RESEARCH COMMUNICATION

Estimation of the free $[Ca^{2+}]$ gradient across endoplasmic reticulum membranes by a null-point method

Alan P. DAWSON,* Gillian T. RICH and Jeff W. LOOMIS-HUSSELBEE

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The rate of unidirectional efflux of ^{45}Ca from rat liver microsomal vesicles loaded with ^{45}Ca and then treated with thapsigargin is not inhibited by increased $[Ca^{2+}]$ in the external medium, although the net efflux rate is substantially inhibited. We have used this property to measure the electrochemical gradient of Ca^{2+} from the inside to the outside of the vesicles at a series of Ca^{2+} loadings, by measuring the external $[Ca^{2+}]_{free}$ at which there is

zero net efflux. At a loading of 7.9 ± 0.6 nmol/mg of microsomal protein, the apparent internal $[Ca^{2+}]_{free}$ is 21 ± 1.6 μM . As the loading is increased, the internal $[Ca^{2+}]_{free}$ increases linearly up to a value of 47 ± 3.6 μM at a loading of 21.6 ± 1.6 nmol/mg. Using a similar technique, the value for $[Ca^{2+}]_{free}$ in the endoplasmic reticulum of permeabilized L1210 cells was found to be 12.5 μM .

INTRODUCTION

There is now an abundance of evidence that in animal cells the endoplasmic reticulum, or a specialized region of it, acts as an intracellular Ca^{2+} store. Ca^{2+} can be released from this store following generation of the second messengers $Ins(1,4,5)P_3$ and cyclic ADP-ribose, which open Ca^{2+} channels in the membrane of the endoplasmic reticulum [1]. The subsequent increase in cytosolic $[Ca^{2+}]$ has, in many cases, been shown to be highly organized in both time and space, showing regular wave patterns of Ca^{2+} elevation passing across the cell [2]. It is reasonable to suppose that the rate of rise of the Ca^{2+} signal will depend, among several other parameters, on the electrochemical gradient of $[Ca^{2+}]_{free}$ between the inside of the endoplasmic reticulum and the cytosol. In addition, a Ca^{2+} increase in the lumen of the endoplasmic reticulum has been proposed to sensitize Ca^{2+} release [3], a phenomenon that might be important in wave generation [2]. A knowledge of the $[Ca^{2+}]_{free}$ inside the endoplasmic reticulum is therefore potentially important in predicting the behaviour of $Ins(1,4,5)P_3$ and ryanodine receptors.

Previous measurements of intra-luminal $[Ca^{2+}]_{free}$ have used Ca^{2+} indicators inside the endoplasmic reticulum. Kendall et al. [4] used aequorin, engineered with a KDEL sequence to target it for retention in the endoplasmic reticulum, to measure intra-luminal $[Ca^{2+}]$ in intact COS cells, where they estimated the value to be 0.3–1.0 μM . In contrast, Hofer and Machen [5] used the low-affinity fluorescent indicator mag-fura 2 to measure $[Ca^{2+}]_{free}$ in the endoplasmic reticulum of permeabilized gastric epithelial cells, and found a value of 127 μM , although this was regarded as a maximum value due to possible cross-talk with Mg^{2+} . Given the difference between these two estimates, a new method of measurement appeared necessary. Furthermore, while intra-luminal indicators can measure $[Ca^{2+}]_{free}$, they cannot measure the electrochemical gradient of Ca^{2+} from inside to outside, which might be affected by any membrane potential existing across the endoplasmic reticulum membrane.

In this paper we describe measurement of the electrochemical activity of Ca^{2+} inside the endoplasmic reticulum or endoplasmic reticulum vesicles using a null-point method. When ATP is added to endoplasmic reticulum vesicles or permeabilized cells, Ca^{2+} is accumulated from the medium until a steady state is

reached [6]. Addition of the Ca^{2+} -ATPase inhibitor thapsigargin (Tg) at the steady state causes an outward leak of Ca^{2+} [7]. By measuring the effects of extra-vesicular Ca^{2+} on the rate of this outward leak, we have arrived at an estimate of the electrochemical Ca^{2+} gradient from the inside to the outside. We also present some data relating this parameter to Ca^{2+} loading, which for the first time gives an estimate of the behaviour of the systems responsible for buffering $[Ca^{2+}]_{free}$ inside the endoplasmic reticulum.

EXPERIMENTAL

Materials

ATP and dithiothreitol (DTT) were from BCL (Lewes, E. Sussex, U.K.). Tg, phosphocreatine and creatine kinase were from Sigma (Poole, Dorset, U.K.). Materials for making Ca^{2+} -sensitive electrodes [calcium ionophore ETH 1001, 2-nitrophenyl octyl-ether, sodium tetraphenyl borate and high-molecular-mass poly(vinyl chloride)] were all from Fluka (Gillingham, Dorset, U.K.).

Microsomal preparation

Endoplasmic reticulum vesicles (microsomes) (36000 *g* fraction) were prepared from livers of fed male rats as previously described [6]. Protein concentration was determined by the method of Lowry et al. [8].

Cell growth

Mouse lymphoma L1210 cells were grown essentially as previously described [9], using a system of gas-permeable batch culture. Cells were grown over a 4 day period to a density of 6.0×10^5 cells/ml, as measured on a Coulter electronic cell counter. Cells (42 ml) were centrifuged at 2000 *g* for 5 min in a bench centrifuge at 25 °C. The pellet was resuspended in 25 ml of wash buffer consisting of 0.9% (w/v) NaCl and 20 mM Hepes/KOH, pH 7.0, at 30 °C and centrifuged as above. This wash was repeated before finally resuspending the final pellet of cells in

Abbreviations used: DTT, dithiothreitol; Tg, thapsigargin.

*To whom correspondence should be addressed.

Figure 1 Experimental method for determination of effects of rapid changes in external $[Ca^{2+}]_{free}$ on Ca^{2+} efflux from endoplasmic reticulum vesicles

Microsomal vesicles (4 mg of protein) were incubated in 2.5 ml of 100 mM KCl/20 mM Hepes/KOH (pH 7.0)/2 mM ATP/5 mM $MgCl_2$ /10 mM phosphocreatine/10 μ g/ml creatine kinase/1 mM DTT/2.5 μ M Ruthenium Red at 30 °C for 10 min until a steady state was reached, in this case at a pCa of about 6.9. Tg (0.5 μ M) was added at the arrow marked Tg, and the rate of efflux was measured as soon as a linear rate was established. In parallel experiments Ca^{2+} (as small volumes of 10 mM $CaCl_2$) was added shortly after Tg to displace the external $[Ca^{2+}]_{free}$ to a new value before measurement of the efflux rate. To change the loading of the vesicles, the required amount of extra Ca^{2+} was added at the start of the uptake, 10 min before Tg.

1 ml of assay buffer [100 mM KCl/20 mM Hepes/KOH (pH 7.0)/1 mM DTT/5 mM succinate (K^+ salt)/50 μ g/ml creatine kinase/10 mM phosphocreatine/2 mM $MgCl_2$ /0.7 mM ATP/2.5 μ M Ruthenium Red] to give a cell density of 2.5×10^7 cells/ml.

Ca^{2+} uptake and release

Ca^{2+} movements were measured using Ca^{2+} -sensitive electrodes constructed and calibrated as previously described [10]. For microsomes the uptake medium contained (in a volume of 2.5 ml) 100 mM KCl, 20 mM Hepes/KOH, pH 7.0, 2 mM ATP, 5 mM $MgCl_2$, 10 mM phosphocreatine, 10 μ g/ml creatine kinase, 1 mM DTT, 2.5 μ M Ruthenium Red and approx. 1.5 mg/ml microsomal protein. The temperature was 30 °C. In the absence of the addition of exogenous Ca^{2+} , steady-state accumulation was achieved at a $[Ca^{2+}]_{free}$ of 80–100 nM after 10 min incubation. Ca^{2+} efflux was started by addition of 0.5 μ M (final concentration)

Tg. Total Ca^{2+} content of the incubation mixtures was measured by atomic absorption spectrophotometry after precipitation of protein with 1.7% (w/v) trichloroacetic acid.

When Ca^{2+} movements were followed using ^{45}Ca , the above incubation mixture was supplemented with 8 kBq of ^{45}Ca (Amersham, Amersham, Bucks., U.K.) plus 10 nmol of exogenous Ca^{2+} . At the required time intervals, 50 μ l samples were removed and filtered through 0.25 μ m filters, the filters being washed with 2×1.0 ml aliquots of 100 mM KCl/20 mM Hepes (pH 7.0) at 0 °C. The ^{45}Ca retained on the filter was measured by liquid-scintillation counting. Under these conditions > 95% of the ^{45}Ca is initially accumulated by the vesicles, so it is possible to measure unidirectional ^{45}Ca efflux without a preliminary washing or isotope dilution step.

For Ca^{2+} uptake into permeabilized L1210 cells, permeabilization by digitonin was performed by adding 10 μ g of digitonin (in 1 μ l of dimethyl sulphoxide) per ml of 2.5×10^7 cells suspended in the assay buffer (described above). To increase reproducibility between successive experiments, Ca^{2+} uptake was normally done on a large scale (10–15 ml of 2.5×10^7 cells/ml). After the cells had loaded to steady state, 1.0 ml samples of the incubation mixture were transferred to the Ca electrode vessel and Ca^{2+} release was started by addition of 0.3 μ M Tg. The Ca^{2+} release properties of the cells loaded in large scale were constant for up to 2 h, allowing experiments to be carried out in rapid succession on identical 1 ml samples of cells. Because the rate of ATP use by the cells was considerably lower than that of the microsomes, it was possible to decrease the ATP concentration substantially in this system, thereby decreasing the Ca buffering of the medium and increasing sensitivity.

Calculation of efflux rates

Since Ca electrodes give an output that is linear with pCa, the $\Delta[Ca]_{total}$ corresponding to a particular ΔpCa is very dependent on the absolute pCa value. This is illustrated in the calibration bars shown in Figure 1. To calculate efflux rates as nmol of Ca^{2+} it was necessary to add calibration pulses of Ca^{2+} at different pCa values during the course of the experiments. The relationship between $\Delta[Ca]_{total}$ and pCa was then fitted to a polynomial that enabled determination of efflux rates at any pCa value across the range. The sensitivity in terms of nmol of $[Ca]_{total}/mV$ electrode output decreased by a factor of about 25 between 1 and 40 μ M $[Ca]_{free}$. Accordingly, the recorder sensitivity was increased at higher values of $[Ca]_{free}$.

RESULTS

The experimental approach that we have adopted is illustrated in Figure 1. All incubations contained Ruthenium Red to block uptake into any mitochondria present. Microsomal vesicles were loaded to steady state in the presence of ATP, then Tg was added to cause loss of accumulated Ca^{2+} down its concentration gradient, in this case at a rate of 0.7 nmol/min per mg (calculated as described in the Experimental section). In parallel experiments, shortly after Tg addition, Ca^{2+} was added to the medium to cause a rapid change in $[Ca^{2+}]$. In the example shown, 30 nmol of Ca^{2+} was added, causing a transition from a $[Ca^{2+}]_{free}$ of approx. 1.5×10^{-7} M (pCa 6.8) to 6×10^{-6} M (pCa 5.2). After a short mixing and electrode response artefact, a new rate of Ca^{2+} efflux of 0.37 nmol/min per mg was established.

The decrease in rate of Ca^{2+} outflow on increasing the $[Ca^{2+}]_{free}$ in the medium could have two possible causes. Firstly, it could be due to a decreased driving force for Ca^{2+} efflux effected by the diminished electrochemical gradient. Secondly, it could be due to an effect of external Ca^{2+} on whatever leak or Ca-conducting

Figure 2 Lack of effect of changing external $[Ca^{2+}]_{free}$ on unidirectional efflux of ^{45}Ca

Microsomal vesicles (4 mg of protein) were incubated as described in the Experimental section. The external $[Ca^{2+}]_{free}$ was followed with a Ca^{2+} -sensitive electrode. After the vesicles were fully loaded (approx. 10 min), $0.5 \mu M$ Tg (○) or $0.5 \mu M$ Tg + $40 \mu M$ $CaCl_2$ (●) were added. In the latter case, the initial pCa was changed from 6.8 to 4.8. The data shown are typical of three separate experiments.

pathway is allowing Ca^{2+} efflux. The second possibility is ruled out by the experiment shown in Figure 2, where instead of net efflux, the unidirectional outward flux of ^{45}Ca was measured. Inhibition of an efflux route by increased external Ca^{2+} would also inhibit this unidirectional flux. However, it is clear that increasing the external $[Ca^{2+}]_{free}$ to $> 1.5 \times 10^{-5} M$ has no effect on the rate of loss of ^{45}Ca (the initial rapid decrease in retained counts on addition of Ca^{2+} to the outside of the vesicles is probably due to displacement of labelled Ca^{2+} from external binding sites). Thus the reduction in rate of Ca^{2+} outflow caused by an increase in extravesicular $[Ca^{2+}]$ provides a method for measurement of the electrochemical gradient of $[Ca^{2+}]$ from inside to outside and hence the activity of intravesicular $[Ca^{2+}]$.

For inhibition of efflux due to a decrease in the electrochemical gradient, the net efflux rate would be expected to decrease linearly with an increase in external Ca^{2+} , reaching zero when the gradient from inside to outside becomes zero. Behaviour of this sort is shown in Figure 3. At the lowest Ca^{2+} loading (7.8 ± 0.6 nmol/mg, $n = 3$) the initial rate of Ca^{2+} efflux is approx. 0.7 nmol/min per mg and this efflux is completely inhibited at an external $[Ca^{2+}]_{free}$ of $21 \pm 1.6 \mu M$. The r value for the regression line is 0.93 . Thus the initial $\Delta[Ca^{2+}]_{free}$ between inside and outside is $21 \pm 1.6 \mu M$, and since initially the external $[Ca^{2+}]_{free}$ at this Ca^{2+} loading was $< 0.1 \mu M$, which is very small compared with $21 \mu M$, this gives a value of $21 \mu M$ for the initial activity of the intravesicular $[Ca^{2+}]$. A similar straight line fit ($r = 0.89$) is obtained at the higher initial Ca^{2+} loading of 12.2 ± 2 nmol/mg, and the intersection point on the horizontal axis gives an activity of intravesicular $[Ca^{2+}]_{free}$ of $30 \pm 2 \mu M$. At the highest loading used (21.6 ± 1.6 nmol/mg) there is substantially more scatter on the data ($r = 0.85$). This may be because the loading is close to its limiting value [6] and the vesicles are becoming

Figure 3 Effect of external $[Ca^{2+}]_{free}$ on the Ca^{2+} efflux rate from microsomal vesicles at different initial Ca^{2+} loadings

Efflux at different initial Ca^{2+} loadings was measured after rapid transition to different external $[Ca^{2+}]_{free}$ values (plotted on the horizontal axis) as described in the Experimental section and in the legend to Figure 1. The initial Ca^{2+} loadings were (in nmol/mg): ○, 7.9 ± 0.6 ; ●, 12.2 ± 2.3 ; and □, 21.6 ± 1.6 . Each line contains data combined from separate experiments on, in each case, three different batches of membranes. The lines were fitted by linear regression. Values for r and the intercepts are given in the text.

Figure 4 Dependence of apparent electrochemical gradient of internal to external $[Ca^{2+}]_{free}$ on Ca^{2+} loading

The data for the electrochemical gradient of Ca^{2+} and Ca^{2+} load are taken from the experiments shown in Figure 3. Horizontal error bars represent S.E.M. ($n = 3$) for Ca^{2+} loading of the vesicles. Vertical error bars represent S.E.M. of intercepts from data of Figure 3.

unstable. Furthermore, at the higher values of $[Ca]_{free}$, the value of $\Delta mV/nmol$ of Ca^{2+} is smaller (see the Experimental section), and hence the intrinsic error in measurement of efflux rate is larger. However, extrapolation of the line gives a value for intravesicular $[Ca^{2+}]_{free}$ of $47 \pm 3.6 \mu M$. A graph of loading against activity of intravesicular $[Ca^{2+}]$ (Figure 4) shows a

Figure 5 Effect of external $[Ca^{2+}]_{free}$ on the Ca^{2+} efflux rate from digitonin-permeabilized L1210 cells

The experiments were carried out as described in the Experimental section and the legend to Figure 1. The different symbols represent data collected from three different batches of cells and the line was fitted by linear regression to the complete data set. Values for r and the intercept are given in the text.

straight-line relationship. The data suggest that, at least over this range, $[Ca^{2+}]$ is buffered in a linear way with no sign of saturation.

While liver endoplasmic reticulum vesicles are very convenient for this sort of study because it is straightforward to work out the Ca^{2+} loading level on an organelle basis, they are somewhat removed from endoplasmic reticulum *in situ*. In this respect, even permeabilized hepatocytes in suspension are not very suitable since the endoplasmic reticulum has been shown to be fragmented in this preparation [11]. However, digitonin-permeabilized L1210 cells seem to maintain a substantial degree of integrity of the endoplasmic reticulum, based on retention of a very high level of $Ins(1,4,5)P_3$ sensitivity [12]. Figure 5 shows an experiment similar to that shown in Figure 2 but carried out on digitonin-permeabilized L1210 cells. The cells were washed and suspended in a low- Ca^{2+} medium to minimize changes in store loading during permeabilization, although some increase due to uptake of trace contaminating Ca^{2+} is inevitable. However, once again, a decrease in the rate of Ca^{2+} outflow is a linear function of extravesicular $[Ca^{2+}]$ ($r = 0.94$) and the intercept on the x -axis gives a value of $12.5 \pm 0.8 \mu M$ for the activity of $[Ca^{2+}]$ inside the endoplasmic reticulum.

DISCUSSION

Hepatocyte endoplasmic reticulum *in situ* contains about 8–10 nmol of Ca^{2+} /mg dry weight [13], in reasonable agreement with the value of 10 nmol/mg of protein that we obtained for microsomal vesicles loaded to steady state at an external $[Ca^{2+}]_{free}$ of 70 nM [6]. On this basis, a value for chemical activity of Ca^{2+} inside vesicles of $21 \mu M$ at a loading of approximately 8 nmol/mg may represent a realistic estimate of the level *in vivo*. It is also close to the value of $12.5 \mu M$ obtained for permeabilized L1210 cells where the endoplasmic reticulum should be more intact.

It is important to note that we are measuring the electrochemical gradient and hence chemical activity of Ca^{2+} inside the endoplasmic reticulum. Since Ca^{2+} is bivalent, a change in membrane potential of 29 mV across the membrane would give

a 10-fold change in this value. However, endoplasmic reticulum membranes appear to be permeable to both Cl^- and K^+ [14], so any membrane potential across the membrane would be expected to be rather small, at least *in vitro* in a KCl-based medium. Also, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA)-type ATPases appear to work by an electrogenic mechanism [15], so the absolute value of the electrochemical gradient of Ca^{2+} established by the pump should be independent of any standing membrane potential, although loading and the value of intravesicular $[Ca^{2+}]_{free}$ will change with membrane potential. It is notable that Hofer and Machen [5], using mag-fura 2, found that the $[Ca^{2+}]_{free}$ inside endoplasmic reticulum was decreased in the absence of Cl^- , which can act as a counter-ion for Ca^{2+} accumulation [14], as can phosphate, which could also buffer intravesicular Ca^{2+} [16]. It may be that small differences in membrane potential account for the difference between the results obtained in intact cells [4] and those obtained in permeabilized cells and endoplasmic reticulum vesicles ([5] and this paper). In any case, since the values we obtain by the null-point method are essentially midway between the previously published values, we are not in serious disagreement with either. What is clear from all these results is that the electrochemical gradient of Ca^{2+} from the endoplasmic reticulum to the cytosol is very much less than that from the outside of the cell to the cytosol.

From the relationship between intravesicular $[Ca^{2+}]$ and Ca^{2+} load shown in Figure 4, it appears that, at least over this range, the Ca^{2+} buffers in the lumen of the endoplasmic reticulum are operating over a linear region; in other words, they are a long way from saturation. This means that as store loading changes during, for example, $Ins(1,4,5)P_3$ -dependent Ca^{2+} release, the $[Ca^{2+}]_{free}$ in the endoplasmic reticulum is likely to change in a smoothly graded fashion. Any control of the $Ins(1,4,5)P_3$ receptor by luminal Ca^{2+} [3] might thus require co-operative effects of Ca^{2+} binding, possibly to an accessory protein, on the luminal face of the $Ins(1,4,5)P_3$ receptor. What is now becoming clearer is the affinity range in which such binding sites would have to operate.

We thank the Wellcome Trust for financial support and Judith Nuttall for expert technical assistance.

REFERENCES

- Berridge, M. J. (1993) *Nature (London)* **365**, 388–389
- Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Kendall, J. M., Badminton, M. N., Dormer, R. L. and Campbell, A. K. (1994) *Anal. Biochem.* **221**, 173–181
- Hofer, A. M. and Machen, T. E. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2598–2602
- Dawson, A. P. (1982) *Biochem. J.* **206**, 73–79
- Thastrup, O., Cullen, P. J., Drabak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Cullen, P. J., Irvine, R. F., Drabak, B. K. and Dawson, A. P. (1989) *Biochem. J.* **259**, 931–933
- Drabak, B. K., Bush, D. B., Jones, R. L., Dawson, A. P. and Ferguson, I. B. (1992) in *Molecular Plant Pathology, A Practical Approach*, vol. 2 (Gurr, S. J., McPherson, M. J. and Bowles, D. B., eds.), pp. 159–194, IRL Press, Oxford
- Renard-Rooney, D. C., Hajnoczky, G., Seitz, M. B., Schneider, T. G. and Thomas, A. P. (1993) *J. Biol. Chem.* **268**, 23601–23610
- Loomis-Husselbee, J. W. and Dawson, A. P. (1993) *Biochem. J.* **289**, 861–866
- Somlyo, A. P., Bond, M. and Somlyo, A. V. (1985) *Nature (London)* **314**, 622–625
- Muallem, S., Schoeffield, M., Pandolf, S. and Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4433–4437
- Eisenrauch, A. and Bamberg, E. (1990) *FEBS Lett.* **268**, 152–156
- Fulceri, R., Bellomo, G., Gamberucci, A., Romani, A. and Benedetti, A. (1993) *Biochem. J.* **289**, 299–306