Kinetic Studies of Calcium Distribution in Ventricular Muscle of the Dog

By G. A. Langer, M.D.

With the technical assistance of S. D. Serena

Previous studies have indicated the presence of at least three kinetically-defined phases for calcium movement in mammalian cardiac tissue. It has been assumed that the most rapidly exchanging fraction derives from a tissue compartment or space which is significantly different from that which might be represented by the "sucrose space" or interstitium. The basis for this assumption has been the demonstration of a prolonged time constant (τ ~ 7 min) for the calcium phase when compared to that for sucrose (τ ~ 1.7 min). The two more slowly exchanging phases have been assumed to represent, for the most part, intracellular calcium.

Niedergerke has implied that a portion of the rapidly exchangeable fraction represents a "superficial binding site" and is important in the transport of that fraction of calcium which facilitates contraction. On the basis of the early work of Daly and Clark, Wilbrandt et al., and Luttgau and Niedergerke, the importance of the ratio [Ca\textsuperscript{2+}]/[Na\textsuperscript{+}] in the determination of contractile tension and contracture was noted. An increase of this ratio produced by altering either ionic concentration resulted in an inotropic effect and contracture. Niedergerke proposed a competition between Na\textsuperscript{+} and Ca\textsuperscript{2+} ions for a place on the cell surface and showed an increased uptake of calcium by frog ventricle during perfusion with sodium-depleted fluids. He has recently proposed a model for this transport system.

It is the purpose of this study to define further the calcium kinetics in mammalian ventricular tissue and to study the movements of calcium which are associated with the inotropism produced by reduction of [Na\textsuperscript{+}]. The definition of these Ca\textsuperscript{2+} movements provides further evidence for a "specialized" calcium transport system. Moreover, the study provides the basis for a comparison between the effect of reduction in [Na\textsuperscript{+}] and the previously described effect of increased frequency of contraction on Ca\textsuperscript{2+} transport.

The isolated, arterially perfused dog papillary muscle and the arterially perfused dog interventricular septum were the experimental preparations used for this purpose.

Methods

Adult mongrel dogs of either sex, weighing 10 to 15 kg, were anesthetized with pentobarbital (30 mg/kg iv). This was followed by 75 to 100 mg of sodium heparin administered intravenously to prevent intravascular clotting. The dogs were sacrificed 15 to 20 minutes after the administration of heparin by overdosage of pentobarbital, the thorax rapidly opened, and the heart excised in its entirety. The technique of dissection, arterial cannulation, and excision of the isolated, perfused papillary muscle has been described in detail previously.

Isotope Studies

The papillary muscle was suspended as illustrated in figure 1. When mounted in this manner and perfused through its artery, it was possible to record a number of parameters continuously and simultaneously: (1) frequency of contraction with the rate controlled, when desired,
FIGURE 1

System of perfusion and recording. The suspended papillary muscle was perfused from either reservoir while rate of contraction, tension developed and isotopic activity of the muscle were recorded simultaneously. Venous effluent could be collected directly into planchets, droplet by droplet, for later counting. (Figure reproduced with permission from the Journal General Physiology.)

external stimulation; (2) isometric systolic and diastolic tensions with a Schilling isometric capacitance transducer; (3) isotopic activity of the muscle as monitored by the G-M probe.* The activity of the muscle was recorded at one-minute intervals, using an 8270 series ultrascaler II † throughout the course of the perfusion with background from the venous effluent eliminated by a shielded drain during isotopic loading; (4) isotopic activity of the effluent droplets collected during washout of isotope. The frequency of sampling of venous effluent isotopic activity depended upon the droplet rate which varied between two and six drops per minute in the various muscles but did not vary more than ± 2% in any one experiment despite alterations in frequency of contraction or diastolic tension.

During isotopic loading of the muscle its surface was washed continuously by a constant drip of fluid of the same composition as the vascular perfusate. This prevented evaporation and concentration of the isotope on the surface of the muscle. The type of perfusate could be changed within five to ten seconds by switching from one reservoir to the other.

The perfusate used was of two types:

(1) 144 mm sodium: NaCl, 130 mm; KCl, 4 mm; CaCl₂, 5 mm; NaHCO₃, 14 mm; NaH₂PO₄, 0.435 mm; MgCl₂ • 6H₂O, 1.0 mm; and glucose, 5.58 mm.
(2) 36 mm sodium: exactly as (1) except NaCl, 22 mm; sucrose, 216 mm.

Both perfusates were equilibrated to 98% O₂—2% CO₂ at 24°C and contained a minimum of 0.04 ml O₂/ml at a pH of 7.3 to 7.4 at the 150 mm Hg perfusion pressures used. Perfusion rate was approximately 1 ml/g tissue/min. The perfusates were labeled with isotopic Ca⁴⁺ to a specific activity of approximately 25 μc/ml. Ca⁴⁺ labeled perfusates of types (1) and (2) were matched carefully for identical specific activity when used in the same muscle.

When muscles were adequately perfused with 144 mm sodium solution tension developed was greater than 2 g/mm² cross-sectional area and remained stable for five to six hours of perfusion at stimulation frequencies up to 10 beats/min.

Following perfusion the muscles were cut down, analyzed for water content (vide infra), digested in concentrated HNO₃ and the digest distributed in planchets for counting of total isotopic activity of the tissue. The isotopic activity of the effluent droplets collected during washout was plotted. These curves were analyzed graphically according to the method described by Solomon.⁹

A total of 24 successful papillary perfusions were done employing varying sequences of perfusate for varying periods of time as well as, in some instances, varying rates of stimulation.

QUANTITATIVE CALCIUM ANALYSIS

The tissue of the interventricular septum of the excised dog heart was perfused (nonisotopically) via the septal artery as previously described.² This enabled serial sampling of the tissue, at varying times for five to six hours, during the course of perfusion with either 144 mm or 36 mm sodium. Tissue samples from the perfused septum weighed 25 to 35 mg (wet). Each group of muscles included samples which were weighed in predried porcelain crucibles. The samples were then dried at 90 to 100°C until weight-stable (four to six hours) and the percentage tissue water calculated. All tissue samples were then ashed at 600°C for 12 to 15 hours.

The ashed samples were analyzed for total Ca²⁺ content according to a modification of the method described by Bachra et al.¹⁰ The ash was dissolved in 0.5 ml 6 N HCl. The solution was then gently boiled to dryness and 0.2 ml 50 mm sodium citrate was added to prevent a drift of the end point of the titration due to the presence of phosphate. 1.0 ml 0.8 N NaOH and 3 drops of sodium-1-(2-hydroxy-1-naphthylazo)-2 naphthol-
CALCIUM KINETICS IN VENTRICULAR MUSCLE

A semilogarithmic plot of tissue activity (•) in counts/min and simultaneous effluent activity (∗) in counts/min/min during washout of a papillary muscle which had been loaded with Ca⁺⁺ for 52 minutes. The resolution of the effluent curve into phases 0 to 3 with their respective rate constants (λ) is indicated.

Graphical analysis of the effluent curve indicates that it may be resolved into 4 exponentially defined phases (0 to 3). This pattern was defined in 9 muscles isotopically loaded for periods varying between 22 and 132 minutes and washed out for periods of 82 to 92 minutes. The following phase rate constants were found (respective time constants (τ) in parentheses).

0 = 3.5 ± 0.4* min⁻¹ (τ = 0.29 min).
1 = 0.59 ± 0.14 min⁻¹ (τ = 1.72 min).
2 = 0.116 ± 0.012 min⁻¹ (τ = 8.62 min).
3 = 0.021 ± 0.001 min⁻¹ (τ = 47.6 min).

It should also be noted in figure 2a that the tissue and effluent plots remained divergent at end-washout. This indicates that the phases defined by the effluent plot do not account for all of the tissue Ca++. Analysis of the tissue plot indicates the presence of at least one additional phase, phase 4, with a rate constant of approximately 0.004 min⁻¹ (τ = 250 min).

Relation of Ca⁺⁺ Exchange to Na⁺ Concentration of Perfusate and Mechanical Activity

Figure 3 illustrates the uptake of Ca⁺⁺ as correlated with mechanical events following 36 mM sodium perfusion in seven papillary muscles. Upon institution of low sodium perfusion there was an immediate and sustained

* 1 SE of the mean.
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(at least 40 min) increase in Ca\textsuperscript{44} uptake. Coincident with the institution of the low sodium perfusion there were progressive increments in peak isometric tension (mean + 60\%) and in maximum rate of isometric tension development (mean + 87\%). It is to be noted, however, that the mechanical events had stabilized at a mean 10.3 minutes following onset of 36 mM sodium perfusion and remained fixed despite continued marked increase in Ca\textsuperscript{44} uptake. No muscle required more than 14 nor less than 6 minutes for mechanical stabilization. All muscles also demonstrated a variable increase in diastolic tension upon institution of low sodium perfusion. The time course for complete development was similar to the other mechanical events in most preparations, though considerably more prolonged in some.

Increased frequency of contraction up to ten times control rate in three muscles failed to produce any additional increment in Ca\textsuperscript{44} uptake during 36 mM sodium perfusion.

Upon return to normal Na\textsuperscript{+} perfusion, isometric tensions and rates of tension development returned to control values over the same time course noted for their increase. Diastolic tension usually fell much less rapidly. The course of Ca\textsuperscript{44} uptake is illustrated in figure 4. It should be noted there was no gain in Ca\textsuperscript{44} activity in the perfused muscle for a period of seven to eight minutes following return to 144 mM sodium perfusion. Following this plateau in isotopic activity the muscle resumed a rate of uptake much reduced as compared to the low sodium period and similar to the control rate of uptake.

Figure 5 illustrates tissue and effluent washout curves from a muscle loaded for 60 minutes with 144 mM sodium solution con-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Relationship of sodium concentration in perfusate, mechanical function and Ca\textsuperscript{44} uptake in seven papillary muscles. The ordinate values are relative ones for the successive periods of time 0 to 80 minutes. --- indicates the mean; ........ indicates extremes.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Relation between Ca\textsuperscript{44} uptake (counts/min) in a papillary muscle and sodium concentration of the loading solution. Broken lines indicate extrapolations of the Ca\textsuperscript{44} uptake with 144 mM sodium perfusion and 36 mM sodium perfusion.}
\end{figure}
CALCIUM KINETICS IN VENTRICULAR MUSCLE

Isotopic washout was initiated with 144 mM sodium perfusate but switched to 36 mM at 12.2 minutes without alteration in perfusion rate. Other than a marked transient increase in effluent activity there was no demonstrable change in the basic washout characteristics of the muscle as evidenced by either the effluent or tissue curves. In the muscle whose washout is illustrated in figure 5 there was greater than 3.5 g/g tissue increase in diastolic tension coincident with the onset of 36 mM sodium perfusion. The pattern illustrated was repeated in a second muscle. Perfusion rates were not significantly altered throughout the course of the experiments.

Ca++ Concentration in Phases 0 to 4

The total Ca++ concentration represented by a phase can be calculated from the relation:

$$\text{Ca}^{++} \text{ concentration} = \frac{Y_0/\lambda}{f \times S_0}$$  \hspace{1cm} (1)

where $Y_0 =$ counts/min/min at the instant washout was commenced. This is the intercept value for the phase being calculated.

$\lambda =$ rate constant (min$^{-1}$) for phase — defined graphically.

Integration of the formula for washout of a phase ($Y = Y_0e^{-\lambda t}$) from beginning of washout to infinite time gives $Y_0/\lambda$.

$f =$ fraction of phase loaded immediately prior to washout. This value of $f$ is derived from the equation for the uptake curve of the phase and equals $1-e^{-\lambda t}$ where $t =$ total period of isotopic loading.

$S_0 =$ specific activity of the perfusate (counts/min/mM Ca$^{++}$).

In each case the muscle was isotopically loaded for a period long enough to insure an $f$ value of at least 0.9 for the phase being analyzed. Total perfusion periods were also essentially the same. The Ca++ concentration of phase 0 was not calculated from equation 1 because of difficulty in ascertaining its exact intercept value since the first few effluent droplets after commencing washout contain some loading solution from the perfusion system. Concentration of phases 3 and 4 was not derived directly from equation 1 because of the very prolonged perfusion times required to achieve $f > 0.9$ in addition to defining the $\lambda$ values during washout.

Analysis of three muscles by equation 1 indicated mean values for phase 1 of 1.8 mM/liter and for phase 2 of 1.7 mM/liter. In a series of muscles perfused for the standard time (five to six hours) with 144 mM Na$^+$ solution and analyzed by the EDTA technique the total tissue Ca++ was 7.5 ± 0.19 mM/liter. This would indicate that phases 0, 3, 4 and any undefined phase or phases account for some 4.0 mM/liter Ca++ under the defined perfusion conditions. To define further the Ca++ content of these phases two papillary muscles were perfused for a total of 5 hours and isotopically loaded for 180 minutes in order to assure near isotopic equilibrium for phases 0 to 3 based upon the mean $\lambda$ values derived from washout. Phase 4 (assuming $\lambda_4 = 0.004$) would be 50% loaded. The labeled Ca++ accounted for 6.7 mM/liter or approximately 90% of the total mus-

* 1 SE of the mean.
cle Ca++ in both muscles. This indicated that phase 4 accounted for approximately 1.6 mM/liter Ca++ if there were no additional, more slowly exchanging phases present. By subtraction then, phases 0 and 3 accounted for 2.4 mM/liter Ca++ between them.

Movement of Ca++ Associated with Inotropism

A perfused papillary muscle with a consistent spontaneous rate of 2 to 3 beats/min was isotopically loaded for exactly 40 minutes while being perfused with 144 mM sodium solution. It was then washed out with 144 mM sodium perfusate for 85 minutes. A portion of this washout curve with its exponential resolution is illustrated in figure 6a. At end-washout the muscle was again loaded isotopically for 40 minutes, but the last 10 minutes were with 36 mM sodium perfusate. A portion of this second loading curve is shown in figure 6b. The period of 36 mM Na+ perfusion was associated with a marked increase in isotopic activity of the muscle, representing a gain of approximately 2 mM Ca++/liter tissue H2O. Coincident with this increment in isotopic activity there occurred a 63% increase in peak isometric tension and an 81% increase in rate of tension development. The mechanical changes reached stable levels 6 to 7 minutes after institution of the 36 mM Na+ perfusion. Following the 10-minute loading with 36 mM Na+ the muscle was once again washed out with 144 mM sodium for 85 minutes. A portion of this washout curve with its exponential resolution is also illustrated in figure 6a.

It is to be noted that there was no significant difference between the two washout curves except during the period of 2 to 16 minutes where the difference was highly significant (greater than 20 SD of the count through much of this period). The remaining portions of the two curves were virtually superimposable throughout the 85 minutes of isotopic washout. The resolution of these curves into their respective exponential components indicates that they were identical except for intercept and exchange constant of phase 2. Following the 10-minute low sodium perfu-
CALCIUM KINETICS IN VENTRICULAR MUSCLE

144 MU N¬ I 3t aW Ha* I
—T  H
PEBTUIIOH | PERFUSION | M 1 N
50
UT ES
33
TENS
43
OK
FIGURE 6b
uptake (counts/min) and mechanical function of papillary muscle immediately prior to the recording of the second washout curve illustrated in figure 6a.

sion there was an increase in exchangeable isotopic activity completely accountable for in kinetic phase 2. In this muscle the additional exchangeable Ca++ was 1 mM/liter tissue H2O which represented approximately only 50% of the additional isotope taken up during the 36 mM Na+ perfusion period. Since further additional loss was not demonstrated in the washout, approximately 1 mM/liter Ca++ was irreversibly bound during the 10-minute 36 mM Na+ perfusion. The inotropism noted during uptake completely disappeared within 10 minutes of the onset of washout with 144 mM Na+, coincident with the period of greatest loss of Ca++ from the tissue. Results from experiments with a second muscle corroborated these findings in that an exchangeable increment in Ca++, again amounting to approximately 1 mM/liter, could be localized to phase 2 following a 20-minute period of 36 mM Na+ perfusion. This second muscle demonstrated the typical inotropic response coincident with initiation of the low Na+ perfusion. The mechanical changes reached stable levels at 7.5 minutes after institution of the 36 mM Na+ perfusion.

QUANTITATIVE Ca++ STUDIES

The quantitative studies are summarized in figure 7. Thirty samples of right ventricular muscle taken immediately following sacrifice of the dog and prior to perfusion contained 1.95 ± 0.08 * mM Ca++/liter tissue H2O and 75.4 ± 0.6% H2O. Perfusion for 40 minutes with 144 mM Na+ perfusate containing 5 mM Ca++ increased these values to 3.08 ± 0.10 mM and 78.8 ± 0.5% respectively. During the next 40 minutes the muscles were perfused with 36 mM Na+ solution during which time the Ca++ concentration increased 2.7-fold to 8.34 ± 0.17 mM and total tissue water fell to 77.4 ± 0.4%. Return to 144 mM Na+ perfusion for 40 minutes resulted in a mean fall of 0.7 mM in Ca++ concentration to 7.63 ± 0.15 associated with an increase in tissue water to 81.4 ± 0.5. This represented an 8.5% fall in Ca++ concentration and a 5.2% gain in tissue H2O. Correction for the dilution effect produced by the gain in tissue H2O (containing 5 mM Ca++) left 0.53 mM/liter absolute loss in tissue Ca++ during the 40-minute return to 144 mM Na+ perfusion. This is significant (t = 2.38, P < 0.025).

In the group of hearts perfused for 150 minutes with 144 mM Na+ solution followed by successive 10-minute periods of 36 mM Na+ and 144 mM Na+ perfusion there was a 0.40 mM loss coincident with the return to 144 mM Na+ solution for 10 minutes. The Ca++ loss, again corrected for dilution, was significant (t = 2.0, P < 0.05) and noted to be 80% of that lost in the muscles subjected to a similar sequence of low and normal sodium perfusion but for periods 4 times as long.

It was observed that failure to acidify the tissue ash prior to EDTA titration in the con-

* 1 SE of the mean.

1 The time course of the transient increment in effluent activity following institution of 36 mM Na+ perfusion (fig. 5) indicates that any fluid shift which occurs is probably followed closely in time by Ca++ movement. It is assumed, therefore, that any net change in tissue water occurring over a perfusion period greater than 2 minutes is at a Ca++ concentration equal to that of the vascular perfusate, i.e., 5 mM.
trol, nonperfused muscle samples resulted in a 16% loss in titratable Ca++. This is in contrast to an 80% loss in titratable Ca++ which occurred in the 36 mM Na+ perfused muscles if the ash was not acidified prior to analysis.

Discussion

**KINETICALLY-DEFINED Ca++ PHASES**

From a kinetic viewpoint the calcium of mammalian ventricular myocardium is heterogeneous. There are at least five (0 to 4) exponentially-defined phases.

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**FIGURE 7**

Ca++ concentration and water content of dog interventricular septal tissue perfused with the solutions indicated for the time periods indicated. "Control" refers to nonperfused right ventricular muscle. Portions A and B represent separate series of experiments. Vertical line, and horizontal limits, at top of each bar, indicate 2 SE of mean.
Phase 0

The exchange constant for phase 0 ($\lambda_0 = 3.5$) is 6 times larger than that of the next most rapidly exchanging phase. The value of $\lambda_0$ is similar to the exchange constant noted for $^{131}$I labeled albumin ($\lambda = 4.1$) in the perfused papillary muscle. This suggests that phase 0 is predominantly representative of Ca++ in the vascular compartment which was previously measured at 1% of the wet weight of the perfused papillary muscle. At a Ca++ concentration of 5 mM in the perfusate this would account for approximately 0.4 mM Ca++/liter tissue water.

Phase 1

This phase has, heretofore, been unrecognized kinetically. $\lambda_1$ (mean = 0.59) is essentially equal to that noted previously for sucrose (0.53) in the perfused papillary muscle. In addition the total Ca++ ascribable to this phase was a mean 1.8 mM/liter. If the interstitial space is approximated at 30% wet weight in these muscles perfused for some 5 hours (containing 83% water) and it is assumed that the 1.8 mM Ca++ is localized to the interstitium then this space is calculated to have a Ca++ concentration of 5 mM/liter. This is the concentration to be expected for the interstitial space in muscles perfused with 5 mM Ca++ solution. These considerations suggest that kinetically defined phase 1 is predominantly representative of Ca++ in the interstitium.

Phase 2

This phase has been consistently defined for Ca++ in mammalian cardiac tissue. Its exchange constant (mean = 0.116) is 20% that for phase 1 or significantly different from that which is assumed to represent Ca++ in the interstitium. The total ascribable to this phase in three muscles was a mean of 1.7 mM/liter. It has been shown that this phase can increase its concentration by 0.5 to 1.0 mM/liter when [Na+], in the perfusate is reduced by 75% and that the turnover of Ca++ in association with phase 2 correlates closely in time with inotropic changes. This would suggest that phase 2 is the kinetic representation of a specialized Ca++ transport system. Much evidence at present 11,12 indicates that elements of the sarcoplasmic reticulum may be responsible for the specialized transport dependent upon a marked ability for pumping Ca++. Weber et al. have shown, in vitro, that the vesicular fraction of the sarcoplasmic reticulum of rabbit muscle is capable of establishing a concentration ratio of $10^5$ between Ca++ accumulated in the vesicles and Ca++ in the medium. The present study indicates that phase 2 is capable of achieving a Ca++ concentration of 2.7 mM under the conditions of perfusion with a solution containing 36 mM Na+ and 5 mM Ca++. No definitive values are available for the relative volume of sarcoplasmic tubular structure in mammalian cardiac tissue but Johnson and Simonds suggest that as much as 30% of the sucrose-measured extracellular space may represent sarcoplasmic reticulum in rabbit heart. This would indicate a concentration of approximately 20 mM if phase 2 were considered to be predominantly representative of the sarcoplasmic reticulum. This is within the concentrating ability of the sarcoplasmic vesicles which achieved a 24 mM Ca++ concentration under the in vitro conditions of Weber's study. Much further correlation is needed but evidence suggests that kinetic phase 2 may be predominantly representative of Ca++ in the sarcoplasmic reticulum.

Phase 3

Phase 0 and phase 3 were calculated to contain 2.4 mM Ca++/liter. Since phase 0 content has been approximated at 0.4 mM, phase 3 contains 2.0 mM/liter. It is probable that this phase is predominantly representative of exchangeable Ca++ which is intracellular in location.

Phase 4

If this slowly exchanging phase ($\lambda$ approx 0.004) accounts for all of the remaining myocardial Ca++ then its content would approximate 1.6 mM Ca++/liter. It is impossible to determine in the present study whether additional phases exist. However it is evident that under the perfusion conditions employed,
TABLE 1

Calcium Phases of Dog Papillary Muscle

<table>
<thead>
<tr>
<th>Phase</th>
<th>Rate constant</th>
<th>Ca++ perfusion Ca++ conc.</th>
<th>Probable origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.5 ± 0.37*</td>
<td>0.4</td>
<td>vascular</td>
</tr>
<tr>
<td>1</td>
<td>0.59 ± 0.14</td>
<td>1.8</td>
<td>interstitial</td>
</tr>
<tr>
<td>2</td>
<td>0.110 ± 0.012</td>
<td>1.7</td>
<td>Ca++ transport system (Psarcopl. reticulum)</td>
</tr>
<tr>
<td>3</td>
<td>0.021 ± 0.001</td>
<td>2.0</td>
<td>intracellular</td>
</tr>
<tr>
<td>4</td>
<td>approx 0.004</td>
<td>1.6</td>
<td>? intracellular</td>
</tr>
<tr>
<td></td>
<td>or less</td>
<td>or less</td>
<td>? connec. tissue</td>
</tr>
</tbody>
</table>

* 1 SE of mean.

some 20 to 25% of the tissue Ca++ is very slowly exchangeable. The origin of this fraction cannot be derived with any assurance. However, phase 4 has a rate constant similar to that found for a portion of connective tissue by Winegrad and Shanes and a portion of the slowly exchanging fraction may be representative of this segment of tissue. The characteristics and suggested origin of phases 0 to 4 are summarized in table 1.

It should be noted that variation in fiber diameter would be expected to contribute to the kinetic inhomogeneity demonstrated. It is, however, not responsible for a significant contribution. As noted previously the fiber radii have, at their extremes, a 1.8-fold variation. It has been ascertained that this radial variation is normal in distribution. Even if the distribution were bimodal, concentrating about the extremes, it could induce only a 1.8-fold variation in rate constants according to the relation: \( \lambda \propto 1/\text{radius} \). Since the successive rate constants differ by a factor of at least five, it is not possible to explain their difference by variation of fiber size alone.

INOTROPISM AND Ca++ MOVEMENTS

The inotropic effects of low sodium perfusion are well defined. Muscles perfused with a solution in which 75% of the NaCl has been substituted with an isosmolar quantity of sucrose consistently showed an increase in peak isometric tension, rate of tension development and diastolic tension. The first two parameters attain their maximum values within 10 minutes of onset of low Na+ perfusion and remain stable for as long as the muscles are perfused with the low Na+ solution. Despite this, however, calcium uptake continues at an undiminished rate for at least 40 minutes at which time tissue calcium concentration has been increased by some 5 mM/liter. This increment in tissue Ca++ is dependent upon an augmented net influx of Ca++ with efflux rate remaining unchanged (fig. 5). The transient spike in effluent activity (of slightly more than 1 min duration) corroborates the invariable finding of a decrease in tissue water following institution of low sodium perfusion. The rapid onset of these changes correlates well with the onset of increasing diastolic tension and development of partial contracture in the muscles studied. Contracture of the muscle would be expected to produce a reduction or "squeezing out" of interstitial volume with an attendant fall in tissue water content and loss of isotope from the interstitial space. The direct tissue count (fig. 5) does not give evidence of this isotopic loss since this space contains very little isotopic activity relative to the more slowly exchanging portions of the muscle at the time low Na+ perfusion was instituted. Only the monitoring of effluent activity, a much more sensitive measurement of transients, would show evidence of this proposed diminution in interstitial volume.

Most of the calcium representing the net gain during low sodium perfusion for 40 minutes has very little inotropic effect. Moreover 90% of it remains in the muscle despite a 40-minute perfusion with normal sodium solution (fig. 7). Corroboration of the strong
binding of 90% of the calcium taken up is the isotope experiment illustrated in figure 4. Upon reinstitution of 144 mM Na+ perfusion following the 30-minute 36 mM Na+ perfusion the muscle activity should have returned toward a point on the extrapolated basal uptake curve if the extra calcium taken up were reversibly bound. Instead there occurred only a transient plateau on the curve before it returned to a basal uptake rate. The 7- to 8-minute period of unchanging isotope activity does, in fact, represent a small net loss in calcium from a portion of the tissue. This is explained by the fact that there is occurring at this time both an increase in tissue water of 4% (containing isotopically labeled Ca++) and a continuing uptake of Ca++ in a muscle not yet loaded to isotopic equilibrium. The “8-minute plateau” is consistent with the 0.53 mM loss shown in figure 7(A). It also indicates that this loss occurred within ten minutes following return to 144 mM Na+ perfusion, which is corroborated by the quantitative studies summarized in figure 7(B).

It was noted that approximately 90% of the calcium in muscles perfused with 36 mM Na+ solution for 40 minutes was “untitratable” with EDTA unless the ashed muscle samples were acidified with HCl and redried. This may suggest the nature of the “irreversibly bound” calcium in these muscles. The only compound likely to interfere with complete titration of the calcium present is its phosphate salt. The presence of significant quantities of Ca₃(PO₄)₂ with a dissociation constant (K) = 10⁻²³ (ref 15) would not allow the formation of Ca²⁺EDTA, with K = 10⁻¹¹ (ref. 16) to progress to completion. None of the other Ca++ complexes likely to be present, e.g., Ca++ proteinate, in the muscle would interfere with the titration.¹⁰ This suggests that the strongly bound and physiologically “inactive” calcium may be in the form of its phosphate salt.

Although 90% of the Ca++ appears irreversibly bound during 40 minutes of low sodium perfusion, at least 0.5 mM/liter remains in a reversible or labile state. The time for this fraction of the tissue calcium to attain equilibrium seems to be within 10 minutes as indicated by the quantitative studies and by the “8 minute plateau” for tissue activity illustrated in figure 4. It is noteworthy that the time required for equilibrium of the mechanical changes secondary to changes in sodium concentration is similar to that for the labile component of Ca++ and suggests that this calcium fraction is closely related to the mechanical activities of the cardiac muscle.

Results also indicated that large increases (up to 10 times) in rate of contraction during low sodium perfusion failed to produce any additional increment in Ca⁴⁴ uptake. This suggests that whichever “system” is involved in augmented calcium uptake secondary to rate increases² is also involved in the response to low sodium perfusion.

**THE "LABILE CALCIUM" AND ITS PHASE**

The isotope experiments indicate that the “labile Ca++ fraction” associated with the development or subsidence of inotropism is associated predominantly with a portion of the muscle manifesting itself kinetically as phase 2. The “labile Ca++” amounted to approximately 1 mM/liter which is comparable to the highest values obtained in the EDTA-analyzed quantitative series.

It is noteworthy that more than 20 minutes are required for phase 2 to achieve essential Ca++ equilibrium following a change in concentration. This is consistent with λ₂ = 0.116. It also suggests that the changes in Ca++ flux associated with increased frequency of contraction noted in a previous study² may involve phase 2. The “rate responsive system” described in this study required some 20 to 25 minutes to reach equilibrium.

The evidence indicates that a portion of the ventricular muscle which manifests itself kinetically as phase 2 is operative in that fraction of Ca++ exchange which is of importance in both the response of the muscle to low Na+ concentrations as well as to changes in frequency of contraction. Therefore the possibility that entry and exit of Ca++ into and out of this “specialized sys-
tern" may be secondary to changing concentrations of Na+ in the same system should be considered. It is more than likely that these concentration changes would be produced in the region of the "specialized system" by the marked reduction of [Na+] used in the present study. Is there any reason to suspect that a rate increase could induce a shift of Na+ in the tissue which might result in a temporary depletion of Na+ in the "specialized system"? A mechanism which might account for this is the lag in Na+ pump activity following rate increase which has been postulated by Woodbury. Based on the assumption that a fraction of the Na+ moving into the cell from this "specialized system" with each beat could not be returned because of "Na+ pump lag," it is possible that a transient deficit in Na+ would be produced in the system following a rate increase. This would allow Ca++ to move into the sites left open until Na+ was pumped back into the system secondary to a gradually increasing Na+ pump activity. As Na+ returned Ca++ would be displaced and the tissue would return to its prestimulation Ca++ content. The postulated transient Ca++ concentration changes have been demonstrated by Langer and Brady. In this manner the competition for sites in the "specialized system" (possibly the sarcoplasmic reticulum) between Na+ and Ca++ would be the basis for one of the major links in the chain of excitation-contraction coupling.

Summary

Five phases (0 to 4) were defined kinetically for calcium in the dog papillary muscle vascularly perfused for five to six hours with perfusate containing 5 mM/liter calcium. The mean rate constant, λ, (min⁻¹), approximate calcium concentration (mM/liter muscle water) and suggested origin of each phase are respectively: (0) λ₀ = 3.5, 0.4, vascular; (1) λ₁ = 0.59, 1.8, interstitial; (2) λ₂ = 0.116, 1.7, "specialized" calcium transport system (?sarcoplasmic reticulum); (3) λ₃ = 0.021, 2.0, intracellular; (4) λ approx 0.004, <1.6, intracellular and/or connective tissue. Inotropism, occurring coincidentally with institution of perfusion by solution containing 25% normal sodium concentration, was marked by a mean 60% increase in peak isometric tension and a mean 87% increase in rate of tension development. These inotropic changes stabilized within 10 minutes despite maintenance of low sodium perfusion and regressed within 10 minutes upon return to normal sodium perfusion. There was a mean 0.5 mM/liter change in calcium concentration of the muscle associated with the inotropic changes. This fraction of tissue calcium could be ascribed entirely to kinetically-defined phase 2 and was freely exchangeable. Prolonged (40-minute) low sodium perfusion resulted in no inotropic changes beyond the first 10 minutes but was associated with a continued marked net increase in muscle calcium. This fraction of tissue calcium was irreversibly bound and inexchangeable. It was suggested that this fraction of calcium may be bound as its phosphate salt.

The possibility that the Ca++ content of the "specialized system" represented by phase 2 is controlled by movements of Na+ into and out of this system is discussed. Ca++ movements associated with increased frequency of contraction are also discussed in the context of their possible relation to Na+ exchange.

Acknowledgment

The author gratefully acknowledges the invaluable advice, assistance, and encouragement of Drs. W. F. H. M. Mommaerts and A. J. Brady.

References

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