Biochimica et Biophysica Acta 893 (1987) 43–48
Elsevier

BBA 42566

Na^+/adenosine co-transport in *Vibrio parahaemolyticus*

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(Received 10 February 1987)

Key words: Adenosine; Sodium ion; Co-transport; *(V. parahaemolyticus)*

Adenosine transport in *Vibrio parahaemolyticus* was studied. Na^+ greatly stimulated adenosine uptake. Addition of adenosine to a cell suspension under anaerobic conditions elicited Na^+ uptake, and the Na^+ uptake was inhibited by monensin, an Na^+ ionophore. Imposition of an electrochemical potential of Na^+ or a membrane potential in energy-depleted cells elicited adenosine uptake. Therefore, adenosine transport in this organism was concluded to proceed by an Na^+/adenosine co-transport mechanism. The Na^+/adenosine co-transport system was induced when cells were grown in the presence of adenosine, and repressed by glucose. Although Na^+ uptake elicited by adenosine was reduced by glucose, it was enhanced by methyl α-glucoside, which reduced the intracellular ATP level. Thus, the effects of glucose and the glucoside on the Na^+/adenosine co-transport system did not seem to be due to inducer exclusion, but to be related to the intracellular ATP level.

Introduction

Vibrio parahaemolyticus, a slightly halophilic marine bacterium, can grow with ATP, ADP, AMP or adenosine as the sole source of carbon. This organism, like other *Vibrio species*, possesses outwardly oriented 5′-nucleotidase in its cell membrane. Thus, extracellular 5′-nucleotides are dephosphorylated by the 5′-nucleotidase to adenosine, which is then utilized as a nutrient [1]. Therefore, cells of *V. parahaemolyticus* presumably have very high activity for adenosine uptake, which is sufficient to support cell growth on adenosine.

Na^+/substrate co-transport is a main mechanism of nutrient transport in halophilic bacteria [2]. Most amino acid transport systems and the sucrose transport system in *Vibrio alginolyticus* are reported to be Na^+-dependent [3]. We found that *V. parahaemolyticus* has an Na^+/serine co-transport system [4]. However, we found that this organism also has H^+/substrate co-transport systems (Toda, K. and Tsuchiya, T., unpublished data), like *Escherichia coli* [5]. Since *V. parahaemolyticus* possesses a high ability to utilize adenosine, we became interested in energy coupling in adenosine transport in this organism.

Here we report that *V. parahaemolyticus* possesses an Na^+/adenosine co-transport system and describe the properties of this system.

Materials and Methods

V. parahaemolyticus AQ3334 was grown aerobically at 37 °C in medium S2 consisting of 50 mM Mop-Tris (pH 7.5)/0.2 M NaCl/25 mM $MgSO_4$/10 mM KCl/1 mM $CaCl_2$/0.01 mM $FeSO_4$/0.33 mM K_2HPO_4/10 mM $(NH_4)_2SO_4$

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; NMe_4OH, tetramethylammonium hydroxide; Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium ion.

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supplemented with 0.5% polypeptone and 20 mM potassium lactate. Unless otherwise indicated, 3 mM adenosine was added to the medium. Cells were harvested in the late exponential phase, and washed twice with buffer consisting of 0.2 M Mops-Tris (pH 7.5)/10 mM $MgSO_4$. For measurement of Na^+ uptake, cells were washed twice with 0.2 M Mops/NMe_4OH (pH 7.5). For studies on adenosine transport driven by artificial driving forces, cells were treated as follows. After harvest, they were washed twice with 10 mM Mops-Tris (pH 7.5)/25 mM $MgSO_4$/0.2 M Na_2SO_4, suspended in the same buffer containing 5 mM dinitrophenol, and shaken at 37° C for 30 min to starve them of energy. They were then divided into two portions. One portion was washed twice with buffer consisting of 50 mM Mes-Tris (pH 6.5)/10 mM $MgSO_4$/0.2 M Li_2SO_4, and the other portion was washed with the same buffer but with Na_2SO_4 instead of Li_2SO_4. The cells were then incubated at 37° C for 10 min to load them with Li^+ or Na^+, and then each portion was further divided into two portions. The Li^+-loaded cells were washed either with 50 mM Mes-Tris (pH 6.5)/10 mM $MgSO_4$/0.2 M Li_2SO_4 or with 50 mM Tricine-Tris (pH 8.5)/10 mM $MgSO_4$/0.2 M Li_2SO_4, and suspended in the same buffer containing 2 mM KCN and 2 μM CCCP. The Na^+-loaded cells were washed with similar buffer containing Na_2SO_4 instead of Li_2SO_4, and suspended in the same buffer containing KCN and CCCP.

Assay mixtures for adenosine transport contained 0.2 M Mops-Tris (pH 7.5)/10 mM $MgSO_4$/20 mM Tris-lactate and cells (about 0.1 mg protein/ml). Transport was initiated by addition of [^{3}H]adenosine (0.1 mM), and samples were taken at intervals, and filtered. In the case of the artificially driven system, transport was started by diluting each type of cell with assay mixture consisting of 50 mM Tricine-Tris (pH 8.5)/10 mM $MgSO_4$/0.2 M Na_2SO_4/10 mM KCN/20 μM CCCP/50 μM [^{3}H]adenosine.

Na^+ uptake was measured in reaction mixture consisting of 0.2 M Tricine-NMe_4OH (pH 8.5)/0.1 mM NaCl and cells (2–3 mg protein/ml), using an Na^+-selective electrode, as described previously [6].

Protein contents [7], ATP contents [8] and the intracellular water space [9] (2.9 μl/mg cell protein) were determined by published procedures. [2-^{3}H]Adenosine was purchased from Amersham.

Results

Effect of Na^+ on adenosine uptake

If adenosine transport is coupled to Na^+, its transport should be stimulated by Na^+. Therefore, we measured adenosine transport in cells in the absence and presence of Na^+. We observed much greater uptake of adenosine in the presence of 10 mM Na^+ than in its absence (Fig. 1). Fig. 2 shows the effects of the external Na^+ concentration on the transport of adenosine. Maximal stimulation was attained at about 10 mM Na^+. At higher Na^+ concentrations, the transport activity decreased to some extent, and at 100 mM Na^+ the activity was about 50% of the maximum (data not shown). For some unknown reason, a double-reciprocal plot was not linear, so the K_m could not be determined, but it was estimated to be a few mM.

We tested the effects of various salts on adenosine uptake (Table I). The transport was stimulated about 4-fold by NaCl, and slightly by KCl, LiCl and NH_4Cl. Na_2SO_4 and $NaNO_3$ had similar effects to NaCl, while $NaOCOCH_3$ caused slightly less stimulation. Thus, the stimulation of adenosine transport was due specifically to Na^+. It should be pointed out, however, that Na^+ stimulates the respiratory chain of *V. parahaemolyticus* [4], so its stimulation of transport of adenosine might have been direct or indirect.

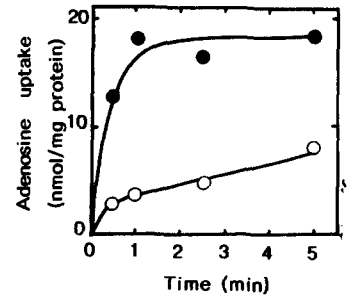

Fig. 1. Stimulation of adenosine transport by Na^+. Uptake of adenosine was measured in assay mixture consisting of 0.2 M Mops-Tris (pH 7.5)/10 mM $MgSO_4$/20 mM Tris-lactate and 0.13 mg protein/ml of cells and 0.1 mM [^{3}H]adenosine, with (●) or without (○) 10 mM NaCl.

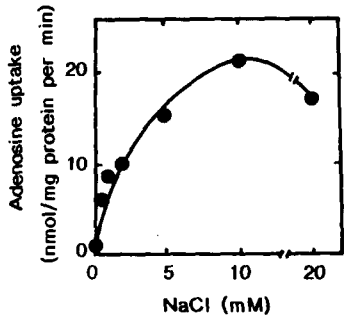

Fig. 2. Effect of Na^+ concentration on adenosine transport. Adenosine uptake was measured in the same assay mixture as for Fig. 1, but with the indicated concentrations of NaCl.

Na^+/adenosine co-transport

If the observed stimulation of adenosine transport by Na^+ is due to Na^+/substrate co-transport, then we should observe (1) Na^+ uptake caused by substrate influx, and (2) substrate uptake driven by an artificially imposed electrochemical potential of Na^+ in energy-starved cells. Demonstrations of these two phenomena should be the most convincing lines of evidence for Na^+/substrate co-transport.

First, we tested whether addition of adenosine to a cell suspension under anaerobic conditions elicited Na^+ uptake. As shown in Fig. 3, when a small volume of anaerobic adenosine solution was added to cells, we detected Na^+ uptake by the cells. Addition of 0.1 mM adenosine resulted in a velocity of the Na^+ uptake of about 10 ng ions/min per mg cell protein. This Na^+ uptake was not observed when monensin, an Na^+ ionophore, was present (20 μM) in the assay mixture.

TABLE I

EFFECTS OF VARIOUS SALTS ON ADENOSINE UPTAKE

Cells were preincubated in assay mixtures containing various salts (10 mM) at 25 °C for 5 min, and [^{3}H]adenosine (0.1 mM) was added to initiate transport. Samples were taken after 30 s for measurement of adenosine uptake.

Salt	Adenosine uptake (nmol/mg protein at 30 s)
Control	2.9
NaCl	12.7
KCl	5.5
LiCl	5.2
NH_4Cl	5.0
Na_2SO_4	13.4
$NaNO_3$	12.2
$NaOCOCH_3$	9.7

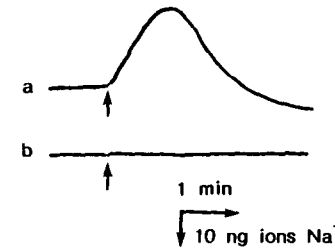

Fig. 3. Uptake of Na^+ elicited by adenosine. Cells were suspended in 0.2 M Tricine-NMe_4OH (pH 8.5)/0.1 mM NaCl in the absence (a) or presence (b) of 20 μM monensin, and incubated at 25 °C under anaerobic conditions. The concentration of extracellular Na^+ was monitored with an Na^+-selective electrode. An anaerobic solution of adenosine was added to a final concentration of 0.1 mM at the times indicated by arrows. Upward deflection represents decrease of extracellular Na^+.

We next tested whether an artificially imposed electrochemical potential of Na^+ caused adenosine uptake. For this, cells were shaken in a buffer containing 5 mM dinitrophenol for 30 min to starve them of energy. This treatment drastically reduced the intracellular ATP level from a few mM in unstarved cells to less than 0.1 mM. Then we imposed a membrane potential ($\Delta\psi$; interior negative) as a diffusion potential of H^+ in the presence of the H^+ ionophore CCCP. A concentration gradient of Na^+ (Δp*Na*; inwardly directed) was imposed by diluting cells that had been shaken and washed in the absence of Na^+ with buffer containing a high concentration of Na^+. The membrane potential drove transient adenosine uptake, and greater uptake was observed when an Na^+ gradient was imposed simultaneously (Fig. 4). Imposition of an Na^+ gradient alone did not drive significant adenosine uptake. However, when the Na^+ gradient was imposed in the presence of TPP^+, a membrane-permeable cation, some uptake of adenosine was observed.

Thus, we obtained the two most convincing lines of evidence for Na^+/adenosine co-transport in *V. parahaemolyticus*.

Induction and repression of the Na^+/adenosine co-transport system

In the experiments described above, we used cells grown in the presence of adenosine. These

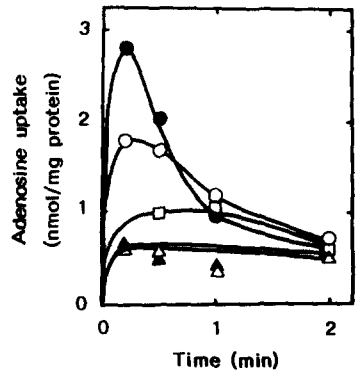

Fig. 4. Adenosine uptake elicited by artificial driving forces. Artificial driving forces were imposed on energy-starved cells and then their adenosine uptake was measured. A membrane potential ($\Delta\psi$; inside negative) was imposed as a diffusion potential of H^+ in the presence of CCCP. An inwardly directed concentration gradient of Na^+ (ΔpNa) was imposed by the addition of Na^+ to the external medium. For details, see Materials and Methods. Symbols: △, control (no driving force); ▲, ΔpNa; □, ΔpNa in the presence of 1 mM TPP^+; ○, $\Delta\psi$; ●, $\Delta\psi + \Delta pNa$.

cells showed fairly high activity of Na^+/adenosine co-transport. However, when cells were grown in the absence of adenosine, their co-transport activity was very low (Fig. 5). When the co-transport activity was measured as Na^+ uptake caused by adenosine, the initial velocity was about 4-times more in cells grown in the presence of adenosine than in cells grown in its absence. Thus, we concluded that the Na^+/adenosine co-transport system was induced by adenosine.

Many metabolic enzymes and transport carriers are repressed when cells are grown in the presence of glucose, so we tested whether the Na^+/adenosine co-transport system was also subject to glu-

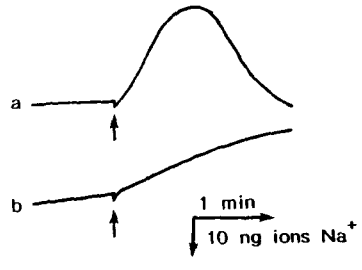

Fig. 5. Induction of the Na^+/adenosine co-transporter by adenosine. Uptake of Na^+ elicited by the addition of adenosine was measured as for Fig. 3 in cells grown in the presence (a) or absence (b) of 3 mM adenosine.

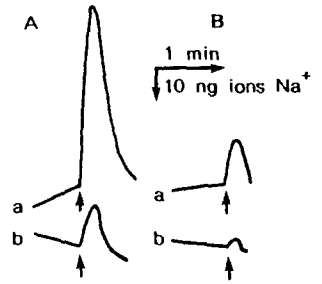

Fig. 6. Effect of glucose on the Na^+/adenosine co-transport system. Uptake of Na^+ elicited by the addition of adenosine was measured as for Fig. 3. A, Cells grown in the presence of 3 mM adenosine were used. Uptake of Na^+ was measured in the absence (a) or presence (b) of 10 mM glucose. B, Cells grown in the presence of both adenosine (3 mM) and glucose (10 mM) were used. The effect of glucose was tested as in A.

cose repression. Fig. 6 shows that cells grown in the presence of glucose possessed much lower activity of Na^+/adenosine co-transport. Furthermore, the co-transport was much less when glucose was present in the assay mixture. Thus, the Na^+/adenosine co-transport system was repressed by glucose, and glucose seemed to inhibit the adenosine carrier. Namely, the carrier seemed to be subject to inducer exclusion [10]. If this were the case, methyl α-glucoside should also inhibit the carrier. However, methyl α-glucoside did not inhibit Na^+/adenosine co-transport but, in fact, markedly stimulated adenosine-induced Na^+ uptake (Fig. 7). Thus, the effects of glucose and methyl α-glucoside were not consistent with inducer exclusion. We do not know how glucose inhibited Na^+/adenosine co-transport and how methyl α-glucoside enhanced it, but we obtain

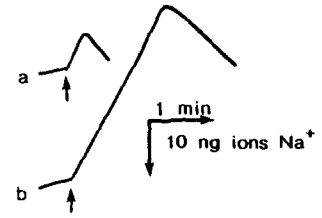

Fig. 7. Stimulation of Na^+/adenosine co-transport by methyl α-glucoside. Uptake of Na^+ elicited by adenosine was measured in cells grown in the presence of both adenosine (3 mM) and glucose (10 mM). Adenosine (final concentration, 0.1 mM) was added at the times indicated by arrows in the absence (a) or presence (b) of 10 mM methyl α-glucoside.

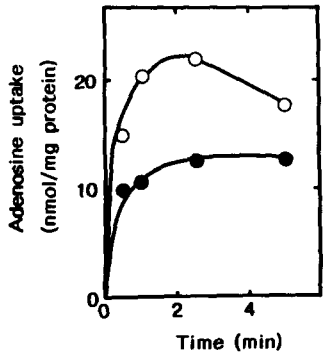

Fig. 8. Effect of monensin on adenosine uptake. Adenosine transport was measured for Fig. 1 in the presence of 10 mM NaCl. Symbols: ○, control; ●, plus 0.1 mM monensin.

indicative data. Namely, we found that the intracellular ATP level of cells incubated in the presence of glucose was 5.3 mM, whereas that of cells incubated with methyl α-glucoside was 0.2 mM. The ATP level of untreated cells was 1.3 mM. Thus, it seems likely that ATP or a closely related compound regulates the activity of the Na^+/adenosine co-transport system. It should be pointed out that incubation with methyl α-glucoside is a recognized procedure for energy-starvation [11].

Possible existence of another adenosine transport system

Unexpectedly, we found that monensin completely inhibited Na^+ uptake induced by adenosine (Fig. 3). This indicates that in this organism monensin acts as an Na^+ ionophore, although it has no effect in whole cells of most Gram-negative bacteria. However, it caused only about 50% inhibition of adenosine uptake (Fig. 8). This finding suggests the presence of another transport system for adenosine that is not coupled to Na^+. With respect to this possibility, the data in Table I are indicative of the presence of another system: namely, addition of Cl^- (KCl, LiCl or NH_4Cl) to the assay mixture slightly increased adenosine uptake over the control level (in SO_4^{2-}).

Discussion

Cells of *V. parahaemolyticus* can grow with adenosine as the sole source of carbon. From this fact, we thought that this organism might have high activity for adenosine transport, so we investigated its adenosine transport. Since many transport systems for nutrients in halophilic bacteria are Na^+/substrate co-transport systems [2], we tested whether adenosine transport proceeded by an Na^+/adenosine co-transport mechanism. The results described in this paper are consistent with the view of Na^+/adenosine co-transport. We observed large transient uptake of adenosine when an artificial electrochemical potential of Na^+ was imposed, but not when an Na^+ gradient alone was imposed. The reason for this is not clear. However, similar results have been reported for the Na^+/α-aminoisobutyric acid co-transport of *V. alginolyticus* [12]. Perhaps, when an Na^+ gradient is the driving force, a back pressure (reverse membrane potential) produced by Na^+/adenosine co-transport would have to be dissipated. Our observation that adenosine was taken up to some extent when an Na^+ gradient was imposed in the presence of TPP^+, a membrane-permeable cation which is expected to dissipate the membrane potential, supports this view.

Adenosine is reported to be taken up by facilitated diffusion by *Vibrio harveyi* cells [13], as by animal cells [14]. *E. coli* is reported to possess at least two energy-dependent transport systems for adenosine [15–17], which are distinguishable by their sensitivities to the adenosine analogue antibiotic showdomycin. Osmotic shock of *E. coli* cells causes reduction in the transport of adenosine [18]. Membrance vesicles prepared from *E. coli* show limited transports of cytidine and uridine, but not of adenosine [18]. Thus, the driving force for adenosine transport in *E. coli* does not seem to be an ion-motive force. In other words, it is probably not due to an Na^+/adenosine or H^+/adenosine co-transport system. Munch-Petersen and Pihl [19] reported that addition of glucose to the assay mixture lowered the uptake of purine nucleoside by *E. coli*. They found that the intracellular ATP level was closely correlated with the purine nucleoside transport activity, the transport activity being low when the ATP level was high. Our result reported here seem to represent a similar phenomenon. The mechanism of this effect of the ATP level on adenosine transport is unknown.

E. coli cells possess another mechanism for the

utilization of adenosine [20]; that is, adenosine is first cleaved, and then adenine is taken up by adenine phosphoribosyltransferase [21]. Since *V. parahaemolyticus* is unable to grow with adenine as the sole source of carbon (unpublished observation), it seems that it does not have this system.

Nevertheless, the present results suggest the existence of another transport system(s) for adenosine besides the Na^+/adenosine co-transporter in *V. parahaemolyticus*. We are now characterizing such a system.

Acknowledgments

We thank Dr. Sumio Shinoda of this Faculty for providing *V. parahaemolyticus* AQ3334. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Mishima Foundation.

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