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Adenosine Stimulates Guanylate Cyclase Activity in Vascular Smooth Muscle Cells*

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Good evidence exists to indicate that the vasodilating effect of adenosine is mediated by cell surface receptors on vascular smooth muscle cells. The mechanism of transmembrane signal transduction for adenosine, however, is not fully understood. Since cGMP is a second messenger known to mediate vasodilation, I have examined the effect of adenosine on the intracellular concentration of cGMP in vascular smooth muscle cells from rat aorta. I found that adenosine at 10^{-9} to 10^{-5} M led to an increase in intracellular cGMP levels in a dose-dependent fashion. The effect of adenosine on cyclic guanosine inorganic monophosphate (cGMP) could be mimicked by the A-type receptor agonists N^6-cyclohexyladenosine and 5′-*N*-ethylcarboxamidoadenosine and was attenuated by the A-receptor antagonist theophylline. The order of potency of the adenosine analogues was N^6-cyclohexyladenosine > 5′-*N*-ethylcarboxamidoadenosine > adenosine. These findings suggest that the effect of adenosine on $cGMP_i$ is mediated by A_1-type cell surface receptors. Concerning the mechanism by which adenosine could elevate $cGMP_i$, I found that the effect of adenosine on $cGMP_i$ was potentiated by the cGMP phosphodiesterase-specific inhibitor M & B 22948. Moreover, I found that N^6-cyclohexyladenosine, 5′-*N*-ethylcarboxamidoadenosine, and adenosine stimulated a guanylate cyclase in homogenates of the cultured smooth muscle cells in a dose-dependent fashion with the same order of potency as their effects on $cGMP_i$. Further evidence was obtained to indicate that adenosine and its analogues stimulated a particulate guanylate cyclase activity, whereas they did not alter soluble guanylate cyclase activity.

Since cGMP is known as a second messenger mediating relaxation of vascular smooth muscle cells, the results obtained in this study could suggest that adenosine exerts its vasorelaxing effect by activating an A_1-receptor-linked guanylate cyclase.

Adenosine is considered an important regulator of blood flow in a variety of organs including heart (1), brain (2), skeletal muscle (3), and adipose tissue (4). This function of adenosine is based on its vasorelaxant effect on arteries. The vasodilatory effect of adenosine on blood vessels can be observed at 10^{-8} to 10^{-5} M both in *in vivo* and *in vitro* preparations (5–7). Furthermore, there is good experimental evidence to indicate that adenosine exerts its vasorelaxant effect by occupying cell surface receptors on vascular smooth muscle cells (8–10). The mode of transmembrane signal transmission, however, according to which activation of adenosine receptors on the cellular plasma membrane leads to relaxation of vascular smooth muscle cells, is not well understood (1–4). Two possible mechanisms by which adenosine could induce vasorelaxation have been discussed. First, results obtained with mechanical (11), electrophysiological (12, 13), and cell culture (14) studies have indicated that adenosine attenuates calcium influx into muscle cells. Therefore, it has been speculated that adenosine might act as a calcium channel blocker. Recent experimental evidence, however, clearly argues against a role of adenosine as a calcium channel blocker (15–17).

The second line of evidence is based on the observation that adenosine is capable of influencing adenylate cyclase activity in a variety of tissues (18). It has been found that cell surface receptors for adenosine are linked to the adenylate cyclase in a way that occupancy of A_1-receptors inhibits adenylate cyclase activity, whereas occupancy of A_2-receptors stimulates this activity (19). Since cAMP is known as a second messenger that mediates relaxation of smooth muscle cells (20), it has been speculated that the vasodilatory effect of adenosine could be mediated by a receptor-linked activation of the adenylate cyclase (21). A strong argument against such signal transmission is, however, that no correlation has been found between vasodilation and tissue levels of cAMP in the presence of adenosine (1, 6, 22). Moreover, adenosine has been found to elevate levels of cAMP only at concentrations that are higher than 10^{-4} M, whereas the full relaxant effect of adenosine can be observed at concentrations that are 2 orders of magnitude lower (6).

A third signal transmission system that mediates vasorelaxation involves cGMP that is generated via an activation of guanylate cyclase (23). Nitrocompounds (23) and atrial natriuretic peptide (24), for example, are thought to cause vasodilation by stimulating a soluble and a membrane-bound guanylate cyclase, respectively. However, a possible role of $cGMP_i$ [1] in the signal transmission of adenosine in vascular smooth muscle has not been considered so far. To my knowledge, the effect of adenosine on cGMP on the level of isolated vascular smooth cells has not been investigated to date. Therefore, the objective of this study was to investigate whether or not adenosine affects the intracellular level of cGMP in vascular smooth muscle cells and, if so, to clarify the mechanism by which adenosine influences $cGMP_i$. Using cultures of smooth muscle cells derived from rat aorta, clear evidence was obtained that adenosine stimulated a particulate guanylate cyclase in these cells. Since the activation of guanylate cyclase and the rise in cGMP occurred at concentrations of adenosine that are typical for its vasorelaxant effect, stim-

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[1] The abbreviations used are: $cGMP_i$, cyclic guanosine inorganic monophosphate; SMC, smooth muscle cells; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHA, N^6-cyclohexyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine.

ulation of the guanylate cyclase could be a signal transmission system which mediates adenosine's vasorelaxant effect.

MATERIALS AND METHODS

Cell Culture—Cultures of smooth muscle cells (SMC) from rat aorta were prepared using the modification described by Ross (26). Aortas were taken from male Sprague-Dawley rats (200–300-g body weight). The vessels were thoroughly freed from adventitial tissue and cut into small pieces. Tissue pieces were seeded in 7-cm^2 Petri dishes (Greiner, Nürtingen, Federal Republic of Germany) together with 1 ml of culture medium (RPMI 1640, 25 mM Hepes, 1 mg/ml insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum, all from Boehringer Mannheim) and incubated in a humidified atmosphere containing 5% CO_2 in air. Medium was removed after 7 days, and tissue explants were detached using a solution of Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free) supplemented with 5 mM EDTA and 0.25% trypsin (bovine pancreas, Sigma). Single cells were separated from tissue pieces by unit gravity sedimentation. Single cells were cloned by cell dilution using 24-microwell plates. Clones with apparent smooth muscle cell morphology were used for further processing. Clonal cells were grown in 7-cm^2 Petri dishes using the same culture conditions as described above. Cells were passaged after they had reached confluency. For experiments, confluent cultures between passages 6 and 10 were used.

Immunofluorescence Staining against Intermediate Filament Desmin—Demonstration of the intermediate filament desmin, which is considered to be specific for myogenic cells (27), was done according to Osborn and Weber (28). Upon passage, part of the cells were seeded on coverslips. Two hours after seeding, the cells were fixed with methanol, washed, and incubated with monoclonal antibody against desmin for 2 h at 37 °C. Mouse monoclonal antibody against porcine desmin (29) was a generous gift from Dr. Mary Osborn. Thereafter, the cells were washed and incubated with fluorescein isothiocyanate-labeled goat antibody mouse γ-globulin for 1 h at 37 °C. Immunofluorescence was examined using a Ployvar fluorescence microscope (Reichert-Jung, Austria).

Determination of cGMP Levels—The medium was removed from the dishes, and cells were washed two times with prewarmed buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.3). Then, 1 ml of this buffer with or without agents was added, and the dishes were placed on a heater maintaining a temperature of 37 °C. The incubation was stopped by removing the buffer and the addition of 1 ml of ice-cold buffer (5 mM potassium phosphate, 0.2 mM EDTA, 0.5 mM 3-isobutylmethylxanthine, 150 mM KCl, pH 6.8). This buffer was quickly removed, and the dishes were placed on an ice block. Finally, the cells were scraped off in 0.4 ml of this buffer using a Teflon policeman. The obtained suspension was subsequently sonicated (50 watts for 30 s), boiled at 95 °C for 5 min, and centrifuged at 10,000 × *g* for 5 min. The supernatant was stored at −80 °C until assay of cGMP. The sediment was lysed with 1 N NaOH and used for protein determination. Protein determination was done according to Lowry *et al.* (30) using bovine serum albumin (Sigma) as a standard. cGMP was assayed with a commercially available radioimmunoassay (New England Nuclear). Cross-reactivity between adenosine and cGMP was checked. Below 10^{-4} M adenosine, no evidence for cross-reactivity was obtained.

Guanylate Cyclase Assay—Guanylate cyclase activity was determined according to Nesbitt *et al.* (31) as modified by Ardaillou *et al.* (32). Guanylate cyclase activity was measured in homogenates of cultured smooth muscle cells, rat abdominal aorta, and bovine coronary artery. Cultured cells (10^7/ml) were sonicated at 50 watts for 30 s in 2 ml of homogenization buffer (250 mM sucrose, 1 mM dithiothreitol, 25 mM Tris-HCl, pH 7.8). Aortas from male Sprague-Dawley rats and pieces from bovine coronary arteries were thoroughly freed from adventitial tissue and subsequently homogenized in the homogenization buffer using a Polytron homogenizer. The homogenate thus obtained was further homogenized in a Dounce homogenizer (tight-fitting) with 30 strokes. In order to obtain a particulate and a soluble fraction, the homogenates were centrifuged at 100,000 × *g* for 1 h. The supernatant was taken as soluble fraction. The pellet was resuspended in fresh homogenization buffer, sonicated, and taken as particulate fraction.

Twenty microliters of homogenate or particulate/soluble fraction containing between 30 and 90 μg of protein were added to 100 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.8, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM phosphocreatine, 150 μg of creatine kinase, 0.5 mM GTP, 2.5 M cGMP, 0.25 mM papaverine, 0.1% bovine serum albumin, and 0.1 μCi of [α-^{32}P]GTP. Adenosine and its analogues were added in 10 μl of water. Incubation was performed for 10 min at 37 °C. The reaction was stopped by adding 20 μl of 100 mM EDTA plus 100 nCi of [^{3}H]cGMP. The solution was boiled and centrifuged. [^{32}P]cGMP was extracted from the supernatant using Dowex AG 50WX4 (Bio-Rad) and neutral alumina (M. Woelm, Eschwege, Federal Republic of Germany) columns exactly as described by Nesbitt *et al.* (31). ^{32}P and ^{3}H radioactivity were determined using Aquasol-2 (New England Nuclear) as a scintillant and a dual-label program on a Rackbeta β-counter (LKB). Each experiment with the guanylate cyclase assay as shown under "Results" represents the mean of quadruplicates. Fig. 1 shows the time dependence and dependence on cellular protein of cGMP formation in assays performed in quadruplicates.

Agents—Adenosine, N^6-cyclohexyladenosine (CHA), and theophylline were purchased from Sigma. 5′-*N*-Ethylcarboxamidoadenosine (NECA) and M & B 22948 were generous gifts from Byk-Gulden Lamberg Chemische Fabrik, GmbH (Konstanz, Fedral Republic of Germany) and the May & Baker Co. (London, Great Britain), respectively.

RESULTS

In order to ensure that the cells used for the experiments were SMC, the existence of the intermediate filament desmin in the cultured cells was checked. Desmin is considered to be specific for myogenic cells *in vivo* and *in vitro* (28) and does not occur in endothelial cells or fibroblasts. I found that more than 90% of the cultured cells stained positive for desmin, confirming that the cells used for experiments are SMC.

Cultured SMC were incubated with various concentrations of adenosine for 2, 5, and 15 min, and the cellular content of cyclic GMP was determined (Fig. 2). It can be seen from Fig. 1 that adenosine led to a transient rise in cGMP levels at concentrations of adenosine ranging from 10^{-9} to 10^{-5} M. The rise in $cGMP_i$ occurred as soon as 2 min after addition of adenosine, which was the first time point examination.

Fig. 3 shows the dependence of cGMP levels observed after 5 min on the concentration of adenosine and of the adenosine analogues CHA and NECA. It can be seen that adenosine led to a 2.5-fold increase of $cGMP_i$ in a dose-dependent fashion. In order to find out whether or not the effect of adenosine is mediated by cell surface receptors, the effect of theophylline (a specific antagonist for A-type receptors (33)) was examined. It can be seen in Fig. 2 that in the presence of theophylline (5×10^{-5} M), the dose-response curve of adenosine for $cGMP_i$ was shifted to the right by 2 orders of magnitude. Fig. 3 further clarifies that the effect of adenosine on cGMP could be mimicked by the A-type receptor agonists CHA and NECA

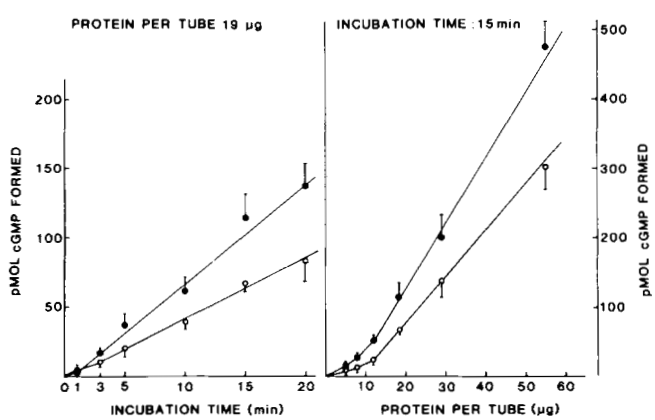

FIG. 1. **Formation of cGMP in guanylate cyclase assay system as functions of incubation time (*left*) and amount of added cellular protein (*right*).** Data are shown as mean ± S.E. of quadruplicates. ○, experiments done in the absence of adenosine; ●, experiment done in presence of adenosine (10^{-6} M). Lines are linear regression curves.

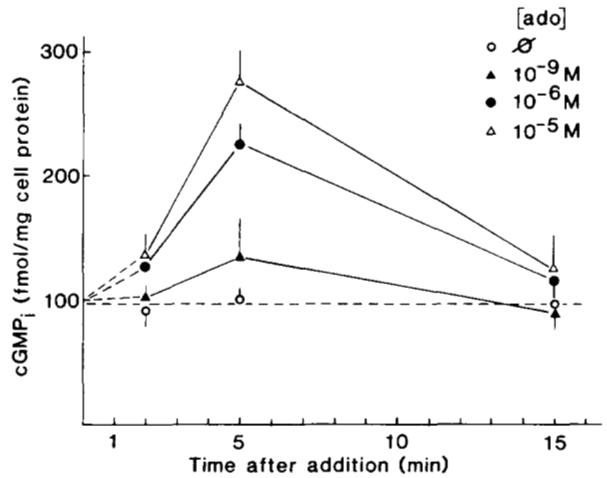

FIG. 2. **Time course of intracellular cGMP after addition of various concentrations of adenosine (*ado*).** Data are mean ± S.E. of 10 experiments.

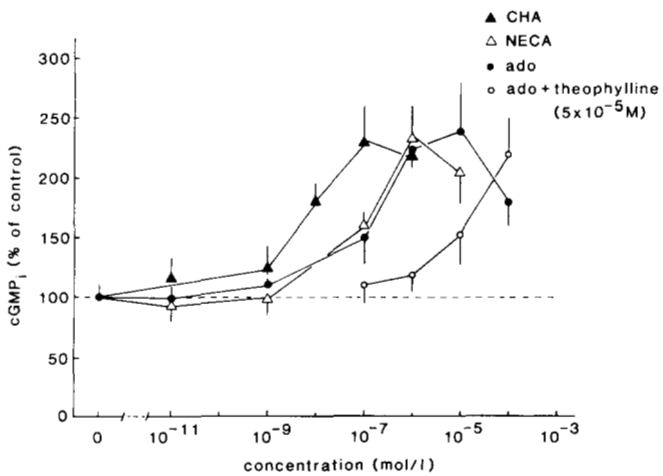

FIG. 3. **Dose dependence of intracellular cGMP on concentration of CHA, NECA, and adenosine (*ado*) in absence and presence of theophylline (5×10^{-5} M).** Cyclic nucleotides were determined 5 min after the addition of the agents. Data ($X \pm$ S.E. of 10 experiments) are presented as percentage of the mean of control (*i.e.* absence of agents). This form of plotting was chosen because of the scatter of control values among the different sets of experiments. Means of control ranged from 70 to 170 fmol/mg cGMP.

(33). Concentrations required for the half-maximal effect on cGMP$_i$ were 6×10^{-9} M for CHA and 1.2×10^{-7} M for both adenosine and NECA. Since the effect of adenosine on cGMP levels was observed exactly in the concentration range of adenosine that is typical for vasodilation (5–7), further experiments were done in order to clarify the mechanism by which adenosine could raise cGMP levels.

The cellular cGMP level in general is determined by the activity of guanylate cyclases on the one hand and by the activity of cGMP phosphodiesterases on the other. To find out whether or not adenosine influences a cGMP phosphodiesterase, combination experiments of adenosine with the cGMP phosphodiesterase-specific inhibitor M & B 22948 (34) were performed (Table I). As it can be seen from Table I, M & B 22948 (1 mM) alone had no significant effect on cGMP$_i$. CHA (10^{-7} M) elevated cGMP levels to around 150% of control. Combination of M & B 22948 and adenosine led to an increase of cGMP to around 400% of control. From the finding that inhibition of cGMP phosphodiesterase by M & B 22948 potentiated the effect of adenosine on cGMP levels, one might conclude that adenosine elevates intracellular

TABLE I

Effect of cGMP phosphodiesterase inhibitor M & B 22948 on rise in cGMP$_i$ evoked by adenosine

cGMP$_i$ was determined 5 min after the addition of the agents. Data are mean ± S.E. of eight experiments.

	cGMP$_i$	% of control
	fmol/mg protein	
Control	93.5 ± 16.3	100
M & B 22948 (1 mM)	111.7 ± 15.2	119
Adenosine (10^{-7} M)	146.7 ± 26.3	156
Adenosine (10^{-7} M) plus M & B 22948 (1 mM)	383.2 ± 31.6	406

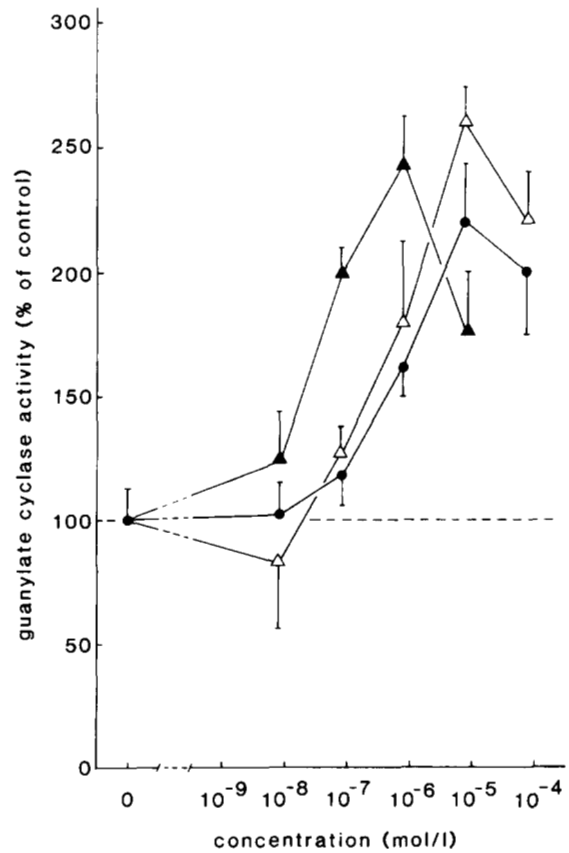

FIG. 4. **Dose dependence of guanylate cyclase activity in homogenates of cultured SMC on concentration of adenosine, CHA, and NECA added.** Data (mean ± S.E. of five experiments) are presented as the percentage of the mean of control (*i.e.* absence of agents). Means of control ranged from 80 to 150 fmol of cGMP formed per min/μg of protein.

cGMP by the stimulation of a guanylate cyclase rather than by the inhibition of a phosphodiesterase. Therefore, the effect of adenosine and its analogues (CHA and NECA) on guanylate cyclase activity of homogenated cultured SMC was investigated (Fig. 4). It is evident from Fig. 4 that adenosine, CHA, and NECA led to a 2–2.5-fold increase in guanylate cyclase activity in a dose-dependent fashion. Half-maximal activation was observed at 4×10^{-8} M for CHA and 8×10^{-7} M for both adenosine and NECA. Fig. 5 demonstrates that the stimulation of guanylate cyclase activity by the different adenosine analogues (each at 10^{-6} M) was prevented in the presence of low concentrations of theophylline (5×10^{-5} M).

Guanylate cylase is known to occur in both a cytoplasmic (soluble) and a membrane-bound (particulate) form within vascular smooth muscle cells (24). In order to find out whether activation of cellular guanylate cyclase activity by adenosine

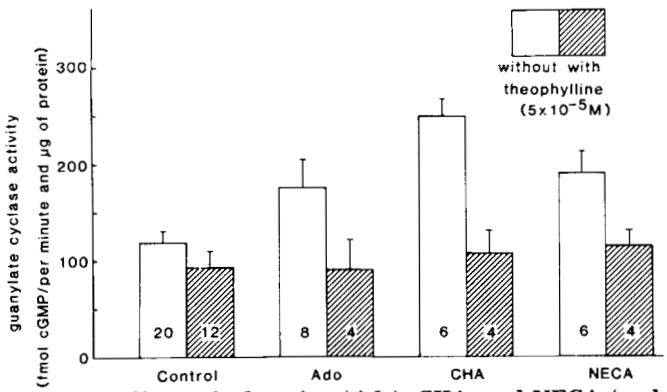

FIG. 5. **Effects of adenosine (*Ado*), CHA, and NECA (each at 10^{-6} M) on guanylate cyclase activity in homogenates of SMC in absence and presence of theophylline (5×10^{-5} M).** Data are mean ± S.E. The *numbers* at the bottom of the columns indicate number of experiments.

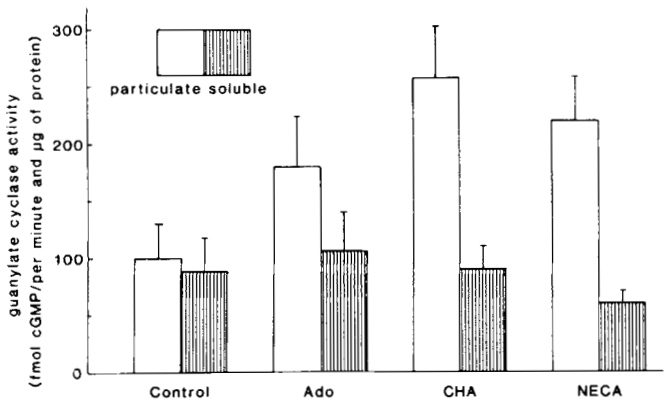

FIG. 6. **Effects of adenosine (*Ado*), CHA, and NECA (each at 10^{-6} M) on guanylate cyclase activity present in supernatant (soluble) and particulate fractions of homogenized cultured SMC.** Data are mean ± S.E. of 10 experiments.

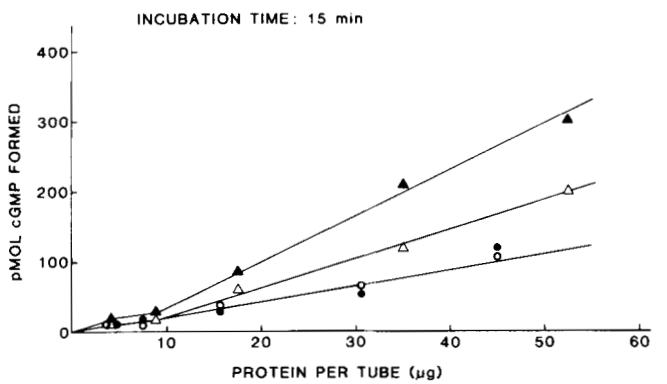

FIG. 7. **Formation of cGMP in guanylate cyclase assay assay system by soluble (*circles*) and particulate (*triangles*) guanylate cyclases as a function of added protein.** *Open symbols*, experiments done in the absence of adenosine; *closed symbols*, experiments done in the presence of adenosine (10^{-6} M). Data are mean of quadruplicates.

is due to the stimulation of a soluble or a particulate guanylate cyclase activity, the effects of adenosine, CHA, and NECA on guanylate cyclase activity present in the supernatant and particulate fractions of the cell homogenate was further examined. Fig. 6 shows that adenosine, CHA, and NECA (all 10^{-6} M) stimulated guanylate cyclase activity in the particulate fraction and failed to affect guanylate cyclase activity in the supernatant fractions. Fig. 7 documents that cGMP formation in the guanylate cyclase assay by both the soluble and particulate guanylate cyclases was linearly dependent on the amount of protein.

DISCUSSION

The objective of this study was to find out whether or not an alteration of the intracellular level of cGMP could be a way of signal transmission through which adenosine could induce relaxation in vascular smooth muscle cells. Since cGMP is known as a second messenger for vasodilation, the effect of adenosine on the cellular level of this cyclic nucleotide was investigated in cultures of vascular SMC from rat aorta.

The relaxant effect of adenosine typically occurs at 10^{-8} to 10^{-5} M (5–7). Clear evidence is provided in the present study that adenosine elevates cGMP levels in this concentration range (Fig. 3). Adenosine was found to elevate cGMP about 2–3-fold over control. Sodium nitroprusside (10^{-6} M) elevated $cGMP_i$ around 3-fold over control in the same cultures (data not shown), and this is the value found for nitrocompounds in vascular tissues *in vitro* (35). If one accepts that the vasorelaxant effect of nitrocompounds is mediated by a rise in $cGMP_i$, then the rise of $cGMP_i$ observed in the presence of adenosine would be sufficient to explain its relaxant effect. It was further found in this study that the effect of adenosine could be mimicked by CHA and NECA, which are adenosine analogues that specifically bind to A-type receptors (33) (Fig. 3). In addition, it was demonstrated that the effect of adenosine on $cGMP_i$ could be antagonized by low concentrations of theophylline, which is considered an antagonist of A-type cell surface receptors of adenosine (33). Taken together, both findings suggest that the effect of adenosine on $cGMP_i$ is mediated by A_1-type cell surface receptors. This conclusion would be in harmony with the experimental evidence that the vasorelaxing effect of adenosine is mediated by cell surface receptors (8, 9) that can be antagonized by theophylline (10).

A-type receptors are separated in two subclasses: A_1- and A_2-receptors. So far, highly specific agonists for both receptor types are not available. It has been shown, however, that there exists an order of specificity of receptor binding among different adenosine derivatives. CHA has been found to have a higher affinity for A_1-receptors than does NECA, whereas the opposite is true for A_2-receptors (33). The finding that the dose-response curve of CHA for $cGMP_i$ is shifted to the left in comparison to that of NECA (Fig. 3) suggests that the effect of adenosine on $cGMP_i$ is mediated by A_1-receptors.

Three pieces of evidence were obtained in this study to indicate that adenosine raises $cGMP_i$ levels by the stimulation of a membrane-bound guanylate cyclase. First, inhibition of cGMP phosphodiesterase potentiated the effect of adenosine on $cGMP_i$ (Table I). Second, adenosine, CHA, and NECA stimulated cellular guanylate cyclase activity in a dose dependent fashion (Fig. 4). The stimulatory effect of adenosine and its derivatives on guanylate cyclase activity could be prevented by the A-type receptor antagonist theophylline (Fig. 5). In parallel with the effect on $cGMP_i$, the order of potency on guanylate cyclase activity again was CHA > NECA. Third, adenosine, CHA, and NECA stimulated guanylate cyclase activity in the particulate fraction but failed to affect soluble guanylate cyclase activity (Fig. 6). Stimulation of a membrane-bound guanylate cyclase by adenosine would in turn be in harmony with the evidence that the vasorelaxing effect of adenosine is mediated by cell surface receptors. Taken together, the data obtained in this study strongly suggest that occupancy of A_1-receptors stimulates a guanylate cyclase in vascular smooth muscle cells. Since cGMP is known

as a second messenger mediating vasorelaxation, my results indicate that cGMP could have an important role in the vasodilation induced by adenosine. In order to find out whether or not activation of guanylate cyclase by adenosine is a more general vascular phenomenon, I have also examined the effect of adenosine on guanylate cyclase activity in homogenates of rat aorta, bovine coronary artery, and cultured SMC from calf aorta (data not shown). Also in these preparations, I found a dose-dependent stimulation of guanylate cyclase activity by adenosine. Concentrations of adenosine required for half-maximal effects were 2×10^{-7}, 2.5×10^{-6}, and 4×10^{-7} M for rat aorta, bovine coronary artery, and SMC from calf aorta, respectively.

Whether or not a coupling between A_1-receptors and guanylate cyclase also exists in cell types different from typical vascular smooth muscle cells remains to be clarified. If so, then it would be interesting to test whether or not A_1-receptor-mediated effects of adenosine, such as inhibition of renin release from renal juxtaglomerular cells (36) or the negative chronotropy in myocardial cells (37), are due to the activation of a guanylate cyclase. It might be of interest in this context that cGMP has recently been found to be a strong inhibitory signal for renin release from renal juxtaglomerular cells (38).

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