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Na^+-DRIVEN Ca^{2+} TRANSPORT IN ALKALOPHILIC *BACILLUS*

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Summary

Ca^{2+} transport was studied in membrane vesicles of alkalophilic *Bacillus*. When Na^+-loaded membrane vesicles were suspended in $KHCO_3$/KOH buffer (pH 10) containing Ca^{2+}, rapid uptake of Ca^{2+} was observed. The apparent K_m value for Ca^{2+} measured at pH 10 was about 7 μM, and the K_m value shifted to 24 μM when measured at pH 7.4. The efflux of Ca^{2+} was studied with Ca^{2+}-loaded vesicles. Ca^{2+} was released when Ca^{2+}-loaded vesicles were suspended in medium containing 0.4 M Na^+.

Ca^{2+} was also transported in membrane vesicles driven by an artificial pH gradient and by a membrane potential generated by K^+-valinomycin in the presence of Na^+.

These results indicate the presence of Ca^{2+}/Na^+ and H^+/Na^+ antiporters in the alkalophilic *Bacillus* A-007.

Introduction

An alkalophilic *Bacillus* sp. (A-007), which was isolated from soil, requires an extreme alkaline environment for optimal growth. Transport activities of organic nutrients in the organism were found to be greatest in alkaline pH and the systems were found to be Na^+-dependent [1], although the intracellular pH in this organism has been shown to be neutral.

Ca^{2+} is one of the physiologically important cations for *Bacillus*, therefore, we studied the Ca^{2+}-transport system in alkalophilic *Bacillus* A-007.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Materials and Methods

Cells and cultivation. Alkalophilic *Bacillus* A-007 was grown in GPY * medium aerobically at 42°C and cells were obtained in a manner similar to that described earlier [1].

Preparation of membrane vesicles. Membrane vesicles were prepared by using the lysozyme-protoplasts method [1]. Cells were suspended in 0.1 M sodium phosphate buffer (pH 6.8) containing 10 mM $MgCl_2$ and lysozyme (0.2 mg/ml), and incubated at 37°C. After about 10 min of incubation, almost all the cells were converted to protoplasts. Protoplasts were precipitated by centrifugation (30 000 × *g*, 10 min) and the precipitates were suspended in 25 mM Hepes/KOH buffer (pH 7.4) containing 10 mM $MgCl_2$ and deoxyribonuclease (1 μg/ml), and homogenized by using Teflon homogenizer. Unbroken cells were precipitated by low-speed centrifugation (800 × *g*, 10 min), the supernatant centrifuged (30 000 × *g*, 10 min) and the precipitate (membrane) washed three times with 25 mM buffer (Hepes/KOH for pH 7.4 and $KHCO_3$/KOH for pH 10.0) containing 10 mM $MgCl_2$ and 0.4 M NaCl (for Na^+-loaded vesicles). For preparation of acid-loaded vesicles, the membranes were washed with 25 mM Hepes/KOH buffer (pH 6.8) containing 10 mM $MgCl_2$, 5 mM NaCl and 0.4 M sucrose.

Measurement of Ca^{2+} transport

Transport of Ca^{2+} into membrane vesicles. Membrane vesicles were suspended in a medium (total 1 ml) containing 25 mM buffer (Hepes/KOH for pH 7.4 and $KHCO_3$/KOH for pH 10.0), 10 mM $MgCl_2$, 0.4 M sucrose, 0.1 or 0.2 mM $^{45}CaCl_2$ (50 Ci/mol) (at 37°C). At intervals, 100 μl of the mixture were filtered through a membrane filter (pore size 0.45 μm, Toyo Roshi Co. Ltd., Japan), washed four times with the medium without substrate (total 15 ml) and dried. The radioactivity on the filter was measured by a gas-flow counter. The initial rate of transport was estimated from the linear portion of uptake curves (usually within 40 s), and the activity was expressed as nmol/mg protein per min.

Efflux of Ca^{2+} from membrane vesicles. Ca^{2+}-loaded vesicles were obtained from a Ca^{2+}-transport experiment in Na^+-loaded vesicles and the vesicles were washed once with 25 mM $KHCO_3$/KOH buffer (pH 10) containing 0.4 M sucrose and 10 mM $MgCl_2$. Ca^{2+}-loaded vesicles were suspended in 1 ml of medium containing 25 mM $KHCO_3$/KOH (pH 10), 10 mM $MgCl_2$ and 0.4 M sucrose or NaCl. In some cases, an ionophore or 2 mM $CaCl_2$ was added to the medium. Efflux was started by the addition of Ca^{2+}-loaded vesicles. At intervals, 100 μl of the mixture were filtered through a membrane filter and the radioactivity on the filter was measured by a gas-flow counter.

Estimation of protein concentration. Protein was determined by using the method of Lowry et al. [2] using bovine serum albumin as a standard.

Radioisotope, ionophores and other chemicals. Carrier-free $^{45}CaCl_2$ (20 Ci/mg) was purchased from New England Nuclear (U.S.A.). FCCP was obtained from Boeringer Mannheim (F.R.G.) and valinomycin from Sigma Chemical Co.

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* GPY, 10 g glucose, 5 g polypeptone, 5 g yeast extract, 1 g K_2HPO_4, 2 g $MgSO_4 \cdot 7\,H_2O$ and 20 g Na_2CO_3 in 1 l.

(U.S.A.). Monensin was a generous gift from Dr. N. Ootake of our institute and A-23187 was also presented by Eli Lilly Co. (U.S.A.). Other chemicals used were the best grade commercially obtainable.

Results

Transport of Ca^{2+} into membrane vesicles driven by an Na^+ gradient at alkaline pH

Previous studies with membrane vesicles of alkalophilic *Bacillus* A-007 have shown that the uptak of organic nutrients in this organism is driven primarly by an Na^+ gradient. Therefore, we assumed the presence of an Na^+-coupled Ca^{2+}-transport system in A-007 cells. For measurement of Ca^{2+} uptake, gradients of Na^+ opposite to normal ($[Na^+]_{in} > [Na^+]_{out}$) were arranged by loading Na^+. Vesicles loaded with 0.5 M NaCl were suspended in 25 mM $KHCO_3$/KOH (pH 10.0) containing varying concentrations of NaCl. In this manner, various magnitudes of the Na^+ gradient were established.

Fig. 1 shows Ca^{2+}-uptake activity at varying magnitudes of the Na^+-gradient. The initial rates of transport were proportional to the magnitudes of the Na^+ gradient imposed. Fig. 1 also shows that about 7 nmol of Ca^{2+} were bound per mg protein of membrane vesicles. Uptake activities, from which bound Ca^{2+} was subtracted, are shown in the following figures.

The effect of various ionophores on Na^+-driven Ca^{2+} transport is shown in Figs. 2 and 3. Neither FCCP (proton conductor) nor valinomycin (K^+ ionophore) inhibited the reaction. Monensin (Na^+ ionophore) and A-23187 (Ca^{2+} ionophore) inhibited the activity.

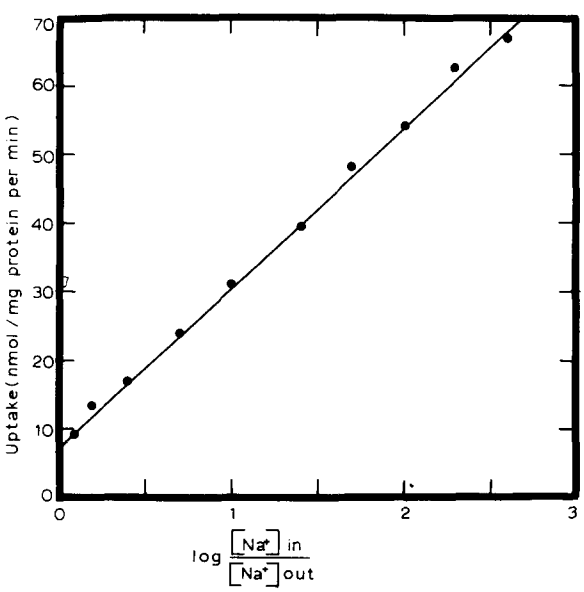
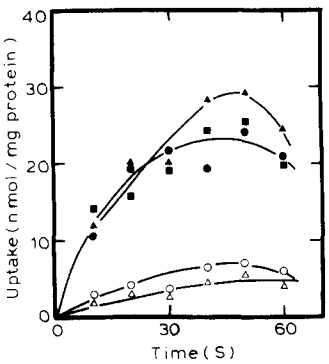


Fig. 1. Effect of NaCl on transport of Ca^{2+}. Membrane vesicles were prepared in 25 mM $KHCO_3$/KOH (pH 10.0) containing 10 mM $MgCl_2$ and 0.5 M NaCl. Transport was assayed in 25 mM $KHCO_3$/KOH (pH 10.0) containing 10 mM $MgCl_2$, membrane vesicles (0.5 mg protein), 0.2 mM $^{45}CaCl_2$, and NaCl and sucrose (total 0.5 M).



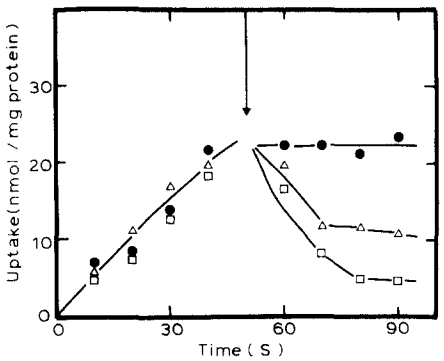


Fig. 2. Effect of ionophores on Ca^{2+} transport driven by an Na^+ gradient. Membrane vesicles were prepared in 25 mM Hepes/KOH (pH 7.4), 10 mM $MgCl_2$ and 0.4 M NaCl. Transport was assayed in 25 mM Hepes/KOH (pH 7.4), 10 mM $MgCl_2$, 0.4 M sucrose, membrane vesicles (0.5 mg protein), 0.2 mM $^{45}CaCl_2$ and ionophore. ●, without ionophore; ▲, FCCP (4 μg/ml); ■, valinomycin (4 μg/ml); △, gramicidin (4 μg/ml); ○, 0.4 M NaCl was added outside vesicles to abolish the Na^+ gradient.

Fig. 3. Effect of A-23187 and monensin on Ca^{2+} transport driven by an Na^+ gradient. Membrane vesicles were prepared in 25 mM $KHCO_3$/KOH (pH 10.0), 10 mM $MgCl_2$ and 0.4 M NaCl. Transport was assayed in 25 mM $KHCO_3$/KOH (pH 10.0), 10 mM $MgCl_2$, 0.4 M sucrose, membrane vesicles (0.5 mg protein) and 0.2 mM $^{45}CaCl_2$. At the time indicated by the arrow, A-23187 (□, 10 μg/ml) or monensin (△, 4 μg/ml) was added. ●, without ionophore.

Effect of pH on the kinetics of Ca^{2+} transport

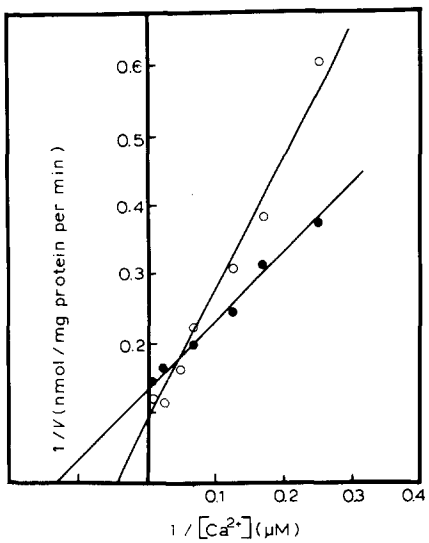
Since Ca^{2+}-uptake activity is dependent on the pH values in the vesicles and of the medium, we tried to determine the effect of pH on the kinetics of transport. Lineweaver-Burk plots of the transport activity at pH 7.4 and 10.0 (both sides of the vesicles) are shown in Fig. 4. The K_m value of the transport system for Ca^{2+} decreased about 4-fold at pH 10.0 compared with at pH 7.4 ($6.7 \cdot 10^{-6}$ M at pH 10.0, $2.4 \cdot 10^{-5}$ M at pH 7.4), although the maximum velocity changed slightly ($7.7 \cdot 10^{-9}$ mol/mg protein per min at pH 10.0, $12.5 \cdot 10^{-9}$ mol/mg protein per min at pH 7.4).

Ca^{2+} transport driven by $\Delta\psi$ or ΔpH

$\Delta\psi$ or ΔpH alone could not drive Ca^{2+} transport (data not shown), and the Ca^{2+}-transport process was obligatorily coupled to the antiport of Na^+. If, however, Na^+/H^+ antiport is present in the vesicles of this organism, Ca^{2+} must be accumulated by $\Delta\psi$ or ΔpH in the presence of Na^+ by circulation of Na^+ via Na^+/H^+ antiport. For this purpose, we prepared acid-loaded (for pH gradient) and K^+-loaded (for $\Delta\psi$) vesicles. These vesicles were used for the transport experiment. As shown in Figs. 5 and 6, Ca^{2+} was accumulated in the vesicles in the presence of Na^+ (in = out).

Efflux of Ca^{2+} from Ca^{2+}-loaded vesicles

Membrane vesicles were isolated by centrifugation after the maximum level of $^{45}Ca^{2+}$ accumulation was reached in Na^+-driven Ca^{2+} transport. The vesicles were washed once with 25 mM $KHCO_3$/KOH (pH 10.0) containing 0.4 M sucrose and 10 mM $MgCl_2$. The efflux of Ca^{2+} from the $^{45}Ca^{2+}$-loaded vesicles was examined. One example of the efflux experiments is shown in Fig. 7.



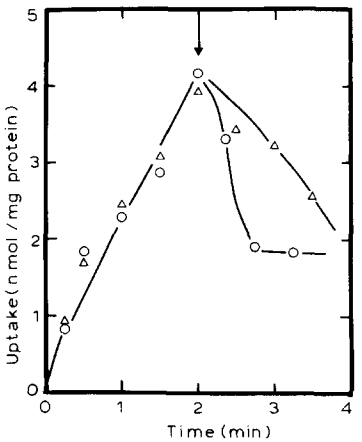
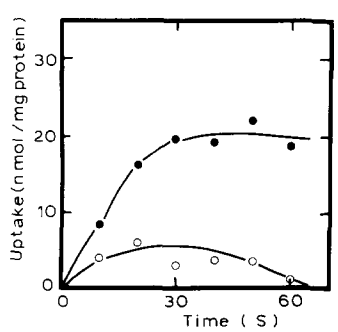


Fig. 4. Effect of Ca^{2+} concentration on Ca^{2+}-transport activity driven by an Na^+ gradient. Experimental conditions were similar to those of Fig. 3, except that the Ca^{2+} concentration was varied. ○, at pH 7.4; ●, at pH 10.0.

Fig. 5. Ca^{2+} transport driven by a proton gradient in the presence of Na^+. Membrane vesicles were prepared in 25 mM Hepes/KOH (pH 6.8) containing 10 mM $MgCl_2$, 5 mM NaCl and 0.4 M sucrose. Transport was assayed in 25 mM $KHCO_3$/KOH (pH 10.0), 10 mM $MgCl_2$, 5 mM NaCl, 0.1 mM $^{45}CaCl_2$ and membrane vesicles (0.25 mg protein). At the time indicated by the arrow, A-23187 (○, 10 μg/ml) was added. △, control.

Release of $^{45}Ca^{2+}$ was limited when the vesicles were suspended in 25 mM $KHCO_3$/KOH (pH 10.0) containing 0.4 M sucrose and 10 mM $MgCl_2$. However, when 0.4 M sucrose was replaced by 0.4 M NaCl, rapid release of $^{45}Ca^{2+}$ was observed. The accumulated $^{45}Ca^{2+}$ was also released when a large excess of



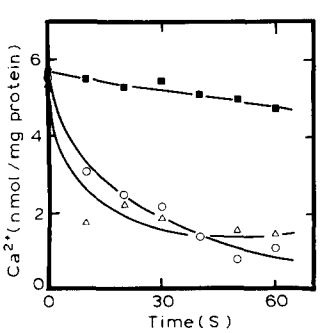


Fig. 6. Transport of Ca^{2+} into membrane vesicles driven by a membrane potential in the presence of Na^+. Membrane vesicles were prepared in 25 mM Hepes/KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM NaCl, and 0.4 M KCl. Transport was assayed in 25 mM Hepes/KOH (pH 7.4) containing 10 mM $MgCl_2$, 10 mM NaCl, 0.4 M sucrose and 0.2 mM $^{45}CaCl_2$. The reaction was started by the addition of valinomycin (4 μg/ml, ●). ○, control.

Fig. 7. Effect of Na^+ and Ca^{2+} on the Ca^{2+} efflux from Ca^{2+}-loaded vesicles at alkaline pH. Ca^{2+} was transported into membrane vesicles driven by an Na^+-gradient for 50 s at pH 10.0. Ca^{2+}-loaded vesicles were washed and resuspended in a small amount of a medium of the following composition; $KHCO_2$/KOH (25 mM, pH 10.0), $MgCl_2$ (10 mM) and sucrose (0.4 M). ■, control; △, sucrose was replaced by NaCl. ○, $CaCl_2$ (2 mM) was added to the medium, For further details, see Materials and Methods.

$CaCl_2$ (2 mM) was added. Monensin and gramicidin inhibited the Na^+-dependent release of Ca^{2+}, and FCCP had no effect on the release (data not shown).

Discussion

The results of the present study indicated that Ca^{2+} was transported in membrane vesicles of alkalophilic *Bacillus* A-007. The transport was driven by an Na^+-gradient (in > out), and the system was active at alkaline pH. Ca^{2+} uptake followed Michaelis-Menten kinetics, and the K_m value for Ca^{2+} at pH 7.4 was $2.4 \cdot 10^{-5}$ M and $6.7 \cdot 10^{-6}$ M at pH 10.0, although had the same magnitude at both these pH values. These results indicate that the Ca^{2+}/Na^+ antiport system, which requires alkaline pH for its activity, is present in the alkalophilic *Bacillus* A-007.

Ca^{2+} transport was also demonstrated in membrane vesicles by an artificial pH gradient and by a membrane potential generated by K^+-valinomycin in the presence of Na^+. In these studies, Na^+ circulation via an Na^+/H^+ antiporter may play a key role in Ca^{2+} accumulation. Ca^{2+} accumulated in the vesicles is largely in the free form, since accumulated Ca^{2+} exists easily when Na^+ is added to the external medium.

An Na^+-dependent Ca^{2+}-transport system in bacteria has been reported only in *Halobacterium halobium* [3]. Other systems are H^+-dependent [4,5] or dependent on the energy of ATP hydrolysis [6,7]. However, in eukaryotic cells, several examples of Na^+-dependent Ca^{2+}-transport systems have been reported [8]. The similarity of the system for Ca^{2+} transport in alkalophilic *Bacillus* to the systems in eukaryotic cells may be an example of convergent evolution between these different membranes. The Ca^{2+}-transport system in the alkalophilic *Bacillus* may provide a excellent tool for the study of the Na^+/Ca^{2+} antiporter.

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