The presence of H^+ and Na^+-linked Ca^{2+} extruding systems in *Methanobacterium thermoautotrophicum*

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Abstract The effects of monovalent cations (Na^+, K^+ and choline$^+$) and the uncoupler 3,3′,4′,5-tetrachlorosalicylanilide (TCS) were tested on $^{45}Ca^{2+}$ uptake by non-energized cells of *Methanobacterium thermoautotrophicum*. $^{45}Ca^{2+}$ uptake was stimulated by the addition of K^+ and (less) by choline$^+$ while Na^+ slowed down and even reversed it, thereby mimicking the energization of cells. The uncoupler agent, TCS, suppressed $^{45}Ca^{2+}$ uptake in non-energized cells in the presence or absence of Na^+ but in cells energized in an atmosphere of CO_2+H_2 it exerted a stimulating effect. Uncoupled $^{45}Ca^{2+}$ efflux was measured in cells pre-loaded with $^{45}Ca^{2+}$ by means of the divalent ionophore A23187 following its washing out by buffer containing serum albumin. The efflux was temperature-dependent and was stimulated by external $^{40}Ca^{2+}$ and Na^+. In the absence of Na^+, the uncoupled efflux was completely inhibited by TCS, whereas in the presence of Na^+, TCS was without any effect. The results are in agreement with the model in which the Ca^{2+} influx pathway is represented by a membrane potential-driven uniport whereas Ca^{2+} efflux is mediated by two transport systems – Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters – whose participation in the total efflux is dependent on the energy of the corresponding gradients of driving ions.

Key words: Ca^{2+} transport; Methanogen; *Methanobacterium thermoautotrophicum*

1. Introduction

Calcium ions play pivotal roles in the regulation of various cellular processes in eukaryotic as well as prokaryotic cells. Intracellular free calcium concentrations are generally maintained at very low levels, (0.1–1 μM) and are regulated either by secondary transport systems or by ATP-dependent calcium pumps [1]. Few data on Ca^{2+} homeostasis are available in the Archaea which have been previously classified as the third kingdom of life [2].

Only recently has evidence been obtained in *Halobacterium halobium* [3] and *Methanobacterium thermoautotrophicum* [4], belonging to the Archaea, that they also possess the transport system(s) for Ca^{2+} ions. The transport of Ca^{2+} in *H. halobium* was shown to proceed by Na^+/Ca^{2+} antiport, which is a eukaryotic mechanism and unexpected in bacteria. On the basis of our experimental results, we have suggested that cells of *M. thermoautotrophicum* possess Ca^{2+} transport systems across the cytoplasmic membrane which involves movements of Ca^{2+} in both directions [4]. In this connection, it should be noted that the energetics of some methanogens, including *M. thermoautotrophicum*, possess special features: their ATP synthesis can be driven by H^+ and Na^+ electrochemical gradients via two (Na^+ and H^+) selective ATP synthases [5,6]. These findings have raised two essential questions: (1) What are the components of Ca^{2+} homeostasis in *M. thermoautotrophicum*? (2) What are the factors controlling Ca^{2+} movement across the cytoplasmic membrane? The results presented here provide some answers to these questions which could also shed some light on the evolutionary aspects of Ca^{2+} transporting systems in general.

2. Materials and methods

M. thermoautotrophicum strain ΔH was used in experiments and was cultivated as described previously [4].

For $^{45}Ca^{2+}$ uptake measurement the procedure described in [4] was used.

$^{45}Ca^{2+}$ loading and measurement of $^{45}Ca^{2+}$ efflux were performed as follows: the suspension of bacteria in 50 mmol l^{-1} Tris-HEPES buffer, pH 7.0, sealed with a butyl-rubber stopper (gas phase, Ar), was supplemented with A23187 (10 μmol l^{-1}) (Calbiochem, Luzern, Switzerland) and $^{45}CaCl_2$ (Radiochemical Centre, Amersham, UK) was immediately added (25 μmol l^{-1}, 40 000 cpm nmol^{-1}). The suspension was incubated for 1 min at 60°C followed by the addition of 0.1 ml per ml of the suspension of cold sterile medium containing BSA (50 mg ml^{-1}) which was prebubbled with argon. Cells were pelleted by centrifugation at 2500 rpm at 4°C. The supernatant was aspirated and the washing procedure was repeated twice with the same medium and, finally, the pellet was resuspended in medium without BSA to a final concentration of about 1 mg protein ml^{-1}. The suspension was kept at room temperature and used immediately for experiments. 100 μl aliquots of the suspension contained about 30 000 cpm and the label was contained in the EGTA-inaccessible compartment.

1-ml aliquots of the $^{45}Ca^{2+}$-labelled suspension were transferred to 5 ml rubber-stoppered vials and preincubated for 5 min at 60°C with additional supplements (see figure legends) (time zero). At the times indicated in the figures, 100-μl aliquots were withdrawn from the vials and mixed with an equal volume of stopping solution containing 3 mM EGTA in the medium which was placed on the surface of 100 μl of a dibutylphthalate:dinonylphthalate (3:1) mixture in an Eppendorff tube and immediately centrifuged in a microcentrifuge for 1 min. Supernatants and pellets were taken for liquid scintillation counting, the latter after deproteinization with 100 μl of 5% trichloroacetic acid. Experiments were performed in duplicate.

Proteins were measured using the method of Lowry et al. [7].

3. Results

Under our experimental conditions (60°C, pH 7.0, protein concentration about 1 mg ml^{-1}, 30 μmol l^{-1} of Ca^{2+} and specific activity of the radionuclide about 20 000 cpm nmol^{-1}, non-energized state of cells) [1], $^{45}Ca^{2+}$ uptake could also be observed in 50 mmol l^{-1} Tris-HEPES buffer. The addition of chlorides of monovalent cations, however, changed its properties selectively depending on the cation. Both K^+ and choline$^+$ stimulated $^{45}Ca^{2+}$ uptake showing a similar time course (Fig. 1), the effect of the latter cation being smaller. Na^+, however, exerted a more complex effect. In general, Na^+

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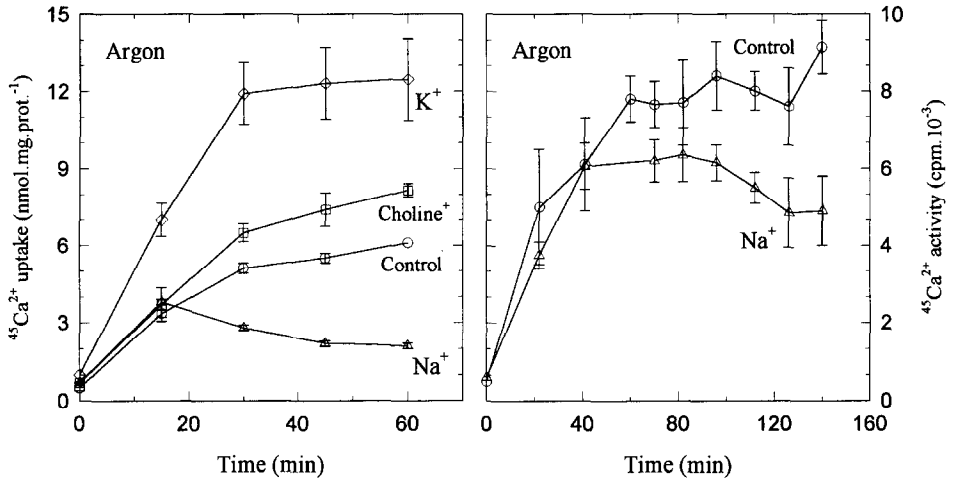

Fig. 1. Effects of monovalent cations on the $^{45}Ca^{2+}$ uptake by non-energized *M. thermoautotrophicum* cells. (Left, A) $^{45}Ca^{2+}$ uptake was measured in 50 mmol l^{-1} Tris-HEPES buffer, pH 7.0 (control) or in the presence of 50 mmol l^{-1} salts (control, ○; Na^+, △; K^+, ◇; choline$^+$, □). (Right, B) Conditions as above but at 45 min NaCl (50 mmol l^{-1}) was added to one sample (control, ○; Na^+, △).

inhibited uptake. However, the early phase of uptake in some experiments remained unchanged or even showed stimulated uptake with inhibition only occurring later (Fig. 1). Pre-incubation of cells with Na^+ up to 70 min before the addition of radionuclide did not significantly change the time course of $^{45}Ca^{2+}$ uptake (not shown). In other experiments, however, only the monophasic inhibitory response was observed in the presence of Na^+ (Fig. 2). The dependence of the inhibition on Na^+ concentration showed an IC_{50} in the range 10^{-3} mol l^{-1}. In the energized state (in the presence of CO_2+H_2) its inhibitory effect persisted in spite of the lower extent of $^{45}Ca^{2+}$ uptake (Fig. 2).

TCS, an uncoupler, had a major effect on $^{45}Ca^{2+}$ uptake which was dependent on the state of energization (Fig. 2). In argon-flushed cells, TCS (10 μmol l^{-1}) inhibited $^{45}Ca^{2+}$ uptake in the absence or presence of monovalent ions (only Na^+ shown) (Fig. 2). In energized cells (i.e. flushed with the CO_2+H_2 mixture), TCS surprisingly stimulated $^{45}Ca^{2+}$ uptake in both the absence and presence of Na^+ (Fig. 2B).

The results suggest that $^{45}Ca^{2+}$ homeostasis in *M. thermoautotrophicum* is accomplished by two Ca^{2+}-transporting systems dependent on the force imposed by the proton gradient and/or membrane potential operating in opposite directions and having different sensitivities to uncoupler. In order to investigate this possibility, uncoupled $^{45}Ca^{2+}$ efflux was measured from cells loaded with $^{45}Ca^{2+}$ by means of A23187. The properties of uncoupled $^{45}Ca^{2+}$ efflux are presented in Fig. 3.

$^{45}Ca^{2+}$ efflux from pre-loaded cells was completely blocked at 25°C as compared to 60°C (Fig. 3A). At 60°C efflux was stimulated by external $^{40}Ca^{2+}$ or Na^+ (Fig. 3A). TCS completely inhibited $^{45}Ca^{2+}$ efflux in the absence of monovalent ions in either energized or non-energized cells (Fig. 3B,C). $^{45}Ca^{2+}$ efflux induced by external Na^+, however, remained unchanged in the presence of TCS, or was even stimulated.

4. Discussion

The effects of monovalent cations on passive $^{45}Ca^{2+}$ uptake

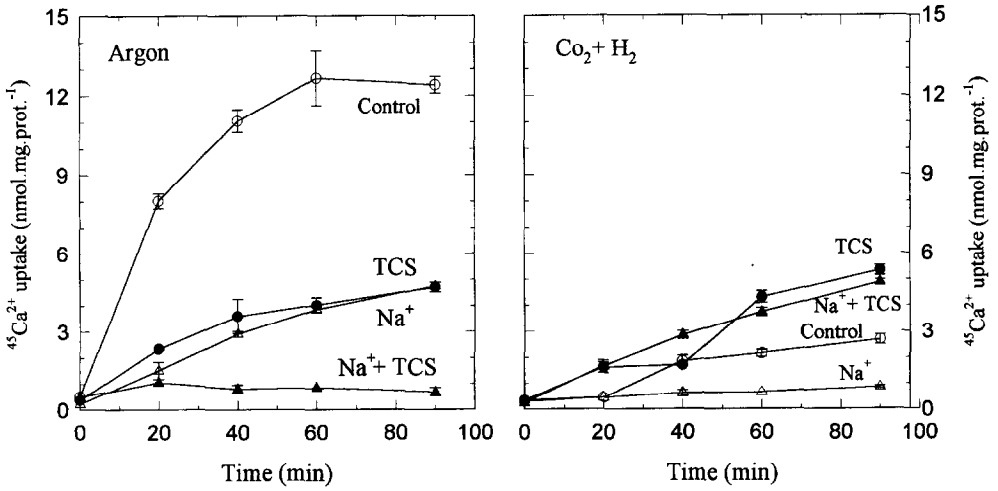

Fig. 2. Effects of NaCl and TCS under non-energized and energized conditions. (Left, A) Conditions as in Fig. 1 (control, ○; Na^+, △) or in the presence of 10 μmol l^{-1} TCS (closed symbols). (Right, B) Energized cells; other conditions as in (A).

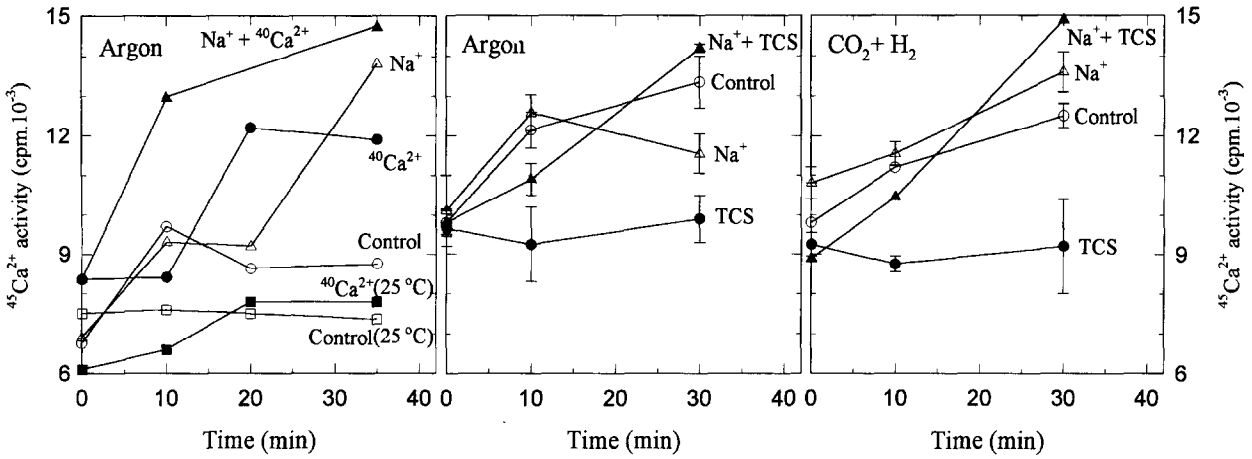

Fig. 3. $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$ pre-loaded cells under various conditions. (Left, A) Pre-loaded non-energized cells were supplemented with Na^+ (triangles) or $^{40}Ca^{2+}$ (closed symbols) (controls, ○), incubated at 60°C and radioactivity in the medium was measured at the times indicated. Controls measured at 25°C (□) and with $^{40}Ca^{2+}$ (■). (Middle, B) Radioactivity released from non-energized cells supplemented with Na^+ (triangles) or controls (circles) in the absence (open symbols) or presence (closed symbols) of 10 μmol l^{-1} TCS at the times indicated. (Right, C) Conditions as in (B) but energized cells were used.

in *M. thermoautotrophicum* are similar to those which could be observed in animal cells deprived of Na^+. The addition of Na^+ is (in animal cells) expected to stimulate the outward-directed activity of the Na^+/Ca^{2+} antiporter and to compensate the passive Ca^{2+} influx. The biphasic effect of Na^+ observed in some experiments (Fig. 1) could be ascribed to the re-equilibration of the Na^+ gradient which precedes the stimulation of Ca^{2+} efflux. Thus, this experiment could indicate the presence of the Na^+/Ca^{2+} antiporter in *M. thermoautotrophicum* membranes. The stimulatory effects of K^+ and choline$^+$ were not identical as the former was more effective at stimulation, suggesting that membrane depolarization might be involved in the stimulation of $^{45}Ca^{2+}$ uptake while the lesser effect of choline$^+$ could be a surface rather than a transmembrane phenomenon. These possibilities are currently being studied in our laboratories.

Besides Na^+ and K^+, H^+ is the cation which is involved in Ca^{2+} transport in bacterial [8], plant [9] or animal [1] cells. The involvement of H^+ as the driving force was assessed by studying the effect of TCS. Its effect was dependent on the energy state of the cell. Inhibition by TCS of $^{45}Ca^{2+}$ uptake in the non-energized state suggests that the latter is dependent on the outward-directed proton-motive force and may represent a Ca^{2+} uniporter driven by an inside-negative membrane potential, maintained in part by the proton-motive force. The reverse action of TCS in the energized state could hardly be interpreted without the additional involvement of the outward-directed transport driven by the proton-motive force as well. Measurement of uncoupled $^{45}Ca^{2+}$ efflux (Fig. 3) clearly showed its presence and sensitivity to the uncoupler. The nature of the underlying transport system, however, is uncertain but it could be through a $Ca^{2+}/(2)H^+$ antiporter.

The problem of the co-existence of Na^+ and H^+ energetics emerged recently in eubacteria [10–12] as well as in Archaea [5] including *M. thermoautotrophicum* [6]. The biphasic kinetics of $^{45}Ca^{2+}$ uptake in the presence of Na^+ (Fig. 1A) and the reversal of $^{45}Ca^{2+}$ uptake after addition of Na^+ (Fig. 1B) indicate the presence of an Na^+-linked outward-directed Ca^{2+}-transport system which was confirmed by the measurement of uncoupled $^{45}Ca^{2+}$ efflux (Fig. 3). This transport was uncoupler-insensitive (Fig. 3) and may be represented by the $Ca^{2+}/(2)Na^+$ antiporter. It is improbable that the effect of Na^+ is mediated by the Na^+/H^+ antiporter as this mechanism is supposed to be sensitive to uncoupler. Thus, both Na^+- and H^+-linked Ca^{2+}-extruding systems are present in the membrane of *M. thermoautotrophicum*. In experiments on [^{3}H]tetraphenylphosphonium chloride distribution which are not detailed here we found that the addition of Na^+ depolarizes the membrane from about −(100–120) mV to about −60 mV. Thus, the activation of the Ca^{2+} extrusion by Na^+ is not coupled to the membrane potential change but directly to the electrochemical potential of Na^+. To exclude the possibility that outward-directed transport is mediated by Ca^{2+}-ATPase (P-type), we found that the total ATPase activity in permeabilized cells of *M. thermoautotrophicum* is insensitive to its inhibitor vanadate (not shown). Moreover, our preliminary results showed that polyclonal antibodies raised against the eukaryotic P-type ATPases did not cross-react with membrane proteins of these cells. On the basis of these results, and the well known statement by Eigen that the absence of evidence is evidence of absence, we might conclude that P-type ATPase in these cells is missing and cannot be involved in the explanation of the presented results.

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