Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 441-447

ATP-INDUCED STIMULATION OF CALCIUM BINDING TO CARDIAC SARCOLEMMA

Jaime MAS-OLIVA, Alan J. WILLIAMS and Winifred G. NAYLER

Cardiothoracic Institute, University of London,
2, Beaumont Street, London W1N 2DX, England

Received January 23,1979

SUMMARY: Sarcolemma vesicles isolated from rabbit ventricular muscle showed a high degree of purity when assessed in terms of calcium binding and marker enzyme activity. Calcium binding to the sarcolemma increased as the free calcium concentration of the medium increased. ATP-independent (passive) and ATP-dependent (active) calcium binding activities were observed. The active binding was associated with an increase in the calcium stimulated ATPase activity of the preparation. A Lineweaver-Burke plot of the ATP-dependent calcium binding data was linear over the whole range of calcium concentrations (20-200 μM) investigated.

Recently studies have been undertaken to investigate the involvement of extracellularly located calcium in excitation contraction coupling in cardiac muscle and it has been suggested that the sarcolemma may regulate the supply of calcium from this source (1). This theory gains support from the fact that isolated fragments of the sarcolemma bind calcium in a concentration-dependent and saturatable manner (2, 3). There is evidence that the basement coat of the sarcolemma is of importance in this process (4, 5, 6), the negative charged residues of carbohydrates (5, 7), the amino acids of the superficially-located proteins and the phospholipids (7) all being considered as possible calcium storage sites. Although a number of workers have investigated sarcolemmal calcium binding, disagreement exists concerning the mechanisms that are involved. It is generally accepted that isolated sarcolemmal fragments are capable of binding appreciable amounts of calcium in the absence of an added energy source such as ATP (2). Some workers have reported that adding ATP to such a system enhances its calcium binding (3), whilst others have found

0006-291X/79/060441-07$01.00/0

either no stimulation or in some cases a decrease in the amount of calcium bound (8). In this communication we report attempts to elucidate the process of calcium binding to isolated cardiac sarcolemma by measuring both passive and energy-dependent binding in the same system.

MATERIALS AND METHODS

Isolation of cardiac sarcolemma. The isolation procedure used is based on that of St. Louis et al. (9) and involves the solubilization of the contractile proteins in combination with differential centrifugation and separation of the remaining membrane components by density gradient centrifugation using either swing out or zonal rotors. The sucrose concentrations for the first gradient were 1.17, 1.39, 1.60 and 1.83 M. The fraction banding between 1.39 - 1.60 M was layered on a second gradient made up of 1.39, 1.53, 1.60 and 1.83 M sucrose. The fraction banding between 1.53 and 1.60 M showed sarcolemmal properties and was characterized by establishing its ATPase activity (Table 1), comparing its enzymatic properties with sarcoplasmic reticulum and mitochondrial preparations (Table 2) and by electron microscopic examination using both negatively stained and sectioned material (data not shown). Cardiac muscle sarcoplasmic reticulum was prepared by the method of Harigaya and Schwartz (10) as modified by Nayler et al (11). Cardiac mitochondria were isolated as described by Williams and Barrie (12). ATPases were assayed according to the method of Matsui and Schwartz (13), and liberated Pi determined by the method of Fiske and Subbarow (14), modified by Price and Williams (15). Cytochrome oxidase was measured as described by Cooperstein and Lazarow (16). Protein concentrations were determined using an adaptation of the method described by Bradford (17).

Calcium binding. Sarcolemmal calcium binding was investigated isotopically using a rapid sampling technique followed by Millipore filtration. Calcium binding was studied over a range of free calcium concentrations using either nitrilotriacetic acid (NTA) or a mixture of NTA and ATP as calcium buffers. In each experiment approximately 250 µg of sarcolemmal protein was pre-incubated for one minute in a medium containing 2.5 mM NTA, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl at pH 7.4, and 25°C. Passive calcium binding was initiated by adding a known amount of $CaCl_2$ containing approximately 2.5 µCi $^{45}Ca^{2+}$ in a final volume of 1.675 ml. 200 µl samples of this system were withdrawn after 5, 25 and 45 seconds and immediately filtered through 0.45 µm Millipore filters and washed with 7 ml of a buffer containing 100 mM KCl, 50 mM Tris-HCl at pH 7.4. After 45 seconds of passive binding ATP-dependent binding was initiated by adding 10 µl of a solution containing 2.71 µ moles of ATP (pH 7.4), giving a final [ATP] of 2.5 mM. Five, 25, 45 and 65 seconds later 200 µl samples were withdrawn and filtered. The filters were dried for 30 minutes before mixing with scintillation fluid. Radioactivity was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. The experiments in which calcium binding and Ca^{2+} stimulated ATPase activities were measured in parallel were carried out using the system developed for calcium binding measurements, Ca^{2+} stimulated ATPase activity being assayed after adding unlabelled calcium. 200 µl samples were taken and added to 1 ml aliquots of deionized boiling water. Inorganic phosphate concentrations were then determined as described previously. Variations in the free calcium and magnesium concentrations of the reaction media caused by adding ATP were calculated using an ion-complexes

in solution computer programme and available stability constants (18). In each experiment the free calcium concentration remained constant following the addition of ATP. The free magnesium concentration deviated by no more than 150 μM from the original concentration of 2.5 mM.

RESULTS AND DISCUSSION

Myocardial sarcolemma isolated as described appeared to be essentially free from sarcoplasmic reticulum and mitochondrial contamination, as shown by the different distribution of marker enzymes (Tables 1 - 2) and its appearance in the electron microscope. Passive calcium binding to the sarcolemma increased as the free calcium concentration of the medium was increased (Fig. 1). At all the calcium concentrations investigated, but particularly at the higher Ca^{2+} concentrations (100 - 200 μM), the initial passive binding after five seconds incubation was high and decreased over the next 20 seconds of incubation. Increasing the calcium buffering capacity of the medium or extending the pre-incubation time, increased the initial binding (data not shown). This initial phase of rapid passive calcium binding may be related to the endogenous calcium content of the sarcolemma which may be influenced by the NTA buffer system. This possibility is now under investigation. Adding ATP increased the amount of calcium bound to the sarcolemma at all free calcium concentrations examined (Fig. 1).

The relationship between calcium binding and calcium stimulated ATPase activity of the sarcolemma was investigated. The increased binding observed after adding ATP was associated with a marked but transient increase in calcium stimulated ATPase activity. Following this short period of ATP hydrolysis the calcium bound to the sarcolemmal preparations continued to increase but at a decreased rate relative to that observed during ATP hydrolysis (Fig. 2). Initial rates of ATP-stimulated calcium binding, calculated on the basis of results obtained during the first five seconds of the ATP-supported reaction, are shown in Figure 3; a double reciprocal

Table 1

ATPase activities of purification steps.

	(n=6) Mg^{2+} -ATPase	(n=6) Na^+, K^+ stimulated-ATPase			(n=6) Ca^{2+} stimulated-ATPase		
	Basal	Control	Control + Ouabain	Control + NaN_3	Control	Control + Ouabain	Control + NaN_3
Homogenate	4.30 ± .51	7.19 ± .47	5.78 ± .81	6.06 ± .73	9.16 ±1.12	8.97 ± .98	6.58 ± .84
Washed particles	5.17 ± .63	7.38 ± .83	5.03 ± .65	6.47 ± .69	8.01 ± .73	7.92 ± .75	7.00 ± .81
Sarcolemma	5.81 ± .39	8.76 ± .51	6.10 ± .43	7.89 ± .38	6.21 ± .50	6.18 ± .43	5.90 ± .60

Homogenate refers to the crude homogenate; washed particles to the fraction before the sucrose gradients and isolated sarcolemma to the fraction banding between 1.53 - 1.60 M in the second gradient. ATPase activity expressed as μ moles Pi/mg/hr. Ouabain 1×10^{-5} M. Sodium azide (NaN_3) 3×10^{-3} M. n = 6 with 5 different preparations. Values ± S.E.M.

Table 2

Distribution of marker enzymes in isolated fractions

	(n=6) SL	(n=3) SR	(n=3) M
Na^+, K^+-ATPase	9.76 ± 1.38	0.04 ± 0.005	0.07 ± 0.005
Cytochrome oxidase	10.0 ±1.79	46.0 ±5.13	1541.0 ± 74.0

Cardiac sarcolemma (SL), sarcoplasmic reticulum (SR) and mitochondria (M). ATPase activity expressed as μ moles Pi/mg/h and cytochrome oxidase activity as n moles of cytochrome c oxidized/mg/min. Values ± S.E.M.

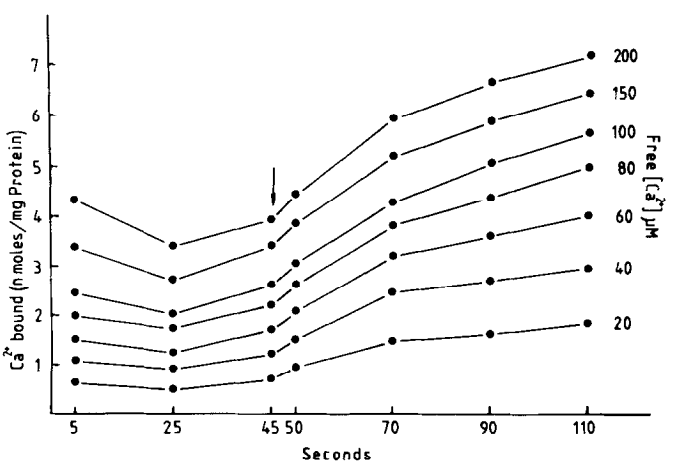

FIGURE 1

Time course and free calcium concentration dependence of the ATP-independent and ATP-dependent calcium binding by cardiac sarcolemma. Samples were withdrawn after the indicated times, filtered and washed with cold buffer containing 100 mM KCl, and 50 mM Tris-HCl, pH 7.4. The arrow represents the addition of 10 μl of a solution containing 2.71 μmoles of ATP to give a final concentration of 2.5 mM. Each point represents the mean of six different experiments with S.E.M. values < 0.5 n moles/mg protein. For details see experimental section.

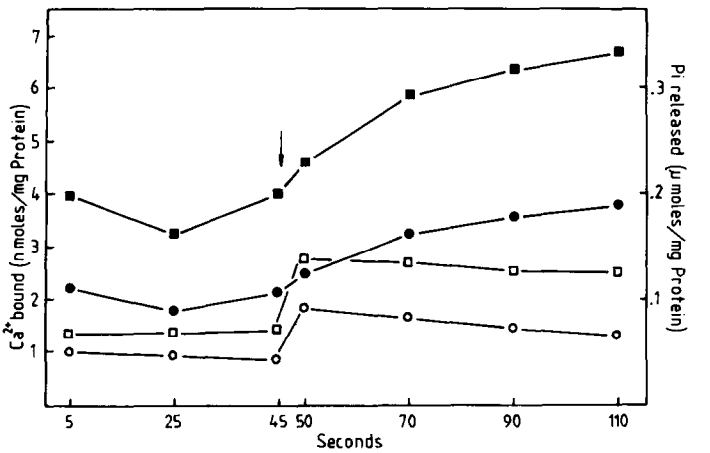

FIGURE 2

Relationship between calcium binding and Ca^{2+} stimulated ATPase activities in rabbit cardiac sarcolemma. Calcium binding assays were performed under the same conditions as Fig. 1. For Ca^{2+} stimulated ATPase determinations samples were withdrawn at the indicated times and added to deionized boiling water before inorganic phosphate (Pi) determinations were made. Each point represents the mean of 5 different experiments, with S.E.M. values for calcium binding of < 0.5 n moles/mg protein and < 0.04 μ moles/mg protein for Ca^{2+} stimulated ATPase. Ca^{2+} binding, (●) 60 μM and (■) 150 μM free$[Ca^{2+}]$. Ca^{2+} stimulated-ATPase, (○) 60 μM and (□) 150 μM free $[Ca^{2+}]$.

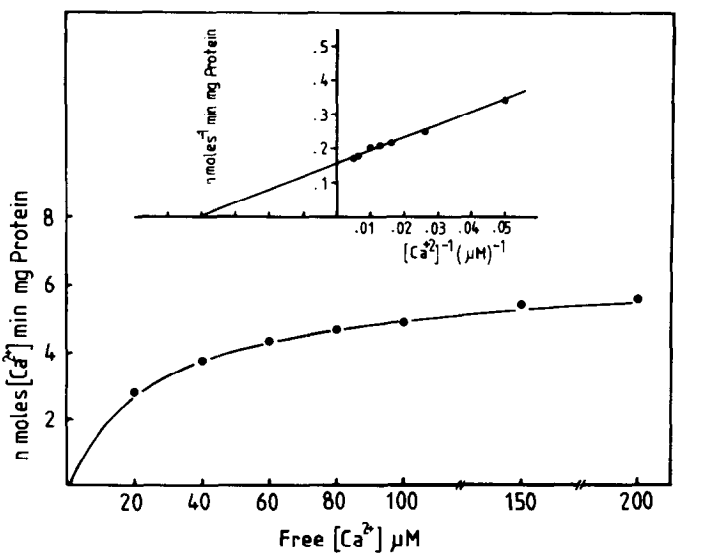

FIGURE 3

Calcium concentration dependence of the initial rate of sarcolemma ATP-dependent calcium binding (conditions as Fig. 1). Each point represents the mean of six different experiments with S.E.M. values < 0.7 n moles/mg protein. Inset: Reciprocal plot of the initial rates at the different free calcium concentrations, with an apparent Km for the calcium binding of 25 μM with a Vmax at 6.3 n moles calcium/mg/min.

plot of this data was linear over the whole range of calcium concentrations investigated, producing an apparent Km of 25 μM and Vmax of 6.3 n moles min^{-1} mg^{-1}.

Our results suggest that ATP does increase calcium binding to isolated cardiac sarcolemma. Details of the mechanism involved in this process and the significance of energy-dependent calcium binding in terms of regulation and control of excitation contraction coupling in cardiac muscle are still under investigation.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Medical Research Council. Dr. J. Mas-Oliva has been supported by grants from the British Council and the National University of Mexico.

REFERENCES

1. Langer, G.A., (1973) Ann. Rev. Physiol. 35, 55-86.
2. Williamson, J.R., Woodrow, M.L. and Scarpa, A. (1975) in: Recent Advances on Cardiac Structure and Metabolism. Basic Functions of Cations in Myocardial Activity (Fleckenstein, A. and Dhalla, N.S. eds.) Vol. 4, pp 61-71, University Park Press, Baltimore.
3. Sulakhe, P.V., Leung, N.L.-K. and St. Louis, P.J. (1976) Can. J. Biochem. 54, 438-445.
4. Frank, J.S., Langer, G.A., Nudd, L.M. and Seraydarian, K. (1977) Circ. Res. 41, 702-714.
5. Jackes, L.W., Brown, E.B., Barret, J.M., Brey, W.S. Jr. and Weltner, W. Jr. (1977) J. Biol. Chem. 252, 4533-4538.
6. Nayler, W.G. (1975) in: Recent Advances on Cardiac Structure and Metabolism. Basic Function of Cations in Myocardial Activity (Fleckenstein, A. and Dhalla, N.S. eds.) Vol. 4, pp 73-79, University Park Press, Baltimore
7. Limas, C.J. (1977) Arch. Biochem. Bioph. 179, 302-309.
8. Dhalla, N.S., Anand, M.B. and Harrow, J.A.C. (1976) J. Biochem. 79, 1345-1350.
9. St. Louis, P.J. and Sulakhe, P.V. (1976) Int. J. Biochem. 7, 547-558.
10. Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 21-33.
11. Nayler, W.G., Dunnett, J. and Berry, D. (1975) J. Mol. Cell Card. 7, 275-288.
12. Williams, A.J. and Barrie, S.E. (1978) Biochem. Biophys. Res. Commun. 84, 89-94.
13. Matsui, H. and Schwartz, A. (1966) Biochem. Biophys. Acta 128, 380-390.
14. Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375-400.
15. Price, N.R. and Williams, A.J. (1977) Biochem. Med. 17, 153-157.
16. Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665-670.
17. Bradford, M.M. (1976) Anal. Biochem. 72, 246-254.
18. Sillén, L.G. and Martell, A.E. (1971) Stability Constants on Metal-ion Complexes. Supplement 1. Special publication 25, The Chemical Society, London.