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Energetics of Calcium Efflux from Cells of *Escherichia coli*

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Intact cells of a H^+-translocating ATPase-deficient strain of *Escherichia coli* were starved of endogenous energy reserves and passively loaded with $^{45}CaCl_2$. Energy-dependent efflux of calcium was observed upon addition of glucose or respiratory substrates. Addition of cyanide or uncouplers prevented efflux. It is concluded that calcium efflux in intact cells is coupled to the proton motive force via secondary calcium-proton exchange.

Ion extrusion in bacteria can be catalyzed by either secondary porters or primary pumps (12, 15). Secondary systems couple ion extrusion to the electrochemical proton gradient or proton motive force established by primary proton pumps. The transport systems function as antiporters by exchange with H^+. In halobacteria, where an electrochemical sodium gradient replaces the electrochemical proton gradient, ion extrusion occurs by exchange with Na^+ (3). Recently, several bacterial ion pumps have been described, including the K^+ pump of *Escherichia coli* (7); the K^+ (1; M. Solioz, personal communication), Na^+ (8), and Ca^{2+} (9) pumps of *Streptococcus faecalis*; and the plasmid-encoded arsenate pumps of *E. coli* (13, 17) and *Staphylococcus aureus* (17). Whereas the potassium pumps catalyze uptake, the others are all extrusion systems.

In one of the initial studies of calcium metabolism in bacteria, Silver and Kralovic (18) demonstrated that the calcium content of *E. coli* cells was dependent on temperature, where less calcium was associated with the cells at physiological temperatures than those at lower temperatures, and suggested that this was the consequence of an energy-dependent calcium extrusion system. (For a recent review of bacterial calcium metabolism, see reference 14.) Encouraged by this report, we searched for energy-dependent uptake of $^{45}Ca^{2+}$ into everted membrane vesicles from *E. coli* (16) and found that vesicles have a Ca^{2+}-H^+ antiporter which catalyzes the uptake of calcium in exchange for protons. The energy for uptake is derived from the proton motive force (5, 21). Similar conclusions were drawn for both intact cells and vesicles of *Bacillus subtilis* and *E. coli* (19). Later, Kobayashi et al. (9) demonstrated that calcium transport in *S. faecalis* was catalyzed by a primary calcium pump, functioning to extrude calcium from intact cells and accumulate it in everted membrane vesicles. It appeared that calcium extrusion could be accounted for by primary pumps in some bacteria and secondary antiporters in others. However, Heefner and Harold (8) subsequently showed that sodium extrusion in intact *S. faecalis* cells was primary, yet uptake into everted vesicles could be either primary or secondary. They suggested that an ATP-driven sodium pump could be converted by proteolysis into a Na^+-H^+ antiporter. The only demonstration of the energy coupling of calcium transport in *E. coli* has been with the in vitro vesicle system. Given the possibility of artifactual generation of antiporters, limited conclusions concerning the mechanisms of calcium transport can be drawn from the vesicle studies without information about calcium extrusion in vivo. We report here that calcium extrusion from intact cells requires a proton motive force but not ATP, suggesting that the mechanism of in vivo calcium extrusion is by exchange with protons.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Cultures were grown overnight in LB medium (11). *E. coli* K-12 strain AN120 (*uncA401 argE3 thi-1 rpsL*) (6) was kindly provided by F. Gibson (Australian National University, Canberra).

Starvation of cells. Cells were depleted of endogenous energy reserves by a modification (13) of the method of Berger (4). Overnight cultures (100 ml) were centrifuged at room temperature and suspended in 100 ml of 10 mM Tris containing 140 mM KCl, 1 mM KH_2PO_4, and 1 mM $MgCl_2$, adjusted to pH 7.5 with HCl (buffer A). 2,4-Dinitrophenol (Na^+ salt, pH 7.5) was added to a concentration of 5 mM, and the cells were incubated for 2 h with shaking at 37°C. The starved cells were centrifuged and washed three times at room temperature with buffer A to remove the dinitrophenol. Cells at this point were essentially depleted of ATP (less than 0.1 nmol/mg of cellular protein) and exhibited no endogenous respiration (13).

Transport assays. Cells (4 mg of protein) were suspended in 0.2 ml of buffer A containing 0.25 mM $^{45}CaCl_2$ (except for the experiments described in Fig.

3) and allowed to passively equilibrate at room temperature. As seen in Fig. 1, the equilibration process was complete by 1 h. Calcium-loaded cells were diluted 100-fold into room temperature buffer A (0.05 ml into 5.0 ml). After 30 min, an energy source was added to initiate energy-dependent efflux. Transport was measured by filtration of a portion of the cells (0.02 ml of undiluted or 1 ml of diluted cells) through 0.45-μm-pore-size nitrocellulose filters. The filters were washed with 5 ml of the same buffer and dried, and radioactivity was measured by liquid scintillation counting. Where indicated, inhibitors were added 5 min before the addition of an energy source.

Variations of the loading and transport procedures included higher and lower pH (from 5.6 to 8.5), higher and lower concentrations of $MgCl_2$ (from 0 to 10 mM), addition of nonradioactive calcium to the wash buffer, higher and lower ionic strength (from 10 to 500 mM KCl), and substitution of different monovalent cations for K^+. In general, the procedure outlined above gave optimal results. Mg^{2+} was especially important for reducing nonspecific binding of Ca^{2+}.

Other methods. Protein was determined by the method of Lowry et al. (10), using bovine serum albumin as a standard. $^{45}CaCl_2$ was obtained from New England Nuclear Corp. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone was a gift from E. I. du Pont de & Nemours Co. Other chemicals were reagent grade and purchased from commercial sources.

RESULTS

Energy-dependent calcium efflux from intact cells. Silver et al. (19) reported that unstarved *B. subtilis* cells could be passively loaded with $^{45}Ca^{2+}$ at 4 or 20°C in the presence of 2,4-dinitrophenol but that at 20°C in the absence of uncoupler calcium was excluded from the cells. A temperature shift from 0 to 20°C resulted in a loss of cell-associated calcium. They mentioned that qualitatively similar results were obtained with *E. coli*; however, the data were not presented. Our attempts to repeat these experiments with non-energy-depleted cells have been hampered by an apparently non-energy-dependent binding of calcium to the cells. This binding was affected both by temperature and concentration. Low to high temperature shifts were accompanied by a decrease in bound calcium. Dilution of uncoupler-treated cells also led to a redistribution of bound radioactivity.

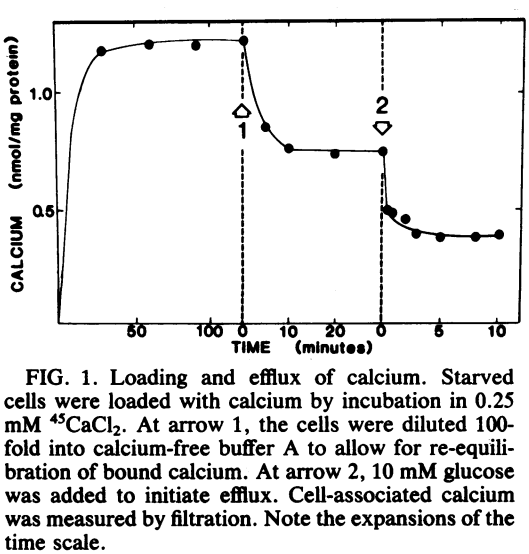

FIG. 1. Loading and efflux of calcium. Starved cells were loaded with calcium by incubation in 0.25 mM $^{45}CaCl_2$. At arrow 1, the cells were diluted 100-fold into calcium-free buffer A to allow for re-equilibration of bound calcium. At arrow 2, 10 mM glucose was added to initiate efflux. Cell-associated calcium was measured by filtration. Note the expansions of the time scale.

We recently found that cells depleted of endogenous energy reserves could be reproducibly loaded with $^{74}AsO_4$ for efflux assays (13). Starved cells could similarly be loaded passively with $^{45}Ca^{2+}$ (Fig. 1). A redistribution of radioactivity occurred upon 100-fold dilution of the cells into calcium-free buffer. We have no information concerning what the released calcium was bound to. After about 10 min, cell-associated $^{45}Ca^{2+}$ reached a new steady-state level. Addition of an energy source such as glucose resulted in rapid efflux of about half of the remaining $^{45}Ca^{2+}$. The residual $^{45}Ca^{2+}$ could be removed only by washing with nonradioactive calcium. Thus, of the approximately 1.2 nmol of calcium per mg of cellular protein associated with starved cells, 40% was removed by dilution. About 20% of the initially associated calcium was removed by energy-dependent efflux. This 20% of the calcium is assumed to be internal and represents a nominal initial concentration of approximately 0.2 mM, suggesting equilibration with external calcium. After dilution, a 100-fold concentration gradient would exist, and this gradient was stable in the absence of an energy source. That this calcium was unbound or in rapid equilibrium with unbound internal calcium is suggested by the release of radioactivity produced by treatment of the cells with toluene (Fig. 2). On the other hand, the radioactivity which was not actively extruded was likewise not released by toluene and was probably bound, although to what is unknown. This residual binding was subtracted when half-times for efflux were calculated.

When cells were loaded with different concentrations of calcium, the cells exhibited energy-dependent efflux (Fig. 3). These assays are not sufficiently quantitative to allow for measurement of efflux kinetics, so no K_m for efflux was determined.

Energetics of calcium extrusion. Energy depleted cells of the H^+-ATPase-deficient strain AN120 were loaded with $^{45}Ca^{2+}$, and the ability of different energy sources to stimulate efflux was examined (Fig. 4). Glucose, lactate, and reduced phenazine methosulfate were similarly effective in supporting efflux, with half-times of 23, 25, and 34 s, respectively. Under these

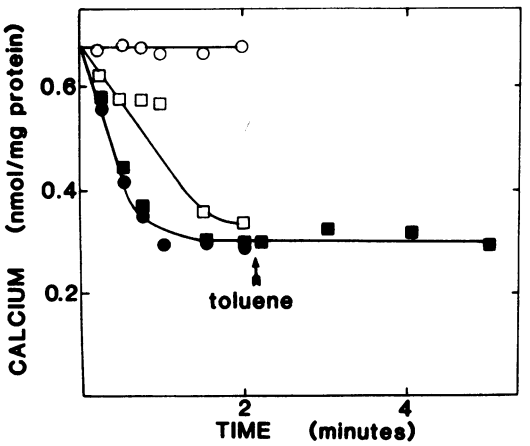

FIG. 2. Release of loaded calcium with toluene. Starved cells loaded with 0.25 mM $^{45}CaCl_2$ were diluted 100-fold into calcium-free buffer A. After 30 min, either 10 mM glucose (●, ■) or 0.7% (vol/vol) toluene (□) was added. After glucose-stimulated efflux was complete, 0.7% (vol/vol) toluene was added (■). The endogenous (no addition after dilution) levels were also determined (○).

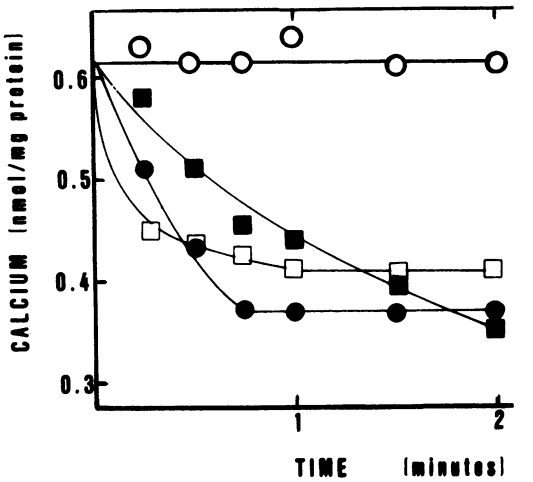

FIG. 4. Effect of energy sources on calcium efflux. Starved cells loaded with 0.25 mM $^{45}CaCl_2$ were diluted 100-fold into calcium-free buffer A. After 30 min, an energy source was added to initiate efflux: ○, endogenous levels; ●, 10 mM glucose; □, 10 mM Tris D,L-lactate; ■, 0.14 mM phenazine methosulfate (PMS) and 20 mM sodium ascorbate.

conditions, lactate and reduced phenazine methosulfate should produce a proton motive force but no ATP, whereas glucose metabolism results in both ATP and a proton motive force. In the presence of cyanide, neither glucose nor lactate could drive efflux (Fig. 5). Cyanide completely blocks respiration and prevents formation of a proton motive force without affecting ATP levels (13). The uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone also blocked calcium efflux (data not shown).

Effect of phosphate on calcium efflux. Calcium efflux occurred equally efficiently in the absence of added phosphate and in the presence of 1 or 10 mM phosphate. We found that the cells were not depleted of internal inorganic phosphate even by starvation in the absence of exogenous phosphate, so no conclusion can be drawn about the requirement for internal phosphate. Loading the cells in the presence of various levels of phosphate during the starvation period and subsequent washes had no effect.

DISCUSSION

Previously it was shown that calcium is transported into everted membrane vesicles of *E. coli*

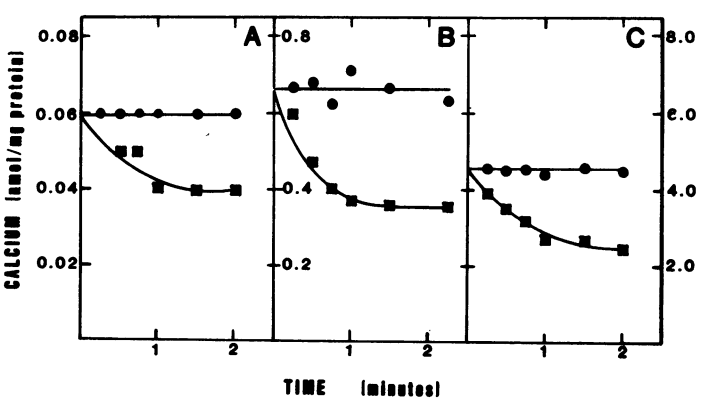

FIG. 3. Concentration dependence of calcium efflux. Loading concentration of $^{45}CaCl_2$; (A) 0.025 mM; (B) 0.25 mM; (C) 2.5 mM. Starved cells loaded with $^{45}CaCl_2$ were diluted 100-fold into calcium-free buffer A. After 30 min, 10 mM glucose was added to initiate efflux (■). The endogenous levels were also determined (●). Note the different scales on the ordinate.

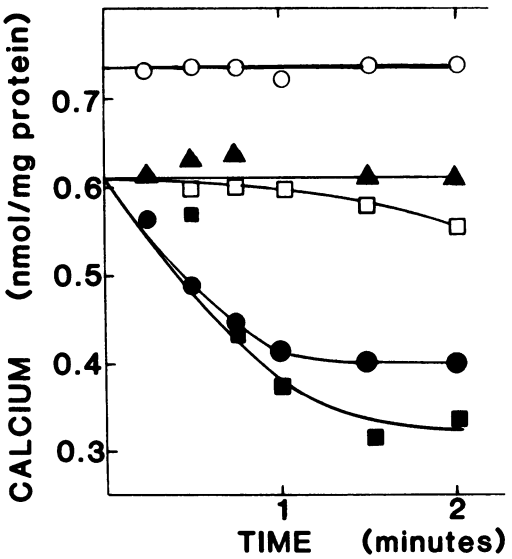

FIG. 5. Effect of cyanide on calcium efflux. Starved cells loaded with 0.25 mM $^{45}CaCl_2$ were diluted 100-fold into calcium-free buffer A. After 25 min, 10 mM NaCN was added (○, □); 5 min later, an energy source was added to initiate efflux: ▲, endogenous levels; □, ■, 10 mM glucose; ○, ●, 10 mM Tris D,L-lactate.

by a secondary transport system which utilizes the proton motive force established by either of the two primary proton pumps: the respiratory chain or the H^+-ATPase (5, 16, 21). It has been assumed that calcium uptake by everted vesicles reflects calcium extrusion from intact cells, yet the only studies of calcium extrusion from intact bacteria have been merely suggestive of an efflux system in *E. coli* (18, 19).

Here we demonstrate that the assumption was a correct one: (i) energy-dependent calcium extrusion occurs and (ii) the driving force is the proton motive force. Our former difficulties in observing efflux were due to the high degree of calcium binding by cells. After removing most of the bound calcium, efflux dependent on cellular energy was observed. Using an adaptation (13) of the protocol of Berger (4), we were able to establish conditions where the only form of cellular energy was phosphate bond energy, mostly in the form of ATP (glucose metabolism in the presence of cyanide), or where a proton motive force existed without substantial intracellular ATP (metabolism of respiratory substrates). Even when intracellular ATP levels were high, efflux did not occur in the absence of respiration. Respiration in the absence of ATP was effective in driving efflux. Clearly, phosphate bond energy is not necessary for efflux, and just as clearly, a proton motive force is sufficient. At pH 7.5 there is no transmembrane pH gradient (20); the membrane potential is the sole component of the proton motive force (22). Thus, a potential alone is sufficient for calcium efflux. In everted membrane vesicles, either a pH gradient or a membrane potential is sufficient (S. Ambudkar and B. P. Rosen, unpublished data), suggesting that the antiporter is electrophoretic (that is, moves in response to a charge gradient), carrying more proton charges than calcium charges. The simplest electrophoretic stoichiometry is 3 H^+-Ca^{2+}.

In these experiments, only efflux down a concentration gradient was examined. We were not able to observe efflux against a concentration gradient because of binding of calcium to the cells. It is possible that uphill efflux might have a different energy requirement from downhill efflux, although we consider that unlikely.

Why is there a difference between *S. faecalis*, which uses a primary ATP-driven pump for calcium efflux (9), and other bacteria, which apparently use secondary systems (14)? Heefner and Harold (8) postulate that *S. faecalis* has a greater dependency on primary pumps because it is physiologically an anaerobe, with less capacity to produce a proton motive force. However, glycolyzing *S. faecalis* maintains a proton motive force with the H^+-translocating ATPase (1), which is quantitatively similar to that of respiring *E. coli* (22). It is apparently more efficient in aerobes to use this one generator of electrochemical energy to couple to a variety of secondary exchange systems than to use individual primary pumps for each. The evolutionary choice between a primary and secondary system for ion transport may depend on two factors. (i) The cells need to maintain a constant internal concentration of ion. It is advantageous to use a pump when the internal concentration of ion must be constant, even though the concentration of that ion in the external environment is variable. This may explain the need for primary K^+ pumps, since maintenance of a relatively constant internal concentration of potassium is required (7). (ii) The cells need to adapt to environmental stress. *E. coli* responds to the stress of osmotic up-shocks by accumulating K^+ with the ATP-driven potassium pump (7). A primary pump for arsenate efflux allows for maintenance of low internal arsenate, independent of the external concentration (13, 17), even when the proton motive force varies.

Does the fact that calcium is extruded by a secondary process in *E. coli* mean that calcium-proton exchange functions simply to prevent accumulation of calcium in the cytosol? Not necessarily; other roles of cations, such as the need for an electrochemical sodium gradient for amino acid and sugar transport, can be fulfilled by secondary systems, e.g., the sodium-proton

antiporter of *E. coli* (2, 15). Knowledge of the mechanism of transport does not always allow us to infer the role of the transported solute in the physiology of the cell.

ACKNOWLEDGMENTS

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