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PRESENCE OF "Ra" AND "P"-SITE RECEPTORS FOR ADENOSINE COUPLED TO ADENYLATE CYCLASE IN CULTURED VASCULAR SMOOTH MUSCLE CELLS[1]

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SUMMARY: The existence of adenosine receptors coupled to adenylate cyclase in cultured vascular smooth muscle cells from rat aorta is demonstrated in these studies. Adenosine, N^6-phenylisopropyladenosine, adenosine N-oxide and 2-chloroadenosine stimulated adenylate cyclase in a concentration dependent manner. The stimulation was dependent on the presence of guanine nucleotides and was blocked by 3-isobutyl-1-methylxanthine. In contrast, 2'deoxyadenosine inhibited adenylate cyclase activity. Adenosine and 2-chloroadenosine showed a biphasic effect on adenylate cyclase, stimulation occurred at low concentrations. The activation of adenylate cyclase by N^6-phenylisopropyladenosine was also dependent on the Mg^{2+} concentration. The data suggest that vascular smooth muscle cells have both "Ra" and "P" receptors for adenosine, and it can be postulated that the relaxant effect of adenosine on vascular smooth muscle may be mediated by its interaction with "Ra" receptors associated with adenylate cyclase.

INTRODUCTION

Adenosine is a potent vasodilator. It is released into coronary circulation in large quantities during hypoxia and has been proposed to regulate blood flow particularly in heart (1). Adenosine has been shown to alter cAMP levels and adenylate cyclase in numerous tissues (2-5). Adenosine stimulated (6-9), inhibited (10-11), or had a biphasic effect (5,7) on adenylate cyclase. These different actions of adenosine on adenylate cyclase appear to be mediated through extracellular R or A sites and an intracellular P site distinguishable by various analogs of adenosine. The R or A sites have been further classified as "Ra" and "Ri" sites (13) (or A_2 and A_1 sites (14)) denoting activating and inhibitory effects on adenylate cyclase respectively. The mechanism of action of adenosine in the relaxation of smooth muscle is not clear. Recently, a close relationship between the relaxant

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Abbreviations used: PBS, phosphate buffered saline; DMEM, Dulbeccos modified Eagles medium; PIA, N^6-phenylisopropyladenosine; IBMX, 3-isobutyl-1-methylxanthines; GMP-P(NH)P, guanyl-5'-yl-(-β-γ-imino)diphosphate.

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effect of adenosine and increased adenylate cyclase activity and cAMP levels has been demonstrated in coronary arteries (15) but effects of adenosine or its analogs on adenylate cyclase from vascular smooth muscle have not been reported, and in general enzyme from smooth muscle has been found to have little or no sensitivity to hormones. We have conducted the present studies to demonstrate if adenosine sensitive adenylate cyclase is present in cultured smooth muscle cells with the eventual purpose of determining the possible role of adenosine in the pathophysiology of hypertension.

MATERIALS AND METHODS

Culture of vascular smooth muscle cells. Vascular smooth muscle cells were cultured from explants of rat aorta by the method of Ross (16). Colonies of vascular smooth muscle cells grew out from some of the explants within 7-14 days. When sufficiently confluent, the colonies were trypsinized with 0.05% trypsin in PBS (Ca^{2+} and Mg^{2+} free, containing 0.02% EDTA). The resulting suspension of smooth muscle cells was centrifuged and the cell pellet dispersed in a small volume of DMEM supplemented with 10% calf serum, 1 mM Hepes and antibiotics and plated at a cell density of 1×10^5 - 1×10^6 cells in 9 cm glass petri dishes. The medium was changed after attachment of the cells and twice weekly thereafter. The cells were again passaged when they reached confluence.

Preparation of particulate fraction. Confluent smooth muscle cells were rinsed twice with PBS to remove culture medium. The cells were then removed from the petri dish with a rubber policeman. The cells were centrifuged and then homogenized in a Dounce homogenizer in a buffer containing 10 mM Tris, 1 mM EDTA and 1 mM DTT pH 7.5. The homogenate was centrifuged at 5000 x g for 10 min. The supernatant was discarded and the pellet was homogenized in the same buffer by hand with a glass teflon homogenizer. This preparation was used for adenylate cyclase assay.

Adenylate cyclase activity determination. Adenylate cyclase activity was determined by measuring [^{32}P] cAMP formation from [α-^{32}P] ATP as described previously (5,17). Typical assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM Mg ATP, [α^{32}P]ATP (1-1.5 x 10^6 CPM), 10 mM $MgCl_2$ (in excess of the ATP concentration), 0.5 mM cAMP, 5 U adenosine deaminase per ml, 1 mM DTT, 10 μM GTP, an ATP regenerating system consisting of 2 mM creatine phosphate, 0.1 mg creatine kinase per ml, and 0.1 mg myokinase per ml in a final volume of 200 μl. Incubations were initiated by the addition of the particulate fraction (30-80 μg of protein) to the reaction mixture which had been thermally equilibrated for 2 min at 37°C. Reactions were conducted in triplicate for 10 min at 37°C. Reactions were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by co-precipitation of other nucleotides with $ZnCO_3$ by the addition of 0.5 ml of 144 mM Na_2CO_3 and subsequent chromatography by the double column system as described by Salomon et al (18). Under the assay conditions used adenylate cyclase activity was linear with respect to protein concentration and time of incubation.

Protein was determined essentially as described by Lowry et al (19), with crystalline bovine serum albumin as standard.

Materials. Adenosine deaminase (EC 3.5.4.4), 2-chloroadenosine, adenosine N'-oxide, GTP, GMP-P(NH)P (guanyl-5'-yl-β-γ-imino diphosphate), ATP, cyclic AMP were purchased from Sigma, St.Louis, Missouri. Adenosine, 2'deoxyadenosine, N^6-phenylisopropyladenosine, creatine kinase (EC 2.7.3.2) and myokinase (EC 2.7.4.3) were purchased from Boehringer Mannheim, Canada. 3-Isobutyl-1-methylxanthine was purchased from Aldrich Chemical Corp. Milwaukee, Wisconsin. [α^{32}P]ATP was purchased from Amersham, DMEM, trypsin and calf serum were from Gibco.

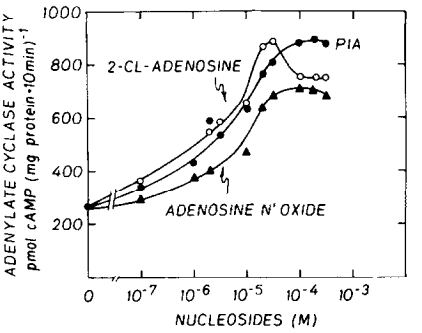

Figure 1 Stimulation of adenylate cyclase activity by increasing concentration of PIA (●-●), 2-cl adenosine (o-o) and adenosine N-oxide (▲-▲) in particulate fraction from cultured vascular smooth muscle cells. Adenylate cyclase activity was measured as under "Methods". Values are the means of triplicate determinations from one of the two experiments.

RESULTS

Effect of adenosine analogs on adenylate cyclase activity. In order to determine the presence of adenosine sensitive adenylate cyclase in cultured vascular smooth muscle cells, the ability of various analogs of adenosine to stimulate adenylate cyclase was studied. The results are shown in Figure 1. PIA, 2-chloroadenosine and adenosine N^1oxide all stimulated adenylate cyclase activity in a concentration dependent manner. It was also of interest to note that there was less stimulation of adenylate cyclase by adenosine analogs when adenosine deaminase was absent from the assay medium (data not shown).

The stimulation of adenylate cyclase by PIA was also dependent on guanine nucleotides such as GTP or GMP-P(NH)P as shown in Table 1. About 35% stimulation of adenylate cyclase was observed with PIA alone which was increased to about 250% in the presence of GTP or GMP-P(NH)P. GTP was more effective than GMP-P(NH)P in enhancing both basal and PIA stimulated adenylate cyclase activity.

To determine the existence of the "P"-site for adenosine action in cultured smooth muscle cells, the effect of adenosine and 2' deoxyadenosine on adenylate cyclase activity was investigated and the results are shown in Figs. 2 A & B. Adenosine had a biphasic effect on adenylate cyclase activity (Fig. 2A). Adenosine increased adenylate cyclase activity at low concentrations (1-50 μM) but the stimulation was decreased at higher concentrations of adenosine. In contrast 2' deoxyadenosine, only inhibited adenylate cyclase activity (Fig. 2B).

TABLE 1 Effect of guanine nucleotides on PIA-sensitive adenylate cyclase in particulate fraction from cultured vascular smooth muscle cells.

Guanine nucleotides (μM)	Adenylate cyclase activity pmol cAMP (mg protein.10 min)$^{-1}$ Basal	PIA (30μM)
None	60 ± 6	80 ± 7
GTP 0.1	65 ± 17	174 ± 1
1.0	225 ± 2	579 ± 34
10.0	600 ± 30	1205 ± 80
GMP-P(NH)P		
0.1	72 ± 10	107 ± 3
1.0	119 ± 6	243 ± 7
10.0	364 ± 45	731 ± 32

Adenylate cyclase activity was determined as given under "Methods" except that GTP was omitted from the assay mixture. The values represent the mean ± S.E.M. of triplicate determinations from one of two experiments.

Effect of 3-isobutyl-1-methylxanthine (IBMX). Since methylxanthines have been shown to block the stimulatory effect of adenosine on adenylate cyclase by competing for the "R"-site, it was of interest to examine the effect of methylxanthines on adenosine sensitive adenylate cyclase in vascular smooth muscle cells. Fig. 3 shows the effect of various concentrations of IBMX on basal and PIA-sensitive adenylate

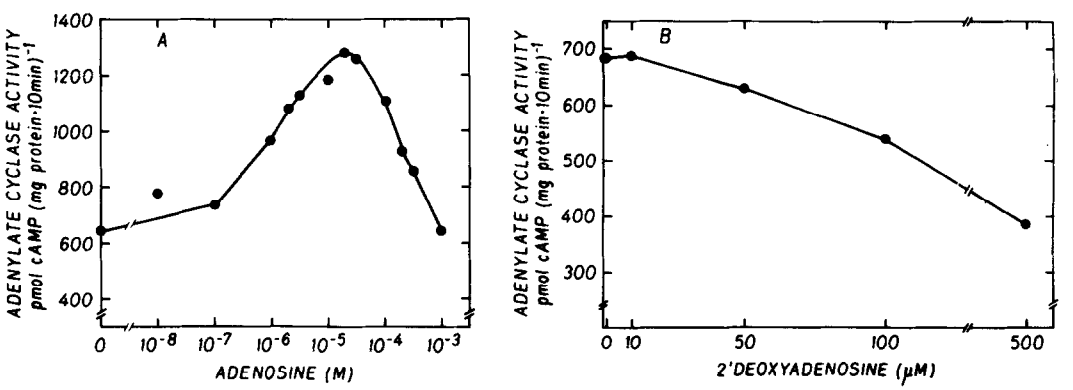

Figure 2 Effect of adenosine (A) and 2'deoxyadenosine (B) on adenylate cyclase activity in particulate fraction from cultured vascular smooth muscle cells. Adenylate cyclase activity was measured as under "Methods" in the absence of ADA. Each value is the mean of triplicate determinations from one of two experiments.

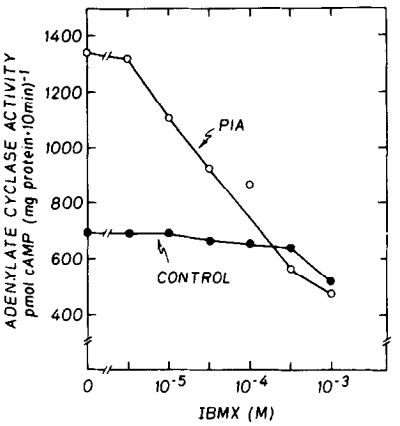

Figure 3 Effect of various concentrations of 3-isobutyl-1-methylxanthine on adenylate cyclase activity in particulate fraction from cultured vascular smooth muscle cells. Activity was determined in the absence (•-•) or presence (o-o) of 30 μM N^6-phenylisopropyladenosine. Values are the means of triplicate determinations from one of two experiments.

cyclase activity. IBMX inhibited PIA-sensitive adenylate cyclase in a concentration dependent manner. At a concentration of 0.5 mM IBMX, the activation of adenylate cyclase by PIA was completely inhibited, while IBMX had little effect on basal adenylate cyclase activity. In addition, 1 mM IBMX was also able to inhibit the stimulatory effect of adenosine on adenylate cyclase activity (data not shown).

Effect of divalent cations. The dependence of adenosine stimulation of adenylate cyclase on the divalent cation concentration has been reported in various systems (3,5,7). In cultured vascular smooth muscle cells, PIA-stimulation of adenylate cyclase also appears to be dependent on the Mg^{2+} ion concentration (Table 2). With increasing concentrations of Mg^{2+}, the basal as well as PIA-sensitive adenylate cyclase activity was increased but the degree of stimulation by PIA was greater (3.5 fold) at low Mg^{2+} concentrations (1 mM) and was decreased to about 90% at 20 mM Mg^{2+}. Similar results were also obtained with Mn^{2+} (data not shown).

DISCUSSION

The present study demonstrates the presence of adenosine sensitive adenylate cyclase in cultured vascular smooth muscle cells. The response to adenosine was biphasic suggesting the presence of both "Ra" (stimulatory) and "P" (inhibitory) site receptors for adenosine in cultured vascular smooth muscle cells. IBMX prevented the stimulation by adenosine and "R" site analogs indicating that adenosine receptors

TABLE 2 Effect of various concentrations of Mg^{2+} on adenylate cyclase activity in the absence and presence of PIA in particulate fraction from cultured vascular smooth muscle cells.

Mg^{2+} (mM)	Adenylate cyclase activity pmol cAMP (mg protein.10 min)$^{-1}$ Basal	PIA	Activity ratio PIA/BASAL
1	9 ± 1	32 ± 1	3.6
3	138 ± 1	329 ± 17	2.4
5	199 ± 8	461 ± 27	2.3
10	267 ± 16	576 ± 4	2.1
20	241 ± 12	465 ± 2	1.9

Adenylate cyclase activity was determined as given under "Methods". The values represent the mean ± S.E.M. of triplicate determinations from one of two experiments.

in smooth muscle cells are of the "Ra" (13) or "A_2" (14) type. Adenosine analogs that interact specifically with "P" site receptors such as 2'deoxyadenosine were only inhibitory to adenylate cyclase in cultured vascular smooth muscle cells.

The inability to observe any stimulatory effect of adenosine analogs on adenylate cyclase in other smooth muscle preparations (20,21) may be due to the possibility that in these enzyme systems bound adenosine was already present at saturating levels. In the present study however, the lower stimulation of adenylate cyclase seen with adenosine (2-fold) (Fig. 2A) as compared to 3.5-fold with "R" site analogs (Fig. 1) suggest that some bound adenosine is present in vascular smooth muscle but may not occupy all the receptors, perhaps because the vascular smooth muscle cells are maintained in tissue culture and therefore not exposed to the normal circulating levels of adenosine. Our results are in agreement with recent observations by Webster and Olsson (22) who demonstrated stimulation of adenylate cyclase by adenosine in dog cardiac membranes.

The stimulation of vascular smooth muscle adenylate cyclase by PIA was dependent on the presence of guanyl nucleotides indicating that the guanine nucleotide binding protein is required for coupling of the "Ra" receptor to the catalytic moiety of adenylate cyclase as has been demonstrated in other tissues (3,5,7). The fact that PIA stimulation of adenylate cyclase activity in cultured vascular smooth muscle cells was greater at low concentrations of Mg^{2+} is in agreement with the earlier findings

made in rat brain striatum (5), liver (23) and platelets (3) and is consistent with the hypothesis that adenosine binds to or interacts with the divalent cation binding site of adenylate cyclase.

It is concluded from these studies that adenosine sensitive adenylate cyclase is present in cultured vascular smooth muscle cells, and it can be hypothesized that the known relaxant effect of adenosine on vascular tissue may be mediated via its direct interaction with the "Ra" receptors associated with adenylate cyclase.

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