Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 13-17

THE BINDING OF CA^{2+} TO CA^{2+}-TRANSPORTING MICROSOMES DERIVED FROM BOVINE UTERINE SARCOPLASMIC RETICULUM

Jordan D. Miller and Mary E. Carsten

Departments of Anesthesiology and Obstetrics and Gynecology, School of Medicine, UCLA, Los Angeles, California

Received June 29, 1987

SUMMARY: An obligatory early step in the transport of calcium across the internal membranes of smooth muscle cells is the binding of calcium to the Ca,Mg-ATPase. The characterization of calcium binding to sarcoplasmic reticulum from smooth muscle has not been reported. Calcium binding to a bovine myometrium preparation was investigated using Scatchard analysis and a computer program utilizing weighted least squares curve fitting and an exact mathematical model of binding. This permitted objective measurement of goodness of fit and showed that best fit was obtained using a two site model. Magnesium did not change the affinity for calcium of the two sites; but reduced the number of low affinity sites to half.

The sarcoplasmic reticulum of smooth muscle plays a major role in the control of intracellular free calcium and thereby of smooth muscle contraction. The uptake of calcium into the sarcoplasmic reticulum depends on the presence of a calcium transporting Mg-ATPase, and multi-step reaction schemes have been proposed (1). An understanding of the events involved in the transport of calcium by the sarcoplasmic reticulum membranes requires the examination of partial reactions which are easier to interpret. Using a well characterized microsomal preparation from bovine myometrial sarcoplasmic reticulum we were able to demonstrate the phosphorylation of the Ca transporting Mg-ATPase (1), and transport of calcium to the inside of the vesicles (2). An obligatory early step, which has not been characterized in smooth muscle, is the binding of calcium.

Magnesium is required for the phosphorylation of the enzyme and calcium transport; however, it decreases the amount of calcium bound to these membranes in the absence of ATP (3). The release of bound calcium by magnesium could occur by a decrease in number and/or affinity of calcium binding sites.

In this study we have used atomic absorption spectroscopy to examine the calcium binding. Scatchard analysis using weighted least squares curve fitting and an exact mathematical model of binding permitted objective measurement of goodness of fit for several models. Our findings offer the first evidence for the specific binding of calcium to smooth muscle sarcoplasmic reticulum and demonstrate the existence of two binding sites.

0006-291X/87 $1.50

Materials and Methods

Uteri, obtained at the slaughterhouse from close-to-term pregnant cows, were immediately dissected and the myometrium carefully stripped free of endometrium as described (4). The muscle strips were rinsed, immersed in ice-cold buffer (0.3 M sucrose, 0.01 M glucose, 5 mM dithiothreitol, 0.02 M Tris, pH 7.2), and transported on ice to the laboratory. The muscle tissue was diced with scissors, minced in a meat grinder, and homogenized in a Waring Blender first for 15 s, and again for 10 s. All operations were carried out in the cold room at 0-4°C. Differential centrifugation was at 2,500 g for 20 min, 15,000 g for 20 min. in a Sorvall GS-3 rotor and at 40,000 g for 90 min.in a Spinco 21 rotor. The final pellets were suspended in 0.08 M NaCl, 0.005 M sodium oxalate and placed on a sucrose density gradient consisting of layers of 35, 45, and 55% sucrose. After 3 h of centrifugation in a Spinco 27.1 swinging bucket rotor (average force 63,000 g), the main protein layer was isolated from the 35% sucrose layer (density of 1.136). The protein was stored at 4°C and used the following day. Protein concentration was determined by the method of Lowry et al (5). Protein was incubated at 37°C in an incubation medium containing 20 mM imidazole, pH 7.0, 10 mM KCl, 2.5 mM Na azide, 0.1 M sucrose, 0.5 mg/ml protein. Duplicate samples were incubated at ten calcium concentrations from 0.2 - 200 uM free calcium, using 2 mM total calcium and adjusting the free calcium with 1.8 - 6.8 mM EGTA. An additional sample was incubated in 9.1 mM EGTA without added calcium to determine calcium binding in the presence of less than 10^{-8} M free calcium. This was used as blank and subtracted from the value of all other samples. In the presence of Mg the free Mg concentration was kept constant at 5.0 mM, using 5 - 5.8 mM total Mg. Free metal concentrations were calculated according to Fabiato and Fabiato (6). After incubation the samples were rapidly cooled to 0°C in an iced slush, placed in a Beckman TI 50 centrifuge and spun at 165,000 g for 30 minutes. The pellets were washed and analyzed for calcium by atomic absorption spectroscopy by a method previously described (4). Analysis of the results were by Scatchard plot and the use of the program "Ligand" as modified for the Apple computer (7,8).

Results and Discussion

Calcium binding at less than 10^{-8} M free calcium amounted to 3-4 nmol/mg. In order to permit extrapolation to a reliable total receptor capacity high enough concentrations must be used. This is verified by the presence of an inflection point with several points above that value when the bound calcium vs free calcium is plotted on semilogarithmic coordinates (9,10) and is seen in Fig. 1. The average binding of calcium in the absence of magnesium is shown in Fig. 2 using a Scatchard plot. In all cases the program was asked for a best fit using one binding site, one binding site and non-specific binding, two binding sites and two binding sites with non-specific binding. The best fit was chosen based on both the runs test and the F parameter. We justified the use of a more complex model when the goodness of fit as shown by the F parameter was at the $p < 0.05$. The best fit required two binding sites and non-specific binding. The binding affinities were $K_1 = 8.4 \pm 0.4 \times 10^6$ M^-, $K_2 = 0.11 \pm 0.02 \times 10^6$ M^-; maximum number of binding sites $r_1 = 3.9 \pm 0.4 \times 10^{-6}$ moles/g, $r_2 = 10.5 \pm 0.5 \times 10^{-6}$ moles/g and non-specific binding = 0.021. The double binding site model significantly improved the fit $F[2,5] = 65$ and a $p < 0.01$ as compared to the single binding site model.

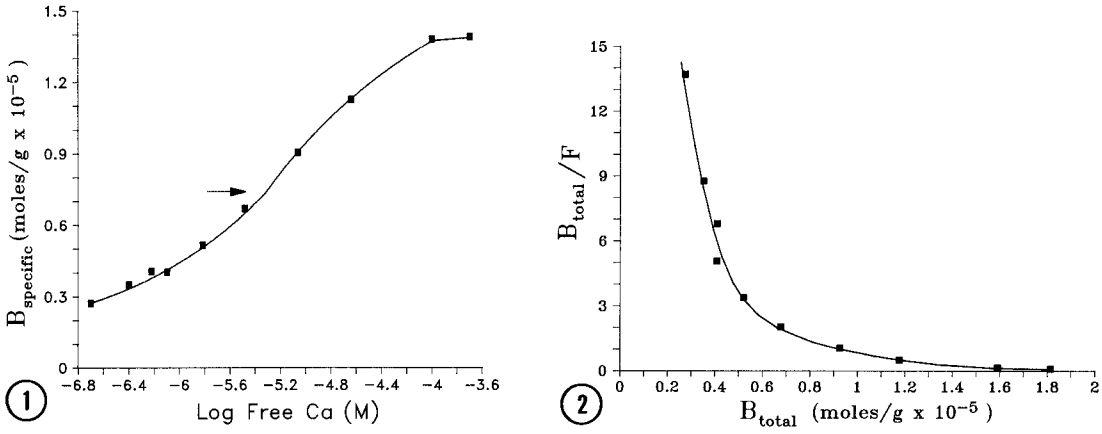

Fig. 1. Semilogarithmic plot of calcium bound to membranes from bovine myometrial sarcoplasmic reticulum against the concentration of free Ca. Each point represents the average of 14 experiments. The arrow is at the inflection point.

Fig. 2. Scatchard plot of the same data as Fig. 1. The line drawn is one calculated using the "best fit" parameters for association constant of the high and low affinity binding sites and maximum number of high and low affinity sites, respectively) and non-specific binding.

To compare the effect of magnesium on the binding of calcium five paired experiments were performed. Each experiment was performed using the same protein on the same day. The best fit required the use of two binding sites and non-specific binding. The constraining of the binding constants to those reported for all 14 experiments did not change the fit $F[2,5] = 0.9$ and 0.3 $p > 0.1$ for absence and presence of magnesium respectively. The maximum number of high affinity sites was 2.47 ± 0.2 and $2.24 \pm 0.1 \times 10^{-6}$ moles/g for calcium binding in the absence and presence of magnesium; whereas the maximum number of binding sites at the low affinity site was 12 ± 0.8 and $5.3 \pm 0.4 \times 10^{-6}$ moles/g, respectively. Magnesium reduced the number of low affinity sites without changing receptor affinity or the number of high affinity sites see Fig. 3.

The use of a calcium ionophore allows us to see whether our results are influenced by the entrapment of calcium within the vesicles. It also will distinguish between calcium binding sites located on the inside and outside of the membranes if the majority of the vesicles are intact. Thus we have measured calcium binding in the presence and absence of the calcium ionophore A23187 (0.2 uM). Eight paired experiments were performed. There was no significant difference in either the affinity or number of binding sites in the presence or absence of the ionophore. The number of high affinity binding sites was 2.89 and low affinity binding sites 13.6×10^{-6} moles/g protein. Since many of the vesicles present are probably open, (we can harvest less than ten percent by increasing the density by Ca oxalate driven uptake (2)) we would not expect to

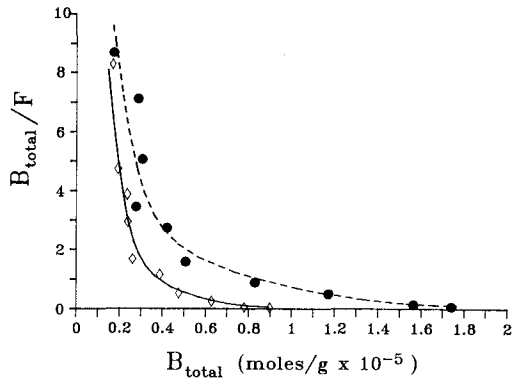

Fig. 3. Comparison of the binding of calcium to microsomes in the presence ◇ and absence ● of 5 mM free magnesium. The data are plotted on Scatchard coordinates. Each point represents the average of 5 experiments. The lines are calculated using the same affinity constants found in the previous graph but allowing the maximum number of binding sites to vary.

see any change in the presence of an ionophore. These results confirm that internally trapped calcium is not responsible for the results obtained. It is not practical, at this time, to measure calcium binding in a preparation of intact vesicles obtained by Ca oxalate loading, since large quantities of intrinsic calcium would obscure any binding (2).

The bovine myometrial microsomal fraction used here has been well characterized in our laboratory and its origin from sarcoplasmic reticulum rather than sarcolemma established. The microsomes show oxalate stimulation of ATP-dependent Ca^{+2} uptake (2), inositol trisphosphate induced calcium release (11), presence of a Ca,Mg-ATPase with a molecular weight of 100,000 to 110,000 (1), absence of a 130,000 molecular weight Ca,Mg-ATPase characteristic of sarcolemma, lack of calmodulin or lanthanum effect on ATPase phosphorylation (12). These properties distinguish the sarcoplasmic reticulum from cell membrane (13).

Prior work demonstrated that calcium equilibrates with the vesicles in less than one minute (4), thus the choice of eight minutes incubation time would give excellent conditions for reaching equilibrium. We chose to use an atomic absorption spectrophotometric method for measuring calcium. This method measures the total calcium present in the microsomes. The use of radioactive tracer Ca^{45} has several problems. Intrinsic Ca^{40} exchanges with the radioactive tracer giving a false estimate of the true binding (4), unless all intrinsic calcium is removed (14); which might introduce other errors.

The use of a computerized weighted least squares curve fitting algorithm for the estimation of calcium binding allows for greater accuracy and at the same time permits statistical analysis to justify the use of more complex models (two vs one binding site) and comparison of constrained vs unconstrained variables (7).

The physiological significance of our findings remains to be determined. The high affinity site may well be associated with the Ca transport ATPase, since its affinity is in the correct range to permit the reduction of free calcium to resting levels. The lower affinity site may represent another calcium binding protein, or a conformational change of the enzyme. Such a change has been hypothesized as the means of releasing calcium to the inside of the vesicle. The fact that there is no change in either the affinity or number of high affinity Ca binding sites in the presence of Mg is consistent with the requirement of the Ca,Mg-ATPase for Mg. A reduction in the number of low affinity sites may be involved in the unloading of calcium to the inside of the vesicles. The use of only intact vesicles with the correct orientation is necessary to reach more detailed conclusions.

References

1. Carsten, M.E., and Miller, J.D. (1984) Arch. Biochem. Biophys. 232, 616-623.
2. Carsten, M.E., and Miller, J.D. (1984) Gynecol. Obstet. Invest. 17, 73-83.
3. Miller, J.D., and Carsten, M.E. (1984) INSERM 124, 265-272.
4. Carsten, M.E., and Miller, J.D. (1977) J. Biol. Chem. 252,1576-1581.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
6. Fabiato, A., and Fabiato, F. (1979) J. Physiol. Paris 75, 463-505.
7. Munson, P.J., and Rodbard, D. (1980) Analyt. Biochem. 107, 220-239.
8. Teicher, M. (1982) MED-58:LIGAND (Applesoft) (Adaptation of Munson's and Rodbard's LIGAND program to the Apple II + microcomputer). Biomedical computing Technology Information Center, Vanderbilt Medical Center, Nashville, TN.
9. Klotz, I.M. (1982) Science 217, 1247-1249.
10. Faguet, G.B. (1986) J. Cell. Biochem. 31, 243-250.
11. Carsten, M.E., and Miller, J.D. (1985) Biochem. Biophys. Res. Commun. 130, 1027-1031.
12. Carsten, M.E., and Miller, J.D. (1985) Soc. Gyn. Invest. 32, 216.
13. Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H., and Casteels, R. (1984) Biochem. J. 224, 445-451.
14. Chevalier, J., Butow, R.A. (1971) Biochemistry 10, 2733-2737.