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THE REGULATION AND LOCALIZATION OF ADENOSINE RECEPTORS IN RENAL EPITHELIAL CELLS

By

David Grant LeVier

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ABSTRACT

THE REGULATION AND LOCALIZATION OF ADENOSINE RECEPTORS IN RENAL EPITHELIAL CELLS

By

David Grant LeVier

The research presented in this thesis explores the regulation and localization of G-protein linked adenosine receptors in renal epithelial cells. Desensitization of adenosine receptors in primary cultures of rabbit cortical collecting tubule (RCCT) cells involves the altered function of both A_1 and A_2 adenosine receptor subtypes. Additionally, altered function of the regulatory G-protein, G_s, confers a heterologous component to this desensitization. The adenosine receptor-mediated pathways were localized by functional criteria to either the apical or basolateral plasma membrane domains of porcine LLC-PK_1 renal cells in monolayer culture. The adenosine receptor mediated pathway that stimulates inositol phosphate turnover was localized to the basolateral surface of LLC-PK_1 cells. The inhibitory adenosine A_1 receptor pathway was also localized to the basolateral aspect of the cell monolayer, although a minor apical component could not be ruled out. The stimulatory adenosine A_2 receptor pathway was found on both sides of the monolayer, although the major component was apical with a smaller, albeit significant, component residing on the basolateral side of LLC-PK_1 cells. Desensitization of stimulatory adenosine A_2 receptors on both sides of LLC-PK_1

cells, following selective exposure of adenosine to either the apical or basolateral side of the monolayer, was found to be homologous. This pattern of homologous desensitization was the same irrespective of the side of the monolayer on which the receptors were exposed to adenosine. This observation suggested that adenosine A_2 receptors on the side of the monolayer opposite that of adenosine exposure, which were presumably unoccupied/unactivated, could be desensitized independently of other unoccupied/unactivated G-protein linked receptors.

This dissertation is dedicated to my parents, Robert and Vivian, my brother, Steven, and most especially to Sue. Only with their love and constant encouragement was I able to complete this work.

With all my love, Dave

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I. Introduction

Adenosine is a catabolic product of adenine nucleotides which was first proposed as a retaliatory metabolic regulator of coronary blood flow by Burne in 1963. Since then, adenosine has been implicated as a physiological regulator of a number of physiological phenomena including several aspects of renal function (reviewed by Spielman et al. 1987; Spielman and Arend 1991). During increased metabolic demand and/or reduced availability of oxygen, catabolism of ATP is increased leading to an increase in adenosine production. Adenosine then exits the cell and acts on cell surface receptors. Adenosine is thought to act as a local hormome, because it is rapidly degraded by adenosine deaminase present in tissues and blood (Conway and Cooke 1939). Adenosine released into the interstitium is thought to act in a feedback loop to re-establish the appropriate level of energy utilization and/or oxygen demand for the current physiological conditions.

Adenosine receptors that, when activated, stimulate and inhibit adenylyl cyclase activity have been shown to exist in renal epithelial cells (Arend et al. 1987). Adenosine also has been shown to stimulate the turnover of inositol phosphates and to mobilize calcium in renal epithelial cells (Arend et al. 1988; Arend et al. 1989; Weinberg et al. 1989). Despite this characterization, little work

has been done to elucidate the regulation or localization of adenosine receptors in these epithelia.

This dissertation presents research on the regulation and localization of adenosine receptors in renal epithelial cells grown in culture. The first chapter is a literature review, the first two sections of which discuss the metabolism of adenosine and the cell surface receptors through which it acts, while the following three sections cover receptor regulation and localization as they relate to the research presented in the thesis. The three chapters that follow the literature review encompass the three research projects that comprise the thesis. Each consists of an Introduction, Methods, Results and Discussion section. The first of these presents research on the desensitization of adenosine receptors in primary cultures of rabbit cortical collecting tubule cells. The second presents results of investigation of the spatial localization of adenosine receptors in the porcine renal cell line, LLC-PK_1. The last chapter extends that preceding it with an investigation of the desensitization of adenosine receptors in the LLC-PK_1 cell line with regard to their spatial distribution. The final chapter summarizes the research presented in this thesis.

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II. Literature Review

A. Adenosine Metabolism

Adenosine, a purine nucleoside, is both a percursor and metabolite of the adenine nucleotides (Figure 1). Adenosine is also coupled to the production of S-adenosylhomocysteine by SAH hydrolase. The equilibrium of this reaction favors the production of S-adenosylhomocysteine, although under normoxic conditions the low intracellular concentrations of adenosine and homocysteine that prevail actually contribute to the hydrolysis of S-adenosylhomocysteine and the production of adenosine (Lloyd and Schrader 1987; Porter and Boyd 1992). In spite of this, SAH hydrolase is inhibited by adenosine and inosine and is assumed not to play an important role in the production of adenosine under hypoxic conditions (Eloranta 1977; Lloyd and Schrader 1987; Schatz et al. 1977). Therefore, the majority of adenosine produced both intracellularly and extracellularly in response to increased oxygen demand and/or hypoxic conditions is thought to arise from the catabolism of adenine nucleotides.

Adenosine originates from the dephosphorylation of 5'-AMP by 5'-nucleotidase which is found as both a cytosolic enzyme and an ectoenzyme (Dendorfer et al. 1987; Frick and Lowenstein 1976; Pearson et al. 1980). Cytosolic 5'-nucleotidase is thought to be responsible for the increase in adenosine observed under hypoxic conditions (Altschuld et al. 1987; Collinson et al. 1987; Frick and Lowenstein 1976; Newby et al. 1987; Shryock et al. 1988). Ecto 5'-nucleotidase

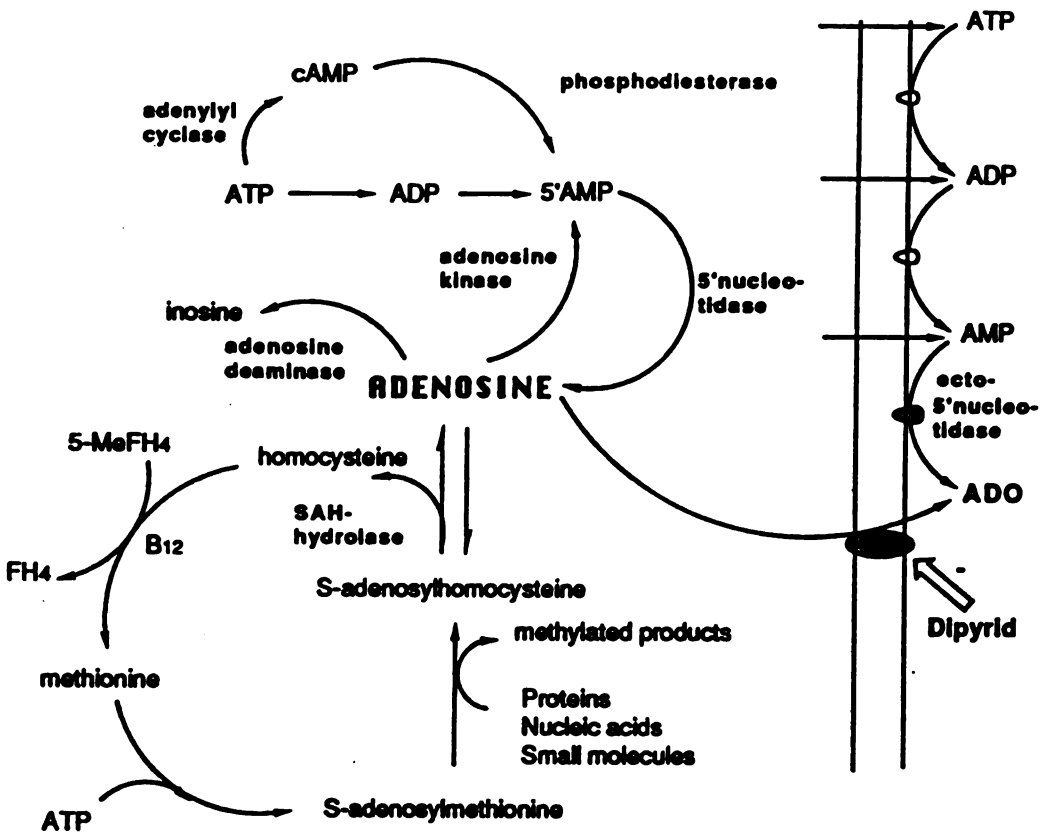

Figure 1. Flow diagram of adenosine sources and sinks.

is thought to be important in terminating the action of extracellular ATP on P_2 purinergic receptors (Gordon 1986). The close association of ecto-ATPase, -ADPase and -5'-nucleotidase along with the nucleoside transporter has been linked to the conversion of extracellular ATP to adenosine and the subsequent translocation of adenosine into the cytosol in the rat heart (Frick and Lowenstein 1978).

Adenosine that is released into or generated in the extracellular compartment can be removed by two mechanisms; 1) conversion to inosine by adenosine deaminase and 2) uptake via facilitated diffusion by the nucleoside transporter into cells and subsequent phosphorylation by adenosine kinase to 5'-AMP(Plagemann and Wohlhueter 1980). The nucleoside transporter is relatively non-selective for adenosine or inosine and allows the bidirectional passage of these nucleosides down their respective concentration gradients. Adenosine deaminase is found both intra- and extracellularly (Baer et al. 1966; Conway and Cooke 1939), and will deaminate adenosine to inosine. Due to the ubiquitous nature of this enzyme, it is thought that any adenosine that enters the circulation will be converted quickly to inosine. Additionally, red blood cells have been shown to take up adenosine and metabolize it (Meyskens and Williams 1971). Adenosine deaminase (EC 3.5.4.4) has a relatively high K_m (35 μM), but it also has a high V_{max}, which allows it to effectively reduce adenosine concentrations to below 1 μM (Manfredi and Holmes 1985; Sparks and Bardenheuer 1986). Adenosine kinase is an intracellular enzyme

that phosphorylates adenosine to 5'-AMP. Adenosine kinase has a low Km (0.4 μM) and is inhibited by high concentrations of adenosine (Miller et al. 1979). Consequently, the majority of adenosine at high concentrations (>50 μM), which occur during increased energy demand and/or hypoxia, is removed by the action of adenosine deaminase. At low concentrations (< 1 μM) prevalent under normoxic conditions, adenosine is phosphorylated to 5'-AMP by adenosine kinase.

The kidney has been shown to both take up and release adenosine (Thompson et al. 1985). It also has been conclusively established that the ischemic kidney will produce adenosine (Miller et al. 1978; Osswald et al. 1980). However, the specific sites of uptake and release of adenosine in the kidney have not been clearly elucidated. Using stop-flow techniques, Miller et al. (1978) suggested that the proximal tubules make a significant contribution to the extracellular adenosine produced in the ischemic kidney. In regard to the uptake of adenosine and its conversion into the nucleotide pool, experiments using isolated renal proximal tubules have shown that exogenous ATP is more effective in increasing intracellular ATP pools than is exogenous adenosine (Weinberg and Humes 1986; Weinberg et al. 1988). Adenosine at a concentration of 250 μM was preferentially deaminated to inosine, which is in keeping with substrate inhibition of adenosine kinase by adenosine. Adenine nucleotides at a concentration of 250 μM lead to increases in intracellular ATP which could be blocked by 5-iodotubercidin, an adenosine kinase inhibitor. These observations concur with those of Frick and

Lowenstein (1978) in suggesting that adenosine production by ecto-5'-nucleotidase in the rat heart is coupled to the uptake of adenosine.

Recent advances in immunohistochemistry have enabled researchers to localize ecto-5'-nucleotidase in frozen sections of rat kidney. Using a monoclonal antibody, ecto-5'-nucleotidase was localized to the apical aspect of proximal tubules, the apical aspect of intercalated cells of the collecting tubule, interstitial cells of the cortex, and the surrounding cells of the afferent and efferent arterioles (Gandhi et al. 1990). Whether this ecto-5'-nucleotidase is involved in the production of extracellular adenosine or the vectorial uptake of adenosine via the nucleoside transporter is unknown. While it is clear that adenosine is released by the ischemic kidney, the specific source(s) of this adenosine has yet to be elucidated.

B. Adenosine Receptors

For more than 60 years it has been recognized that adenosine has physiological effects (Drury and Szent-Gyorgi 1929). To cite just a few, adenosine has been linked to changes in coronary blood flow (Burne 1963; Burne 1980), renal function (reviewed by Spielman et al. 1987; Spielman and Arend 1991), release of neurotransmitters (Fredholm et al. 1987), and inhibition of lipolysis (Fain and Malbon 1979). As discussed earlier, adenosine is metabolized quickly and most probably does not act as a blood borne hormone, but rather as an autocrine and

paracrine effector. Adenosine is released into the extracellular space in response to increased energy demand and/or reduced oxygen supply, and acts on cell surface receptors activating second messenger systems to bring about functional changes.

The purinergic receptors, P_1 and P_2, were first described by Burnstock (1978). The P_1 and P_2 classifications are based on the potency of either adenosine or adenine nucleotides at these respective sites; P_1 receptors show an agonist potency profile of adenosine > AMP > ADP > ATP, while P_2 receptors have a potency profile of ATP > ADP > AMP > adenosine. Moreover, the P_1 receptors are antagonized by methylxanthines, such as caffeine and theophylline, while P_2 receptors are not. To date there have been no antagonists developed for the P_2 class of purinergic receptors.

The P_1 receptors are further classified as adenosine A_1 and A_2 receptors (Londos et al. 1980). These cell surface adenosine receptors have typically been distinguished on the basis of their selectivity for adenosine analogs. Three of the adenosine analogs in wide use are, N^6-cyclohexyl adenosine (CHA), R(-)N^6-(2-phenylisopropyl) adenosine (R-PIA), and 5'-N-ethylcarboxamido adenosine (NECA). The adenosine A_1 receptor has an agonist potency profile of CHA > R-PIA > NECA, while the adenosine A_2 receptor has the reverse potency profile of NECA > R-PIA > CHA. Typically, analogs with substitutions at the N^6 position are selective for the adenosine A_1 receptor, while analogs with 5'- and 2-substitutions are selective for the adenosine A_2 receptor (Daly and Padgett 1992).

The prototypical adenosine A_1 receptor is activated by nanomolar concentrations of agonist and is inhibitory towards adenylyl cyclase. The adenosine A_2 receptor is activated by micromolar concentrations of agonist and is stimulatory towards adenylyl cyclase. The adenosine A_2 receptor is coupled to adenylyl cyclase through the regulatory guanine nucleotide binding protein, G_s. In contrast, the adenosine A_1 receptor is coupled to multiple effectors through pertussis toxin-sensitive regulatory G-proteins, presumably of the G_i family. In addition to inhibiting adenylyl cyclase (Table 1), adenosine A_1 receptors stimulate (Arend et al. 1989) and inhibit (Delahunty et al. 1988) inositol phosphate turnover, inhibit chloride transport (Kelley et al. 1990; Schwiebert et al. 1992), activate potassium channels (Kurachi et al. 1986), and stimulate cGMP production (Kurtz 1987). In further distinguishing the A_1 receptor from the A_2 receptor, an antagonist with a 700 fold selectivity for the adenosine A_1 receptor, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), has been developed and proven to be very useful (Lohse et al. 1987). Unfortunately, an antagonist with similar selectivity for the adenosine A_2 receptor has not yet been developed. The best adenosine A_2 antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), has only a 5-20 fold selectivity for the adenosine A_2 receptor (Seale et al. 1988; Sebastiao and Ribeiro 1989).

There is another "adenosine receptor" located on the catalytic subunit of adenylyl cyclase termed the P site. This intracellular P site inhibits adenylyl

Table 1. Coupling of Adenosine Receptors to Effectors

Effect of A_1 stimulation	Tissue
Inhibition of adenylyl cyclase	Adipose Renal Cardiac CNS
Increased inositol phosphate	28A-RCCT cells
Decreased inositol phosphate	GH_3 pituitary cells
Decreased chloride transport	Shark rectal gland 28A-RCCT cells
Activated K^+_{Ach} channels	Atrial cells
Increased cGMP production	smooth muscle cells
Effect of A_2 stimulation	**Tissue**
Stimulation of adenylyl cyclase	RCCT cells 28A-RCCT cells vascular smooth muscle Platlets Striatum

cyclase and is activated by millimolar concentrations of adenosine. In contrast to the extracellular adenosine receptors, the P site is not antagonized by methylxanthines. The physiological function of the P site is still in question, because adenosine kinase and adenosine deaminase maintain intracellular concentrations of adenosine well below the micromolar range. Therefore, it appears unlikely that adenosine plays a role as the endogenous ligand of the P site. A metabolite of nucleic acids, 3'-AMP, however, is a likely candidate as an endogenous ligand because it occurs at sufficient concentrations and has sufficient affinity to activate the P site (Johnson et al. 1989).

Very recently, the structural features of the adenosine receptors have begun to be elucidated. Photoaffinity and purification studies have demonstrated that the adenosine A_1 receptor has an apparent molecular weight of 35,000 daltons (Klotz et al. 1985; Lohse et al. 1986; Nakata 1990). Similar studies also have established that the adenosine A_1 receptor is glycosylated (Klotz and Lohse 1986; Stiles 1986). Furthermore, the adenosine A_1 and A_2 receptors have been cloned and can be distinguished on the basis of different nucleotide and amino acid sequence (Libert et al. 1991; Maenhaut et al. 1990). The clones of the adenosine A_1 and A_2 receptors have seven transmembrane-spanning domains that have become a distinguishing characteristic of G-protein linked receptors. The amino acid sequences of these two adenosine receptors share a 51% identity with one another in their transmembrane domains. The cloned adenosine A_1 receptor has a

molecular weight of 36,000 daltons, while the cloned adenosine A_2 receptor has a molecular weight of 45,000 daltons (Libert et al. 1991; Maenhaut et al. 1990). In addition, the adenosine A_1 receptor has a putative glycosylation site on the second extracellular loop and putative phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) on the third intracellular loop (reviewed by Stiles 1992). The possible significance of phosphorylation sites on these receptors will be discussed in the section on receptor regulation.

C. Regulation of β-Adrenergic Receptors

Quite often, biological systems regulate their responsiveness to stimuli such that a stimulus does not elicit the same response upon a second or prolonged exposure. This phenomenon is often termed tachyphylaxis, indicating a reduced effectiveness of a drug over time. The biochemical basis for this phenomenon has recently gained considerable attention, with a goal being to control tachyphylaxis and extend the usefulness of pharmacologic agents. A large number of pharmacologic agents act through cell surface receptors, of which the β-adrenergic receptor is a well studied example.

The β-adrenergic receptor, like the rhodopsin receptor, belongs to the family of cell surface receptors that are characterized by seven transmembrane spanning domains. These receptors couple to their various effectors through guanine nucleotide binding proteins (G-proteins). The G-protein linked receptors have been

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observed to undergo tachyphylaxis and this has been termed desensitization of receptor response. With regard to desensitization, the most extensively studied of these G-protein linked receptors is the β-adrenergic receptor system (reviewed in Benovic et al. 1988; Hausdorff et al. 1990). The β-adrenergic receptor is linked through G_s to adenylyl cyclase and thus increases the production of cAMP upon activation by an agonist. Consequently, there are at least three points at which altered function could act to bring about desensitization, those being the β-adrenergic receptor, G_s, and adenylyl cyclase.

There are two forms of desensitization, "homologous" and "heterologous". Homologous desensitization is the reduced responsiveness of a specific receptor system following exposure of that system to agonist. In contrast, heterologous desensitization is the reduced responsiveness of multiple distinct receptor systems following exposure of one of those systems to agonist. Both forms of desensitization involve the phosphorylation of the receptor by various kinases, which leads to the rapid uncoupling of the receptor from its G-protein (Nambi et al. 1985).

Heterologous desensitization of the β-adrenergic receptor occurs at low (nanomolar) concentrations of agonist and involves the phosphorylation of the receptor (Clark et al. 1989; Loshe et al. 1990a). This phosphorylation appears to be responsible for a rightward shift of the concentration-response curve but does not alter the maximum response to agonist (Hausdorff et al. 1989). The rightward

shift of the concentration-response has been speculated to be due to reduced coupling efficiency of the β-adrenergic receptor to G_s, although the phenomenon of "spare" receptors allows the system to continue to respond maximally at high concentrations of agonist. There are two consensus sites, delineated by the amino acids RRSS, on the β-adrenergic receptor for phosphorylation by cyclic AMP dependent protein kinase (PKA) (Hausdorff et al. 1990). One site is found in the third intracellular loop and the other is in the proximal portion of the carboxy-terminus tail. Using site-directed mutagenesis, the site in the third intracellular loop has been identified as important in the PKA-induced desensitization of β-adrenergic receptors (Clark et al. 1989). In contrast, the PKA phosphorylation site in the proximal portion of the carboxy-terminal tail can be altered without effect on heterologous desensitization of the β-adrenergic receptor. This is consistent with the observation that the third intracellular loop of the β-adrenergic receptor is important in coupling to G_s (Strader et al. 1987; O'Dowd et al. 1988). Therefore, the phosphorylation of β-adrenergic receptors via PKA and subsequent reduction in receptor-G-protein coupling efficiency appears to be an important component in the heterologous desensitization of this receptor.

In addition to phosphorylation by PKA, the purified β-adrenergic receptor has been reported to be phosphorylated by the Ca^{++} phospholipid-dependent, diacylglycerol-activated kinase, protein kinase C (PKC) in a cell free system (Bouvier et al. 1987). While agonist occupation of the β-adrenergic receptor

increased phosphorylation by PKA, phosphorylation of the receptor by PKC was not influenced by agonist occupation. This suggests that, while PKC can phosphorylate β-adrenergic receptors *in vitro*, it does not participate in the regulation of adenylyl cyclase-coupled β-adrenergic receptor function *in vivo*. There are no reports on the functional consequences of PKC phosphorylation of β-adrenergic receptors.

In contrast to heterologous desensitization, homologous desensitization of the β-adrenergic receptor occurs at high agonist concentrations (micromolar) and has a faster time of onset (minutes as opposed to hours). In addition to a rightward shift of the agonist dose response curve, as in heterologous desensitization, homologous desensitization is distinguished by a decrease in the maximal response to agonist. This decrease in the maximal response is thought to occur through a complete uncoupling of phosphorylated β-adrenergic receptor from G_s. Homologous desensitization has been characterized by rapid uncoupling of the receptor and sequestration from the cell surface followed by a slower down-regulation of receptor number. In addition to phosphorylation by PKA, a second receptor-specific kinase termed β-adrenergic receptor kinase (βARK) has been identified (Benovic et al. 1986). This kinase has been implicated in the rapid uncoupling of β-adrenergic receptors during homologous desensitization by phosphorylating only the agonist-occupied form of receptor (Lohse et al. 1989). βARK is a cytosolic protein that translocates to the plasma membrane upon agonist

occupation of a receptor. Strasser et al. (1986) reported that agonist stimulation of both β-adrenergic and prostaglandin E_1 receptors was associated with the translocation of βARK from the cytosol to the plasma membrane. Additionally, βARK has been implicated in the short term agonist promoted desensitization of inhibitory α_2-adrenergic receptors (Liggett et al. 1992). These studies support the hypothesis that βARK may be an adenylyl cyclase-coupled receptor kinase that can regulate the function of many species of both stimulatory and inhibitory adenylyl cyclase-coupled receptors.

The site(s) that βARK phosphorylates are distinct from those that PKA phosphorylates (Hausdorff et at 1989). These sites are thought to be located in a serine, threonine rich area at the distal end of the carboxy-terminal tail. When the three serines and one threonine in the amino acid sequence 355 to 364 of the carboxy-terminal tail are replaced with either alanine or glycine, the ability of the β-adrenergic receptor to undergo homologous desensitization and rapid sequestration is abolished. Paradoxically, down-regulation of the β-adrenergic receptor still takes place with prolonged (24 hour) exposure to 10 μM isoproterenol (Hausdorff et al. 1991). Classically, the rapid sequestration of β-adrenergic receptors from the cell surface, as defined by decreased binding of hydrophilic radioligands, following phosphorylation by βARK was thought to be an early step in the down-regulation of receptors exposed to agonist for long periods of time. These mutations in the carboxy-terminal tail prevented the phosphorylation and

rapid sequestration of the β-adrenergic receptors. However, mutations in the carboxy-terminal tail did not prevent long term down-regulation of the β-adrenergic receptor. This suggests that long term agonist-induced down-regulation of β-adrenergic receptors occurs through a mechanism independent of either phosphorylation or rapid sequestration.

Purified βARK retained its ability to phosphorylate β-adrenergic receptors in a cell free system. Surprisingly, this did not lead to desensitization of β-adrenergic receptor mediated activation of adenylyl cyclase. Nevertheless, phosphorylation of β-adrenergic receptors with crude extracts of βARK uncoupled the receptor from G_s. These results led investigators to propose that a cofactor was involved in the homologous desensitization of β-adrenergic receptors. In an analogous receptor system, the light sensitive receptor rhodopsin, binding of an additional cytosolic protein called arrestin following receptor phosphorylation is required for desensitization to occur. An analogous protein has been cloned called β-arrestin that regulates β-adrenergic receptors much as arrestin regulates rhodopsin. β-arrestin is thought to bind to the phosphorylated carboxy-terminal tail and prevent the interaction of β-adrenergic receptors with G_s (Lohse et al. 1990b). This mechanism is consistent with the diminished maximal response to β-adrenergic agonists following homologous desensitization.

In addition to desensitization, β-adrenergic receptor function is altered through a mechanism termed cross-regulation. Cross-regulation refers to the

influence of inhibitory receptor activation on subsequent stimulatory receptor function. Persistent (48 hour) activation of the inhibitory adenosine A_1 receptor pathway in smooth muscle DDT_1-MF2 cells caused the increased expression of the stimulatory β-adrenergic receptor and a 50 fold decrease in the ED_{50} for isoproterenol. This cross-regulation was accompanied by an increase in the mRNA level for β-adrenergic receptors and a decrease in the level of $G_{i\alpha 2}$-protein (Hadcock et al. 1991). In a second study, acute activation (60 min) of the inhibitory adenosine A_1 receptor resulted in an increase in the maximal response to β-agonist (Port et al. 1992). The increase in maximal response was attributed to a decrease in basal β-adrenergic receptor phosphorylation. Activation of adenosine A_1 receptors lowers cAMP levels and reducs PKA activity, thereby reducing the basal level of β-adrenergic receptor phosphorylation. β-adrenergic receptor cross-regulation of other receptors also has been observed. Short term stimulation of β-adrenergic receptors has been shown to increase mRNA levels of the α_1-adrenergic receptor (Morris et al. 1991). This effect of β-adrenergic receptors could be mimicked by forskolin or cAMP analogs, indicating that this may be a PKA-mediated event.

D. Regulation of Adenosine Receptors

Far less is known about regulation of adenosine receptors than β-adrenergic receptors. While desensitization of the stimulatory adenosine A_2 receptor and the

inhibitory adenosine A_1 receptor has been observed, very little is known about the biochemical mechanisms that are responsible for these phenomena.

Homologous desensitization of the stimulatory adenosine A_2 receptor has been reported in rat brain striatum (Hawkins et al. 1988; Porter et al. 1988), rat vascular smooth muscle cells (Anand-Srivastava et al. 1989), hamster DDT_1 MF-2 smooth muscle cells (Ramkumar et al. 1991), and rat kidney fibroblasts (Newman and Levitzki 1983). Adenosine A_2 receptors are found in abundance in the straitum of the brain. Prolonged exposure (24 hours) to the adenosine analog NECA induced a homologous pattern of desensitization of adenosine A_2 receptors in the striatum (Porter et al. 1988). This desensitization was accompanied by a reduction in adenosine A_2 receptor stimulated adenylyl cyclase activity and a reduction in receptor number. Although the adenosine analog NECA is not selective for adenosine A_1 versus A_2 receptors, it did not induce a desensitization of adenosine A_1 receptors in the striatum within the 24 hour exposure time. Additionally, the response of G_s to stimulation by NaF was not affected by desensitization of adenosine A_2 receptors. This suggests that the homologous desensitization of adenosine A_2 receptors in striatum was due to altered function of the receptor itself (Porter et al. 1988).

Desensitization of adenosine A_2 receptors in cultured vascular smooth muscle cells was found to be both time- and concentration-dependent. Micromolar concentrations of the adenosine analog NECA were required to induce

desensitization and the effect was rapid, with full desensitization occurring within 60 minutes. Adenylyl cyclase activity stimulated by GTP, forskolin, and isoproterenol was unaffected, indicating a homologous desensitization of adenosine A_2 receptors in vascular smooth muscle cells (Anand-Srivastava et al. 1989). As in vascular smooth muscle cells, the desensitization of adenosine A_2 receptors in DDT_1 MF-2 cells was rapid and homologous. DDT_1 MF-2 cells are derived from hamster vas deferens smooth muscle. The $t_{1/2}$ for desensitization was 45 minutes and was also rapidly reversible with 80% of control adenylyl cyclase activity returning four hours after the removal of agonist. In contrast to desensitization of adenosine A_2 receptors of rat striatum, the adenosine A_2 receptors of DDT_1 MF-2 cells did not undergo down-regulation (Ramkumar et al. 1991). Consequently, the desensitization of adenosine A_2 receptors appears to be rapid and homologous in most of the tissues and cell systems studied.

In contrast to adenosine A_2 receptors, the desensitization of inhibitory adenosine A_1 receptors has a slower time of onset (days versus hours). Although the majority of desensitization studies of adenosine A_1 receptors have been carried out with adipocytes (Green 1987; Green et al. 1990; Green et al. 1992; Hoffman et al. 1986; Longabaugh et al. 1989; Parsons and Stiles 1987), studies also have been carried out with DDT_1 MF-2 cells (Ramkumar et al. 1991), and embryonic chicken heart (Shryock et al. 1989).

Desensitization of adenosine A_1 receptor-mediated inhibition of lipolysis in

adipocytes isolated from rats following a six day infusion of the adenosine analog R-PIA was first described by Hoffman et al. (1986). Upon further investigation using this *in vivo* model, desensitization of adenosine A_1 receptors was found to be heterologous, involving changes in adenosine A_1 receptor number and G-protein levels (Longabaugh et al. 1989; Parsons and Stiles 1987). These investigators found a 30% to 50% reduction in adenosine A_1 receptor number. Western blots of the α subunits of G_s, G_{i1}, G_{i2}, and G_{i3} revealed an increase in G_s, no change in G_{i3} and a decrease in G_{i1} and G_{i2}. There was no change in the mRNA levels for any of the α subunits of these G-proteins, suggesting a change in the post-transcriptional processing of these proteins. Additional work carried out with isolated adipocytes in culture has supported the *in vivo* studies (Green 1987; Green et al. 1990; and Green et al. 1992). These changes in G-protein levels therefore constitute the basis, at least in part, for the heterologous desensitization of inhibitory pathways towards adenylyl cyclase with prolonged exposure of the adenosine A_1 receptor to agonist in adipocytes.

Desensitization of adenosine A_1 receptors also has been reported in DDT_1 MF-2 smooth muscle cells. The time of onset of this desensitization ($t_{1/2}$, 8 hours) was shorter than that reported for adipocytes and involved a 40% reduction in adenosine A_1 receptor number. This is the only study to show an increase in the phosphorylation state of an adenosine receptor following desensitization. The ability of NaF, forskolin and isoproterenol to stimulate adenylyl cyclase was

unaltered in this study, suggesting a homologous pattern of adenosine A_1 receptor desensitization in DDT_1 MF-2 cells (Ramkumar et al. 1991). Furthermore, adenosine A_1 receptors have been shown to become desensitized in cardiac tissue following 44 hours of exposure to the adenosine analog R-PIA. This desensitzation was homologous and was characterized by a 60% decrease in receptor number (Shryock et al. 1989).

Therefore, the desensitization of adenosine A_1 receptors is characterized by a long time of onset (days versus hours) and a down-regulation of receptor number. Additionally, there is at least one report of adenosine A_1 receptor phosphorylation following desensitization (Ramkumar et al. 1991). In all tissues other than adipocytes, desensitization of adenosine A_1 receptors appears to be homologous. In contrast, the desensitization of this receptor in adipocytes is heterologous and involves changes in the levels of G_s, G_{i1} and G_{i2}, along with a down-regulation in receptor number. The levels of G_s were increased, leading to an increased sensitivity to stimulatory agonists, while G_{i1} and G_{i2} were decreased, leading to a desensitization of inhibitory inputs.

E. Receptor Localization

The distribution of many hormone receptors in various tissues has been investigated. For example, adenosine A_1 receptors in the kidney were found to be concentrated in the medulla by autoradiographic visualization of ^{125}I-HPIA binding

sites (Weber et al. 1988). While similar studies of many hormones in many tissues have been carried out, only recently have researchers begun to investigate the distribution of hormone receptors on epithelial cells. Epithelial cells, whose major function is the vectoral transport of water and solutes, can be grown as polarized monolayers (reviewed by Rodriguez-Bolan and Nelson 1989). That is, epithelial cell monolayers have an apical and basolateral side separated by tight junctions. When epithelial cells grow in culture, they orient with their basolateral sides attached to the substratum and their apical sides up to the media. The tight junctions function as a barrier, segregating the integral membrane proteins on either side of the cellular monolayer. Thus, an integral membrane protein targeted to one or the other side cannot cross the tight junction barrier. Some integral membrane proteins, such as the Na^+/K^+ ATPase, which is basolaterally located, are expressed on only one side of the epithelial cell monolayer (Rodriguez-Bolan and Nelson 1989). This polar distribution of transport proteins is the very basis for the vectoral transport of water and solutes. With this in mind, researchers have very recently begun investigating the distribution of hormone receptors on polarized epithelial cells that may regulate the activity of these transport proteins.

E.1. Peptide Hormone Receptor Localization

It has been known for some time that arginine-vasopressin (AVP) stimulates cAMP production only when presented to the basolateral side of isolated kidney

collecting tubules (Grantham and Burg 1966; Schwartz et al. 1974). This increase in cAMP is thought to mediate the increase in water permeability of collecting tubules in response to AVP. For much of this time it has been assumed that there is no effect of luminal AVP in the collecting tubule. However, recently there have been reports of AVP initiating responses from the apical side of the cellular monolayer. Apically applied AVP was shown to be involved in the release of PGE_2 (Garcia-Perez and Smith 1984) from primary cultures of canine cortical collecting tubule (CCCT) cells. Additionally, AVP exposed to the luminal side of isolated rabbit cortical collecting tubules stimulated the sustained hyperpolarization of transepithelial potential differance (Ando et al. 1991). Therefore it would appear that while AVP stimulates cAMP production only from the basolateral side of isolated collecting tubules, it is also involved in other signal transduction pathways on the luminal side.

Bradykinin, a nine amino acid peptide of the kinin family, has been shown to stimulate the release of PGE_2 only when exposed to the apical surface of CCCT cells (Garcia-Perez and Smith 1984). In primary cultures of canine tracheal epithelia, bradykinin stimulated arachidonic acid release only from the apical side, but stimulated inositol phosphate turnover from both sides (Denning and Welsh 1991). These two studies suggest that the ability of bradykinin to stimulate arachadonic acid release, and therefore prostaglandin production, from only the apical surface of epithelial cells may be a general feature of bradykinin receptor

organization. However, in one of these studies bradykinin also stimulated the turnover of inositol phosphates from either side of the epithelium (Denning and Welsh 1991). This suggests that there may be two types of bradykinin receptors, or alternatively that there is one type of bradykinin receptor linked to two signal transduction pathways via distinct G-proteins. That is, there may be a G-protein involved in the release of arachadonic acid that is isolated to the apical aspect of epithelia and is responsive to bradykinin receptor stimulation.

Localization of vasoactive intestinal peptide (VIP) receptors has been investigated in differentiated human colonic adenocarcinoma cells (HT29-D4) (Fantini et al. 1988). This cell line differentiates when grown in a glucose-free, galactose-containing media. As HT29-D4 cells differentiated and reached confluency, the binding of ^{125}I-VIP applied to the apical surface declined to non-specific background. Cells grown on permeable supports, however, displayed sustained binding of ^{125}I-VIP to their basolateral aspect. Moreover, VIP could elicit the stimulation of cAMP production when presented to the basolateral, but not the apical side of differentiated HT29-D4 cells. Therefore it was concluded that the VIP receptors of differentiated HT29-D4 cells are located exclusively to the basolateral side of the monolayer (Fantini et al. 1988).

These studies suggest that several hormone receptors have differential distributions on polarized epithelial cell monolayers. The distribution of a hormone receptor on a monolayer of epithelial cells may be indicative of the side on which

the hormone is most likely to be present *in vivo*, and is most certainly important in the functional response of a particular epithelium to a hormone signal.

E.2. Adenosine Receptor Localization

The distribution of adenosine A_2 receptors has been investigated in several epithelial cell preparations (Barrett et al. 1989; Husted et al. 1990; Pratt et al. 1986). The adenosine analog 2-chloroadenosine was employed to study the polarity of adenosine A_2 receptors in canine tracheal epithelium (Pratt et al. 1986). 2-Chloroadenosine was found to stimulate the secretion of Cl^- from either side of the epithelium. The stimulation of Cl^- secretion is thought to occur via adenosine A_2 receptor mediated increases in cAMP production. The short circuit current (I_{sc}) elicited by 2-chloroadenosine stimulation of Cl^- secretion was greater on the apical side than on the basolateral side. Apical addition of 2-chloroadenosine following basolateral stimulation could further increase the change in I_{sc}. In contrast, the basolateral addition of 2-chloroadenosine following apical stimulation could not cause any further increase in I_{sc}. When the adenosine receptor antagonist 8-phenyltheophylline was used to antagonize this effect, it only blocked when presented to the apical surface of the monolayer. This led the authors to suggest that the adenosine A_2 receptor is localized to the apical aspect of canine tracheal epithelium (Pratt et al. 1986).

The adenosine analog NECA was shown to stimulate chloride secretion

(measured as I_{sc}) when applied to either side of monolayers of the colonic cell line T_{84} (Barrett et al. 1989). The rank order of potency of several adenosine analogs to stimulate I_{sc} was consistent with stimulation of adenosine A_2 receptors. While the kinetics of the change in I_{sc} was the same when stimulated from either side, addition of NECA to the basolateral side elicited a greater change in I_{sc} than did apical exposure. Furthermore, the ability of NECA to stimulate cAMP production in T_{84} cells was markedly greater when presented to the basolateral side. When cells were preincubated with NECA for 7 days, the cAMP response and the apical I_{sc} response were completely desensitized. However, the basolateral I_{sc} response was only reduced by 50%. From this it was suggested that while the adenosine A_2 receptor appears to reside on both sides of the T_{84} cell monolayer, it may be coupled to different signal pathways. Additionally, there appears to be a subset of adenosine receptors coupled to the stimulation of I_{sc} on the basolateral side of the cell that are resistent to desensitization (Barrett et al. 1989).

Adenosine A_2 receptor stimulated cyclic AMP production was shown to be greater in response to apically applied NECA than basolaterally applied NECA in primary cultures of rat inner medullary collecting tubule cells (Husted et al. 1990). These studies suggest differential distributions of adenosine A_2 receptors on several types of epithelia. Although it is still unclear whether this receptor is exclusively localized to only one side of epithelial monolayers or simply has a higher density on one side. One caveat to both this study (Husted et al. 1990) and the one

preceeding it (Barrett et al. 1989), is that NECA has a lipophilic nature that may allow it to cross a confluent monolayer of cells and stimulate receptors on the contralateral side. In regard to the inhibitory adenosine A_1 receptor, there are as yet no published reports of the differential distribution of this receptor on epithelia.

III. Desensitization of Adenylyl Cyclase Coupled Adenosine Receptors in Rabbit Cortical Collecting Tubule Cells

A. Introduction

Adenosine, a product of purine nucleotide metabolism, has been shown to alter the cellular transport properties of several types of epithelial cells such as amphibian A6 cells and secretory cells of the shark rectal gland (Forrest et al. 1980; Forrest et al. 1982; Lang et al. 1985). Adenosine also has been shown to alter renal function by mediating changes in renal hemodynamics, renin release, and tubule function (Spielman et al. 1980; Spielman and Thompson 1982; Spielman 1984). More recently, Dillingham and Anderson (1985) documented the inhibition of AVP-stimulated water permeability following administration of adenosine analogs to isolated rabbit cortical collecting tubules. Previous work from our laboratory has shown that rabbit cortical collecting tubule (RCCT) cells grown in culture possess adenylyl cyclase-coupled adenosine A_1 and A_2 receptors (Arend et al. 1987). Activation of the inhibitory adenosine A_1 receptor has been shown to attenuate AVP stimulated cAMP production, whereas activation of the stimulatory adenosine A_2 receptor increases cAMP production (Arend et al. 1987).

Recently, our attention has focused on the processes of adenosine receptor-coupled signal transduction in renal tubule cells. Collecting tubule cell adenosine receptors are coupled to adenylyl cyclase via the guanine nucleotide binding

proteins, G_i and G_s. Adenosine A_1 receptors, which are coupled to an inhibitory G_i protein, inhibit adenylyl cyclase whereas adenosine A_2 receptors, which are coupled to the stimulatory G_s protein, stimulate adenylyl cyclase (Londos et al. 1978; Londos et al. 1980). The two receptor subclasses are further distinguished by their relative affinites for adenosine. Adenosine A_1 receptors are activated by adenosine in nanomolar concentrations whereas adenosine A_2 receptors are stimulated by adenosine at micromolar concentrations.

Extensive work has been done to characterize the mechanism(s) of desensitization in ß adrenergic receptor-coupled adenylyl cyclase systems (Benovic et al. 1988; Nambi et al. 1985; Sibley et al. 1986). Using amphibian erythrocytes and avian erythrocytes as cell models to investigate ß adrenergic receptor desensitization, Sibley et. al. (1986) concluded that ß adrenergic receptor desensitization in the amphibian erythrocyte occurs by concurrent receptor phosphorylation and sequestration from the cell surface. In the avian erythrocyte, however, desensitization is characterized by ß adrenergic receptor phosphorylation and uncoupling from adenylyl cyclase with no concomitant sequestration from the cell surface (Nambi et al. 1985). In contrast, desensitization of chinese hamster fibroblast ß adrenergic receptors involved receptor phophorylation and regulation of mRNA levels (Bouvier et al. 1989). These results indicate that while the mechanisms of desensitization may vary from system to system, altered receptor function remains the common endpoint.

Although considerable information is known about ß adrenergic receptor desensitization, much less is known about desensitization of the adenosine receptor-coupled adenylyl cyclase systems. Desensitization of the stimulatory adenosine A_2 receptor system has been reported in rat kidney fibroblasts and vascular smooth muscle cells from rat aorta (Anand-Srivastava et al. 1989; Newman and Levitzke 1983). Desensitization of stimulatory adenosine A_2 receptors in rat brain striatum also has been reported (Hawkins et al. 1988; Porter et al. 1988). In all cases, desensitization of adenosine A_2 receptors was found to be homologous. Desensitization of inhibitory adenosine A_1 receptors has been reported in rat adipocytes, where loss of high affinity binding sites along with decreases in G_i and increases in G_s were reported (Hoffman et al. 1986). This desensitization of adenosine A_1 receptors in adipocytes was reported to lead to decreased responsiveness of inhibitory receptors and increased responsiveness of stimulatory receptors towards adenylyl cyclase (Green et al. 1990; Parsons and Stiles 1987).

The purpose of the present study, therefore, was to investigate desensitization of adenosine receptors coupled to adenylyl cyclase as a means of further elucidating the mechanism(s) of adenosine receptor signal transduction in rabbit cortical collecting tubule (RCCT) cells.

B. Methods

Materials: Cell culture reagents were obtained from Gibco BRL, (Grand Island, NY). Adenosine analogs, NECA and CHA, and the adenosine receptor antagonist 1,3-diethyl-8-phenylxanthine (DPX) were gifts from Warner Lambert Co.,(Ann Arbor, MI). The adenosine receptor antagonist 8-cyclopentyl-1,3-diphenylxanthine (DPCPX) was purchased from Research Biochemicals Inc., (Natick, MA). Pertussis toxin was purchased from Peninsula Laboratories, Inc., (Belmont, CA). The phosphodiesterase inhibitor RO 20-1724 was obtained from Biomol Research Labs, Inc., (Plymouth Meeting, PA). All other agents were of reagent grade and obtained from standard sources.

RCCT Cell Immunodissection: Immunodissection of cortical collecting tubule cells was carried out as described previously (Smith and Garcia-Perez 1985; Spielman et al. 1986). In brief, six polystyrene culture dishes were treated with 0.2 mg each of a rabbit collecting tubule cell-specific antibody (Rct-30) in 3 ml of phosphate buffered-saline (PBS) for two to three hours. The dishes were then washed three times with 3 ml of 1% bovine serum albumin (BSA) in PBS to block any non-specific protein binding sites.

A six week old New Zealand White rabbit was euthanized by administering an intraperitoneal overdose of sodium pentobarbitol. The kidneys were excised using aseptic technique, the renal capsules were removed, and the kidneys were placed in ice cold PBS. The renal cortex was then removed and minced into $1 mm^3$

cubes using a sterile razor blade. The resulting cortical "mash" was incubated in 0.1% collagenase in Krebs buffer [mM: 118 NaCl, 25 $NaHCO_3$, 14 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 4.7 KCl, 2.5 $CaCl_2$, 1.8 K_2HPO_4, and 1.8 $MgSO_4$, pH 7.4] at 37°C for 40 minutes. At 10 minute intervals the cortical mixture was pipetted up and down (2-3 times) in a 25 ml pipet to aid in the break up of cortical tissue. At the end of the 40 minute collagenase incubation, the tissue was centrifuged (500 x g) for five minutes. The supernatant was aspirated and the pellet resuspended in 10 ml of 0.2% NaCl for 30 seconds to lyse red blood cells. An equal volume of 1.6% NaCl was then added, followed by an equal volume of PBS in order to bring the solution to isotonicity. The tissue mixture was then filtered through 250 µm Gelman filters to remove any large tissue pieces. The resulting suspension of single cells and tubule fragments was centrifuged (500 x g), resuspended in 10% BSA in PBS, and recentrifuged to remove cellular debris. The final pellet, consisting of single cells and tubule fragments, was resuspended in 6 ml of PBS.

Following cortical cell preparation, 1 ml aliquots of the cell suspension were added to each of six Rct-30 antibody treated-culture dishes. The cellular suspension was allowed to remain on the culture dishes for two to three minutes to allow Rct-30 antibody to bind to collecting tubule cells. The culture dishes were then washed three to five times with 3 ml of PBS to remove any unbound cells and tubule fragments. Media containing Dulbecco's modified eagle's medium

(DMEM), 10% decomplemented fetal bovine serum (FBS), 2mM glutamine, and 50 µg/ml of penicillin and streptomycin was added to the dishes (8 ml per dish) and the RCCT cells were grown to confluency (4 to 5 days).

At confluency, RCCT cells were removed from the culture dishes with 0.1% trypsin + 0.05% (Ethylenedinitrilo)tetraacetic acid (EDTA) in PBS and reseeded onto 24 well culture plates in 1 ml aliquots per well. Typically, one 100 mm culture dish of confluent RCCT cells was resuspended into 50 ml of culture media. This RCCT cell suspension was then added to the wells of two 24 well plates in 1 ml aliquots. The cells were again allowed to grow to confluency (4 to 5 days) before undergoing any experimental procedure.

Desensitization Procedure: All experimental pretreatments and treatments were done in triplicate (i.e. three culture wells per treatment regimen). For cells receiving 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclohexyladenosine (CHA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), or 1,3-diethyl-8-phenylxanthine (DPX) pretreatment, culture media was aspirated and replaced with 300 µl of DMEM containing the specific effector. Control wells had their culture media removed and replaced with 300 µl of DMEM. Following three hours of incubation at 37°C in a 7% CO_2 atmosphere, all cells (pretreated and control) were washed with 1 ml of Krebs buffer. Cells in control wells were incubated for one hour at 37°C in 300 µl of Krebs buffer containing 100 µM RO 20-1724, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. Cells in

pretreatment wells also were incubated for one hour at 37°C in 300 µl of Krebs buffer containing RO 20-1724 along with the same concentration of pretreatment effector used in the previous three hour incubation.

Following the four hour desensitization period described above, all cells were washed once with 1 ml of Krebs buffer. Specific adenosine receptors were then stimulated with the appropriate agent, nanomolar concentrations of CHA for A_1 stimulation and micromolar concentrations of NECA for A_2 stimulation, at 37°C. In addition, cholera toxin was used to directly stimulate adenylyl cyclase to assess the effect of pretreatment on G_s. CHA, NECA or cholera toxin were added in 300 µl of Krebs buffer containing 100 µM RO 20-1724. At the end of a 30 minute stimulation period, all cells were treated with 75 µl of 0.2 N HCl to terminate cellular processes. Cells in four control wells received 300 µl of 8% TCA and were subsequently used for protein determination. Culture plates were then frozen at -80°C to lyse the cells, thawed, and 200 µl of 0.5 M Na_2HPO_4 was added to each well to neutralize the acidic supernatant. A radioimmunoassay was performed immediately to determine cellular cAMP content (Frandsen and Krishna 1976). Cyclic AMP values were expressed as pmol cAMP/mg protein. Protein content was determined by a modified Lowry's assay (Markwell et al. 1978).

Statistics: Data were analyzed by analysis of variance (ANOVA) for dose response of desensitization, effects of DPX, DPCPX, and pertussis toxin. In addition, the effect of a NECA pretreatment on arginine-vasopressin (AVP)

response in RCCT cells were also analyzed by ANOVA. If a difference among the means was indicated, Scheffe's multiple comparisons test was used to determine which means were statistically different. The CHA inhibition of basal cAMP production, time course to desensitization, and cholera toxin pretreatment effects were analyzed by Student's t-test. Statistical significance was ascribed for $p < 0.05$ and all values are expressed as mean ± SEM. Statistical analysis was performed using Apple MacIntosh software (StatView™ 512+ from BrainPower, Inc., Calabasas, CA).

C. Results

Figure 2 illustrates the time course of desensitization for adenosine A_2 receptor-stimulated cAMP production. After 30, 60 and 120 minutes of pretreatment with 10 µM NECA, 67%, 33%, and 0% of control stimulated cAMP production was observed. No further alteration of the adenylyl cyclase-coupled adenosine A_2 receptor system was evident following three and four hours of pretreatment (data not shown). The $T_{1/2}$ for this desensitization response was 45 minutes.

The four hour desensitization dose response relationship using 10 nM to 10 µM NECA is shown in Figure 3. Pretreatment with 10 nM NECA had no effect on the subsequent ability of 10 µM NECA to stimulate cAMP production in RCCT cells. Following 40 nM NECA pretreatment, 72% of control stimulated cAMP was

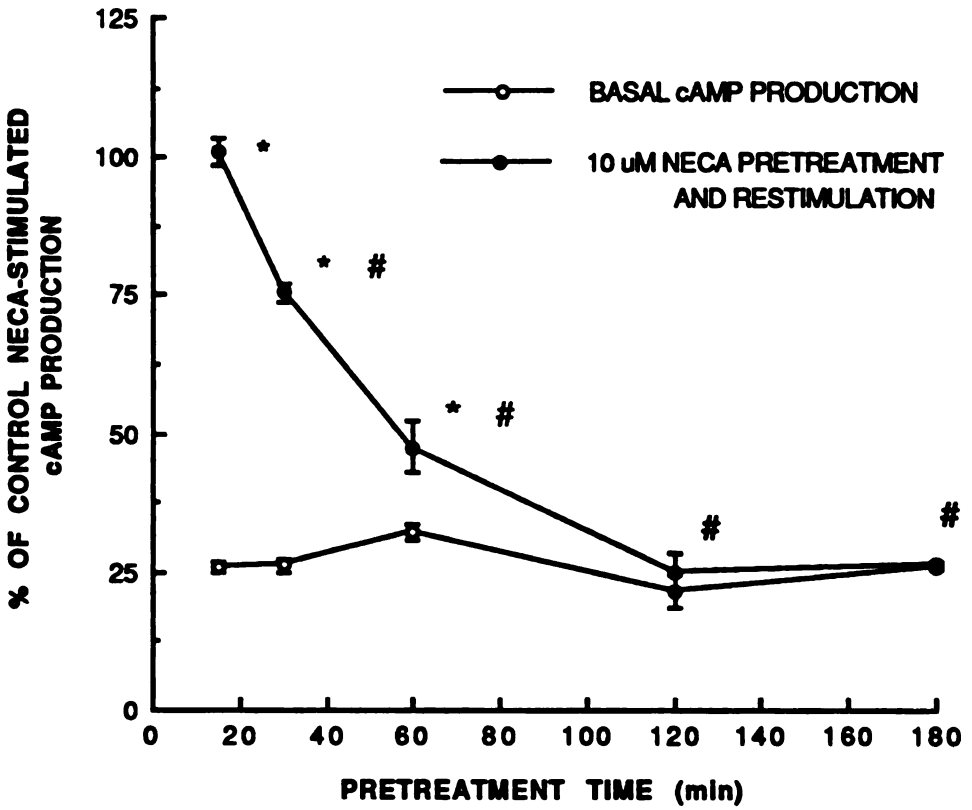


Figure 2. Time course of desensitization of adenosine A_2 receptors in RCCT cells. Cells were restimulated with 10 μM NECA following pretreatment for the indicated times with 10 μM NECA. Values are expressed as the mean ± SEM of two experiments in triplicate. The mean basal and stimulated cAMP values without pretreatment were 19.6 ± 5.9 and 75.6 ± 23 pmol cAMP/mg protein, respectively. * $p < 0.05$ (Student's t-test; two tailed) indicates a significant difference from basal. # $p < 0.05$ indicates a significant difference from 100%.

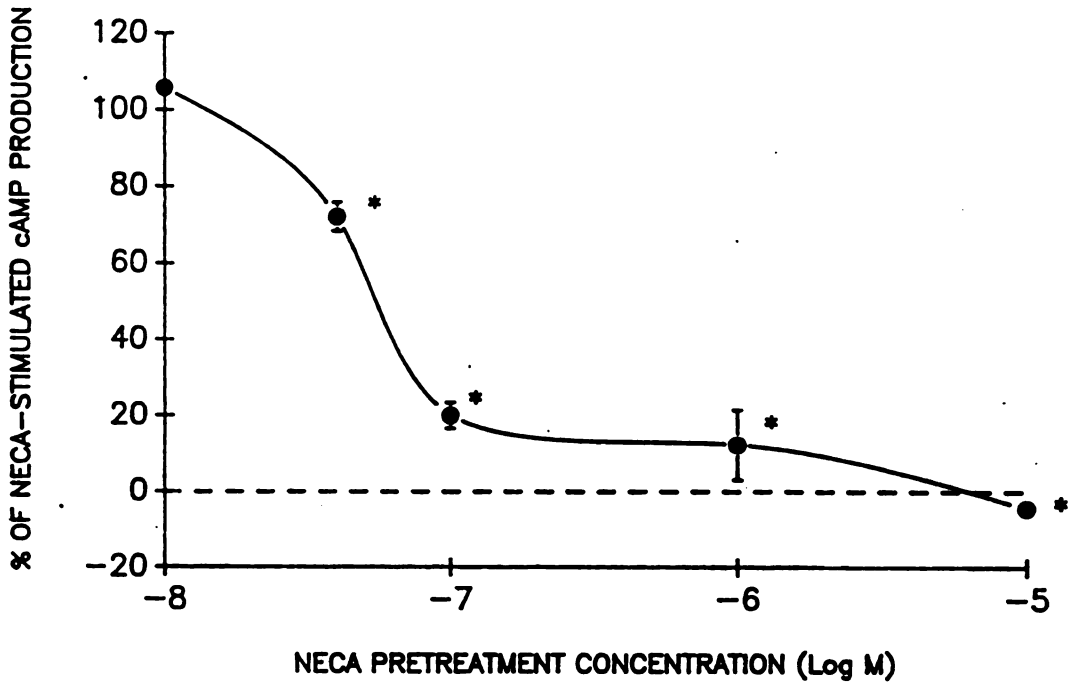


Figure 3. Effect of four hour NECA pretreatment (10 nM to 10 μM) on desensitization of adenosine A_2 receptor stimulated cAMP production in RCCT cells. Values are expressed as mean ± SEM of two experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from control NECA-stimulated cAMP production.

recovered. After 100 nM NECA pretreatment, 20% of control (10 μM NECA) stimulated cAMP production could be regained. Following pretreatment with 1 μM NECA, only 12% of 10 μM NECA stimulated cAMP production could be regained, whereas pretreatment with 10 μM NECA resulted in subsequent 10 μM NECA stimulated cAMP production below basal. The IC_{50} for this response was 80 nM NECA.

Figure 4 shows the effect of adenosine receptor antagonism on adenosine A_2 receptor desensitization. When 10 μM DPX , an adenosine receptor antagonist, was added concurrently with 10 μM NECA during the pretreatment period, 54% of control (10 μM NECA) stimulated cAMP production was retained. This is in contrast to only 11% retention of control-stimulated cAMP production following adenosine A_2 receptor desensitization with 10 μM NECA alone, which is not above basal production. DPX at concentrations lower than 10 μM had no effect on A_2 receptor desensitization (data not shown).

When 1 μM DPCPX , a specific A_1 adenosine receptor antagonist, was added concurrently with 10 μM NECA, NECA-stimulated cAMP production was increased above that of non-DPCPX treated cells, yet adenosine A_2 receptor desensitization was still complete (Figure 5). Basal cAMP levels in DPCPX treated RCCT cells were significantly higher than in non-DPCPX treated cells. Figure 5 also shows the effect of 24 hour of pertussis toxin (1 μg/ml) treatment on adenosine A_2 receptor desensitization. Although basal cAMP production was increased after

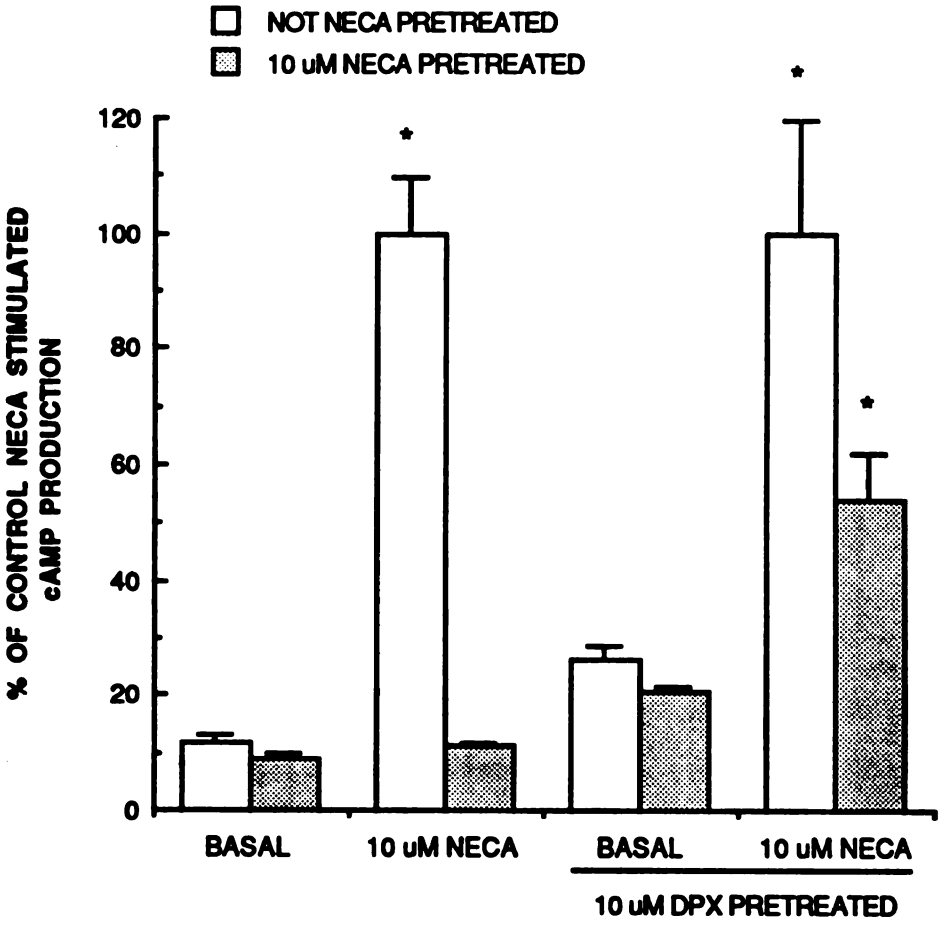


Figure 4. Effect of four hour 10 μM NECA pretreatment with and without 10 μM 1,3-diethyl-8-phenylxanthine (DPX) on desensitization of adenosine A_2 receptors in RCCT cells. Values are expressed as mean $\pm$ SEM of two experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from basal cAMP production.

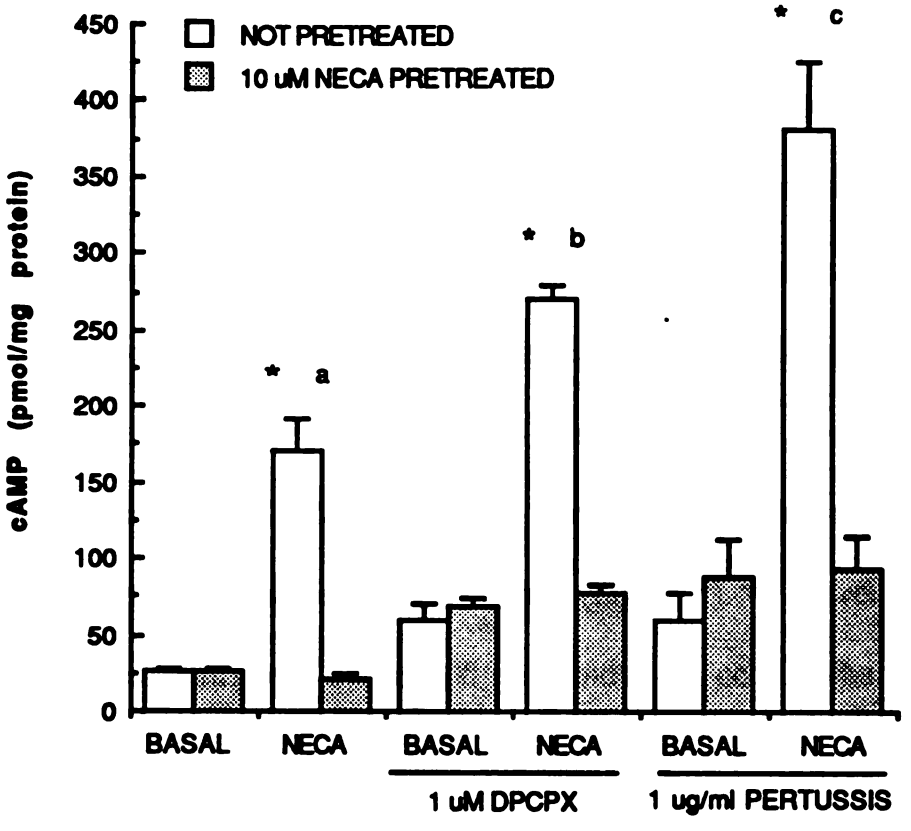


Figure 5. Effect of four hour 10 μM NECA pretreatment with and without 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or 24 hour pertussis toxin treatment (1 μg/ml) on desensitization of adenosine A_2 receptors in RCCT cells. Values are expressed as mean ± SEM of two experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from basal cAMP production of the respective treatment group. Letters are significantly different at a $p < 0.05$.

24 hours of pertussis toxin exposure and 10 μM NECA-stimulated cAMP production was significantly higher than that of control cells, 10 μM NECA-induced adenosine A_2 receptor desensitization was still complete. Pretreatment with 10 μM NECA for four hours did not alter forskolin-stimulated cAMP production in RCCT cells (Figure 6).

A possible role for G_s in adenosine A_2 receptor desensitization was investigated by stimulating G_s with cholera toxin following pretreatment with 10 μM NECA (Figure 7). At both concentrations investigated cholera toxin stimulated cAMP production, albeit to levels that were significantly less than control.

Four hour pretreatment with CHA, an adenosine A_1 receptor specific agonist, at concentrations of 100 nM or less did not diminish adenosine A_1 receptor mediated inhibition of basal cAMP production (Figure 8). However, pretreatment with CHA at concentrations above 100 nM, known to occupy the adenosine A_2 receptor, eliminated the ability of CHA (50nM) to inhibit basal cAMP production.

The effect of adenosine A_2 receptor desensitization on AVP-stimulated cAMP production is shown in Figure 9. Following a four hour pretreatment with 10 μM NECA, the response to 1 μM AVP was significantly reduced to 47% of control AVP-stimulated cAMP production, although NECA pretreated cells could still significantly increase AVP stimulated cAMP production above basal levels.

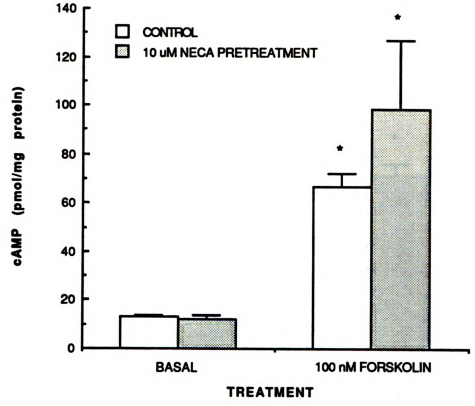


Figure 6. 10 μM NECA four hour pretreatment of 100 nM forskolin stimulation of cAMP production in RCCT cells. Values are expressed as mean ± SEM of two experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from basal cAMP production.

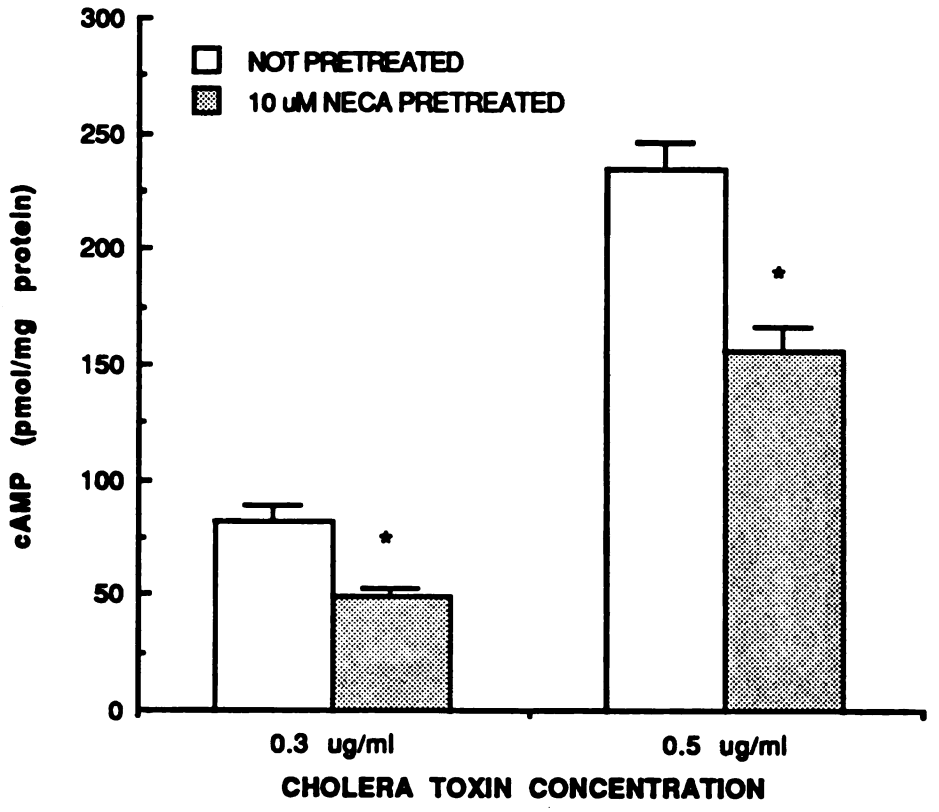


Figure 7. Effect of four hour 10 μM NECA pretreatment on cholera toxin stimulated cAMP production in RCCT cells. Values are reported as mean ± SEM of two experiments in triplicate. * $p < 0.05$ (Student's t-test; two tailed) indicates a significant difference from non-pretreated cholera toxin stimulated cAMP production.

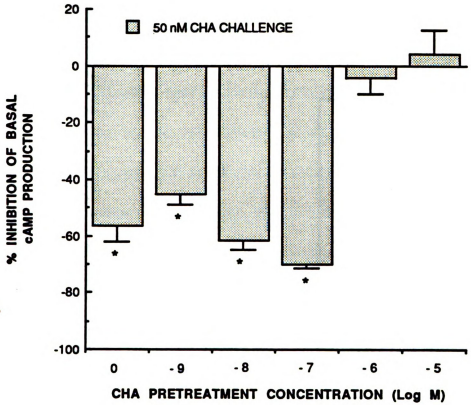


Figure 8. Effect of four hour pretreatment with CHA (1 nM to 100 μM) on adenosine A_1 receptor inhibition of basal cAMP production in RCCT cells. Following pretreatment with CHA, basal production of cAMP was inhibited with 50 nM CHA. Values are expressed as mean ± SEM of one experiment in triplicate. The 0 CHA pretreament group is the mean ± SEM of six treatment groups (n-18). * $p < 0.05$ (Student's t-test; one tailed) indicates a significant decrease from basal cAMP production.

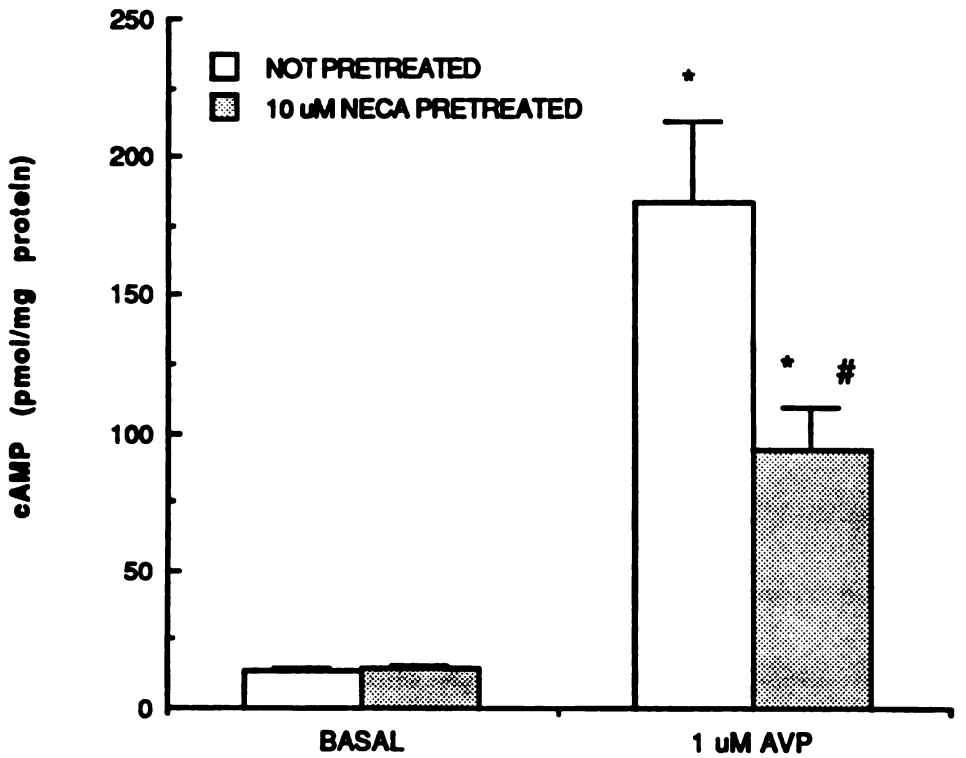


Figure 9. Effect of four hour 10 μM NECA pretreatment on arginine vasopressin (AVP) stimulated cAMP production in RCCT cells. Values are expressed as mean ± SEM of three experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from basal cAMP production. # $p < 0.05$ indicates a significant difference from non-pretreated AVP-stimulated cAMP production.

D. Discussion

Our laboratory has shown that primary cultures of RCCT cells possess both inhibitory adenosine A_1 and stimulatory adenosine A_2 receptors coupled to adenylyl cyclase (Arend et al. 1987). The present study sought to determine whether adenosine receptors in RCCT cells are always fully functional or, if they are regulated by prior exposure to agonist. The desensitization of hormone-stimulated adenylyl cyclase systems, such as the β-adrenergic receptor system, has been well documented (Benovic et al. 1988; Bouvier et al. 1989; Nambi et al. 1985; Sibley et al. 1986). Moreover, desensitization of adenosine A_2 receptors in rat kidney fibroblasts (Newman and Levitzke 1982), vascular smooth muscle (Anand-Srivastava et al. 1989), vas deferens smooth muscle (Ramkumar et al. 1991) and striatum (Hawkins et al. 1988; Porter et al. 1988) have been reported. In all cases, the desensitization of adenosine A_2 receptors was reported to be homologous. Desensitization of adenosine A_1 receptors in rat adipocytes has also been described (Green et al. 1990; Hoffman et al. 1986; Parsons and Stiles 1987). The desensitization of adenosine A_1 receptor has also been reported in smooth muscle cells (Ramkumar et al. 1991). The present report, however, is the first to document concurrent adenosine A_1 and A_2 receptor desensitization in primary cultures of cortical collecting tubule cells.

Desensitization of adenosine A_2 receptors in RCCT cells grown in culture was both time and concentration dependent. A significant reduction in 10 μM

NECA-stimulated cAMP production occurred within 30 minutes, with complete desensitization of adenosine A_2 receptor stimulated cAMP production occurring within two hours. Adenosine A_2 receptor desensitization occurred only when pretreatment concentrations of NECA approached micromolar concentrations. These results are consistent with the hypothesis that adenosine A_2 receptor occupation and activation are necessary to initiate desensitization. These results also suggest that adenosine A_1 receptor occupation and activation, which presumably occurs at lower concentrations of NECA, does not produce adenosine A_2 receptor system desensitization within the four hour exposure time frame.

To further probe the involvement of adenosine A_1 receptors in adenosine A_2 receptor desensitization, the specific adenosine A_1 receptor antagonist DPCPX was administered during adenosine A_2 receptor desensitization. When 1 μM DPCPX was present, adenosine A_2 receptor desensitization remained intact. This finding is consistent with the dose dependency of adenosine A_2 receptor desensitization, in that agonist occupation/activation of the adenosine A_1 receptor is not necessary for desensitization. Moreover, addition of DPCPX significantly raised basal cAMP production suggesting the removal of a tonic inhibitory input by adenosine A_1 receptors to adenylyl cyclase.

Pertussis toxin, which inactivates the G_i subfamily of G-proteins, did not prevent adenosine A_2 receptor desensitization, suggesting that G_i is not primarily involved. However, cells treated with pertussis toxin had significantly higher

NECA-stimulated cAMP production than non-pertussis toxin treated cells. Pertussis toxin also raised basal cAMP production, which again suggests the removal of a tonic inhibition on adenylyl cyclase. Not surprisingly, the DPCPX and pertussis toxin results appear to be qualitatively similar. Both interventions appear to remove a tonic inhibitory influence on the adenylyl cyclase system, but do not alter adenosine A_2 receptor desensitization. The blockade of adenosine A_1 receptors with DPCPX allowed for a significant increase in 10 µM NECA-stimulated cAMP production over control. Pertussis toxin inactivation of G_i resulted in an even larger significant increase of control 10 µM NECA-stimulated cAMP production. These two observations suggest that the adenosine A_1 receptor system is only one of many that may be involved in the tonic inhibition of adenylyl cyclase in RCCT cells. Desensitization of adenosine A_2 receptors did not significantly alter the ability of forskolin to stimulate cAMP production. Together, the forskolin and pertussis toxin experiments suggest that neither adenylyl cyclase nor G_i are directly involved in adenosine A_2 receptor desensitization. Because neither DPCPX nor pertussis toxin administration significantly altered the pattern of adenosine A_2 receptor desensitization, it would appear that the mechanism responsible for this phenomenon resides within the stimulatory pathway of the adenosine receptor-adenylyl cyclase system.

Ten micromolar DPX, an adenosine A_1 and adenosine A_2 receptor antagonist at this concentration, presented concurrently with 10 µM NECA pretreatment

significantly attenuated adenosine A_2 receptor desensitization. With 10 µM DPX present, 54% of control NECA-stimulated cAMP production was regained as opposed to only 11% without DPX present. Basal cAMP production was 11% of control 10 µM NECA stimulated cAMP production. Pretreatment with DPX alone at concentrations higher than 10 µM produced adenosine A_2 receptor desensitization (data not shown). The desensitization of adenosine A_2 receptors by DPX at concentrations higher than 10 µM may be due to inhibition of phosphodiesterases and, therefore, increased cellular cAMP levels. Alternatively, DPX may act as a partial adenosine A_2 receptor agonist at concentrations higher than 10 µM. Nevertheless, agonist occupation/activation of adenosine A_2 receptors appears to be necessary for adenosine A_2 receptor desensitization.

Following pretreatment of RCCT cells with 10 µM NECA, cholera toxin, a potent activator of G_s, retained its ability to stimulate cAMP production, albeit, to levels that were significantly attenuated as compared to non-pretreated cells. Cholera toxin at concentrations of 0.3 µg/ml and 0.5 µg/ml stimulated cAMP production to 37% and 62%, respectively, of those levels achieved in the absence of NECA pretreatment. A reduction in functional G_s for cholera toxin induced ADP-ribosylation may explain the attenuation in cAMP production following pretreatment with 10 µM NECA. Indeed, Edwards et al. (1987) observed decreased ADP-ribosylation of the α-subunit of G_s-protein following prolonged exposure of platelets to the prostacyclin analog iloprost.

The effect of pretreatment with CHA on adenosine A_1 receptor responsiveness in RCCT cells also was investigated. While desensitization of the adenosine A_1 receptor system's ability to inhibit basal cAMP production was observed, adenosine A_1 receptor desensitization did not occur until pretreatment concentrations of CHA reached concentrations (1 μM) known to occupy/activate the A_2 adenosine receptor. This provides further support for the hypothesis that adenosine A_2 receptor occupation/activation is needed to initiate desensitization of both adenosine A_2 and adenosine A_1 receptors in RCCT cells.

The ability of AVP to increase cAMP production was attenuated following desensitization of the adenosine receptor system with 10 μM NECA. In this experiment, 1 μM AVP-stimulated cAMP production was 47% of control. However, pretreatment of RCCT cells with NECA at nanomolar concentrations, which activates only adenosine A_1 receptors, did not alter adenylyl cyclase responsiveness to 1 μM AVP. This is not surprising because adenosine A_1 receptor stimulation by CHA at these concentrations did not desensitize the adenosine receptor system. Taken together, these results suggest that adenosine A_2 receptor-induced heterologous desensitization of AVP responsiveness in RCCT cells had occurred.

In summary, it appears that neither adenylyl cyclase nor G_i are directly involved in adenosine receptor-coupled adenylyl cyclase system desensitization in RCCT cells. Desensitization of this system appears to require agonist

occupation/activation of the adenosine A_2 receptor subclass and results in a reduced availability of functional G_s. The desensitization of adenosine receptor-coupled adenylyl cyclase is additionally characterized by a reduced responsiveness to AVP. This reduced responsiveness of AVP receptor-coupled adenylyl cyclase following adenosine receptor desensitization may, in part, be due to altered function of G_s and constitutes a heterologous desensitization. Desensitization of this system is further characterized by the inability of adenosine A_1 receptor stimulation to inhibit basal cAMP production following prolonged occupation/activation of adenosine A_2 receptors. Because pertussis toxin inactivation of G_i does not affect the pattern of adenosine A_2 receptor desensitization, and adenylyl cyclase appears not to be affected by desensitization, it seems reasonable to speculate that altered function of the adenosine A_1 receptor has occurred.

In all of the experiments where adenosine receptor desensitization occurred, the stimulatory adenosine A_2 receptor-coupled adenylyl cyclase system was occupied/activated. Prolonged occupation/activation of the adenosine A_2 receptor system lead to the reduced ability of cholera toxin to stimulate the regulatory G_s protein. Moreover, the heterologous desensitization of AVP receptor-coupled adenylyl cyclase is supportive of altered function of G_s. In conclusion, desensitization of adenosine receptor-coupled adenylyl cyclase in RCCT cells is characterized by alterations in the function of adenosine A_2 and adenosine A_1 receptors and the regulatory protein G_s.

IV. The Functional Localization of Adenosine Receptor Mediated Pathways in the LLC-PK$_1$ Renal Cell Line

A. Introduction

The purine nucleoside, adenosine, is known to regulate a large number of physiological functions including the regulation of neurotransmitter release, vasodilation, and inhibition of lipolysis in adipose tissue (Daly et al. 1981; Londos et al. 1978). In the kidney, adenosine is known to alter renal function through changes in hemodynamics, renin release and tubule function (Dillingham and Anderson 1985; Spielman 1984; Spielman et al. 1980; Spielman and Thompson 1982).

These physiological effects of adenosine are thought to be mediated primarily by its interaction with extracellular adenosine receptors. Adenosine receptors are coupled to adenylyl cyclase through the guanine nucleotide regulatory proteins G_i and G_s.(Arend et al. 1987; Londos et al. 1978; Londos et al. 1980) These receptors are divided into two subclasses based on the following criteria: 1) ligand/receptor pharmacology, 2) coupling to G-proteins, 3) second messenger systems, and 4) structures deduced from molecular cloning (Libert et al. 1991; Londos et al. 1978; Londos et al. 1980). The adenosine A_1 receptor is known to act through an inhibitory G-protein (G_i) to inhibit adenylyl cyclase activity with the following adenosine analog potency profile: CHA > PIA > NECA. The adenosine

A_1 receptor has a high affinity for adenosine analogs and is stimulated at nanomolar concentrations. The adenosine A_2 receptor acts through a stimulatory G-protein (G_s) to stimulate adenylyl cyclase activity. This subclass of adenosine receptor has an adenosine analog potency profile of NECA > PIA > CHA, and is of lower affinity, responding to micromolar concentrations. A third adenosine receptor mediated pathway found in renal epithelial cells stimulates inositol phosphate turnover with a subsequent release of calcium from intracellular stores (Arend et al. 1988; Arend et al. 1989; Weinberg et al. 1989). This pathway is blocked by the specific adenosine A_1 receptor antagonist DPCPX and is pertussis toxin sensitive. However, unlike the adenosine A_1 and A_2 receptors, this receptor mediated pathway has an adenosine analog potency profile of CHA = PIA = NECA. Therefore, this receptor mediated pathway has been suggested to be a subtype of the adenosine A_1 receptor (Arend et al. 1988; Arend et al. 1989).

Transporting epithelial cells separated by tight junctions are known to grow in culture as a polarized monolayer, displaying both an apical and a basolateral side (Pfaller et al. 1990; Rodriguez-Bolan and Nelson 1989). These epithelia express transport proteins such as Na^+/K^+ ATPase at their cell surface in a polarized manner. This polarity is the basis for the vectoral transport of solutes and water. Recently, studies have focused on the spatial localization of receptors that regulate transport in several types of epithelial cells. For example, the vasoactive intestinal peptide (VIP) receptor has been localized to the basolateral side of the colonic cell

line HT29-D4 (Fantini et al. 1988). In addition, the majority of adenosine receptors responsible for changes in short circuit current in the T_{84} colonic cell line were also found to be basolaterally located (Barrett et al. 1989). However, in canine tracheal epithelium, adenosine applied to the mucosal surface was found to stimulate chloride secretion through an apically located adenosine receptor (Pratt et al. 1986).

Renal tubule cells are exposed to two spatially separate fluid compartments; the interstitial fluid (basolateral) compartment, and the tubular lumen or urine compartment (apical). Adenosine in these two extracellular compartments represents separate pools that may act differentially to regulate tubular epithelial transport via spatially distinct receptors. Therefore, the purpose of these studies was to localize the adenosine receptor mediated pathways in the LLC-PK_1 renal cell line.

B. Methods

Cell Culture: LLC-PK_1 cells (passage number 194) were obtained from American Type Culture Collection, Rockville Maryland. Cell culture reagents were obtained from Gibco BRL, Grand Island NY. The cells were cultured in a media consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml each of penicillin and streptomycin at 37°C in a 7% CO_2 atmosphere. Cells were routinely passaged

every 7 days.

Gelatin Substrate Coating of Culture Inserts: Cell culture inserts (12 mm diameter, Millicell^{tm}-CM; obtained from Millipore Corp. Bedford MA.) were coated with gelatin (Millipore technical brief: Lit. No. TB017 copyright 1989) as an extracellular matrix upon which LLC-PK_1 cells adhere and grow. Using aseptic techniques in a laminar flow hood, inserts were placed in a sterile 24 well plate and wetted with 0.5 ml of phosphate buffered saline (PBS mM; 138 NaCl, 3 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). A solution consisting of 5% w/v gelatin and 5% w/v sucrose in dH_2O was made. The resulting slurry was heated in a boiling water bath to allow the gelatin and sucrose to go into solution. The PBS was aspirated and the inserts were coated with 200 µl of warm gelatin solution. If the inserts were not pre-wetted with PBS, the gelatin solution would form as a droplet and not coat the insert evenly. The gelatin solution was immediately aspirated and the remaining gelatin film was allowed to air dry on the culture inserts in a laminar flow hood. After drying, the gelatin film was rehydrated with 0.5 ml PBS per insert for 10 minutes. The PBS was aspirated and the gelatin film was fixed with 0.5 ml of 1% glutaraldehyde in PBS for 30 minutes. This fixation procedure also had the advantage of sterilizing the gelatin film. For reasons that are unclear, LLC-PK_1 cells do not grow well on unfixed gelatin. Inserts were washed twice with 0.5 ml of PBS to remove the glutaraldehyde and the fixed gelatin film was treated with 0.5 ml of 0.1 M glycine in PBS for 30 minutes to quench reactive groups

introduced by the glutaraldehyde. Inserts then were washed twice with 0.5 ml PBS and placed in a sterile 24 well plate containing 0.5 ml of culture media per well. This media served as the basolateral culture media for the cells. Following two washes with 5 ml of PBS, a confluent monlayer of LLC-PK_1 cells was removed from a 100 mm culture plate by a 3 minute incubation at 37°C with 4 ml of 0.2% trypsin, 0.05% (Ethylenedinitrilo)tetraacetic acid (EDTA) in PBS. The resulting suspension of cells was sedimented by centrifugation (500 x g) for five minutes. The supernatant was discarded and the cells were diluted 1:20 (~10^5 cells/ml) with culture media. Three hundred microliters (300 µl) of the cell suspension was then transfered to the inside of each insert. Epithelial cells seeded in this manner will orient apical side up and basolateral side down on the gelatin substrate in the insert (Figure 10). Cells were allowed to grow to confluency for 5 to 7 days before each experiment.

Permeability of a LLC-PK Cell Monolayer to ^{14}C-Sucrose: Once confluent, the permeability of the LLC-PK_1 cell monolayer to ^{14}C-sucrose was assessed. Inserts with and without cells were carefully washed with Krebs buffer and then placed in a new 24 well plate containing 0.5 ml of Krebs buffer (basolateral side). Three hundred microliters (300 µl) of Krebs buffer containing 1 µM ^{14}C-sucrose was added to the inside (apical side) of the inserts. The 24 well plate was then placed on an orbital shaker at 37°C and shaken gently. At timed intervals (0 to 60 minutes at ten minute intervals), 50 µl aliquots were removed from buffer

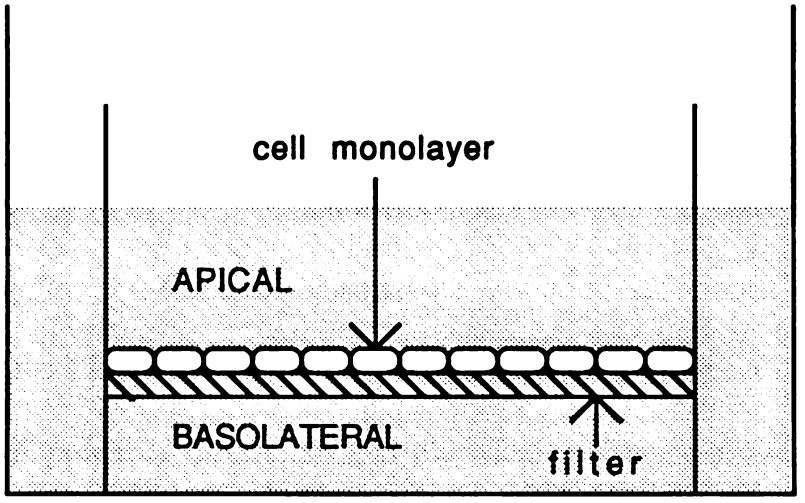


Figure 10. Diagram of LLC-PK_1 cells grown on a Millicell^{tm}-CM insert.

surrounding the inserts (basolateral side) and placed in glass scintillation vials containing 5 ml of scintillation cocktail. The volume that was removed from the outside of the inserts was immediately replaced with 50 µl of Krebs buffer to maintain the outside volume at 0.5 ml. The radioactivity in these samples was counted for three minutes by a LKB 1209 Rackbeta beta counter. The concentration of ^{14}C-sucrose that passed through the inserts was calculated and expressed as percent of the equilibrium concentration, the concentration that would result from free mixing of the apical and basolateral volumes. The converse experiment of measuring the flux of ^{14}C-sucrose from outside (basolateral) the insert to inside (apical) was performed as described above.

Measurement of the potential difference and electrical resistance of a LLC-PK$_1$cell monolayer: Once confluent, the potential difference and electrical resistance of the LLC-PK$_1$ cell monolayer was measured to assess the monolayer's integrity. All potential difference and electrical resistance measurements were made immediately before any experimental procedure was undertaken. For this purpose, an Epithelial Voltohmmeter (EVOM; World Precision Instruments, Inc., New Haven CT.) was used. In a laminar flow hood, EVOM electrodes were sterilized in 95% ethanol and rinsed with culture media. Each of the electrodes contains a pellet of Ag/AgCl for the measurement of voltage and silver wires for the passage of current to measure electrical resistance. The potential difference and electrical resistance of each LLC-PK$_1$ monolayer was measured by placing the

anode in the media on the inside of the insert (apical) and the cathode in the media on the outside of the insert (basolateral). Because the Millicell-CM inserts have a surface area of 1.13 cm^2, electrical resistances were multiplied by 1.13 to express resistance as ohm x cm^2. Typical potential differences ranged from 1.0 to 2.0 mV (apical side negative), while the electrical resistances were approximately 250 ohm x cm^2.

Adenosine A_1 and A_2 Receptor cAMP Pathway Localization: Following 5 to 7 days of culture on Millicell^{tm}-CM filter inserts, LLC-PK_1 cells were exposed to adenosine (30 nM or 100 µM) in an attempt to study the sidedness of inhibitory adenosine A_1 and stimulatory A_2 receptor mediated pathways respectively. For the localization of adenosine A_2 receptors, 100 µM adenosine was used to stimulate cAMP production in LLC-PK_1 cells. Inserts were washed with Krebs buffer and incubated for 30 minutes at 37°C in Krebs buffer (300 µl apical, 500 µl basolateral) containing 100 µM RO 20-1724, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. This buffer was aspirated from the inserts and specific cell surfaces were stimulated with 100 µM adenosine in Krebs buffer containing 100 µM RO 20-1724 for an additional 30 minutes at 37°C. Krebs buffer containing 100 µM RO 20-1724 and 1.5 U/ml adenosine deaminase (ADA) was placed on the contralateral side to deaminate any adenosine that might cross the monolayer. The stimulation of adenylyl cyclase by A_2 adenosine receptors was terminated by adding 75 µl of 0.2 N HCl to the inside (apical) of each insert. The

inserts along with the upper volume of buffer were then frozen at -80°C.

The sidedness experiments for the inhibitory adenosine A_1 receptor were performed essentially as described above for the adenosine A_2 receptor, except that 30 nM adenosine was used to inhibit forskolin-stimulated cAMP production. Forskolin was used to directly stimulate adenylyl cyclase, because inhibition of stimulated cAMP levels can be more clearly resolved than inhibition of basal cAMP levels. Confluent LLC-PK_1 cells on inserts were washed with Krebs buffer and incubated with Krebs buffer containing RO 20-1724 for 30 minutes at 37°C as described above. The specific cell surfaces (apical or basolateral) were then stimulated with Krebs buffer containing RO 20-1724 and 1 µM forskolin with or without 30 nM adenosine for 30 minutes at 37°C. Forskolin was present on both sides of the monolayer. Krebs buffer containing RO 20-1724 and 1.5 U/ml ADA was placed on the contralateral side. The inhibition of forskolin stimulated cAMP was terminated by adding 75 µl of 0.2 N HCl to the inside of the insert. The inserts were frozen along with the inner volume of buffer at -80°C.

cAMP Determination: Samples from sidedness experiments were thawed at room temperature and neutralized by adding 200 µl of 0.5 M Na_2HPO_4. The cAMP levels in the supernatant of each sample were measured via radioimmunoassay as described by Frandsen and Krishna (1976). Cyclic AMP levels were expressed per mass of cell protein as measured by a modified Lowry's assay (Markwell et al. 1978).

Adenosine Stimulation of Intracellular Calcium Release: Adenosine stimulation of free intracellular calcium $[Ca^{+2}]_i$ release by LLC-PK_1 cells in suspension was performed as described by Grynkiewicz et al. (1985). A confluent monolayer of cells was removed from a culture dish with a 3 minute incubation with 0.2% trypsin and 0.05% EDTA in PBS at 37°C. The cells were centrifuged (500 x g) for five minutes and the cellular pellet resuspended in 300 µl of simplified saline (SSS mM; 145 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.4). The cells were loaded with the fluorescent calcium chelator Fura-2AM, at a concentration of 10 µM with 0.02% pluronic acid in SSS for 30 minutes at 37°C in a shaking water bath. The pluronic acid was utilized to aid in the loading of Fura-2AM into the cells. After loading, the cells were washed twice with 10 ml SSS and resuspended in 400 µl of SSS. Samples of this cell suspension used for calcium measurements were diluted 1:100 into a 1.5 ml volume of SSS in a quartz cuvette. The cuvette was thermostated at 37°C on a magnetic stirrer in a dual excitation wavelength spectrofluorometer (SPEX Industries, Inc., Metuchen, NJ). The cell suspension in the cuvette was continuously stirred and effectors were added at a 1:100 dilution to their final concentrations. Cells were excited at 340 nm and 380 nm, while emmision was monitored at 505 nm. The ratio of fluorescence (R) emitted by exciting at 340 nm/380 nm was used to calculate $[Ca^{+2}]_i$ as described by Grynkiewicz et al. (1985):

$[Ca^{+2}]_i = K_d \times (R-R_o/R_s-R) \times (F_o/F_s)_{380}$

The effective K_d of Fura-2AM for calcium, 224 nM, as determined by Grynkiewicz et al. (1985) was used. R is the experimental fluorescence ratio, R_o is the fluorescence ratio at zero free calcium and R_s is the fluorescence ratio at saturating free calcium. F_o is the fluorescence emitted by 380 nm excitation at zero free calcium and F_s the fluorescence at saturating free calcium. Digitonin (50 µM) was used to measure the fluorescence to saturating concentrations of calcium, while 5 mM Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was used to measure the fluorescence at zero free calcium.

Adenosine Receptor Mediated Inositol Phosphate Pathway Localization: A confluent 100 mm plate of LLC-PK_1 cells was incubated for 6 hours in 5 ml of Medium 199 supplemented with 2 mM glutamine, 1 µM dexamethasone, 5 pM triiodothyronine, and 5 nM insulin with 6 µCi/ml of myo-[2-^{3}H(N)]inositol. This incubation was utilized to load the phosphotidylinositol pools with myo-[2-^{3}H(N)]inositol. Cells were removed from the plate by a 3 minute incubation with 0.2% trypsin, 0.05% EDTA in PBS. The cells were washed from the plate with 5 ml PBS and centrifuged at 500 x g for 5 minutes. The resulting cellular pellet was resuspended in 10 ml of lithium buffer (mM; 58 NaCl, 60 LiCl, 4.7 KCl, 1.5 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose, and 20 HEPES. pH 7.4), and incubated for 20 minutes at 37°C in a water bath with constant shaking. Following two washes in

10 ml of lithium buffer, the cells were resuspended in 15 ml of lithium buffer and 400 µl aliquots were added to microcentrifuge tubes. Hormones (adenosine or AVP) at 5x their final concentration were added to the microcentrifuge tubes in 100 µl aliquots (500 µl final volume). The stimulation of inositol phosphates was allowed to occur for 10 minutes at 37°C, followed by termination of the reaction with 500 µl of 10% trichloroacetic acid (TCA). The reaction mixture was then frozen at -80°C. After thawing at room temperature, individual reaction mixtures were centrifuged and the supernatant applied to anion exchange columns (Bio-Rad, AG-1X8, 100- to 200-mesh formate form). The inositol phosphates were then separated as described by Berridge et. al. (1983). Following three washes with 3 ml distilled water and two washes with 3 ml 50 mM ammonium formate plus 5 mM sodium borate, increasing concentrations of ammonium formate (200 mM, 400 mM, 1 M) containing 100 mM formic acid (2x 1 ml) were used to elute the inositol phosphates; 200 mM ammonium formate for IP_1, 400 mM ammonium formate for IP_2, and 1 M ammonium formate for IP_3. The lipids were extracted from the TCA induced precipitate with methanol and chloroform. The radioactivity in the inositol phosphate pools were normalized to the radioactivty in the lipid fraction and expressed as percent of control (unstimulated cells).

Experiments for the sidedness of the inositol phosphate response were carried out as described above with slight modifications. Inserts were placed in supplemented Medium 199 containing 6 µCi/ml of myo-[2-^{3}H(N)]inositol (300 µl

apical, 500 µl basolateral) for 6 hours. The inserts were carefully washed once with lithium buffer and incubated in lithium buffer at 37°C for 20 minutes and again washed carefully with lithium buffer. Lithium buffer containing hormone (10 µM adenosine or 1 µM AVP) was then applied to specific cell surfaces (apical or basolateral). Adenosine deaminase (1.5 U/ml) in lithium buffer was introduced on the contralateral side as described previously. At the end of the 10 minute stimulation period, 300 µl of 10% TCA was introduced to the inside of each insert. The inserts were removed from the 24 well plate and the monolayers along with the upper (apical) volume of buffer were frozen at -80°C. After thawing the cells and supernatant, the supernatant was run over an anion exchange column as described above. Filters with the lysed cells attached were cut from their supports and the lipids were extracted with methanol and chloroform. Again the radioactivity of the inositol phosphate fractions were normalized to the radioactivity in the lipid fraction. The level of inositol phosphate was expressed as percent of control.

Statistics: Data were analyzed by analysis of variance (ANOVA) for sidedness effects, time course effects, and agonist concentration effects. If a difference among the means was indicated, Scheffe's multiple comparisons test was used to determine which means were statistically different. Analyses of inositol phosphate data (sidedness) was performed by Student's t-test. Statistical significance was ascribed for $p < 0.05$ and all values are expressed as mean $\pm$

SEM. Statistics were performed using Apple MacIntosh software (StatView^{tm} 512+, BrainPower, Inc., Calabasas, CA).

C. Results

The permeability of gelatin-coated Millicell^{tm}-CM filter inserts to ^{14}C-sucrose was determined (Figure 11). In the absence of a LLC-PK$_1$ cell monolayer, ^{14}C-sucrose placed in either the upper or lower chamber reached steady state with the contralateral chamber within 30 minutes. Approximately 11% of the total radioactivity added at the beginning of the experiment was removed in the course of measuring ^{14}C-sucrose flux across an insert without cells. However, in the presence of a confluent monolayer of LLC-PK$_1$ cells ^{14}C-sucrose reached less than 10% of the steady state concentration on the contralateral side at the end of a 60 minute time course.

Adenosine (10 µM) was found to elevate intracellular calcium in LLC-PK$_1$ cells in a dose dependent manner (Figure 12) as previously reported by Weinberg et. al. (1989). The response consisted of two phases. The increase in calcium in the initial phase was 100% over baseline (94 $\pm$ 9.3 nM), followed by a sustained phase with calcium elevated by 55% over basal when cells in suspension were exposed to 10 µM adenosine. Additionally, 1 µM AVP stimulated calcium release to 200% over baseline. Chelating extracellular calcium with 5 mM EGTA did not prevent the adenosine-induced peak increase in intracellular calcium but did abolish

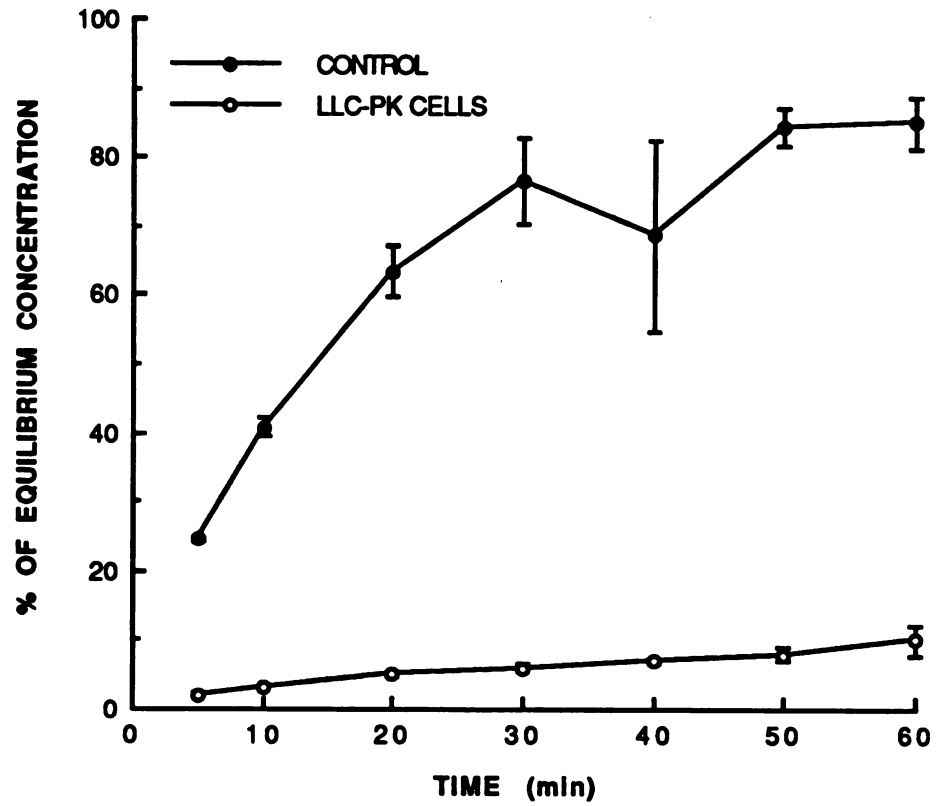


Figure 11. Permeability of LLC-PK$_1$ cells grown on gelatin coated Millicell^{tm}-CM inserts to 1 µM ^{14}C-sucrose. Inserts with (open circles) or without (closed circles) a confluent monolayer of LLC-PK$_1$ cells had 1 µM ^{14}C-sucrose placed on the outside (basolateral) of the insert and 50 µl aliqout samples were taken at the indicated time points from the inside (apical) of the inserts. Representative graph of two experiments in duplicate.

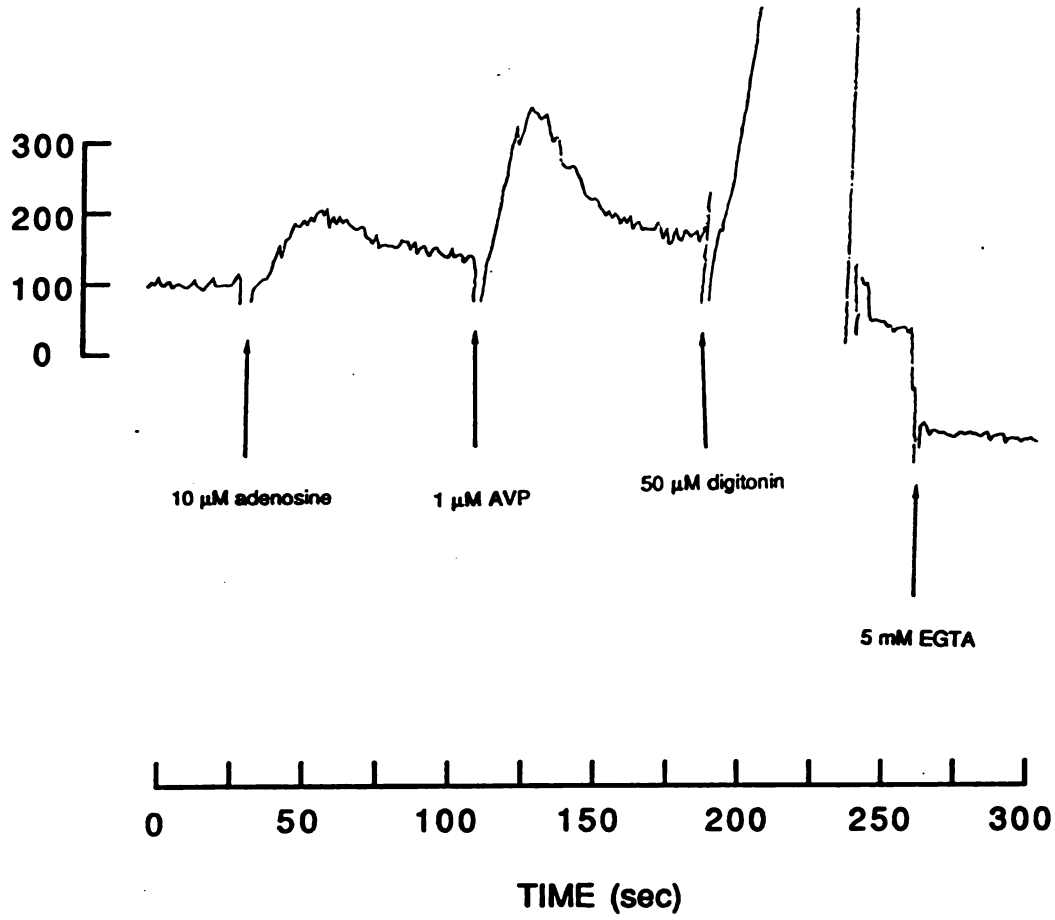


Figure 12. Spectrofluorometer generated tracing of intracellular free calcium concentration in LLC-PK_1 cells in response to adenosine and AVP. The ordinate is expressed as intracellular free calcium concentration in nM.

the sustained phase (Figure 13), thereby suggesting that calcium is released from an intracellular store. Moreover, Weinberg et al. (1989) have shown that mobilization of calcium in LLC-PK_1 cells by adenosine is blocked by the adenosine receptor antagonist, DPCPX, and is pertussis toxin sensitive. Because inositol phosphate stimulation is known to increase the cytosolic free calcium concentration, it may be that the adenosine-induced calcium release in LLC-PK_1 cells is mediated by inositol triphosphate stimulation. A time course for the stimulation of inositol phosphates was determined for LLC-PK_1 cells in suspension (Figure 14). Inositol phosphate levels reached peak values at 10 minutes following stimulation by 10 µM adenosine and returned to the same level as the control by 20 minutes. All remaining inositol phosphate measurements were made using a 10 minute time course. DPCPX blockade of adenosine stimulated inositol phosphate turnover in cells in suspension is shown in Figure 15. Adenosine (10 µM) stimulated inositol phosphate production in a dose dependent manner. However, 1 µM DPCPX blocked adenosine stimulated inositol phoshpate production. Figure 16 shows results of inositol phosphate stimulation by 10 µM adenosine applied to either the apical or basolateral side of cells grown on Millicell^{tm}-CM inserts. None of the inositol phosphate species was increased above control when cells were exposed to adenosine on the apical side. However, all three inositol phosphate species were significantly elevated by 10 µM adenosine applied to the basolateral side of the monolayer.

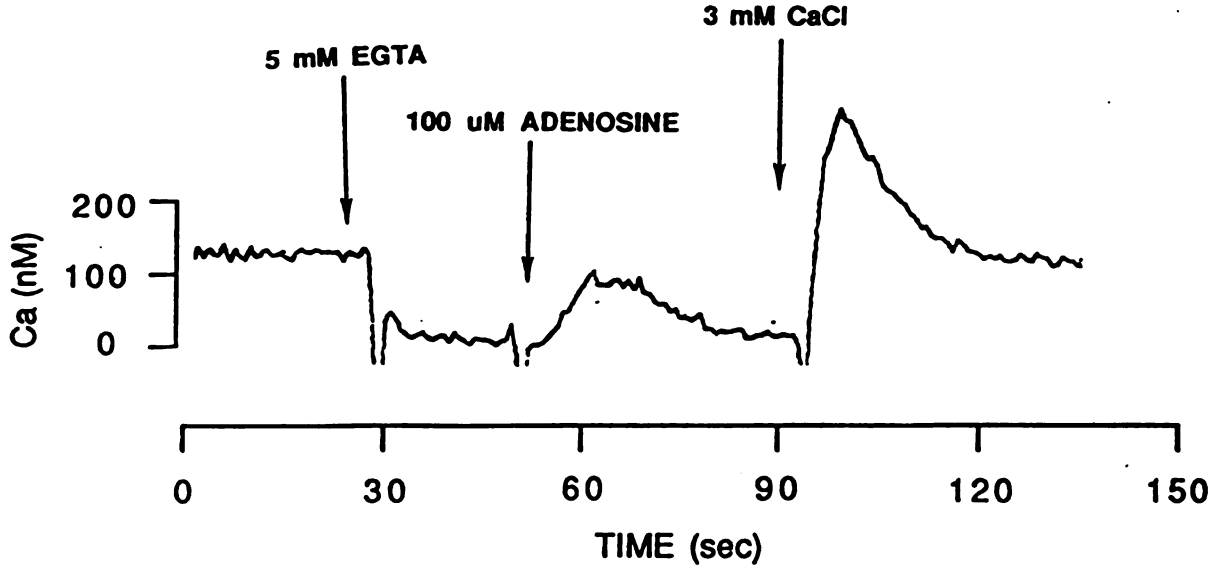


Figure 13. Spectrofluorometer generated tracing of intracellular free calcium concentration stimulated by adenosine in LLC-PK_1 cells following chelation of extracellular calcium with EGTA.

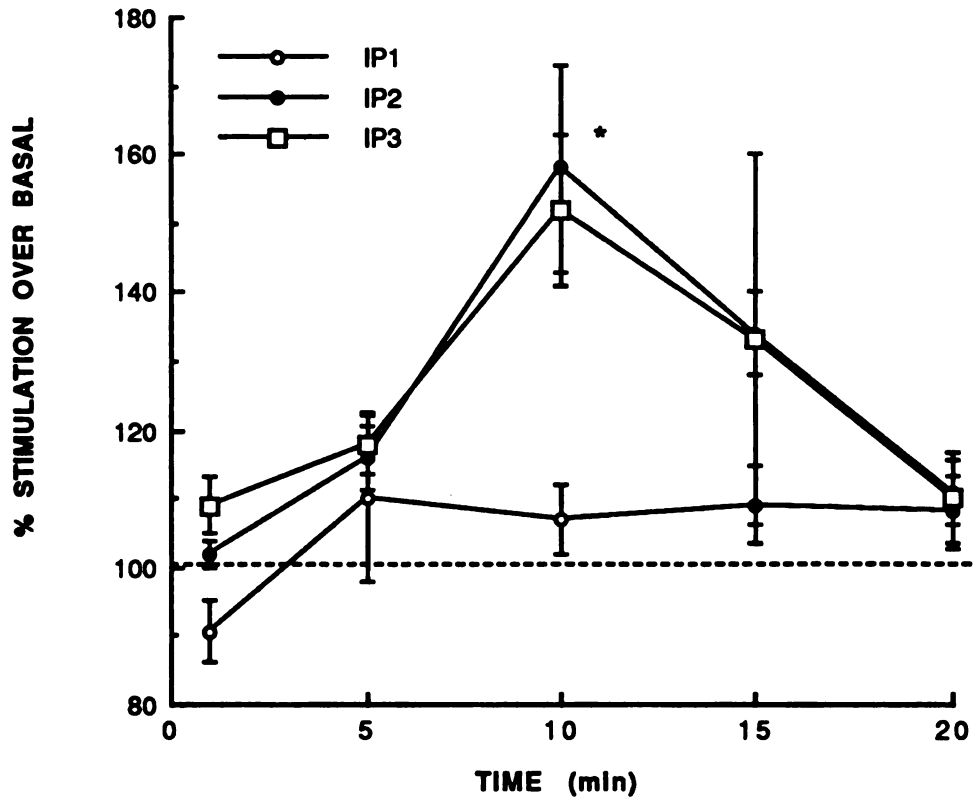


Figure 14. Adenosine stimulation of inositol phosphates in LLC-PK_1 cells in suspension. LLC-PK_1 cells were incubated in the presence of 10 µM adenosine for the indicated times. Inositol phosphates were collected and fractionated as described in "Methods". Values are expressed as mean $\pm$ SEM of three experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from one minute time point.

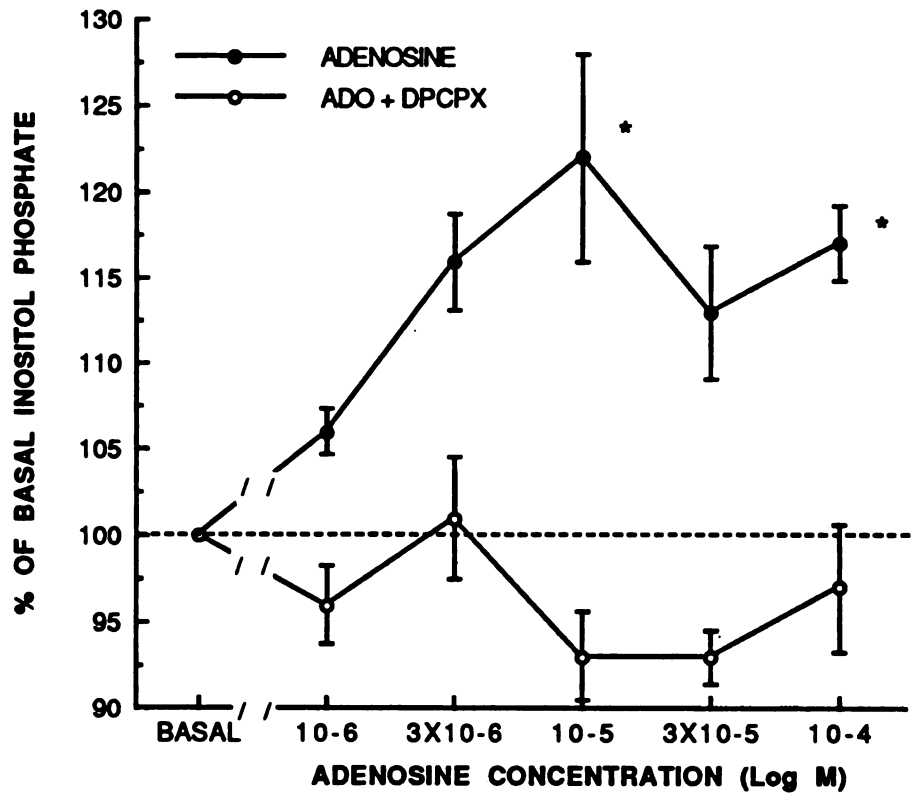


Figure 15. Adenosine dose response stimulation of inositol phosphate (IP1) in LLC-PK$_1$ cells with and without 1 µM DPCPX. LLC-PK$_1$ cells were incubated with increasing concentrations of adenosine in the presence or absence of 1 µM DPCPX for ten minutes. Inositol phosphate (IP1) was collected as described in "Methods". Values are expressed as mean $\pm$ SEM of three experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from 1 µM adenosine stimulated cAMP production.

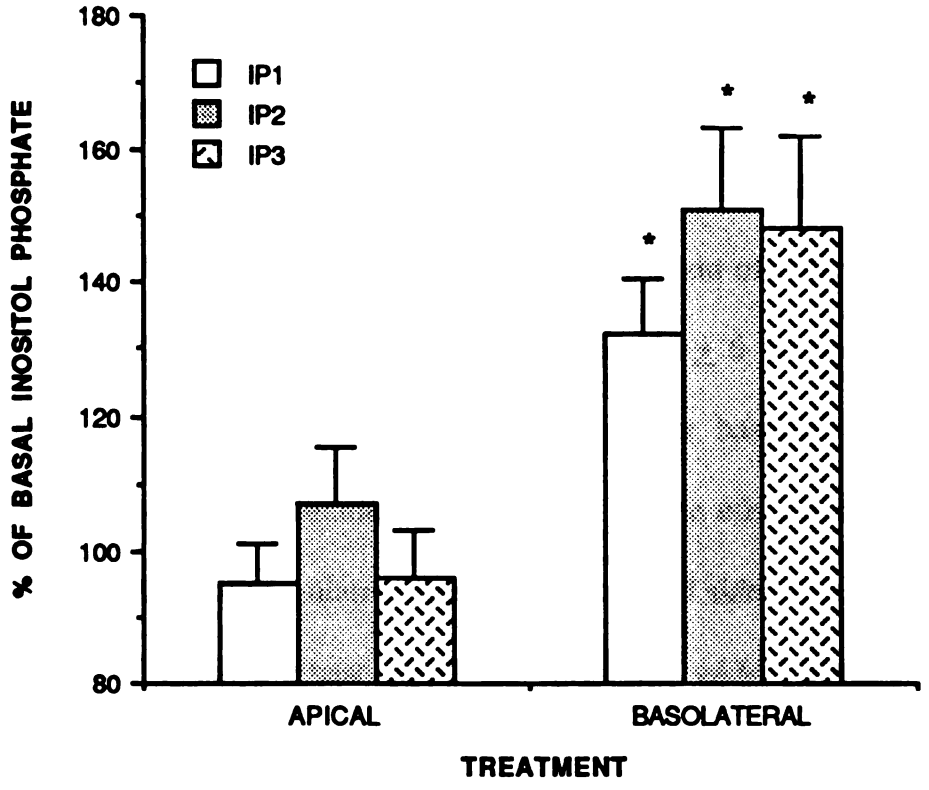


Figure 16. Apical versus basolateral 10 μM adenosine stimulation of inositol phosphates in LLC-PK_1 cells grown on Millicell™-CM inserts. LLC-PK_1 cells were exposed to 10 μM adenosine on either the apical or basolateral side for ten minutes. Inositol phosphates were collected and fractionated as described in "Methods". Values are expressed as mean ± SEM of three experiments in triplicate. * $p < 0.05$ (Student's t-test; two tailed) indicates a significant difference from the corresponding apical exposure.

LLC-PK_1 cells were stimulated with 1 μM forskolin and concurrent 30 nM adenosine was applied to either the apical or basolateral side of the monolayer (Figure 17). Forskolin stimulated cAMP levels were unchanged by apically applied adenosine (30 nM). Cells exposed to 30 nM adenosine on the basolateral side exhibited a significant 23% inhibition of forskolin stimulated cAMP production.

The results of adenosine A_2 receptor localization in LLC-PK_1 cells stimulated with 100 μM adenosine on the apical, basolateral or apical and basolateral sides are shown in Figure 18. Stimulation of either the apical (129 $\pm$ 14 pmol cAMP/mg protein) or basolateral side (58 $\pm$ 6 pmol cAMP/mg protein) evoked significant increases in cAMP levels over baseline (13.5 $\pm$ 1.8 pmol cAMP/mg protein). Stimulation from the apical side elicited significantly higher levels of cAMP than stimulation from the basolateral side. In addition, concurrent stimulation of apical and basolateral sides produced cAMP levels (189 $\pm$ 15 pmol cAMP/mg protein) that were the numerical sum of each of the sides stimulated independently.

The time course of 100 μM adenosine stimulation of cAMP production by apical or basolateral adenosine A_2 receptors is shown in Figure 19. Both apical and basolateral stimulation of cAMP production reached a plateau at 30 minutes. The absolute level of cAMP produced by apical and basolateral stimulation was significantly different at all times (Figure 19A). However, when cAMP levels were expressed as percent of cAMP present at 30 minutes, there was no difference

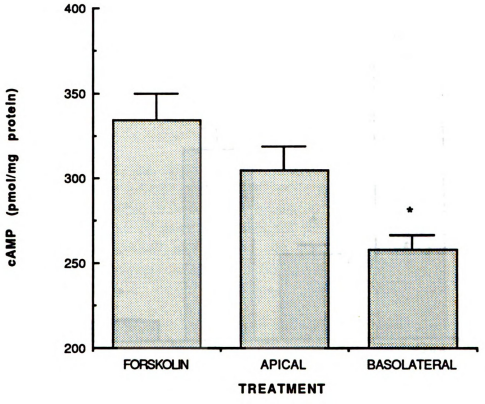


Figure 17. Apical versus basolateral 30 nM adenosine inhibition of 1 μM forskolin stimulated cAMP production in LLC-PK_1 cells grown on Millicell^{tm}-CM inserts. LLC-PK_1 cells were treated with 1 μM forskolin $\pm$ 30 nM adenosine on either the apcial or basolateral side for 30 minutes. Cyclic AMP was measured as described in "Methods". Basal cAMP production was 7.0 $\pm$ 0.4 pmol/mg protein. Values are expressed as mean $\pm$ SEM of three experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from forskolin stimulated cAMP levels.

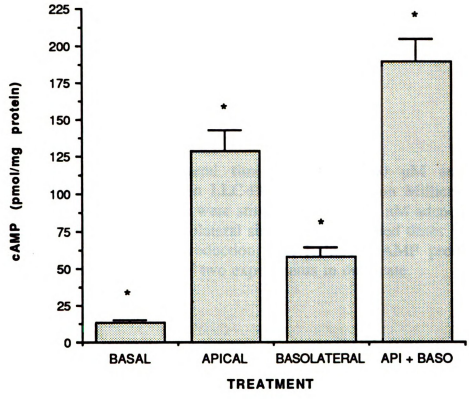
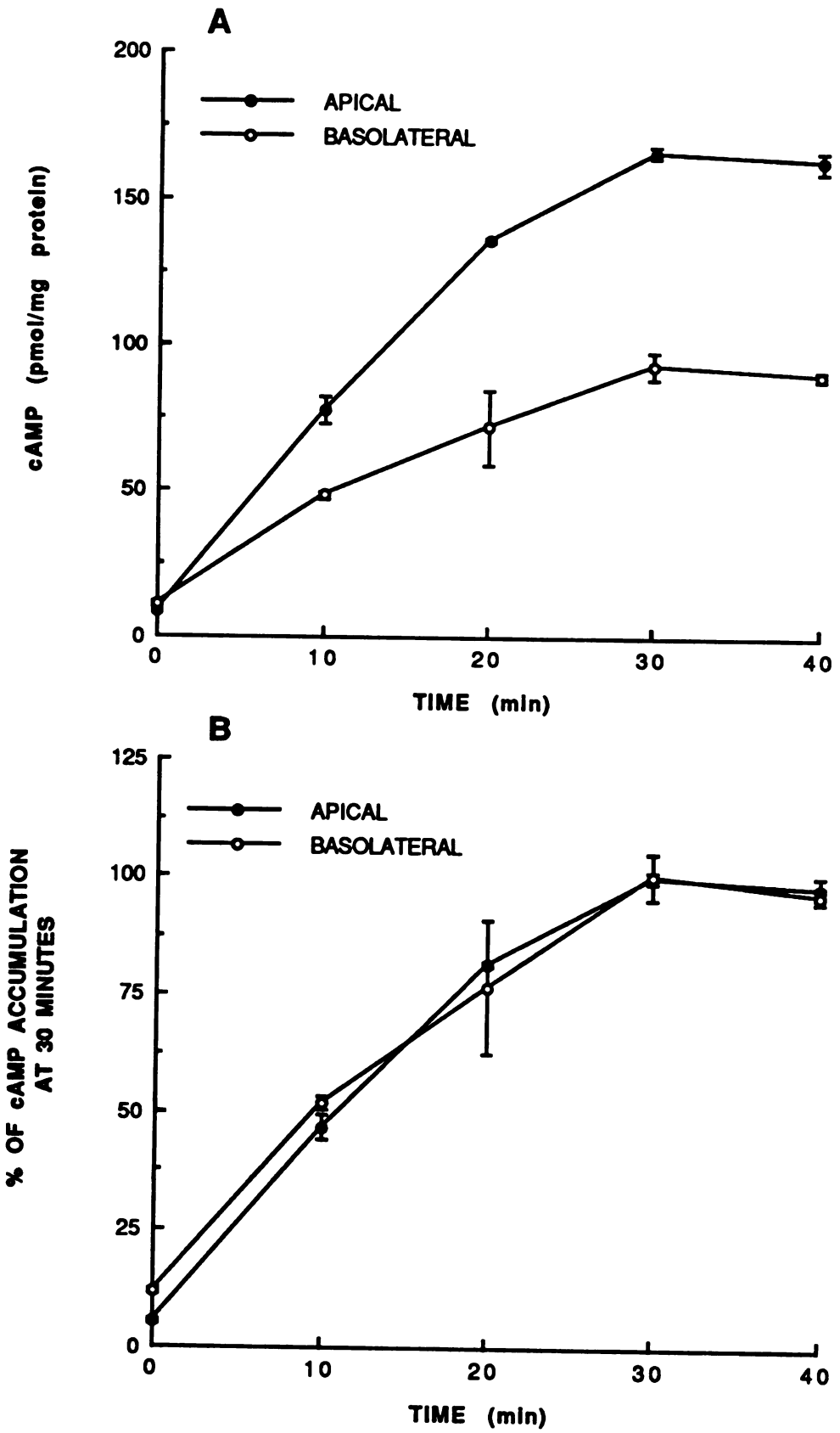


Figure 18. Apical versus basolateral 100 µM adenosine stimulation of cAMP in LLC-PK_1 cells grown on Millicell^{tm}-CM inserts. LLC-PK_1 cells were stimulated with 100 µM adenosine on either the apical, basolateral or both sides for 30 minutes. Cyclic AMP was measured as described in "Methods". Values are expressed as mean $\pm$ SEM of four experiments in duplicate. * $p < 0.05$ (ANOVA) indicates a significant difference of each treatment from the others.

Figure 19. Apical versus basolateral time course to 100 µM adenosine stimulation of cAMP in LLC-PK_1 cells grown on Millicell^{tm}-CM inserts. LLC-PK_1 cells were stimulated with 100 µM adenosine on either the apical or basolateral side for the indicated times.
A: Absolute cAMP production. **B**: Relative cAMP production. Representative graph of two experiments in duplicate.


A
200
150
100
50
0
APICAL
BASOLATERAL
cAMP (pmol/mg protein)
0
10
20
30
40
TIME (min)
B
125
100
75
50
25
0
APICAL
BASOLATERAL
% OF cAMP ACCUMULATION AT 30 MINUTES
0
10
20
30
40
TIME (min)

between the responsiveness of the apical and basolateral sides (Figure 19B). A 30 minute stimulation with supramaximal doses (100, 300 and 1000 μM) of adenosine showed a marked difference in the absolute levels of cAMP produced via apical and basolateral stimulation (Table 2). Neither 100 nM DPCPX nor 24 hour pretreatment with 0.1 μg/ml of pertussis toxin altered the pattern of responses elicited by adenosine A_2 receptor stimulation in LLC-PK_1 cells (data not shown).

D. Discussion

Transporting epithelia display a polarity consisting of an apical and a basolateral side separated by tight junctions (Pfaller et al. 1990). The integral membrane proteins involved in the transport of solutes and water are expressed asymmetrically in these epithelia (Rodriguez-Bolan and Nelson 1989). This asymmetric distribution of transport proteins is the basis for the vectoral transport of solutes and water. There is increasing evidence that the receptor systems regulating transport are also distributed in a polarized manner (Barrett et al. 1989; Fantini et al. 1988; Pratt et al. 1986). While there is growing data on the actions of adenosine receptor systems in renal and other transporting epithelia, very little is known about the spatial location of adenosine receptors on these epithelia.

The permeability of a confluent monolayer of LLC-PK_1 cells was assessed using ^{14}C-sucrose (1 μM). While ^{14}C-sucrose freely crossed a gelatin treated filter insert without cells and reached steady state within 30 minutes, it did not cross a

Table 2. Apical versus basolateral supramaximal adenosine stimulation of cAMP production in LLC-PK_1 cells.

Adenosine Concentration (Log M)	Apical	Basolateral
	(pmol/mg protein)	
10^{-4}	104 ± 13	51.8 ± 4.7*
$3x10^{-4}$	130 ± 15	54.2 ± 6.4*
10^{-3}	141 ± 17	57.2 ± 7.4*

The mean ± SEM of cAMP levels of two experiments in duplicate.
* Significantly different from apical cAMP levels at a $p < 0.05$.

confluent monolayer of cells to any significant degree (< 10% of the equilibrium concentration of 370 nM). Sucrose is thought to be neither transported nor metabolized by these cells and has a molecular weight similar to adenosine. Both uptake and metabolism of adenosine occurs in these cells, therefore, the crossing of ^{14}C-sucrose should represent an over-estimate of the permeability of the monolayer to adenosine. In addition, adenosine deaminase was present on the contralateral side of LLC-PK$_1$ monolayers to further ensure that any adenosine crossing the monolayer was deaminated to inosine. Although the K_m of adenosine deaminase is 35 µM, it has a relatively high V_{max}, 1 unit = 1 µmole/minute at pH 7.4. The ratio of enzyme activity/substrate flux gave a 40 fold higher adenosine deaminase activity than the predicted flux rate of adenosine to the contralateral side (Appendix A). Therefore, we feel that any adenosine that may have crossed the monolayer was quickly deaminated to the inactive compound, inosine. Moreover, non-metabolizable analogs of adenosine were not used in this study. Those adenosine analogs possess varying degrees of lipophilicity and, therefore, may partition into the plasma membrane and cross the monolayer.

Adenosine was found to stimulate the turnover of inositol phosphates in the LLC-PK$_1$ cell in a dose dependent manner. This effect was blocked by the adenosine receptor antagonist, DPCPX, indicating that adenosine acts through a receptor mediated mechanism to stimulate inositol phosphate production. When cells were exposed to adenosine on the apical or basolateral side independently,

only exposure on the basolateral side elicited a stimulation of inositol phosphates. It may be argued that the signal transduction machinery for the stimulation of inositol phosphates is not present in the apical membrane of this cell line. However, 1 μM vasopressin (AVP) was found to stimulate inositol phosphate turnover when presented to either the basolateral or the apical side (data not shown). This finding suggests that the adenosine receptor that mediates inositol phosphate turnover, and thus calcium release from intracellular stores, is located in the basolateral membrane of LLC-PK_1 cells.

The adenosine A_1 receptor is known to mediate inhibition of adenylyl cyclase concurrently activated by stimulatory receptors, such as the AVP receptor (Arend et al. 1987; Dillingham and Anderson 1985; Roy 1984). However, when assessing the functional location of the adenosine A_1 receptor, we chose to look at the inhibition of forskolin stimulated cAMP levels to avoid the further complication of the possible polarity of a stimulatory receptor system. When applied to the basolateral side, 30 nM adenosine inhibited forskolin stimulated cAMP levels by 23%. In contrast, exposure of 30 nM adenosine to the apical side caused a non-significant change (~ 8% decrease) in forskolin stimulated cAMP levels. This finding suggests that inhibitory adenosine A_1 receptors are located primarily basolateral, but that the presence of a minor component on the apical side cannot be completely eliminated.

The functional localization of the stimulatory adenosine A_2 receptor was

assessed by spatially stimulating cAMP production with 100 μM adenosine. Exposure of adenosine to either side of the monolayer elicited a significant elevation in cAMP levels above control. Stimulation of the apical side produced significantly higher levels of cAMP than stimulation of the basolateral side. Concurrent stimulation of both apical and basolateral sides produced cAMP levels that were the numerical sum of either side stimulated independently.

Because the inhibitory adenosine A_1 receptor is located basolaterally, we explored the possibility that the lesser degree of increased cAMP production by basolateral adenosine A_2 receptor stimulation may be due to concurrent inhibition by adenosine A_1 receptors. The specific adenosine A_1 receptor antagonist, DPCPX (100 nM), was used to block adenosine A_1 receptors while adenosine A_2 receptors were stimulated with 100 μM adenosine. Blockade of the adenosine A_1 receptors did not alter the pattern of adenosine A_2 receptor-stimulated cAMP production. Moreover, when cells were pretreated for 24 hours with 0.1 μg/ml pertussis toxin to block any other inhibitory influence that may occur via G_i, the pattern of adenosine A_2 receptor stimulated cAMP production did not change. These findings suggest that the lower cAMP levels produced by basolateral adenosine A_2 receptor stimulation are not due to inhibitory influences that may predominate on that side.

The disparity between cAMP production induced by apical versus basolateral receptor stimulation may be explained in part by the inability of adenosine to access the basolateral cell surface because of the mechanical hinderance of the filter

insert and gelatin substrate required for cell growth. When a time course to stimulate cAMP production was performed, the absolute levels of cAMP were different between apical and basolateral sides at all time periods. However, the levels of cAMP reached a plateau at 30 minutes in both cases. When cAMP levels were expressed as a percent of levels reached at 30 minutes, the rate of stimulation from both sides was identical. In addition, supramaximal concentrations of adenosine continued to stimulate cAMP production to different degrees when applied to the apical and basolateral sides. These data suggest that the lower cAMP levels resulting from basolateral adenosine receptor stimulation are not due to a significant hinderance of adenosine access to the basolateral side. Further support for the primary functional localization of adenosine A_2 receptors to the apical cell surface and adenosine A_1 receptors to the basolateral cell surface can be seen from the results of inositol phosphate turnover experiments. Using a 10 minute time course, adenosine stimulated inositol phosphate turnover when applied to the basolateral side only. Interestingly, there was a significant difference in apical verses basolateral stimulation of cAMP production at the 10 minute time point in experiments examining the time course of adenosine A_2 receptor stimulated cAMP production. Taken together, these results suggest that the majority of adenosine A_2 receptors reside in the apical membrane, whereas only a minor complement of adenosine A_2 receptors reside in the basolateral membrane of LLC-PK_1 cells.

From the results discussed above, we have developed a model of the functional localization of adenosine receptors in the LLC-PK_1 cell (Figure 20). This model suggests that the majority of stimulatory adenosine A_2 receptors are exposed to the apical surface, while the majority of inhibitory adenosine A_1 receptors are exposed to the basolateral surface of the cell. In addition, the adenosine receptor mediated pathway that stimulates inositol phosphate turnover and presumably Ca^{++} release from intracellular stores is exposed to the basolateral cell surface. If the adenosine receptors have a similar distribution in the renal tubule, adenosine A_2 receptors would be exposed to the tubular lumen, while the adenosine receptor mediated pathway that stimulates inositol phosphate turnover and adenosine A_1 receptors would face the extracellular space on the vascular side of the tubule. However, it should be cautioned that the functional distribution of adenosine receptors on the LLC-PK_1 cell may be reflective of aberrant targeting of these receptors by cells in culture. It remains to be determined if a functional separation of adenosine receptors is observed *in vivo*.

The tubular epithelium of the kidney is bathed by two distinct extracellular fluid compartments, one being interstitial fluid (basolateral) and the other being tubular fluid (apical). There is evidence to suggest that the concentration of adenosine in renal blood and urine is different under normal resting conditions (Spielman and Thompson 1982). Those investigators reported that the adenosine concentration of arterial and venous renal blood was ~50 nM, whereas the

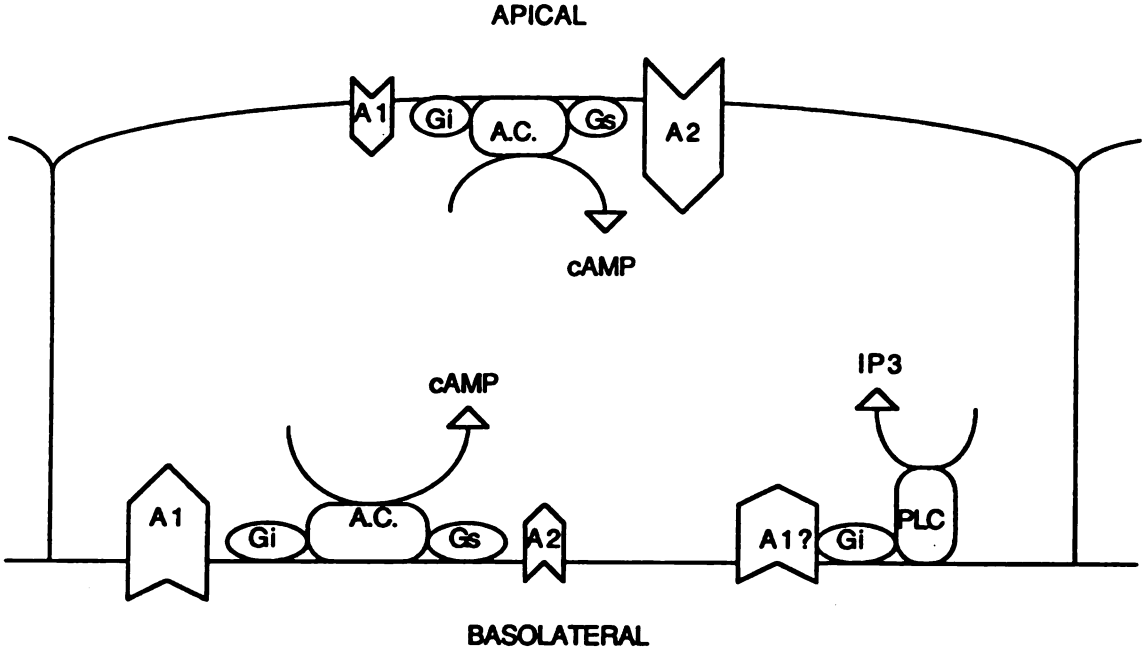


Figure 20. Model of adenosine receptor functional localization in LLC-PK$_1$ cells. This figure shows a model of the spatial localization of the adenosine receptor mediated pathways in the LLC-PK$_1$ cell. The relative size of each "receptor" indicates the predominance of that receptor pathway on either the apical or basolateral side.

concentration in the urine was ~300 nM. If adenosine receptors are globally distributed about the tubule cell, then adenosine in the tubular lumen would have an overriding influence on the resulting signal transduction. However, we report here that adenosine receptors have a polarized distribution in the LLC-PK_1 renal cell line and that this distribution may be reflective of the adenosine concentrations that are present on either side of the epithelium.

V. Desensitization of Adenosine A_2 Receptors in the LLC-PK_1 Renal Cell Line: Homologous Desensitization Can Occur Independently of Receptor Occupation/Activation

A. Introduction

The prolonged exposure of cell types or tissues that predominantly express the stimulatory adenosine A_2 receptor have shown a homologous pattern of desensitization. Homologous desensitization of adenosine A_2 receptors has been seen in vascular smooth muscle from rat aorta (Anand-Srivastava et al. 1989), rat kidney fibroblasts (Newman and Levitzki 1983), and rat brain striatum (Hawkins et al. 1988; Porter et al. 1988). Ramkumar et al. (1991) observed the desensitization of both adenosine A_1 and A_2 receptors in the DDT_1 MF-2 smooth muscle cell line to specific agonists for each of the two receptors. However, the time course for desensitization of the adenosine A_1 receptor had a $t_{1/2}$ of 8 hours, while the $t_{1/2}$ for the adenosine A_2 receptor was 45 minutes. In contrast, the adenosine A_1 and adenosine A_2 receptors in RCCT cells in culture desensitized at essentially the same concentration and time course of exposure to agonist (Arend, personal communication; LeVier et al. submitted).

Recently the idea of spatial separation of cell surface receptors on a polarized monolayer of epithelial cells has received attention. Polarized epithelia are structured such that tight junctions between the apical and basolateral sides

separate the membrane bound proteins on one side from the other (Rodriquez-Bolan and Nelson 1989). This polarization of epithelial monolayers and therefore the proteins responsible for solute transport is the basis for vectoral transport of solute and water across the monolayer. Several researchers have demonstrated the polar distribution of membrane bound receptors on epithelial cells. These include the bradykinin (Denning and Welsh 1991), VIP (Fantini et al. 1988), AVP (Ando et al. 1991), and adenosine receptors (Barrett et al. 1989; Husted et al. 1990; Pratt et al. 1986) which have been found to have a polarized distribution on several epithelial cell types. This laboratory has recently established the functional localization of adenosine receptor pathways in the LLC-PK_1 renal cell line (LeVier et al. in press). The inhibitory adenosine A_1 receptor pathway was found to be basolaterally located, although a minor component located on the apical side could not be discounted. In contrast, the stimulatory adenosine A_2 receptor pathway was localized to both the apical and basolateral side of the LLC-PK_1 monolayer with the major component residing on the apical side. The present study investigated the effect of selective exposure of adenosine to either the apical or basolateral side of a monolayer of LLC-PK_1 cells on the subsequent responsiveness of adenylyl cyclase-coupled adenosine receptors.

B. Methods

Cell Culture: LLC-PK_1 cells (passage number 194) were obtained from American Type Culture Collection, (Rockville Maryland). Cell culture reagents were obtained from Gibco BRL, (Grand Island NY). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml each of penicillin and streptomycin at 37°C in a 7% CO_2 atmosphere. Cells were routinely passaged every 7 days. Cells for experiments in 24 well plates were lightly trypsinized from one 100 mm culture dish with a solution of 0.2% trypsin and 0.05% EDTA in PBS and sedimented by centrifugation (500 x g) for five minutes. The resulting pellet was then resuspended in 25 ml of culture media. This cell suspension was then distributed to one 24 well plate at 1 ml per well. The cells were then allowed to grow to confluency before an experiment was undertaken.

Gelatin Substrate Coating of Culture Inserts: Cell culture inserts (12 mm diameter, Millicell™-CM; obtained from Millipore Corp., Bedford MA.) were coated with gelatin (Millipore technical brief: Lit. No. TB017 copyright 1989) as an extracellular matrix upon which LLC-PK_1 cells adhere and grow. The inserts were then prepared for cell culture as described in Methods of chapter IV (Figure 9).

Measurement of the Potential Difference and Electrical Resistance of a LLC-PK_1 Cell Monolayer: Once confluent, the potential difference and electrical

resistance of the LLC-PK_1 cell monolayer was measured to assess the monolayer's integrity. All potential difference and electrical resistance measurements were made before any experimental procedure was undertaken. The measurement of potential difference and electrical resistance was carried out as described in Methods of chapter IV.

Desensitization Protocol: LLC-PK_1 cells were grown to confluency on either Millicell^{tm}-CM inserts or 24 well plates. All cells were washed with Krebs buffer. Cells grown in 24 well plates were pretreated for 1 hour with 100 µM adenosine in Krebs buffer (500 µl). Cells grown on filter inserts were pretreated for 1 hour with 100 µM adenosine in Krebs buffer on either the apical or basolateral side of the monolayer (300 µl apical, 500 µl basolateral). Krebs buffer with 1.5 U/ml of adenosine deaminase (ADA) was placed on the contralateral side of pretreated inserts. Control groups were pretreated for 1 hour with Krebs buffer without adenosine. Following the pretreatment time period, the monolayers were washed with Krebs buffer. Each monolayer was then stimulated for 30 minutes with 100 µM adenosine in Krebs buffer (300 µl in wells; 300 µl apical, 500 µl basolateral in inserts) in the presence of 100 µM RO 20-1724, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. The protein kinase C inhibitor, staurosporine (1 µM), was included in the pretreatment regime of cells on a 24 well plate to investigate the possible involvement of PKC in the signal pathway for desensitization. The stimulation of cAMP production was halted by adding 75 µl

of 0.2 N HCl to each well or upper side of inserts. The cells were then frozen at -80°C to lyse cells and release cellular contents into the supernatant for subsequent measurement of cAMP production.

cAMP Determination: Samples were thawed at room temperature and the pH was neutralized by adding 200 µl of 0.5 M Na_2HPO_4. The cAMP levels in the supernatant of each sample were measured via radioimmunoassay as described by Frandsen and Krishna (1976). Cyclic AMP levels were expressed per mass of cell protein as measured by a modified Lowry's assay (Markwell et al. 1978).

Adenylyl Cyclase Activity: A highly specific competitive protein kinase A (PKA) inhibitor, Rp-cAMPS (from BioLog life science institute in LaJolla CA.), was used to specifically inhibit PKA and investigate its role in the desensitization of adenosine A_2 receptors. LLC-PK_1 cells grown to confluency on 60 mm culture dishes were incubated in the presence or absence of 100 µM adenosine ± 10 µM or 100 µM Rp-cAMPS in Krebs buffer at 37°C for 1 hour. The cells were preincubated for 10 minutes with Rp-cAMPS prior to the introduction of 100 µM adenosine. The cells were washed twice with 5 ml PBS and removed from the culture dishes by incubation at 37°C for 3 minutes with 0.2% trypsin, 0.05% EDTA in PBS. The cells were collected by washing the dish with 5 ml PBS and centrifuged (500 x g) for 5 minutes. The resulting cellular pellet was resuspended in 2 ml of cyclase buffer (mM: 50 Tris-HCl, 5 $MgCl_2$, pH 7.4) and gently homogenized with a small glass-teflon homogenizer. The ensuing homogenate was

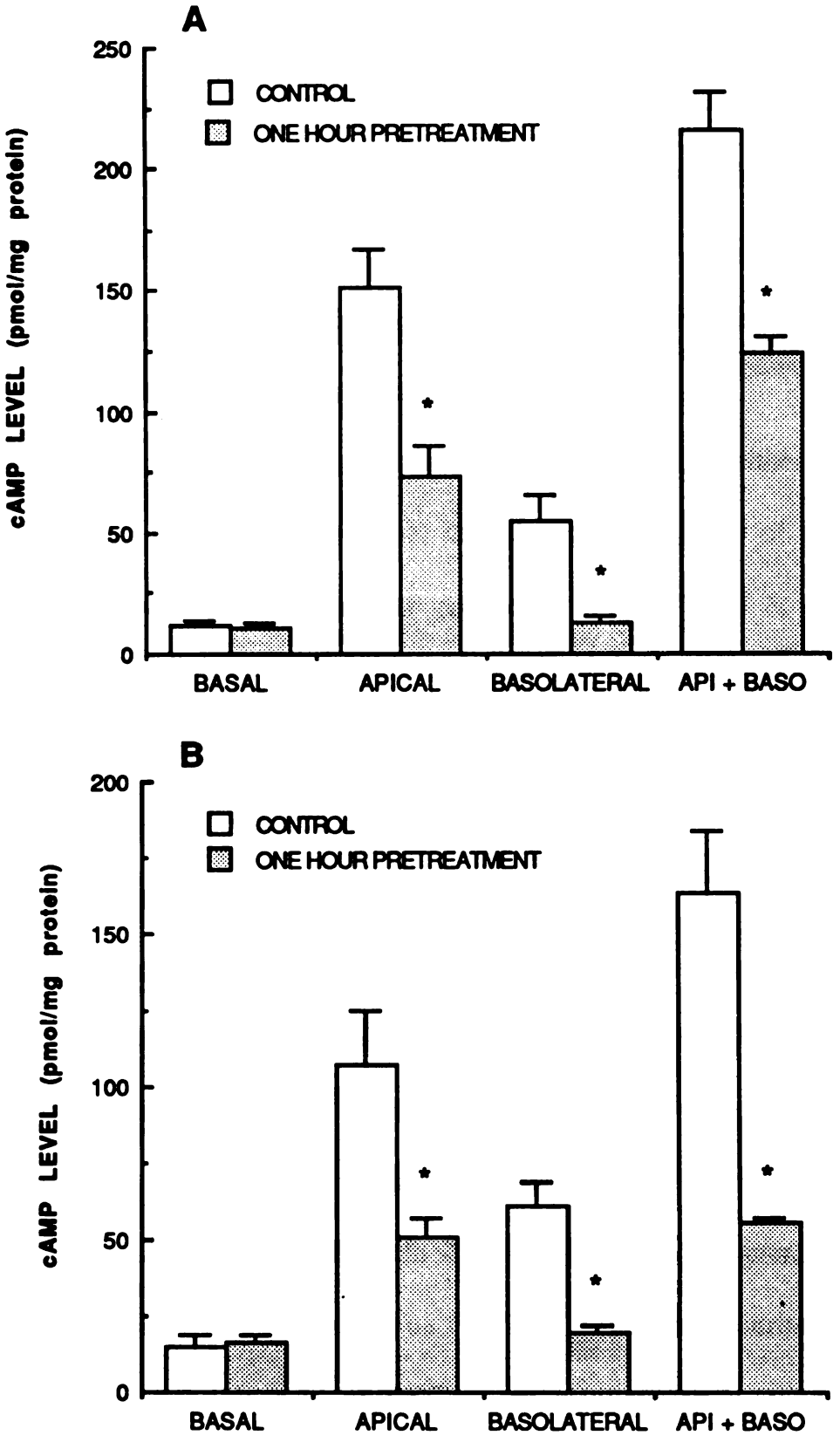
centrifuged at 12,000 RPM in a Beckman JA-20 rotor for 15 minutes at 4°C. The resulting crude membrane pellet was resuspended in 0.5 ml cyclase buffer so as to yield a protein concentration of ~ 1 mg/ml. Adenylyl cyclase activity was initiated by adding ten microliters (~20 µg membrane protein) of the crude membrane preparation to microcentrifuge tubes in a final reaction volume of 250 µl. The final concentration of reaction mixture components were: 0.31 mg/ml creatine kinase, 1.68 mg/ml creatine phosphate, 0.3 mg/ml BSA, 5 units/ml adenosine deaminase, 0.25 mM ATP, 0.25 mM cAMP, 0.5 mM EGTA, 25 µM GTP, 0.25 mM RO 20-1724, and 10 µCi/ml α^{32}P-ATP in cyclase buffer. Adenosine A_2 receptor stimulated adenylyl cyclase activity was allowed to run for 30 minutes at 30°C in the presence or absense of 10 µM NECA and terminated by adding 250 µl 6.7% TCA. Adenylyl cyclase activity in the membranes was determined as described by Salomon et al. (1974).

Statistics: All data sets were analyzed by analysis of variance (ANOVA). If a difference among means was indicated, Scheffe's multiple comparisons test was used to determine which means were statistically different. Statistical significance was ascribed for a $p < 0.05$. All values are expessed as mean ± SEM. Statistics were performed using Apple MacIntosh software StatView^{tm} 512+ from BrainPower, Inc., Calabasas, CA.

C. Results

As has been previously demonstrated (LeVier et al. in press) the functional response of adenosine A_2 receptors in LLC-PK_1 cells shows a marked polarity. Adenosine applied to either side of the monolayer stimulates cAMP production via the adenosine A_2 receptor with the apical side responding to a greater degree than the basolateral side. Moreover, the response to adenosine presented to both sides simultaneously is the numerical sum of either side stimulated independently. A pretreatment time of one hour was chosen to minimize the possible flux of adenosine across the cellular monolayer. Following a one hour pretreatment with adenosine on either the apical or basolateral side of a LLC-PK_1 cell monolayer, the ability of selectively applied 100 µM adenosine to restimulate cAMP production was significantly blunted as compared to control on either side of the monolayer (Figure 21 A & B). Apical pretreatment lead to a 51% decrease of apical and a 42% decrease of the combined apical and basolateral adenosine A_2 receptor stimulated cAMP production. Furthermore, apical pretreatment induced a complete desensitization of the adenosine A_2 receptor response on the basolateral side. Basolateral pretreatment also lead to a complete desensitization of adenosine A_2 receptor stimulated cAMP production on the basolateral side. In addition, basolateral pretreatment lead to a 53% decrease of apical and 66% decrease of combined apical and basolateral adenosine A_2 receptor stimulated cAMP production.

Figure 21. Selective 100 µM adenosine pretreatment of apical or basolateral adenosine A_2 receptors in LLC-PK_1 cells. Cells grown to confluency on Millicell^{tm}-CM inserts were pretreated with 100 µM adenosine for one hour on either the (**A**) apical or (**B**) basolateral side. The cells were then rechallenged with 100 µM adenosine for 30 minutes on either the apical, basolateral or both sides. Cyclic AMP was measured as described in "Methods". Values are reported as mean $\pm$ SEM of two experiments in duplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from the corresponding control value.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cAMP LEVEL (pmol/mg protein)
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API + BASO
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cAMP LEVEL (pmol/mg protein)
CONTROL
ONE HOUR PRETREATMENT
*
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BASAL
APICAL
BASOLATERAL
API + BASO

The adenosine A_1 receptor selective agonist, R-PIA, was used to inhibit cAMP production stimulated by forskolin following pretreatment with adenosine. A one hour pretreatment with 100 µM adenosine on either the apical or basolateral side did not blunt the ability of 30 nM R-PIA to inhibit 1 µM forskolin stimulated cAMP production via the adenosine A_1 receptor (Figure 22). Control, apically and basolaterally pretreated cells all responded to 30 nM R-PIA by inhibiting 1 µM forskolin stimulated cAMP production by 40%.

A one hour pretreatment with 100 µM adenosine on either the apical or basolateral side of the cell monolayer did not alter the pattern of 1 nM arginine vasopressin (AVP) stimulated cAMP production (Figure 23 A & B). LLC-PK_1 cells were equally responsive to 1 nM AVP added to either the apical or basolateral side of the monolayer. In addition, 1 nM AVP added to both sides concurrently stimulated cAMP production that was the numerical sum of the two sides stimulated independently.

Forskolin was used to explore the effect of elevated cAMP levels on adenosine A_2 receptor function in the LLC-PK_1 cell (Figure 24). Forskolin (1 µM) increased cAMP production to levels that were similar to those stimulated by 100 µM adenosine. Following pretreatment for one hour with 1 µM forskolin, 100 µM adenosine stimulation of cAMP production on the apical, basolateral or both sides of LLC-PK_1 cells was unaltered. The PKC inhibitor, staurosporine, was used to investigate the possible role of PKC in the desensitization of adenosine A_2

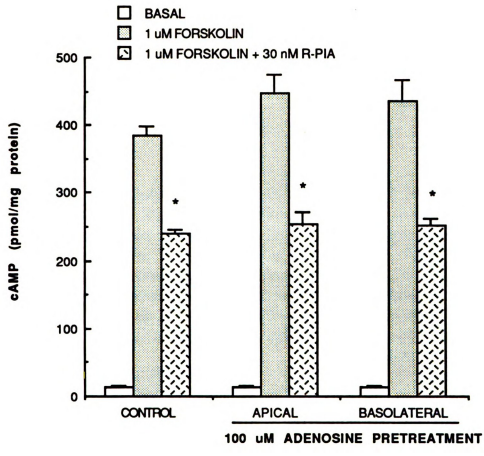
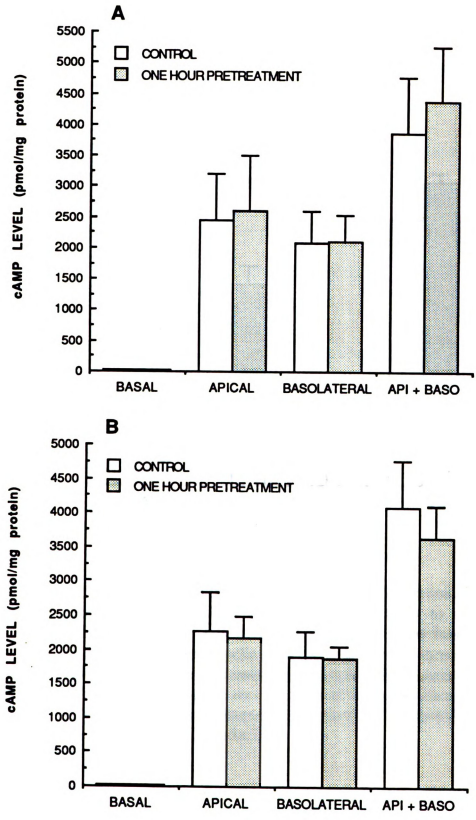


Figure 22. Apical versus basolateral 100 µM adenosine pretreatment effect on R-PIA inhibition of 1 µM forskolin stimulated cAMP production in LLC-PK_1 cells. Following pretreatment with 100 µM adenosine on either the apical or basolateral side for one hour, LLC-PK_1 cells were treated with 1 µM forskolin $\pm$ 30 nM R-PIA for 30 minutes. Cyclic AMP was measured as described in "Methods". Values are expressed as mean $\pm$ SEM of four experiments in duplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from forskolin stimulated cAMP levels.

Figure 23. Effect of apical versus basolateral 100 µM adenosine pretreatment on AVP-stimulated cAMP production in LLC-PK_1 cells. Cells grown to confluency on Millicell^{tm}-CM inserts were pretreated for one hour with 100 µM adenosine on the (A) apical or (B) basolateral side. Cyclic AMP production was stimulated with 1 nM AVP for 30 minutes on the apical, basolateral or both sides. Values are expressed as the mean $\pm$ SEM of two experiments in duplicate. Cyclic AMP was measured as described in "Methods". An ANOVA was performed as described.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CONTROL
ONE HOUR PRETREATMENT
cAMP LEVEL (pmol/mg protein)
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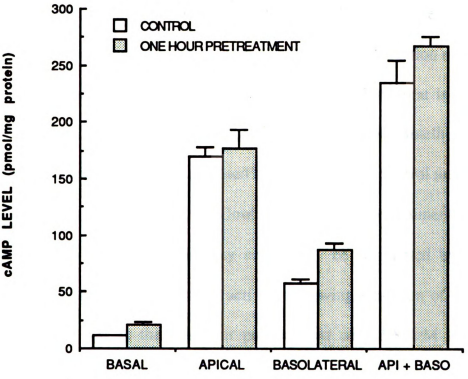

Figure 24. Effect of pretreatment with 1 μM forskolin on adenosine A_2 receptor responsiveness in LLC-PK_1 cells. Cells grown to confluency on Millicell^{tm}-CM inserts were pretreated with 1 μM forskolin on both sides for one hour. Cyclic AMP production was stimulated with 100 μM adenosine for 30 minutes on the apical, basolateral or both sides. Values are reported as mean $\pm$ SEM of two experiments in duplicate. Cyclic AMP was measured as described in "Methods". An ANOVA as performed as described.

receptors (Figure 25). When 1 μM staurosporine was added concurrently with 100 μM adenosine during a one hour pretreatment of LLC-PK_1 cells grown on a 24 well culture plate, the pattern of adenosine A_2 receptor desensitization was not altered.

The cell permeant cAMP analog Rp-cAMPS was used to assess the contribution of protein kinase A in the homologous desensitization of adenosine A_2 receptors (Figure 26). Rp-cAMPS is an analog of cAMP that is a competitive inhibitor of PKA with an IC_{50} of approximately 10 μM (Botelho et al. 1988). Adenylyl cyclase activity was increased by 85 $\pm$ 3% when control membranes were stimulated with 10 μM NECA. Following a one hour pretreatment with 100 μM adenosine, adenylyl cyclase activity could only be stimulated by 30 $\pm$ 3.7%. NECA stimulated adenylyl cyclase activity following inclusion of Rp-cAMPS at 10 μM or 100 μM in the one hour pretreatment with 100 μM adenosine was increased by 34 $\pm$ 4.7% or 34 $\pm$ 2.6% respectively.

D. Discussion

While desensitization of receptor coupled-adenylyl cyclase systems has been thoroughly researched, the idea of spatial effects on desensitization is something new. Virtually none of the studies in polarized epithelia have tested the effects of the polar distribution of a receptor its desensitization. The adenosine A_2 receptor has been reported to be desensitized in a number of cell and tissue types, however

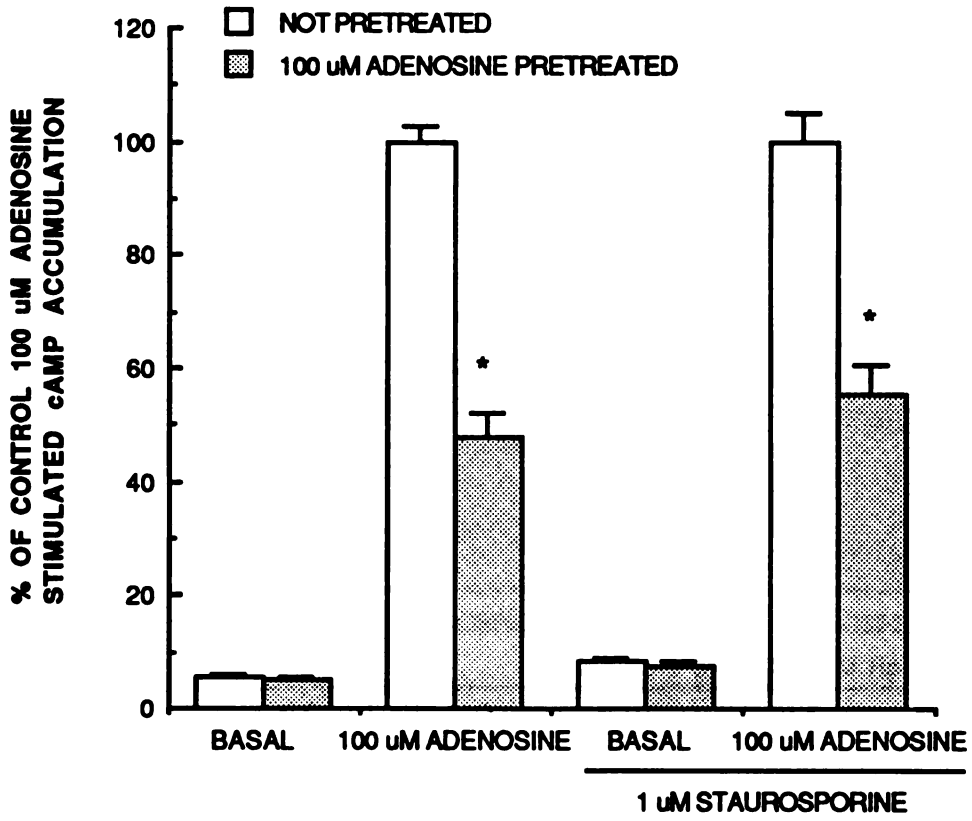

Figure 25. Effect of pretreatment with 100 μM adenosine ± 1 μM staurosporine on adenosine A_2 receptor desensitization in LLC-PK_1 cells. The PKC inhibitor, staurosporine (1 μM), was added concurrently with 100 μM adenosine in a one hour pretreatment of cells grown on a 24 well plate. The cells were then rechallenged with 100 μM adenosine for 30 minutes. Values are reported as mean ± SEM of two experiments in triplicate. Cyclic AMP levels were measured as described in "Methods". * $p < 0.05$ (ANOVA) indicates a significant difference from control stimulated cAMP production.

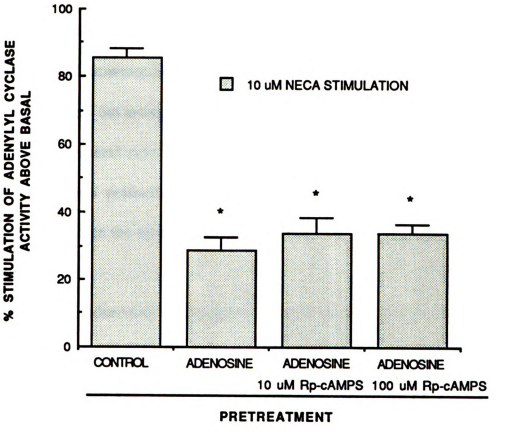

Figure 26. Effect of the PKA inhibitor, Rp-cAMPS, on adenosine A_2 receptor desensitization induced changes in adenylyl cyclase activity in LLC-PK_1 cell membranes. Cells grown to confluency on a culture dish were concurrently pretreated with 100 µM adenosine and the competitive PKA inhibitor Rp-cAMPs at concentrations of 10 µM or 100 µM for one hour. Membranes were prepared and adenylyl cyclase activity measured as described in "Methods". Values are expressed as mean $\pm$ SEM of two experiments in triplicate. * $p < 0.05$ (ANOVA) indicates a significant difference from control stimulated adenylyl cyclase activity.

none of these cells or tissues are epithelial in nature (Anand-Srivastava et al. 1989; Hawkins et al. 1988; Newman and Levitzki 1983; Porter et al. 1988; Ramkumar et al. 1991). In contrast, this laboratory has reported the desensitization of adenosine A_1 and adenosine A_2 receptors in renal cortical collecting tubule cells (Arend personal communication; LeVier et al. submitted). The afore mentioned work was carried out using adenosine analogs that are sufficiently lipophilic to access the basolateral side of cells grown to confluency on a culture dish. The present work was performed using the hydrophilic ligand adenosine exposed selectively to either the apical or basolateral aspect of cells grown on Millicell™-CM filter inserts.

Recently, adenosine A_2 receptor function was determined to be present on both apical and basolateral sides of LLC-PK_1 cells (LeVier et al. in press). The major component of adenosine A_2 receptors was found to be apical with a minor component on the basolateral side. In addition, concurrent stimulation of adenosine A_2 receptors on both sides resulted in an increase in cAMP production that was the numerical sum of either side stimulated independently. Nonetheless, adenosine placed on either side evoked a significant increase in cAMP production over basal. This differential distribution of adenosine A_2 receptors provided an opportunity to selectively occupy and activate a subpopulation of receptors on the same cell.

In preliminary experiments on the desensitization of adenosine receptors performed on cells grown to confluency on a solid plastic support (24 well plate),

a significant reduction in adenosine A_2 receptor responsiveness (50%) had occurred within 30 minutes of exposure to 100 µM adenosine and continued to decline for up to four hours (data not shown). Following selective exposure of either the apical or basolateral side to 100 µM adenosine for 1 hour, the adenosine A_2 receptor response on the basolateral side was abolished. However, the response on the apical side was reduced by 50%. The 50% reduction in response on the apical side is strikingly similar to that seen with desensitization of LLC-PK_1 cells grown to confluency on a plate. It would appear that a subpopulation of adenosine A_2 receptors on the apical side of LLC-PK_1 cells is refractory towards desensitization over the time course studied. Surprisingly, selective exposure of either side of the monolayer to 100 µM adenosine resulted in the same pattern of desensitization of adenosine A_2 receptors.

Previous work established that confluent monolayers of LLC-PK_1 cells are tight to the flux of ^{14}C-sucrose, a sugar of comparable molecular weight to adenosine. Additionally, adenosine deaminase was included in the buffer on the contralateral side of monolayers pretreated with adenosine. The adenosine deaminase was present with a 40 fold higher activity than predicted adenosine flux across the cellular monolayer (Appendix A). Therefore, the adenosine receptors on the contralateral side of the cell monolayer presumably were not occupied/activated during the one hour exposure time course, yet this subpopulation of receptors became desensitized. A heterologous pattern of desensitization could

explain such a phenomenon. However, the responsiveness of the stimulatory AVP receptor was not affected by one hour exposure to 100 μM adenosine in the LLC-PK_1 cell. Interestingly, AVP evoked cAMP production on either side of the cell monolayer to an equal degree. Monolayers of LLC-PK_1 cells did not allow ^{3}H-AVP to cross at a time course of up to one hour (data not shown). Moreover, the response to AVP exposed to both sides concurrently was the numerical sum of either side stimulated independently. This suggests that there are equal numbers of AVP receptors on either side of the LLC-PK_1 cell monolayer. The ability of 30 nM R-PIA to inhibit forskolin stimulated cAMP production via the adenosine A_1 receptor also was not affected by a one hour exposure to 100 μM adenosine. These results argue for an alteration of adenosine A_2 receptor function and not G-protein or adenylyl cyclase function, as the responsiveness of other receptors coupled to the stimulation or inhibition of adenylyl cyclase are unaffected. Consequently, these results suggest that subpopulations of unoccupied/non-activated adenosine A_2 receptors can undergo homologous desensitization.

The homologous desensitization of the β-adrenergic receptor has been associated with the phosphorylation of the carboxy-terminus tail of the receptor by a receptor specific kinase termed βARK (Benovic et al. 1988, Hausdorff et al. 1990). βARK has been shown to preferentially phosphorylate the occupied form of the receptor. Additionally the β-adrenergic receptor has also been shown to be phosphorylated by PKA under conditions where heterologous desensitization occurs

(Bouvier et al. 1989). Although currently there is no information on the phosphorylation state of adenosine A_2 receptors under either basal or desensitizing conditions, it seems reasonable to postulate that the altered function of adenosine A_2 receptors may involve their phosphorylation. Therefore, we chose to investigate the possibilities that either PKA or PKC are involved in the homologous desensitization of adenosine A_2 receptors.

It has been reported that protein kinase C can phosphorylate the adenylyl cyclase-coupled β-adrenergic receptor in vitro (Bouvier et al. 1987). While the phosphorylation of the β-adrenergic receptor by protein kinase A was stimulated by receptor occupancy, protein kinase C mediated phosphorylation of the β-adrenergic receptor was not. Previously, the stimulation of inositol triphosphate turnover by 10 μM adenosine was shown to occur via a receptor mediated mechanism in LLC-PK_1 cells (LeVier et al. in press). This adenosine receptor mediated release of inositol triphosphate is thought to occur through the stimulation of phospholipase C. The concentration (100 μM) of adenosine used to desensitize adenosine A_2 receptors would also elicit an adenosine receptor stimulated release of diacylglycerol (DAG) via phospholipase C. Diacylglycerol is known to activate protein kinase C. Additionally, cAMP has been shown to activate protein kinase C via protein kinase A in the LLC-PK_1 cell line (Anderson and Breckon 1991). However, inclusion of 1 μM staurosporine did not alter the pattern of homologous desensitization of the adenosine A_2 receptor. This suggests that protein kinase C

is not involved in the homologous desensitization of adenosine A_2 receptors.

Forskolin was used to stimulate the intracellular level of cAMP in LLC-PK_1 cells for one hour. This elevation of cAMP level within the cell and presumed increase in protein kinase A activity did not alter the pattern of homologous desensitization of adenosine A_2 receptors. This suggests that protein kinase A is not involved in the desensitization of adenosine A_2 receptors that are not occupied/activated. In order to investigate the possibility that protein kinase A may be involved in the desensitization of occupied/activated adenosine A_2 receptors, the competitive protein kinase A inhibitor Rp-cAMPS was employed. Rp-cAMPS has been shown in several cell systems to have a K_i ranging from 1 μM to 10 μM (Botelho et al. 1988). Preincubation with Rp-cAMPS at a concentration of 10 μM or 100 μM followed by exposure of cells to 100 μM adenosine for 1 hour did not alter the pattern of adenosine A_2 receptor desensitization. This implies that protein kinase A is not involved in the desensitization of the occupied/activated adenosine receptor either. Taken together, the results of these experiments suggest that the homologous desensitization of adenosine A_2 receptors in LLC-PK_1 cells is not primarily mediated by protein kinase A or protein kinase C.

Unfortunately, we were unable to investigate the possibility that a βARK-like kinase may be involved in the homologous desensitization of adenosine A_2 receptors. Other researchers have used heparin to inhibit βARK in permeabilized cell systems (Hausdorff et al. 1990). When LLC-PK_1 cells were permeabilized the

adenylyl cyclase activity of subsequently isolated membranes could not be stimulated using adenosine receptor agonists. Apparently the permeabilization process interferes with the ability of adenosine A_2 receptors to stimulate adenylyl cyclase in LLC-PK_1 cells. Therefore we were unable to investigate the possibility that a βARK-like kinase may be involved in the homologous desensitization of adenosine A_2 receptors. The development of cell permeant specific inhibitors of βARK-like activity would be of great value in investigating this phenomenon. Although our data cannot confirm the occupational state of contralateral receptors there is, nonetheless, the exciting possibility that homologous desensitization of adenosine A_2 receptors in LLC-PK_1 cells is accompanied by the altered function of unoccupied/non-activated adenosine A_2 receptors on the contralateral side.

VI. Summary and Conclusions

The research presented in this thesis suggests that:

1. Desensitization of adenosine receptors in primary cultures of RCCT cells involves the altered function of adenosine A_1 and A_2 receptors. Additionally, altered function of the regulatory G-protein, G_s, confers a heterologous component to this desensitization.
2. The adenosine receptor mediated pathways were functionally localized to either the apical or basolateral side of the LLC-PK_1 renal cell line. The adenosine receptor mediated pathway that stimulates inositol phosphate turnover was localized to the basolateral aspect of LLC-PK_1 cells. The inhibitory adenosine A_1 receptor pathway was localized to the basolateral aspect of the cell monolayer, although a minor apical component could not be ruled out. The stimulatory adenosine A_2 receptor pathway was found to reside on both sides of the monolayer. However, the major component of the adenosine A_2 receptor pathway was apical with a smaller, albeit significant, component residing on the basolateral side of LLC-PK_1 cells.
3. Desensitization of stimulatory adenosine A_2 receptors on both sides of LLC-PK_1 cells, following selective exposure of adenosine to either the apical or basolateral side of the monolayer, was found to be homologous. This

pattern of homologous desensitization was the same irrespective of the side on which the receptors were exposed to adenosine. This suggested that adenosine A_2 receptors on the contralateral side of adenosine exposure, which were presumably unoccupied/unactivated, could be desensitized independently of other unoccupied/unactivated G-protein linked receptors.

The desensitization of adenosine receptors in primary cultures of RCCT cells did not occur until concentrations of agonist reached levels known to occupy/activate the stimulatory adenosine A_2 receptor. Lower concentrations of agonist which occupy/activate the inhibitory adenosine A_1 receptor did not induce desensitization out to a time course of 4 hours. Neither adenylyl cyclase nor the regulatory G_i-protein appeared to be involved in adenosine receptor desensitization, suggesting that altered function of both adenosine A_1 and A_2 receptors had occurred. Cholera toxin stimulated cAMP production was attenuated following exposure to adenosine agonists. This decreased stimulation by cholera toxin suggested an altered function of the regulatory G_s-protein, and forms the basis for the observed heterologous desensitization of AVP receptors.

The functional localization of adenosine receptors in cultured renal epithelial cells is an important first step in the physical localization of these receptors both in culture and *in vivo*. The functional localization of adenosine receptor mediated pathways in the LLC-PK_1 cell line revealed that the majority of inhibitory

adenosine A_1 receptors were basolateral, while the majority of stimulatory adenosine A_2 receptors were apical. The adenosine receptor mediated pathway responsible for the stimulation of inositol phosphate turnover was basolateral. Additionally, concurrent stimulation of adenosine A_2 receptors on both sides of the monolayer produced cAMP levels that were the numeric sum of either side when stimulated independently. This, along with the presence of adenosine deaminase on the contralateral side in sufficient amounts to give an enzymatic activity 40 fold higher than the predicted flux of adenosine across the monolayer (Appendix A), suggests that adenosine receptors on the contralateral side were not occupied/activated during the course of an experiment.

The above results allowed for the selective exposure of a subset of adenosine A_2 receptors on a monolayer of LLC-PK_1 cells. Following a one hour exposure of adenosine receptors on either the apical or basolateral side to a stimulatory concentration of adenosine, the adenosine A_2 receptors were desensitized. The pattern of desensitization was the same regardless of the side of exposure, with the basolateral adenosine A_2 receptors being completely desensitized and the apical adenosine A_2 receptors desensitized by ~50%. Neither adenosine A_1 receptors nor AVP receptors were desensitized, marking this as a homologous desensitization of adenosine A_2 receptors. Various kinases have been implicated in the altered function of receptors (β-adrenergic receptors for example) following prolonged exposure to agonists. Further investigation revealed that protein kinase A and

protein kinase C were not primarily involved in the homologous desensitization of adenosine A_2 receptors. As outlined in the literature review, a βARK-like kinase may be involved in the homologous desensitization of occupied G-protein linked receptors, however we have no evidence of this in LLC-PK_1 cells. Nevertheless, the exciting possibility exists that homologous desensitization of a subset of adenosine A_2 receptors that are unoccupied/unactivated can occur in the LLC-PK_1 renal cell line. Because neither PKA or PKC are involved, and βARK preferentially phosphorylates occupied receptors, a novel second messenger system may be implicated in the homologous desensitization of unoccupied/unactivated adenosine A_2 receptors.

In conclusion, the findings summarized above indicate that the adenosine receptor systems present in renal epithelial cells have a polar distribution and are regulated by prior exposure history to agonist. The polar distribution of adenosine receptor mediated pathways on a renal epithelium has functional consequences dependent upon the side on which such an epithelium might be presented with adenosine. That is, adenosine on the interstitial side could bring about functional changes that are different than those brought about by adenosine present on the lumenal side of renal tubular epithelium. It should be cautioned, however, that the localization and regulation of these adenosine receptor systems in cell culture might not be directly reflective of those that exist *in vivo*. Nonetheless, the results of these three projects will serve as a solid data base upon which future *in vivo* work

can draw.

Appendix

Appendix A

The velocity of an enzymatic reaction is defined as:

$v = (V_{max} \times [c]) / (K_m + [c])$

V_{max} is the velocity of the enzymatic reaction at infinite substrate concentration.

K_m is the concentration of substrate where $v = V_{max}/2$.

[c] is the concentration of substrate at time t.

The K_m of adenosine deaminase (EC 3.5.4.4) is 35 µM.

The V_{max} of adenosine deaminase is 1 unit = 1 µmole of adenosine deaminated per minute at pH 7.4 at 25°C.

Starting with an adenosine concentration of 100 µM, the predicted concentration of adenosine that may have crossed from the apical (upper) side of the monolayer to the basolateral (lower) side at 30 minutes was 5% (1.88 µM) of the steady state concentration of 37.5 µM.

The concentration of adenosine deaminase used was 1.5 U/ml.

The basolateral volume was 0.5 ml, thus 0.75 units of ADA was present.

v = 0.75 µmole/min x 1.88 µM / 35 µM + 1.88 µM = 0.038 µmole/min.

Amount of adenosine predicted to have crossed at 30 minutes;
1.88 µM x $5x10^{-4}$L = 0.00094 µmole.

Ratio of ADA activity to adenosine flux:
0.038 µmole/min / 0.00094 µmole = 40 min^{-1}.

A similar set of calculations for the ADA activity added to the apical (upper) side of the monolayer versus the predicted flux of adenosine from the basolateral (lower) side gives a ratio of;
0.037 µmole/min / .00093 µmole = 40 min^{-1}.

Therefore there was ~ 40 fold more adenosine deaminase activity present on the contralateral side than adenosine flux to that side.

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VII. Literature Cited

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