Calcium controls phage T5 infection at the level of the *Escherichia coli* cytoplasmic membrane

Mélanie Bonhivers, Lucienne Letellier*

Laboratoire des Biomembranes, URA CNRS 1116, Université Paris Sud, Bât. 433, 91405 Orsay cedex, France

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Abstract Phage T5 requires 0.1 mM calcium to produce phage progeny in *Escherichia coli* cells. Decreasing calcium below 0.1 mM at time phage DNA was transferred depleted the bacteria of K^+, caused membrane depolarization, perturbation of phage DNA transfer and resulted in a low internal ATP level. Our data suggest that calcium controls the conformation of the channel involved in the transfer of phage DNA through the host envelope and that below 0.1 mM calcium the channel remains open. This creates an energetic state of the host unfavorable to the synthesis of phage components and leads to abortion of the infectious process.

Key words: Phage T5; Abortive infection; Channel; Calcium

1. Introduction

Infection of *Escherichia coli* by the tailed phages such as T-phage and λ shares common features: after random collision between the virion and the bacterium, the phage irreversibly attaches to a specific receptor in the outer membrane, the DNA is ejected from the phage capsid, crosses the envelope and enters the cytoplasm. Based on data obtained with phage T4 it was proposed that transfer of phage DNA through the inner membrane is channel-mediated [1,2].

Unlike T4, T5 possesses a flexible non-contractile tail [3]. Binding of the phage to its outer membrane receptor (the FhuA protein) [4,5] is followed by the transfer of the double-stranded DNA (121 kbp) through the envelope. This transfer occurs in two steps. 8% of the T5 chromosome (FST DNA) enters the cytoplasm during the first 2 min [6]. After a pause of about 5 min the remaining 92% of the DNA (SST DNA) is transferred [7]. The two stages in phage DNA transfer are associated with an efflux in two steps of K^+. It was proposed that this efflux is due to the opening of a channel in the inner membrane, the function of which is to allow the transport of the DNA [8]. In vitro and in vivo experiments suggest that the phage protein pb2, located in the distal part of the phage tail, forms the DNA channel [9,10].

Transfer of FST and SST DNA is associated with the synthesis of three classes of phage proteins [7,11]. Class I proteins are synthesized from about 1 to 6 min after infection and are encoded by the FST DNA. Two of these proteins (A1 and A2) are in particular necessary for the transfer of the SST DNA. Synthesis of class II proteins is initiated at about 5 min and lasts about 20 min. Starting between 8 and 12 min, class III proteins and phage DNA are synthesized and this synthesis continues until cell lysis.

T5 infective process requires calcium to produce progeny phage [12,13]. Below a threshold of 0.1 mM calcium little or no class I proteins are produced and infected cells enter a dormant state. Subsequent addition of 1 mM calcium, even 15 min after infection, allows synthesis of class II and III proteins [14,15]. However, the mechanism by which calcium regulates the infection process is unknown. We investigated the effect of calcium on the membrane permeability changes associated with the transfer of the FST and SST DNA. Our findings indicate that the absence of calcium perturbs the transfer of phage T5 DNA and prevents correct resealing of the envelope.

*Corresponding author. Fax: (33) (1) 6985-3715.

Abbreviations: FST DNA, first step transfer DNA; SST DNA, second step transfer DNA; MOI, multiplicity of infection di-I-C1–5: 3,3′-dimethylindodicarbocyanine iodide.

2. Experimental

2.1. Growth of bacteria and phage preparation

E. coli F was used for phage production [12]. Host cells were grown and infected in LB medium supplemented with 1 mM $CaCl_2$. Phage stocks were purified as described [16]. Phage with radioactively labelled DNA were obtained as described previously [10,17]. [^{3}H]Uridine (1 TBq·mmol^{-1}; 39 nM) was added at the time of infection.

2.2. Preparation of FST complexes

E. coli cells were grown to an A_{650} of 0.2 in LB medium, washed and suspended to a density of 2×10^9 cells·ml^{-1} in 10 mM HEPES, 150 mM NaCl pH 7.2, 0.2% glucose, 50 μg·ml^{-1} chloramphenicol and incubated for 5 min at 37°C in the presence or absence of 1 mM $CaCl_2$. Then phage T5 labelled with [^{3}H]uridine was added at a multiplicity of infection of 5. After 15 min incubation the infected cells were repeatedly pipetted and centrifuged for 15 min at $6000 \times g$ to break away the non-transferred DNA which was eliminated by a DNAse treatment [18]. The pellet obtained from the fifth centrifugation was counted for radioactivity.

2.3. K^+ efflux experiments

Bacteria were grown to an $A_{650}$of 0.2 (2×10^8 cells·ml^{-1}) in LB medium, centrifuged, the pellet was washed once and resuspended in 10 mM HEPES, 150 mM NaCl pH 7.2, 0.8 mM $(NH_4)_2SO_4$ ('phage buffer') to an A_{650} of 30. K^+ efflux experiments were performed at 37°C using a K^+-valinomycin selective electrode as described previously [1,8]. Phage buffer was supplemented with 0.2% casamino acids, 0.2% glucose and with 0.6 mM KCl ('phage medium'). The concentration of KCl was chosen to take in account the K_m of the K^+ constitutive transport system TrkA (1.5 mM) [19] and the characteristics of the electrode.

2.4. Determination of intracellular and extracellular ATP concentration

Cells (A_{650} = 2; 2×10^9 cells·ml^{-1}) were incubated for 15 min at 37°C in phage medium. ATP was determined as described [20]. ATP and K^+ efflux experiments were carried out simultaneously.

2.5. Membrane potential measurements

Cells were first permeabilized towards the membrane potential ($D\Psi$) probes by a Tris-EDTA treatment [21]. The permeabilized cells (2×10^8 cells·ml^{-1}) were incubated in phage buffer containing 0.2% glucose and 0.6 mM KCl for 10 min at 37°C. Then the fluorescence probe di-I-C1–5 (0.17 μM, final concentration) was added. When a steady state of

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fluorescence was attained, phage T5 was added and the fluorescence was measured continuously at 640 nm (excitation wavelength, 600 nm). The fluorescence signal was calibrated in terms of $D\Psi$ as described [8,22].

3. Results

3.1. Effect of calcium and other di- and trivalent cations on phage T5-induced K^+ efflux

E. coli cells incubated in phage medium and in the presence of an energy source (glucose) retained 400 nmol·mg^{-1} K^+ whether or not incubated with calcium (up to 3 mM) (Fig. 1). Addition of phage T5 (MOI = 3) resulted in an efflux of K^+ in one or two steps depending on the concentration of added calcium. For calcium concentrations equal or higher than 0.1 mM efflux was two-step. The timing of the two effluxes correlated well with the timing of transfer of the FST and SST DNA [8]. We shall call the first and second efflux, FST and SST efflux respectively. Decreasing the calcium concentration below 1 mM considerably increased the amplitude and the rate of the FST efflux. Below 0.1 mM calcium only the FST efflux was observed. The initial rate of the FST efflux was 10 times higher in the absence than in the presence of 1 mM calcium (718 compared to 71 nmol·mg^{-1}·min^{-1}). The presence of 2 mM EDTA increased this rate to 1031 nmol·mg^{-1}·min^{-1} (data not shown). Interestingly, several divalent cations and even a trivalent cation had effects similar to those of calcium. The initial rate of the FST efflux was reduced by 50% in the presence of 2.7 mM Mg^{2+}, 0.16 mM Mn^{2+}, 0.07 mM Ca^{2+} or 0.01 mM Gd^{3+} (data not shown).

3.2 Reversibility of the effect of divalent cations on K^+ efflux

Phage T5 was added to *E. coli* cells incubated in phage medium containing or not chloramphenicol (50 μg·ml^{-1}), and 1 mM calcium was added at various times after initiation of FST efflux. The addition of calcium immediately reduced the initial rate of K^+ efflux and even allowed a slight reuptake of K^+ (Fig. 2). However, the SST efflux (which takes place solely in cells not treated with chloramphenicol) was only observed if calcium was added within 2 min following addition of phage T5. The reversibility of the Ca^{2+}/EDTA effect on K^+ efflux was tested (Fig. 3). *E. coli* cells were infected in phage medium containing 1 mM Ca^{2+}. In a first experiment, EDTA (2 mM) was added at the end of the FST efflux. This resulted in a large increase of the K^+ efflux. EDTA also promoted this K^+ efflux if added to chloramphenicol-treated cells and at the end of the FST efflux (not shown). In a second experiment, EDTA was added after both the FST and SST efflux had taken place (i.e. when the phage DNA was fully transferred). A new efflux of K^+ started immediately. Addition of 5 mM Ca^{2+} after EDTA allowed the cells to reaccumulate K^+. Control experiments (in the absence of phage) showed that neither Ca^{2+} nor EDTA had any effect on the accumulation or on the steady state level of K^+ in *E. coli* cells (data not shown).

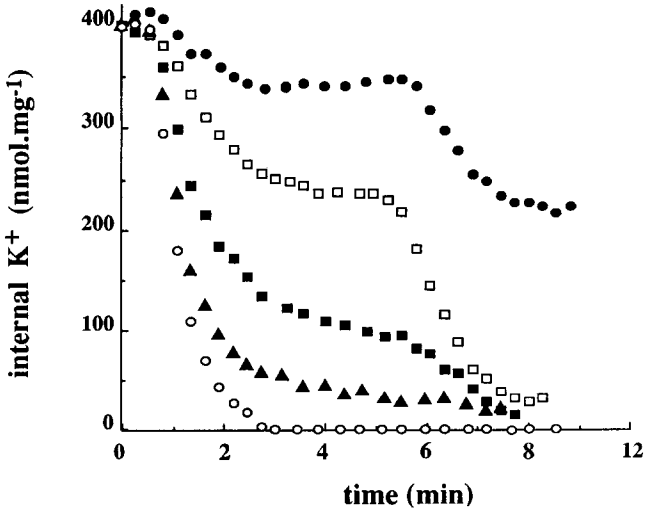

Fig. 1. K^+ movements induced by T5 phage as a function of calcium concentration: *E. coli* cells (2×10^9 cells·ml^{-1}; 2.5 ml) were incubated at 37°C in phage medium containing various concentrations of calcium. Phage T5 (MOI = 3) was added at time 0. The phage medium contained a few μM calcium due to the presence of casamino acids. Added calcium concentrations (mM): (○) 0; (▲) 0.03; (■) 0.1; (□) 0.5; (●) 1 and 3.

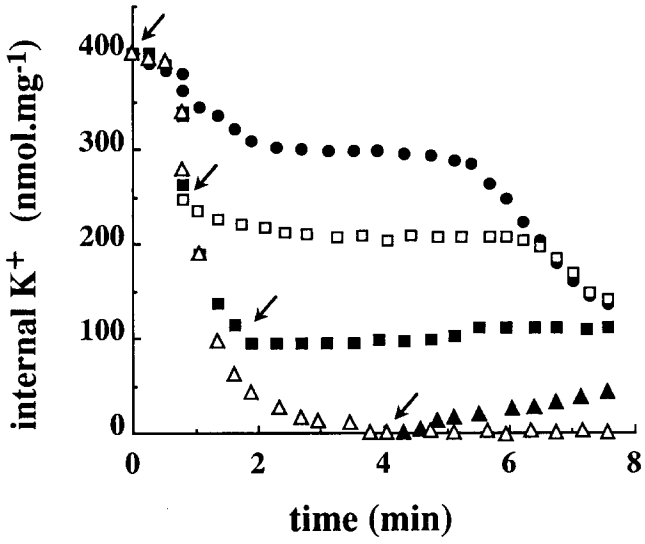

Fig. 2. Effect of addition of calcium at different times on the T5-induced K^+ effluxes: experimental conditions were similar to those described in the legend to Fig. 1 except that calcium (1 mM) was added at the times indicated by the arrow: (●) 0 min; (□) 0.8 min; (■) 2 min; (▲) 3.6 min; (△) without calcium.

3.3. Effect of calcium on the membrane potential changes accompanying T5-induced K^+ efflux

E. coli cells incubated in phage buffer containing 1 mM Ca^{2+} and 1 mM Mg^{2+} become partially and transiently depolarized upon addition of phage T5: depolarization starts with the onset of FST DNA transfer and of FST efflux and repolarization coincides with the arrest of the FST efflux and arrest of the FST DNA transfer [8,21,23]. The experiments described here suggest that Ca^{2+} and EDTA affect the permeability of the cytoplasmic membrane of T5-infected cells. We therefore tested the effects of these ions on $\Delta\Psi$. $\Delta\Psi$ of uninfected cells was 165 mV (negative inside) (Fig. 4). Addition of phage T5 resulted in a transient depolarization to a minimum of 80 mV if cells were incubated with Ca^{2+} and Mg^{2+} (1 mM) and a total depolarization not followed by repolarization if cells were incubated in the absence of these cations. The effect of Ca^{2+} was reversible:

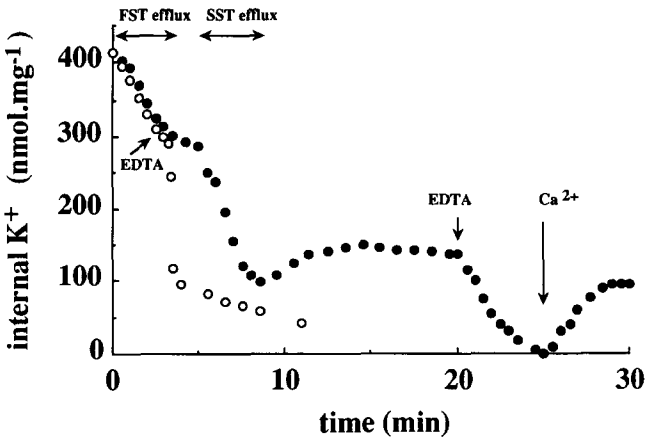

Fig. 3. Effect of the successive addition of EDTA or calcium on the T5-induced K^+ effluxes: experimental conditions were similar to those described in the legend to Fig. 1: (●) EDTA (2 mM) added at t = 20 min and calcium (5 mM) at t = 25 min. (○) EDTA (2 mM) added at t = 3 min in cells treated or not treated with chloramphenicol.

1 mM EDTA was added to phage-bacteria complexes incubated with 1 mM Ca^{2+} once the steady state of $\Delta\Psi$ was attained. The result was total depolarization (not shown). Addition of 1 mM Ca^{2+} to cells infected in the absence of Ca^{2+} and during the depolarization stopped the depolarization and even allowed repolarization (Fig. 4).

3.4. ATP content of T5-infected cells treated with calcium

The ATP content (ATP_{in}) of the cells and the presence of ATP in the external medium were determined during each of the different stages of transfer of T5 DNA. ATP was not found in the external medium whether cells were incubated in the presence or absence of calcium (not shown). However, the ATP content of the infected cells depended on calcium concentration (Fig. 5). Above 0.1 mM calcium, ATP_{in} first increased and then decreased during the first 4 min. Starting 4 min after addition of phage, ATP_{in} increased very rapidly in cells incubated with 1 mM calcium (to 180% of the basal level). ATP_{in} of cells incubated with 0.1 mM calcium was not different from that of uninfected cells and was 3 times lower in the absence than in the presence of 1 mM calcium.

3.5. Effect of Ca^{2+} on phage DNA transfer

Since the two-step efflux of K^+ was not observed in the absence of calcium, this suggested that the transfer of phage DNA might have been perturbed. Transfer of radioactively labelled phage DNA was determined in chloramphenicol-treated cells. Under these conditions, and if calcium is present, only the FST DNA is transferred. Successive centrifugations and pipetting of these phage–bacterium complexes break the tail of the adsorbed phages and the non-transferred DNA which remains exposed to the external medium can then be eliminated by DNAse treatment [17,18]. The radioactivity associated to the phage–bacterium complex after 5 successive centrifugations was compared to that initially present after the first centrifugation and corresponding to that of total adsorbed phage. The infected cells incubated in the presence of 1 mM Ca^{2+} retained 6.3 ± 2.0% of the initial radioactivity (results of three independent experiments). This is consistent with the phage transferring its FST DNA [18]. If infected cells were incubated without Ca^{2+} they retained 19.1 ± 3.6% of the initial radioactivity. This suggests that the arrest at the FST stage had not taken place. Control experiments indicate that the efficiency of phage adsorption was the same whether or not Ca^{2+} was present.

4. Discussion

We report that calcium regulates the membrane permeability changes induced by phage T5 upon successive transfer of the FST and SST DNA. At calcium concentrations higher than 0.1 mM the membrane reseals after the FST and the SST effluxes of K^+. Furthermore, the partial depolarization which accompanies the FST efflux is followed by repolarization. Decreasing the calcium concentration below 0.1 mM has several consequences: (i) the FST efflux is not arrested and K^+ continues to leak out for up to 8 min; (ii) the SST efflux is not detectable; and (iii) the cytoplasmic membrane is totally depolarized. This suggests that the membrane does not reseal. However, resealing followed the simple addition of calcium during the FST efflux as evidenced by both the arrest of this efflux and the partial repolarization. This resealing takes place in chloramphenicol-treated cells and is therefore independent of the synthesis of phage proteins. Conversely, chelation of calcium by EDTA was sufficient to reinitiate K^+ efflux and membrane depolarization, and further addition of calcium allowed the membrane to seal again. Resealing was possible for at least 25 min following infection (i.e. even after the transfer of the DNA was completed). The reversibility of the 'calcium effect' and its timing rule out an abnormal phage-cell attachment in the absence of calcium and indicate that the 'calcium effect' occurs independently of the stage of penetration of phage DNA. However, the absence of calcium prevented the arrest of DNA transfer at the

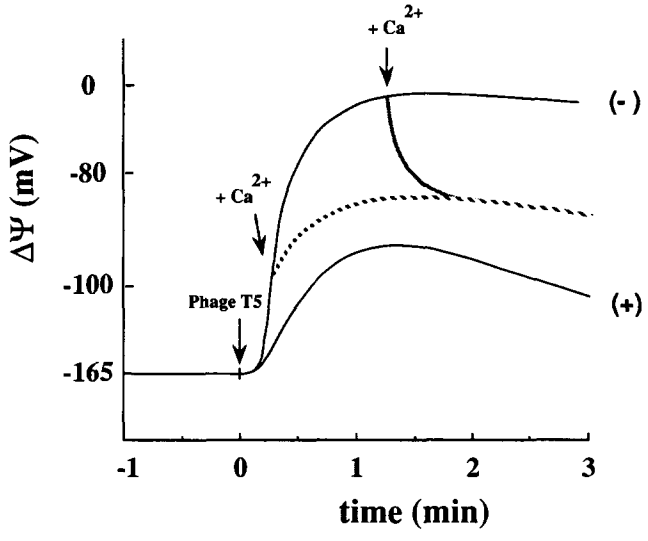

Fig. 4. Effect of calcium on the membrane potential changes induced by phage T5: $\Delta\Psi$ changes were measured with the fluorescent cyanine dye di I-C1-5, EDTA-treated cells were incubated in buffer containing 1 mM Ca^{2+} and Mg^{2+} (+) or no divalent cations (−). Phage T5 (MOI = 5) was added where indicated. In the experiment (−Ca^{2+}), 1 mM Ca^{2+} was added either at t = 0.25 min (∿∿∿) or at t = 1.25 min (∿∿∿).

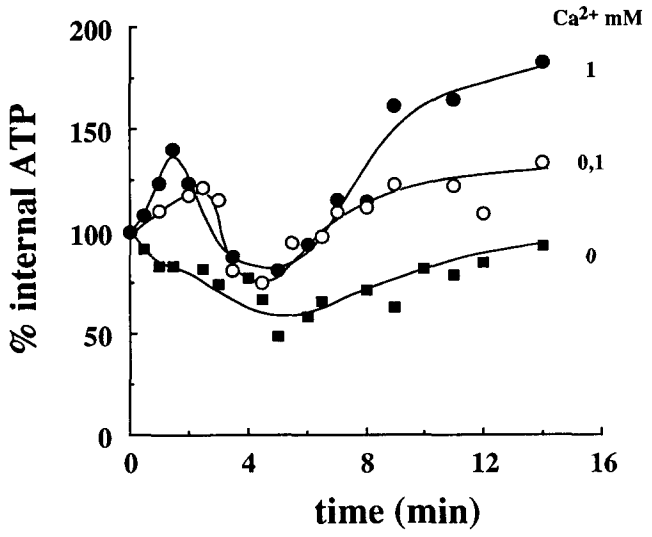

Fig. 5. Effect of calcium on the ATP content of T5-infected *E. coli* cells: experimental conditions were similar to those described in the legend to Fig. 1. Phage buffer contained (■) 0, (○) 0.1, or (●) 1 mM calcium. Uninfected cells contained 6 nmol·ATP·mg^{-1} cell dry weight; this value was defined as 100% ATP.

FST stage: 19.1 ± 3.6% of the DNA was transferred compared to 6.3 ± 2% in the presence of 1 mM calcium.

The increased ion permeability of the inner membrane observed in the absence of calcium correlated with a decrease, but not a leakage, of cytoplasmic ATP to 50% of its original level in uninfected cells. The absence of recovery of ATP is probably due to ATP hydrolysis by the F_1F_0-ATPase as a consequence of membrane depolarization [20]. In contrast, ATP increased to 180% of the basal level when calcium was present and during the synthesis of mRNA and of class II and class III proteins.

What is the reason for the increased ion permeability and what is the target of calcium? The permeability changes could be reinitiated after transfer of the total phage DNA. Direct interaction between calcium and phage DNA is therefore unlikely. The calcium-induced permeability changes were observed only with phage T5 and not with phage T4 or T7 (data not shown) suggesting that calcium is acting on a component (bacterial and/or phage) specifically involved in the first steps of phage T5 infection. The transfer of phage T5 DNA and the efflux of K^+ take place through a proteinous channel located in the contact sites between the inner and outer membrane of *E. coli* [10]. Calcium may act directly on the channel by affecting its conformation. The K^+ efflux experiments suggest the following model: in calcium-containing medium, the channel opens first during the transfer of the FST DNA and then closes. Since at this stage, the DNA crosses the envelope and remains in continuity between the cytoplasm and the capsid [18], this supposes that the closing is triggered by recognition of the channel protein by a specific stop signal on the phage DNA. After synthesis of the FST-DNA encoded proteins the channel would be again transiently reopened during transfer of the SST DNA. In medium not containing calcium the K^+ efflux is 10 times higher and the membrane does not reseal. This suggests that the size of the channel is increased and that it remains in an open conformation. Such a modification of the channel conformation may prevent its correct interaction with the DNA and the recognition of the stop signal region. As a consequence the transfer of the DNA continues beyond the FST DNA. In vitro experiments suggest that a phage protein, pb2, located in the distal part of the phage tail forms a channel [9]. This protein is also involved in the transfer of phage DNA [10]. Determination of a putative interaction between pb2 and the phage DNA would strenghten this hypothesis. Whatever the mode of action of calcium, the conformation of the channel appears to be susceptible to change from an opened to a closed state during all stages of DNA transfer and even after transfer.

These observations give us a framework to explain how the expression of T5 genome is regulated by calcium. We think that calcium indirectly controls the energetic level of the infected bacteria. In its absence the transport of amino acids and the synthesis of proteins and of RNA cannot take place because of the membrane depolarization and the low level of ATP. In particular the synthesis of A1 and A2, involved in degradation of host DNA and proteins, may be prevented. This is in accordance with previous data [14] which indicate that incorporation of [^{14}C]valine into proteins sharply decreased below 0.1 mM calcium. These authors also claimed that protein synthesis ceased after 8 min; this is also the time at which, according to our experiments, the ATP level drops significantly. Membrane depolarization and low ATP are probably also responsible for the inability of infected cells to take up the radioactively labelled precursor used to detect pre-early mRNA synthesis [15]. Phage-bacterium complexes prepared in a medium not containing calcium recover their potentiality to form infective centres following addition of calcium [12]. This is consistent with our observation that calcium allows the closing of the channel, the repolarization of the membrane and therefore allows the cells to recover an energetic state favorable to protein and RNA synthesis.

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