Trans-Sarcolemmal Ca\(^{2+}\) Movements Associated With Contraction of the Rabbit Right Ventricular Wall

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The purpose of this study was to examine the movements of Ca\(^{2+}\) into the myocardium from the extracellular space during the course of muscle contraction. Equilibration of the rabbit right ventricular wall with perfusate containing \(^{45}\)Ca was measured by collecting effluent droplets over time. This protocol was carried out in a quiescent muscle and then repeated 15–20 minutes later in an identical fashion except that halfway through the collection period the muscle was stimulated to contract. We were thus able to quantitate the contraction-dependent changes in \(^{45}\)Ca movements. In control experiments using \(^{34}\)S EDTA as an extracellular space marker, we observed that contraction altered the volume of this space. This alteration in extracellular space was relatively small, and the quantitation of \(^{45}\)Ca movements was corrected for this change. The addition of 1 \(\mu\)M Bay K 8644 to the perfusate stimulated tension and augmented Ca\(^{2+}\) depletion from the extracellular space. The addition of 15 \(\mu\)M nifedipine to the perfusate significantly reduced both tension development and Ca\(^{2+}\) depletion from the extracellular space of the muscle. Net contraction-dependent movement of Ca\(^{2+}\) from the extracellular space into the myocardium under control conditions at 1 mM [Ca\(^{2+}\)] was 10–14 \(\mu\)mol Ca\(^{2+}\)/kg tissue wet wt/beat. This value indicates either a large contribution of Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum and/or significant contribution of sarcolemmal bound Ca\(^{2+}\) to excitation-contraction coupling in the rabbit ventricle. (Circulation Research 1987;61:805-814)
Materials and Methods

Male New Zealand rabbits weighing 2-3 kg were killed by an intravenous injection of pentobarbital (50 mg/kg) with heparin (500 U/kg). The heart was rapidly excised and placed in a beaker containing the perfusion solution (in mM): NaCl 140, KCl 7.5, MgCl 1.0, CaCl 1.0, dextrose 10, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) 6.0 (pH 7.3). The heart was then transferred to a dissection block, the atria trimmed away and discarded, and the right coronary artery cannulated and perfused with the above solution at 37°C. The perfusate was maintained at a constant flow of 1.5 ml/min with the use of a Gilson Minipuls 2 peristaltic pump. The perfusate was directed through one of three stopcocks attached to a common conduit and perfusion cannula (Figure 1). The right ventricular wall was carefully dissected away from the heart, the cannula was tied in with suture thread, and the free right ventricular wall was clamped into the perfusion apparatus diagrammed in Figure 1. The dissection block was removed, and a wide clamp was used to attach the muscle to a force transducer to measure isometric tension development. Resting tension was maintained at about 10 g. The muscle was allowed to equilibrate for approximately 60 minutes during which time the perfusate temperature was gradually reduced. Tissue and perfusate temperatures were monitored with temperature probes. All experiments were performed at a perfusate temperature of 22°C and at 48 beats/minute when stimulated. The lower perfusate temperature was used because it eliminated ectopic activity in the quiescent muscle, eliminated the possibility of perfusate temperature differences in the peristaltic and syringe pump reservoirs, and increased tension development in the muscle, which would optimize our resolution of Ca²⁺ movements associated with contraction. Square wave stimuli of 5-msec duration were used at twice the threshold voltage. The perfusate was continuously bubbled with 100% O₂. The right ventricular wall is stable under these conditions as evidenced by the decline in developed force of less than 5% over 5 hours, and all experiments were completed within this time. For the purposes of our experiments, it was necessary to reduce shunt flow in the preparation. Therefore, we employed a small clip across the base of the tissue to occlude the anterior right atrial branch of the right coronary artery that had been cut in the process of removal of the right ventricular wall.

After the muscle had been equilibrated, the experimental protocol was initiated by turning off the excitation stimulus. The muscle remained quiescent for 2 minutes, after which the peristaltic pump was turned off and one of the syringe pumps (Figure 1) was turned on. The pumps were driven independently by two separate step motors and set to regulate perfusate flow rate at 1.5 ml/min. Two more minutes of quiescence were observed to ensure equilibrium. The syringe pump was then turned off, and this simultaneously activated the second syringe pump. This second syringe contained an identical perfusate except for the addition of 3-6 μCi ⁴⁵Ca/μmol ⁴⁵Ca. Since the stopcocks always remained open and the cannula length from the conduit to the muscle was small, the dead space volume was limited (0.03 mm³). This ensured a complete change from nonisotopic to isotopic perfusate within 5 msec. The use of syringe pumps instead of peristaltic pumps at this crucial time also eliminated any variation in perfusion pressure or flow. Immediately after initiation of perfusion with the isotopic media, venous effluent was collected drop by drop into individual scintillation vials. A digital timer was activated simultaneously with the switch to isotopic perfusion so that drop time and total collection duration could be recorded. Each scintillation vial was weighed before and after the droplet collection to determine droplet weight and, thus, droplet volume (95-125 μl). The droplet was subsequently immersed in scintillation fluid and measured for radioactivity in a Beckman LS-200B scintillation counter. Isotopic perfusion of the quiescent right ventricular wall continued for 3 minutes. Therefore, the total quiescent period was 7 minutes in length. Spontaneous beating was only rarely observed, and an experiment was rejected if one or more spontaneous beats occurred during the quiescent collection period.

The purpose of our study was to examine beat-dependent changes in Ca²⁺ flux. This required obser-
viation of the difference in $^{45}$Ca exchange during a quiescent protocol as described above and during one in which beating was initiated. As shown in Figure 2, three different experimental protocols were employed. The first, as described above, was quiescent throughout. The preparation was perfused via the peristaltic pump and the first syringe pump for 2 minutes each before the isotopic perfusion was begun with the second syringe pump. The droplets were collected for 3 minutes. The muscle was then returned to the peristaltic pump and stimulated again for about 15 minutes. If this protocol was repeated up to 3 times in succession on the same muscle, the $^{45}$Ca-equilibration curves obtained from these three collection periods were found to be superimposable. This established the reproducibility of the equilibration of $^{45}$Ca with the quiescent muscle. Furthermore, the muscle suffered no damage from repeated bouts of quiescence. Returning stimulus to a muscle after it had been challenged with 2–3 bouts of quiescence did not affect force generation (35.9 ± 2.9 g/g wet wt, before and 35.8 ± 2.7 g/g wet wt, after; n = 9). If we wished to examine the effects of beating on $^{45}$Ca flux (protocol 2, Figure 2), the first protocol was repeated in an identical fashion except that at 1.5 minutes into the droplet collection period, electrical stimulus to the muscle was reinitiated. Droplet collection continued for the remaining 1.5 minutes. Thus, in the same muscle, we were able to determine whether beating caused an alteration in efflux $^{45}$Ca-specific activity by comparing isotopic activity in droplets collected from the quiescent period with those collected from the stimulated muscle and to ensure that any differences in the $^{45}$Ca content of the effluent that did occur as a function of beating were not due to changes in the $^{45}$Ca equilibration with the muscle prior to the initiation of the stimulus (because we could compare the $^{45}$Ca-efflux curves during the first 1.5 minutes of the collection period when the muscle was quiescent in both cases). This, then, constituted the basic experimental design for measuring beat-dependent changes in $^{45}$Ca efflux. To gain further confidence in our measurements, we also manipulated inotropy in the muscle in some experiments. In these experiments, the first two protocols were carried out as shown in Figure 2 and as described above. However, after the second protocol, the muscle was returned to electrical stimulus for 15 minutes, and the second protocol was then repeated with the addition of 15 μM nifedipine or 1 μM Bay K 8644 to the perfusate pumped by both syringes. This allowed sufficient time for the drug to reach the muscle and ensured its effect on muscle function. These drugs did not affect $^{45}$Ca efflux from the muscle during the initial 1.5 minutes of collection.

The experimental design described above was devised to optimize the measurement of net $^{45}$Ca (and $^{45}$Ca) movement into the cell. On the basis of existing data, it was decided that 1.5 minutes into the collection period would be the most desirable time to initiate beating since the vascular space would be completely equilibrated with the $^{45}$Ca isotope, the interstitial space would be almost equilibrated, and the myocardial cell minimally labelled. Thus, one can conclude that alterations in $^{45}$Ca-specific activity in the effluent would not reflect movements of $\text{Ca}^{2+}$ in the vascular space, and may reflect $\text{Ca}^{2+}$ movements in the interstitial space but mostly reflect $\text{Ca}^{2+}$ transport into the myocardial cell. However, to ensure that the $^{45}$Ca alterations that we observed due to contraction of the ventricle were solely a result of $\text{Ca}^{2+}$ flux into the myocardial cell and not influenced by changes in isotope specific activity in the interstitial space, an accurate measurement of the extracellular space was
required. Radioactive cobicite EDTA is a marker that is confined to the extracellular space in the heart and, therefore, is an appropriate indicator of change in extracellular volume that may occur as a function of tension generation in the muscle. Synthesis of [58Co]EDTA was carried out as described with the following modifications. The addition of EDTA in the process was increased to 10 mg, potassium acetate was increased to 20 mg, and the CoCl2 was decreased to 12 mg. This was done to optimize the complexation of 58Co with EDTA. The experimental procedure for measurement of extracellular space involved perfusion of the muscle with standard perfusate containing [58Co]EDTA. Control experiments demonstrated that the micromolar concentrations of this compound had no effect on performance. The procedure followed for the perfusion of muscles with [58Co]EDTA was identical to that outlined in protocols 1 and 2 (Figure 2) except [58Co]EDTA replaced 45Ca as the isotope present in the perfusate.

At the end of each experiment, the tissue was trimmed of fat and vascular tissue, blotted dry on tissues, and weighed to obtain the wet tissue weight. The muscle was then dried at 100°C for 24–48 hours and reweighed to obtain dry tissue weight.

All chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Dr. A. Scriabine, Miles Laboratories, New Haven, Conn., generously provided the Bay K 8644 for our use in this study.

Results

Figure 3 presents results from a representative experiment. 45Ca efflux from the right ventricular muscle is plotted over time as a function of beating in the muscle. The results from two separate collection periods obtained from the same muscle are shown. In the first curve, the muscle remained quiescent over the entire 3-minute collection period. In the second curve, the muscle remained quiescent for the first 1.5 minutes before stimulation was initiated. Note the immediate and dramatic decline in effluent 45Ca-specific activity that occurred when stimulation was begun. It is well known that Ca2+ must move from the extracellular space into the muscle cell in order for tension to be generated. Thus, in Figure 3, we attribute the decrease in effluent isotopic activity to a movement of 45Ca from the extracellular space into the myocardial cell. Since the specific activity and the concentration of the extracellular Ca2+ of the perfusate are known, it is possible to quantitate the net Ca2+ movement in micromoles. The amount of ion that moved from the extracellular space into the myocardial cell is measured as the difference in 45Ca-specific activity between the efflux curves during quiescence and stimulation. For example, from the data of Figure 3, the maximal difference at any one collection point was 0.30 μCi/ml. Taking into account the perfusate-specific activity (7.35 μCi/ml) and the concentration of the extracellular Ca2+ (1 μmol/ml), this represents 40.8 nmol Ca2+ or 38.13 μmol Ca2+/kg tissue wet wt. If the difference in 45Ca-specific activity between the efflux curves obtained from the quiescent and beating muscles is summed together from each droplet, the total cumulative net depletion was 202.3 μmol Ca2+/kg wet wt over the first 60 seconds following stimulation.

Our choice of initiating contraction after 1.5 minutes of isotopic perfusion was based on results from preliminary experiments that demonstrated it to be the optimal time point for observing 45Ca movements from the extracellular space into the myocardium. If stimulation was initiated 10 minutes after isotopic perfusion was started, we could not reliably detect 45Ca-efflux changes due to contraction because of the more complete equilibration of 45Ca with the muscle cell. If stimulation was initiated as early as 45 seconds after isotopic perfusion was started, it appeared to increase our power to resolve the changes in 45Ca efflux that occurred due to force development but did not allow sufficient time to unequivocally ensure that the 45Ca efflux pattern prior to stimulation was identical from protocol 1 to 2. These curves must be absolutely superimposable, particularly during the early time
points when the slope is steep, if we were to attribute any changes in these curves to contractile-associated $^{45}$Ca movements. Initiating stimulation 1.5 minutes after isotopic perfusion began allowed us to observe the similarity of the efflux curves in protocol 1 and 2 before stimulation began (Figure 1) in the presence of minimal $^{45}$Ca-labelling of the myocardial pool.

The values obtained from Ca$^{2+}$ flux from the data in Figure 3 were uncorrected for any change in extracellular space that may have occurred as a result of stimulation of the muscle. It is possible that fluid from the myocardial cell that is not fully equilibrated with the isotope may be "squeezed" by the contraction into the extracellular space, which is more completely equilibrated. This would result in a dilutional effect that would cause a decrease in isotopic activity not related to cellular influx. Therefore, an estimate of the change in extracellular volume due to contraction was necessary if the $^{45}$Ca results were to be interpretable.

Figure 4 shows the results obtained from a typical experiment using $[^{58}$Co]EDTA in the perfusate as a marker of extracellular space. $[^{58}$Co]EDTA effluent droplets were collected from the same muscle that had been quiescent during one collection period and quiescent-then-stimulated during another collection period. During the former period, droplets were collected over 10 minutes to establish the equilibration characteristics of the marker with the muscle. As shown in Figure 4, $[^{58}$Co]EDTA achieved a near-asymptotic level in 8–10 minutes. This value is similar to that reported elsewhere for the extracellular space equilibration of $[^{58}$Co]EDTA in interventricular septal muscle.$^{15}$ It should also be emphasized that the general equilibration pattern for $[^{58}$Co]EDTA at the early time points (0–3 minutes) is similar to that observed for $^{45}$Ca. This would suggest that both isotopes equilibrate in the extracellular space in an analogous fashion.

The data from Figure 4 concerning the 10-minute perfusion with $[^{58}$Co]EDTA were replotted semilogarithmically in the inset graph to define the exchange kinetics of $[^{58}$Co]EDTA in the right ventricular wall. The plot indicated two exchange components. In the series, the fast exchange component had a rate constant ($\lambda$) of 8.68 ± 1.5/min and an exchange half-time of 5.13 ± 1.00 sec ($n = 3$). The slow exchange component had a rate constant of 0.70 ± 0.04/min and an exchange half-time of 59.55 ± 3.00 sec ($n = 3$). These characteristics are similar to those found elsewhere for the extracellular space in the interventricular septal muscle.$^{13}$ It is likely that the fast exchange component represents the vascular compartment and the slow exchange component represents the interstitial space.$^{13}$ Our results are consistent with the contention that $[^{58}$Co]EDTA is an appropriate marker of the extracellular space.

Figure 4 also demonstrates the effects of beating on $[^{58}$Co]EDTA efflux from the right ventricular wall. If contraction did not alter extracellular space, the efflux curves for $[^{58}$Co]EDTA would be expected to appear identical in the presence or absence of beating. In the data presented in Figure 4, there was very little effect of beating on effluent isotope activity. Indeed, the most striking difference was an initial but transient increase in effluent isotope activity. These results from a single
We found that the effluent droplet was diluted by a movement of fluid from the muscle cell to the extracellular space. We were able to precisely quantitate the volume of fluid that diluted each droplet in each experiment using \[^{58}\text{Co}\]EDTA according to the following formula:

\[
\frac{1}{1-\text{SA}} \times \text{D\%} \times \text{DV} = \text{dilution volume of the droplet,}
\]

where SA is the percent labelling of the interstitial space (this was calculated for each droplet time point according to the exchange kinetics); D\% is the decrease in isotope-specific activity between the quiescent curve and the stimulated curve divided by the specific activity measured from the quiescent curve; DV is the actual volume of the effluent droplet that was collected. From the exchange kinetics previously reported for the vascular space in our preparation, we assumed it to be fully equilibrated with isotope by 1.5 minutes. Therefore, its influence on the above calculation was negligible, and consequently, it was not included.

Each venous effluent droplet (95–125 \(\mu\)l) was diluted by 1–8 \(\mu\)l over the 1.5 minutes of collection during beating. These values were obtained from separate experiments with nine muscles. This change in extracellular space due to contraction was relatively small, but it does influence the quantification of \(\text{Ca}^{2+}\) movements, as described in Figure 3. Therefore, all measurements of \(^{45}\text{Ca}\) efflux were corrected for the alteration in extracellular space due to contraction of the muscle. Presented in Table 1 are the \(^{45}\text{Ca}\)-depletion results that have been corrected for the change in extracellular space. Maximal depletion (calculated from the largest decrease in isotope-specific activity in a single effluent droplet during contraction as compared with the same time point during quiescence) was 54 \(\mu\)mol \(\text{Ca}^{2+}/\text{kg wet wt}\) or nearly 14 \(\mu\)mol \(\text{Ca}^{2+}/\text{kg wet wt/beat}\). Depletion, of course, was larger on a cumulative basis over time.

To gain further confidence in our technique for measurement of \(\text{Ca}^{2+}\) movements and further characterize these movements, several interventions were carried out to alter the isotropy of the muscle. We hypothesized that if the muscle were exposed to drugs that are known to alter contractile force by affecting trans-sarcolemmal \(\text{Ca}^{2+}\) flux, we would observe appropriate changes in our measurements of the depletion of \(^{45}\text{Ca}\) from the extracellular space. Bay K 8644 is a chemical agent that augments tension generation by increasing \(\text{Ca}^{2+}\) movement into the myocardium via the slow \(\text{Ca}^{2+}\) channels. For these series of experiments, the perfusate \([\text{Ca}^{2+}]_{\text{p}}\) was reduced to 0.5 mM in order to maximize the inotropic effect. As described in "Materials and Methods" and in Figure 2, the procedure consisted of three collection periods: quiescent, stimulated during the second period, and stimulated in the presence of 1 \(\mu\)M Bay K 8644 during the third collection period. The syringes were protected from light exposure to reduce drug degradation. The inotropic effects of Bay K 8644 in a typical muscle experiment are presented in Figure 5. Note the immediate increase in the rates of tension generation and relaxation and total developed force. In a series of these experiments \((n=9)\), total developed force \((g/g \text{ wet wt})\) in control muscles was 24.9 ±3.3, whereas after several minutes of Bay K 8644 exposure, it was increased to 40.7 ± 3.9.

The \(^{45}\text{Ca}\)-efflux characteristics were examined as a function of Bay K 8644, and results from one representative experiment are presented in Figure 6. Similar to the results presented in Figure 3, \(^{45}\text{Ca}\)-specific activity decreased in the effluent with stimulation of the muscle. If stimulation were carried out in the Bay K 8644 pretreated muscle, \(^{45}\text{Ca}\)-specific activity in the effluent decreased to a greater extent. Table 2 summarizes the results from a series of experiments using Bay K 8644. These values were corrected for changes in

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**Table 1. Calcium Depletion From the Extracellular Space During Contraction**

<table>
<thead>
<tr>
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<th>Value</th>
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<tr>
<td>Maximal depletion</td>
<td>54.4 ± 11.4</td>
</tr>
<tr>
<td>Maximal depletion</td>
<td>13.6 ± 2.8</td>
</tr>
<tr>
<td>Cumulative depletion</td>
<td>167.6 ± 28.9</td>
</tr>
<tr>
<td>Cumulative depletion</td>
<td>200.6 ± 34.3</td>
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</table>

Values are presented as mean ± SEM for 14 experiments.

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**Figure 5.** Contractile function of a muscle exposed to control conditions after quiescence or after exposure to Bay K 8644. Developed force \((F)\) and its first derivative \((dF/dt)\) were measured. Note the immediate and substantial increase in both parameters due to Bay K 8644 treatment in comparison with the performance observed in its absence. Perfusate \([\text{Ca}^{2+}]_{\text{p}}\) was 0.5 mM.
extracellular space that occurred during contraction using the same [58Co]EDTA protocol previously described but with the additional condition of (±)-Bay K 8644. 45 Ca depletion in the absence of Bay K 8644 was less than that in the series of experiments reported in Table 1 because, presumably, perfusate [Ca2+] was reduced to 0.5 mM from 1.0 mM. Bay K 8644 increased Ca2+ depletion from the extracellular space by 2–3 times.

To further test the contention that the stimulus-dependent changes in 45Ca efflux represented a movement of Ca2+ from the extracellular space into the myocardium, a negative inotropic agent, nifedipine, was introduced into the perfusate. Nifedipine is a potent antagonist of Ca2+ movement through membrane-slow Ca2+ channels.19 Perfusion of the right ventricular wall with 15 µM nifedipine nearly eliminated tension development (Figure 7), but excitation persisted as indicated by the presence of small force transients. In a series of nine experiments, total developed force declined from 34.1 ± 4.1 g/g wet wt to 2.3 ± 0.4 g/g wet wt in the absence and presence of 15 µM nifedipine, respectively.

The effects of nifedipine on 45Ca efflux from the right ventricular wall are shown in Figure 8. Consistent with the results in Figures 3 and 6, the control muscle demonstrated a significant decrease in effluent isotope activity when stimulated. However, if the same muscle is pretreated with nifedipine, the 45Ca-efflux curve is superimposable upon the curve obtained when the muscle was totally quiescent. This occurred in spite of stimulation to the nifedipine-treated muscle. Table 3 summarizes the results from a series of experiments with nifedipine. Five of the six experiments demonstrated no detectable change in 45Ca efflux due to contraction in the presence of nifedipine. Only in one experiment was there a small depletion of 45Ca from the extracellular space. However, together, the series of experiments demonstrates a dramatic difference in the stimulus-dependent 45Ca depletion from the extracellular space when nifedipine is present.

The isotopic technique of measuring Ca2+ flux in the present investigation is similar to that employed by Lewartowski and colleagues.19 The experimental protocol is, however, different. To more closely approximate the conditions of Lewartowski and coworkers, the right ventricular wall was stimulated at 48 beats/min in nonisotopic perfusate, then perfused for 4 minutes in an identical perfusate containing 45Ca (6 µCi/µmol Ca2+), once in the absence and once in the presence of stimulation. This 45Ca-loading period was followed by 15 minutes of perfusion in nonisotopic medium without stimulation, and the effluent was collected and treated as before. The results from such an experiment are presented in Figure 9. The first minute of washout was the only time when we could detect a depletion of 45Ca from the extracellular space as a function of stimulation of the muscle. The difference in Ca2+ content of the effluent from each collection point during this minute was calculated for the quiescent and stimulated conditions. The cumulative depletion was 81.72 and 106.77 µmol Ca2+/kg wet wt for 30 and 60 seconds, respectively. The maximal depletion for one collection time point was 31.29 µmol Ca2+/kg wet wt or 4.34 µmol Ca2+/kg wet wt/beat. These values are less than but not qualitatively dis-
similar from the results of the present study (Table 1). However, the trans-sarcolemmal Ca$^{2+}$ flux using this protocol is strikingly less than the values reported by Lewartowski and colleagues. For the purposes of comparison, we have included in Figure 9 $^{45}$Ca-effluent content that would have been expected from the data of Lewartowski$^9$ based on a conservative estimate of Ca$^{2+}$ movement into the myocardium from the extracellular space (190 μmol Ca$^{2+}$/kg wet wt). Clearly, isotope activity in the effluent should have been much greater if we were to observe similar effects. Excitation-dependent Ca$^{2+}$-uptake values from 300–500 μmol Ca$^{2+}$/kg wet wt/beat have also been reported using different experimental conditions in other studies by this group,$^{10,11}$ which would only exaggerate the difference in findings. However, it must be kept in mind that these investigators used a higher perfusate Ca$^{2+}$ concentration than was employed in the experiment presented in Figure 9 (1.8 mM as opposed to 1.0 mM), which would contribute to higher influx measurements.

**Discussion**

Our results demonstrate a movement of Ca$^{2+}$ from the extracellular space into the myocardial cell during force generation in the right ventricular wall. Several lines of evidence support this contention. First, the decrease in $^{45}$Ca efflux from the muscle in response to contraction is consistent with an enhanced entry of isotope into the muscle cell to support force generation. Second, the time course of $^{45}$Ca movement is consistent with a role in force generation. The decrease in $^{45}$Ca efflux from the muscle is immediate in response to tension generation. Third, the movement of $^{45}$Ca in our study in response to two inotropic agents correlates well with the known action of these drugs. $^{45}$Ca depletion

**Table 3. Calcium Depletion From the Extracellular Space in Presence or Absence of Nifedipine**

<table>
<thead>
<tr>
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<th>Nifedipine</th>
<th>+ Nifedipine</th>
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<tbody>
<tr>
<td>Maximal depletion (μmol Ca$^{2+}$/kg wet wt)</td>
<td>39.8 ± 12.0</td>
<td>1.3 ± 1.3</td>
</tr>
<tr>
<td>Maximal depletion (μmol Ca$^{2+}$/kg wet wt/beat)</td>
<td>10.0 ± 3.0</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Cumulative depletion (μmol Ca$^{2+}$/kg wet wt/30 sec)</td>
<td>139.5 ± 33.0</td>
<td>5.1 ± 5.1</td>
</tr>
<tr>
<td>Cumulative depletion (μmol Ca$^{2+}$/kg wet wt/60 sec)</td>
<td>157.2 ± 41.3</td>
<td>10.1 ± 10.1</td>
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</table>

Values are expressed as mean ± SEM of 6 experiments.
from the extracellular space increased when Bay K 8644 was present, an agent known to stimulate Ca\(^{2+}\) entry from the extracellular space into the myocardium through the slow Ca\(^{2+}\) channels.\(^{15}\) Ca\(^{2+}\) depletion from the extracellular space decreased when nifedipine was present, an agent known for its Ca\(^{2+}\) channel blocking capacity.\(^{15}\) Fourth, changes in Ca\(^{2+}\) efflux were due to tension generation and not electrical stimulation. Electrical stimulation of the muscle in the presence of nifedipine resulted in no detectable change in Ca\(^{45}\) movement (Figure 8). These observations support the argument that we have measured, isotopically, the movement of Ca\(^{2+}\) from the extracellular space into the myocardial cell coincident with contraction of the muscle.

The decrease in Ca\(^{45}\) efflux in response to force generation cannot be explained by artifactual complications. All of the Ca\(^{45}\)-efflux curves prior to stimulation were superimposable (Figures 3, 6, and 8). Thus, deviations from this equilibration curve would not be expected and must be directly associated with tension generation in the muscle. Another cause for concern was the influence that a change in the extracellular space may have on Ca\(^{45}\) measurements during contraction. Although extracellular space did change with contraction, this was quantitated and the Ca\(^{45}\) results corrected to account for this change. The size of this correction (1–7%) was small and does not threaten the validity of our measurements of Ca\(^{45}\) movement.

Under control conditions, we observed a depletion of 10–14 \(\mu\text{mol} \text{Ca}^{2+}/\text{kg wet wt/beat}\) (Tables 1 and 3) from the extracellular space. This value is subject to several considerations. We have employed a muscle preparation in which vascular shunts are an inevitable consequence of cut edges of tissue. Although major shunts were controlled by clamping (“Materials and Methods”), not all vascular shunts were regulated. This would result in some perfusate interacting with the myocardial cells to a minimal degree, thus tending to mask changes in isotope-specific activity. Because of this, the values above may represent a lower limit. Reflux of isotopic Ca\(^{45}\) from the myocardial cell after its entry would also lower our estimate of Ca\(^{45}\) depletion from the extracellular space during contraction. This would certainly represent a significant factor at the later times of effluent collection in the droplet collection period but would have less effect on the initial few droplets after initiation of contraction. At that time, the high isotope-specific activity outside the myocardial cell and the low isotope-specific activity within the cell greatly favor a net movement of isotope into the cell and very little Ca\(^{45}\) efflux from the cell. Indeed, this relative lack of Ca\(^{45}\) reflux in our study represents an advantage of this technique over microelectrode or absorbance methods of measuring Ca\(^{2+}\) flux.

The trans-sarcolemmal Ca\(^{2+}\) flux that we observed of 10–14 \(\mu\text{mol} \text{Ca}^{2+}/\text{kg wt/beat}\) is in the range of values reported by other investigators at 1–2 mM \([\text{Ca}^{2+}]_0\) using different measurement techniques.\(^{45}\) (McLeod and Bars, submitted manuscript.) Our estimate is less than a tenth of the maximal trans-sarcolemmal Ca\(^{2+}\) movements of 190–359 \(\mu\text{mol}/\text{kg wt/beat}\) reported by Lewartowski and colleagues.\(^{10}\) This discrepancy could not be due to the method of monitoring Ca\(^{2+}\) flux because our study and those cited from the Lewartowski group use an isotopic technique. The difference is unlikely to be due to the experimental protocol since, in preliminary studies, we could not detect large movements of Ca\(^{2+}\) from the extracellular space into the myocardium during contraction using an experimental protocol identical to that employed by these investigators\(^{10}\) (Figure 9). We may only conclude that the discrepancy may be due to some intrinsic differences in the muscle preparations employed. One potentially serious problem with using the Langendorff-type heart preparation\(^{10}\) is the complication of having two fluid-filled chambers in which perfusate may accumulate. Contractile artifacts may occur due to changes in the volume and/or specific activity of isotope in these chambers. This hypothesis must be directly addressed.

The present results confirm the necessity of Ca\(^{2+}\) influx across the sarcolemma in the process of excitation-contraction coupling in the rabbit heart. Previous studies in this species using ryanodine\(^{16,17}\)
indicate that approximately 80% of the Ca\(^{2+}\) reaching the myofilaments is derived directly from trans-sarcolemmal influx. At 1.0 mM [Ca\(^{2+}\)], as used in the present study, the rabbit ventricle generates approximately 50% maximal force. Recent estimates of the amount of Ca\(^{2+}\) required to generate 50% force vary between approximately 60\(^2\) and 120 \(\mu mol/kg\) wet wt.\(^{12}\) Thus, it would seem that an influx between 48 and 96 \(\mu mol\) Ca\(^{2+}/kg\) wet wt/beat should be measured. The present results indicate a value of 14 \(\mu mol Ca^{2+}/kg\) wet wt/beat (Table 1), which is a fraction of the total influx required. There are two possible explanations for the discrepancy: 1) The ryanodine experiments lead to a large underestimation of the contribution of Ca\(^{2+}\)-induced calcium release from the SR in the rabbit ventricle. 2) There is significant binding of calcium at the sarcolemmal surface that serves to buffer and prevent large net changes in interstitial calcium over the course of a contractile cycle; that is, a significant amount of calcium enters from sarcolemmal binding sites and returns to these sites during contraction without affecting interstitial content. Recent studies\(^{16,20-22}\) (Rich et al, submitted manuscript) indicate such sarcolemmal Ca\(^{2+}\) binding and a role for this Ca\(^{2+}\) in the beat-to-beat support of contraction. The possibility that cell depolarization may alter sarcolemmal Ca\(^{2+}\) binding and thereby affect tension generation in the heart is currently controversial.\(^{21,22}\)

Acknowledgments

We express our appreciation to Dr. J. H. B. Bridge for his helpful suggestions regarding the synthesis of \[^{58Co}\]EDTA. The excellent help of Mr. John Parker and Mr. Ivan Whitehorn during the course of this study is also gratefully acknowledged.

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Acknowledgments

We express our appreciation to Dr. J. H. B. Bridge for his helpful suggestions regarding the synthesis of \[^{58Co}\]EDTA. The excellent help of Mr. John Parker and Mr. Ivan Whitehorn during the course of this study is also gratefully acknowledged.

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Key Words • excitation-contraction coupling • trans-sarclemmal Ca\(^{2+}\) flux • Bay K 8644 • nifedipine • rabbit ventricle
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G N Pierce, T L Rich and G A Langer

doi: 10.1161/01.RES.61.6.805

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/61/6/805

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