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Metabolism and Uptake of Adenosine Triphosphate and Adenosine by Porcine Aortic and Pulmonary Endothelial Cells and Fibroblasts in Culture

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SUMMARY Incubation of cultured porcine aortic and pulmonary endothelial cells and mediastinal fibroblasts in the presence of ³H-ATP resulted in the hydrolysis of the nucleotide and the appearance of adenosine, while, simultaneously, a saturable, temperature-dependent uptake of radioactivity was taking place. The same pattern was observed in the three cell types. Adenosine uptake was studied in the same cell populations and also found to be a saturable, temperature-dependent process. The presence of two components for the transport, a high affinity system (K_m of 3 μ M) and low affinity system (K_m of 0.3-1.1 mM), was established in both types of endothelial cells, as well as in fibroblasts (K_m of 8.3 μ M and 0.8 mM). Endothelial cells, however, could be easily differentiated from fibroblasts on the basis of several kinetic features. In the three cell types, adenosine, once taken up, was rapidly phosphorylated under the action of adenosine kinase. No evidence of adenosine deaminase activity was found in intact cells, whereas conversion of adenosine to inosine was observed in a subcellular fraction of sonicated cells. The effect of low temperatures was more marked on adenosine kinase activity than on the uptake process itself. Inosine and adenine had no effect on the transport of adenosine, whereas dipyridamole, at 10^{-6} M, had a very strong inhibitory action. The role of ATP and adenosine in the control of smooth muscle tone could be reexamined in view of their handling by endothelial cells of systemic and pulmonary origins.

IN THE PAST 10 years, several groups of investigators have shown that the lung is capable of degrading and/or taking up various types of substances, among which are biogenic amines such as 5-hydroxytryptamine and norepinephrine, the peptides angiotensin and bradykinin, certain

prostaglandins, and adenine nucleotides.^{1, 2} Binet and Burstein³ were the first ones to report on the disappearance of ATP in the pulmonary circulation. More recently, Ryan and his group investigated this phenomenon, using isolated perfused rat lungs. They found that 5-AMP and ATP were hydrolyzed to yield adenosine and, possibly, deaminated to give rise to inosine, whereas the question of a cellular uptake of these products or of their metabolites was left open.^{4, 5} They also took advantage of the presence of 5'-nucleotidase at the level of the plasma membranes of pulmonary endothelial cells to design a

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Received September 9, 1977; accepted for publication February 10, 1978.

method yielding a preparation of endothelial plasma membranes.⁶

In the course of our systematic study on the differential properties of pulmonary and aortic endothelial cells, we investigated the metabolism and uptake of adenine nucleotides. For this purpose we used cultured endothelial cells taken from the pulmonary artery and the aorta of pigs, as well as mediastinal fibroblasts, and we tested the possible cellular specificity of this phenomenon. The results obtained with ATP indicate not only that there is no difference between endothelial cells of pulmonary and systemic origins, but also that fibroblasts have the same properties as endothelial cells. Furthermore, since it appeared that the uptake of radioactivity by all these cellular populations was associated with the hydrolysis of ATP and the appearance of adenosine in the incubation medium, a more extensive study on the uptake and subsequent intracellular metabolism of this purine nucleoside was undertaken.

Methods

A 0.1% collagenase solution (202 U/ml type I CLS; Worthington Biochemical Corp.) was used to isolate endothelial cells. For subcultures and the isolation of fibroblasts, a 0.2% trypsin solution was made with a buffer containing: NaCl, 137 mM; KCl, 4 mM, Na₂HPO₄, 0.5 mM; KH₂PO₄, 0.15 mM; penicillin, 500 U/ml; and streptomycin, 100 µg/ml. The EDTA solution (0.68 mM) came from Microbiological Associates). Plastic Corning Petri dishes, 35 mm in diameter, and plates of 24 cups, 16 mm in diameter (Costar) were used for cell cultures. The cells were cultured in Dulbecco's medium (Microbiological Associates) containing 7.5% bicarbonate (50 ml/liter), streptomycin (50 µg/ml), penicillin G (50 U/ml), amphotericin (1 µg/ml) (E.R. Squibb), and 20% fetal bovine serum (Microbiological Associates) ("growth medium") or 2% fetal bovine serum ("maintenance medium").

The standard incubation medium consisted of Krebs-Ringer bicarbonate buffer (KRBB) (NaCl, 118 mM; KCl, 4.75 mM; KH₂PO₄, 1.19 mM; MgSO₄, 1.19 mM; CaCl₂, 2.54 mM; NaHCO₃, 25 mM) supplemented with 5 mM glucose and equilibrated with 5% CO₂-95% O₂. The following reagents were bought from Boehringer: adenine, adenosine, disodium salt of adenosine 5'-diphosphate (ADP), inosine, disodium salt of inosine 5'-monophosphate (IMP), disodium salt of inosine 5'-triphosphate (ITP), and disodium salt of adenosine 5'-triphosphate. Disodium salt of adenosine 5'-monophosphate (AMP) came from Fluka A.G., xanthine and hypoxanthine were bought from Sigma Chemical Co., and dipyrindamole was kindly provided by Boehringer. The cellulose F and polyethyleneimine (PEI)-cellulose F thin layer plates came from Merck. [2-³H]adenosine 5'-triphosphate, ammonium salt (16 Ci/mmol), [2-³H]adenosine (24 Ci/mmol), and [8-¹⁴C]inosine (61 mCi/mmol) were purchased from Radiochemical Center.

Isolation of Cells

Isolated aortic and pulmonary endothelial cells were obtained from the descending thoracic aorta and the

pulmonary artery of freshly killed pigs by the collagenase method as previously described.⁷

The fibroblasts were taken from the mesothelium located between the esophagus and the thoracic descending aorta. After dissection, the fragments of tissue were washed three times with phosphate-buffered saline (PBS) (NaCl, 137 mM; KCl, 3 mM; Na₂HPO₄, 8 mM; KH₂PO₄, 1.4 mM; MgCl₂, 0.5 mM; CaCl₂, 1 mM) and centrifuged at 200-300 g. They were exposed to the trypsin solution for a 10-minute period, after which the medium was discarded. The fragments were reexposed to trypsin for about 2 hours; fetal bovine serum was then added to inactivate trypsin and the suspension filtered through gauze. Cells were collected after a 10-minute centrifugation at 200-300 g at room temperature.

Cultures

Endothelial cells and fibroblasts were cultured in Petri dishes in a 5% CO₂-95% air atmosphere, saturated with water vapor, at 37°C, the cellular concentration being always higher than 2 × 10⁵ cells/ml medium. The growth medium was changed 24 hours after plating, and, subsequently, every 5 days, until there was confluence of the cells. At that time, the growth medium was changed to the maintenance medium. Twenty-four hours before the experiment, a last change of maintenance medium was made.

The experiments involving endothelial cells were made on primary cultures only. For fibroblasts, however, cells of various passages (up to 12) were used. For the subcultures, the fibroblasts were rinsed once with the EDTA solution (0.68 mM) and then exposed to a mixture of 0.2% trypsin solution and EDTA (vol/vol 1:4), until the cells came off. Cells were resuspended in growth medium and plated at a maximal 1:5 split ratio.

Measurement of converting enzyme activity was made directly in the Petri dish for the cultured endothelial cells and fibroblasts as previously described.⁸

Experimental Protocol

The incubations were made in the Petri dishes under constant agitation. The culture medium was discarded and the dishes were washed three times with KRBB + 5 mM glucose at 37°C. At the beginning of the experiment, appropriate amounts of substrate were added to the incubation medium, kept at a temperature varying from 4° to 37°C. In the experiments related to ATP hydrolysis, a 100-µl sample of the incubation medium, out of an initial volume of 2 ml, was collected after 5 minutes. At the end of the incubation period, the medium was collected, the cells were washed three times with KRBB at 4°C, and the radioactivity was extracted with 0.4 N perchloric acid. Since 95 ± 3% of the radioactivity (mean ± SE of eight determinations) was recovered in the perchloric acid extract, this extract was judged to be representative of the uptake process. Protein determination was made on the perchloric acid extract after careful scraping of the Petri dishes to detach adhering cells.

In the experiments in which dipyrindamole was used, the drug was added to the incubation medium at the same

time as adenosine. The same sequence was observed when the effect of a substance was tested on the uptake of another compound.

In the experiments in which the efflux of ^3H was measured, cells were incubated in the presence of labeled adenosine for 60 minutes. At the end of this period, the incubation medium was discarded, and the cells were exposed to three rapid washings with KRBB + 5 mM glucose at 37°C. Finally, fresh incubation medium, with or without adenosine, was added, and 10- μl samples were taken at various times for the measurement of their radioactive content. The radioactivity released in the incubation medium was related to the calculated uptake of ^3H before the beginning of the efflux experiment. This value was obtained by adding the ^3H remaining in the cells at the end of the experiment to the total amount of radioactivity released.

Kinase Assay

We used the method of Steck et al.⁹ The endothelial cells were sonicated in 100 mM acetate buffer, pH 6.8, containing 5 mM MgCl_2 and 15 mM NaF. They were then submitted to centrifugation at 30,000 g for 10 minutes at 4°. One ml of the supernatant fluid was incubated for 30 minutes at 4°, 15°, and 37°C, in the presence of labeled adenosine and 5 mM ATP. At the end of the incubation period, 0.5 ml of the incubation medium was added to 0.5 ml of ethanol. The analysis of the radioactivity of the supernatant fluid was made by thin layer chromatography. Protein concentration of the remainder of the suspension was $44 \pm 16 \mu\text{g/ml}$ (mean \pm SE of 20 measurements).

Analytical Procedures

Analysis of the radioactive samples was made by thin layer chromatography on PEI-cellulose and cellulose F plates.^{10, 11} Four systems were used, either separately or sequentially, for the separation of adenine, inosine, adenosine, hypoxanthine, xanthine, and the related nucleotides: for PEI-cellulose F plates, formate-formic acid (0.5 M), LiCl (1 M), and ammonium sulfate (1.5 M); for cellulose F plates we used bidistilled water.

Samples of the incubation and of the cellular extracts were mixed with the scintillation fluid Instagel (Packard). Measurement of radioactivity on the thin layer plates was made by scraping the spots corresponding to the markers and measuring their radioactivity in toluene containing 4% Cab-o-sil, 0.5% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene (Merk). An external standard was used for the measurement of counting efficiency and results were adjusted accordingly. Protein was determined according to the method of Lowry et al.¹²

Results

The identity of cultured endothelial cells was checked according to their morphological appearance by light (phase contrast) microscopy and by the presence of converting enzyme. The activity of this enzyme was 47 ± 7 nmol His-Leu/mg protein per 60 minutes for the aortic endothelial cells ($n = 22$), 170 ± 37 for the pulmonary

endothelial cells ($n = 27$), and 32 ± 23 ($n = 13$) for the fibroblasts (mean \pm SE).

Figures 1-3 illustrate the disappearance of extracellular ATP, when incubated in the presence of pulmonary endothelial cells, aortic endothelial cells, and fibroblasts, and the progressive appearance of adenosine in the incubation medium. An identical pattern can be observed for the three kinds of cells. Thus, about 90% of the ATP initially present was hydrolyzed to yield mainly adenosine, but also 5'-AMP (16-21% of the radioactivity present in the medium at the end of the incubation period). After a 15-minute incubation period, up to one-third of the radioactivity initially present in the incubation medium was found in the cells. This process was dependent on the substrate concentration and on the temperature, as shown by Table 1. Chromatographic analysis of the cell extract indicated that the radioactivity present in the cells was essentially due to ATP (more than 90%). Inosine, adenosine, or hypoxanthine could not be found in the incubation medium or inside the cell.

Since the uptake of radioactivity was taking place at a time when extracellular ATP was rapidly hydrolyzed to give rise to adenosine, it could be inferred that adenosine was the substrate used for the transcellular transport, with a subsequent intracellular rephosphorylation. A more extensive study of adenosine transport and metabolism was therefore undertaken.

The rate of uptake of 10^{-6} M adenosine, as a function of time, was measured in the three kinds of cells at 37°C

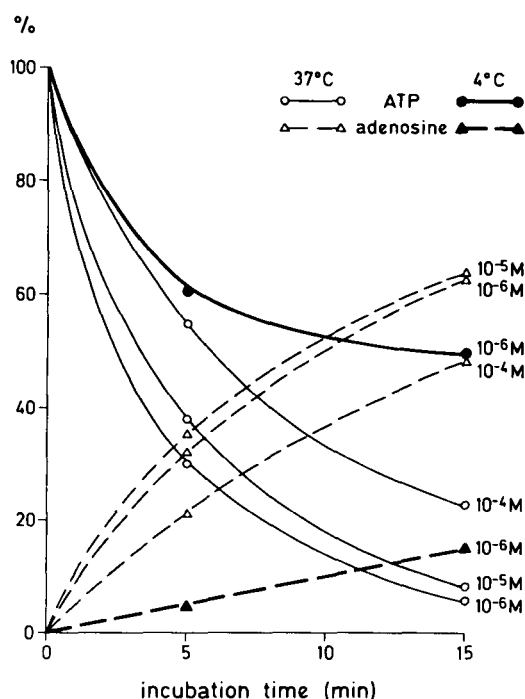


FIGURE 1 Hydrolysis of ATP and production of adenosine by cultured pulmonary endothelial cells. Various concentrations of ^3H -ATP, at 4°C and 37°C, were added to the incubation medium, and the rates of disappearance of ATP and of appearance of adenosine were followed over a 15-minute period. Each point represents the mean of three determinations.

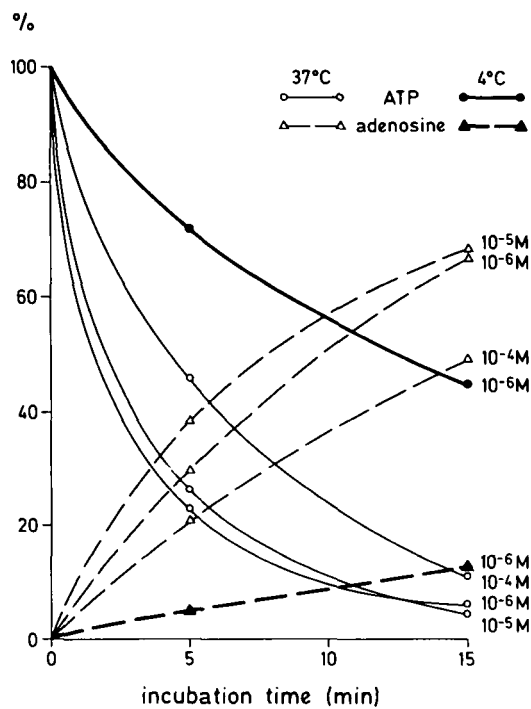


FIGURE 2 Hydrolysis of ATP and production of adenosine by cultured aortic endothelial cells. Various concentrations of ^3H -ATP, at 4°C and 37°C , were added to the incubation medium, and the rates of disappearance of ATP and of appearance of adenosine were followed over a 15-minute period. Each point represents the mean of three determinations.

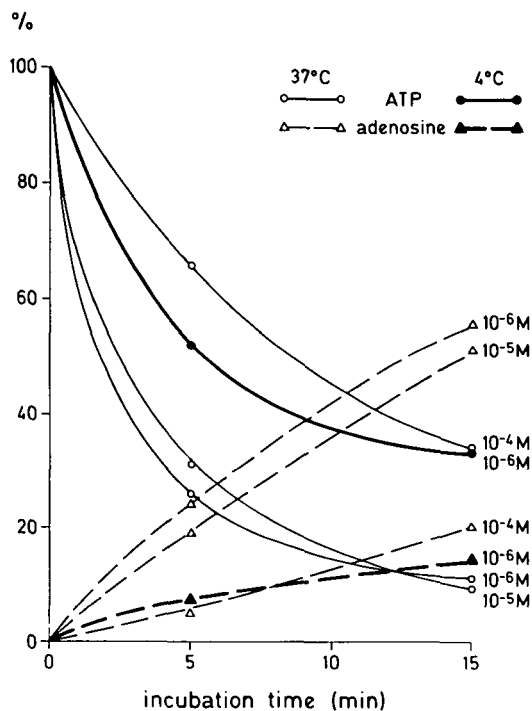


FIGURE 3 Hydrolysis of ATP and production of adenosine by cultured mediastinal fibroblasts. Various concentrations of ^3H -ATP, at 4°C and 37°C , were added to the incubation medium, and the rates of disappearance of ATP and of appearance of adenosine were followed over a 15-minute period. Each point represents the mean of three determinations.

TABLE 1 Concentration of Intracellular ^3H after a 15-Minute Incubation Period in Presence of ^3H -ATP

Substrate concentration (M)	Temperature	Pulmonary endothelial cells	Aortic endothelial cells	Mediastinal fibroblasts
10^{-6}	4°C	0.3	0.3	0.1
10^{-6}	37°C	3.8	3.9	1.8
10^{-5}	37°C	1.6	2.6	1.2
10^{-4}	37°C	1.0	1.0	0.5

After a 15-minute incubation period, the radioactivity present in the cells was related to the radioactive content of the medium. The values are expressed as $\text{dpm}\cdot\text{mg protein}^{-1}/\text{dpm}\cdot\text{ml medium}^{-1}$. Each value represents the mean of two experiments.

(Table 2). In view of the results obtained, a 1-minute incubation period was chosen for the measurement of the initial rate of uptake of adenosine in both types of endothelial cells, whereas, for fibroblasts, a 5-minute incubation period was taken, the uptake being linearly related to the duration of incubation.

The effect of various concentrations of adenosine on the rate of uptake of ^3H measured at 4°C indicated that the concentration of radioactivity was a linear function of the substrate concentration, whereas evidence for saturability was obtained in the experiments done at 37°C . The values measured at 4°C were therefore subtracted from those obtained at 37°C , and a double reciprocal plot was used to analyze the relationship between substrate concentration and the corrected uptake of radioactivity at 37°C (Figs. 4 and 5).

For pulmonary and aortic endothelial cells, there were clearly two different systems for uptake: one with a high affinity (K_m of 3.2×10^{-6} M and 3.0×10^{-6} M and V_{max} of 0.61 and 0.61 nmol/mg protein per minute for the pulmonary and aortic endothelial cells, respectively) for low substrate concentrations, and the other one, with low affinity, operating for substrate concentrations higher than $20 \mu\text{M}$ (K_m of 1.1 mM and 0.3 mM and V_{max} of 6.8 and 5.9 nmol/mg protein per minute). For fibroblasts, a similar pattern was found, with the presence of a high affinity system (K_m of 8×10^{-6} M and V_{max} of 0.50 nmol/mg protein per minute) and of a low affinity system, for adenosine concentrations higher than 0.2×10^{-3} M (K_m of 0.8 mM and V_{max} of 4.0 nmol/mg protein per minute). Analysis of the radioactivity extracted from the endothelial cells and the fibroblasts revealed that, even after 1 minute, ATP accounted for 75% of the intracellular radioactivity, a value of 93% being reached after a 60-minute incubation.

TABLE 2 Rate of Uptake of 10^{-6} M Adenosine as a Function of the Duration of the Incubation Period, at 37°C

Incubation period (min)	Rate of uptake of adenosine (nmol/mg protein per min)		
	Pulmonary endothelial cells	Aortic endothelial cells	Mediastinal fibroblasts
1	0.23 (11)*	0.25 (9)	0.07 (3)
5	0.20 (2)	0.16 (2)	0.07 (3)
15	0.17 (4)	0.20 (4)	0.06 (3)
60	0.20 (3)	0.19 (3)	0.07 (3)

*Numbers in parentheses = the number of experiments.

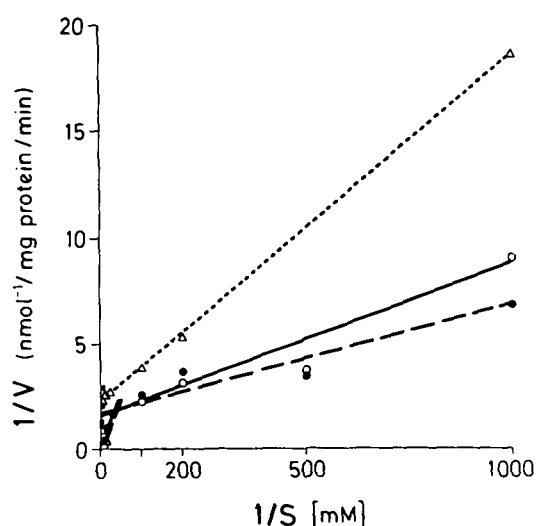


FIGURE 4 Double reciprocal plot relating adenosine concentration to its rate of uptake by porcine pulmonary (○) and aortic (●) endothelial cells and by mediastinal fibroblasts (Δ). Values obtained at 4°C were subtracted from those measured at 37°C. Each point represents the mean of 4–11 determinations.

Because of the subsequent phosphorylation of intracellular adenosine by a kinase system, the rate-limiting step in the uptake of adenosine could have been determined by the kinase activity and not by the uptake process itself. We attempted to dissociate these two elements by measuring the effects of various temperatures on the adenosine kinase activity in the supernatant extract of sonicated cells and on the rate of accumulation of radioactivity by the cells. Table 3 shows the results of these experiments for two concentrations of adenosine, within the range of the high affinity system. Lowering the temperature from 37° to 15°C produced a more important decrease in the kinase activity than in the uptake, particularly at 10^{-5} M adenosine. Not all of the radioactivity analyzed in the measure-

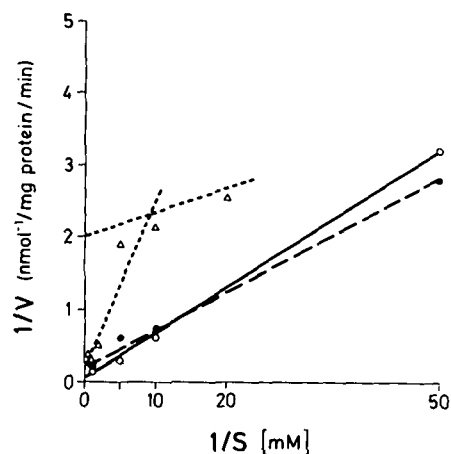


FIGURE 5 Double reciprocal plot relating adenosine concentration (between 0.02 and 2 mM) to its rate of uptake by porcine pulmonary (○) and aortic (●) endothelial cells and by mediastinal fibroblasts (Δ). Values obtained at 4°C were subtracted from those measured at 37°C. Each point represents the mean of 4–8 determinations.

ment of kinase activity could be recovered as adenosine or adenine nucleotides. The remainder was inosine, which accounted for about 30% of the total ^3H at 37°C.

Another possibility for the transport of adenosine inside the cells would have been the formation of inosine, after deamination and dephosphorylation of AMP or deamination of adenosine, followed by the transcellular transport of inosine and its subsequent rephosphorylation and amination. This hypothesis was tested by measuring the effect of inosine at 10^{-6} and 10^{-4} M on the rate of uptake of adenosine by endothelial cells. Table 4 shows the absence of effect of inosine at 10^{-6} M and its slight action at 10^{-4} M, whereas the uptake of inosine was clearly inhibited in the presence of high concentrations of adenosine; most of the radioactivity present in the cells following the incubation in the presence of ^{14}C inosine was recovered as ATP (87% and 88%). Adenine and adenosine had no effect on the uptake of each other (Table 4).

Dipyridamole, a known inhibitor of adenosine uptake,¹³ also acted very efficiently on endothelial cells (Table 4), as well as on fibroblasts (unpublished results).

Finally, experiments were done to test the mobility of the ATP formed, following adenosine uptake. After incubation with ^3H -adenosine which resulted, as shown before, in the intracellular formation of ^3H -ATP, the cells were exposed either to fresh medium alone or to a medium containing unlabeled adenosine at 10^{-6} and 10^{-4} M. The efflux of radioactivity into the incubation medium was followed over a 15-minute period. Spontaneous release of radioactivity was rather slow (20% in 15 minutes) and was not accelerated in the presence of low and high concentrations of adenosine in the medium (Fig. 6).

Discussion

The use of cultured cells was judged necessary to obtain intact, viable cells, without any release of cytoplasmic enzymes that could interfere with the physiological pattern. Thus, adenosine deaminase, although present in endothelial cells, as can be inferred from the production of inosine by a suspension of sonicated cells, does not seem to act on the adenosine taken up. Different K_m values for adenosine deaminase and adenosine kinase or a phenomenon of compartmentalization of these enzymes^{14, 15} may be the reason for this lack of involvement of adenosine deaminase. The measurement of converting enzyme activity, a marker for the identity of endothelial cells,¹⁶ was used to validate these preparations of cultured cells, and the results indicate that pulmonary endothelial cells could be easily differentiated from fibroblasts. The low enzyme activity measured in aortic endothelial cells could be explained by their utilization at a stage of nonoptimal confluence, since recent experiments (unpublished observations) indicate that converting enzyme activity increased abruptly only several days after cultured endothelial cells became confluent. Examination of the data in Tables 1 and 2 and Figures 4 and 5 also suggests that both aortic and pulmonary endothelial cells, because of their comparable behavior, can be considered as similar, homogeneous cell populations, differing from fibroblasts in several respects.

TABLE 3 *Effect of Temperature on the Rate of Uptake of Adenosine and on Adenosine Kinase Activity*

	10 ⁻⁶ M Adenosine			10 ⁻⁵ M Adenosine		
	37°C	15°C	4°C	37°C	15°C	4°C
Adenosine uptake (nmol/mg protein per min)						
Pulmonary endothelial cells	0.23 ± 0.03 (11)*	0.10 ± 0.01 (3)	0.04 ± 0.01 (3)	0.60 ± 0.07 (8)	0.40 ± 0.02 (3)	0.23 ± 0.05 (4)
Aortic endothelial cells	0.25 ± 0.05 (9)	0.19 ± 0.09 (3)	0.05 ± 0.01 (3)	0.60 ± 0.05 (10)	0.56 ± 0.07 (3)	0.21 ± 0.03 (3)
Kinase activity (pool pulmonary artery-aorta)						
% Adenosine	7 ± 2	49 ± 3	76 ± 2	31 ± 4	76 ± 2	90 ± 1
% Nucleotides	60 ± 4 (5)	13 ± 2 (5)	4 ± 0 (4)	28 ± 2 (4)	4 ± 1 (4)	4 ± 2 (3)

The uptake of adenosine was measured during a 1-minute incubation period. For the measurement of kinase activity, a subcellular suspension of endothelial cells was incubated in the presence of 10⁻⁶M ³H-adenosine for 30 minutes and the percentage of adenosine transformed into adenine nucleotides was measured. The data obtained from pulmonary and aortic cells were pooled. Values are expressed as means ± se.

* Numbers in parentheses = the number of experiments.

Analysis of the results obtained with ATP clearly demonstrates a comparable pattern with all these three cell populations. Presence of phosphatase and of 5'-nucleotidase activity cannot be restricted to endothelial cells, even on a quantitative basis; the existence of the pulmonary endothelial specificity of the histochemical reaction reported by Ryan and Smith⁶ therefore can be questioned. We obtained the same histochemical results with cultured fibroblasts, in accord with our metabolic measurements (unpublished observations).

The uptake of radioactivity during incubation with ³H-ATP generally is thought to result from the transport of adenosine after the hydrolysis of ATP, and this phenomenon has also been observed and extensively analyzed by other investigators^{17, 18} in other cellular systems. Only Chaudry et al.¹⁹ obtained evidence for a direct transport of ATP by kidney slices. The very rapid rate of formation of extracellular adenosine in our preparations also made likely the existence of a transport system operating for adenosine, and the presence of such a process has been amply demonstrated in the subsequent experiments on adenosine uptake.

The handling of ATP by these cells might be important

to consider when the physiological effect of extracellular adenine nucleotides is considered. ATP has been proposed by Burnstock²⁰ to be the possible mediator of the purinergic system or nonadrenergic inhibitory system. The presence of endothelium and fibroblasts in the vicinity of the ATP released by nerves endings of this system would therefore alter the effect of this mediator by shortening the duration of its action. This situation would be analogous to the extraneuronal uptake of norepinephrine, as defined by Iversen.²¹

Adenosine uptake by various cell types recently has been the object of a number of investigations, and the type of transport involved has been characterized. For red cell ghosts, the transcellular passage of adenosine seems to be due to facilitated diffusion for low concentrations (less than 10 μM), with a K_m of 7.5 μM, and to simple diffusion for higher concentrations.²² There is also evidence for a more complex system with two components, as demonstrated by Strauss et al.²³ with murine lymphocytes. They found two saturable types of transport, with a K_m of 12 and 400 μM; there is a striking similarity to our results obtained with endothelial cells and fibroblasts. Other investigators have studied the uptake of adenosine

TABLE 4 *Effect of Various Substances on the Uptake of Adenosine, Inosine, or Adenine*

Substrate, 10 ⁻⁶ M	Substance tested	Concentration (M)	Uptake as % of control	
			Pulmonary endothelial cells	Aortic endothelial cells
Adenosine	Inosine	10 ⁻⁶	91 ± 5 (4)*	97 ± 3 (3)
Adenosine	Inosine	10 ⁻⁴	84 ± 4 (4)	87 ± 2 (3)
Inosine	Adenosine	10 ⁻⁶	117 ± 2 (3)	100 ± 2 (4)
Inosine	Adenosine	10 ⁻⁴	23 (2)	37 ± 2 (4)
Adenosine	Adenine	10 ⁻⁶	112 ± 17 (3)	92 ± 5 (3)
Adenosine	Adenine	10 ⁻⁴	102 ± 23 (4)	104 ± 17 (3)
Adenine	Adenosine	10 ⁻⁴	107 ± 3 (3)	81 (2)
Adenosine	Dipyridamole	10 ⁻⁶	5 ± 1 (3)	5 ± 1 (4)
Adenosine	Dipyridamole	10 ⁻⁷	100 (2)	98 (2)

The effect of various substances, at different concentrations, on the uptake of substrates at 10⁻⁶ M was tested during a 1-minute incubation period. Results are expressed as percentage of the value obtained in the control experiment, where the uptake of the substrate alone was measured (means ± se).

* Numbers in parentheses = the number of experiments.

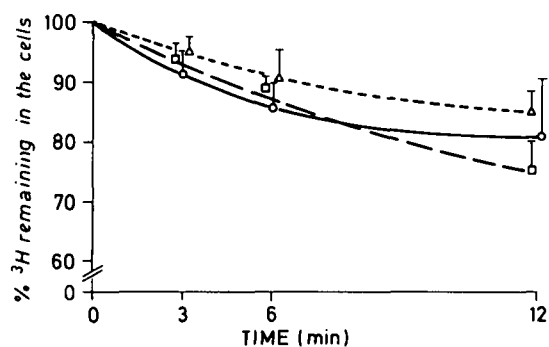


FIGURE 6 Efflux of ^3H from cultured pulmonary endothelial cells. After a 60-minute incubation period in the presence of 1×10^{-6} M adenosine, the initial medium was replaced by fresh medium alone (O) or a medium containing 10^{-6} M (Δ) or 10^{-4} M (\square) adenosine, and the release of ^3H was followed over a 15-minute period. Each point represents the mean \pm SD of three determinations.

by chick fibroblasts,²⁴ rabbit polymorphonuclear leukocytes,²⁵ Novikoff rat hepatoma cells,²⁶ and human lymphocytes,¹⁸ and found a single saturable system with a K_m value between 1.2 and 10 μM . In a whole organ (heart),¹⁵ a K_m value of 11.6 μM was measured. In a few of these studies, however, was the effect of substrate concentrations higher than 10^{-4} M tested, so that the existence of a low affinity system could have been missed.

In nearly all the cellular systems tested, adenosine uptake was followed by rapid phosphorylation under the effect of adenosine kinase, and the product found intracellularly was ATP. The general agreement is that transport, rather than phosphorylation, is the rate-limiting step for the uptake of adenosine. This view is based on the inhibitory effect of dipyridamole on transport and the absence of an effect of this drug on adenosine kinase,¹⁷ the different K_m found for transport and for kinase activity, and on the fact that other nucleosides are transported in a similar fashion when cells are devoid of kinase activity or are ATP depleted.^{17, 27} In our study on endothelial cells, we also obtained indirect evidence that transport was the rate-limiting step by showing the differential effect of temperature on the rate of uptake and on kinase activity, the latter being much more sensitive to the lowering of temperature than the transport process. Scholtissek,²⁸ working on chick fibroblasts, reached similar conclusions.

Other possibilities were considered: adenosine could have been transformed into adenine as with *Escherichia coli*²⁹ by a purine nucleotide phosphorylase, but no adenine could be detected, either in the medium or in the cell, and adenine at high concentration had no effect on adenosine uptake, as already shown by Holmsen and Rozenberg³⁰ in platelets. However, unlike the results reported for platelets, an inhibitory effect of adenosine on adenine uptake could not be found in endothelial cells. Adenosine could have been deaminated to yield inosine, which would have been the substrate for the transport process. However, no evidence for deaminase activity, i.e., the presence of inosine, could be found in the incubation medium except when broken cells were used,

and inosine, at high concentrations, had no major effect on adenosine transport.

Finally, mention can be made of the relative lack of mobility of the ATP formed from the transport of adenosine. The radioactive ATP newly formed was released at a slow rate into the extracellular medium, and the addition of an excess of precursor did not accelerate the efflux. Further studies on the utilization of the ATP thus formed and its possible conversion to cyclic AMP under the effect of adenylyl cyclase, known to be present in endothelial cells,³¹ are in progress.

Although it might be hazardous to extrapolate from our results that endothelial cells and fibroblasts elsewhere in the body, or in other species, share the same properties as the ones we have studied, this possibility certainly exists. The fact that endothelial cells are ubiquitous, and particularly well placed to control the inflow of mediators, may lead to a reconsideration of their function in the physiological and pharmacological action of adenosine. This substance is known to affect the coronary circulation and skeletal muscle flow,³²⁻³⁴ the tone of smooth muscle,³⁵ the formation of cyclic AMP,³⁶ the release of insulin by the pancreas,³⁷ and the control of norepinephrine release.³⁸ The question of the role of adenosine and of adenosine triphosphate as mediators can be reexamined in view of their fate in the circulation at the endothelial level. The hypothesis already suggested by others,^{39, 40} according to which endothelial cells could act as couplers between circulating substances and the target cell or organ, is in line with the handling of vasoactive agents by endothelial cells, but the proof of the existence of receptors at the level of these cells and of the coupling mechanisms still must be established. Alternatively, the hydrolysis of ATP and the uptake of adenosine by endothelial cells could be considered as important processes in the local control and modulation of the effects of these mediators.

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Circ Res. 1978;42:869-876

doi: 10.1161/01.RES.42.6.869

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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