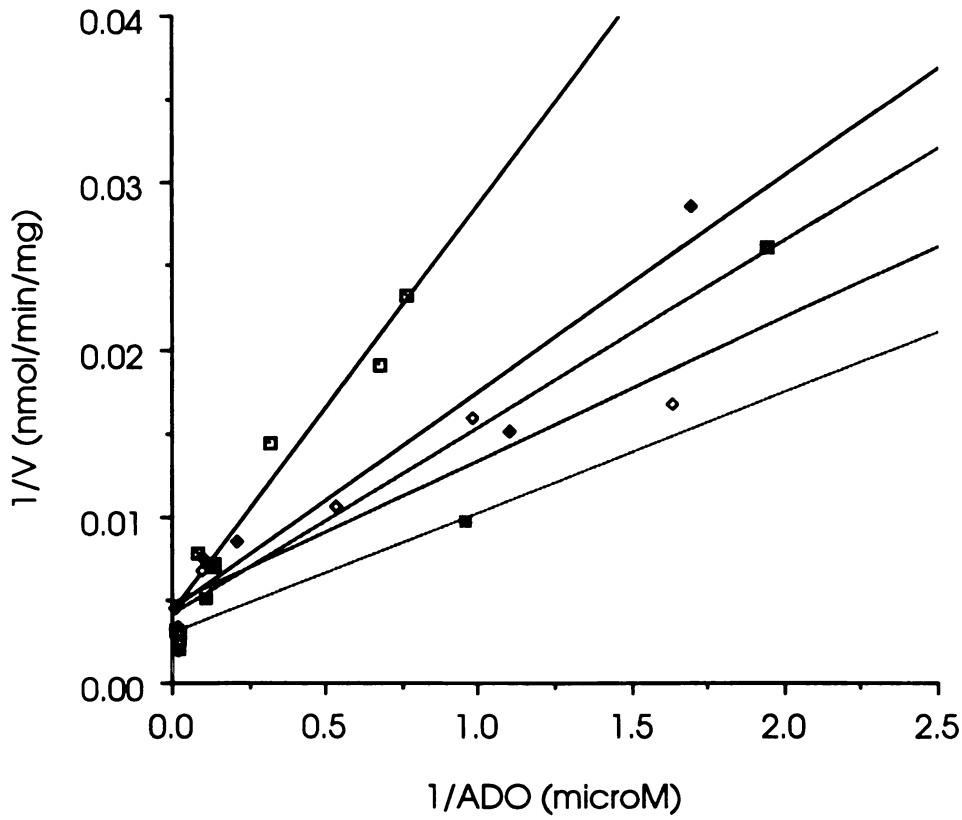

Figure 6. Activity of adenosine kinase purified from CPAE. A double reciprocal plot. Each line represents a separate experiment (n=5). Vmax=236.3±19.7 nmol/min/mg, Km=2.8±0.6 μM for ADO.

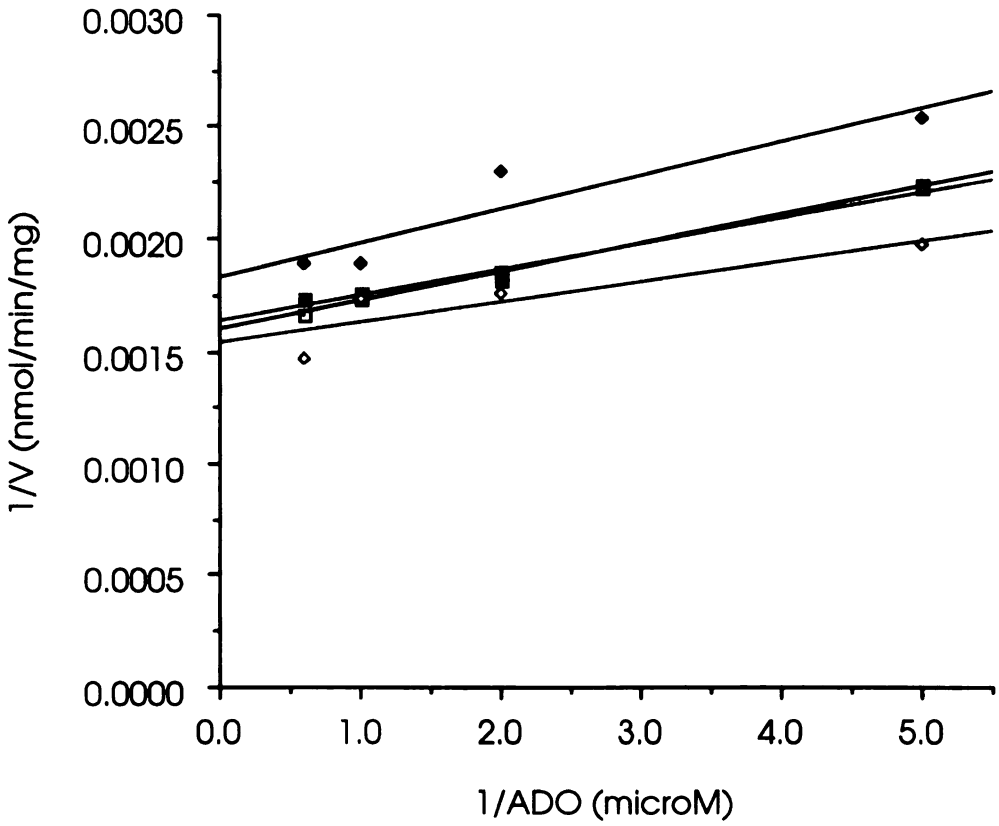

Figure 7. Activity of adenosine kinase purified from YPEN-1. A double reciprocal plot. Each line represents a separate experiment (n=4). Vmax=608.1±22.3 nmol/min/mg, Km<0.5 μM for ADO.

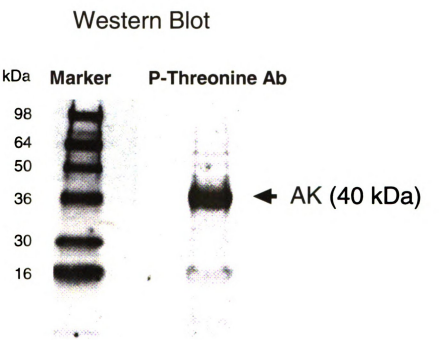

Figure 8. The adenosine kinase purified from CPAE is immunoreactive with a phosphothreonine antibody.

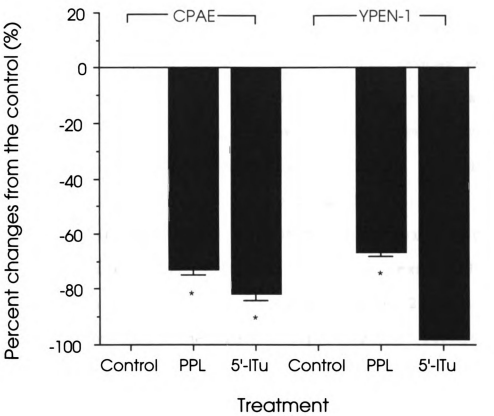

Figure 9. The serine/threonine phosphatase, PPλ decreases purified AK activity. CPAE, cow pulmonary artery endothelial cells; YPEN-1, rat prostate endothelial cells. PPL, protein phosphatase λ; 5'-ITu, 5'-iodotubercidin (8 μM). *, $p<0.05$ vs. control (n=3).

3.2. FK506 induces ADO release from cultured EC in part via inhibition of AK activity

3.2.1. ADO release

In order to evaluate the effect of FK506 on ADO metabolism in EC, we prelabeled adenine nucleotide pools of YPEN-1 with ^{3}H-ADO and then measured the tracer release into the fresh medium with and without drug treatment. FK506 significantly increases tracer release from the cells by 72% (Fig. 10). This increased release is blocked by dipyridamole, an ADO transporter inhibitor. We used a specific AK inhibitor, 5'-NDADO as a positive control for ADO release from the cells. As expected, 5'-NDADO substantially increases tracer release (272%), which is also blocked by dipyridamole. Dipyridamole itself does not change tracer release from the cells (Fig. 10). These results indicate that FK506 induces an increase in ADO formation in EC. Furthermore, the increase in ADO formation is intracellular, and ADO leaves the cells via the dipyridamole-sensitive purine transporters.

3.2.2. ADO uptake

We used ^{3}H-ADO uptake into EC as an indirect measure of AK activity in the cells. In preliminary experiments, ADO 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 ºC in the absence of EHNA, an ADA inhibitor. This shows the predominant role of AK in ADO uptake even when the degradation of intracellular ADO to inosine is a possibility. When testing the effect of FK506 on ADO uptake, we measured ^{3}H-ADO uptake in the presence of EHNA. This assured that >92% of the observed uptake of ^{3}H-ADO is due to AK activity.

Cells were treated with FK506 following the protocol described in the Methods section and using a concentration, which has been previously demonstrated not to induce endothelial dysfunction in a similar preparation (Benigni et al., 1992). After cells were pulsed with ^{3}H-ADO for 30 min under FK506 treatment, we measured the tracer level in the solubilized cells. In both YPEN-1 and CPAE, FK506 decreases ADO uptake into the cells in a concentration-dependent manner (Figure 11 and 12). In control cells, ADO uptake rates are 0.5±0.04 and 1.8±0.05 pmol/min/10^{6} cells for YPEN-1 and CPAE, respectively. In YPEN-1, FK506 at concentrations of 3 μM and higher is effective; it is slightly less potent in CPAE. An ADO transporter inhibitor, dipyridamole at 10 μM inhibits the ADO uptake into EC by

90%. In separate experiments, CsA also decreased ADO uptake into CPAE at concentrations of 10 μM and higher (Fig. 13). FK506 is more potent than CsA.

In order to evaluate a potential mechanism of action of FK506 in ADO uptake in EC, 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 ADO uptake into both YPEN-1 and CPAE in a concentration-dependent manner (Fig. 14 and 15). This result suggests that there is a mechanism, which does not involve calcineurin, because rapamycin does not inhibit calcineurin.

3.2.3. AK Activity

The uptake data strongly suggest that FK506 inhibits AK activity in intact EC. If AK activity is inhibited by its dephosphorylation, it should be possible to observe decreased activity of AK purified from cells previously treated with FK506. In addition, AK activity in a cytosolic fraction should be decreased by previous treatment of the intact cells with FK506. However, FK506 pretreatment does not change the activity of AK purified from the cytosol of YPEN-1 (Fig. 16). Furthermore, neither FK506 nor rapamycin

pretreatment changes the activity of AK in the cytosolic fraction of the cells (Fig. 17).

It seems possible that a cellular fraction of AK, which modulates uptake of ADO, is associated with the plasma membrane. In this case, FK506 might have no effect on total AK or in cytosolic AK, while inhibiting the activity of membrane-associated AK. Therefore, we tested a new working hypothesis that the activity of AK in cultured EC depends on the activity of the enzyme associated with the cell membrane fraction. YPEN-1 cells were treated with FK506 using the same protocol as in previous experiments. Following treatment, a crude membrane fraction was prepared, and AK activity was measured as described in the Methods section. Indeed, FK506 significantly decreases the activity of AK by 35% in the crude membrane fraction (Fig. 18). When cells were treated with rapamycin using the same protocol, no effect in AK activity of the crude membrane fraction was observed.

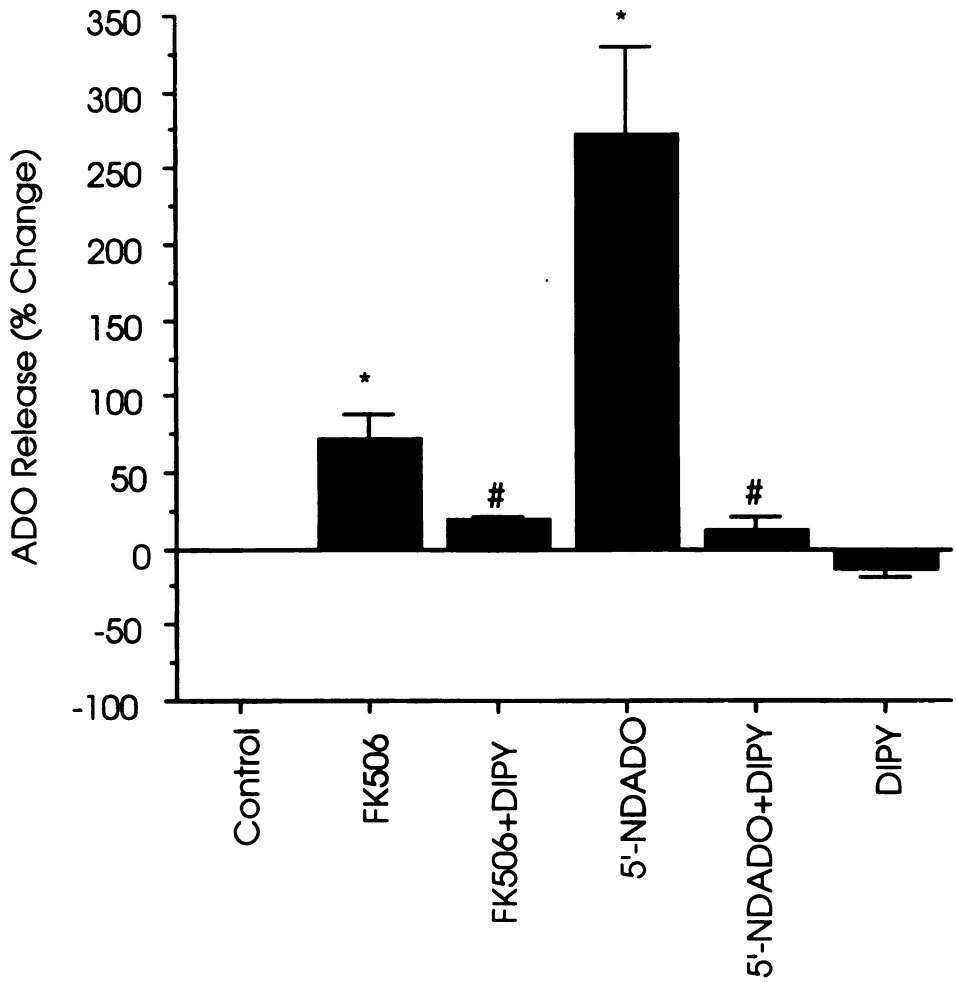

Figure 10. FK506 increases adenosine release from YPEN-1 (n=4 or 5). DIPY, dipyridamole (10 μM); 5′-NDADO, 5′-amino-5′-deoxyadenosine (10 μM). *, $p<0.05$ vs. control; #, $p<0.05$ vs. the respective treatment.

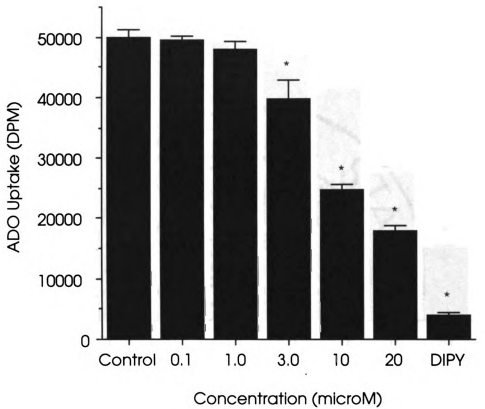

Figure 11. FK506 decreases adenosine uptake into YPEN-1 (n=3). DIPY, dipyridamole (10 μM). *, $p<0.05$ vs. control.

ADO Uptake (DPM)

35
30
25
20
1
1

Figu

*, p

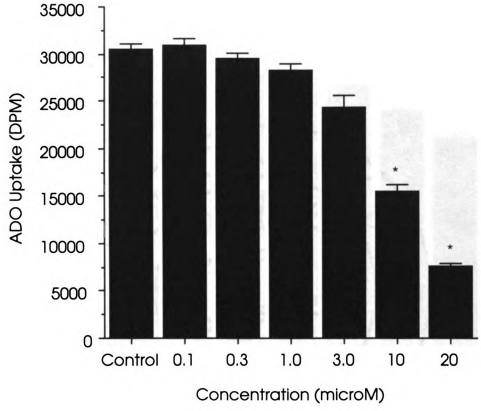

Figure 12. FK506 decreases adenosine uptake into CPAE (n=3).

*, $p<0.05$ vs. control.

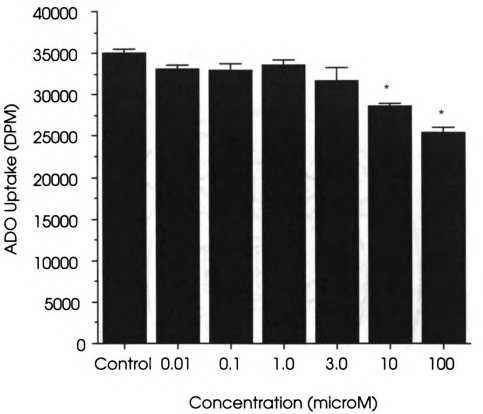

Figure 13. Cyclosporin A decreases adenosine uptake into CPAE (n=3). *, $p<0.05$ vs. control.

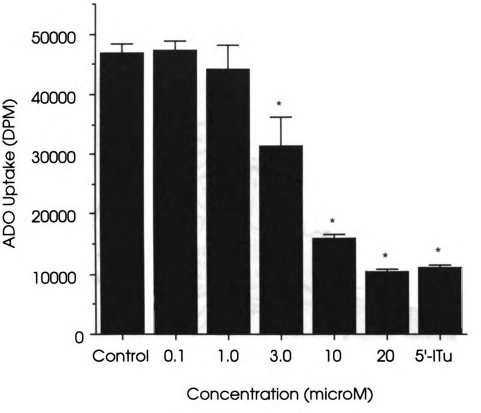

Figure 14. Rapamycin decreases adenosine uptake into YPEN-1 (n=3). 5'-ITu, 5'-iodotubercidin (10 μM). *, $p<0.05$ vs. control.

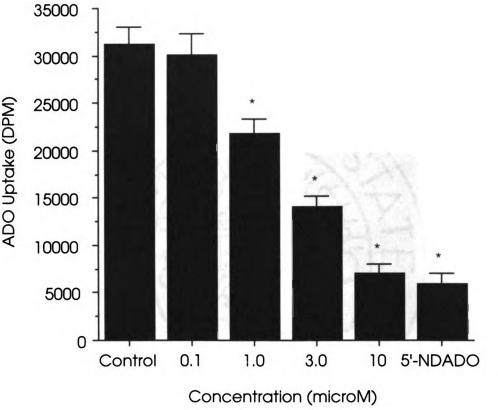

Figure 15. Rapamycin decreases adenosine uptake into CPAE (n=3). 5'-NDADO, 5'-amino-5'-deoxyadenosine (10 μM). *, $p<0.05$ vs. control.

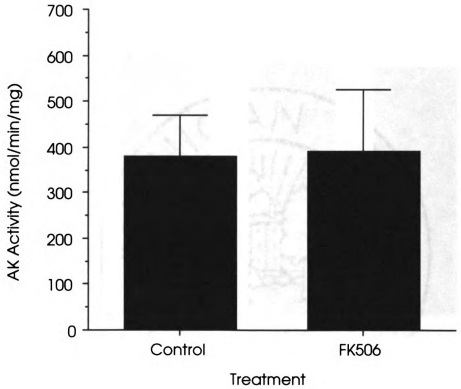

Figure 16. Activity of adenosine kinase purified from YPEN-1 previously exposed to FK506 (n=3). FK506 (10 μM).

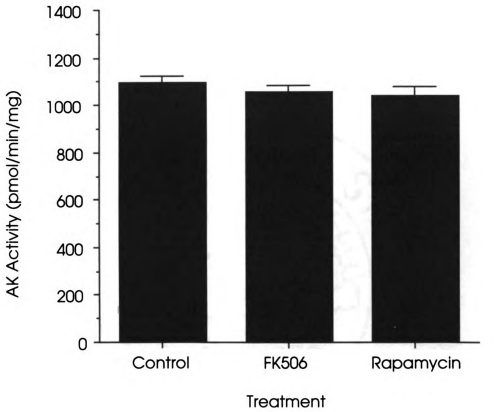

Figure 17. Activity of adenosine kinase in the cytosol fraction of YPEN-1 previously exposed to FK506 or rapamycin. Control, n=8; FK506 (10 μM), n=6; rapamycin (10 μM), n=5.

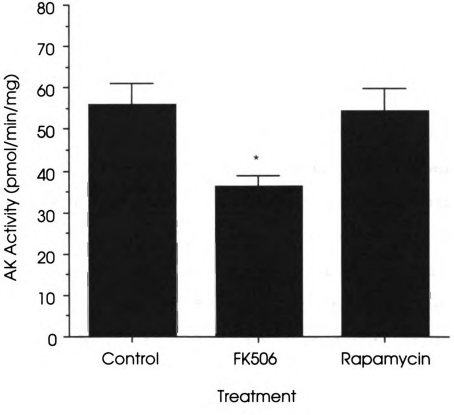

Figure 18. FK506 decreases adenosine kinase activity in the crude membrane fraction of YPEN-1. The membrane fraction was isolated after the intact cells were previously exposed to FK506 (10 μM, n=6) or rapamycin (10 μM, n=5) for 30 min at 37 °C. Control, n=9. *, $p<0.05$ vs. control.

3.3. Effects of cell signaling effectors and inhibitors on ADO uptake and ADO transport in cultured EC

3.3.1. ADO uptake

We further evaluated a potential signaling mechanism involved in AK inhibition induced by FK506. Because FK506 as well as rapamycin and CsA have been shown to modulate MAPK pathways, we tested effects of the specific inhibitors for the MAPK pathways on ADO uptake in EC. In each case, cells were treated with a MAPK inhibitor following the protocol described in the Methods section and using a concentration, which has been previously demonstrated to be effective in a similar preparation (Laird et al., 1998). ADO uptake was used as a proxy for AK activity in these experiments. We found that a tyrosine kinase inhibitor, genistein decreases ADO uptake into YPEN-1 (Fig. 19). The specific p38 MAPK inhibitors, SB203580 and SB202190 also decrease ADO uptake in a concentration-dependent manner (Fig. 20). The inhibitors for MEK, PD098059 and for PI_3K, wortmannin do not change ADO uptake.

3.3.2. Cytosolic and crude membrane fraction AK activity

Since genistein and SB203580 inhibit ADO uptake presumably by effects on a tyrosine kinase and the p38 MAPK pathway, we wanted to know if they affected the activity of

cytosolic and/or membrane fraction AK. To test the possibility, YPEN-1 cells were treated with genistein or SB203580 according to the protocol described in the Methods section. Cell fractionation was carried out as described above. These drugs had no effect on either cytosolic (Fig. 21) or crude membrane fraction (Fig. 22) AK activity. In addition, SB203580 had no effect on the activity of AK purified from the cytosol of YPEN-1 (Fig. 23)

3.3.3. ADO transport

Pretreatment of cells with three of the drugs (rapamycin, genistein and SB203580) which inhibit ADO uptake into EC has no effect on cytosolic or membrane fraction AK activity. A possible explanation for this is that their effects on ADO uptake result from inhibition of ADO transport, not from inhibition of AK activity. To test this possibility, we observed their effect on initial uptake of tracer ADO as described in the Methods section. Dipyridamole at a concentration, which completely inhibits the dipyridamole-sensitive purine transporters, was used as a positive control (Fig. 24). Genistein (100 μM) is almost as effective as dipyridamole in inhibiting ADO transport. Rapamycin and SB203580 are also effective in reducing ADO transport.

3.3.4. ADO release

Inhibition of ADO uptake by rapamycin, genistein and SB203580 is not likely to be the result of AK inhibition because these drugs have no effect on cytosolic or membrane fraction AK activity. Instead their effect on ADO uptake can be explained by inhibition of ADO transport. ADO release experiments provide another way to distinguish between effects on AK activity and ADO transport. Agents, which inhibit AK activity, cause an increase in ADO release. Agents, which inhibit ADO transport, decrease or have no effect on ADO release. They can have no effect on ADO release when the control release is extremely low, as is the case in the EC studied here. When YPEN-1 cells were treated with the above-mentioned drugs, they had no effect on ADO release (Fig. 25). Dipyridamole at a concentration known to block ADO transport, also had no effect (Newby, 1986). By contrast, the AK inhibitor (5'-NDADO), and FK506, which inhibits membrane fraction AK activity, both increase ADO release (Fig. 25).

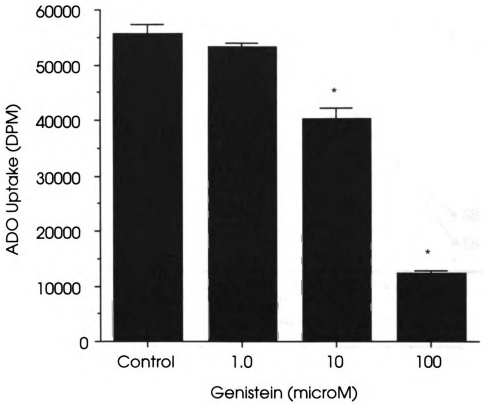

Figure 19. A tyrosine kinase inhibitor, genistein decreases adenosine uptake into YPEN-1 (n=3). *, $p<0.05$ vs. control.

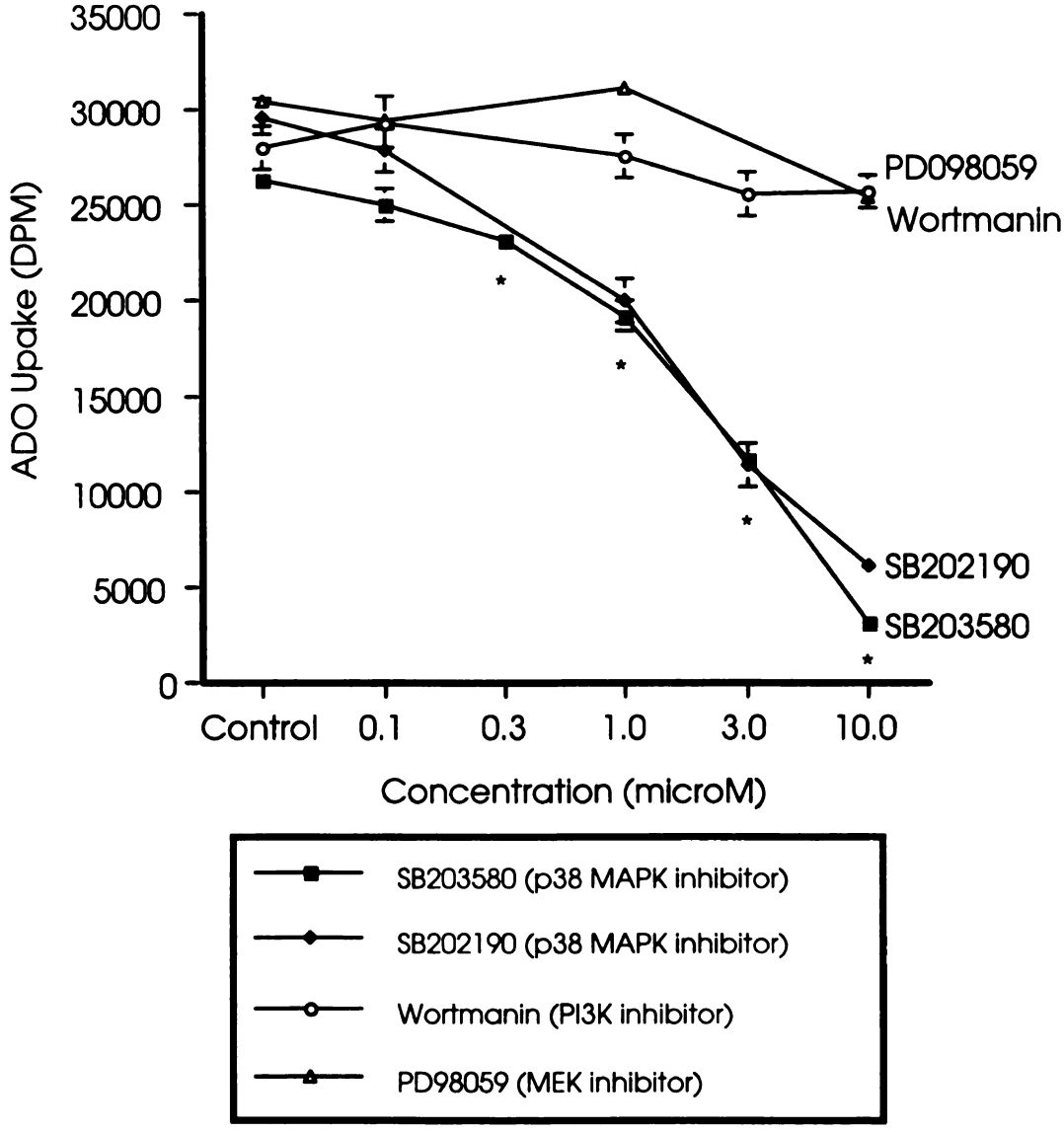

Figure 20. The p38 MAPK inhibitors decrease adenosine uptake into CPAE (n=3). *P<0.05.

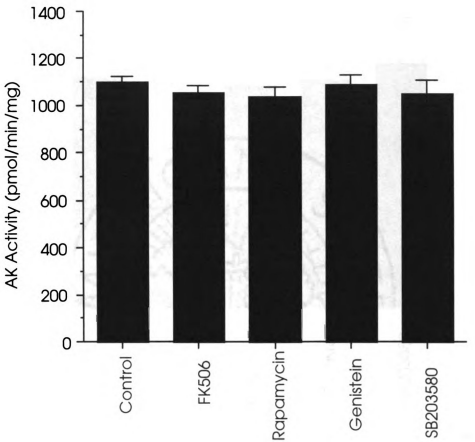

Figure 21. Activity of adenosine kinase in the cytosol fraction of YPEN-1 previously exposed to the different drugs. Control, n=8; FK506 (10 μM), n=6; rapamycin (10 μM), n=5; genistein (100 μM), n=4; SB203580 (10 μM), n=4.

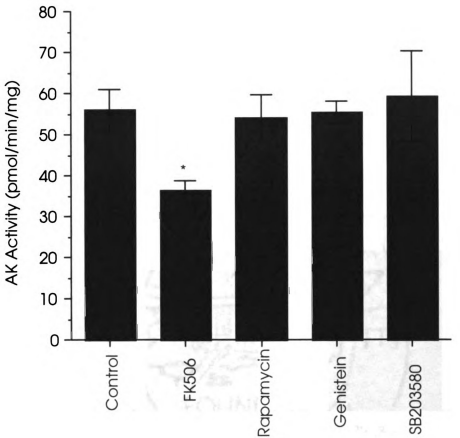

Figure 22. Activity of adenosine kinase in the crude membrane fraction of YPEN-1 previously exposed to the different drugs. Control, n=9; FK506 (10 μM), n=6; rapamycin (10 μM), n=5; genistein (100 μM), n=4; SB203580 (10 μM), n=4. *, $p<0.05$ vs. control.

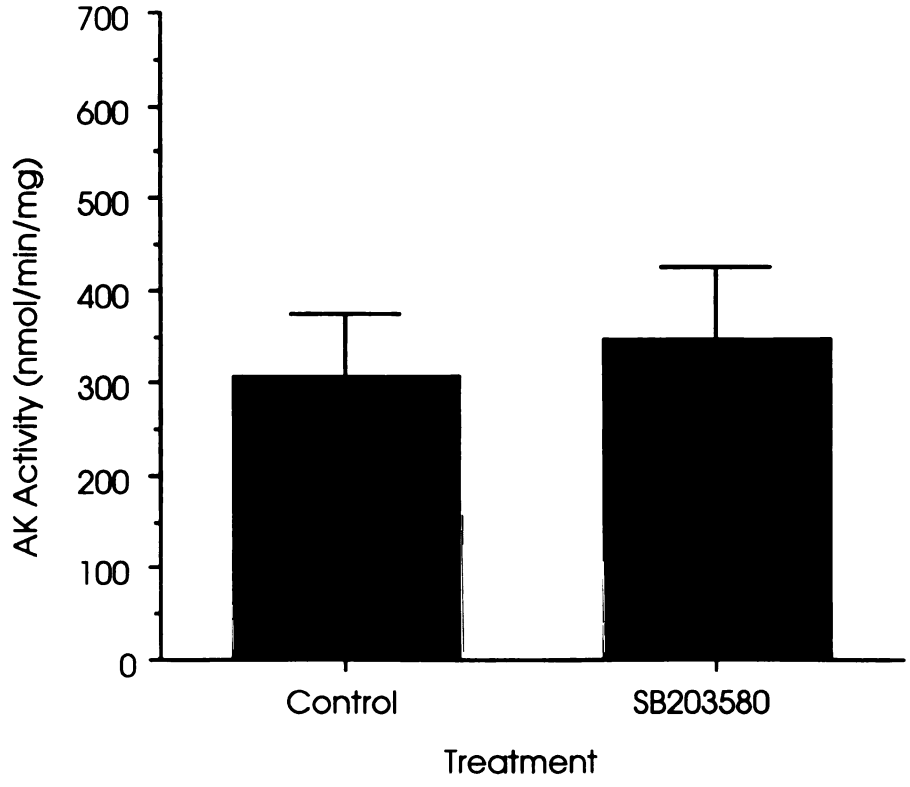

Figure 23. Activity of adenosine kinase purified from YPEN-1 previously exposed to SB203580 (N=3). SB203580 (10 μM).

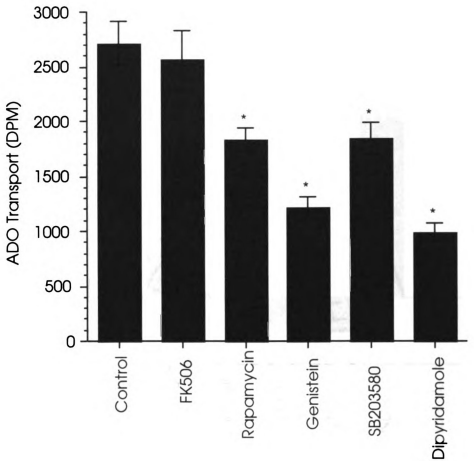

Figure 24. Adenosine transport into YPEN-1 under the different drug treatments (n=4): FK506 (10 μM); rapamycin (10 μM); genistein (100 μM); SB203580 (10 μM); dipyridamole (10 μM). *, $p<0.05$ vs. control.

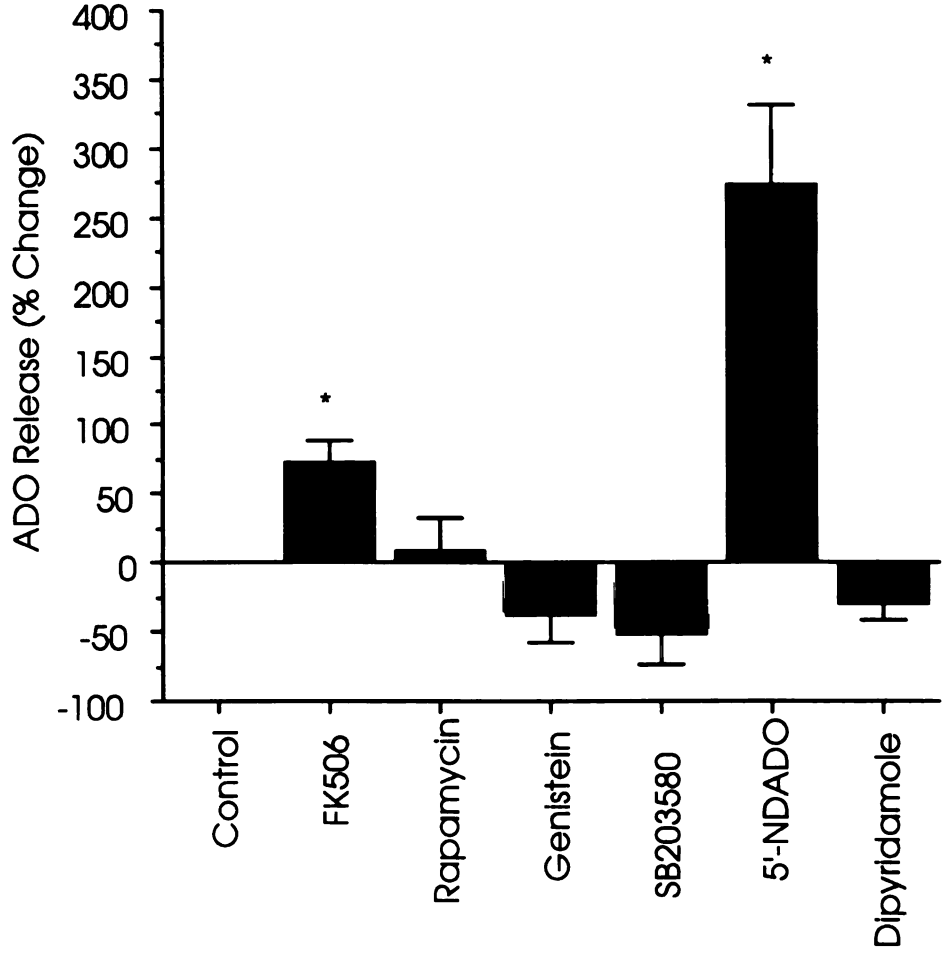

Figure 25. Adenosine release from YPEN-1 under the different drug treatments (n=4 or 5). FK506 (10 μM); rapamycin (10 μM); genistein (100 μM); SB203580 (10 μM); dipyridamole (10 μM). *, $p<0.05$ vs. control.

3.4. Other signaling pathways

We also evaluated effects of three different hormones, and several signaling effectors and inhibitors as pharmacological probes to identify the signaling pathways involved in AK regulation using the ADO uptake assay for a screening purpose. The α and β-adrenergic agonist (norepinephrine) and a receptor tyrosine kinase agonist (insulin) did not change ADO uptake into YPEN-1 (Fig. 26). Another G protein-coupled receptor agonist, thrombin did not change ADO uptake into CPAE (Fig. 27). The adenylate cyclase activator (forskolin) and a PKA inhibitor (H-89) did not change ADO uptake into the cells (Fig. 28). The activator of PKC (PMA) and an inhibitor of PKC (Ro-31-8220) did not change ADO uptake into the cells (Fig. 29). Sodium orthovanadate (an inhibitor of protein tyrosine phosphatases) and okadaic acid (a potent inhibitor of PP1 and/or PP2A at the concentrations used) did not have a significant effect on ADO uptake into YPEN-1 (Fig. 30). Anisomycin, an activator of JNK and p38 MAPK and an inhibitor of protein synthesis did not have a significant effect in CPAE (Fig. 31). Finally, we found that a calcium ionophore A23187 decreased ADO uptake into CPAE at concentrations of 3 μM and higher (Fig. 32). These results are summarized in Table 1.

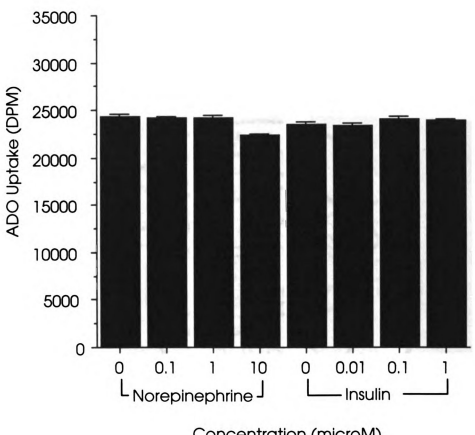

Figure 26. Effects of norepinephrine and insulin on adenosine uptake into YPEN-1 (n=3).

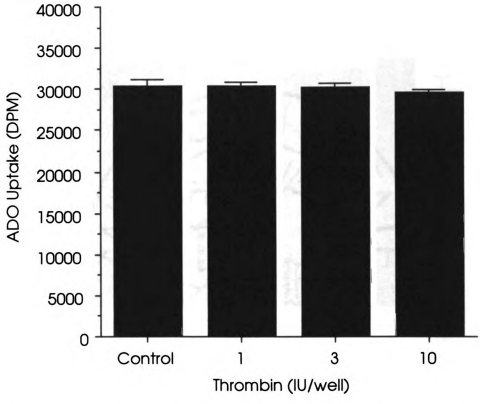

Figure 27. The effect of thrombin on adenosine uptake into CPAE (n=3).

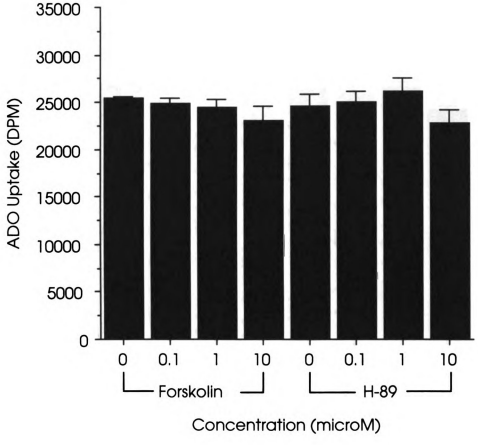

Figure 28. Effects of forskolin and H-89 on adenosine uptake into CPAE (n=3).

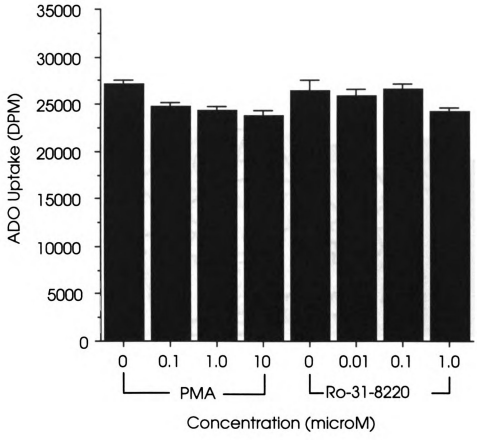

Figure 29. Effects of PMA and Ro-31-8220 on adenosine uptake into CPAE (n=3).

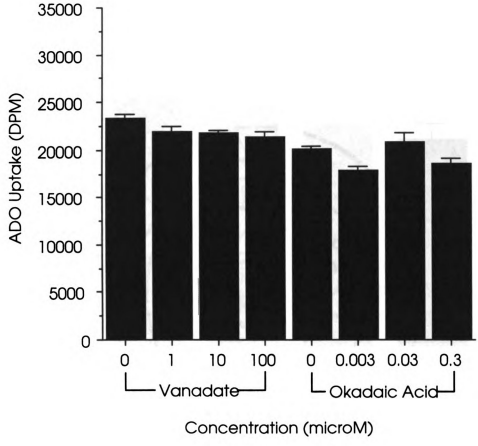

Figure 30. Effects of sodium orthovanadate and okadaic acid on adenosine uptake into YPEN-1 (n=3).

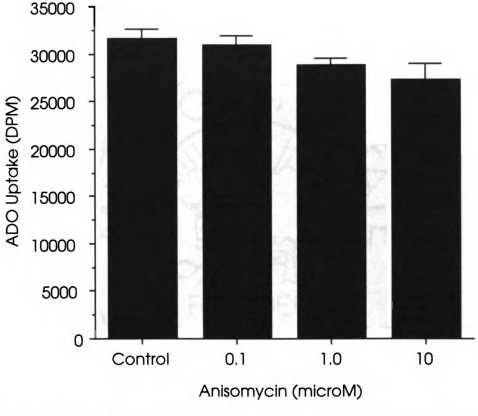

Figure 31. The effect of anisomycin on adenosine uptake into CPAE (n=3).

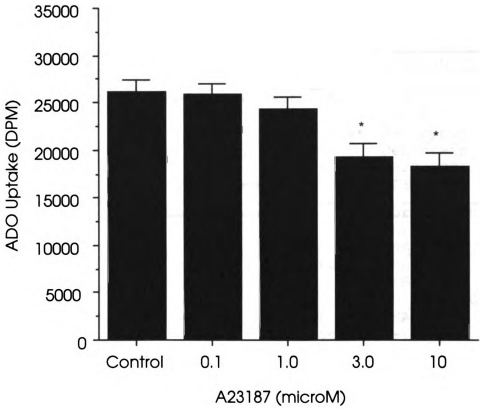

Figure 32. The effect of calcium ionophore A23187 on ADO uptake into CPAE (n=3). *, $p<0.05$ vs. control.

Table 1. Effects of different signaling effectors and inhibitors on ADO uptake.

Effect	Type	CPAE and/or YPEN-1
Activation	None	
Inhibition	Immunosuppressants: PP2B	CsA FK506
	Other	Rapamycin
	MAPK pathway	Genistein SB203580, SB202190
	Calcium signaling	A23187
No effect	Receptor agonists	Norepinephrine Thrombin Insulin
	PKA pathway: activator	Foskolin 8-Br-cAMP
	inhibitor	Ro-31-8220
	PKC pathway: activator	PMA
	inhibitor	H-89
	Kinase inhibitors	PD098059 Wortmannin
	Phosphatase inhibitors	Sodium orthovanadate Okadaic acid
	Stress	Anisomycin Lipopolysaccharide

4. DISCUSSION

4.1. Purification and characterization of AK in cultured EC

Adenosine kinase was purified from both cow pulmonary artery endothelial cells (CPAE) and rat prostate endothelial cells (YPEN-1). When we compared the purified AK activities of cow and rat EC and that of guinea pig heart AK purified by the same method, the Vmax and Km values were considerably different among them. The Vmax values for cow and rat AK were 14 and 36 times higher than Vmax of guinea pig heart AK, respectively, and the Km values were 1/5 and 1/65 of Km of guinea pig heart AK, respectively (Gorman et al., 1997). We then compared Km values of purified AK from several different species and tissues in the literature (Table 2). The Km of AK of cow EC was similar to that of cow liver, and the Km of AK of rat EC was similar to those of rat heart and liver. The Km of guinea pig heart was similar to that of guinea pig liver. These results suggest that AK activity is species-specific.

AK has been purified and cloned from several sources. The sequence analyses have revealed that AK contains significant sequence similarities to microbial sugar kinases such as ribokinase and fructokinase (Singh et al., 1996; Spychala et al., 1996).

It appears that activity of AK is modulated by phosphorylation. Studies in isolated guinea pig and rat hearts have indicated that purified AK contains phosphothreonine residues, phosphorylation of AK is altered under hypoxic condition, and the activity closely correlates with the degree of threonine phosphorylation of the enzyme (Sparks et al., 1998). In line with that, the purified AK from EC also contains phosphoserine and phosphothreonine residues (Fig. 8). The serine/threonine phosphatase, PPλ decreased the activity of AK purified from EC as well as the rat heart AK (Fig. 9). In addition, a sequence analysis (PROWL) of both human and rat AK revealed that AK contains 4-5 putative PKC phosphorylation sites ([ST]-x-[RK]) and 2-3 putative casein kinase II phosphorylation sites ([ST]-x(2)-[DE]). These results suggest that the serine/threonine phosphorylation is an important determinant of AK activity in EC as well as cardiac myocytes. However, we were not able to identify physiological or pathophysiological interventions that can change the phosphorylation level of AK in EC.

4.2. FK506 inhibits AK activity in cultured EC

Three lines of evidence indicate that FK506 inhibits *in situ* AK activity in cultured EC. FK506: (a) inhibits uptake of tracer ADO (Fig. 11 and 12), (b) enhances release of

tracer ADO (Fig. 10) and (c) reduces AK activity of a crude membrane fraction of EC (Fig. 18). In addition, FK506 does not inhibit transport of tracer ADO (Fig. 24). Each of these lines of evidence is evaluated below.

We used ADO uptake as a screening tool to establish the possibility that FK506 inhibits *in situ* AK activity. We did this because our own preliminary experiments, as well as work from other laboratories indicated that it is extremely unlikely that AK could be inhibited without an inhibition of ADO uptake. In preliminary experiments, we found that when cultured EC are exposed to extracellular ^{3}H-ADO, more than 90% of ^{3}H-ADO taken up into EC is in the form of adenine nucleotides. This means that AK predominates over ADA in providing the major pathway for ADO metabolism, once ADO enters the cell. This result is comparable to previous observations in human and porcine EC in culture (Shryock et al., 1988; Smolenski et al., 1994; Sobrevia et al., 1994). In fact, the role of ADO deamination in cultured EC was small even at high concentrations of ADO (10 μM) in the incubation medium (Sobrevia et al., 1994). Nevertheless, we included an inhibitor of ADA, EHNA in all uptake and release experiments so that the sole intracellular sink for ADO would be via AK.

This is not to say that ADA may not be important in ADO actions in other contexts. Among other roles of ADA, ecto-

ADA is involved in cell signaling via enhancing A_1 ADO receptor-mediated second messenger production (Ciruela et al., 1996). An ADA inhibitor deoxycoformycin prevented the interaction between the ecto-ADA and A_1 ADO receptor (Saura et al., 1996).

FK506 induces an increase in ADO release after the adenine nucleotide pools of cultured EC are prelabeled with ^{3}H-ADO (Fig. 10). Because an ADA 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 EC can also release adenine nucleotides and cAMP and can form extracellular ADO via extracellular dephosphorylation of AMP by ecto-5'-NT (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). Therefore, intracellular ADO is the only source for tracer found in the incubation medium in our release experiments.

Intracellular ADO has three possible fates: (a) phosphorylation to AMP, (b) deamination to inosine and (c) exit from the cell by a purine transporter. When AK is inhibited, the importance of the other two pathways will be

increased. Thus, AK inhibition is expected to result in increased ADO release (Decking et al., 1994). Indeed, the AK inhibitors used in our experiments uniformly increase ADO release, as does FK506 (Fig. 10).

Pretreatment of cultured EC with FK506 did not cause inhibition of purified AK or AK activity of the cytosolic fraction. However, FK506 causes a decrease in membrane fraction AK activity of cultured EC (Fig. 18). This finding raises the possibility that FK506 acts either by decreasing the activity of AK associated with the plasma membrane (e.g. dephosphorylation), or by translocating membrane AK to the cytosol. If the latter is the case, one might expect to see a reciprocal change in cytosolic AK activity when membrane AK activity changes. However, cytosolic AK activity is so much greater than membrane AK activity that changes in the cytosolic AK activity due to transfer to or from the membrane would be impossible to detect (Fig. 17).

In order to determine activity of AK associated with the plasma membrane of cultured EC, 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 AK activity is associated with plasma membrane. Spychala

recently found a detectable amount of AK activity in a plasma membrane fraction isolated from lymphocytes. The Km of AK in the membrane fraction was 2-6 times lower than that of AK in the cytosolic fraction, suggesting a modification of AK associated with plasma membrane. It is possible that AK associated with plasma membrane would be easily accessible to both adenine nucleotide pools and ADO transporters on the cell membrane (personal communication). There is no evidence that mitochondria or microsomes contain AK.

Recently, Spychala and Mitchell (1998) also observed a decrease in AK activity in T lymphocytes in response to CsA or FK506 although the change in activity was observed in the cell lysate preparation. The drugs did not change the activity of AK in purified recombinant enzyme preparations or when the drugs were directly added to the cell lysates. These results also indicate that CsA and FK506 promote ADO release by a novel mechanism involving inhibition of the AK activity (Spychala and Mitchell, 1998). Further studies are required to evaluate the biochemical mechanism involved in FK506-induced inhibition of AK associated with the membrane.

In summary, it appears that drugs like FK506 and CsA may act in novel ways to elevate extracellular ADO levels. Therefore, it has been proposed that endogenous ADO action is implicated in FK506 and CsA-mediated immunosuppression

(Guieu et al., 1998). In addition, the anti-inflammatory effect of aspirin is also in part mediated by endogenous ADO action. In a murine air pouch model of acute inflammation, aspirin induced an increase in ADO level at the inflamed site, and the anti-inflammatory effects (e.g. leukocyte accumulation) of aspirin were blunted by removal of ADO or A_2 ADO receptor antagonism. Moreover, this effect of aspirin was retained in cyclooxygenase-2 or NFκB knockout mice, indicating an endogenous ADO-dependent mechanism (Cronstein et al., 1999a; Cronstein et al., 1999b). The effects of elevated ADO are discussed below.

4.3. The role of ADO in preventing the toxic effects of FK506 and CsA

In long-term use, both CsA and FK506 cause nephrotoxicity and hypertension in organ transplant recipients (Ader and Rostaing, 1998; Sander and Victor, 1995). Because FK506 exhibited a similar toxicity profile to that of CsA, inhibition of calcineurin, the same intracellular target of both drugs is implicated to mediate the toxicity (Sander and Victor, 1995). This is opposite to what we may expect from the effects of elevated ADO levels in response to these drugs. It is thought that the drug-induced toxicity may be due to desensitization of ADO receptors after prolonged exposure to elevated levels of ADO

(Guieu et al., 1998). Several studies have indicated that ADO receptor antagonism reduced chronic CsA-induced nephrotoxicity (Ates et al., 1996; Bennett et al., 1992; Carrier et al., 1993). In an animal model of cerebral ischemia, acute administration of an A_1 ADO receptor agonist, N^6-cyclopentyladenosine (CPA) improved both survival and neuronal preservation, and acute treatment with A_1 ADO receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (CPX) significantly deteriorated the outcome. In contrast, chronic administration of CPA did not have a significant effect but chronic CPX treatment resulted in a significant improvement in the outcome, suggesting an A_1 ADO receptor desensitization due to prolonged exposure to the ADO agonist (Von Lubitz et al., 1994). Recent studies have demonstrated the agonist-induced desensitization for A_1, A_{2A}, A_{2B} and A_3 ADO receptor subtypes (Palmer and Stiles, 1997). Our findings in regulation of membrane AK activity may explain the mechanism by which extracellular ADO levels are elevated in response to FK506 and CsA.

4.4. Effects of cell signaling effectors and inhibitors on ADO uptake and ADO transport in cultured EC

We had hoped that the drugs rapamycin, genistein and SB203580 could be used as tools to explore the pathway by which FK506 exerts its effect on AK. Initially, we were

encouraged by the fact that all three of these agents inhibit ADO uptake (Fig. 14, 15, 19 and 20). However, further experiments indicated that these agents do not act on ADO uptake by inhibiting AK activity. They (a) have no effect on cytosolic or membrane fraction AK activity, (b) have no effect on ADO release and (c) inhibit ADO transport into EC (Fig. 24).

Transport of ADO was measured by observing the first seconds of uptake of tracer ADO in the presence of an inhibitor of AK activity, 5'-NDADO. This means that radioactivity associated with the cells gives a measure of ADO entering the cells. The portion of this accumulation blocked by dipyridamole gives an indication of the activity of the dipyridamole-sensitive purine transporter. Genistein was almost as effective in blocking ADO transport as dipyridamole, and both rapamycin and SB203580 had substantial activity (Fig. 24).

In the present study, the mechanism of actions of these drugs on ADO transporters was not explored. It could be either non-specific effects of the drugs on ADO transporters or a cell signaling-mediated effect. It has been shown in cultured chromaffin cells, secretagogues, such as acetylcholine and nicotine, and PKC activators inhibited ADO uptake into the cells via downregulation of high affinity ADO transporters (Delicado et al., 1991). In addition, ADO

transport was decreased in cultured EC isolated from gestational diabetic patients. Given the fact that diabetes manifest some forms of endothelial dysfunction, the role of ADO metabolism in this disease was implicated (Sobrevia et al., 1994).

Another enzyme involved in ADO metabolism, ecto-5'-NT was shown to participate in harnessing the beneficial effects of endogenous ADO both *in vivo* and *in vitro* models of acute inflammation. The anti-inflammatory effect of methotrexate and sulfasalazine, commonly used drugs in rheumatoid arthritis was mediated by the elevated extracellular level of ADO formed via ecto-5'-NT (Morabito et al., 1998). This enzyme was also shown to be activated in canine coronary arteries during hypoxia via activation of PKC (Minamino et al., 1995). In our experimental settings for ^{3}H-ADO uptake and release, however, it might not be possible to detect any change in 5'-NT activity.

The α and β-adrenergic agonist, norepinephrine did not have a significant effect on ADO uptake (Fig. 26). It has been shown that an α_1-adrenergic agonist, methoxamine induced an increase in both adenosine and adenine nucleotides preferentially from EC in segments of rabbit thoracic aorta (Sedaa et al., 1990). In cultured EC from porcine thoracic aorta, however, norepinephrine did not have a significant effect on the purine release (Borst and Schrader, 1991). These results may indicate that EC in

culture behaved differently in response to norepinephrine stimulation or the receptor-transduction system in the cells was not functional. Thrombin also has shown to induce an increase in the purine release from cultured human EC and porcine thoracic aorta EC, and enhanced membrane permeability is implicated as the mechanism (Deussen et al., 1993; Lollar and Owen, 1981) although our study in CPAE did not show a significant effect on ADO uptake.

Calcium ionophore A23187, which is known as a potent stimulus of nitric oxide formation in EC (Bassenge and Heusch, 1990), decreased ADO uptake into CPAE at concentrations of 3 μM and higher (Fig. 32), suggesting a role of calcium signaling in regulation of AK or ADO transporters. This result agrees with that in cultured porcine thoracic aorta EC where A23187 increased adenosine release although enhanced membrane permeability and the extracellular dephosphorylation of adenine nucleotides are implicated as the mechanism (Deussen et al., 1993). A number of signaling effectors and inhibitors for PKA, PKC and MAPK pathways, and kinase and phosphatase inhibitors tested did not have a significant effect on ADO uptake. These results suggest that at least these signaling pathways may not be involved in regulation of AK and ADO transporters in EC. Further studies are required to identify the exact signaling mechanism involved in regulation of AK in EC.

Table 2. Characteristics of purified adenosine kinases from difference sources.

Species	Tissue	Km (μM)	Mr (KDa)	References
Human	Liver	0.2	40	(Yamada et al., 1981)
	Placenta	0.4	38-41	(Palella et al., 1980)
Rat	Brain	0.2	38-41	(Yamada et al., 1981)
	Heart	0.2		(Fisher and Newsholme, 1984)
	Liver	0.3		(Drabikowska et al., 1985)
	YPEN-1	<0.5	40	(Hwang et al.) (In preparation)
Cow	Liver	1.4	38	(Hao and Gupta, 1996)
	CPAE	2.8	40	(Hwang et al., 1998) (Abstract)
Guinea pig	Heart	13	38	(Gorman et al., 1997)
	Brain	20		(Shimizu et al., 1972)

YPEN-1, rat prostate endothelial cells.
CPAE, cow pulmonary artery endothelial cells.

5. SUMMARY AND CONCLUSIONS

5.1. Summary

5.1.1. AK was purified from both cow pulmonary artery endothelial cells (CPAE) and rat prostate endothelial cells (YPEN-1). The identity of purified AK (40 KDa) was confirmed by labeling the enzyme with an AK antibody.

5.1.2. The Vmax and Km for adenosine of purified AK from CPAE and YPEN-1 were determined.

5.1.3. The purified AK was immunoreactive with phosphoserine and phosphothreonine antibodies.

5.1.4. The activity of AK purified from EC was decreased by 73±1.9% and 67±1.3% for cow and rat AK, respectively after treatment with the serine/threonine phosphatase, PPλ.

5.1.5. The immunosuppressant, FK-506 increased ADO release from EC.

5.1.6. FK-506 decreased ADO uptake into EC in a concentration-dependent manner.

5.1.7. The activity of AK in the crude membrane fraction of EC was decreased by previously exposing the intact cells with FK-506.

5.1.8. Another immunosuppressant, rapamycin decreased ADO uptake into EC as a result of ADO transport inhibition.

5.1.9. The p38 MAPK inhibitors, SB203580 and SB202190 and a tyrosine kinase inhibitor, genistein also decreased ADO uptake into EC as a result of ADO transport inhibition.

5.1.10. Hormones, or the signaling effectors and inhibitors involved in PKA and PKC pathways or other protein kinases (MEK and PI_3K) and phosphatases (PP1 and PP2A) did not have a significant effect on ADO uptake, measured as an index of AK activity.

5.2. CONCLUSIONS

5.2.1. The serine/threonine phosphorylation is a potential mechanism for regulation of AK activity in EC.

5.2.2. A potential mechanism of action of an immunosuppressant, FK-506 in ADO metabolism is inhibition of AK in EC.

5.2.3. AK activity associated with the plasma membrane could be responsible for changes in the extracellular ADO levels.

6. LITERATURE CITED

Ader, J.L. and L. Rostaing, 1998, Cyclosporin nephrotoxicity: pathophysiology and comparison with FK-506, Curr Opin Nephrol Hypertens 7, 539.

Andres, C.M. and I.H. Fox, 1979, Purification and properties of human placental adenosine kinase, J Biol Chem 254, 11388.

Ates, E., P. Sharma, S. Erkasap, D. Talbot, E. Ihtiyar, B. Yasar and H. Kiper, 1996, Cyclosporine nephrotoxicity in the ischemic kidney and the protective effect of pentoxifylline--a study in the rat, Transplantation 62, 864.

Balcells, E., J. Suarez and R. Rubio, 1992, Functional role of intravascular coronary endothelial adenosine receptors, Eur J Pharmacol 210, 1.

Bassenge, E. and G. Heusch, 1990, Endothelial and neuro-humoral control of coronary blood flow in health and disease, Rev Physiol Biochem Pharmacol 116, 77.

Becker, B.F., S. Zahler, C. Kupatt, C. Seligmann, B. Heindl and C. Kowalski, 1998, Cardiovascular actions of adenosine. Granulocyte and blood platelet adhesion in the reperfused myocardium, Adv Exp Med Biol 431, 73.

Becker, B.F., S. Zahler, C. Seligmann, C. Kupatt and H. Habazettl, 1996, [Interaction of adenosine with leukocytes and thrombocytes], Z Kardiol 85, 161.

Belardinelli, L., J. Linden and R.M. Berne, 1989, The cardiac effects of adenosine, Prog Cardiovasc Dis 32, 73.

Benigni, A., M. Morigi, N. Perico, C. Zoja, C.S. Amuchastegui, A. Piccinelli, R. Donadelli and G. Remuzzi, 1992, The acute effect of FK506 and cyclosporine on endothelial cell function and renal vascular resistance, Transplantation 54, 775.

Bennett, W.M., L.W. Elzinga, G.A. Porter and S. Rosen, 1992, The effects of pentoxifylline on experimental chronic cyclosporine nephrotoxicity, Transplantation 54, 1118.

Berne, R.M., 1980, The role of adenosine in the regulation of coronary blood flow, Circ Res 47, 807.

Bierer, B.E., P.S. Mattila, R.F. Standaert, L.A. Herzenberg, S.J. Burakoff, G. Crabtree and S.L. Schreiber, 1990, Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin, Proc Natl Acad Sci U S A 87, 9231.

Borst, M.M. and J. Schrader, 1991, Adenine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine, Circ Res 68, 797.

Bouma, M.G., T.M. Jeunhomme, D.L. Boyle, M.A. Dentener, N.N. Voitenok, F.A. van den Wildenberg and W.A. Buurman, 1997a, Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A2 and A3 receptors, J Immunol 158, 5400.

Bouma, M.G., F.A. van den Wildenberg and W.A. Buurman, 1996, Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells, Am J Physiol 270, C522.

Bouma, M.G., F.A. van den Wildenberg and W.A. Buurman, 1997b, The anti-inflammatory potential of adenosine in ischemia-reperfusion injury: established and putative beneficial actions of a retaliatory metabolite, Shock 8, 313.

Brunet, A. and J. Pouyssegur, 1997, Mammalian MAP kinase modules: how to transduce specific signals, Essays Biochem 32, 1.

Bullough, D.A., M.J. Magill, K.M. Mullane and G.S. Firestein, 1996, Carbohydrate- and CD18-dependent neutrophil adhesion to cardiac myocytes: effects of adenosine, Cardiovasc Res 32, 328.

Burkhardt, H. and J.R. Kalden, 1997, Xenobiotic immunosuppressive agents: therapeutic effects in animal models of autoimmune diseases, Rheumatol Int 17, 85.

Carrier, M., L.P. Perrault, F. Tronc, D.J. Stewart and C.L. Pelletier, 1993, Pentoxifylline decreases cyclosporine-induced renal endothelin release and vasoconstriction, Ann Thorac Surg 55, 490.

Ciruela, F., C. Saura, E.I. Canela, J. Mallol, C. Lluis and R. Franco, 1996, Adenosine deaminase affects ligand-

induced signalling by interacting with cell surface adenosine receptors, FEBS Lett 380, 219.

Coughlin, S.R., 1999, How the protease thrombin talks to cells, Proc Natl Acad Sci U S A 96, 11023.

Cronstein, B.N., R.I. Levin, M. Philips, R. Hirschhorn, S.B. Abramson and G. Weissmann, 1992, Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors, J Immunol 148, 2201.

Cronstein, B.N., M.C. Montesinos and G. Weissmann, 1999a, Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFkappaB, Proc Natl Acad Sci U S A 96, 6377.

Cronstein, B.N., M.C. Montesinos and G. Weissmann, 1999b, Sites of action for future therapy: an adenosine-dependent mechanism by which aspirin retains its antiinflammatory activity in cyclooxygenase-2 and NFkappaB knockout mice, Osteoarthritis Cartilage 7, 361.

Cronstein, B.N., E.D. Rosenstein, S.B. Kramer, G. Weissmann and R. Hirschhorn, 1985, Adenosine; a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A2 receptor on human neutrophils, J Immunol 135, 1366.

Decking, U.K., C. Alves, R. Spahr and J. Schrader, 1994, 2-Fluoroadenosine uptake by erythrocytes and endothelial cells studied by 19F-NMR, Am J Physiol 266, H1596.

Decking, U.K., G. Schlieper, K. Kroll and J. Schrader, 1997, Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release, Circ Res 81, 154.

Del Vecchio, P.J. and J.R. Smith, 1981, Expression of angiotensin-converting enzyme activity in cultured pulmonary artery endothelial cells, J Cell Physiol 108, 337.

Delicado, E.G., R.P. Sen and M.T. Miras-Portugal, 1991, Effects of phorbol esters and secretagogues on nitrobenzylthioinosine binding to nucleoside

transporters and nucleoside uptake in cultured chromaffin cells, Biochem J 279, 651.

Detmers, P.A., D. Zhou, E. Polizzi, R. Thieringer, W.A. Hanlon, S. Vaidya and V. Bansal, 1998, Role of stress-activated mitogen-activated protein kinase (p38) in beta 2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst, J Immunol 161, 1921.

Deussen, A., B. Bading, M. Kelm and J. Schrader, 1993, Formation and salvage of adenosine by macrovascular endothelial cells, Am J Physiol 264, H692.

Deussen, A., G. Moser and J. Schrader, 1986, Contribution of coronary endothelial cells to cardiac adenosine production, Pflugers Arch 406, 608.

Downey, J.M., G.S. Liu and J.D. Thornton, 1993, Adenosine and the anti-infarct effects of preconditioning, Cardiovasc Res 27, 3.

Drabikowska, A.K., L. Halec and D. Shugar, 1985, Purification and properties of adenosine kinase from rat liver: separation from deoxyadenosine kinase activity, Z Naturforsch [C] 40, 34.

Duchen, M.R., O. McGuinness, L.A. Brown and M. Crompton, 1993, On the involvement of a cyclosporin A sensitive mitochondrial pore in myocardial reperfusion injury, Cardiovasc Res 27, 1790.

Ely, S.W. and R.M. Berne, 1992, Protective effects of adenosine in myocardial ischemia, Circulation 85, 893.

Ely, S.W., G.P. Matherne, S.D. Coleman and R.M. Berne, 1992, Inhibition of adenosine metabolism increases myocardial interstitial adenosine concentrations and coronary flow, J Mol Cell Cardiol 24, 1321.

Firestein, G.S., D. Boyle, D.A. Bullough, H.E. Gruber, F.G. Sajjadi, A. Montag, B. Sambol and K.M. Mullane, 1994, Protective effect of an adenosine kinase inhibitor in septic shock, J Immunol 152, 5853.

Firestein, G.S., D.A. Bullough, M.D. Erion, R. Jimenez, M. Ramirez-Weinhouse, J. Barankiewicz, C.W. Smith, H.E. Gruber and K.M. Mullane, 1995, Inhibition of neutrophil adhesion by adenosine and an adenosine kinase inhibitor. The role of selectins, J Immunol 154, 326.

Fisher, M.N. and E.A. Newsholme, 1984, Properties of rat heart adenosine kinase, Biochem J 221, 521.

Gawaz, M., F.J. Neumann, I. Ott, A. Schiessler and A. Schomig, 1996, Platelet function in acute myocardial infarction treated with direct angioplasty [see comments], Circulation 93, 229.

Gorman, M.W., M.X. He, C.S. Hall and H.V. Sparks, 1997, Inorganic phosphate as regulator of adenosine formation in isolated guinea pig hearts, Am J Physiol 272, H913.

Grisham, M.B., L.A. Hernandez and D.N. Granger, 1989, Adenosine inhibits ischemia-reperfusion-induced leukocyte adherence and extravasation, Am J Physiol 257, H1334.

Gu, J.G., A. Nath and J.D. Geiger, 1996, Characterization of inhibitor-sensitive and -resistant adenosine transporters in cultured human fetal astrocytes, J Neurochem 67, 972.

Guerini, D., 1997, Calcineurin: not just a simple protein phosphatase, Biochem Biophys Res Commun 235, 271.

Guieu, R., B. Dussol, C. Devaux, J. Sampol, P. Brunet, H. Rochat, G. Bechis and Y.F. Berland, 1998, Interactions between cyclosporine A and adenosine in kidney transplant recipients, Kidney Int 53, 200.

Gupta, R.S., 1996, Adenosine-AMP exchange activity is an integral part of the mammalian adenosine kinase, Biochem Mol Biol Int 39, 493.

Hao, W. and R.S. Gupta, 1996, Pentavalent ions dependency of mammalian adenosine kinase, Biochem Mol Biol Int 38, 889.

Hirschhorn, R., 1995, Adenosine deaminase deficiency: molecular basis and recent developments, Clin Immunol Immunopathol 76, S219.

Hourani, S.M. and N.J. Cusack, 1991, Pharmacological receptors on blood platelets, Pharmacol Rev 43, 243.

Hsueh, W.A. and R.E. Law, 1999, Insulin signaling in the arterial wall, Am J Cardiol 84, 21J.

Huang, S., S. Apasov, M. Koshiba and M. Sitkovsky, 1997, Role of A2a extracellular adenosine receptor-mediated

signaling in adenosine-mediated inhibition of T-cell activation and expansion, Blood 90, 1600.

Hwang, K., C.S. Hall, W.S. Spielman and H.V. Sparks, 1998, Characteristics of adenosine kinase purified from bovine pulmonary artery endothelium, FASEB J (Abstract) 12, A88.

Jiang, N., E.A. Kowaluk, C.H. Lee, H. Mazdiyasni and M. Chopp, 1997, Adenosine kinase inhibition protects brain against transient focal ischemia in rats, Eur J Pharmacol 320, 131.

Jordan, J.E., Z.Q. Zhao, H. Sato, S. Taft and J. Vinten-Johansen, 1997, Adenosine A2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence, J Pharmacol Exp Ther 280, 301.

Kaufmann, S.H., C.M. Ewing and J.H. Shaper, 1987, The erasable Western blot, Anal Biochem 161, 89.

Kitakaze, M., M. Hori, H. Sato, S. Takashima, M. Inoue, A. Kitabatake and T. Kamada, 1991, Endogenous adenosine inhibits platelet aggregation during myocardial ischemia in dogs, Circ Res 69, 1402.

Kochan, Z., R.T. Smolenski, M.H. Yacoub and A.L. Seymour, 1994, Nucleotide and adenosine metabolism in different cell types of human and rat heart, J Mol Cell Cardiol 26, 1497.

Koshiba, M., H. Kojima, S. Huang, S. Apasov and M.V. Sitkovsky, 1997, Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells, J Biol Chem 272, 25881.

Kowaluk, E.A., S.S. Bhagwat and M.F. Jarvis, 1998, Adenosine kinase inhibitors, Curr Pharm Des 4, 403.

Kroll, K., U.K. Decking, K. Dreikorn and J. Schrader, 1993, Rapid turnover of the AMP-adenosine metabolic cycle in the guinea pig heart, Circ Res 73, 846.

Kroll, K., J. Schrader, H.M. Piper and M. Henrich, 1987, Release of adenosine and cyclic AMP from coronary endothelium in isolated guinea pig hearts: relation to coronary flow, Circ Res 60, 659.

Laird, S.M., A. Graham, A. Paul, G.W. Gould, C. Kennedy and R. Plevin, 1998, Tumour necrosis factor stimulates stress-activated protein kinases and the inhibition of DNA synthesis in cultures of bovine aortic endothelial cells, Cell Signal 10, 473.

Lee, J.I. and G.J. Burckart, 1998, Nuclear factor kappa B: important transcription factor and therapeutic target, J Clin Pharmacol 38, 981.

Li, J.M., R.A. Fenton, B.S. Cutler and J.G. Dobson, Jr., 1995, Adenosine enhances nitric oxide production by vascular endothelial cells, Am J Physiol 269, C519.

Liao, J.K., 1998, Endothelium and acute coronary syndromes, Clin Chem 44, 1799.

Lin, B.B., M.C. Hurley and I.H. Fox, 1988, Regulation of adenosine kinase by adenosine analogs, Mol Pharmacol 34, 501.

Lollar, P. and W.G. Owen, 1981, Active-site-dependent, thrombin-induced release of adenine nucleotides from cultured human endothelial cells, Ann N Y Acad Sci 370, 51.

Luscinskas, F.W. and M.A. Gimbrone, Jr., 1996, Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment, Annu Rev Med 47, 413.

Maggirwar, S.B., D.N. Dhanraj, S.M. Somani and V. Ramkumar, 1994, Adenosine acts as an endogenous activator of the cellular antioxidant defense system, Biochem Biophys Res Commun 201, 508.

Massoudy, P., S. Zahler, C. Kupatt, E. Reder, B.F. Becker and E. Gerlach, 1997, Cardioprotection by cyclosporine A in experimental ischemia and reperfusion--evidence for a nitric oxide-dependent mechanism mediated by endothelin, J Mol Cell Cardiol 29, 535.

Millar, C.G., G.F. Baxter and C. Thiemermann, 1996, Protection of the myocardium by ischaemic preconditioning: mechanisms and therapeutic implications, Pharmacol Ther 69, 143.

Miller, L.P., L.A. Jelovich, L. Yao, J. DaRe, B. Ugarkar and A.C. Foster, 1996, Pre- and peristroke treatment with the adenosine kinase inhibitor, 5'-deoxyiodotubercidin, significantly reduces infarct

volume after temporary occlusion of the middle cerebral artery in rats, Neurosci Lett 220, 73.

Minamino, T., M. Kitakaze, K. Komamura, K. Node, H. Takeda, M. Inoue, M. Hori and T. Kamada, 1995, Activation of protein kinase C increases adenosine production in the hypoxic canine coronary artery through the extracellular pathway, Arterioscler Thromb Vasc Biol 15, 2298.

Morabito, L., M.C. Montesinos, D.M. Schreibman, L. Balter, L.F. Thompson, R. Resta, G. Carlin, M.A. Huie and B.N. Cronstein, 1998, Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides, J Clin Invest 101, 295.

Moser, G.H., J. Schrader and A. Deussen, 1989, Turnover of adenosine in plasma of human and dog blood, Am J Physiol 256, C799.

Mullane, K. and D. Bullough, 1995, Harnessing an endogenous cardioprotective mechanism: cellular sources and sites of action of adenosine, J Mol Cell Cardiol 27, 1041.

Nees, S., V. Herzog, B.F. Becker, M. Bock, C. Des Rosiers and E. Gerlach, 1985, The coronary endothelium: a highly active metabolic barrier for adenosine, Basic Res Cardiol 80, 515.

Newby, A.C., 1986, How does dipyridamole elevate extracellular adenosine concentration? Predictions from a three-compartment model of adenosine formation and inactivation, Biochem J 237, 845.

Newby, A.C., 1991, Adenosine: origin and clinical roles, Adv Exp Med Biol , 265.

Olsson, R.A. and J.D. Pearson, 1990, Cardiovascular purinoceptors, Physiol Rev 70, 761.

Palella, T.D., C.M. Andres and I.H. Fox, 1980, Human placental adenosine kinase. Kinetic mechanism and inhibition, J Biol Chem 255, 5264.

Palmer, T.M. and G.L. Stiles, 1997, Structure-function analysis of inhibitory adenosine receptor regulation, Neuropharmacology 36, 1141.

Rosengren, S., G.W. Bong and G.S. Firestein, 1995, Anti-inflammatory effects of an adenosine kinase inhibitor. Decreased neutrophil accumulation and vascular leakage, J Immunol 154, 5444.

Sander, M. and R.G. Victor, 1995, Hypertension after cardiac transplantation: pathophysiology and management, Curr Opin Nephrol Hypertens 4, 443.

Saura, C., F. Ciruela, V. Casado, E.I. Canela, J. Mallol, C. Lluis and R. Franco, 1996, Adenosine deaminase interacts with A1 adenosine receptors in pig brain cortical membranes, J Neurochem 66, 1675.

Sedaa, K.O., R.A. Bjur, K. Shinozuka and D.P. Westfall, 1990, Nerve and drug-induced release of adenine nucleosides and nucleotides from rabbit aorta, J Pharmacol Exp Ther 252, 1060.

Seligmann, C., C. Kupatt, B.F. Becker, S. Zahler and S. Beblo, 1998, Adenosine endogenously released during early reperfusion mitigates postischemic myocardial dysfunction by inhibiting platelet adhesion, J Cardiovasc Pharmacol 32, 156.

Shimizu, H., S. Tanaka and T. Kodama, 1972, Adenosine kinase of mammalian brain: partial purification and its role for the uptake of adenosine, J Neurochem 19, 687.

Shryock, J.C. and L. Belardinelli, 1997, Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology, Am J Cardiol 79, 2.

Shryock, J.C., R. Rubio and R.M. Berne, 1988, Release of adenosine from pig aortic endothelial cells during hypoxia and metabolic inhibition, Am J Physiol 254, H223.

Singh, B., W. Hao, Z. Wu, B. Eigl and R.S. Gupta, 1996, Cloning and characterization of cDNA for adenosine kinase from mammalian (Chinese hamster, mouse, human and rat) species. High frequency mutants of Chinese hamster ovary cells involve structural alterations in the gene, Eur J Biochem 241, 564.

Smolenski, R.T., Z. Kochan, R. McDouall, C. Page, A.L. Seymour and M.H. Yacoub, 1994, Endothelial nucleotide catabolism and adenosine production, Cardiovasc Res 28, 100.

Snyder, F.F. and T. Lukey, 1982, Kinetic considerations for the regulation of adenosine and deoxyadenosine metabolism in mouse and human tissues based on a thymocyte model, Biochim Biophys Acta 696, 299.

Sobrevia, L., S.M. Jarvis and D.L. Yudilevich, 1994, Adenosine transport in cultured human umbilical vein endothelial cells is reduced in diabetes, Am J Physiol 267, C39.

Sparks, H.V., Jr. and H. Bardenheuer, 1986, Regulation of adenosine formation by the heart, Circ Res 58, 193.

Sparks, H.V., Jr., D.F. DeWitt, R.D. Wangler, M.W. Gorman and J.B. Bassingthwaighte, 1985, Capillary transport of adenosine, Fed Proc 44, 2620.

Sparks, H.V., M.W. Gorman and C.S. Hall, 1998, Adenosine release of the isolated guinea pig heart is regulated by phosphorylation of adenosine kinase, FASEB J (Abstract) 12, A670.

Springer, T.A., 1994, Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm, Cell 76, 301.

Spychala, J., N.S. Datta, K. Takabayashi, M. Datta, I.H. Fox, T. Gribbin and B.S. Mitchell, 1996, Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases, Proc Natl Acad Sci U S A 93, 1232.

Spychala, J. and B.S. Mitchell, 1998, Cyclosporin A and FK506 inhibit adenosine uptake and adenosine kinase activity in T-lymphocytes, FASEB J (Abstract) 12, A939.

Spychala, J., B.S. Mitchell and J. Barankiewicz, 1997, Adenosine metabolism during phorbol myristate acetate-mediated induction of HL-60 cell differentiation: changes in expression pattern of adenosine kinase, adenosine deaminase, and 5'-nucleotidase, J Immunol 158, 4947.

Stokoe, D., S.G. Macdonald, K. Cadwallader, M. Symons and J.F. Hancock, 1994, Activation of Raf as a result of recruitment to the plasma membrane [see comments] [published erratum appears in Science 1994 Dec 16;266(5192):1792-3], Science 264, 1463.

Sweeney, M.I., 1997, Neuroprotective effects of adenosine in cerebral ischemia: window of opportunity, Neurosci Biobehav Rev 21, 207.

Tatlisumak, T., K. Takano, R.A. Carano, L.P. Miller, A.C. Foster and M. Fisher, 1998, Delayed treatment with an adenosine kinase inhibitor, GP683, attenuates infarct size in rats with temporary middle cerebral artery occlusion, Stroke 29, 1952.

Thorn, J.A. and S.M. Jarvis, 1996, Adenosine transporters, Gen Pharmacol 27, 613.

Thornton, J., S. Striplin, G.S. Liu, A. Swafford, A.W. Stanley, D.M. Van Winkle and J.M. Downey, 1990, Inhibition of protein synthesis does not block myocardial protection afforded by preconditioning, Am J Physiol 259, H1822.

Vanhoutte, P.M., 1997, Endothelial dysfunction and atherosclerosis, Eur Heart J 18 Suppl E, E19.

Von Lubitz, D.K., R.C. Lin, N. Melman, X.D. Ji, M.F. Carter and K.A. Jacobson, 1994, Chronic administration of selective adenosine A1 receptor agonist or antagonist in cerebral ischemia, Eur J Pharmacol 256, 161.

Wang, J., L. Drake, F. Sajjadi, G.S. Firestein, K.M. Mullane and D.A. Bullough, 1997, Dual activation of adenosine A1 and A3 receptors mediates preconditioning of isolated cardiac myocytes, Eur J Pharmacol 320, 241.

Yamada, Y., H. Goto and N. Ogasawara, 1981, Adenosine kinase from human liver, Biochim Biophys Acta 660, 36.

Yamazaki, K. and K.J. Pienta, 1995, Establishment of an immortalized Copenhagen rat prostate epithelial cell line, In Vivo 9, 427.

Zhao, Z.Q., H. Sato, M.W. Williams, A.Z. Fernandez and J. Vinten-Johansen, 1996, Adenosine A2-receptor activation inhibits neutrophil-mediated injury to coronary endothelium, Am J Physiol 271, H1456.