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MODULATION OF ADENYLATE CYCLASE ACTIVITY IN CULTURED BOVINE PULMONARY ARTERIAL ENDOTHELIAL CELLS

EFFECTS OF ADENOSINE AND DERIVATIVES

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Abstract—We studied the effects of adenosine and adenosine derivatives on adenylate cyclase activity in cultured endothelial cells from bovine pulmonary artery. Basal and stimulated enzyme activities were measured in membrane preparations using [alpha-^{32}P]ATP as the substrate and chromatographic isolation of formed [^{32}P]cAMP. Basal cyclase activity was 11 ± 1 (mean ± SEM) pmol/mg protein/min. Forskolin, 5′-guanylylimidodiphosphate (Gpp(NH)p) and (−)isoproterenol stimulated adenylate cyclase in a concentration-dependent manner, producing maximal stimulations of three, seven and four times the basal activity respectively. In the presence of adenosine deaminase, cyclohexyladenosine, an A_1 agonist, had no effect on basal and forskolin- or Gpp(NH)p-stimulated activities, whereas 5′-(*N*-ethyl)-carboxamidoadenosine (NECA), an A_2 agonist, had a small stimulatory effect (52% increase over basal). In the presence of IBMX, adenosine and two P-site agonists, 2′,5′-dideoxyadenosine (DDA) and 2′-deoxyadenosine-3′-monophosphate (2′-deoxy-3′-AMP), inhibited forskolin (30 μM)-stimulated adenylate cyclase activity with an order of potency of 2′-deoxy-3′-AMP > DDA > adenosine. DDA and 2′-deoxy-3′-AMP were also able to inhibit cyclase activity stimulated by Gpp(NH)p (10^{-5} M) or isoproterenol (10^{-6} M) with the same order of potency. Only 2′-deoxy-3′-AMP inhibited the stimulated adenylate cyclase activity by more than 50% (IC_{50} = 19–32 μM). These findings indicate that (1) long-term cultured endothelial cells from bovine pulmonary artery express A_2 and beta-adrenergic receptors which stimulate adenylate cyclase activity through G_S transducer proteins, and (2) the natural compound and P-site agonist, 2′-deoxy-3′-AMP, is a potent inhibitor, and possibly a natural regulator, of adenylate cyclase activity in this tissue.

Pulmonary vascular endothelium participates in the control of vascular tone by several mechanisms, including metabolism of vasoactive peptides and/or their precursors by ecto-enzymes [1], uptake of drugs and endogenous compounds [2], and release of vasoactive substances, such as prostaglandins [3] and EDRF‡ [4], as well as interactions with other cell types such as platelets [5] and leucocytes [6]. While several vasoactive agents induce endothelial responses by activating a variety of cellular receptors, including alpha$_2$-adrenergic, histaminergic, and serotonergic receptors [7–11], the intracellular signals governing these actions are poorly understood.

Adenosine exerts several effects in the circulation including vasodilation in systemic [12] and pulmonary vessels [13] and inhibition of platelet aggregation [14]. While endothelial cells are a prominent target for the initial actions of adenosine [15–17], the intracellular transduction of such effects remains unclear. There is little information on the nature of endothelial adenosine receptors, and conflicting evidence has been reported on the effects of adenosine on adenylate cyclase activity in endothelium and vessels from different species and tissues: for example, adenosine was found to increase cAMP in intact cultured endothelial cells [18] and microvessels [19, 20], but also reported to inhibit adenylate cyclase in disrupted cultured endothelial cells [21]. Modulation of cAMP levels by adenosine could result in diverse effects such as alteration of adherence of poly-morphonuclear leucocytes to endothelium [22], modification of endothelial permeability to macromolecules [23], or modification of PGI_2 synthesis [19, 24].

In certain cells (such as adipocytes), adenosine binds to high affinity A_1 receptors producing a decrease in adenylate cyclase activity, whereas in other cell types (such as hepatocytes) adenosine

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‡ Abbreviations: EDRF, endothelium derived relaxing factor; DDA, 2′,5′-dideoxyadenosine; 2′-deoxy-3′-AMP, 2′-deoxyadenosine-3′-monophosphate; CHA, cyclohexyladenosine; NECA, 5′-(*N*-ethyl)-carboxamidoadenosine; TED buffer, 0.06 M Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5; IBMX, 3-isobutyl-1-methylxanthine; Gpp(NH)p, 5′-guanylylimidodiphosphate; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

binds to low affinity A_2 receptors producing an increase in enzyme activity [25–31]. These effects are mediated by interactions of the adenosine receptor with the GTP-dependent transducer proteins G_I and G_S [28, 29, 31, 32]. Additionally, adenosine exerts a direct inhibitory effect on the catalytic subunit, acting at an intracellular locus known as the "P site" [31, 33–35]. Relatively selective agonists for the A_1 and A_2 receptors are known [25, 36]; methylxanthines, such as 3-isobutyl-1-methylxanthine (IBMX), are non-specific A_1 and A_2 inhibitors [25], but they have no action at the P site [37]. On the other hand, several analogs of adenosine modified on the ribose moiety possess P-site activity [27, 34, 35].

The aim of this study was to characterize the adenylate cyclase system in cultured pulmonary arterial endothelial cells and to study the effects of adenosine on this system. We examined cyclase activity in membranes isolated from cultured pulmonary arterial endothelial cells. We first established that these long-term cultured cells maintained the cyclase stimulatory system, including catalytic subunit, guanine nucleotide stimulation, and coupling to beta-adrenergic receptors. We then studied the effects of (1) adenosine, (2) two P-site agonists, 2′,5′-dideoxyadenosine (DDA) and 2′-deoxyadenosine-3′-monophosphate (2′-deoxy-3′-AMP), (3) an A_1 receptor selective agonist, cyclohexyladenosine (CHA), and (4) an A_2 receptor agonist, 5′-(*N*-ethyl)-carboxamidoadenosine (NECA), on the enzyme system. Adenosine analogs were found to either stimulate or inhibit adenylate cyclase depending on their relative actions at the P site and A_2 receptor.

MATERIALS AND METHODS

Materials. DDA was a gift from the National Cancer Institute. Forskolin was purchased from Calbiochem (LaJolla, CA), cationic exchange resin AG 1 × 4 from BioRad (Rockville Centre, NY), and [alpha-^{32}P]ATP from ICN (Irvine, CA). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). M199 culture medium penicillin and streptomycin were purchased from Hazleton Research Products Inc. (Denver, PA); fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT), gentamycin from ESI Pharmaceuticals (Cherry Hill, NJ), and fungizone from Flow Laboratories (McLean, VA).

Cell culture and membrane preparation. Bovine pulmonary arterial endothelial cells were harvested mechanically and identified as previously described [38]. Cells were grown in M199 medium supplemented with fetal bovine serum (10–20%) and antibiotics (fungizone, 500 μg/liter; gentamycin, 40 mg/liter; penicillin, 10,000 units/liter; streptomycin, 10 mg/liter). The cultures were maintained at 37° with 5% CO_2 in air. Medium was changed every 3–4 days, and cells reached confluence 6–10 days after seeding. The cells were subcultured (1:2) for up to 50 times at or after confluence using a rubber policeman to harvest the cells. The cells were never exposed to proteolytic enzymes.

Unless indicated otherwise, experiments were performed 1–8 days after confluence. Cells were scraped mechanically in M199 medium and washed twice in cold TED buffer (0.06 M Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5). After centrifugation at 1000 *g* for 10 min, the pellet was homogenized with a Teflon/glass tissue grinder and centrifuged at 33,000 *g* for 25 min at 4°. The pellet was suspended in TED buffer and used without further treatment.

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Salomon *et al.* [39] with minor modifications. Each assay tube contained the following reagents in a final volume of 250 μl: 60 mM Tris–HCl, pH 7.5, 1 mM EGTA, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM ATP, 20 mM creatine phosphate, 10 units creatine phosphokinase, 0.5 to 1 μCi [alpha-^{32}P]ATP, 1 mM IBMX (or 1 mM cAMP), various concentrations of GTP and Mg^{2+}, and 50–150 μg membrane protein. The mixture was incubated at 37° for 10 min, and the reaction was stopped by immersion in a boiling water bath for 1 min. Centrifugation for 20 min at 4000 *g* provided the supernatant fraction for [^{32}P]cAMP isolation, which was carried out according to the method of Mao and Guidotti [40]. An aliquot of the supernatant fraction was loaded onto a neutral alumina column equilibrated with 60 mM Tris–HCl, pH 7.5, and eluted with 5 ml of the equilibration buffer. The eluate was loaded onto a Bio-Rad AG 1X4 formate column equilibrated with water. The column was washed twice with water and the bound cAMP eluted with 5 ml of 1 N formic acid. [^{32}P]cAMP was quantified using a scintillation spectrometer. Eighty percent of applied [^{3}H]cAMP was routinely recovered from the column. Protein was measured by the method of Lowry *et al.* [41].

Calculations and statistics. All experiments were performed in quadruplicate. The data are presented as the mean ± SEM of the indicated number of observations. Significance of concentration-related effects was assessed by correlation between log concentration and enzyme activity. The significance of differences between several concentration–effect curves or of individual concentration effects was assessed by analysis of variance (ANOVA). Order of potency was assessed by comparison of the slopes of the lines obtained from linear regression of log-concentration versus effects.

The effect of time after confluence on the basal activity was tested by ANOVA.

RESULTS

Basal adenylate cyclase activity in the crude membrane preparation from cultured endothelial cells varied from 1 to 20 pmol of cAMP/min/mg protein with a mean (± SEM) of 11 ± 1 (132 observations) for the entire series of experiments. Basal enzyme activity was independent of the time after confluence was reached (Table 1).

Inclusion of forskolin during the 10-min incubation period caused a concentration-dependent increase in cyclase activity in the membrane ($r = 0.74$, $P < 0.01$; Fig. 1). This increase was significant with as little as 1 μM forskolin ($P < 0.05$); maximal stimulation at 100 μM forskolin was three times basal activity.

The stable GTP analog 5′-guanylylimidodiphosphate (Gpp(NH)p) increased enzyme activity in a

Table 1. Influence of time after confluence on adenylate cyclase activity in pulmonary arterial endothelial cells

Days after confluence	Adenylate cyclase activity (pmol/min/mg protein)	N
1	15.92 ± 3.29	7
2	10.20 ± 1.75	12
3	15.39 ± 0.286	8
4	10.36 ± 1.90	8
5	15.48 ± 3.76	8
6	19.34 ± 0.86	4
8	18.35 ± 2.70	16
15	9.65 ± 2.60	7

Each value is the mean ± SEM for N observations. (One-way ANOVA: $F_{7,62} = 1.95$, $P = 0.075$.)

concentration-dependent manner ($r = 0.82$, $P < 0.01$), and the maximal stimulation at 100 μM Gpp(NH)p was nearly seven times the basal activity. At concentrations of 10^{-5} M or greater, Gpp(NH)p was a more potent stimulator of adenylate cyclase activity than forskolin (Fig. 2).

The beta-adrenergic receptor agonist (−)isoproterenol also increased cyclase activity (Fig. 3). Maximal stimulation was observed with 100 μM (−)isoproterenol and was four times the basal activity. This increase was also concentration dependent ($r = 0.70$, $P < 0.01$).

In the presence of the phosphodiesterase inhibitor and nonspecific (A_1 and A_2) adenosine receptor antagonist IBMX, adenosine and two P-site agonists, DDA and 2′-deoxy-3′-AMP, decreased forskolin (30 μM)-stimulated cyclase activity (Fig. 4). Statistical analysis by two-way ANOVA indicated that the three substances had significantly different effects on the enzyme. The order of potency for enzyme inhibition was 2′-deoxy-3′-AMP > DDA > adenosine. Only 2′-deoxy-3′-AMP reduced enzyme activity to pre-forskolin, basal levels. The IC_{50} for 2′-deoxy-3′-AMP was 52 μM.

Similarly, in the presence of IBMX, both 2′-deoxy-3′-AMP and DDA reduced Gpp(NH)p (10 μM)-stimulated adenylate cyclase activity (Fig. 5). As

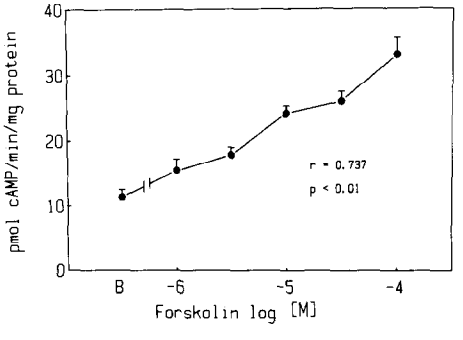

Fig. 1. Forskolin stimulation of adenylate cyclase activity in membranes from cultured bovine pulmonary arterial endothelial cells. Reaction medium contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SEM (N = 12). B = basal activity in the absence of forskolin; r = correlation coefficient; and P = significance of r.

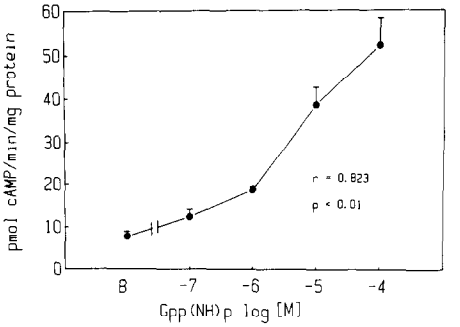

Fig. 2. Effect of Gpp(NH)p on adenylate cyclase activity in membranes from cultured bovine pulmonary arterial endothelial cells. Reaction medium contained 1 mM IBMX and 5 mM Mg^{2+}. Values are means ± SEM (N = 8). B = basal activity; r = correlation coefficient; and P = significance of r.

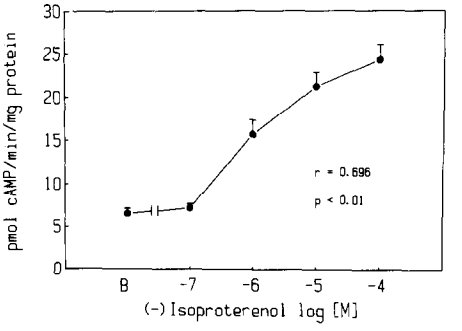

Fig. 3. Effect of (−)isoproterenol on adenylate cyclase activity in membranes from cultured bovine pulmonary arterial endothelial cells. Reaction medium contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SEM (N = 12). B = basal activity; r = correlation coefficient; and P = significance of r.

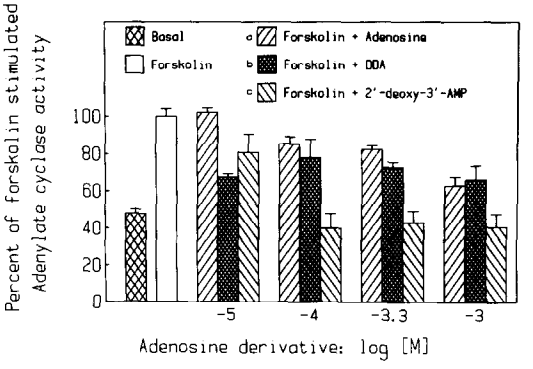

Fig. 4. Effects of adenosine, 2′,5′-dideoxyadenosine (DDA) and 2′-deoxyadenosine-3′-monophosphate (2′-deoxy-3′-AMP) on adenylate cyclase activity in membranes from cultured bovine pulmonary arterial endothelial cells stimulated with 30 μM forskolin. Activity is expressed as percent of adenylate cyclase activity stimulated by 30 μM forskolin. Reaction medium contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± 1 SEM (N = 4). Comparison between groups: a ≠ b, $P < 0.02$; a and b ≠ c, $P < 0.001$ by two-way ANOVA. Concentration effect within concentration group estimated by linear regression: (a) $r = -0.71$, $P < 0.001$; slope = −6.46. (b) $r = -0.75$, $P < 0.001$; slope = −8.20. (c) $r = -0.83$, $P < 0.001$; slope = −16.52.

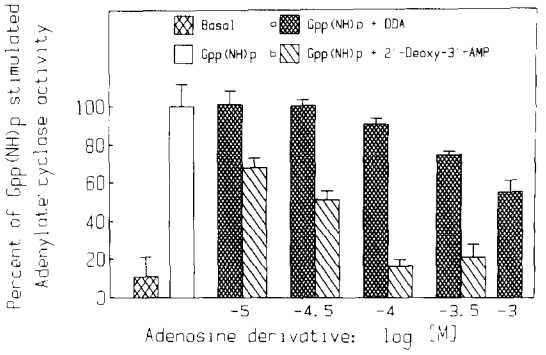

Fig. 5. Effects of DDA and 2′-deoxy-3′-AMP on adenylate cyclase activity in membranes from cultured pulmonary arterial endothelial cells stimulated by 10 μM Gpp(NH)p. Reaction medium contained 1 mM IBMX and 5 mM Mg^{2+}. Values are means ± SEM (N = 8). Comparison between groups: a ≠ b, $P < 0.001$ by two-way ANOVA. Concentration effect within each group estimated by linear regression: (a) $r = -0.32$, $P < 0.05$; slope = −10.16. (b) $r = -0.89$, $P < 0.001$; slope = −23.59.

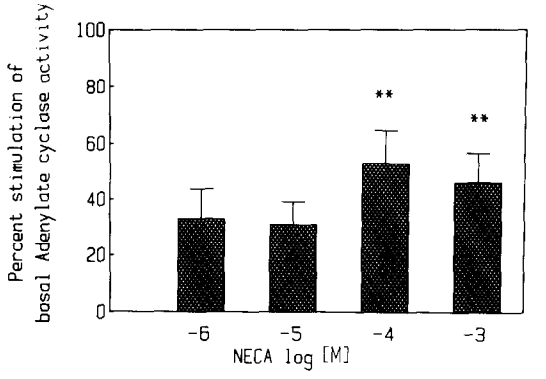

Fig. 7. Effect of 5′-(*N*-ethyl)-carboxamidoadenosine (NECA) on adenylate cyclase activity in membranes from cultured bovine pulmonary arterial endothelial cells. Reaction mixture contained 1 mM cAMP, 2 μM GTP, 5 mM Mg^{2+} and 10 I.U./ml adenosine deaminase. Values are means ± SEM (N = 12). Key: (**) different from basal activity level ($P < 0.01$) by one-way ANOVA and Dunnett's *t*-test.

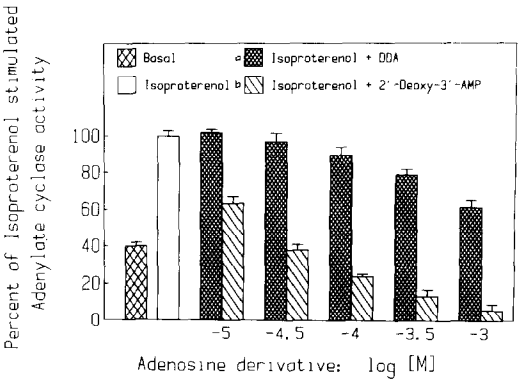

Fig. 6. Effects of DDA and 2′-deoxy-3′-AMP on adenylate cyclase activity in membranes from cultured pulmonary arterial endothelial cells stimulated by 10 μM (−)isoproterenol. Reaction mixture contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SEM (N = 4). Comparison between groups: a ≠ b, $P < 0.001$ by two-way ANOVA. Concentration effect within each group estimated by linear regression: (a) $r = -0.40$, $P < 0.02$; slope = −8.53. (b) $r = 0.97$, $P < 0.001$; slope = −24.7.

with forskolin, 2′-deoxy-3′-AMP was a more potent inhibitor of Gpp(NH)p-stimulated adenylate cyclase activity than DDA; the IC_{50} for 2′-deoxy-3′-AMP was 32 μM.

In the presence of IBMX, both 2′-deoxy-3′-AMP and DDA inhibited (−)isoproterenol (10 μM)-stimulated adenylate cyclase activity (Fig. 6). Again, 2′-deoxy-3′-AMP was the more potent inhibitor, with an IC_{50} of 19 μM.

To determine the effects of A_1 and A_2 agonists on cyclase activity, large amounts of cAMP were included in the assay medium in lieu of IBMX, to minimize the effect of degradation of [^{32}P]cAMP by phosphodiesterase (IBMX is a nonspecific adenosine receptor antagonist). Adenosine deaminase was included in these assays to prevent accumulation of adenosine. In the presence of cAMP (1 mM), GTP (2 μM), adenosine deaminase (10 I.U./ml) and Mg^{2+} (5 mM), cyclohexyladenosine an A_1 selective

Table 2. Effect of cyclohexyladenosine (CHA) on unstimulated and on forskolin- and Gpp(NH)p-stimulated adenylate cyclase activity in membranes from cultured pulmonary arterial endothelial cells

	cAMP (pmol/min/mg protein)					
CHA (M)	Control	N	Forskolin (30 μM)	N	Gpp(NH)p (10 μM)	N
0	13.50 ± 1.29	4	21.43 ± 0.626	8	56.69 ± 3.06	8
10^{-8}	15.65 ± 2.03	4	20.62 ± 1.68	4	62.50 ± 2.40	4
10^{-7}	15.38 ± 2.30	4	25.35 ± 1.84	4	56.03 ± 1.94	4
10^{-6}	12.10 ± 5.05	4	22.21 ± 1.72	4	55.12 ± 2.44	4
10^{-5}	14.47 ± 1.87	4	23.78 ± 1.39	4	54.03 ± 2.77	4
10^{-4}	—		21.35 ± 0.30	4	—	
5×10^{-4}	—		20.88 ± 0.74	4	—	
10^{-3}	—		20.02 ± 0.99	4	—	
ANOVA:	$F_{4,15} = 0.89$ $P = 0.49$		$F_{7,28} = 2.02$ $P = 0.088$		$F_{4,19} = 1.09$ $P = 0.39$	

Reaction medium contained 1 mM cAMP, 2 μM GTP, 5 mM Mg^{2+} and 10 I.U./ml adenosine deaminase. Values are means ± SEM of four observations.

agonist, did not affect basal, forskolin- or Gpp(NH)p-stimulated adenylate cyclase activity in endothelial cell membranes (Table 2). In contrast, under the same conditions, 5′-(*N*-ethyl)-carboxamidoadenosine, an A_2-agonist, produced a small but consistent stimulation of basal cyclase activity (Fig. 7). Analysis of variance followed by Dunnett's *t*-test indicated that, at 0.1 and 1.0 mM, NECA increased enzyme activity ($P < 0.01$). The maximum increase (52%) was found with 0.1 mM NECA.

DISCUSSION

Endothelial cells possess transmitter and hormonal receptors for a variety of agents, and they play an integral role in vascular smooth muscle activity. The biochemical mechanisms subserving these effects are incompletely understood. The aim of this study was to characterize the adenylate cyclase system in membranes from cultured bovine pulmonary artery endothelial cells and to identify the effects of adenosine and adenosine derivatives on this system. We confirmed the presence of beta-adrenergic receptors coupled to adenylate cyclase through G_S transducer protein, and we found that, depending on their site of action, adenosine analogs either stimulate or inhibit cyclase activity. Furthermore, a naturally occurring nucleotide, 2′-deoxy-3′-AMP, had a strong inhibitory effect on adenylate cyclase activity in these cells.

Beta-adrenergic receptor and adenosine receptor effects: Stimulation of adenylate cyclase activity. Previous reports have demonstrated that adrenergic agonists increase cAMP levels in cultured endothelial cells from bovine pulmonary artery [42, 43]. Strong evidence for the presence of beta-adrenergic receptors in endothelial cells from other tissues and species emerges from (1) the increase of cAMP production induced by beta-adrenergic agonists in intact cells or membranes isolated from human umbilical cells [24, 44], bovine [44] and rabbit [45] aortic endothelial cells, and in microvessel preparations from tissues where endothelial cells predominate [46], and (2) the specific binding of beta-adrenergic radioligands to bovine aortic endothelial cells [10]. Our results confirm the presence of a beta-adrenoceptor coupled to adenylate cyclase in chronically cultured pulmonary artery endothelial cells, as assessed by the concentration-dependent stimulatory effect of isoproterenol. The degree of stimulation of cAMP accumulation by isoproterenol in the present experiments (5-fold) was similar to that reported recently by Grigorian and Ryan [43] in the same cells, even though basal activity was considerably higher in the present study. Two methodological differences which may account for this are (1) our inclusion of 1 mM DTT in all tissue preparation medium and (2) the use of a much higher concentration of GTP by Grigorian and Ryan in their incubation medium (1 mM vs 2 μM). Adenylate cyclase activity is depressed by high concentrations of guanine nucleotides in a variety of cells. Nevertheless, our results are qualitatively similar, and isoproterenol stimulated adenylate cyclase over similar concentration ranges.

Our results also provide evidence for the presence of A_2 adenosine receptors on endothelial cells from bovine pulmonary artery: NECA, one of the most potent A_2 agonists [36], stimulated adenylate cyclase, whereas CHA, the most potent and selective A_1 agonist [36], had no effect on cyclase activity, suggesting the absence of either (1) A_1 receptors or (2) G_I proteins (or their functional dissociation from the receptors). The most effective concentration of NECA was 100 μM, which is consistent with its action on a low affinity A_2 receptor. The stimulatory effect of NECA on adenylate cyclase was apparent only in the presence of adenosine deaminase, which metabolizes the adenosine produced by degradation of the substrate or of the cAMP produced or added. Adenosine deaminase has been used for this purpose by several investigators [20, 29, 37] since many adenosine analogs are not substrates for this enzyme [36]. Goldman *et al.* [18] have found that adenosine induces an increase in cAMP in intact cultured endothelial cells from swine aorta, which is consistent with the presence of A_2 receptors on these cells. Adenosine and its analogs, such as NECA and 2-chloroadenosine, also increase cAMP in membranes and whole cells from endothelium-rich preparations, such as microvessels from rabbit [19], guinea pig [20] and rat [47] brain.

The magnitude of the stimulation of adenylate cyclase by NECA was modest compared to the effect of isoproterenol. The weaker effect of A_2 receptor stimulation compared to beta-receptor stimulation could reflect a lower density or intrinsic activity of the A_2 receptor, and raises the question of whether long-term cultured cells maintain the characteristics of *in vivo* cells. However, the NECA effect in our preparation is comparable to results obtained with noncultured cells from cerebral capillaries [47]. The present work indicates that long-term culture does not cause a deletion of either beta-adrenergic or A_2 adenosine receptors. The mechanical technique of passage and avoidance of proteolytic enzymes may have contributed to the preservation of surface receptors during long-term culture.

Physiological roles have been described for several of the receptors which have been identified on endothelial cells. Histamine, for example, induces an endothelium-dependent vasodilation by triggering the secretion of EDRF and prostacyclin by endothelial cells [4, 8]. Similarly, A_2 and beta-adrenergic receptors are involved in an endothelium-dependent vasodilatory response in dog coronary arteries [10]. This involvement has not been observed in other dog systemic arteries [48, 49], but has been reported in rat femoral artery [16]. Thus, in addition to its aforementioned diverse cellular effects, endothelial adenylate cyclase may play a role in certain endothelium-dependent vascular relaxing responses to adenosine.

P-site effects: Inhibition of adenylate cyclase activity. The adenylate cyclase system possesses several sites of action for adenosine derivatives (Fig. 8). A_1 and A_2 receptors face the exterior of the cell, and, like many other surface receptors, modulate adenylate cyclase activity through interactions with guanine nucleotide-dependent proteins (G proteins) [32]. While the control of adenylate cyclase by extracellular messengers has received much attention, enzyme activity can also be modulated by adenosine derivatives acting in the interior of the cell. Sahyoun

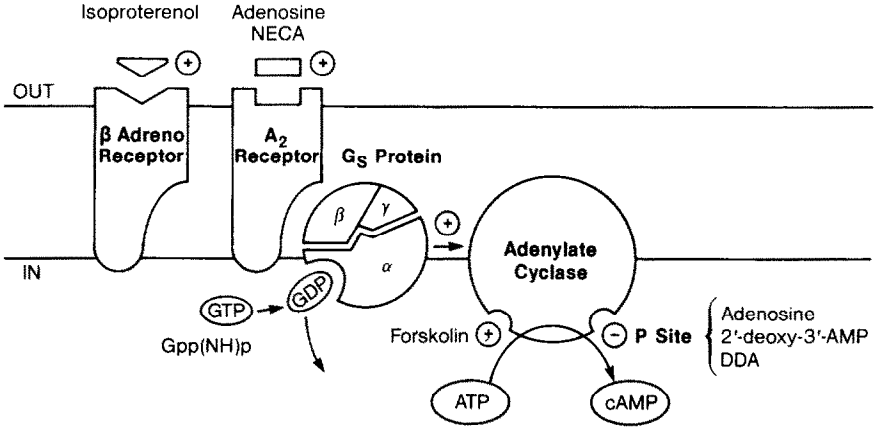

Fig. 8. Summary of drug interactions with the A_2, receptor-G_S-adenylate cyclase system. Binding of an A_2 agonist promotes the association of receptor with G_S which, in turn, promotes a GTP/GDP exchange on the alpha subunit of G_S. GTP binding occasions an uncoupling of the receptor–G_S complex and a dissociation of G_S subunits, one of which (G_S-alpha$_{GTP}$) interacts with adenylate cyclase, thereby stimulating activity. Subsequent to GTP hydrolysis, the G protein subunits reassociate and are free to interact with the receptor. (Variations on this sequence of events are possible; see Ref. 32). Four sites of action of the drugs used in this study are indicated: (1) the agonist binding site of the A_2 receptor (adenosine, NECA), (2) the guanine nucleotide binding site on the alpha subunit of G_S (Gpp(NH)p), (3) the forskolin binding site on adenylate cyclase, and (4) the P site on adenylate cyclase (DDA, 2'-deoxy-3'-AMP, adenosine). P-site agonists inhibit cyclase activity.

et al. [50, 51] identified 2'-deoxy-3'-AMP as a natural inhibitor of adenylate cyclase in membranes of toad erythrocytes and rat spleen. The adenosine moiety is required for this inhibitory effect. Several biosynthetic pathways of this compound have been proposed, including DNA breakdown, transfer of a phosphate from the 5'-position of a nucleotide to the 3'-position on an acceptor nucleoside, and degradation of cyclic deoxyadenosine-3',5'-monophosphate [50, 51]. Londos and Wolff [33] subsequently described the inhibitory effect of adenosine and several of its derivatives on adenylate cyclase in membranes from a number of different cell types. Since this activity did not tolerate any modification of the purine moiety of adenosine, the site at which these derivatives act was termed the P site [33]. P-site agonists also act on solubilized adenylate cyclase, confirming the presence of the P site on the catalytic subunit of the enzyme [52].

Our results demonstrate that, in the presence of IBMX, an inhibitor of A_1 and A_2 receptors [25], the P-site agonists DDA and 2'-deoxy-3'-AMP inhibited adenylate cyclase. DDA had a weak inhibitory effect on enzyme activity stimulated by forskolin, Gpp(NH)p or isoproterenol. This is in agreement with the modest inhibitory effect of DDA on Gpp(NH)p-stimulated cyclase activity in Leydig tumor cell membranes in the absence of Mn^{2+} [53].

2'-Deoxy-3'-AMP is clearly a much more potent inhibitor of adenylate cyclase than DDA in membrane preparations (i.e. in preparations where 2'-deoxy-3'-AMP is freely accessible to the P site) [54]. The natural presence of 2'-deoxy-3'-AMP inside different cell types [50, 51] raises the question of the physiological role of the P site and of its possible natural agonist(s). While adenosine is the native agonist for A_1 and A_2 receptors, its role at the P site is uncertain in light of its weak P-site activity. Even if it is actively transported into the cells, it is unlikely to reach an effective concentration inside the cells since it is metabolized rapidly by several pathways [36]. Compounds such as DDA, on the other hand, are powerful P-site agonists under certain conditions (notably, at $[Mn^{2+}] = 0.1$ to 1 mM). DDA is able to cross cell membranes, but has not been identified as a natural compound. Our findings are consistent with the suggestion of Sahyoun *et al.* [50, 51] that 2'-deoxy-3'-AMP is the natural P-site modulator of adenylate cyclase.

The physiological effect of P-site stimulation in the endothelium is unclear. Collis and Brown [55] demonstrated that beta-*d*-xylofuranosyladenosine, a P-site agonist, inhibits contraction of guinea pig aorta with intact endothelium. This inhibition is reversed by dipyridamole, a purine uptake inhibitor, but not by 8-phenyltheophylline, an A_1 and A_2 [36] receptor antagonist, suggesting a P-site target for this action. Thus, a decrease in intracellular endothelial cAMP could, at least partly, account for the aforementioned vascular property of beta-*d*-xylofuranosyladenosine and perhaps other P-site agonists which can enter the cell. It is conceivable that endothelium-dependent relaxation of pig aorta induced by high doses of adenosine [7] may be related to a P-site effect.

In summary, long-term cultured endothelial cells from bovine pulmonary artery express adenylate cyclase which was modulated by adenosine A_2 receptors and P-site agonists. Either stimulation or inhibition of endothelial adenylate cyclase by A_2 or P-site agonist, respectively, can be involved in important homeostatic processes. It is conceivable that the naturally occurring nucleotide, 2'-deoxy-3'-AMP, a particularly effective P-site agonist, may be a natural regulator of adenylate cyclase activity *in vivo*.

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