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Facilitated Transport of Calcium by Cells and Subcellular Membranes of *Bacillus subtilis* and *Escherichia coli*

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The level of calcium in growing cells is lower than that in the growth medium. Non-energy-dependent uptake of ^{45}Ca by log-phase cells of *Bacillus subtilis* occurs under two conditions: at 0 C or in the presence of *m*-chlorophenyl carbonylcyanide hydrazone. Similar uptake, but quantitatively less, occurs with *Escherichia coli* cells under the same conditions. Membrane vesicles prepared from *B. subtilis* or *E. coli* accumulate ^{45}Ca by a process that does not depend on added energy sources and is not inhibited by the respiratory poison cyanide. The properties of calcium transport in all cases is consistent with carrier-mediated, facilitated transport with specificity $Ca^{2+} > Sr^{2+} > Mn^{2+} > Mg^{2+}$. Upon transfer of cells from 0 C to 20 C, pre-accumulated ^{45}Ca is released. Heat-killed cells do not accumulate ^{45}Ca and calcium is released by cells upon addition of toluene (under conditions that do not cause visible lysis). These results suggest that the facilitated uptake of calcium may be utilizing a transport system that normally is responsible for the energy-dependent excretion of calcium from the cells.

Energy-dependent, highly specific, active transport systems for many cations, including potassium (8, 11), magnesium (23, 25, 26), manganese (9, 27), iron (14, 18), and zinc (6), have been described. In each case the system is dependent upon metabolic energy, generally derived from respiration or adenosine triphosphate (e.g., reference 7), and is oriented in the cell membrane so as to bring the cation from the outside of the cell into the cell. Calcium active transport into bacterial cells has been reported (4, 5, 10) only during the stage of sporulation when massive amounts of calcium and dipicolinic acid (together as a chelate) are accumulated (21). Growing cells of *Bacillus subtilis* do not accumulate calcium, and preliminary evidence was suggestive of metabolically active calcium efflux with *Escherichia coli* (28).

We report data in this paper suggesting the energy-independent, facilitated transport of calcium by cells and membranes of *B. subtilis* and *E. coli*. "Energy-independent" means that we measure calcium accumulation with intact cells under non-energized conditions including incubation at 0 C or in the presence of uncouplers such as *m*-chlorophenyl carbonylcyanide hydrazone (CCCP). With subcellular membrane vesicles, calcium accumulation is not dependent upon the presence of respiratory substrates. By "facilitated" we mean that the accumulation shows substrate specificity and saturation kinetics—which are considered diagnostic of transport across the membranes by highly specific "carrier" molecules, usually thought to be either membrane-embedded proteins or ionophores. In these respects, calcium accumulation by bacterial cells is being studied in the wrong direction, since we are measuring movement from the outside to the inside but believe that the physiologically significant transport is generally from the inside of the cell to the outside. The situation in bacterial cells is thus very similar to that described with animal cells (2, 3, 24), where there are data suggestive of an energy-dependent calcium extrusion system. Our studies are also similar, in this respect, to those of Harold and Levin (13), who measured lactic acid accumulation by *Streptococcus faecalis* cells, mediated by a system they consider primarily involved in extrusion of lactate.

MATERIALS AND METHODS

B. subtilis W23 was grown to 0.25 mg (dry weight) per ml in K^+- and Mg^{2+}-supplemented tryptone broth containing 10^{-4} M calcium as described previously (8, 10). *E. coli* K-12 cells and membrane vesicles were grown and prepared as described previously (1, 26, 27).

Intact cells were concentrated by centrifugation and resuspension in 1/50th the original culture volume, followed by 50-fold dilution into either fresh broth or 15 mM glycylglycine buffer (pH 7.0) containing 0.1 M NaCl, and salts or inhibitors as indicated in the figure legends. Samples (0.2 ml) were periodically removed, filtered through membrane filters (type HA, Millipore Corp., Bedford, Mass.), and washed twice with 5 ml of incubation medium (at room temperature or at 0 C). Radioactivity was counted in a gas-flow counter.

Membrane vesicles were prepared from log-phase cells (1) by the standard techniques of Kaback (15, 16) and as modified for use with *B. subtilis* (17). The vesicles used in these experiments were stored for over a year in 0.25 M sucrose at −70 C. Vesicles were thawed rapidly, centrifuged, and suspended (at 4 mg of protein per ml) in a buffer of 0.2 M sucrose plus 10^{-2} M KPO_4 (pH 7.0). After the additions indicated in the figure legends and the final addition of radioactivity, 50-μl samples were distributed into small "polyvials" and incubated at room temperature (20 to 25 C) without special oxygenation. The respiratory energy source added to membranes was 20 mM sodium ascorbate plus 0.1 mM phenazine methosulfate (PMS).

RESULTS

Membrane vesicles. ^{45}Ca and amino acid uptake by *B. subtilis* vesicles is shown in Fig. 1. The accumulation of ^{45}Ca was slightly greater in the absence of ascorbate and PMS than in their presence (Fig. 1A). In other experiments, the initial rate of ^{45}Ca uptake was also more rapid in the absence of ascorbate and PMS. This is in contrast to the ^{45}Ca uptake experiments with fresh membrane vesicles (29), where we reported greater uptake rates in the presence of ascorbate and PMS (see also reference 12).

[^{14}C]proline uptake was completely dependent upon the presence of the respiratory substrate ascorbate-PMS and was eliminated by addition of the respiratory poison cyanide. ^{45}Ca uptake was slightly stimulated upon addition of cyanide to the vesicles and 10 μM CCCP did not inhibit ^{45}Ca uptake (data not shown). In Fig. 1, the contrast between energy-dependent active transport (of proline) and non-energy-dependent uptake (^{45}Ca) is particularly striking. At least 80% of the ^{45}Ca accumulated by vesicles can be released (exchanged) upon the addition of excess nonradioactive calcium (Fig. 1A). The highest calcium accumulation in Fig. 1A would represent a 20-fold concentration gradient assuming 4 μl of vesicular water per mg of protein and assuming that the accumulated calcium was free in solution. Although it is not possible to measure the free calcium directly, we assume that the larger fraction of the calcium is bound internally and is not free, simply because we can not envisage a non-energy-dependent mechanism leading to a true concentration gradient of this magnitude.

Calcium accumulation qualitatively similar to that with *B. subtilis* membrane vesicles has been obtained with *E. coli* membrane vesicles, but quantitatively the *E. coli* membranes accumulate only 20% as much calcium as the *B. subtilis* membranes (Fig. 2). A comparable, lesser, calcium accumulation was also found with *E. coli* cells compared with *B. subtilis* cells (see below).

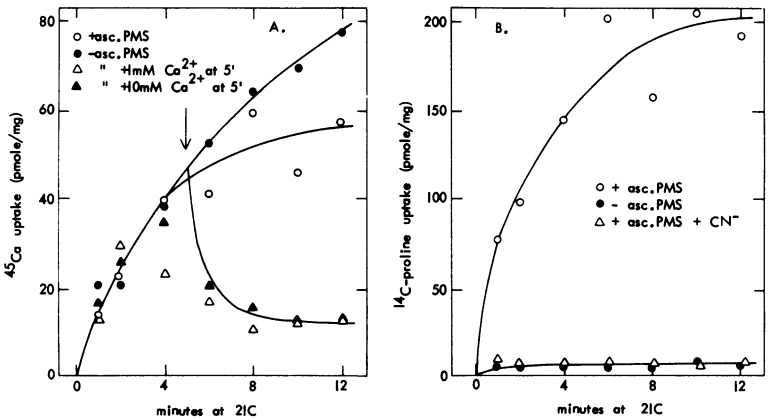

FIG. 1. *Energy independence of calcium uptake in B. subtilis vesicles. (A) Sodium ascorbate (20 mM) and PMS (0.1 mM) were added to one sample, followed by addition of 1 μM ^{45}Ca (1 μCi/ml) to all. At 5 min, additional nonradioactive $CaCl_2$ was added to two series of samples. (B) Ascorbate-PMS was added to two of the three volumes, and 10 mM NaCN was added to one of these just prior to the addition of 4.3 μM [^{14}C]proline (1 μCi/ml) at time zero.*

Although calcium accumulation by membrane vesicles is not energy dependent, the accumulation of another divalent cation, Mn^{2+}, is completely energy dependent (1; Bhattacharyya, manuscript in preparation) and behaves, in this regard, just like proline uptake.

Intact cells. The experiments shown in Fig. 3 demonstrate the two conditions under which we observe calcium uptake by intact cells of *B. subtilis*. In the presence of 50 μM CCCP, the cells steadily accumulate more than 25-times more ^{45}Ca than in the absence of the uncoupler at 20 C (Fig. 3A). The specificity of this uptake can be seen from the strongly inhibitory effect of adding nonradioactive 1 mM Ca^{2+}, compared with the lack of effect of Mg^{2+} and the somewhat lesser effects of adding excess nonradioactive Mn^{2+} or Sr^{2+}. Toluene (1% vol/vol) caused the rapid release of more than 90% of the ^{45}Ca accumulated presence of 50 μM CCCP or at 0 C (data not shown). Heat-killed cells (80 C for 10 min) do not accumulate ^{45}Ca in the presence of CCCP (data not shown). ^{45}Ca accumulation by cells at 0 C and effects of temperature shifts are shown in Fig. 3B. The scale in Fig. 3B is greater than that in Fig. 3A because 3 μM $^{45}Ca^{2+}$ was used rather than 0.1 μM. The fractional accumulation of ^{45}Ca is about the same under these two conditions (4 to 5% of the added calcium by 0.25 mg [dry weight] cells per ml). More than 80% of the accumulated calcium was exchangeable upon addition of excess nonradioactive calcium, with the rate and extent being dependent upon the concentration added. Also note in Fig. 3B that switching a culture that had accumulated calcium at 0 C to room temperature resulted in a gradual release of most of the accumulated calcium. We have been less successful in experiments moving *B. subtilis* cultures from 25 C to 0 C after 30 min, however, since visible cell lysis occurs and the partially lysed cells take up smaller and more variable levels of ^{45}Ca than they would have if exposed earlier. In similar experiments with *E. coli* cells, transfer from 25 C to 0 C after 30 min of incubation with ^{45}Ca resulted in accumulation comparable to that with cells

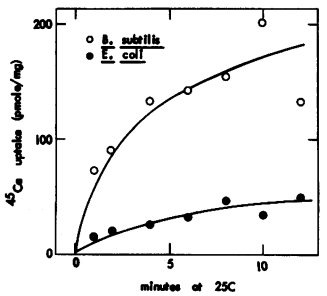

FIG. 2. *Calcium uptake by membrane vesicles from E. coli and B. subtilis. Ascorbate, PMS, and 15 μM ^{45}Ca were added.*

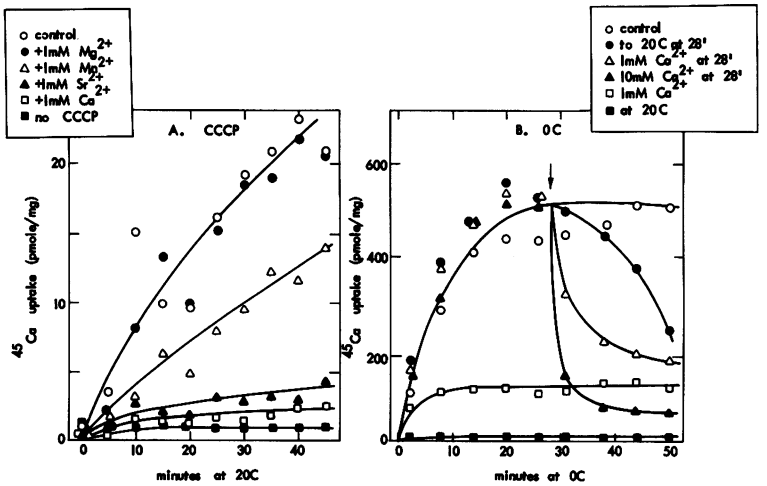

FIG. 3. *Calcium uptake by B. subtilis cells with added CCCP or at 0 C. (A) Cells were grown, centrifuged, resuspended at 12.5 mg/ml, and diluted 50-fold into glycylglycine-sodium chloride buffer containing 50 μM CCCP and ^{45}Ca (0.1 μM, 0.1 μCi/ml). The flasks also contained 1.0 mM chloride salts of Mg^{2+}, Mn^{2+}, Ca^{2+}, or Sr^{2+} as indicated. A control flask without CCCP was included in this experiment. (B) Cells were grown, centrifuged, and diluted into glycylglycine-sodium chloride buffer with ^{45}Ca (3.0 μM; 0.14 μCi/ml) at 0 or 20 C. $CaCl_2$ (1.0 mM) was added to one flask (□); at 28 min, Ca^{2+} was added to two flasks, and a third flask was moved into the 20 C bath.*

initially exposed to 0 C conditions (data not shown). *E. coli* cells accumulate ^{45}Ca both at 0 C (28) and in the presence of uncouplers. However, the accumulation by these cells is quantitatively much less than by a comparable cell mass of *B. subtilis*. *Rhodopseudomonas capsulata* cells also accumulate low levels of calcium with CCCP (Jasper, manuscript in preparation), suggesting that the quantitative difference may be characteristic of gram-negative bacteria when compared with gram-positive bacteria.

The initial rate of calcium uptake by whole cells is a function of the concentration of CCCP added (Fig. 4) and, to a lesser degree, this is also true of the net accumulation after 30 to 60 min. The two experiments (Fig. 4) were run under different conditions, one in glycylglycine buffer and trace calcium and the other in tryptone broth containing 10^{-4} M Ca^{2+}. Nevertheless, the results can be represented by a single curve (Fig. 4) with maximum rate of calcium uptake at 40 to 60 μM CCCP. The rate of calcium uptake then starts to decrease, but even at 200 μM CCCP the rate of calcium uptake is still half of the peak rate. The other "uncouplers" of oxidative phosphorylation, pentachlorophenol and tetrachlorosalicylanilide, have similar effects to CCCP and stimulate the accumulation of ^{45}Ca by *B. subtilis* cells in glycylglycine buffer, but higher concentrations of pentachlorophenol and tetrachlorosalicylanilide are needed for comparable rates of calcium uptake than with CCCP (data not shown). Fluorophenyl carbonylcyanide hydrazone (FCCP), which is a more potent uncoupler than CCCP, brings about the same level of ^{45}Ca uptake as does CCCP, but with fluorophenyl carbonylcyanide hydrazone the optimum concentration is about 2 μM (data not shown). This correlation between the potency as an uncoupler and its potency in stimulating calcium uptake adds to the argument that the phenomena are related.

CCCP-dependent calcium accumulation follows Michaelis-Menten saturation kinetics (Fig. 5), with a K_m of 0.36 mM and a V_{max} of 330 nmol/min per g (dry weight) of cells at 20 C with 50 μM CCCP present. In replicate experiments of this type, the V_{max} was relatively reproducible, but the apparent K_m varied by a factor of 10 although the data in each separate experiment appeared to lie on a rectangular hyperbola as required for saturation kinetics. We do not know the basis for this lack of reproducibility, but a similar lack of reproducible K_m characterized earlier studies of the uptake of calcium (10, 12; unpublished data). Inhibition studies, with addition of 1.0 mM

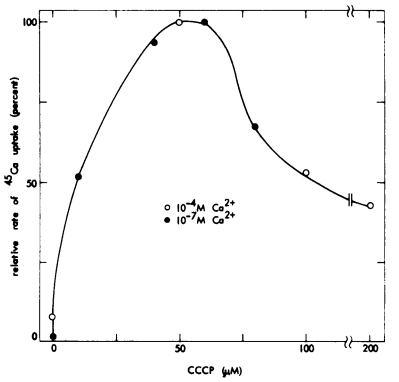

FIG. 4. *Effect of CCCP on calcium uptake by B. subtilis in either tryptone broth (10^{-4} M Ca^{2+}, ○) or glycylglycine buffer (10^{-7} M added Ca^{2+}, ●). At the beginning of the experiment, cells were diluted 50-fold into the medium containing ^{45}Ca and the indicated concentration of CCCP. The CCCP was dissolved in dimethylsulfoxide, which was added to the test solutions at a constant 2% (vol/vol). The initial rates of calcium uptake were normalized to the highest rate in each medium (200 pmol/min per mg in tryptone or 0.49 pmol/min per mg in glycylglycine-sodium chloride buffer).*

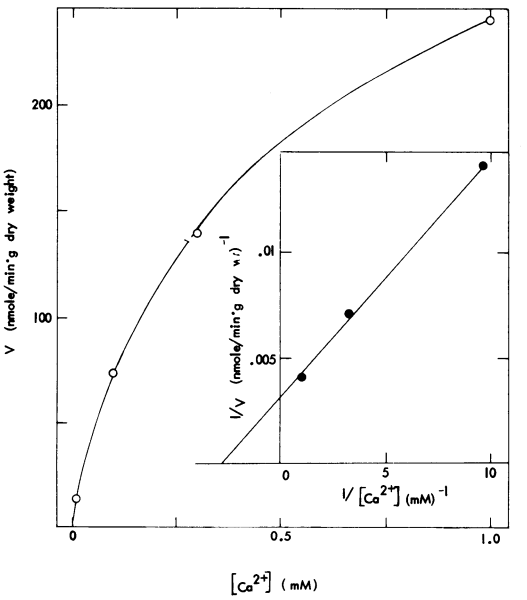

FIG. 5. *Concentration dependence of calcium uptake by B. subtilis cells with added CCCP. The initial rates of uptake of calcium in glycylglycine-sodium chloride buffer containing 50 μM CCCP and various concentrations of calcium and ^{45}Ca were determined as in Fig. 3. The inset shows the reciprocal rates of uptake and concentration.*

nonradioactive salts of divalent cations, showed the same sequence of specificity with intact cells and CCCP as previously shown with membrane vesicles (Fig. 1)—that is, Ca^{2+} was more inhibitory of ^{45}Ca uptake than Sr^{2+}, which was more inhibitory than Mn^{2+}, and Mg^{2+} was essentially without effect (data not shown). $LaCl_3$ (100 μM) strikingly inhibits the ^{45}Ca uptake (1 μM) both at 0 C and with CCCP, but we have not pursued the question as to whether the mechanism is similar to that reported with mitochondria (19).

Our attempts to measure the intracellular calcium level in bacteria metabolizing at 25 or 37 C have been frustrated both by binding to the cell surface and by lysis of *B. subtilis* during extensive washing. *E. coli* cells bound approximately 10% of the ^{45}Ca added to tryptone broth during growth (50 nmol/mg [dry weight] of cells). Extensive washing by centrifugation or on membrane filters reduced this value to less than 0.02% (100 pmol/mg). When nonradioactive cells were exposed to trace ^{45}Ca in glycylglycine buffer, the fraction bound to unwashed cells and remaining fraction bound to washed cells were about the same. Estimating 4 μl of cell water per mg (dry weight) and with the unsatisfactory assumptions that the residual calcium was free in the cell water and that none has been lost during washing, we can calculate that the intracellular calcium was, perhaps, 25% of the extracellular concentration. Most of the residual calcium would be expected to be tightly surface bound or bound to intracellular structures, which would lead to an overestimate of intracellular calcium. Indeed, our best guess, based on experience with the other divalent cations (9, 25–29), is that no more than $1/10$ of the intracellular calcium would be expected to be unbound. This then leads to an estimate (although only an order-of-magnitude estimate) that the free intracellular calcium level is a few percent of the extracellular level. Losses of intracellular free cation during filter washing was not likely to be a serious problem, since we did not find this with isotonic washing during potassium, magnesium, and manganese measurements (8, 9, 25–28).

DISCUSSION

We have looked at Ca^{2+} uptake under three conditions: in cells at 0 C, in cells at 20 C with CCCP, and in membrane vesicles in the absence of respiratory substrates. The specificity, as measured by inhibition of ^{45}Ca uptake by addition of nonradioactive divalent cations, seems to be the same under all conditions, i.e., Ca^{2+} over Sr^{2+} over Mn^{2+} over Mg^{2+}. These three conditions permit energy-independent accumulation of Ca^{2+} by a process showing substrate specificity and saturation kinetics as a function of Ca^{2+} concentration. These properties are generally diagnostic of carrier-mediated, facilitated transport, although they do not offer tight proof of such.

We propose that the system generally functions in the reverse direction, taking Ca^{2+} out of the cell to maintain a low intracellular concentration of Ca^{2+}. The effect of the uncouplers and low temperature then would be to slow or stop the normal metabolically active calcium efflux, allowing calcium movement by facilitated diffusion via the same calcium carrier molecules. A more direct experimental approach would be to preload cells with ^{45}Ca and measure the characteristics of the excretion process. However, we have found this experimentally difficult.

The situation in *E. coli* and *B. subtilis* is similar to that in mammalian cells, where ^{45}Ca extrusion has been studied (2, 3, 24). Organelles, such as mitochondria (19) and the sarcoplasmic reticulum (20), show energy-dependent calcium uptake mediated by highly calcium-specific systems. The result of extrusion from the cells plus accumulation by organelles appears to be a universal lowering of the calcium level within all cell types (3).

After these experiments were completed, we exchanged manuscripts with two laboratories involved in related series of experiments. Golub and Bronner (12) have been studying ^{45}Ca accumulation with membrane vesicles prepared from log-phase *B. megaterium* and find energy-dependent calcium accumulation with freshly prepared membrane vesicles. Silver et al. (29) similarly found ascorbate-PMS stimulation of ^{45}Ca uptake with fresh membrane vesicles from *B. subtilis*; neither laboratory has pursued the basis for the change from energy-dependent transport to energy-independent transport. Rosen and McClees (23) studied calcium transport in two types of membrane vesicles from *E. coli*. They found little or no calcium uptake by vesicles prepared by osmotic lysis procedures equivalent to those we used, but did find high-level energy-dependent calcium transport by "everted" membranes prepared by passage through a French pressure cell. Rosen and McClees (23) interpret their data as being consistent with a calcium transport system in *E. coli* that is oriented in the membrane to carry out energy-dependent transport from inside the cell to the outside, as suggested earlier on less direct evidence (28). In summary, studies from

several laboratories indicate the existence of highly specific carrier-mediated transport systems for calcium in bacterial cells.

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