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THE RELATIONSHIP BETWEEN CALCIUM FLUX AND BEATING IN HYPERPERMEABLE HEART MUSCLE CELLS

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Summary

Beating hyperpermeable myocardial fragments, suspended in fresh medium, show a Ca uptake rate which greatly exceeds the release rate. Under these conditions, Ca influx and contraction frequency co-vary with temperature. Ca efflux, however, is not temperature responsive. In addition, influx, but not efflux, is ATP-dependent. This implies that influx, but not efflux, is a controlled process, the activity of which has the same determinants as does contraction. However, under conditions approximating the steady state, the Ca influx rate was found to decrease, while the efflux rate increased. This suggests the possibility, at least, that the source of contraction-activating Ca can vary with the prevailing conditions.

Introduction

It is generally accepted that in skeletal muscle activation of contraction depends on the release of Ca^{2+} from intracellular sites [1,2]. In the case of cardiac muscle, however, there have been contradictory findings. The contractile behavior of skinned heart muscle cells suggests that a small amount of Ca influx ('trigger Ca') evokes release of a larger amount of (contraction-activating) Ca from intracellular stores [3]. By contrast, experiments with more intact preparations have suggested that contraction-activating Ca is primarily derived from extracellular sources [4,5]. The high ^{45}Ca influx rates seen with myocardial fragments composed of beating hyperpermeable heart muscle cells [6] is also compatible with this latter mechanism. The efflux rate of ^{45}Ca from these cells, which would be of value in determining the relative roles of intracellular and extracellular Ca in the contraction process, has not been measured. These cells are particularly interesting since they show free sarcolemmal permeability to divalent cations, while their intracellular organelles are intact [6–9]. Ca flux thus reflects function of the intracellular organelles.

In this paper we examine the initial velocities of Ca influx and efflux as functions of temperature, and compare these flux rates to contraction frequency.

Methods

Myocardial fragments were prepared as previously described [6,7]. Saline-washed ventricular muscle from 1—6 mice was disaggregated in a Virtis-45 homogenizer for 8 s at 8000 rev./min in ice-cold phosphate-buffered medium (KP_i) containing (mM): KCl, 100; $MgCl_2$, 3; ATP · 2Na, 3; NaCl, 7; potassium phosphate buffer, pH 6.8, 20; potassium succinate, 10. This medium had a Ca content of 8.7 ± 1.3 μM (mean ± S.E.), as determined by atomic absorption spectroscopy. The cold homogenate was passed through a plastic screen (Swinnex filter holder, Millipore Corp.) to remove large fragments. The myocardial fragments were then allowed to sediment, on ice, for 15 min at 1 × *g*, and the subcellular debris was discarded with the supernatant fluid. The fragments obtained from this procedure showed a length of 272 ± 139 μm (mean ± S.D.) and a width of 70 ± 58 μm. The myocardial fragments were resuspended in fresh medium and samples taken for protein determination.

To load with ^{45}Ca prior to washout studies, 0.1 mCi of carrier-free ^{45}Ca, in 0.1 ml, was added to 22 ml of suspended myocardial fragments. A 1.5 ml sample was removed and filtered through a Millipore SC filter (8 μm pore size). The ^{45}Ca content of the resulting filtrate was used to calculate the specific activity of the medium. The preparation was then incubated, with agitation, for 60 min at 25° C. A second 1.5 ml sample was then removed and filtered. The loss of ^{45}Ca from this filtrate was used to calculate the amount of calcium taken up during the loading period. The 60 min uptake period was more than twice the time interval to Ca steady state [6], insuring uniform labelling of various intracellular pools. ^{45}Ca-loaded myocardial fragments were sedimented at 140 × *g* for 2 min at 25° C and resuspended in 30 ml washout medium. The suspension was placed in a water bath shaker and 1 ml samples filtered as described above. The ^{45}Ca content of each filtrate, after subtraction of the zero-time level, was used to calculate the amount of isotope which have moved from the intracellular to the extracellular space. The amount of ^{45}Ca washed out was plotted as a function of time, and the initial slope of this curve was used to estimate the initial velocity of efflux. Myocardial fragments to be used for ^{45}Ca influx measurements were treated in the same manner as those used for efflux studies, except for the time of isotope addition. The methods used to estimate initial velocity of ^{45}Ca uptake and contraction frequency (beats per min) were previously described [6,7].

Results

^{45}Ca flux in phosphate-buffered medium. When ^{45}Ca-loaded myocardial fragments were resuspended in KP_i, an initial period of isotope efflux into the medium was noted, followed by a period of reversal of the ^{45}Ca movement back into the myocardial fragments (Fig. 1). This is to be expected if net influx exceeds efflux. When P_i was omitted from the medium and Tris buffer added in its place, the initial velocity of efflux was slightly increased and net return

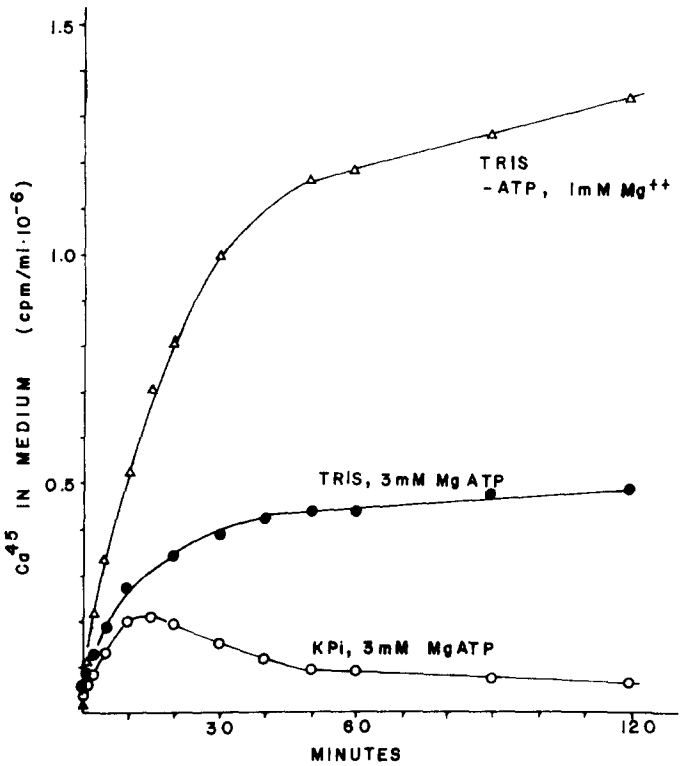

Fig. 1. Effects of P_i and ATP on ^{45}Ca efflux. Myocardial fragments were preloaded with ^{45}Ca for 1 h in P_i-buffered medium at 25° C. They were then resuspended in unlabelled medium, buffered with either Tris or P_i, as indicated, and containing the indicated concentrations of ATP and $MgCl_2$. The concentration of ^{45}Ca in the medium was measured, at the indicated times, after removal of myocardial fragments by filtration. The initial velocity of Ca efflux (V_o) was found from the initial slope of curves such as these.

of isotope to the myocardial fragments did not occur. This is consistent with the view that sequestration of calcium as the phosphate precipitate allows establishment of a steeper gradient than is otherwise possible [10]. When ATP was omitted from Tris-buffered medium, the initial velocity of efflux was similar to that occurring in the presence of ATP. Without ATP in the medium, however, ^{45}Ca washout continued at close to the initial velocity over a longer time, so that there was greater net efflux. This indicates that efflux is not energy dependent, and occurs in the absence of contractile activity, since myocardial fragments do not beat in the absence of added ATP [7].

To test the relationship between contractile activity and calcium flux we compared the effects of temperature on the initial beats per min to its effects on the initial velocity of influx (V_i) and the initial velocity of efflux (V_o). Both V_i and beats per min increased with temperature over the range 10—25° C, but V_o remained essentially unaltered over the entire range 0—35° C (Fig. 2). Although there are variations among these measurements, there is no doubt that both beats per min and V_i increased with temperature over the range 10—25° C while V_o remained unchanged. The correlation coefficient squared (r^2) for beats per min and V_i over the range 0—35° C was 0.71, but only 0.14 for

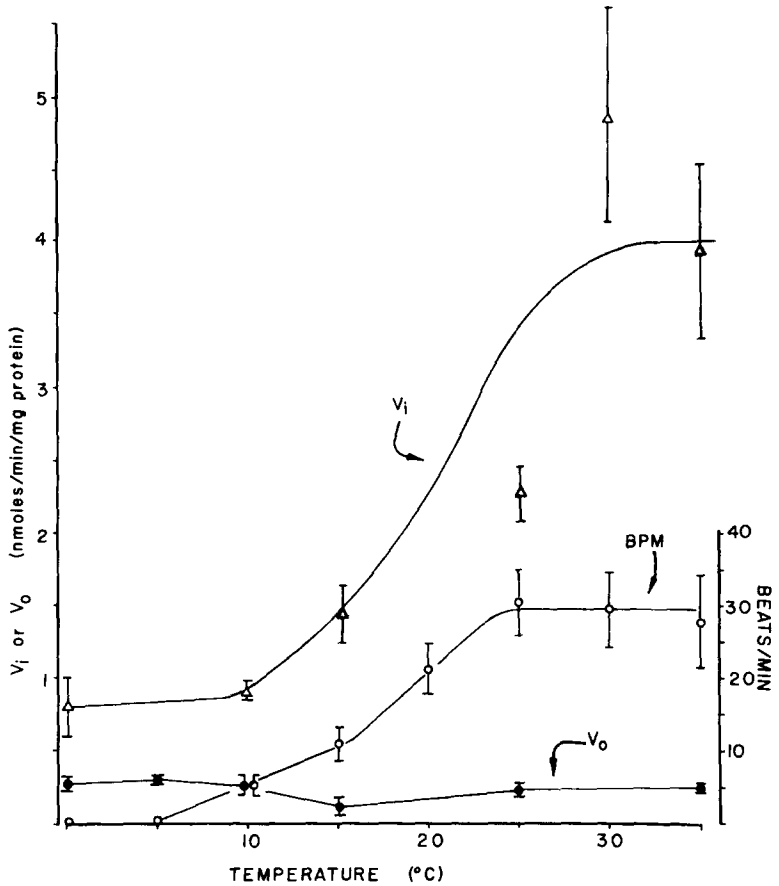

Fig. 2. ^{45}Ca influx velocity, efflux velocity and contraction frequency as functions of temperature. Myocardial fragments were suspended in P_i-buffered medium and incubated at 25°C, with agitation, for 1 h (preincubation). They were then sedimented and resuspended in fresh P_i-buffered medium at the indicated temperature. ^{45}Ca was added either at the beginning of the preincubation, in preparation for washout studies, or at the time of final resuspension at the indicated temperature, in the case of uptake studies. The isotope content was then assayed in samples of medium after filtration to remove myocardial fragments. V_o indicates the initial rate of appearance of isotope in the medium from preloaded myocardial fragments. V_i is the initial rate of isotope disappearance in the uptake studies. Contraction frequency, or beats per min (BPM) was measured in samples from parallel (unlabelled) incubation vessels. These samples were placed on a microscope stage at the same temperature as the incubation vessel. The time for 10 consecutive contractions of the first myocardial fragments noted in the microscope field was used for estimation of beats per min.

beats per min and V_o. If the range was limited to 10—25°C, then r^2 for beats per min and V_i was 0.97, but for beats per min and V_o it was 0.00. Although the immediate source of effluent ^{45}Ca is the cytosol, efflux of ^{45}Ca from the myocardial fragments reflects movement of isotope from sequestered sites into the cytosol, and thence into the medium. The constancy of V_o, as temperature and contraction frequency vary, therefore implies that ^{45}Ca release from sequestered sites does not parallel contraction frequency.

^{45}Ca flux in depleted medium. The high V_i/V_o demonstrated by this preparation under the conditions described above, obviously reflects a non-steady

TABLE I

^{45}Ca UPTAKE AND RELEASE WITH FRESH OR "DEPLETED" MEDIUM

Myocardial fragments were preincubated for 60 min at 25°C with agitation. They were then sedimented and resuspended in either fresh or "depleted" P_i-buffered medium at 25°C. Depleted medium is medium in which myocardial fragments were incubated 60 min at 25°C, after which they were removed by centrifugation. ^{45}Ca was added either at the beginning of the preincubation (efflux studies), or at the time of final resuspension (influx studies). In either case, samples of the myocardial fragments suspension were periodically filtered to remove myocardial fragments and the isotope content of the filtrate determined. V_i and V_o are the initial velocities of ^{45}Ca influx and efflux respectively.

Medium	(nmol/min per mg protein) (mean ± S.E. (*N*))	
	Uptake rate (V_i)	Washout rate (V_o)
Fresh	2.2 ± 0.19 (10)	0.22 ± 0.03 (7)
Depleted	1.2 ± 0.15 (4)	0.88 ± 0.11 (4)

state. However, a steady state is attained after about 30 min of incubation at 25°C, when a high but stable $[Ca]_i/[Ca]_o$ is reached [6]. Both the $[Ca]_i/[Ca]_o$ and beats per min remain constant from 30 min to at least 2 h following initiation of the incubation period. On the basis of the ^{45}Ca distribution, together with atomic absorption measurements made on the original medium, the $[Ca]_o$ at equilibrium can be estimated to be about 1 μM. Thus, the steady state is observed with myocardial fragments that are 'Ca loaded' and a medium that is 'Ca depleted'.

In order to study Ca flux under conditions closer to the steady state, we resuspended myocardial fragments, after the 1 h preincubation, in 'depleted medium'. This was prepared by harvesting the KP_i medium in which myocardial fragments had been suspended for 1 h, at 25°C, at a protein concentration of 1 mg/ml. This medium is referred to as depleted because its calcium content was about 10% that of fresh medium. In a steady state, the net influx and efflux rates must be equal. In the prevent case, this could come about through a reduction of V_i, an elevation of V_o, or both. The results shown in Table I indicate that the last of these alternatives was observed. That is, when myocardial fragments were suspended in depleted medium, in order to approximate the steady-state condition, the V_i was lower and the V_o was higher than when resuspension was in fresh medium.

Discussion

Under the pre-steady state conditions studied here, the co-variation of Ca influx velocity and contraction frequency as functions of temperature suggests that Ca influx plays a critical role in beating of the hyperpermeable cells. Conversely, the failure of Ca efflux to co-vary with contraction frequency implies that Ca release from intracellular storage sites is less important. These findings are in basic agreement with previous studies on Ca flux in hyperpermeable heart muscle cells [6], and with the conclusions reached by Langer on the role of extracellular or 'superficial' Ca stores in myocardial contractility [4,5]. It is

difficult, however, to reconcile the pre-steady state data presented here with demonstrations that intracellular stores provide contraction-activating Ca [3,11].

In particular, Fabiato and Fabiato [3] measured contractile force of skinned heart muscle cells while the $[Ca^{2+}]_o$ was varied. The finding of a large increment in contractile force with a relatively small increment in $[Ca^{2+}]_o$ led them to conclude that Ca influx evokes Ca release from intracellular stores. De Clerck et al. [11], using a similar preparation, found that mechanical performance varied with the concentration of Ca in the medium, but not with the degree of loading. This was interpreted as indicating an extracellular origin for the contraction-activating Ca. Thus, in spite of the convincing data of Fabiato and Fabiato, there is reason to suspect that activator Ca may be derived from extracellular sources under some conditions. One possible explanation for the divergent results and views is that there is more than one mechanism controlling Ca oscillations: one which involves periodic Ca release from intracellular organelles, and another which involves transsarcolemmal influx as the source of contraction-activating Ca. The latter mechanism is strongly supported by the results obtained when myocardial fragments are resuspended in fresh phosphate-buffered medium. However, the tendency for V_i to decrease while V_o increased at near-steady-state suggests that a second mechanism could be operative under these (steady-state) conditions.

The existence of more than one mechanism governing $[Ca^{2+}]_i$ oscillations is entirely reasonable, given the experimental findings of various investigators described above. In skeletal muscle more than one mechanism exists for triggering Ca release from sarcoplasmic reticulum. Here, the degree of Ca loading determines which mechanism is operative [1]. Recently, Scharff [13,14] demonstrated that for erythrocyte membrane-bound $(Ca^{2+} + Mg^{2+})$-dependent ATPase, the K_m for Ca^{2+} varies with the $[Ca^{2+}]$ to which the membrane was exposed during preparation. Other kinetic differences were also apparent. These findings are particularly relevant, since the $[Ca^{2+}]$ to which the heart muscle cells were exposed varied greatly in the works under discussion. In particular, the cells used by Fabiato and Fabiato were exposed to high concentrations of ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid and very low $[Ca^{2+}]$. The cells used in the work presented here were studied at a low $[Ca]_o$, compared to physiologic levels, but never to the very low values used by Fabiato and Fabiato [3]. In the work of Langer [5], the extracellular $[Ca^{2+}]$ was close to physiologic.

Acknowledgements

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