Characterization of a calcium porter of *Streptococcus pneumoniae* involved in calcium regulation of growth and competence

MARIE-CLAUDE TROMBE*

Centre de Recherches de Biochimie et Génétique Cellulaires du CNRS et Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cédex, France

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It is shown that *Streptococcus pneumoniae* possesses a Ca\(^{2+}\) transporter, sensitive to the amiloride derivative 2',4'-dimethylbenzamil (DMB), which is essential for grown at high Ca\(^{2+}\)-concentrations, and which mediates the triggering by Ca\(^{2+}\) of competence for genetic transformation in the exponential phase and autolysis in the late exponential phase. DMB inhibited both Ca\(^{2+}\) transport and the Ca\(^{2+}\) response. Kinetic analysis of \(^{45}\)Ca\(^{2+}\) transport in ATP-depleted *S. pneumoniae* revealed an electrogenic influx sensitive to DMB. This transport was cooperative with respect to Ca\(^{2+}\) concentration, and exhibited a Hill coefficient (n\(_H\)) of 2. In bacteria pre-loaded with \(^{45}\)Ca\(^{2+}\), a DMB-sensitive efflux could be triggered by an imposed Na\(^{+}\) gradient. The efflux kinetics showed the same cooperativity profile as Ca\(^{2+}\) concentration and a similar n\(_H\) value to that of influx, suggesting a possible Na\(^{+}\)/Ca\(^{2+}\) antiport. Cooperativity of transport was lowered (n\(_H\) = 1) by a mutation that confers resistance to DMB and abolishes the Ca\(^{2+}\) response. These results demonstrate that DMB-sensitive Ca\(^{2+}\) transport is essential for growth and competence regulation. The role of the DMB-sensitive porter involved in Ca\(^{2+}\) circulation and in Ca\(^{2+}\) homeostasis and its possible regulation by competence factor are discussed.

Introduction

The roles of Ca\(^{2+}\) in eukaryotes, including yeast, are diverse and important, ranging from activation of enzymes to hormone release and cell cycle control. Both Ca\(^{2+}\)-porters and Ca\(^{2+}\)-binding proteins, (calmodulins) have been described (for reviews see Campbell, 1983; Carafoli, 1987, 1988). In prokaryotes, Ca\(^{2+}\) is also involved in various processes (for reviews, see Norris et al., 1991; Onek & Smith, 1992), and calmodulin-like proteins have been characterized in several species (Inouye et al., 1983; Fry et al., 1983; Swan et al., 1987, 1989; Falah et al., 1988). Ca\(^{2+}\)-transporters including pumps and channels, have been identified (Kobayashi et al., 1978; Vrij et al., 1985; Ambudkar et al., 1986). So far, the role played by Ca\(^{2+}\) in bacteria is poorly understood. It is reported that Ca\(^{2+}\) activates auto-phosphorylation of the heat-shock protein, DnaK, in *Escherichia coli* (Cegielska & Georgopoulos, 1989), and that it stimulates expression of the virulence factor haemolysin in *Actinobacillus pleuropneumoniae* (Frey & Nicolet, 1988).

In *Streptococcus pneumoniae*, Ca\(^{2+}\) is an obligatory cation for growth. However, high Ca\(^{2+}\) (1 mM) triggers, via an exported protein (competence factor, CF), the induction of competence for genetic transformation in exponentially growing cultures and lysis in early stationary phase. DNA uptake at competence prevents lysis in early stationary phase, and it has been proposed that DNA transport constitutes a homeostatic response to elevated Ca\(^{2+}\) levels (Trombe et al., 1992). On the other hand, autolysis, dependent on the activity of the N-acetylmuramidase encoded by *lytA* (Sanchez-Puelles et al., 1986), is involved in the pathogenicity of smooth derivatives of *Streptococcus pneumoniae* (Berry et al., 1989). Interestingly, competence induction and culture lysis are prevented by the Ca\(^{2+}\)-channel inhibitor 2',4'-dimethylbenzamil (DMB) (Trombe et al., 1992). In the present report, the kinetic parameters of the DMB-sensitive component of calcium transport have been established, both in the wild-type strain and in a DMB-resistant mutant of *S. pneumoniae*, and correlated with the Ca\(^{2+}\) response.

Methods

**Strains.** The RX derivative of *Streptococcus pneumoniae*, strain Cp1015, bearing the *strR* mutation, was used as the standard wild-type strain (Morrison et al., 1984). The required mutations were
introduced into the wild-type background by genetic transformation using DNA from the mutant strains. Two rounds of transformation with non-saturating levels of DNA were performed to reduce the chance of multiple gene transfer. The mutation in the com locus of strain Cp1322 was introduced with DNA from strain omega 22, a competence-defective mutant selected after insertional mutagenesis (Morrison et al., 1984). The relevant phenotypes of Cp1322 are its inability to produce CF (Morrison et al., 1984) and its resistance to Ca\(^{2+}\)-induced lysis (Trombe et al., 1992).

Screening of Cp1015 for spontaneous mutants resistant to DMB was done on plates containing 15 \( \mu \)g DMB ml\(^{-1}\). Their frequency in a culture was between 10\(^{-2}\) and 10\(^{-4}\). After characterization of their natural transformability, clones altered for competence regulation were selected. Mutations conferring DMB resistance were transferred to the wild-type background by genetic transformation. Bacteria transformed to DMB resistance (DMB\(^R\) transformants) were selected on plates containing 15 \( \mu \)g DMB ml\(^{-1}\), as described previously (Trombe et al., 1992). Strain Cp2200 was taken as a representative of pleiotropic mutations in which the DMB\(^R\) phenotype was associated with an alteration of competence regulation.

**Specific materials.** The source of [\(^3\)H]DNA was the thymidine-auxotrophic strain R119, which also carries the rII-32 mutation (Tiraby & Fox, 1973). Amilorides were from Merck Sharp & Dohme, USA, and were provided by Dr E. J. Cragoe. DMB was prepared specifically for this study. Nutrients used for growth media were from Difco. \(^{45}\)Ca\(^{2+}\) was from CEA, France. Luciferase-luciferin and ATP were from Sigma.

**Growth and competence induction.** Growth conditions and media were identical for the wild-type and mutant strains, as described previously (Clave et al., 1987). Briefly, stock cultures were grown at pH 7.5 in a complex medium containing (g l\(^{-1}\)): NaCl, 5; yeast extract, 1; tryptone, 5; enzymic casein hydrolysate, 10; glucose, 2; K\(_2\)HPO\(_4\), 3. This medium contained 120 mm-Na\(^+\) and 0.2 mm-Ca\(^{2+}\) as measured by atomic absorption spectrometry. Growth curves were measured and autolysis tests were done as described previously (Trombe et al., 1992). For transport experiments, a culture frozen at an OD\(_{550}\) of 0.4 was thawed, centrifuged at 4°C and washed with uptake medium; the pellet was kept at 4°C before use (5-30 min). Such bacteria had an ATP pool of 3 \( \mu \)mol compared with 0.25 \( \pm \) 0.05 mmol in exponentially growing bacteria. They were taken as ATP-depleted cells. ATP measurements were made using a luciferase-luciferin assay as described by Lopez et al. (1989). The intracellular Na\(^+\) content was estimated at 85 \( \pm \) 5 mmol by atomic absorption spectrometry.

**Transport measurements.** In general, values for uptake at 15 s (\(V_{15}\)) were taken to approximate to the initial rate of transport, and these values are referred to as initial rates throughout the paper. Kinetic measurements (0-60 s) were made to verify the linearity of the response during this interval. Ca\(^{2+}\) transport was assayed using the radioactive isotope \(^{45}\)Ca\(^{2+}\). In streptococci, ion pumps, including a Ca\(^{2+}\)-pump, are well known (for reviews, see Heefner, 1982; Rosen, 1987). The Ca\(^{2+}\)-pump expels Ca\(^{2+}\) from the cells. Therefore, ATP-depleted bacteria were used to avoid interference between the activities of these putative pumps and of the porter being studied. ATP-depleted bacteria were suspended in the appropriate uptake medium at room temperature to give a suspension of OD\(_{550}\) between 2 and 5. A Ca\(^{2+}\) gradient (in < out) was imposed by addition of a \(^{45}\)Ca\(^{2+}\) solution and 0.1 ml samples were filtered at intervals through 0.45 \( \mu \)m Gelman Metricel membranes. After three washes with medium containing 0.5 mg CaCl\(_2\) ml\(^{-1}\), the radioactivity retained on the filters was quantified by liquid scintillation as previously described (Trombe et al., 1984). It was verified that Ca\(^{2+}\) washes lowered non-specific absorption on the filters without significant displacement of intracellular \(^{45}\)Ca\(^{2+}\). Transport of \(^{45}\)Ca\(^{2+}\) was measured at pH 8, in bacteria incubated either in the growth medium or in a Tris-based medium at the required Na\(^+\) concentration (Trombe et al., 1984).

When the effect of amilorides was examined, these were added to the bacterial suspension before \(^{45}\)Ca\(^{2+}\) addition. The initial rate of \(^{45}\)Ca\(^{2+}\) transport by DMB-sensitive bacteria was taken as the difference between values obtained in the absence and in the presence of 10 \( \mu \)g DMB.

For \(^{45}\)Ca\(^{2+}\)isoleucine transport measurements in ATP-depleted bacteria, the same conditions as for fully energized bacteria (Trombe et al., 1984) was used. The initial rates of uptake (determined in the first 15 s) were 20-30 times lower than the values obtained in fully energized cells (Trombe et al., 1984). Calculations were done on the basis that 1 ml of suspension at an OD\(_{550}\) of 1 represents an intracellular free space of 1 ml and contains 0.33 mg protein (Trombe et al., 1984).

**Genetic analysis.** Transformation was used to carry out genetic transfers. The recipient strain was transformed with DNA extracted from the donor under conditions described previously (Trombe et al., 1992). Selection of transformants was done on plates containing the selective agent. Transformed bacteria for one given marker were tested for a second marker by streaking the colonies on the appropriate selective plates containing either 2 \( \mu \)g erythromycin ml\(^{-1}\) or 15 \( \mu \)g DMB.

**Results**

**Evidence for a DMB-sensitive Ca\(^{2+}\) transport involved in homeostasis at high Ca\(^{2+}\).**

When a \(^{45}\)Ca\(^{2+}\) gradient ([Ca\(^{2+}\)\(_{\text{out}}\) > [Ca\(^{2+}\)\(_{\text{in}}\)]) was imposed on ATP-depleted bacteria (see Methods) a net influx could be measured. At 1-4 mm-Ca\(^{2+}\), uptake was linear for 30 s and was followed by efflux with a plateau corresponding to approximately 1 mm between 3 and 5 min. The Ca\(^{2+}\)-channel inhibitor DMB decreased the initial rate of influx and the steady state was not reached (Fig. 1). Initial rates of influx in media containing increasing DMB concentrations showed (Fig. 2a) 50%
DMB-sensitive Ca\textsuperscript{2+} transporter of S. pneumoniae

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\[ \text{Fig. 2. Inhibition by DMB of Ca}\textsuperscript{2+} \text{ transport (a) and growth (b, c). (a) Initial rate of Ca}\textsuperscript{2+} \text{ transport (\(\square\), 45Ca}^\text{2+} \text{ concn 1-2 mM, sp. act. 220 fmol = 1 c.p.m.; \(\surd\), 45Ca}^\text{2+} \text{ concn 0-4 mM, 190 fmol = 1 c.p.m.) determined in the presence of various concentrations of DMB. The vertical bars represent SEM (n = 3). (b, c) Growth measurements were made in media containing 0.2 mM-Ca}\textsuperscript{2+} (b) or supplemented with 1 mM-Ca\textsuperscript{2+} (c) and the following DMB concentrations (\(\mu\text{M})\): \(\bullet\), 0; \(\bigcirc\), 2; \(\surd\), 3; \(\Delta\), 4; \(\blacksquare\), 5; \(\square\), 6; \(\bullet\), 10 (c only). \]

inhibition at 4 \(\mu\text{M} (I_{90})\) and a plateau between 8 and 12 \(\mu\text{M}\). Residual uptake, at the plateau, presumably depends on a DMB-resistant system(s). When added to cultures at high Ca\textsuperscript{2+}, DMB slowed the growth rate, with total inhibition at 10 \(\mu\text{M}\) (Fig. 2b), while at low Ca\textsuperscript{2+}, the effect of DMB on bacterial growth related to the duration of the lag phase, rather than the growth rate (Fig. 2c). Such an effect of Ca\textsuperscript{2+} on growth-inhibition was not observed in the case of amiloride or its derivative 5-(N,N'-hexamethylene)amiloride (HMA), which did not affect the initial rate of 45Ca\textsuperscript{2+} uptake (data not shown). Altogether, these data point to a DMB-sensitive function involved in Ca\textsuperscript{2+} circulation. This function is essential for homeostasis in high Ca\textsuperscript{2+} media.

Kinetics of calcium transport

A plot showing the initial rate for the DMB-sensitive component of 45Ca\textsuperscript{2+} influx, as a function of the external Ca\textsuperscript{2+} concentration, exhibits sigmoidicity, with Hill coefficients (nH) of 1-9 (Fig. 3a). Similar nH values were obtained from uptake experiments performed in the
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Fig. 4. Effect of Ca\(^{2+}\) on the electrogenic transport of \[^{14}\text{C}\]isoleucine. Energy-depleted bacteria were suspended in Tris-based medium at OD\(_{550}\) 0.3 and incubated at room temperature in the presence of \[^{14}\text{C}\]isoleucine (10^{-2} \text{ mM}, 50 fmol \equiv 1 \text{ d.p.m.}). At intervals, 0.1 ml samples were withdrawn, filtered and rinsed as already described. Results are expressed as d.p.m. per \(\mu\)g intracellular free space (see Methods). □, Uptake in Ca\(^{2+}\)-free medium; △, ●, simultaneous addition of \[^{14}\text{C}\]isoleucine and 1.2 mM-CaCl\(_2\); (▲, 10 \(\mu\)M-DMB was added to the suspension before \[^{14}\text{C}\]isoleucine and CaCl\(_2\)). Inset, \[^{14}\text{C}\]isoleucine transport in a Ca\(^{2+}\)-free medium, in the absence (●) or in the presence (□) of 10 \(\mu\)M-DMB. The scale in the inset is the same as in the main figure. The initial rate of transport in Ca\(^{2+}\)-free medium (\(V_{15}\)) was 0.18 nmol min\(^{-1}\) (mg protein\(^{-1}\)).

Electrogenic transport

Electrogenicity of \(\text{Ca}^{2+}\) influx was checked using the electrogenic uptake of isoleucine as a probe (Trombe et al., 1984; Trombe, 1984). Inhibition of isoleucine uptake by \(\text{Ca}^{2+}\) influx should reflect membrane depolarization. Addition of \(\text{Ca}^{2+}\) to the uptake medium abolished \[^{14}\text{C}\]isoleucine uptake after 15 s (Fig. 4). This delay corresponds to the kinetics of \(\text{Ca}^{2+}\) influx (Fig. 1). DMB suppressed the \(\text{Ca}^{2+}\) effect. In a Ca\(^{2+}\)-free medium, DMB (10 \(\mu\)M) did not alter the rate of isoleucine transport (Fig. 4, inset). This suggests that \(\text{Ca}^{2+}\) inhibition of isoleucine uptake is due to electrogenic \(\text{Ca}^{2+}\) influx, via the DMB-sensitive porter.

Genetic correlation between \(\text{Ca}^{2+}\) transport, growth, competence induction and autolysis

Among spontaneous mutants resistant to 15 \(\mu\)M-DMB (DBM\(^{R}\)), clone DMB\(^{R}\) was chosen because it showed a reduced rate of \(^{45}\text{Ca}^{2+}\) transport. Mutant CP2200 was constructed by transformation of the wild-type strain Cp1015 with DNA from mutant DMB\(^{R}\) and its growth inhibition by DMB was verified (Fig. 5). The uptake kinetics of \(^{45}\text{Ca}^{2+}\) showed a lower cooperativity than the wild-type strain, \(n_{H} = 1.3\) (Fig. 6). This mutant did not develop natural competence in high-Ca\(^{2+}\) medium (20 independent experiments) and was resistant to autolysis. This provides genetic evidence to implicate the kinetics of \(\text{Ca}^{2+}\) transport in the \(\text{Ca}^{2+}\) response. Interestingly, resistance to autolysis and the loss of natural transformability in mutant Cp2200 are reminiscent of the characteristics of the competence-defective (Com\(^{−}\)) mutant Cp1322 (Morrison et al., 1984; Trombe et al., 1992); however, strain Cp1322, selected for its defect in natural transformability, was not DMB-resistant. Genetic
analysis was performed to determine whether the gene conferring the DMBR\(^{1}\) phenotype, carried by DNA of strain CP2200, and comA::ermAM, carried by the recipient strain Cp1322 (Ery\(^{R}\)), were alleles. No Ery\(^{S}\) transformant was found among 100 DMB\(^{R}\) transformants after transformation of strain Cp1322 by DNA extracted from mutant Cp2200, suggesting that these markers were not carried by the same DNA transforming molecule. The average size of a transforming molecule being estimated around 5 kb, it is likely that the mutation responsible for the DMBR\(^{1}\) phenotype defines a new gene which regulates the Ca\(^{2+}\) response, including competence induction.

**Discussion**

Kinetic analysis of \(^{45}\)Ca\(^{2+}\) transport in ATP-depleted *S. pneumoniae* provides evidence for Ca\(^{2+}\) transport down its electrochemical potential (Figs 1, 3a) and in response to an imposed Na\(^{+}\) gradient (Fig. 3b). This transport is sensitive to the amiloride derivative DMB (Figs 1, 2a). Neither amiloride itself nor HMA inhibited the initial rate of \(^{45}\)Ca\(^{2+}\) transport. Thus *S. pneumoniae* probably possesses a DMB-sensitive Na\(^{+}/Ca^{2+}\) antiporter. Using electrogenic isoleucine transport (Trombe et al., 1984) as a reporter activity, it was shown that the DMB-sensitive component of the Ca\(^{2+}\) transport was electrogenic (Fig. 4). In prokaryotes, electrogenic Ca\(^{2+}\) transport has already been described for several species (Tsujibo & Rosen 1983; Vrij et al., 1985), and observed in ATP-depleted *Streptococcus faecalis* (Kobayashi et al., 1978). However, no specific inhibitor was characterized. In *S. pneumoniae*, DMB defines a function which probably corresponds to a Na\(^{+}/Ca^{2+}\) antiporter essential for growth at high Ca\(^{2+}\) concentrations. The DMB concentration (10 \(\mu\)M) at which the plateau of uptake-inhibition is observed, suggesting a total block of the porter (Fig. 2a), corresponds to total inhibition of growth at high Ca\(^{2+}\) (Fig. 2c). The most likely hypothesis is that, in *vivo*, the porter mediates both influx and efflux of Ca\(^{2+}\) by antiport of Na\(^{+}\) and thus adjusts the intracellular Ca\(^{2+}\) concentration to a level compatible with growth.

On the other hand, the DMB concentration that lowers by 50% the rate of transport (\(I_{50} = 4 \mu\)M) (Fig. 2a) corresponds to the inhibitory concentration for competence induction and for autolysis (Trombe et al., 1992; Fig. 2b). Finally, the reduced value for the initial rate of Ca\(^{2+}\) transport at high Ca\(^{2+}\) in mutant Cp2200 due to a loss of cooperativity (Fig. 6) can be correlated with a deficiency in natural competence and with resistance to autolysis. This shows that the initial rate of Ca\(^{2+}\) uptake via the DMB-sensitive porter is involved in the regulation of competence and of autolysis (Trombe et al., 1992).

It is noteworthy that in the wild-type strain, cooperativity of transport has an \(n_{H}\) value of 2 for influx and efflux (Fig. 3a,b). Indeed, the Ca\(^{2+}\) concentration of 1 mM, where the inflexion point is observed in kinetics studies, falls in the same order of magnitude as the critical concentration at which the Ca\(^{2+}\) response is induced (Trombe et al., 1992) and corresponds to the Ca\(^{2+}\) concentration of body fluids that constitute the natural habitat of *S. pneumoniae*. Considering the transport kinetics, in this range of values tiny changes in Ca\(^{2+}\) concentration should result in large shifts of the rate of transport. This constitutes a valuable property for a putative sensor of Ca\(^{2+}\) concentration in the medium or in the cytoplasm.

The consequences of growth at high Ca\(^{2+}\) characterized to date are autolysis activation and competence induction (Trombe et al., 1992). Indeed, competence has been associated with cytoplasmic alkalinization, stimulation of glycolysis (Lopez et al., 1989), induction of specific oligopeptides (Morrison & Baker, 1979), activation of a membrane endonuclease (Lacks et al., 1975; Puyet et al., 1990) and DNA transport activity (Clavé et al., 1987; Clavé & Trombe, 1989). Thus competence might be considered as a ‘specialized’ physiological state associated with ionic signals and triggered by Ca\(^{2+}\) influx. Such a description of competence does not exist for other systems such as *Bacillus subtilis*, where the cascade of gene regulation implicated in the onset of competence is well established (Dubnau, 1991).

Amiloride-sensitive ion channels have been extensively described in eukaryotes (Cuthbert & Fanelli, 1978; Kaczorowski et al., 1985; Simchowitz & Cragoe, 1986). Na\(^{+}\) circulation, through an amiloride-sensitive Na\(^{+}/H^{+}\) antiporter, appears to be a critical event in generating ionic signals. These signals are represented by an increase in cytoplasmic pH and Na\(^{+}\) (Paris & Pouyssegur, 1983; Pouyssegur et al., 1984). These signals, associated with an elevation of free cytoplasmic Ca\(^{2+}\), are ubiquitous in the response of eukaryotic cells to growth factors and hormones (for reviews, see Rozengurt, 1980; Metcalf et al., 1985). On the other hand, it has been proposed that Ca\(^{2+}\) uptake is the determining step in cell differentiation and hence in the cell cycle (Miyakama et al., 1985; Iida et al., 1990).

The Ca\(^{2+}\) effect in *S. pneumoniae* is controlled by an exported protein (Trombe et al., 1992) named competence factor (CF) (Tomasz & Hotchkiss, 1964). CF probably recognizes a specific receptor on the cytoplasmic membrane (Ziegler & Tomasz, 1970). It has been shown that CF stimulates a DMB-sensitive \(^{45}\)Ca\(^{2+}\) influx but does not lower the Ca\(^{2+}\) requirement for competence induction (Trombe et al., 1992). It is proposed that CF activation of the Ca\(^{2+}\) response occurs through the stimulation of Ca\(^{2+}\) influx via the DMB-sensitive porter.
The cooperativity level, as regards Ca\(^{2+}\), that modulates the rate of cation uptake might constitute the critical parameter where regulation occurs. The behaviour of strain Cp2200, which carries a pleiotropic mutation that lowers cooperativity of Ca\(^{2+}\) transport and alters the regulation of competence and of autolysis, appears to fit this hypothesis. Other mutants showing an enhanced nH value for Ca\(^{2+}\) transport have been isolated and are now being studied.

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References


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