Maintenance of Intracellular Calcium in Escherichia coli*

Preeti Gangola and Barry P. Rosen‡

From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

Recently a series of fluorescent calcium indicator dyes have been developed for measurement of free intracellular calcium in eukaryotic cells. Here we report the use of one such dye, fura-2, for the study of intracellular calcium levels in the prokaryote Escherichia coli. Cells of E. coli were loaded with the membrane-permeable acetoxyethyl ester of fura-2, which was cleaved intracellularly to give the free pentaacid. The concentration of free [Ca2+]i, in unstarved cells was maintained at 90 ± 10 nM, irrespective of the Ca2+ concentration in the extracellular medium. Cells of a strain lacking the H+-translocating ATPase were depleted of endogenous energy reserves and loaded with calcium. In this strain oxidative phosphorylation is uncoupled, so ATP is not produced by respiration. In starved cells [Ca2+]i varied from 0.2 to 0.7 μM when the loading Ca2+ concentration varied from 10 μM to 10 mM. Addition of glucose lowered the Ca2+ levels to 90 nM. Addition of respiratory substrates as energy donors produced cyanide-sensitive efflux. Total cell Ca2+ increased in parallel to the extracellular calcium, but the pool of free calcium did not equilibrate with the total cellular pool. These results demonstrate that 1) the pool of total Ca2+ in the bacterial cell is large and responds to extracellular calcium, 2) the free [Ca2+]i is independent of extracellular calcium, and 3) energy in the form of a proton motive force is required for maintenance of the free intracellular pool of calcium.

Calcium ion plays a pivotal role in the regulation of various cellular processes in eukaryotic cells. Although little is known about the physiological role of the ion in prokaryotes, calcium transport systems have been found in all bacterial species investigated so far (1, 2). The transport of calcium in bacterial cells was first demonstrated in Escherichia coli, where it was demonstrated that Ca2+ is extruded from the cells in a temperature-dependent fashion (3). The existence of a metabolically linked efflux system was suggested. Tsujibo and Rosen (4) showed that calcium extrusion is driven by a proton motive force. Detailed studies with everted membrane vesicles of E. coli (5–7) demonstrated that the calcium extrusion occurs via a secondary Ca2+/H+ exchange catalyzed by a calcium/proton antiporter. A second system for calcium extrusion, a calcium phosphate symporter/proton antiporter, has also been identified in everted membrane vesicles (8).

From studies with E. coli (4–8) and other bacterial species (9–12), it has been estimated that, in analogy to eukaryotes, the cytosolic Ca2+ concentration is maintained well below the extracellular medium. However, no actual measurement of free Ca2+ in cytosol has been performed. The radioisotope technique used in previous studies (4) reports the total Ca2+ associated with the cells. Use of calcium-sensitive dyes in bacteria has been hampered by the impermeability of the dyes and/or the small size of the cells. The recent development of the fluorescent dye fura-2 (13) has enabled us to measure the intracellular Ca2+ levels in E. coli. In this communication we report the first measurement of intracellular free Ca2+ in bacterial cells. This study shows that in E. coli the level of cytosolic calcium is highly regulated and confirms the previous suggestion that calcium extrusion is catalyzed by a Ca2+/H+ antiporter driven by the proton motive force.

MATERIALS AND METHODS

Growth of Cells—E. coli K-12 strain AN120 (uncA401 argE3 thi-1 rpsL) (14) cultures were grown at 37 °C in minimal medium (15) supplemented with 0.5% glycerol, vitamin B1 (1.25 μg/ml), and casamino acids (0.1%).

Starvation and Permeabilization of Cells—Overnight cultures (100 ml) were centrifuged at room temperature and washed two times with 100 ml of 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl2 (buffer A). The cells were depleted of endogenous energy reserves by incubation with 5 mM 2,4-dinitrophenol in 100 ml of buffer A at 37 °C for 2 h (16). The starved cells were centrifuged and washed two times with buffer A at room temperature. The cells were made permeable to fura-2-pentaacetoxyethyl ester and ionophores by treatment with EDTA (17). Washed cells (starved or unstarved) obtained from 100 ml of culture were resuspended in 3 ml of 0.12 M Tris-HCl, pH 8.0. The suspension was added to 50 ml of warm (37 °C) solution of 0.12 M Tris-HCl, pH 8.0, containing 0.2 mM EDTA and shaken at 37 °C. After 2 min the reaction was stopped by addition of MgCl2 to a final concentration of 1 mM. The cells were then centrifuged and washed once with buffer A at room temperature.

Fura-2 Loading in Cells—For most experiments permeabilized cells were suspended in 2 ml of buffer A and incubated with 5 μM fura-2 pentaacetoxyethyl ester at 37 °C. After 2 h the cells were diluted with 50 ml of buffer A and centrifuged. The cells were washed three times with 50 ml of buffer A, each wash, and suspended in 2 ml of buffer A. For fluorescence measurements, 50 μl of this suspension was diluted to 2 ml with buffer A, with or without CaCl2, as indicated. Starved cells were loaded with Ca2+ by adding desired concentrations of CaCl2 in the incubation medium during the dye loading. All steps were performed at room temperature unless otherwise noted.

Fluorescence Measurements—Fluorescence measurements were performed with an Amino-Bowman spectrophotofluorometer interfaced with an AT&T 6300 computer. The analog signal from the fluorometer converted to a digital output using the data acquisition program Vacuum MkII (Micro Based Computers, Baltimore, MD). Data analysis was done with Lotus 123.

The standard monochromator settings were at wavelengths of 340 nm for excitation and 510 nm for emission with a 5.5-mm band pass. During the measurements the cell suspension was continuously stirred. Reagents were added with a microliter syringe through a light-protected port.

* This work was supported by United States Public Health Service Grant DE06071 from the National Institutes of Health and Grant PCM-8516039 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom requests for reprints should be addressed: Dept. of Biochemistry, Wayne State University, School of Medicine, Scott Hall of Basic Medical Sciences, 540 East Canfield, Detroit, MI 48201.
where $F$ is the fluorescence intensity of dye in the cells, $F_{\text{max}}$ and $F_{\text{min}}$ are the intensities at saturating and zero calcium concentrations, respectively, and $K_d$ is the dissociation constant for fura-2 determined under the appropriate conditions. The $K_d$ was determined to be 315 nM by titration of $1 \mu M$ fura-2 in buffer A. After the measurement of fluorescence intensity under desired conditions, cells were permeabilized with $25 \mu M$ gramicidin or $10 \mu M$ ionomycin. $F_{\text{max}}$ was measured by addition of $1 \mu M$ CaCl$_2$, and $10 \mu M$ EGTA was added to determine $F_{\text{min}}$.

Measurement of Total Cell Ca$^{2+}$—Total cellular calcium was measured using the radioactive tracer $^{45}$Ca. Cells loaded with fura-2 were suspended in 0.2 ml of buffer A containing the desired concentrations of CaCl$_2$ and allowed to passively equilibrate at room temperature for 1 h. Calcium-loaded cells were diluted 100-fold with buffer A to minimize the extracellular binding (4). Portions (1 ml) of the cells were filtered through 0.45-µm pore size nitrocellulose filters. The filters were washed three times with 5 ml each time of the same buffer, and radioactivity was measured by liquid scintillation counting. Where indicated, 10 mM glucose was added at 30 min after dilution of cells, and 1-ml portions were filtered after 10 min and washed as described above.

Measurement of Cytosolic Volume—The cytosolic volume was measured by the distribution of $^{3}H_2O$ and $^{[14]C}$sucrose (19). The cytosolic volume was determined to be $0.9 \mu l/g$ total cell protein and was not significantly altered by starvation of cells, EDTA treatment, or addition of glucose.


developed from E. coli.


distinguishable from that of ester, peaking at 370 nm, to that of Ca$^{2+}$ indicator dye, lowered the apparent values of $[Ca^{2+}]_{i}$, resulting in a shift in the excitation spectrum.


cells were loaded with fura-2 pentaacetoxymethyl ester by addition of $1 mM$ CaCl$_2$, and $10 mM$ EGTA was added to determine $F_{\text{min}}$.


to the cytosol and was hydrolyzed. Hydrolysis was monitored by the gradual shift in the excitation spectrum from that of ester, peaking at 370 nm, to that of Ca$^{2+}$ indicator dye. These mixed acid esters gave rise to excitation spectra peaking at intermediate wavelengths and were insensitive to free calcium inside the cells. The concentration of free fura-2 pentaacetoxymethyl ester inside the cells was determined as described (18). Washed cells were permeabilized with $25 \mu M$ gramicidin, and the resulting fluorescence intensity in presence of excess CaCl$_2$ (corrected for the autofluorescence of permeabilized unloaded cells) was compared with the standards containing known concentrations of fura-2. With a loading concentration of $5 \mu M$ fura-2 pentaacetoxymethyl ester, the free acid trapped in the cells was determined to be 70-95 µM. Leakage of dye from the cells was found to be less than 1% h$^{-1}$.

$[Ca^{2+}]_{i}$ in E. coli—The cytosolic concentration of free Ca$^{2+}$ in the unstarved E. coli cells was determined to be $90 \pm 9 \text{ nM}$ ($n = 6$). In order to examine whether the buffering of free calcium by the dye lowered the apparent values of $[Ca^{2+}]_{i}$, cells were loaded with fura-2 pentaacetoxymethyl ester concentrations ranging from 1.0 to 10 µM, producing free dye concentrations of 20 to 100 µM, $[Ca^{2+}]_{i}$ was determined to be independent of dye concentration over that range. Furthermore, the basal $[Ca^{2+}]_{i}$ level was maintained constant over a range of medium calcium from $10 \mu M$ to $10 \mu M$ CaCl$_2$ (Fig. 2 and Table 1).

Effect of Ionophores on $[Ca^{2+}]_{i}$—When cells were exposed to ionomycin or gramicidin, $[Ca^{2+}]_{i}$ increased as a function of the concentration of ionophore and the medium $[Ca^{2+}]_{i}$. At $0.5 \mu M$ ionomycin $[Ca^{2+}]_{i}$ increased gradually as a function of external calcium (Fig. 2). At $5 \mu M$ ionomycin fura-2 fluorescence increased to a maximum within seconds. At this point the intracellular calcium concentration was assumed to be equal to the extracellular, as no further increase in the fluo-
Intracellular free and total calcium as a function of extracellular [Ca$^{2+}$] in control and energy-depleted cells of E. coli

| [Ca$^{2+}$]$_{ec}$ | [Ca$^{2+}$]$_{ic}$ | [Ca$^{2+}$]$_{total}$ | [Ca$^{2+}$]$_{total}$ |
|------------------|------------------|-----------------|-----------------|
| mM               | mM               | mM              | mM              |
| 0.08             | 0.094            | 0.012           | 0.21            |
| 0.18             | 0.098            | 0.048           | 0.34            |
| 0.33             | 0.100            | 0.181           | 0.41            |
| 1.08             | 0.094            | 0.350           | 0.54            |
| 5.08             | 0.110            | 0.670           | 0.69            |
| 10.08            | 0.120            | 1.390           | 0.70            |

* Control cells were not depleted of endogenous energy reserves and assayed without addition of exogenous energy source.
* Starved cells were depleted of endogenous energy reserves and assayed without addition of exogenous energy source.

Free intracellular calcium was determined from fura-2 fluorescence.

Total intracellular calcium was determined from $^{40}$Ca$^{2+}$ distribution and expressed as a nominal concentration using a value of 0.9 μl of cell water/mg of cell protein.

**Fig. 3. Energy dependence of calcium efflux from starved cells.** Cells were depleted of endogenous energy reserves and loaded with fura-2 pentaacetoxymethyl ester and 0.25 mM CaCl$_2$. Loading conditions were as described in the legend to Fig. 2. Curve A, no addition; curve B, 10 mM glucose added at 50 s; curve C, 10 mM NaCN added 5 min prior to beginning of recording and 10 mM glucose added at 50 s. For clarity curves A and C have been offset upwards by 0.5 and 1%, respectively.

**Energetics of Ca$^{2+}$ Efflux—**We have reported previously (4) that E. coli cells depleted of endogenous energy reserves could be passively loaded with calcium. These starved cells are unable to extrude calcium, but calcium efflux could be initiated by addition of an energy source. However, those experiments measured only total calcium with $^{40}$Ca$^{2+}$. In order to examine the free [Ca$^{2+}$], during calcium efflux, it was necessary first to deplete the cells of endogenous energy reserves by incubation with dinitrophenol (16). The starved cells were then loaded with varying concentrations of Ca$^{2+}$, and the cytosolic free [Ca$^{2+}$] was assayed by fura-2 fluorescence. In energy-depleted cells [Ca$^{2+}$], varied proportionally with the loading concentration of Ca$^{2+}$ (Table I). However, even though [Ca$^{2+}$] increased, the levels attained by starved cells after 2 h of incubation with calcium never exceeded 0.1% of the external concentration, suggesting a very low passive permeability to Ca$^{2+}$. After 2 h of loading, addition of EGTA to remove extracellular Ca$^{2+}$ did not alter [Ca$^{2+}$]. Addition of glucose as an energy source produced a rapid decrease in [Ca$^{2+}$], to the level observed in the unstarved cells (Fig. 3). In energized cells efflux was observed when either 1 mM EGTA or 1 mM CaCl$_2$ was present in the assay medium. These results are consistent with our earlier report showing that extrusion of Ca$^{2+}$ from cells, even down a concentration gradient, requires energy (4). It should be noted that the initial increase in the fluorescence intensity following the addition of glucose appears to be an artifact and not the result of a transient increase in [Ca$^{2+}$], because a similar increase in autofluorescence was observed in cells not loaded with fura-2. This increase in autofluorescence probably results from a change in light scattering. No corresponding change in cell volume was found after glucose addition, so the cause of the increase in light scattering is unknown.

The effect of various energy sources on Ca$^{2+}$ efflux was examined. Because E. coli strain AN120 lacks the proton-translocating ATPase, there is no interconversion of ATP and the proton motive force (16). Therefore, conditions can be established to produce either chemical or electrochemical energy in the absence of the other. Metabolism of glucose produces both ATP through glycolytic metabolism and a proton motive force by respiration. In the presence of cyanide to inhibit respiration, glucose metabolism produces only ATP without a proton motive force. On the other hand, respiratory substrates such as lactate and succinate produce only proton motive force with little or no substrate level phosphorylation. Inhibition of respiration by cyanide prevented efflux of Ca$^{2+}$ from the cells when glucose was present as an energy source (Fig. 3), even though the ATP levels are unaffected by cyanide treatment (16). Addition of the respiratory substrates lactate or succinate also produced calcium efflux, although the rate was slower (Fig. 4). After reaching a steady state, the final level of free intracellular calcium was approximately the same with the three energy donors (Table II). These results confirm the earlier suggestions that Ca$^{2+}$ efflux in E. coli is coupled to the proton motive force (4) and catalyzed by Ca$^{2+}$/H$^+$ antiporters (5-8).

**Relation of [Ca$^{2+}$], and Total Cytosolic Ca$^{2+}$—**Total intracellular Ca$^{2+}$ in energy-depleted cells was measured with $^{40}$Ca under conditions identical to those used for fura-2 measurements of free [Ca$^{2+}$]. The contribution of extracellularly bound Ca$^{2+}$ was minimized by 100-fold dilution of cells in Ca$^{2+}$-free buffer and rigorous washing after filtration (4). In energy-depleted cells both [Ca$^{2+}$], and total [Ca$^{2+}$] (expressed as a nominal concentration) increased with increasing extracellular Ca$^{2+}$ concentration (Table I). Total intracellular Ca$^{2+}$ was equal to the extracellular Ca$^{2+}$ concentration. On the other hand, free [Ca$^{2+}$], increased only 5-fold over the entire
with 0.25 mM CaCl₂. This is far in excess of what a proton motive force can support for a single secondary exchanger of reasonable H⁺:Ca⁺ stoichiometry (a ratio of 3); has been proposed for the calcium/proton antiporter (8). However, the second calcium extrusion system, which exchanges protons for the neutral [CaHPO₄] complex, is coupled to both the proton motive force and the phosphate gradient (8). If the concentration of phosphate is higher inside the cell than out, the phosphate gradient could contribute substantially to the extrusion of calcium. Another possibility is that extrusion is also coupled to ATP, as in the case of the streptococci (11). However, extrusion was observed when only electrochemical energy was available in the absence of ATP and not when ATP was present in the absence of a proton motive force (Figs. 3 and 4). Thus, only electrochemical energy and not chemical energy is required for extrusion. It is not clear whether the thermodynamic calculations are meaningful in this case since the system is displaced from the steady state. Calculations of the relationship of concentration ratios and electrochemical potentials assume equilibrium or, at worst, a steady state close to equilibrium. Clearly, the passive permeability of calcium is so low and the calcium buffering so great that a steady state so low and the calcium buffering so great that a steady state is not attained even in starved cells, where extrusion does not occur. Thus, for a system displaced far from the steady state equilibrium thermodynamic calculations may have little relevance.

In conclusion, the present study demonstrates that cells of E. coli maintain an intracellular [Ca⁺²] similar to that of eukaryotic cells. Three factors contribute to the low [Ca⁺²]:

1. restricted entry of the ion into cytosol due to low passive permeability,
2. a large pool of bound Ca⁺², and
3. efficient extrusion of calcium via energy-dependent antiport systems.

Not only is [Ca⁺²] kept low, but it is maintained at a constant value of 10⁻⁶ M, implying a finely tuned regulatory process. Why this should be so is not clear, since calcium has not been demonstrated to participate in any intracellular event in bacteria (1, 2). The role of calcium in regulating a variety of physiological processes in eukaryotic cells has been well established. The similarity in the maintenance of [Ca⁺²] between E. coli and eukaryotes suggests that a regulatory role for intracellular Ca⁺² may exist in prokaryotes.

REFERENCES

1. Rosen, B. P. (1982) in Membrane Transport of Calcium (Carafoli, E., ed.) pp. 187–216, Academic Press, New York
2. Rosen, B. P. (1987) BBA Rev. Biomembr. 906, 101–110
3. Silver, S., and Kralovic, M. L. (1969) Biochem. Biophys. Res. Commun. 34, 640–645
4. Tsujishita, H., and Rosen, B. P. (1989) J. Bacteriol. 154, 854–858
5. Rosen, B. P., and McCles, J. S. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 5042–5046
6. Brey, R. N., Beck, J. C., and Rosen, B. P. (1978) Biochem. Biophys. Res. Commun. 83, 1588–1594
Intracellular Calcium in E. coli

7. Brey, R. N., and Rosen, B. P. (1979) *J. Biol. Chem.* **254**, 1957–1963
8. Ambudkar, S. V., Zlotnick, G. W., and Rosen, B. P. (1984) *J. Biol. Chem.* **259**, 6142–6146
9. Bronner, F., Nash, W. C., and Golub, E. E. (1975) in *Spores VI* (Gerhardt, P., Costilow, R. N., and Sadoff, H. L., eds) pp. 356–361. American Society for Microbiology, Wash. D. C.
10. Bhattacharyya, P., and Barnes, E. M., Jr. (1976) *J. Biol. Chem.* **251**, 5614–5619
11. Kobayashi, H., Van Brunt, J., and Harold, F. M. (1978) *J. Biol. Chem.* **253**, 2085–2092
12. Belliveau, J. W., and Lanyi, J. K. (1978) *Arch. Biochem. Biophys.* **186**, 98–105
13. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
14. Butlin, J. D., Cox, G. B., and Gibson, F. (1971) *Biochem. J.* **124**, 75–81
15. Tanaka, S., Lerner, S. A., and Lin, E. C. C. (1967) *J. Biol. Chem.* **93**, 642–648
16. Berger, E. A., and Heppel, L. A. (1974) *J. Biol. Chem.* **249**, 7747–7755
17. Leive, L. (1968) *J. Biol. Chem.* **243**, 2373–2380
18. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
19. Stock, J. B., Rauch, B., and Roseman, S. (1977) *J. Biol. Chem.* **252**, 7850–7861
20. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 535–541
21. Gennaro, R., Pozzan, T., and Romeo, D. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1416–1420
22. Poenie, M., Alderton, J., Tsien, R. Y., and Steinhardt, R. A. (1985) *Nature* **315**, 147–149