Calcium Flux in the Mammalian Ventricular Myocardium

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ABSTRACT The exchange of Ca^{45} was studied in dog myocardium by means of a newly developed perfusion technique whereby an excised papillary muscle was perfused through its own artery. This makes possible the sequential and simultaneous correlation of ionic flux with ventricular myocardial function with each muscle serving as its own control. Calcium exchange has the following characteristics: (*a*) The major component of calcium flux is independent of the frequency of contraction. It demonstrates a rapidly equilibrating phase (half-time, 4 to 6 minutes) and a more slowly equilibrating phase with a progressively decreasing rate constant. The flux characteristics of the more rapidly equilibrating compartment are determined by a factor or factors, in addition to simple diffusion, which increase the time required for this compartment to achieve a steady-state with respect to the vascular compartment. (*b*) A lesser component of exchange is stimulus–rate dependent and is characterized by an alteration in the rate of calcium turnover such that the altered influx:efflux ratio requires 20 to 25 minutes to achieve equilibrium. After this time, despite the higher stimulus rate, there is no evidence of change in total tissue calcium. (*c*) The initial rate of the transient response is approximately proportional to the change in stimulus rate.

The central role of calcium in muscular excitation–contraction coupling has been emphasized since the observations of Heilbrunn and Wiercinski (1). Bianchi and Shanes (2, 3) have demonstrated a correlation between changing contractility and changing calcium flux in frog skeletal muscle. Winegrad and Shanes (4) have shown essentially the same relationship for guinea pig atrial tissue. These last studies demonstrated an increment in calcium influx per beat associated with short periods (10 minutes) of increased contractility but no increase in total calcium associated with longer periods of increased contractility.

The development of a new perfusion technique has made possible the se-

quential and simultaneous measurement of ionic fluxes with tension and frequency of contraction in the dog papillary muscle. The sequential calcium flux has been continuously measured for periods of 1 to 4 hours and correlated directly with changes in frequency of contraction. This has provided a means of measuring some of the kinetics of calcium movement in cardiac ventricular muscle with each muscle serving as its own control.

MATERIALS AND METHODS

Dogs of intermediate size (10 to 15 kg) possessed the optimal ventricular septal morphology for this technique. Adult mongrel dogs of either sex were anesthetized with intravenous pentobarbital (30 mg/kg) followed by 75 to 100 mg of sodium heparin IV. 15 to 20 minutes after the administration of heparin the dogs were sacrificed by overdosages of pentobarbital, the thorax rapidly opened, and the heart excised in its entirety. The whole heart was immediately plunged into a tray of continuously oxygenated standard perfusate, equilibrated to 98 per cent O_2–2 per cent CO_2, at 24°C. This solution was of the following composition: NaCl, 130 mM; KCl, 4 mM; $CaCl_2$, 5 mM; $NaHCO_3$, 14 mM; NaH_2PO_4, 0.435 mM; $MgCl_2 \cdot 6H_2O$, 1.0 mM, and glucose, 5.56 mM. The Ca:K concentration ratio was selected to reduce spontaneity and maintain adequate contractility. While immersed in the tray the entire right ventricular wall was excised exposing the right side of the interventricular septum with its array of papillary muscles. The heart (now minus the right ventricular wall) was rapidly transferred to a dissection well containing the oxygenated bathing solution. The total elapsed time from sacrifice of the animal to this stage was approximately 1 minute. Further description is best divided into three stages.

1. *Septal Artery Cannulation* In preliminary experiments, the course of the septal artery in several hearts had been delineated with a vinylite plastic injection technique. This technique has recently been well described by Blair (5). In most hearts the septal artery can be visualized readily through the septal endocardium in its middle one-third. The septal artery was dissected to a point proximal to the origin of its anterior branch and cannulated at this point (Fig. 1). This branch arises in the initial one-third of the artery which is usually moderately deep in the septal tissue. The septal cannula was drawn from polyethylene tubing (P.E. 20) tapering to a flanged tip of approximately 0.5 mm diameter. It was attached to a tapered No. 22 spinal needle which, in turn, was connected to a three-way stopcock and the entire cannula assembly mounted on a micromanipulator. One arm of the stopcock admitted perfusate from the pressurized reservoir while the remaining arm served as an exit for flushing the system and as a bubble trap for gases coming out of solution. Reservoir pressure was maintained in two serially connected systems at approximately 200 mm Hg with 98 per cent O_2–2 per cent CO_2 bubbling continuously into the perfusate *via* a constant pressure valve. At the operating pressure, which gave approximately 150 mm Hg at the cannula tip, and at 24°C, the perfusate contained a minimum of 0.04 ml/O_2 ml perfusate at a pH of 7.35–7.40. The septal perfusate also contained 1.0 ml/100 ml of

0.5 per cent aqueous solution Evan's blue dye (T-1824). 3 to 5 minutes elapsed from sacrifice of the animal to the stage of septal artery cannulation.

As noted by Blair (5) the septal artery supplies approximately 75 per cent of the septum. This has been corroborated in the present group of experiments. The presence of Evan's blue dye provided an excellent demarcation of the area being perfused from the septal artery. The primary purpose of the dye, however, was to stain the arterial supply of the individual papillary structures. At this stage then, the portion of the interventricular septum supporting the papillary muscles was continuously perfused and oxygenated, and the arterial supply of the papillary muscles was usually well outlined.

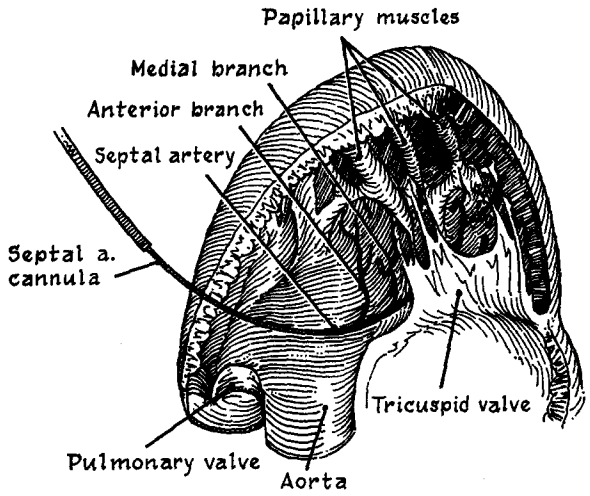

FIGURE 1. Septal artery cannulation. The cannula is inserted proximal to the origin of the anterior branch. The perfused area includes the papillary muscles arising from the right side of the interventricular septum.

2. *Cannulation of the Papillary Arterial Supply* The papillary muscle morphology, particularly the vascular supply, varied extremely. The number of papillaries varied from one to four and they were often bi- or trilobed. The larger muscles almost always exhibited a multiple arterial supply so that total perfusion would have required multiple cannulations. The most anterior papillary always beat spontaneously and later presented a problem for controlled stimulation. This observed spontaneity is most probably due to the termination of the moderator band, with its concentration of Purkinje fibers, in the base of the anterior papillary.

When the best available muscle had been selected, a tie (5-0 silk) was placed at the point of tendinous insertion at the distal end. With the chordae cut, the muscle could be partially retracted so as to expose its base at all points. Further dissection necessitated the aid of the dissecting microscope.

The small, blue-stained papillary artery could often be seen lying superficially at the superior aspect of the base of the muscle. However, careful dissection was fre-

quently required to isolate the vessel. The size of the artery varied from 100 to 200 μ in diameter. The vessel was dissected clean and a loose tie of 13/15 denier silk placed at the site where the cannula was to be tied in place. It is to be emphasized that the muscle was being continuously perfused while this dissection was being completed.

At this point a second flanged polyethylene cannula, selected from a number of predrawn (from P.E. 10 polyethylene tubing) cannulas, was brought into position. With the papillary cannula mounted in the same manner as the septal artery cannula, the cannula tip (100 to 200 μ in diameter) was brought into close alignment with the artery at the selected site of cannulation. The arterial wall was then pierced with a specially tapered No. 30 gauge needle. The septal artery flow kept the papillary artery distended and the arterial puncture open by the exit of perfusate. Both these factors aided insertion of the papillary cannula. Immediately following insertion of the cannula, flow was initiated and the cannula tied in place. Perfusion through the septal artery was discontinued and the septal unit freed for later use (see below).

The papillary muscle, at this stage, was being perfused *via* its feeding artery while still attached to the septum. A portion of its venous drainage could be seen to flow from Thebesian orifices at its base.

3. *Excision and Mounting of the Muscle* The papillary was dissected from the septal wall making certain that the plane of dissection was deep to the site of entry of the cannulated artery. Dissection too close to the site of entry ran the risk of cutting across an arterial branch which would produce a partial shunting of the perfusate. Once the papillary was dissected free a tie was placed so as to hold a small segment of tissue and endocardium at the cut end. This would later serve as an anchoring tie.

At this time a preliminary evaluation of the adequacy of the perfusion could be made by noting the following: (*a*) The entire surface of the muscle should remain moist without external washing. (*b*) The blue staining of the tissue should partially clear. (The perfusate no longer contains Evan's blue dye.) (*c*) The perfusion rate (with approximately 150 mm Hg pressure at the cannula tip) should very closely approximate 1.0 ml/min./gm of tissue and remain unaltered by changes in frequency of contraction. (*d*) Stimulation of the muscle should evoke a vigorous contractile response. The muscle was then mounted in its chamber (Fig. 2). The distal tendinous tie was attached to an RCA transducer (5734) whose output was fed into one channel of a direct writing Sanborn recorder (model 320). The opposite end was anchored by means of the previously attached tie. Platinum stimulating electrodes were attached as illustrated. The tension developed by the muscles was of the order of 2 gm per mm^2 cross-sectional area. This is comparable to contractile tensions noted by others (6) using bath-perfusion techniques and smaller muscles. Tension development remained stable for 5 to 6 hours at stimulation frequencies of five to six beats per minute. An increase in frequency of contraction was matched by approximately the same increase in developed tension per unit time at frequencies up to 15 to 20 per minute for periods of at least 30 minutes.

For the purpose of monitoring isotopic activity a small Geiger-Müller probe was placed within 0.3 cm of the side of the muscle as illustrated. This probe had a 0.25 inch diameter mica window of 1.4 mg/cm^2 thickness (model 222A Geiger tube,

Atomic Accessories, Inc., Valley Stream, New York). The output of the probe was fed into a decade scaler (model 186A-Nuclear-Chicago Corporation, Chicago) and, on some occasions, simultaneously fed into an analytical ratemeter (model 1620 BS, Nuclear-Chicago). The output of the ratemeter was recorded on the second channel of the Sanborn recorder. Depending upon the experimental protocol, the venous effluent was (*a*) allowed to drop freely into a funnel drain at the bottom of the chamber if the muscle was subjected to periodic washing and isotopic counting;

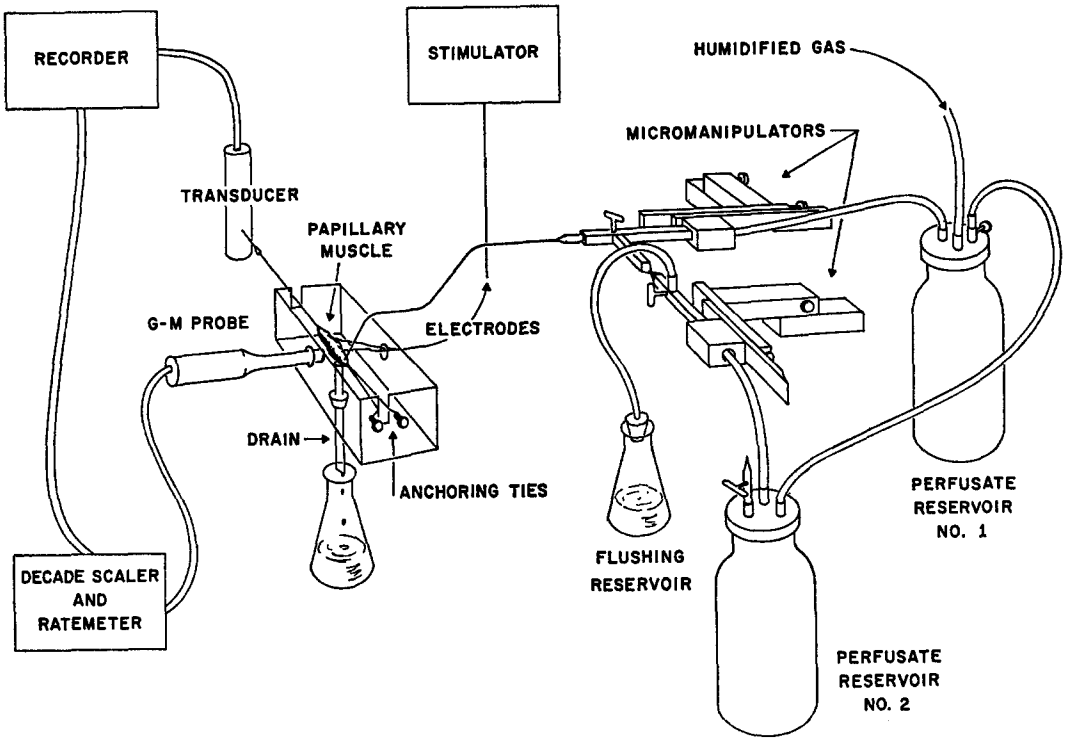

FIGURE 2. System of perfusion and recording. The suspended papillary muscle is perfused from either reservoir while rate of contraction, tension developed, and isotopic flux are recorded simultaneously.

(*b*) collected through a shielded wick drain at the muscle surface if the perfusion and counting were continuous; or, (*c*) collected, droplet by droplet, in planchets during washout for later counting.

Once the mounting was completed moistened gauze was placed at the bottom of the chamber and the chamber covered to minimize evaporation. The second perfusion unit (used originally to supply the septal artery) was now connected to the open arm of the stopcock of the unit in use and its gas supply reinitiated (Fig. 2). This provided a means of alternating perfusate.

The reliability of surface counting as a measure of total tissue activity when using isotopes of low energy (0.15 to 0.25 Mev) β-emission was checked by construction of a

"phantom" papillary muscle. The phantom consisted of a hollow cylinder of 0.5 mil polyethylene film (Dow) which absorbed less than 7 per cent of the Ca^{45} emission. The cylinder was filled with fluids of known total activity (Atomic Energy Commission Standard, Oak Ridge, Tennessee) and surface counts were made with the G-M probe. There was found to be a linear relationship between activity recorded by the probe and total activity of the phantom muscle over the experimental counting range from 100 to 15,000 CPM. The probe recorded 0.05 to 0.06 per cent of the total activity of Ca^{45} within the phantom when the probe was oriented, with respect to the phantom, exactly as with the papillary muscle.

Several groups of experiments were done.

1. EXTRACELLULAR SPACE MEASUREMENTS

(*a*) *Total Extracellular Space* This was measured with sucrose-C^{14}. The sucrose space was loaded to a steady-state with a standard solution of sucrose-C^{14} of known specific activity. The muscle was then cut down, digested with strong acid, and analyzed for total activity. The sucrose space was calculated from the relation:

$$\text{Sucrose space} = \frac{\text{Activity of muscle} \times \text{volume of standard}}{\text{Activity of standard}}$$

(*b*) *Vascular Space* Measured with I^{131}-labeled human albumin in exactly the same manner as above.

2. FLUX MEASUREMENTS

All flux measurements were made in essentially the same manner. Following a period of stabilization of the muscle in the chamber and with a continuous record of frequency of contraction as well as level of tension, the isotopically labeled perfusate was started. Ionic flux during loading and washout, was measured in one of three ways: (*a*) Stopping the perfusion at either 5 or 10 minute intervals, washing the muscle with 3 ml of non-radioactive perfusate (requiring 10 seconds), blotting, and counting for 1 minute; (*b*) continuous perfusion with shielded drainage with continuous counting and rate recording; or, (*c*) perfusion with continuous washing and shielded drainage for 5 minute periods followed by a 1 minute count during which perfusion, wash, and stimulation were all stopped. The fluxes measured were: (*a*) I^{131}-labeled albumin flux, (*b*) sucrose-C^{14} flux, (*c*) sucrose flux with increased frequency of contraction, (*d*) calcium^{45} loading, (*e*) calcium^{45} washout, (*f*) calcium flux with increased frequency of contraction.

RESULTS

Extracellular Space Measurements

SUCROSE C^{14} FLUX Washout of the sucrose space in a typical experiment is illustrated in Fig. 3A. The uptake curve gave an almost identical time con-

stant except for its initial portion. This region was distorted by dilution of the isotope wave front in the perfusion system which occurs on the initial switch from one perfusion system to another. It should be noted that the muscle had attained a steady-state with respect to sucrose-C^{14} activity at the start of the

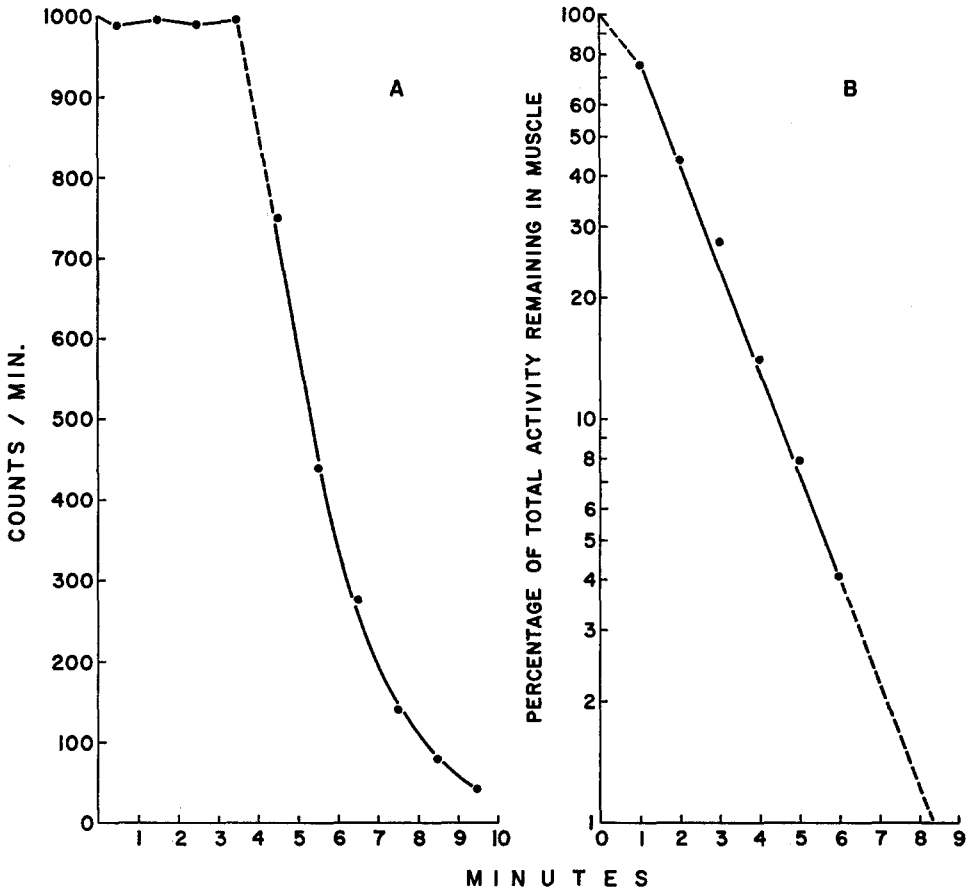

FIGURE 3A. Linear plot of sucrose-C^{14} washout in a typical experiment. Washout commenced at 3.5 minutes at which time the muscle had reached a steady-state of activity for sucrose-C^{14} at 990 CPM.

FIGURE 3B. Semilogarithmic plot of washout curve in Fig. 3A. The broken lines indicate extrapolations from measured solid line curves.

washout. Ninety-six per cent of the total activity was washed out in 6 minutes. A semilogarithmic plot of the washout is illustrated in Fig. 3B. The half-time for washout of the sucrose space was 1.2 minutes. These time characteristics were essentially identical in two muscles. In a third muscle previously equilibrated to the same unlabeled sucrose concentration the half-time for washout was 1.6 minutes.

Measurement of the sucrose space in a typical experiment in which the muscle had attained a steady-state with respect to sucrose-C^{14} activity gave a value of 0.28 ml/gm tissue.

I^{131}-LABELED ALBUMIN FLUX There was a well defined phase of rapid loading and washout of the albumin space. For three sequences of loading-washout in the same muscle, the half-time for loading or washout of this space was 10 seconds.

The volume of the vascular space was assumed equivalent to the volume of the rapidly loading I^{131} albumin space. In a muscle perfused at 175 mm Hg pressure with a perfusion rate of 1.4 ml/gm/minute the vascular space was 0.07 ml/gm tissue.

INTERSTITIAL SPACE Subtracting the volume of the vascular space from the sucrose space leaves a volume of 0.21 ml/gm tissue for the interstitial space. These values are from muscles which were perfused by the standard technique. Variations would be expected to occur dependent upon perfusion pressure and total perfusion time.

CALCIUM FLUX

Ca^{45} Loading The basic curve for loading of Ca^{45} in the papillary muscle is illustrated in Fig. 4, which represents a typical experiment. Curves of this type were obtained from ten papillary muscles which were perfused with a solution containing 5 mM calcium. The median semilogarithmic plot for loading of Ca^{45} in five muscles indicated that there were at least two kinetically distinct systems, one of which ("fast phase") loaded much more rapidly than did the other ("slow phase"). The median slope for fast phase loading indicated a half-time of 6 minutes (extremes of 4 and 8 minutes) and for the slow phase a half-time of 70 minutes (extremes of 44 and 130 minutes). A more accurate evaluation of the kinetics is gained from analysis of washout curves.

Ca^{45} Washout Fig. 5 illustrates the washout curve as plotted simultaneously from a direct count of a papillary muscle and from the activity of each droplet of effluent collected and counted in planchets. The muscle had been loaded with Ca^{45} for 130 minutes and was stimulated at a frequency of 6 beats/minute. The experimental effluent curve can be resolved into a fast and slow phase by assuming a slow phase with a half-time of 38 minutes (represented by the 55 to 80 minute washout period) and extrapolating the slow phase plot to zero time. The counts contributed by this component are then subtracted from the experimental semilogarithmic plot to give a fast phase with a half-time of 6 minutes, which is comparable to the value noted for uptake.

The effluent curve alone, like the loading curves, does not, however, ac-

count for the exchange characteristics of all of the tissue calcium. The fact that the simultaneous tissue and effluent plots are still widely divergent at the termination of the perfusion indicates that the exchange characteristics of a substantial fraction of the slow phase have yet to become manifest in the effluent plot and that a portion of the tissue calcium washes out with a half-

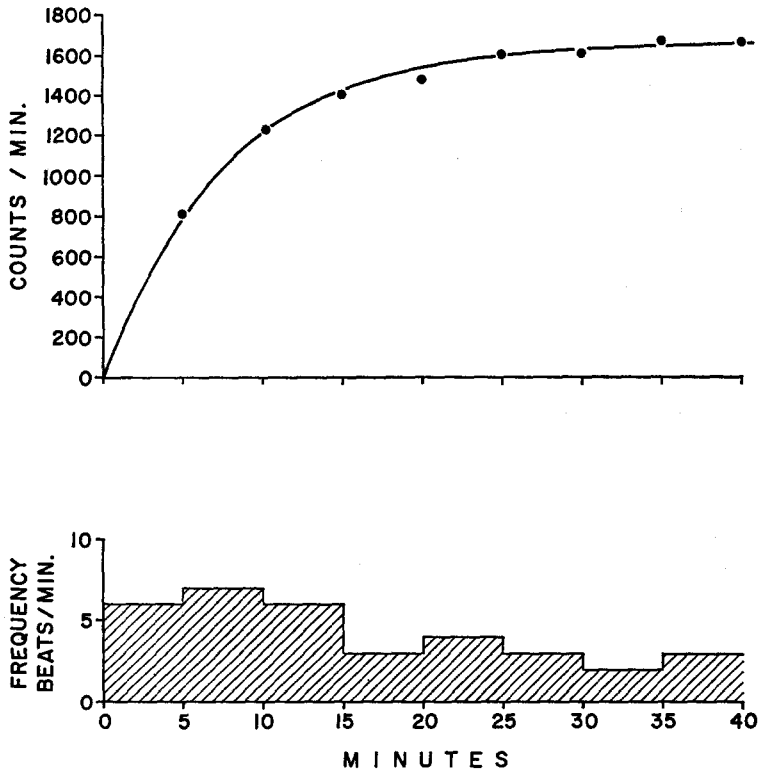

FIGURE 4. Linear plot of Ca^{45} loading in a typical experiment with a plot of the average frequency of contraction for 5 minute periods during loading.

time well in excess of 4 hours (indicated by the slope of the tissue plot). A second papillary muscle showed essentially the same pattern.

It should be noted that the fast phase for calcium loaded or washed out at a rate only 20 to 30 per cent that of the sucrose space.

FLUX WITH INCREASED FREQUENCY OF CONTRACTION The sequence with respect to Ca^{45} flux following an increment in rate of contraction during loading is illustrated in Fig. 6. This represents a typical experiment. Following a fourfold increase in rate of contraction (matched by a fourfold increase in tension developed per unit time) there occurred, simultaneously, an increment in the rate of Ca^{45} uptake. This increased rate of uptake returned to the

original slope after 10 to 15 minutes though the increased frequency of contraction was maintained. This is shown by the leveling off of the uptake curve. The uptake curve then showed a definite reversal in direction during the 15 to 20 minute period following onset of increased rate of contraction. Efflux of isotope exceeded influx during this phase so that total activity of the muscle

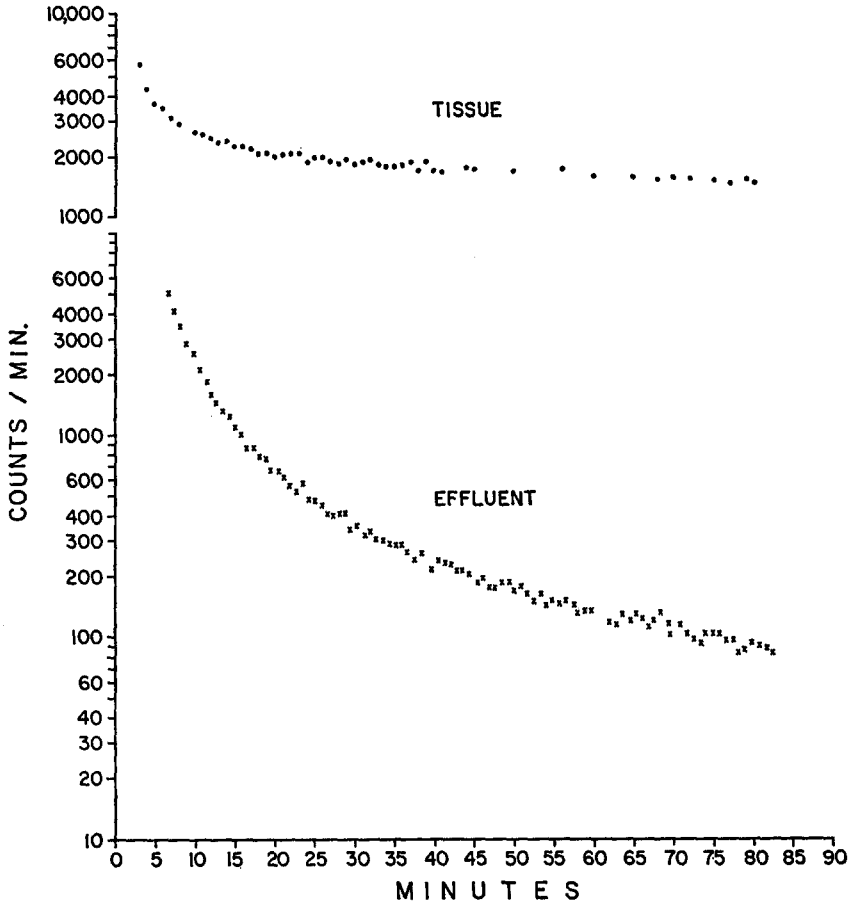

FIGURE 5. Semilogarithmic plot of the washout of Ca^{45} in a typical experiment plotted from direct tissue count (solid circles) and from the activity (×) of each effluent droplet.

returned to a point on the extrapolation of the original uptake curve prior to stimulus change (broken lines in Fig. 6). This same basic sequence following an increase in contraction frequency occurred six times in four papillary muscles. Three other muscles demonstrated the initial increase in Ca^{45} uptake coinciding with increased frequency of contraction but flux measurements were not continued for an extended period following the increase in stimulus rate. This prevented a complete definition of flux reversal in these muscles.

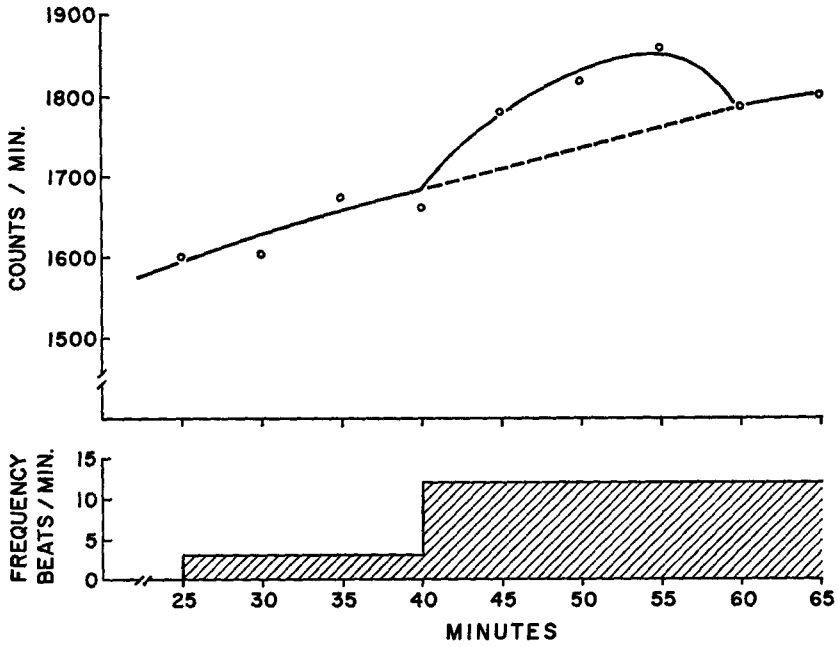

FIGURE 6. Ca^{45} loading in a typical experiment with a plot of the average frequency of contraction for 5 minute periods. There is a fourfold increase in rate of contraction at 40 minutes. The broken line represents the standard loading curve in the absence of increased stimulation.

The flux changes coincident with change in contraction frequency are summarized in Table I. It should be noted that the frequency of contraction was increased an average of 3.7 times above the original rate. This coincided with

TABLE I

RELATION OF Ca^{45} UPTAKE TO FREQUENCY OF CONTRACTION

Muscle	Control rate of contraction	Increase in frequency of contraction (× control rate)	Observed maximum increase in uptake rate of Ca^{45} (× control rate)	Time to achieve maximum increment in Ca^{45} uptake	Time to return to control Ca^{45} uptake rate
	beats/min.			*min.*	*min.*
1	4	9.0	7.2	10	—
2	11	3.7	4.0	10	—
3	5	2.6	1.3	20	—
4	3	3.7	4.3	5	20
	12	1.7	4.0	5	25
5	6	1.9	1.8	15	20
6	5	3.0	1.6	15	20
Mean		3.7	3.5	11.4	21.3

a mean increase in Ca^{45} uptake 3.5 times that of the original rate of uptake. The maximum increment in uptake was achieved at a mean of 11.4 minutes and had returned to the original rate of uptake by a mean time of 21.3 minutes. In a muscle which showed the typical flux sequence with regard to increased stimulation, the specific activity of the muscle for Ca^{45} was ascertained by digestion of the muscle and then counting by means of a calibrated planchet technique. The fiber diameter of the dog papillary was assumed to be 16.9 μ

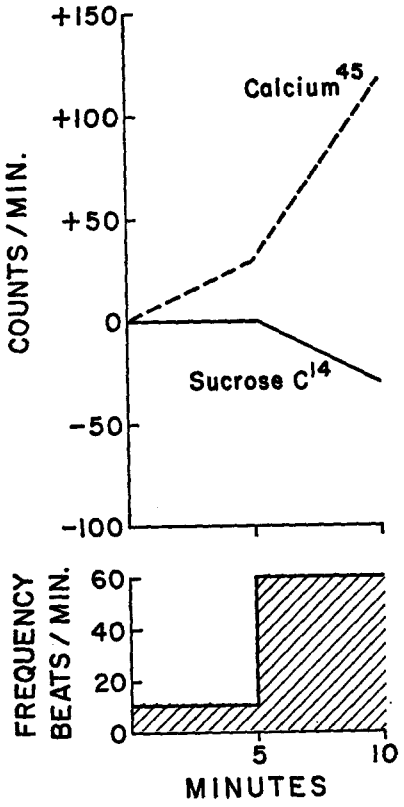

FIGURE 7. Sucrose-C^{14} flux in a typical experiment with a plot of the average frequency of contraction for 5 minute periods. The zero point in the uppermost graph represents 990 CPM sucrose-C^{14} activity. Frequency of contraction was increased sixfold at 5 minutes. The typical response in Ca^{45} count (broken lines) to increased stimulation is illustrated for comparison.

(mean value of 100 fibers in a formalin-fixed hematoxylin and eosin–stained section), which gave a fiber surface area of 97 cm^2 in the muscle analyzed (fibers were assumed to be cylindrical). The point at which the maximum increment in uptake occurred (12 minutes following onset of stimulation at 12 beats/minute in the previously unstimulated muscle) represented 132 $\mu\mu M$ Ca^{++}/cm^2 or 0.92 $\mu\mu M$ Ca^{++}/cm^2/beat.

In all the experiments the increase in frequency of contraction was matched by approximately the same increase in developed tension per unit time.

During Ca^{45} washout, increase in contraction frequency failed to induce any significant changes in the rate of washout of the slow phase in eight ex-

periments. The increased rate of stimulation was continued for as long as 25 minutes and no significant alteration in washout rate could be seen.

The sequence with respect to sucrose-C^{14} flux following increments in rate of contraction is illustrated in Fig. 7. The muscle was contracting at a rate of 10 beats/minute during the period after which it had reached a steady-state with respect to sucrose-C^{14} activity. The count had stabilized at a level of 990 CPM. Upon increasing the frequency of contraction sixfold to 60 beats/minute there was a diminution in recorded activity over the next 5 minutes to 960 CPM. The typical alteration in Ca^{45} uptake with similar rate change, as recorded in other muscles, is plotted for comparison.

DISCUSSION

The development of the physiological perfusion technique described makes possible sequential and simultaneous correlation of ionic flux with ventricular myocardial function. Each papillary muscle acts as its own control. That the tissue, under the conditions of the perfusion, closely approximates the conditions of the ventricular myocardium *in vivo* is supported by the stability of contractile tension and by the absence of edema.

Vascular Space The vascular space value of 0.07 ml/gm tissue represents 25 per cent of the total measured extracellular space. This value is slightly greater than that reported by others for dog heart (0.06 ml/gm (7)) and is expected to be so because of the relatively high perfusion pressures employed.

The volume of the vascular space indicates that the mean transit time of the vascular system is approximately 4 seconds at the average flow rate established by the perfusion (1.0 ml/min./gm). The time required to establish a steady-state for I^{131}-labeled albumin is 1 minute indicating that the equilibration of the vascular space is extremely rapid relative to the other spaces measured. It is to be assumed, therefore, that any substance once it has arrived at the distal tip of the perfusing cannula is distributed equally throughout the entire vasculature of the papillary within a maximum of 1 minute under average perfusion conditions.

Interstitial Space The sucrose space of the papillary muscle (0.28 ml/gm) is in accord with other studies of mammalian myocardium (4). The volume of the interstitial space (sucrose space minus albumin space) of 0.21 ml/gm indicates that the perfusion pressures used do not produce a significant degree of interstitial edema during the course of an experiment. This is to be expected from the studies of Salisbury *et al.* (8) indicating no edema formation with non-pulsatile perfusion pressures below 150 mm Hg.

The sucrose space washout is understandably more rapid with this technique than with soaking procedures. The rapid phase included 96 per cent

of the space and had a half-time of 1.2 minutes as compared to 80 per cent and 3.7 minutes reported by Winegrad and Shanes (4).

Extracellular Space and Contraction Though it was considered very unlikely that the extracellular space should increase in volume coincidently with increase in frequency of contraction, the possibility was ruled out by the measurement of sucrose-C^{14} flux before and after increase in stimulus rate. The diminution in isotopic activity indicated a small *decrease* in the extracellular compartment associated with increased rate of contraction. That there is no *increase* in extracellular volume under these conditions is important when interpretations of changes in ionic flux are made.

Calcium Flux The basic pattern for loading and washout of calcium in the papillary muscle is similar to that reported by others for frog skeletal muscle (9) and guinea pig atria (4). An initial rapid phase followed by a slower phase has been described. It is of note that the initial or fast phase loading rate with respect to Ca^{45} was, in all muscles, slower than that with respect to sucrose-C^{14}. On the basis of simple diffusion, the half-time for the fast phase calcium loading should be 2.8 times as rapid as for sucrose (10). This suggests a fast phase of complex composition which exchanges calcium at a rate significantly different from that of simple diffusion into the interstitial area. This was suggested by Winegrad and Shanes (4) and Niedergerke (11).

The pattern of washout indicates that the slower phase of calcium flux is not defined by first-order kinetics. The divergence of the simultaneous plots of tissue activity and effluent activity is evidence for the complexity of the flux kinetics. This has been discussed in detail by Persoff (12). The curve derived from the effluent droplet activity is the best representation of the kinetics of the system at any one time whereas the curve derived directly from the tissue is dominated by the more slowly exchangeable (or relatively inexchangeable) portion of the tissue calcium.

One would expect multiple rate constants on the basis of differences in muscle fiber diameters. In 100 fibers measured there was a range in diameter between 12 and 22 μ. Though this variation contributes to the complexity of the system, it is unlikely that it is sufficient to explain the wide range of slow phase exchange.

The Ca^{45} loading and washout studies indicate that there are at least three calcium phases: (*a*) a vascular phase, (*b*) a fast phase of composition significantly different from a simple interstitial space operating under the laws of simple diffusion. The kinetics of this system indicate an approximate half-time of 4 to 6 minutes and, (*c*) a slow phase, representing intracellular calcium with a progressively decreasing exchange constant such that a portion of the intracellular calcium might be regarded as "non-exchangeable." Other

studies (4, 9) have indicated that the non-exchangeable calcium might account for 30 to 37 per cent of the total muscle calcium.

Flux with Increased Frequency of Contraction The sequence of changes in flux of Ca^{45} following increase in stimulation indicates that if the rate increase is maintained for at least 20 to 25 minutes there is no increase in absolute quantity of muscle calcium. Under conditions of increased stimulation the average muscle demonstrates an increased influx/efflux isotope ratio for 11.4 minutes and then a reversal of this ratio until 21.3 minutes when it reaches equilibrium. There are a number of mechanisms to be considered in explanation of these results.

1. If the increased rate of contraction had augmented the equilibration rate to the 5 mM calcium solution this would produce an increment in the muscle specific activity. Measurement of total Ca using the EDTA analysis (13, 14) after 4 to 6 hours' perfusion with 5 mM Ca showed an increase of 20 to 30 per cent in exchangeable Ca. At the time the stimulus rate was changed in the present experiments the increase in total Ca must have been about 80 per cent complete. Therefore, if the increase in stimulation rate resulted in a rapid equilibration of the cellular Ca with the 5 mM perfusate Ca, a maximum increase in specific activity of 5 to 6 per cent would be expected. This is within the range found in these experiments. However, the increment should be sustained if this were the operative mechanism and such is not the case.

2. It is possible that the increased influx of labeled calcium is immediately matched by an efflux of an equivalent amount of unlabeled calcium. The source of this unlabeled calcium would have to be the intracellular volume which had not yet reached a steady-state with respect to the perfusate. This mechanism is not supported by the results obtained when increased stimulation is applied during washout of the Ca^{45}. At this time the major source of isotopic activity is the intracellular phase, and no increased rate of decline in isotopic activity could be demonstrated following increased rate of contraction, even though increased stimulation was maintained for as long as 25 minutes.

3. The possibility that increased rate of contraction produces an absolute increase in extracellular volume and therefore a larger calcium fast phase has been ruled out by the sucrose-C^{14} study.

None of the preceding mechanisms is supported by experimental evidence. The remaining and most likely explanation is that increased frequency of contraction initiates a process of augmented calcium turnover with the influx first exceeding efflux. The efflux rate then increases to exceed influx for a brief period before this "rate-responsive system" reaches equilibrium which

requires some 20 to 25 minutes following the onset of increased stimulation. At this time, the basal loading rate again becomes manifest. This does not deny the possibility that the rapid calcium turnover continues for as long as the increased rate of contraction is maintained.

This explanation is in accord with other studies. Winegrad and Shanes (4) found an increment in Ca^{45} uptake in guinea pig atria stimulated for 10 minutes and then analyzed. However, in spite of this, it was found that stimulation for as long as 1 hour did not change the calcium content of the tissue. It is likely that the rate-responsive system had not come to equilibrium at 10 minutes but had certainly achieved equilibrium at 1 hour. Analysis of the maximum increment in Ca^{++} uptake following increased stimulation in a typical muscle gave a value of 0.92 $\mu\mu$M Ca^{++}/cm^2/beat. Winegrad and Shanes found an average additional calcium influx of 0.55 ± 0.14 $\mu\mu$M Ca^{++}/cm^2/beat in guinea pig atria stimulated at 30 beats/minute for 10 minutes in 2.5 mM Ca^{++}. These values are comparable when one considers that the dog papillary muscle was perfused with 5 mM Ca^{++}. Also, it is likely that Winegrad and Shanes analyzed their muscles at a state when the increment in Ca^{++} uptake was near maximum. Henrotte's *et al.* (15) observations on turtle ventricle are also consistent in that no modification of calcium exchange was produced in ventricles stimulated for 1 hour as compared to those at rest. Niedergerke's observations are conflicting. He first reported no appreciable change in rate of Ca^{45} movement in frog ventricle coincident with a 10 minute period of stimulation as compared to a resting ventricle (11) but later stated (16) that periods of electrical stimulation increase the uptake of labeled calcium.

The present sequential observations of calcium flux in the mammalian ventricular myocardium lend additional support to the concept of the central role of calcium in excitation-contraction coupling. Moreover, it seems quite likely that a distinct calcium transport system is operative in supporting increased contractility.

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REFERENCES

1. Heilbrunn, L. V., and Wiercinski, F. J., *J. Cell. and Comp. Physiol.*, 1947, **29**, 15.
2. Bianchi, C. P., and Shanes, A. M., *J. Gen. Physiol.*, 1959, **42**, 803.

3. SHANES, A. M., and BIANCHI, C. P., *J. Gen. Physiol.*, 1960, **43**, 481.
4. WINEGRAD, S., and SHANES, A. M., *J. Gen. Physiol.*, 1962, **45**, 371.
5. BLAIR, E., *Circulation Research*, 1961, **9**, 333.
6. ABBOTT, B. C., and MOMMAERTS, W. F. H. M., *J. Gen. Physiol.*, 1959, **42**, 533.
7. GIBSON, J. G., II, SELIGMAN, A. M., PEACOCK, W. C., AUB, J. C., FINE, J., and EVANS, R. D., *J. Clin. Inv.*, 1946, **25**, 848.
8. SALISBURY, P. F., CROSS, C. E., KATSUHARA, K., and RIEBEN, P. A., *Circulation Research*, 1961, **9**, 601.
9. GILBERT, D. L., and FENN, W. O., *J. Gen. Physiol.*, 1957, **40**, 393.
10. International Critical Tables, National Research Council, New York, McGraw-Hill Book Co. Inc., 1929, **5**, 66–71.
11. NIEDERGERKE, R., *J. Physiol.*, 1957, **138**, 506.
12. PERSOFF, D. A., *J. Physiol.*, 1960, **152**, 354.
13. BACHRA, B. N., DAUER, A., and SOBEL, A. E., *Clin. Chem.*, 1958, **4**, 107.
14. PAPPENHAGEN, A. R., and JACKSON, H. D., *Clin. Chem.*, 1960, **6**, 582.
15. HENROTTE, J. C., COSMOS, E., and FENN, W. O., *Am. J. Physiol.*, 1960, **199**, 779.
16. NIEDERGERKE, R., *Experientia*, 1959, **15**, 128.