ATP-DEPENDENT CALCIUM RELEASE FROM BINDING SITE IN *STREPTOCOCCUS BOVIS*: BOUND VERSUS FREE POOLS

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The introduction of the acetoxymethyl ester of fura-2 (fura-2/AM) has made possible the measurement of the concentration of intracellular free calcium (free $[Ca^{2+}]_i$) in bacteria. We report here a study of intracellular calcium levels and exclusion mechanisms in *Streptococcus bovis* using fura-2/AM and $^{45}CaCl_2$. The total $[Ca^{2+}]_i$ in cells not energized with glucose increased in proportion to external Ca^{2+} concentration over the range 0.1–0.5 mM, the intra- and extracellular levels being approximately equal. In contrast, the free $[Ca^{2+}]_i$ remained at 71.1 ± 1.9 nM in the face of extracellular concentrations from 0.1 to 5 mM. Energizing by the addition of glucose produced a dramatic increase in the intracellular concentration of ATP and a marked decrease in total $[Ca^{2+}]_i$, but had no effect on the free $[Ca^{2+}]_i$ regardless of whether extracellular calcium was high or low. Total $[Ca^{2+}]_i$ was affected little, if at all, by the metabolic inhibitors FCCP and DCCD, implying that neither proton motive force nor $F_0F_1H^+$ -ATPase is necessary for calcium exclusion. In contrast, total $[Ca^{2+}]_i$ and ATP were both greatly reduced by iodoacetate. These results suggest that 1) *S. bovis* has a remarkable calcium buffering capacity, and 2) most of its intracellular calcium exists in a bound form which can be released by energizing via a mechanism coupled to ATP hydrolysis.

Two different mechanisms underlying the exclusion of intracellular calcium ion have been reported to be present in living cells: one involves a primary pump and the other secondary exchangers. Eukaryotes utilize both a primary pump and secondary exchangers (11,18), while most bacteria utilize only secondary exchangers (2,4,5,15,17,19,20,22). However the streptococci, exceptionally among

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the bacteria examined so far, have been found to utilize a primary calcium pump (1, 13, 14). Furthermore, there are species differences in the mechanisms used for calcium exclusion even within one and the same genus: thus, *Streptococcus faecalis*, *S. sanguis* and *S. lactis* exclude calcium using a primary pump, while *S. cremoris* (8) uses secondary exchangers. More work seems to be necessary to fully characterize the mechanisms underlying intracellular calcium ion exclusion among the streptococci.

It is important to know the level of intracellular free calcium ion to elucidate its physiological role in living cells. In prokaryotes, it has been difficult to make actual measurements of the free calcium ion because of the small size of the prokaryote cell and the impermeability of most calcium-sensitive fluorescent dyes. However, the recent development of membrane-permeable calcium-sensitive fluorescent dyes has greatly facilitated the understanding of calcium movement across membranes (see review by Cobbold and Rink (7)). They can be incorporated into small cells in a nondisruptive manner, so that the number of cell types in which the intracellular free calcium ion can be studied has increased dramatically, and they permit continuous monitoring of changes in free $[Ca^{2+}]_i$. The introduction of the acetoxymethyl ester of fura-2 (fura-2/AM), a membrane-permeable derivative of fura-2 (see review by Cobbold and Rink (7)), has now made possible the measurement of free $[Ca^{2+}]_i$ in bacteria (9, 10).

Taking advantage of this development, we have compared the free $[Ca^{2+}]_i$ with the total pool and have observed the effects of glucose as an energy source and of inhibitors on calcium efflux in an attempt to examine and characterize the mechanism, be it primary pump or secondary exchangers, used to exclude calcium from intact cells of *S. bovis*, a facultative anaerobic rumen streptococcus.

MATERIALS AND METHODS

Organisms and growth conditions. *Streptococcus bovis* JB1 was kindly provided by Dr. M. P. Bryant (Departments of Animal Science and Microbiology, University of Illinois, U.S.A.). Cultures were grown anaerobically in a medium similar to the medium 10 broth described by Caldwell and Bryant (6) except that the glucose concentration was 20 mM. The medium was prereduced and inoculated. The gas phase was 100% CO₂. The medium was adjusted to pH 6.8 prior to sterilization and cultures were grown at 37°C.

Fura-2/AM loading into cells and fluorescence measurements. Stationary growing cultures (80 ml) were harvested by centrifugation (3,500 × g, 23°C, 5 min), washed twice and resuspended with 50 ml of 50 mM Tris-HCl, at pH 7.5, containing 100 mM KCl and 1 mM MgCl₂ (buffer A). Fura-2/AM can penetrate the membrane of *S. bovis*, a Gram-positive anaerobic bacterium without an outer membrane, and enter the cytosol, as it does through the eukaryotic plasma membrane; in contrast, cells of *Escherichia coli* need to be made permeable to fura-2/AM by treatment with Tris and EDTA (10). Washed and resuspended cells were

anaerobically incubated under N₂ with 5 μ M fura-2/AM at 37°C with or without 0.25 mM calcium. After 2 h, the cells were washed three times with 50 ml of buffer A and resuspended in the same buffer (approximately 1.0 mg of protein in 1.0 ml). Fluorescence measurements were performed at 23°C using a Hitachi F4000 spectrophotofluorometer interfaced with a computer. The standard settings were at wavelengths of 340 nm for excitation and 505 nm for emission with a 5-nm band-pass. During the measurements, the cell suspension was continuously stirred. Reagents were added using a microliter syringe through a light-protected port. All steps were performed at room temperature unless otherwise noted.

Measurement of free $[Ca^{2+}]_i$. The free $[Ca^{2+}]_i$ was calculated using the following equation (12):

$$[Ca^{2+}]_i = K_d(R - R_{\min}/R_{\max} - R)(F2_{\min}/F2_{\max})$$

where the fluorescence ratio R is the ratio $F1/F2$, where $F1$ and $F2$ are the fluorescence intensities at wavelengths $\lambda_1 = 340$ nm and $\lambda_2 = 380$ nm. K_d is the effective dissociation constant for fura-2 to be used 315 nm in our experiment. Experiments were terminated by the rapid addition of 100 μ M gramicidin. $R_{\max}$ values were calculated from the ratio $F1_{\max}/F2_{\max}$, the fluorescence levels at 340 and 380 nm, respectively, recorded after permeabilization with 100 μ M gramicidin in the presence of excess calcium. The ratio $R_{\min}$ was determined from the fluorescence intensities at wavelengths 340 and 380 nm after the addition of 10 mM EGTA following permeabilization.

Measurement of intracellular total calcium. The concentration of intracellular total calcium (total $[Ca^{2+}]_i$) was measured using the radioactive tracer ⁴⁵Ca. Calcium uptake was initiated by addition to the medium of ⁴⁵CaCl₂ (0.1 mM, unless otherwise noted). ⁴⁵Ca-loaded cells were diluted 50-fold with buffer A containing 0.1 mM LiCl 30 min after the addition of ⁴⁵CaCl₂. Portions (5 μ l) of the cell suspension were filtered through 0.45- μ m pore size Millipore filters. The filters were washed three times with 3 ml each time of the same buffer and radioactivity measured by liquid scintillation counting. All steps were performed at room temperature.

ATP determinations. Cells (approximately 1.0 mg of protein in 1.0 ml) were extracted for 20 min with 0.5 ml of ice-cold 14% perchloric acid which contained 9 mM EDTA; insoluble cell debris was then removed by centrifugation (13,000 $\times g$, 5 min, 23°C). Cells were rapidly pipetted into the extraction reagent and mixed by magnetic stirring during the whole extraction period. The supernatant (1 ml) was neutralized with 0.5 ml of KOH-KHCO₃ (1 M each, 0°C). The extract was assayed for ATP by the firefly luciferase method (16). To this end, neutralized extracts were diluted 50-fold with 40 mM Tris-H₂SO₄ buffer containing 2 mM EDTA, 10 mM MgCl₂ and 0.1% bovine serum albumin (pH 7.75). Next, 500 μ l of diluted extract was added to 100 μ l of purified luciferin-luciferase (Lumit, Nihon Zeneral Co., Tokyo, Japan). Light output was measured with a luminometer (CLD-100 Chemiluminescence detector, Tohoku Electronic Industrial Co., Sendai, Japan) using ATP as a standard.

Other procedures. The content of bacterial protein was determined by a Bio-Rad Protein Assay with bovine serum albumin as the standard.

Chemicals. $^{45}\text{CaCl}_2$ was obtained from NEN Research (Wilmington, DE, U.S.A.). Fura-2/AM and fura-2 were purchased from Dojin Chemical Research Co. (Kumamoto, Japan). Gramicidin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), dicyclohexylcarbodiimide (DCCD), iodoacetate and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and were purchased from commercial sources.

Statistical analysis of data. Where appropriate, data are expressed in the form mean $\pm$ SEM, with the number of experiments indicated by the value of *n*. The significance of differences was assessed using an unpaired Student's *t*-test, with *p* < 0.05 being considered significant.

RESULTS

Loading of fura-2/AM into S. bovis

Internal fura-2/AM is hydrolyzed by intracellular esterase and the free fura-2 cannot then permeate the membrane. Hydrolysis was monitored by the gradual shift in the excitation spectrum from that of the ester, peaking at 380 nm, to that of the free acid, peaking at 340 nm. Increasing the incubation time from 30 to 60 to 90 min (Fig. 1A–C) progressively increased the ratio between the fluorescence levels at 340 and 380 nm (see inset in Fig. 1). A further increase in incubation time caused no further increase in the ratio so that it reached a plateau between 90 and 120 min (Fig. 1C and D), suggesting that the hydrolysis of fura-2/AM was completed somewhere between 90 and 120 min under our experimental conditions. Accordingly, we subsequently used an incubation time of 120 min to ensure the complete hydrolysis of fura-2/AM.

Free Ca^{2+} in S. bovis

The free $[\text{Ca}^{2+}]_i$ in nonenergized cells of *S. bovis* was calculated to be 71.1 ± 1.9 nM (mean $\pm$ SEM, *n* = 4 for each concentration) and it remained constant over a range of calcium concentrations in the external medium from 0.1 to 5 mM (Fig. 2). No significant difference in the free $[\text{Ca}^{2+}]_i$ was found between nonenergized cells and starved cells (data not shown). After 2 h of fura-2/AM and calcium loading, the introduction of 5 mM EGTA into the medium, to remove extracellular calcium, failed to decrease the free $[\text{Ca}^{2+}]_i$ (Fig. 3B). The addition of glucose to the incubation medium likewise had no effect on the free $[\text{Ca}^{2+}]_i$ (Fig. 3A). In contrast, the addition of EGTA after permeabilization with 100 μM gramicidin caused a rapid fall to a lower plateau level, regardless of whether the concentration of extracellular calcium was relatively high (Fig. 3A) or lowered by the initial dose of EGTA (Fig. 3B). Thus, in our experiment, neither EGTA alone nor glucose had any significant effect on free $[\text{Ca}^{2+}]_i$.

Fig. 1. Hydrolysis of fura-2/AM by cells of *Streptococcus bovis*.

Cells were suspended in buffer A (see MATERIALS AND METHODS) at 2–3 mg of cell protein/ml and incubated with $5 \mu\text{M}$ fura-2/AM at 37°C . At various times, $50 \mu\text{l}$ of cell suspension was diluted with 2 ml of buffer A. Hydrolysis was then monitored by measuring the shift in the wavelength for maximum excitation. Incubation time was: curve A, 30 min; curve B, 60 min; curve C, 90 min; curve D, 120 min. Emission wavelength was 505 nm. Inset shows the change in fluorescence ratio $F1/F2$ with time, $F1$ and $F2$ being the fluorescence intensities at wavelengths $\lambda_1 = 340 \text{ nm}$ and $\lambda_2 = 380 \text{ nm}$.

Fig. 2. Effects of changes in the concentration of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) on the concentration of intracellular free (open circles) and total Ca^{2+} ($[\text{Ca}^{2+}]_i$) in *Streptococcus bovis*.

Fura-2/AM and $^{45}\text{Ca}^{2+}$ loading conditions and calculation of free $[\text{Ca}^{2+}]_i$ are as described under "MATERIALS AND METHODS." Total $[\text{Ca}^{2+}]_i$ and fluorescence intensity (for free $[\text{Ca}^{2+}]_i$) were measured 30 min after each addition of calcium.

Total Ca^{2+} in *S. bovis*

We used a value for the ratio of intracellular volume to protein of $2.6 \mu\text{l}/\text{mg}$ for stationary cells (21) to calculate the concentrations of total calcium and ATP. The total $[\text{Ca}^{2+}]_i$ in nonenergized cells increased with increases in the concentration of

Fig. 3. Effect on the concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) of energization and of changing the concentration of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$).

Nonenergized cells suspended in buffer A were loaded with fura-2/AM for 120 min at 37°C with 1 mM Ca^{2+} , washed three times and resuspended in 2 ml buffer A at 23°C ; fluorescence was then recorded. The free $[\text{Ca}^{2+}]_i$ was calculated as described under "MATERIALS AND METHODS." A, glucose was added at 150 s; B, 5 mM EGTA was added at 30 s to remove extracellular calcium and glucose was added 7 min later. In B, the initial addition of 5 mM EGTA produced an extracellular $[\text{Ca}^{2+}]$ lower than that used in the experiment shown in A. Additions were made as indicated: 10 mM glucose, 5 or 10 mM EGTA, $100\text{ }\mu\text{M}$ gramicidin.

extracellular calcium ($[\text{Ca}^{2+}]_o$) (Fig. 2). When such cells were energized with 10 mM glucose, there was a dramatic decrease in $^{45}\text{Ca}^{2+}$ so that total $[\text{Ca}^{2+}]_i$ fell to $30.1 \pm 3.4\text{ }\mu\text{M}$ (mean $\pm$ SEM, $n=7$) (Fig. 4) and there was a marked increase in intracellular ATP, which reached nearly 6.0 mM (Table 1). In contrast, non-energized stationary cultures of *S. bovis* had an ATP content of approximately 0.1 mM (Table 1). The addition of 10 mM glucose before $^{45}\text{Ca}^{2+}$ -loading in stationary cells resulted in low levels of intracellular $^{45}\text{Ca}^{2+}$ at a constant value of $22.4 \pm 2.4\text{ }\mu\text{M}$ (mean $\pm$ SEM, $n=7$) (Fig. 4).

Effects of ionophores and inhibitors on total Ca^{2+}

Glucose metabolism can produce two effects via ATP, one that generates a proton motive force through its hydrolysis and another in which its hydrolysis generates energy directly. When glucose-energized cells were treated with the protonophore FCCP, an uncoupler which collapses the proton gradient, there was little effect on total $[\text{Ca}^{2+}]_i$ (Fig. 5). Treatment with the $\text{F}_0\text{F}_1\text{H}^+$ -ATPase inhibitor, DCCD, had little or no effect on total $[\text{Ca}^{2+}]_i$ (Fig. 5) and only a minimal effect on intracellular ATP (Table 1). On the other hand, iodoacetate, an inhibitor of glycolysis, greatly decreased both total $[\text{Ca}^{2+}]_i$ and intracellular ATP (Fig. 5 and Table 1).

Fig. 4. Energy-dependent calcium efflux from nonenergized cells.

At time 0, 0.5 mM $^{45}\text{CaCl}_2$ was added to cell suspensions without (open circles) or with 10 mM glucose (closed circles); suspensions were then incubated at 23°C and the concentration of intracellular total Ca^{2+} ($[\text{Ca}^{2+}]_i$) measured. After 30 min (arrow), 10 mM glucose was added to cell suspensions previously without glucose.

Table 1. Effect of metabolic inhibitors on intracellular ATP concentration.

Treatment of energized or nonenergized cells	ATP (mM)
Nonenergized stationary cells	0.09
Glucose-energized stationary cells	5.9
250 μM DCCD	6.3
200 μM iodoacetate	0.13
500 μM iodoacetate	0.05

DISCUSSION

In the present study, to enable investigation of calcium exclusion mechanisms in *S. bovis*, the free $[\text{Ca}^{2+}]_i$ was compared with the total $[\text{Ca}^{2+}]_i$ in whole cells loaded with fura-2/AM and $^{45}\text{Ca}^{2+}$ (Fig. 2). The total $[\text{Ca}^{2+}]_i$ increased in proportion to the external concentration over the range 0.1–0.5 mM, the intra- and extracellular levels of total calcium being approximately equal. In contrast, free $[\text{Ca}^{2+}]_i$ was found to be 4–5 orders of magnitude less than the $[\text{Ca}^{2+}]_o$ and it was maintained at a constant value of 71.1 ± 1.9 nM over the same range of $[\text{Ca}^{2+}]_o$. This range, 0.1 to 0.5 mM, was used as the range for our experimental $[\text{Ca}^{2+}]_o$, because such concentrations are routinely used in the growth medium for *S. bovis*, for example 0.2 mM in our study. Even when $[\text{Ca}^{2+}]_o$ was increased outside this range (up to 5 mM) free $[\text{Ca}^{2+}]_i$ remained at a similar constant level. These observations, however, show that the cells of *S. bovis* are able to take up substantial amounts of calcium while maintaining free $[\text{Ca}^{2+}]_i$ at a low and constant level and that most of the calcium in whole cells exists in a bound form. This suggests that *S. bovis* has a remarkable calcium buffering capacity, as does isolated axoplasm (3).

Fig. 5. Effect of metabolic inhibitors (FCCP, DCCD, iodoacetate) on reduction in calcium caused by glucose-energization.

The concentration of intracellular total Ca^{2+} ($[Ca^{2+}]_i$) was measured 30 min after the addition of $^{45}CaCl_2$. Cells were energized by a 30-min incubation with 10 mM glucose. FCCP (10 μM), DCCD (50 and 250 μM), or iodoacetate (200 and 500 μM) were added 15 min prior to glucose addition. The percentage reduction in $[Ca^{2+}]_i$ after energization was calculated as the value of $[(A-B)/A] \times 100\%$, where A is $[Ca^{2+}]_i$ with added inhibitor (or ethanol) but without glucose, B is $[Ca^{2+}]_i$ with added inhibitor (or ethanol) with glucose.

In the case of *E. coli*, the removal of bound calcium from the cell can be achieved merely by diluting calcium-loaded cells 100-fold with calcium-free buffer; the total $[Ca^{2+}]_i$ then reaches a new steady-state level (22). However, in the present experiment with *S. bovis*, most of the bound calcium could not be removed simply by washing with calcium-free buffer (data not shown), suggesting that Ca^{2+} release from binding sites in *S. bovis* is driven by a different mechanism from that in *E. coli*. As we did not measure the concentration of cellular cold Ca^{2+} before the addition of $^{45}CaCl_2$, it remains unknown whether the increase in cellular $^{45}Ca^{2+}$ content was due to increased uptake of $^{45}Ca^{2+}$ into the bound pool which was not still saturated or due to the exchange of nonradioactive bound Ca^{2+} for $^{45}Ca^{2+}$. However, there is a possibility that increased total $[Ca^{2+}]_i$ could be attributed to increased uptake of Ca^{2+} into the bound pool since most of the bound Ca^{2+} could not be removed simply by washing and since the release of cellular $^{45}Ca^{2+}$ occurred only with energization of cells by glucose (Fig. 4).

The addition of glucose caused a dramatic increase in the concentration of intracellular ATP (Table 1) and a marked decrease in total $[Ca^{2+}]_i$ (Fig. 4), but had no effect on the free $[Ca^{2+}]_i$ regardless of whether $[Ca^{2+}]_o$ was relatively high or low (Fig. 3A and B). These results are different from those observed in *E. coli* (10) where the free $[Ca^{2+}]_i$ in cells energy-depleted by incubation with dinitrophenol varied in proportion with the loading concentration of calcium, and the addition of glucose lowered free $[Ca^{2+}]_i$. Moreover, our observation that iodoacetate, an inhibitor of glycolysis, produced a marked reduction in intracellular ATP and an almost complete inhibition of the effect of glucose on the total $[Ca^{2+}]_i$

(Table 1 and Fig. 5) suggests that the decrease in total $[Ca^{2+}]_i$ is due to energization coupled to ATP hydrolysis. This, in turn, suggests that the release of bound calcium from *S. bovis* is ATP-dependent. Our finding that the release of bound calcium caused by energizing is not accompanied by an increase in free $[Ca^{2+}]_i$ might be explained in the following way: 1) in *S. bovis* most of the calcium exists in a tightly bound form and is released from its binding site on energizing with glucose, 2) the pool size of intracellular free calcium is very small, so that the released calcium is expelled very rapidly and the steady state of free calcium is regained almost immediately.

Since the mechanisms underlying calcium efflux have been investigated previously in experiments in which free $[Ca^{2+}]_i$ has not been measured directly, the ATP-dependent calcium efflux reported in streptococci (1, 13, 14) may have actually represented, at least in part, ATP-dependent release of bound calcium. Our findings in the present study that energizing the cells of *S. bovis* caused the release of bound calcium from intracellular stores did not tell us which mechanism is employed for intracellular calcium exclusion. Accordingly, we examined the effects of inhibitors on total $[Ca^{2+}]_i$ to try to determine whether *S. bovis* uses secondary exchangers for this purpose. Since FCCP, which would be expected to collapse the proton gradient, had no effect (Fig. 5), calcium efflux in this organism appears not to be mediated by a calcium-proton antiporter. Moreover, DCCD had little or no effect on either calcium efflux or ATP synthesis (Fig. 5 and Table 1). These results show that neither $F_0F_1H^+$ -ATPase nor proton motive force is necessary for calcium exclusion in *S. bovis*, in agreement with the findings for *S. faecalis*, *S. sanguis* and *S. lactis* (1, 13, 14). That is, it is likely that *S. bovis* uses a mechanism other than the calcium/proton antiporter. This might be, for example, calcium exchange coupled with movement of another ion. However, the free $[Ca^{2+}]_i$ was 10^{-8} M at 10^{-3} M extracellular calcium (Fig. 2): a 10^5 -fold gradient. This gradient is far in excess of that expected if only calcium exchange coupled with another ion is involved in calcium extrusion from *S. bovis*. Moreover, it was not possible to distinguish the ATP used for the release of bound calcium from that used for efflux of free calcium ion. For these reasons, the possibility of the involvement of a primary pump coupled to ATP hydrolysis cannot be excluded. More work is necessary to reach a definite conclusion on this point.

It is not yet possible to finally identify the mechanism underlying calcium efflux from *S. bovis*, but the information obtained in the present study will help us to understand the processes regulating intracellular calcium. The present data do suggest that *S. bovis* has a remarkable calcium buffering capacity and that most of its intracellular calcium exists in a tightly bound form which is released on energizing by a mechanism directly coupled to ATP hydrolysis.

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