INITIAL CALCIUM BINDING RATES OF CANINE CARDIAC RELAXING SYSTEM (SARCOPLASMIC RETICULUM FRAGMENTS) DETERMINED BY STOPPED-FLOW SPECTROPHOTOMETRY

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SUMMARY: Calcium binding by cardiac relaxing system (sarcoplasmic reticulum fragments) from canine ventricle was measured by a dual-beam spectrophotometric technique with manual and with stopped-flow mixing. The ATP-dependent equilibrium Ca^{++} binding level was 68.3 ± 3.2 nmole/mg protein. The initial Ca^{++} binding was 19 nmole/mg protein within 150 msec at 25°; the reaction exhibited a Q_{10} of approximately 2. It is concluded that the cardiac sarcoplasmic reticulum has the binding capacity and sufficient initial rates of Ca^{++} binding to effect relaxation *in vivo*.

The central role of the cardiac sarcoplasmic reticulum in the relaxation of functioning myocardium has been suggested by several authors (1-6). It has been estimated that preparations of cardiac relaxing system (CRS), presumably derived from sarcoplasmic reticulum, have a Ca^{++} binding capacity sufficient to effect relaxation of fully activated fibers (3, 7, 8). However, the accumulation of these amounts of Ca^{++} requires several min of incubation in the presence of ATP. Such slow rates of Ca^{++} binding are clearly incompatible with *in vivo* dynamics because relaxation in most mammalian hearts is essentially complete in approximately 200 msec or less. The present study was undertaken to investigate the binding of Ca^{++} to a highly active canine CRS during the physiologically meaningful time period of less than 200 msec, in an attempt to resolve the inconsistency.

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METHODS

CRS was isolated from the hearts of healthy dogs by means of techniques previously described (3, 9). The final pellet (10 000 - 37 000 x g) was suspended at 12 mg/ml in 50 mM KCl-20 mM Tris-maleate buffer (pH 6.8), and assayed within 3-4 hr. Ca^{++} binding refers to the ATP-dependent accumulation of Ca^{++} in the absence of any added precipitating agent. The overall binding reaction consists of three components. Upon addition of ATP, Ca^{++} binding begins and reaches its peak value within 1-2 min. This level is maintained for a short while and is followed by Ca^{++} release from the CRS. The peak level of binding (when Ca^{++} binding is limited only by the CRS) is referred to in this paper as the equilibrium Ca^{++} binding level. The term maximal is not used because Ca^{++} binding is "maximal" at different levels, depending on the conditions of assay used by different investigators. In addition to the usual Millipore filtration method of assay (3, 9), equilibrium Ca^{++} binding was measured using a dual-beam spectrophotometric method based on the Ca^{++}-chelating dye, murexide (ammonium purpurate), using either manual or stopped-flow mixing, and the recording conditions previously described (3). Changes in the free Ca^{++} concentration were estimated from absorbance changes of murexide at 542 nm, using 507 nm as the isosbestic reference wavelength (3, 10). Identical mixtures were placed in each syringe of the stopped-flow apparatus, except that one contained ATP and Ca^{++} and the other, CRS. Mixing of the two occurred during flow to the cuvette, within 10 msec before observations were taken. The reactions were recorded on a storage oscilloscope with a strip chart recorder placed in parallel to monitor them beyond the time which would be measured on the oscilloscope. The reaction medium contained 0.2 - 0.6 mM murexide, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM Tris- or Na_2-ATP, 20 mM Tris-maleate buffer (pH 6.8), 0.8 mg CRS protein/ml and 20-60 μM Ca^{++}. In some experiments, an ATP-regenerating system consisting of 2 mM phosphoenolpyruvate and pyruvate kinase (0.05 mg/ml) was also included. The temperature was maintained at 25° or 37°.

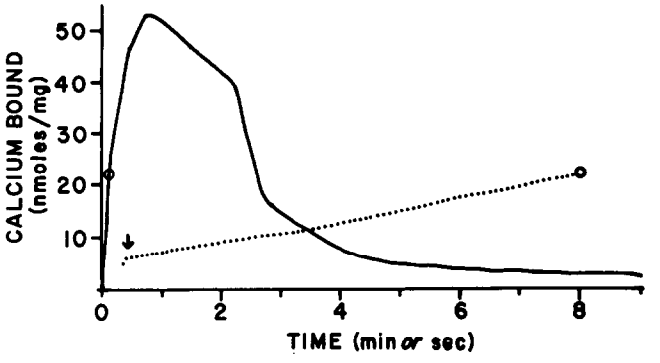

Figure 1: Time course of Ca^{++} binding and release by canine CRS. Abscissa expressed in min for the solid line; in sec for the dotted line. The incubation medium contained 0.3 mM murexide, 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-maleate buffer (pH 6.8), 51 μM $CaCl_2$, 0.2 mM Tris-ATP and 0.8 mg CRS protein/ml. Temp was 25°. Coincident points in time on the solid and dotted lines are indicated by the circles. Similar results were seen in more than 20 such measurements. The arrow provides a 400 msec time mark for the dotted line. Recording prior to this time was limited by the response time of the recorder and by the rates of mixing when stopped-flow mixing was not used. These conditions preclude any estimates of the initial binding rates.

RESULTS AND DISCUSSION

The binding of Ca^{++} by freshly prepared canine CRS measured by the Millipore filtration method averaged 68.3 ± 3.2 nmole/mg protein at equilibrium (N = 9). This mean value somewhat exceeds that previously reported for canine CRS (7, 9) and the highest value reported by Weber (8) for dystrophic chicken CRS.

The solid line of Figure 1 shows the time course of the overall binding and release reaction, measured spectrophotometrically by the murexide method without stopped-flow mixing. The equilibrium Ca^{++} binding level was essentially the same as that found with the Millipore filtration technique. Release was rapid and characteristically triphasic; the pattern was affected by the concentration of Ca^{++}, with the rapid release phase being more pronounced at higher Ca^{++} concentrations. Higher concentrations of ATP (up to 2 mM) delayed the onset of the rapid release phase and the presence of an ATP-regenerating system abolished or masked it, without appreciably slowing the overall release rate (data not shown).

Further analysis of the release phase is in progress. For example we have recently shown that the H^+ concentration strongly affects Ca^{++} release (11).

The dotted line of Figure 1 shows the time course of the binding reaction, on an expanded scale over the first 8 sec of the reaction. With this time resolution (i.e. without stopped-flow mixing) the reaction appears to be linearly related to time, but does not pass through the origin.

The Ca^{++} binding and release reaction was temperature-dependent. Figure 2 shows the overall sequence of the reaction at 25° (upper panel) and 37° (lower panel). Mixing was by stopped-flow. The Q_{10} based on the binding process was 2.3. A similar value has been previously reported (12).

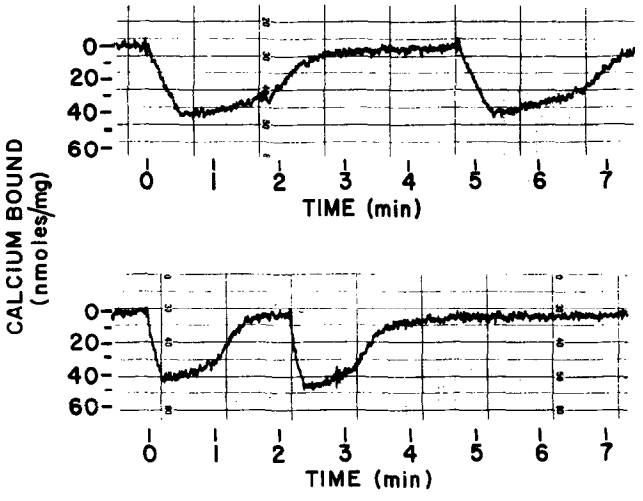

Figure 2: Overall time course of Ca^{++} binding and release by canine CRS after stopped-flow mixing at 25° (upper panel) and 37° (lower panel). The reaction mixture was the same as in Figure 1, except that it contained 44 μM $CaCl_2$. The total reaction sequence of binding and release was faster at the higher temp. The triphasic Ca^{++} release was evident at both temp. The Q_{10} for the binding process was 2.3. Replicate runs are shown at each temp.

The initial Ca^{++} binding reaction was very rapid and could be accurately recorded only on the oscilloscope. The lower panel of Figure 3 shows the

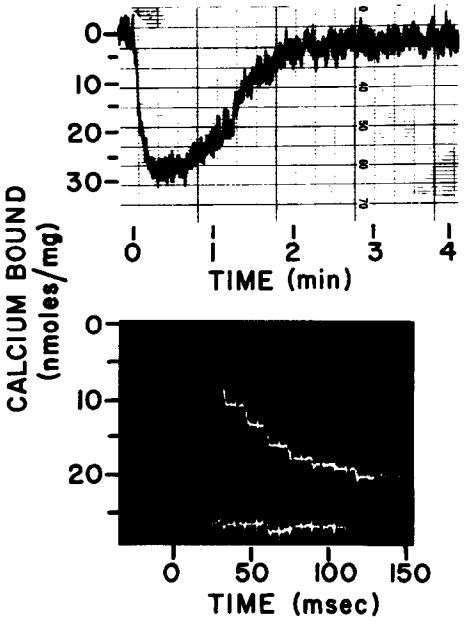

Figure 3: Ca^{++} binding and release by canine CRS after stopped-flow mixing in the presence of limiting amounts of Ca^{++}: total reaction sequence (upper panel) and simultaneous recording of initial binding over the first 150 msec (lower panel). The reaction mixture was the same as for Figure 1, except that the Ca^{++} concentration was 22 μM. The temp was 25°. Horizontal line at the bottom of the lower panel indicates the level of the electronic noise in the absence of the binding reaction. Chopped appearance is due to alternation of the monitoring wavelengths.

first 150 msec of the same binding and release reaction recorded in the upper panel of this figure. Even though there were no abrupt changes in the Ca^{++} binding rate, a single value could not be ascribed to the initial rate because it decreased approximately exponentially with time. For example, over the interval 0-25 msec, 7.44 nmole Ca^{++}/mg CRS protein were bound, yielding an apparent Ca^{++} binding rate of approximately 0.298 nmole/mg/msec; whereas the rate had decreased to 0.062 nmole/mg/msec over the interval 100-125 msec. The average binding rate over the entire interval 0-150 msec was 0.140 nmole/mg/msec. Because the rate was not constant, we have chosen to present both an initial rate of Ca^{++} binding and the initial amount of Ca^{++} bound. The initial rate is defined as the average rate over the arbitrary interval 0-50 msec, and the initial amount bound is the total Ca^{++} bound/mg protein at the end of the interval 0-150 msec.

For the preparation in Figure 3, the initial rate was 0.272 nmole/mg/msec and the initial amount bound was 21.1 nmole/mg, under the conditions where Ca^{++} was present in limiting amounts. For two other preparations, these values were 0.235 and 0.235 nmole/mg/msec, and 16 and 19 nmole/mg. Usually, limiting concentrations of Ca^{++} were used with the stopped-flow method, to provide an additional internal calibration. In this case, all detectable free Ca^{++} is removed from solution by the CRS, providing an accurate relationship between the change in absorbance and the change in the free Ca^{++} concentration. The overall sequence of Ca^{++} binding and release (Figure 3, upper panel) was used in this way to calibrate the trace in the lower panel of this figure. When Ca^{++} was added in sufficient excess that it was not limiting, calibrations were statically done before and after the experiment, using known amounts of Ca^{++} in the absence of ATP. When Ca^{++} was not limiting, the initial rate, for the preparation in Figure 3, was 0.285 nmole/mg/msec and the initial amount bound was 22.3 nmole/mg, though this value was not as precise as when Ca^{++} was limiting. Similar results were found when another system of stopped-flow mixing and optical density recording (Durrum stopped-flow spectrophotometer) was used.

The initial Ca^{++} binding reaction was also temperature-dependent. When a preparation which bound an initial amount of 16 nmoles/mg at 25° was assayed at 37°, it bound 37 nmole/mg during the interval 0-150 msec. The initial Ca^{++} binding rate at 37° was 0.460 nmole/mg/msec and decreased to 0.085 nmole/mg/msec after 100 msec (value over the interval 100-125 msec).

Although no previous report of the initial rate of Ca^{++} binding by CRS has appeared, Ohnishi and Ebashi (10) employing a rapid-flow technique, found that skeletal relaxing system bound 40 nmole Ca^{++}/mg protein after 30 msec at 20°. Our values represent about 20% of this. Langer (13) has estimated that only 15% of the rate of skeletal muscle Ca^{++} binding would be sufficiently rapid to accomplish full relaxation in the cardiac system.

The data obtained using stopped-flow spectrophotometry to measure the amount of Ca^{++} bound during the interval 0-150 msec, indicate that the rate of Ca^{++} binding can account for the rapidity of relaxation as it occurs in cardiac muscle. The average amount of Ca^{++} bound by the end of this time period was 19 nmole/mg protein at 25°. Previous investigators have estimated the sarcoplasmic reticulum content of ventricle between 3.4 mg (8) and 10 mg (5) per g wet weight. Based on these values, the Ca^{++} bound after 150 msec would be between 64 and 190 nmole/g wet weight of ventricle, which is in the range suggested as requisite to effect cardiac relaxation in vivo (3, 8, 13, 14).

The present data thus provide direct confirmation that CRS has both the binding capacity and the rate estimated as requisite to induce relaxation in the cardiac muscle. This is the first study of calcium-sarcoplasmic reticulum interaction using stopped-flow spectrophotometry.

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