Characterization of a Ca^{2+} Uniporter from *Bacillus subtilis* by Partial Purification and Reconstitution into Phospholipid Vesicles

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Ca^{2+} was accumulated by right-side-out membrane vesicles of *Bacillus subtilis* following imposition of a diffusion potential, inside-negative, owing to K^{+} -efflux via valinomycin. Uptake was dependent on the magnitude of the membrane potential. This voltage-dependent Ca^{2+} uptake was inhibited by Ca^{2+} channel blockers such as nitrendipine, verapamil and LaCl_3 , and was competitively inhibited by Ba^{2+} and Sr^{2+} . The system showed saturation kinetics with an apparent K_m for Ca^{2+} of about $250\ \mu\text{M}$. Proteins responsible for the voltage-dependent Ca^{2+} uptake were partially purified by preparative isoelectric focusing in a Sepharose bed. A fraction at pH 5.28–5.33 contained the activity. The characteristics of Ca^{2+} uptake in reconstituted proteoliposomes were the same as those in membrane vesicles (sensitive to Ca^{2+} channel blockers; inhibited by Ba^{2+} and Sr^{2+}). In addition, uptake was not influenced by a pH gradient imposed on the vesicles. The apparent K_m for Ca^{2+} in the reconstituted system was about $260\ \mu\text{M}$. The specific activity was increased about 50-fold by purification with isoelectric focusing.

INTRODUCTION

The study of Ca^{2+} transport systems in bacterial cells has shown that $\text{Ca}^{2+}/\text{H}^{+}$ and $\text{Ca}^{2+}/\text{Na}^{+}$ antiporters are responsible for extruding Ca^{2+} (Brey & Rosen, 1979). Recently, we purified and characterized the antiporter from *Bacillus subtilis* (Matsushita *et al.*, 1986). Conversely, the Ca^{2+} uptake system found in *Azotobacter vinelandii* (Zimniak & Barnes, 1980) and *Streptococcus faecalis* (Kobayashi *et al.*, 1978) was voltage (membrane-potential)-dependent.

Ca^{2+} is indispensable for sporulation in *B. subtilis* and Ordal (1977) reported that Ca^{2+} is required for the chemotactic response of this organism. Therefore, it is important to study the mechanism of Ca^{2+} uptake. Recently, a membrane-potential-dependent Ca^{2+} uptake system in *B. subtilis* was found by de Vrij *et al.* (1985). In this paper we describe the characterization and partial purification of the membrane protein responsible for transport.

METHODS

Preparation of right-side-out membrane vesicles. *Bacillus subtilis* W23 was grown in GYP medium (glucose, 1%; yeast extract, 0.5%; polypeptone, 0.5%), and the cells were harvested in mid-exponential phase by centrifugation. The cells were washed once with 50 mM-potassium phosphate buffer (pH 8.0). Right-side-out vesicles were prepared by the method described by Konings *et al.* (1973), and the final pellet was suspended in 50 mM-potassium phosphate buffer (pH 6.8).

Transport assay. Ca^{2+} uptake was driven by an artificial membrane potential. Membrane vesicles were suspended in 50 mM-potassium phosphate buffer (pH 6.8) containing 0.35 M-KCl, and incubated at 45°C for 20 min (potassium loading). MgCl_2 was then added (final concn 2.5 mM) and the suspension was centrifuged at $105\,000\ g$ for 30 min; the pellet (K^{+} -loaded vesicles) was then suspended in 0.5 ml of a solution containing 50 mM-sodium phosphate buffer (pH 6.8), 0.35 M-choline chloride, 2.5 mM- MgCl_2 and 0.4 mM- $^{45}\text{CaCl}_2$. The suspension

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPMP⁺, triphenylmethylphosphonium ion.

was incubated at 35 °C, and then the reaction was started by the addition of 1 μ l valinomycin (1 mg ml⁻¹). At suitable time intervals, samples (50 μ l) were filtered through membrane filters (0.45 μ m pore size; Toyo Science Co., Tokyo). Membrane vesicles on the filter were washed three times with 2.5 ml of medium without ⁴⁵CaCl₂. The radioactivity on the filter was measured with a gas flow counter. Binding of Ca²⁺ to the membrane vesicles, which reached a maximum value after 30 s at 35 °C [0.50–0.60 nmol Ca²⁺ bound (mg membrane protein)⁻¹] was subtracted from the total uptake value. Initial rates of transport were estimated and expressed as nmol Ca²⁺ transported min⁻¹ (mg protein)⁻¹.

Preparation of membrane protein. Membrane proteins were prepared as described by Matsushita *et al.* (1986). In *B. subtilis*, membrane proteins are released from the membrane and accumulated in the cells as water-insoluble protein aggregates during membrane autolysis (Kusaka *et al.*, 1976).

Cells (wet wt 10 g) were suspended in 0.1 M-sodium phosphate buffer (pH 6.8; total vol. 250 ml) containing 1 mM-ZnCl₂ and lysozyme (150 μ g ml⁻¹). After about 2 h incubation at 37 °C DNAase (0.5 mg) was added and the lysate was centrifuged at 40000 *g* for 25 min. The pellet (membrane protein aggregates) was washed once with 25 mM-Tris/H₂SO₄ (pH 7.2) and suspended in the same buffer containing 6 M-urea (final vol. 100 ml). The suspension was incubated in an ice-bath for 3 h to permit solubilization by urea, and then centrifuged at 156000 *g* for 2 h. The released membrane proteins were recovered in the supernatant.

Preparative isoelectric focusing. Preparative isoelectric focusing in Sepharose CL-6B (Pharmacia) using a flat-bed apparatus FBE 3000 (Pharmacia) was done as described by Radola (1974). About 50 mg protein was mixed with 30 ml Sepharose CL-6B and 2.5 ml Pharmalyte (pI 4.0–6.5; Pharmacia; 40%, v/v). After 12 h electrophoresis, the gel was scraped from the bed, divided into 20 fractions, and eluted with 6 mM urea. Voltage-dependent Ca²⁺ uptake activity was found in the fractions between pH 5.20–5.40. For further purification, the gel (pH 5.20–5.40; a total of 2.2 mg protein in 3.5 ml gel) was transferred into a small trough (1.0 $\times$ 1.0 cm; 6 cm long) and isoelectric focusing was repeated for another 12 h. After electrophoresis, the gel was cut into 10 equal portions and protein was eluted with 1.0 ml 25 mM-Tris/H₂SO₄ (pH 7.2) containing 6 M-urea. The eluted protein was salted out by the addition of 0.55 g solid (NH₄)₂SO₄ to 1 ml of protein solution (above 80% saturation) and Ca²⁺ uptake activity was estimated.

Formation of proteoliposomes and assay of transport activity. Proteins (5–10 μ g) and asolectin (6 mg) were mixed in 25 mM-Tris/H₂SO₄ (pH 7.2) containing 0.4 M-KCl and sonicated for 2 min (10 kHz, 100 W) at room temperature. Then MgCl₂ was added (final concn 2.5 mM) and the sonicate was centrifuged at 105000 *g* for 30 min. The pellet was suspended in 0.5 ml of a solution containing 25 mM-Tris/H₂SO₄ (pH 7.2), 0.4 M-choline chloride, 2.5 mM-MgCl₂ and 0.4 mM-⁴⁵CaCl₂. Ca²⁺ uptake was started by the addition of valinomycin (final concn 1 μ g ml⁻¹) at 30 °C. The other methods were the same as those for the assay of transport in membrane vesicles. Binding of Ca²⁺ to the proteoliposomes (about 80 pmol Ca²⁺ was bound to 1 mg asolectin) was subtracted from the total uptake value. The initial rate of transport was estimated and expressed as nmol Ca²⁺ min⁻¹ (mg protein)⁻¹.

Protein determination. This was by the Lowry method.

Chemicals. ⁴⁵CaCl₂ [18 μ Ci μ mol⁻¹; (666 kBq μ mol⁻¹)] was purchased from New England Nuclear; all other reagents were of analytical grade.

SDS-PAGE. The method of Laemmli (1970) was used.

RESULTS

Ca²⁺ uptake by right-side-out membrane vesicles

Right-side-out membrane vesicles of *B. subtilis* accumulated Ca²⁺ in the presence of 20 mM-ascorbate and 0.1 mM-phenazinemethosulphate as an electron donor system. Uptake was inhibited by the uncoupler FCCP (2 μ M), but not by the electroneutral K⁺/H⁺ exchanger nigericin (data not shown), indicating that the membrane potential was the driving force for Ca²⁺ uptake in the vesicles, as reported by de Vrij *et al.* (1985). This conclusion was supported by Ca²⁺ uptake experiments using membrane vesicles on which artificial membrane potentials were imposed. A K⁺-diffusion membrane potential, inside-negative, was generated in K⁺-loaded vesicles by the addition of valinomycin. This potential drove Ca²⁺ uptake into the vesicles by a process that was inhibited (Fig. 1) by the membrane-permeant cation TPMP⁺.

The rate of Ca²⁺ uptake by the vesicles (estimated from the initial velocity of uptake) was measured over a range of membrane potentials generated by the establishment of a K⁺ gradient across the membrane in the presence of valinomycin. K⁺-loaded vesicles (0.4 M-internal K⁺ concn) were suspended in solutions containing various concentrations of KCl. The rate of Ca²⁺ uptake was proportional to log([K⁺]_{in}/[K⁺]_{out}) (Fig. 2). The membrane potentials generated by

Fig. 1

Fig. 2

Fig. 1. Ca²⁺ uptake driven by a K⁺-diffusion potential in right-side-out vesicles of *B. subtilis*. K⁺-loaded vesicles (1 mg protein) were suspended in 0.5 ml 50 mM-sodium phosphate buffer (pH 6.8) containing 0.35 M-choline chloride (○), plus 1 mM-TPMP⁺ (□). Uptake was initiated by the addition of 1 µg valinomycin. Valinomycin was not added in the control experiment (●). All the experiments were done in the presence of 0.4 mM-⁴⁵CaCl₂ and 2.5 mM-MgCl₂ as described in Methods.

Fig. 2. Voltage-dependency of Ca²⁺ uptake in right-side-out vesicles of *B. subtilis*. K⁺-loaded vesicles were suspended in 0.5 ml 50 mM-sodium phosphate buffer (pH 6.8) containing various K⁺ concentrations (1, 4, 20, 40, 100, 200 and 400 mM); the final cation concentration in the reaction mixture was adjusted to 0.4 M with choline chloride. All the experiments were done in the presence of 0.4 mM ⁴⁵CaCl₂ and 2.5 mM-MgCl₂.

Table 1. Effect of Ca²⁺ channel blockers on the voltage-dependent Ca²⁺ uptake system in *B. subtilis*

Ca ²⁺ channel blocker	Concn (µM)	Uptake activity [nmol Ca ²⁺ min ⁻¹ (mg protein) ⁻¹]	Percentage of control
None	—	1.61	100
Nitrendipine	1.0	0.61	38
	10.0	0.22	14
Verapamil	25.0	1.01	62
	50.0	0.65	40
LaCl ₃	100.0	0.28	17
	250.0	0.05	3

K⁺-diffusion, and estimated from changes in fluorescence of the dye 3,3'-dipropylthiodicarbocyanine [diS-C₃(5)] (Waggoner, 1976), were proportional to log([K⁺]_{in}/[K⁺]_{out}) (data not shown). Therefore, the rate of uptake of Ca²⁺ (Fig. 2) was dependent on the magnitude of the membrane potential (voltage). It is well-known that Ca²⁺ flux through voltage-dependent Ca²⁺ channels in animal excitable membranes is inhibited by blockers such as nitrendipine, verapamil and LaCl₃. Therefore, we investigated the effect of these drugs on Ca²⁺ uptake by membranes of *B. subtilis*. Uptake was driven by K⁺-diffusion potentials and was inhibited by all of these drugs, though verapamil was not so effective as nitrendipine (Table 1).

Ca²⁺ uptake showed saturation kinetics and a Lineweaver-Burk plot of the data (not shown) indicated a *K_m* for Ca²⁺ of about 250 µM. Uptake was competitively inhibited by Ba²⁺ and Sr²⁺, and *K_i* was calculated to be 930 µM for Ba²⁺ and 370 µM for Sr²⁺.

Fig. 3. Patterns of protein distribution after the second isoelectric focusing on Sepharose CL-6B. The pH 5.20–5.40 fraction from the first isoelectric focusing (pH 4.0–6.5) was isolated and isoelectric focusing was repeated in a small trough for another 12 h. Gels were cut into 10 fractions and protein was estimated in each fraction. ●, Protein; ■, pH; ○, Ca²⁺ uptake activity.

Isolation and reconstitution of the protein responsible for the voltage-dependent Ca²⁺ uptake system

Membrane proteins (110 mg) released during membrane autolysis of *B. subtilis* were dissolved in 50 ml 25 mM-Tris/H₂SO₄ (pH 7.2) containing 6 M-urea, and subjected to (NH₄)₂SO₄ fractionation. The fraction precipitated at 35–55% (w/v) saturation (active fraction) was isolated by centrifugation. The pellet (about 50 mg) was dissolved in 2.5 ml distilled water and dialysed against 2 l distilled water. Solid urea was added to the retentate to give a urea concentration of 6 M (final volume was about 4 ml and the pH of solution was 6.4). The protein solution was mixed with 30 ml semi-dried Sepharose CL-6B and Pharmalyte (final concn 3.3%, v/v; pI 4.0–6.5) and subjected twice to isoelectric focusing (Fig. 3). Voltage-dependent Ca²⁺ uptake activity was found after reconstitution of the fractions at pH 5.28–5.33 (see below). SDS-PAGE of these fractions revealed six protein components with molecular masses of 64, 47 (major protein), 42, 36 (major), 32 and 20 kDa (Fig. 4). Further purification is needed to obtain a homogeneous preparation of the Ca²⁺ uptake system.

Proteins of pI 5.28–5.33 (5–10 µg) were reconstituted into asolectin (6 mg) liposomes. Ca²⁺ uptake into these proteoliposomes could be driven by a membrane potential, inside negative, generated by K⁺-diffusion in the presence of valinomycin. Specific activity was increased about 50-fold by the second isoelectric focusing [125 nmol Ca²⁺ min⁻¹ (mg protein)⁻¹].

Bacterial nutrient transport is often coupled to H⁺ flux; therefore we studied the effect of a pH gradient on Ca²⁺ uptake. The gradient was imposed on K⁺-loaded vesicles and voltage-dependent uptake was started by the addition of valinomycin. Uptake was inhibited at pH values under 5.0 and above 8.5. In addition, proteoliposomes were formed in 25 mM-Tris/H₂SO₄, pH 8.0 (pH 8.0 vesicles) or 25 mM-MES/Tris, pH 5.5 (pH 5.5 vesicles); pH 8.0 vesicles were then suspended in 25 mM-MES/Tris (pH 5.5) and pH 5.5 vesicles were suspended in 25 mM-Tris/H₂SO₄ (pH 8.0). Ca²⁺ uptake was not influenced by these pH gradients (the results for pH 8.0 vesicles are shown in Fig. 5).

Uptake activity in the proteoliposomes was inhibited 62% by 10 µM-nitrendipine, 40% by 25 µM-verapamil, 88% by 100 µM-LaCl₃, 50% by 1 mM-BaCl₂ and 75% by 1 mM-SrCl₂ (Fig. 5). This effect was comparable to those found in the membrane vesicles (see Table 1).

The *K_m* for Ca²⁺ in the reconstituted system was about 260 µM, which is close to the value for the membrane vesicles.

DISCUSSION

Ca²⁺ uptake in *B. subtilis* was studied extensively by de Vrij *et al.* (1985). The results in the present paper agree with their work. We have further characterized the transport system and shown it to be sensitive to Ca²⁺ channel blockers of animal excitable membranes.

Fig. 4

Fig. 5

Fig. 4. SDS-PAGE of membrane protein and partially purified Ca²⁺ uniporter (pI 5.28–5.33). Proteins were stained with Coomassie blue. Molecular mass standards are indicated on the left. Lane 1, initial membrane protein; lane 2, pI 5.28–5.33 fraction.

Fig. 5. Ca²⁺ uptake driven by a K⁺-diffusion potential in proteoliposomes containing the pH 5.28–5.33 fraction. K⁺-loaded proteoliposomes (5–10 µg protein) were suspended in 25 mM-Tris/H₂SO₄ (pH 7.2) containing 0.4 M-choline chloride. Uptake was initiated by addition of 1 µg valinomycin. Inhibitors added were nitrendipine (10 µM, ▲); verapamil (25 µM, ■) LaCl₃ (100 µM, □); BaCl₂ (1 mM, △) or SrCl₂ (1 mM, ○). ●, No inhibitor added. Uptake activity was also assayed in K⁺-loaded proteoliposomes in which a pH gradient, inside alkaline, was imposed (▽).

Membrane proteins in *B. subtilis* were readily released during membrane autolysis and isolated as water-insoluble protein aggregates. Using this membrane protein as a starting material, we partially purified a voltage-dependent Ca²⁺ uptake system. This system was further characterized by reconstitution into liposomes, and the sensitivity of Ca²⁺ uptake to Ca²⁺ channel blockers was confirmed. The sample was not homogenous, and comprised six protein components as shown by SDS-PAGE. The activity was stable during purification in 6 M-urea: therefore, membrane protein isolation by the present method could be used for further purification in future work.

Ca²⁺ uptake into the reconstituted proteoliposomes was not influenced by a pH gradient, and appeared to depend only on the membrane potential: therefore, the Ca²⁺ uptake system may be a voltage-dependent uniporter.

The inhibitory effect of the Ca²⁺ channel blockers, especially nitrendipine, was an interesting pointer for future studies.

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