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EFFECTS OF ADENOSINE AND ANALOGS ON ADENYLATE CYCLASE ACTIVITY IN CULTURED BOVINE AORTIC ENDOTHELIAL CELLS

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Abstract—We studied the effects of adenosine and analogs on adenylate cyclase (AC) activity in membranes from long-term cultured bovine aortic endothelial cells, using [α-^{32}]ATP as substrate and chromatographic separation of [^{32}P]cAMP. Compared to our previous findings in cultured bovine pulmonary arterial endothelial cells (Legrand *et al.*, *Biochem Pharmacol* **38**: 423–430, 1989), the present results were qualitatively and quantitatively comparable between the two cell types. In aortic cells, AC activity was stimulated in a concentration-dependent manner by isoproterenol, forskolin and 5′-guanylylimidodiphosphate (Gpp(NH)p), by 2.6-, 5.2- and 4.8-fold respectively. The A_2 adenosine agonist 5′-(*N*-ethyl)-carboxamidoadenosine induced a smaller (60%) increase of AC activity. Adenosine (10^{-3} M) partially inhibited (30%) the Gpp(NH)p-stimulated AC activity. Similarly, adenosine partially reversed, but 2′,5′-dideoxyadenosine (DDA) totally blocked (IC_{50}: 540 μM), the forskolin-induced stimulation of AC activity. DDA and 2′-deoxyadenosine-3′-monophosphate (2′-deoxy-3′-AMP) also inhibited the isoproterenol-induced stimulation of AC activity (IC_{50}: 350 and 23 μM respectively). Adenosine-induced inhibition of stimulated AC activity does not appear to involve adenosine A_1 receptors since the specific A_1 agonist cyclohexyladenosine did not reverse forskolin stimulation of AC activity. Instead, it suggests a direct action of adenosine on the catalytic subunit of the adenylate cyclase (P site). We conclude that membranes from long-term cultured bovine aortic endothelial cells, express β-adrenergic and adenosine A_2 receptors coupled to adenylate cyclase activation. The two P site agonists, DDA and 2′-deoxy-3′-AMP, and, with a weaker effect, adenosine itself, inhibited the activated cyclase at the P site. The natural nucleotide 2′-deoxy-3′-AMP was a strong inhibitor in aortic cell types (as in pulmonary arterial endothelial cells) and may possibly act as a modulator of adenylate cyclase in these cells.

Vascular endothelium is involved in the uptake, metabolism, and release of vasoactive substances [1–4], controls microvascular permeability [5], interacts with blood cells [6, 7], and plays an integral role in cardiovascular homeostasis [8]. These functions are initiated through activation of membrane receptors for a variety of agonists [9–13]. The cellular mechanisms of related signal transduction, however, remain largely unknown.

Adenosine is a potent vasodilator which acts directly on smooth muscle cells [14, 15]; its vascular effects, however, could be partially endothelium dependent [16–18]. Adenosine is the natural agonist for two types of receptors (A_1 and A_2), which inhibit and stimulate adenylate cyclase through G_i and G_s proteins respectively [19–24]. Adenosine also inhibits the catalytic subunit of adenylate cyclase in a variety of cells through direct interaction with a "P site" [25–29].

In a previous study, we demonstrated a dual effect of adenosine on adenylate cyclase in membranes from cultured bovine pulmonary arterial endothelial cells: a stimulatory effect by the A_2 agonist 5′-(*N*-ethyl)-carboxamidoadenosine (NECA‡) and an inhibitory effect by P site agonists [30]. There is increasing evidence that endothelium from different vessels exhibits different functional properties [3, 31] and that adenosine exerts complex vascular effects, including size-dependent relaxation of systemic vessels [32] and even contraction of pulmonary arterial segments [33] via mechanisms not clearly understood. Accordingly, we have characterized the effects of adenosine analogs on adenylate cyclase function in membranes from cultured bovine *aortic* endothelium (BAE) and compared them to our previous findings in bovine *pulmonary* arterial cells [30].

We found that basal activity of adenylate cyclase was slightly lower in bovine aortic endothelial cells than in bovine pulmonary arterial endothelial cells. Modulators of adenylate cyclase, however, produced qualitatively similar effects on bovine pulmonary arterial and aortic endothelial cells: adenylate cyclase was stimulated to comparable degrees by isoproterenol, 5′-guanylylimidodiphosphate (Gpp(NH)p), forskolin and the A_2 agonist NECA.

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‡ Abbreviations: AC, adenylate cyclase; BAE, bovine aortic endothelium; 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(aminoethylether)-tetra-acetate.

Furthermore, stimulated adenylate cyclase activity was inhibited to a comparable extent by adenosine and two P site agonists.

MATERIALS AND METHODS

Cell culture and membrane preparation. Bovine aortic endothelial cells were harvested mechanically and identified as previously described [34]. Cells were grown in M199 medium supplemented with fetal bovine serum [10–20%] and antibiotics (fungizone, 500 μg/L; gentamicin, 40 mg/L; penicillin, 10,000 units/L; streptomycin, 10 mg/L). Cultures were maintained at 37° with 5% CO_2 in air. The medium was changed every 3–4 days, and cells reached confluence 6–10 days after seeding. The cells were subcultured (1:2) at or after confluence, for up to 50 times, using a rubber policeman to harvest the cells. The cells were never exposed to proteolytic enzymes.

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.* [35] 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 [α-^{32}P]ATP; 1 mM IBMX (or 1 mM cAMP); 0 or 2 μM GTP; 5 mM 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 [36]. 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 1 × 4 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.* [37].

Calculations and statistics. All experiments were performed in quadruplicate. The data are presented as the means ± SE of the indicated number of observations. The significance of concentration-related effects was assessed by correlation between log-agonist concentration and enzyme activity. The significance of differences between several concentration–effect curves or individual concentration effects was assessed by the analysis of variance (ANOVA).

RESULTS

Basal and stimulated adenylate cyclase activity. Basal adenylate cyclase activity in the crude membrane preparation from cultured BAE varied from 0.5 to 19 pmol cAMP/min/mg protein with a mean of 8.3 ± 0.44 (N = 138). The presence of isoproterenol in the medium during the 10-min incubation induced a concentration-dependent stimulation of adenylate cyclase activity ($P < 0.001$); maximal stimulation (2.6-fold) was obtained with 10 μM isoproterenol (Fig. 1). Forskolin stimulated adenylate cyclase more strongly than isoproterenol, and maximal stimulation (25.8 pmol cAMP/min/mg protein) was approached at 100 μM forskolin and was five times the basal activity (Fig. 1).

A stimulation comparable to that produced by forskolin was also observed when the stable GTP analog, 5′-guanylylimidodiphosphate (Gpp(NH)p), was included in the medium during the incubation. This stimulation was concentration dependent ($P < 0.001$) and reached levels greater than 5-fold basal activity at 100 μM Gpp(NH)p (Fig. 2).

Effects of adenosine derivatives on adenylate cyclase activity. The effects of adenosine (1 mM) on Gpp(NH)p-stimulated adenylate cyclase activity are shown in Fig. 2. Enzyme activity was dependent on the concentration of Gpp(NH)p ($P < 0.001$); adenosine inhibited adenylate cyclase activity ($P < 0.001$) by 31% at the highest GPP(NH)p dose. The effects of adenosine and the P site agonist 2′,5′-dideoxyadenosine (DDA) on the forskolin (30 μM)-stimulated adenylate cyclase activity are shown in Fig. 3. Both substances inhibited enzyme activity only at concentrations in excess of 100 μM. At 0.5×10^{-3} M, DDA was more potent than adenosine ($P < 0.02$) and was able to bring adenylate cyclase activity back to near basal levels.

The specific A_1 adenosine agonist cyclohexyladenosine (CHA), when incubated without IBMX and with 1 mM cAMP and 10 I.U./mL adenosine deaminase, did not inhibit forskolin-stimulated adenylate cyclase activity. In the absence of CHA, forskolin (30 μM) stimulated adenylate cyclase activity from 9.3 ± 0.8 to 19.3 ± 1.1 pmol cAMP/min/mg protein. These stimulated levels were not affected by 10^{-4} M or 10^{-3} M CHA (20.4 ± 1.0 and 20.1 ± 1.7 pmol cAMP/min/mg protein respectively).

When adenylate cyclase activity was stimulated with isoproterenol (10 μM), DDA and the other P site agonist 2′-deoxy-3′-AMP were similarly able to inhibit enzyme activity (Fig. 4). 2′-Deoxy-3′-AMP was a more potent inhibitor than DDA ($P < 0.001$). The threshold inhibitory concentration of 2′-deoxy-3′-AMP was 10 μM and the IC_{50} was 23 μM, whereas for DDA these values were 100 and 350 μM respectively. At a concentration of 5×10^{-4} M and higher, 2′-deoxy-3′-AMP decreased enzyme activity below basal levels.

When NECA, a potent A_2 adenosine receptor activator, was added to the incubation medium, without IBMX and with 1 mM cAMP and 10 I.U./mL adenosine deaminase, adenylate cyclase activity was stimulated; a maximal effect of 60% stimulation was induced by 100 μM NECA (Fig. 5).

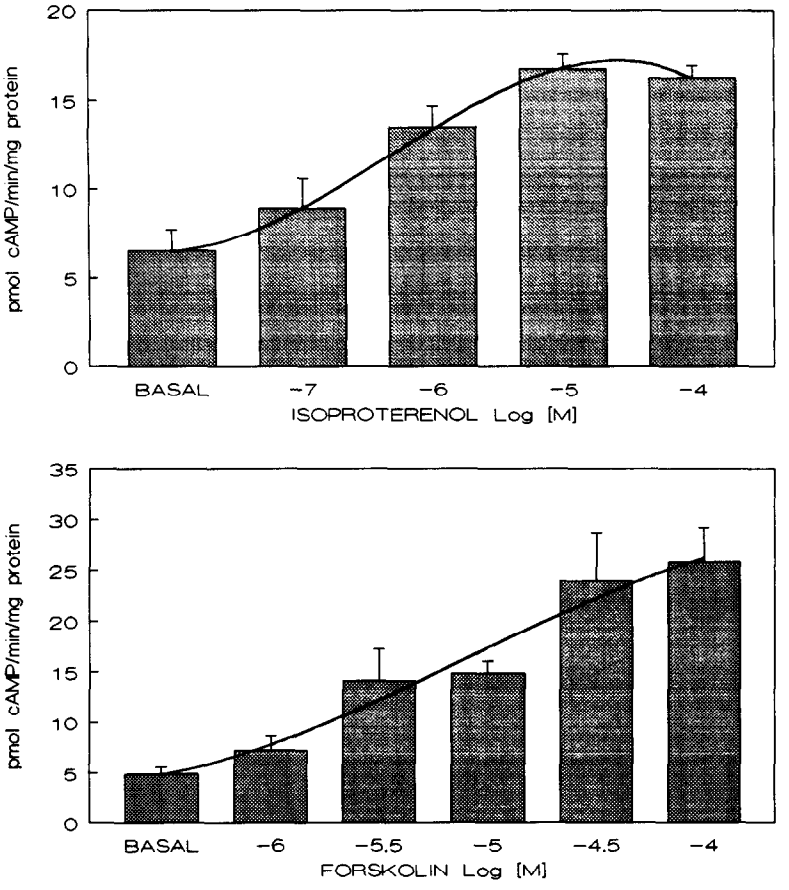

Fig. 1. Effects of isoproterenol (upper panel, N = 12) or forskolin (lower panel, N = 8) on adenylate cyclase activity in membranes from bovine aortic endothelial cells. Reaction media contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SE. $P < 0.01$ from basal levels in each curve with one-way ANOVA.

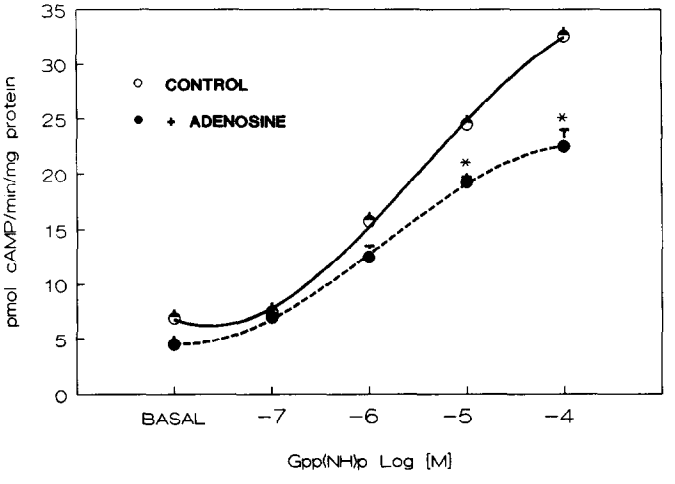

Fig. 2. Effects of Gpp(NH)p in the presence or absence of adenosine (1 mM) on adenylate cyclase activity in membranes from cultured bovine aortic endothelial cells. Reaction media contained 1 mM IBMX and 5 mM Mg^{2+}. Values are means ± SE. $P < 0.001$ from basal levels with two-way ANOVA. * $P < 0.05$ from the corresponding control value with Student's *t*-test.

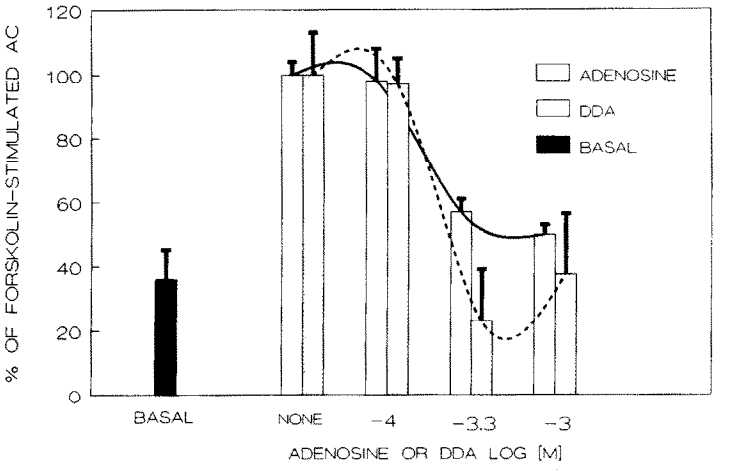

Fig. 3. Effects of adenosine and 2',5'-dideoxyadenosine (DDA) on the forskolin-stimulated adenylate cyclase (AC) activity in membranes from cultured bovine aortic endothelial cells. "Basal" reflects AC levels in the absence of forskolin, adenosine or DDA (7.8 ± 1.6 pmol cAMP/min/mg protein). All other bars represent AC levels in the presence of 30 μM forskolin. Reaction media contained 1 mM IBMX, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SE, N = 4. $P < 0.05$ from forskolin-stimulated levels in the absence of adenosine or DDA ("NONE") with two-way ANOVA.

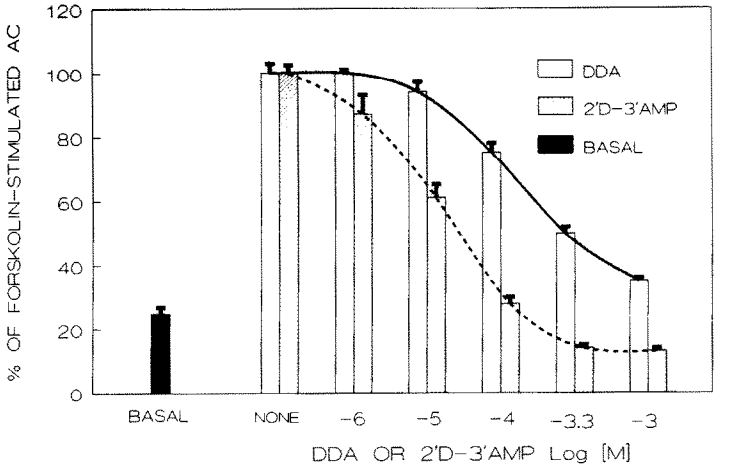

Fig. 4. Effects of 2',5'-dideoxyadenosine (DDA) and 2'-deoxy-3'-AMP (2'D-3'-AMP) on the isoproterenol-stimulated adenylate cyclase (AC) activity in membranes from culture bovine aortic endothelial cells. "Basal" reflects AC levels in the absence of isoproterenol, DDA or 2'D-3'-AMP (10.8 ± 1.3 pmol cAMP/min/mg protein). All other bars represent AC levels in the presence of 10 μM isoproterenol. Reaction media contained 1 mM IBMX, 2 μM GTP and 5 mm Mg^{2+}. Values are means ± SE, N = 8. $P < 0.01$ from isoproterenol-stimulated levels in the absence of DDA or 2'D-3'AMP ("NONE") with two-way ANOVA.

DISCUSSION

Endothelial cells are now known to be much more than a passive barrier between blood and interstitium; they contribute to the regulation of vascular tone through the secretion of a variety of mediators such as prostaglandins [3], endothelium derived relaxing factor (EDRF) [4] or the recently identified endothelin [38]. Endothelial cells are also able to modify their membrane potential and may transmit these electric signals to underlying smooth muscle cells through gap junctions [39, 40]. Additionally, endothelial cells can change their shape, and thus modulate the permeability of the monolayer [5, 41]. All these functions can be triggered by mediators through receptor activation and transduction of the signal through the cell.

In a previous study, we demonstrated that cultured bovine pulmonary arterial endothelial cells express both β-adrenergic and adenosine A_2 receptors which stimulate adenylate cyclase [30]. On the other hand, adenosine P site agonists, including adenosine itself, inhibit stimulated adenylate cyclase in these cells [30]. In the present study, we characterized the

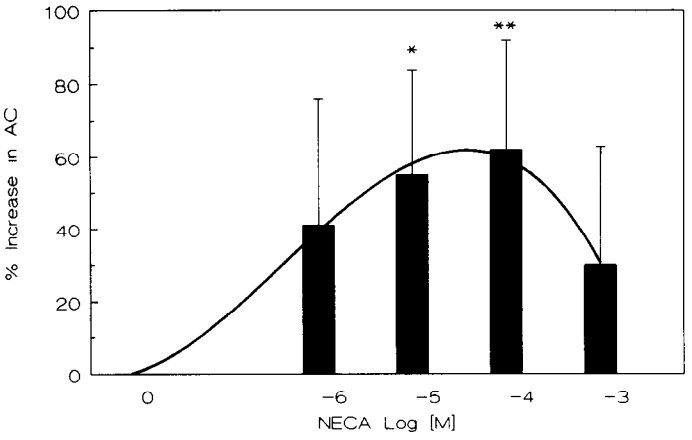

Fig. 5. Effects of NECA on adenylate cyclase activity in membranes from cultured bovine aortic endothelial cells. Basal levels: 5.4 ± 1.3 pmol cAMP/min/mg protein. Reaction media contained 1 mM cAMP, 10 I.U./mL adenosine deaminase, 2 μM GTP and 5 mM Mg^{2+}. Values are means ± SE, N = 12. Key: (*) $P < 0.05$ and (**) $P < 0.01$ from zero with one-way ANOVA and Dunnett's *t*-test.

adenylate cyclase system from cultured bovine aortic endothelium in order to study possible functional differences in cells derived from different vessels.

Bovine aortic endothelial cells resembled bovine pulmonary arterial cells in that (1) adenylate cyclase activity was stimulated by the β-adrenergic agonist isoproterenol and by the adenosine A_2 receptor agonist NECA, (2) adenosine and analogs which are agonists of the P site inhibited isoproterenol-forskolin- or Gpp(NH)p-stimulated adenylate cyclase, (3) the specific adenosine A_1 agonist cyclohexyladenosine did not affect forskolin-stimulated adenylate cyclase activity, and (4) the most active adenosine derivative at the P site of adenylate cyclase was the natural nucleotide 2′-deoxy-3′-AMP. The major difference found is that basal activity of adenylate cyclase was slightly lower in aortic cells compared to pulmonary arterial cells harvested, grown and studied under identical conditions (8.3 ± 0.4 vs 11.1 ± 1 pmol cAMP/min/mg protein; $P < 0.02$). While the basal adenylate cyclase activity was significantly lower in aortic than in pulmonary arterial endothelial cells, the difference was small (25%) and of questionable physiologic significance; furthermore, each agonist used had comparable effects on both cell types when expressed as fold-stimulation of basal activity (Table 1).

This lack of differences can be the result of long-term culture which could obtund the expression of properties stimulated by differences in the physiological milieu *in vivo*, i.e. oxygen tension or blood pressure. However, long-term cultured arterial, venous and capillary endothelial cells are able to maintain other functional differences, as for example the profile of prostaglandins released in response to different stimuli [3].

The modulation of vascular endothelial adenylate cyclase by β-adrenergic or A_2 adenosine receptors could have several important effects: stable cAMP analogs or substances which increase cAMP levels are able to (1) reduce the permeability of endothelium monolayer [42] and capillaries [43], (2) increase the activity of angiotensin converting enzyme [44] and (3) inhibit the adherence of polymorphonuclear leukocytes to endothelial cells [45].

Table 1. Comparison of the stimulations of adenylate cyclase in bovine aortic and pulmonary arterial endothelial cells*

Stimulator	Cell type	S/B	N
Gpp(NH)p (100 μM)	Ao	4.8 ± 1.2	16
	PA	6.8 ± 1.3	8
Forskolin (100 μM)	Ao	5.2 ± 0.7	12
	PA	2.9 ± 0.2	12
Isoproterenol (10 μM)	Ao	2.6 ± 0.1	12
	PA	3.7 ± 0.3	8
NECA	Ao	1.6 ± 0.3	12
	PA	1.5 ± 0.1	12

* Abbreviations: S/B: ratio of stimulated to basal adenylate cyclase activity; PA: pulmonary arterial endothelial cells; and Ao: aortic endothelial cells. Values are means ± 1 SE. Values for PA are from Ref. 30. Basal levels: Ao = 8.3 ± 0.4 pmol cAMP/min/mg protein, PA = 11.1 ± 1 pmol cAMP/min/mg protein, N = 138 each.

Adenosine is a potent vasodilator which acts on the vascular smooth muscle directly [14–15] or via the endothelium [16–18]. When adenosine is present in the blood stream, its fast metabolism and uptake are not likely to allow a large fraction to reach the vascular smooth muscle cells [46, 47], thus reinforcing the role of endothelium in mediating the vascular action of adenosine, *in vivo*. The cell signaling as well as the downstream events mediating such effects remain unknown; endothelium-dependent relaxation induced by several agonists is clearly dependent on a rise in cytosolic calcium [48]; however, endothelial cells secrete a variety of substances with different effects on smooth muscle tone, and it is possible, in fact probable, that endothelium-dependent effects involve several different pathways.

The role of the P site in the endothelium-dependent effects of adenosine on vascular tone are unclear: the P site agonist β-D-xylofuranoyl-adenosine can induce an endothelium-dependent relaxation [49]. However, adenosine itself, because of its weak activity and fast degradation, would hardly reach concentrations that would be active at the P site under physiological conditions. Nevertheless, endothelium-dependent effects of high doses of adenosine could possibly be due to a P site effect [16, 18]. While adenosine is not likely to be a natural modulator at the P site, the nucleotide 2′-deoxy-3′-AMP may be, since it has a higher activity and is naturally present in some cells [50, 51]; there is also evidence that cAMP production must be regulated from the inside of the cell because it is involved in key cell events such as cell proliferation [52].

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