Calcium Flux and Contractility in Guinea Pig Atria

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ABSTRACT The calcium in guinea pig atria can be divided into three components by kinetic studies with Ca^{45}: (*a*) a rapidly exchangeable fraction with a half-time of 4.5 minutes; (*b*) a slowly exchangeable fraction with a half-time of 86 (or 168) minutes; and (*c*) an inexchangeable fraction. In Krebs-Henseleit solution containing 2.5 mM calcium, the calcium content of the tissue at rest remains constant, the flux being about 0.02 $\mu\mu$mol/cm^2-second. An increase or a decrease in extracellular calcium concentration by 1.25 mM causes a proportionate change in influx. A large increase in Ca^{45} entry, equivalent to as much as 0.55 $\mu\mu$mol/cm^2, accompanies a contraction. When the strength of contraction is varied by stimulating at different frequencies or in solutions containing calcium at different concentrations, the increment of Ca^{45} uptake per beat changes proportionally with the strength of the beat. Total atrial calcium is not increased by stimulation; however, the increase in outflux of Ca^{45} during contraction that this constant tissue calcium implies could not be demonstrated under the experimental conditions employed. The observations are discussed in the light of the possible role of calcium transfer in excitation-contraction coupling.

INTRODUCTION

The importance of calcium in the contraction of heart muscle has been known since the work of Ringer (1). It is also known that the strength of contraction of isolated cardiac muscle is dependent on the calcium concentration in the external medium. When calcium is withdrawn from the bathing solution, contractions very rapidly cease whereas electrical activity continues for a significant period of time (29, 30). Subsequent studies have led to the conclusion that calcium is important at several different steps in the contractile process. An effect on the action potential has been demonstrated (16, 17), and more recently the work of Niedergerke (3–10) and others (19, 20, 22, 33)

has focused attention on the possibility that calcium couples excitation with contraction.

The observation that the intracellular injection of a very small quantity of calcium causes localized contracture in frog skeletal muscle (Heilbrunn and Wiercinski (2), Niedergerke (9)) is consistent with the involvement of Ca^{++} in the intracellular events of contraction. This is supported further by the demonstration that the increased Ca^{++} influx associated with contraction and contracture is closely correlated both quantitatively and temporally with the mechanical events (11). These data suggest that a movement of calcium, possibly from the cell surface to its interior, accompanies contraction and links excitation to contraction in skeletal muscle.

The major purpose of this study was to determine whether calcium influx also increases during contraction in cardiac tissue and, further, whether a quantitative relation exists between the influx and the strength of contraction. To this end, an examination has been made of the influence of frequency of stimulation and of extracellular calcium concentration on the strength of contraction and on calcium influx. In order to assure a valid analysis of the data and to complete the general picture, calcium efflux and calcium content have also been studied.

METHODS

I. *General*

Female, hybrid, guinea pigs weighing 275 to 325 gm were decapitated and the left atrial appendages removed. During the entire dissection, which took 1 to 3 minutes, Krebs-Henseleit (abbreviated K-H) solution (pH 7.4, NaCl 118 mM, KCl 4.8 mM, KH_2PO_4 1.0 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 27.2 mM, $CaCl_2$ 2.5 mM, glucose 200 mg per cent (12)), through which 95 per cent O_2–5 per cent CO_2 bubbled, flowed continuously over the entire left atrium. The isolated tissue, weighing from 25 to 50 mg, was suspended in a test tube of K-H solution with one end fixed to a hollow stainless steel rod and the other end connected by means of a gold-nickel alloy chain to a Statham strain gauge. The strain gauge was connected to a Brush oscillograph (model RD 232100) through a Brush carrier amplifier (model RD 561200). Small stainless steel clips designed to produce minimal tissue damage were used for attachment to the edges of the muscle. 95 per cent O_2–5 per cent CO_2 bubbled continuously through the hollow rod, and resting tension was set at approximately 25 to 50 mg, the value selected being that which gave near maximal contractile tensions.

The muscle was permitted to recover at room temperature (24–26°C) without stimulation for 2 hours, the solution being changed every 30 minutes during this period. The period of 2 hours was selected because, in a series of experiments to determine the stability of the preparation, it was observed that twitch tension at moderate rates of contraction sometimes rose during the first 60 minutes following dissection, but then remained essentially unchanged for at least 8 to 10 hours. All

experiments were performed at 24–26°C rather than at a higher temperature because the atria are stable for longer periods of time. Preparations showing a tendency to beat spontaneously were rejected.

II. *Contraction Measurements*

Because the muscle fibers of the atrium are oriented in different directions, two different pairs of sites of attachment were used to eliminate any possibility of de-

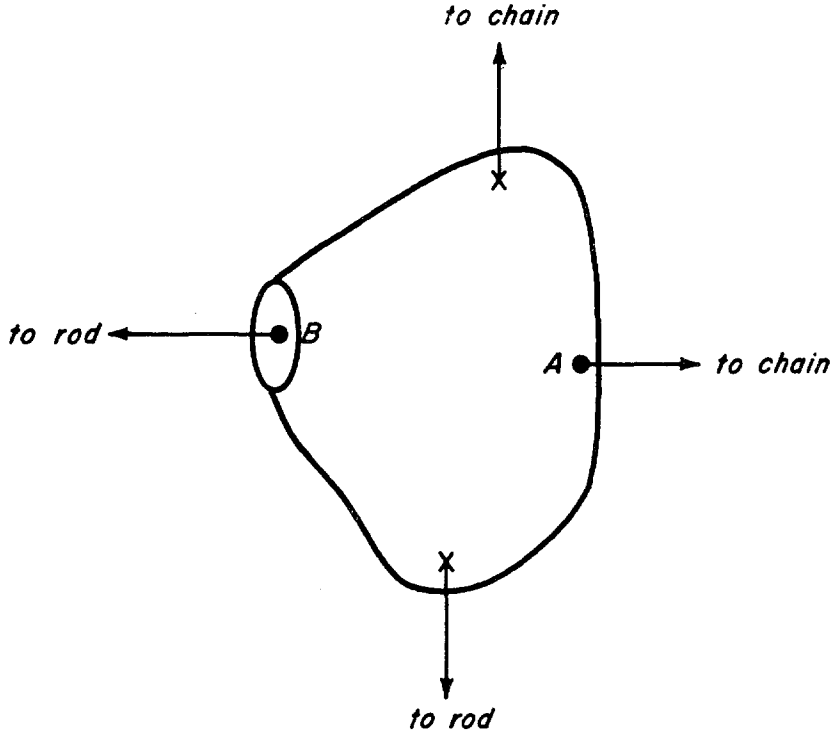

FIGURE 1. Method of attachment of atrium. *A* is the appendicial portion and *B* the basal portion of the atrium. The paired sites of attachment (designated by ● or ×) were alternated in the experiments.

pendence of relative tension development (or isotope movement), under the several conditions studied, on the orientation of the whole tissue (Fig. 1). No difference existed in the relative mechanical (or isotopic) observations made on the atria attached in the two ways.

Isometric twitch tensions at three different rates of contraction, 6/minute, 15/minute, and 30/minute, and in solutions of three different Ca^{++} concentrations, 1.25, 2.50, and 3.75 mM, were measured on each atrium in a series of six experiments to determine the relative force of contraction under different conditions. Thus, each muscle was stimulated for nine separate periods, during which either frequency or

external Ca^{++} concentration was changed, and for a tenth period, which was a repetition of the first condition of stimulation. The last period was a precaution against any possible change in condition of the muscle during the course of the experiment; no such change was observed.

The heterogeneous nature of the fiber alignment within the atrium is such that only volume displacement or luminal pressure measurements can give an accurate value for the total force of the atrium. In these studies, twitch tension relative to that at a standard frequency and at a standard Ca^{++} concentration was more significant than the absolute values. Therefore, regardless of the fraction of the total energy output of the atrium measured, the fraction was constant in a given experiment, and the relative twitch tension at several rates of contraction and at several calcium concentrations is significant. Maximum tensions measured in this way were about 2 gm.

The method of suspension of the tissue results in a twitch which is partly isotonic and partly isometric. However, when shortening rather than tension at different rates of stimulation and in solutions of different Ca^{++} concentration was measured by means of a Schilling variable capacitance transducer (38), the relative length changes did not differ significantly from the relative tension changes.

III. *Isotopic Measurements*

A different group of atria, not stimulated before their soak in Ca^{45}, was used in the isotopic experiments. Muscles employed for relative tension studies were not used because it was not known whether contractile activity is accompanied by any alteration in the form or distribution of tissue calcium. It was felt that less variation in the characteristics of the calcium within the tissues during the exposure to the isotope would exist if the muscle had remained at rest prior to the exposure to Ca^{45}.

(*A*) *Ca^{45} Washout Studies*

After the 2 hour recovery period, individual muscles were suspended in K-H solution to which a tracer amount of Ca^{45} had been added to produce a radioactivity of 1 to 5 μc/ml. The total exposure to the radioactive solution varied from 15 minutes to 10 hours depending on the purpose of the experiment. In some experiments the muscle was stimulated during part or all of the period of exposure to the isotope. At the conclusion of this interval in Ca^{45} solution, the rod and clips suspending the tissue were blotted with filter paper and the muscle and its holder rapidly dipped five times into non-radioactive K-H solution to wash away the superficially adherent isotopic solution. The muscle was then washed out in successive 5 cc volumes of non-isotopic solution for periods up to 5 hours. As can be seen from sucrose washout studies (see below) no isotopic solution was trapped in the lumen of the atrium. The solution was changed at 2, 4, 6, 8, 10, and 15 minutes and every 15 minutes thereafter. Aliquots from the exposed solutions were dried in planchets and the radioactivity counted by means of a Tracerlab automatic gas flow counter. At the end of the washout the tissue was lightly blotted on filter paper and the cut edges and sites of attachment removed for separate analysis. The structure of the atrium makes cut edges unavoidable. It is

essential to remove them for separate counting since they may contain as much as ten times more Ca^{45} than the undamaged tissue.

Each region of the tissue was weighed, dried for 12 hours at 98–104°C, and incinerated for an additional 12 hours at 560°C. The residual ash was taken up in 3 ml of 0.1 N HCl (or distilled, demineralized water when total calcium was measured), the solution dried on planchets, and measured for radioactivity by means of the Tracerlab gas flow counter. The Ca^{45} content found after washout, expressed as the equivalent volume of the medium containing this amount of Ca^{45}, will be referred to as "residual Ca^{45} space." This was added to the activity of the collected samples to reconstruct the decline of the muscle Ca^{45} with time, which is designated the "desaturation curve" (11).

(*B*) *Ca^{45} Uptake Studies*

Resting uptake was measured by exposing the atria to Ca^{45} solution for 15 minutes, washing out the tissue in non-radioactive K-H solution for 3 hours, and analyzing the undamaged tissue for residual Ca^{45}. This method required correction for the Ca^{45} lost during the washout period. However, as pointed out by Bianchi and Shanes (22), the washout is important to eliminate the greater variability and reduced sensitivity that would result from the presence of a large amount of Ca^{45} in the extracellular space. Short periods of exposure to Ca^{45} prevent the occurrence of any significant backflux of Ca^{45} from the cells to the medium during the exposure to radioactive solution. Although the extracellular space does not instantaneously equilibrate with the Ca^{45} bathing solution, this limitation tends to be balanced by the additional exposure to the isotope due to the delay in the emptying of the extracellular space during washout (32).

To determine the effect of contraction on Ca^{45} uptake, the muscle was soaked at rest for 5 minutes in Ca^{45}-Krebs solution and then stimulated with a Grass stimulator with rectangular pulses of 4 msec. duration at suprathreshold voltage for 10 minutes in the same solution. Twitch tension showed no decline during this period of stimulation when the frequency did not exceed 30/minute. The stainless steel rod was used as one electrode and a bare platinum wire in the bathing solution as the other. The muscle was then washed out for 3 hours and treated as in resting uptake studies.

In experiments with radioactive K-H solution containing calcium at concentrations other than 2.5 mM, the muscle was usually exposed for 30 minutes, prior to the soak in the radioactive solution, to a non-radioactive solution containing the same calcium concentration as the Ca^{45} K-H solution to be used. In the several cases in which such prior soaking was not employed, Ca^{45} uptake was the same as in those with prior soaking. Washout in all these experiments was in 2.5 mM Ca^{++} solution for 3 hours.

(*C*) *C^{14}-Sucrose Studies*

The extracellular space was estimated by determining the sucrose space of the tissue. Atria were soaked for 3 hours in K-H solution to which had been added C^{14}-sucrose in tracer amounts and 7 mg per cent penicillin and 15 mg per cent streptomycin to

prevent bacterial metabolism of the sucrose. Then they were dipped five times into non-isotopic solution, blotted, weighed, and soaked overnight in distilled water with antibiotics to leach out the sucrose. The radioactivity of the distilled water and the initial C^{14}-sucrose solution was then counted and the sucrose space calculated in the usual manner.

The rate of loss of sucrose from a C^{14}-sucrose loaded muscle at rest and during activity was determined as for Ca^{45}.

IV. *Total Ca Measurements*

Total calcium content of the undamaged muscle was measured by flame photometry. A Beckman DU spectrophotometer with a photomultiplier and an oxygen-propane flame attachment served to measure the intensity of emission at 556 micra. All glassware used in these experiments was soaked, after the usual cleaning procedures, in a large volume of distilled water which had been purified further by passage through a Barnstead-Bantam demineralizing column to a final conductance of 0.1 part per million (expressed as NaCl). The standard solutions employed to estimate the calcium consisted of various concentrations of $Ca_3(PO_4)_2$ in a solution of Na_3PO_4 and K_3PO_4 at levels corresponding to those in the experimental solutions. It has been shown that, at these concentrations, small differences in Na^+ and K^+ concentrations do not significantly influence the emission at 556 micra (37). The use of PO_4 as the predominant anion was found to be an unnecessary precaution at such ionic levels, for the same calibration curve is obtained with chloride instead of phosphate.

V. *Cell Surface Area Estimation*

Cell surface area per gram of atrium was estimated by measuring the diameters of twenty-five cells in four sections from each of three atria fixed in 10 per cent formalin. The average diameter was 25 μ. The result of the appropriate calculation, after the allotment of 10 per cent of the total cell volume of the atrium for connective tissue, is a value of 1000 cm^2 surface area per gram of atrium. This is about twice the figure available for atria from other mammals (34, 35) and will be employed for approximate estimates of flux.

RESULTS

I. *Desaturation Studies*

GENERAL When the time course of the loss of Ca^{45} from a guinea pig atrium, previously loaded with the isotope, is reconstructed from the activity of washout samples and plotted on semilog coordinates, it becomes apparent that after an initial period of rapid loss the rate of emergence of the isotope becomes much slower and approaches that of a first-order process; *i.e.*, after about 60 minutes the slope of the curve becomes essentially constant (Fig. 2).

If the linear portion of the desaturation curve is extrapolated to zero time and subtracted from the original curve, a second curve with a much greater and varying negative slope remains. Thus, the desaturation curve can be approximated as the sum of at least two separate functions, a slower one with

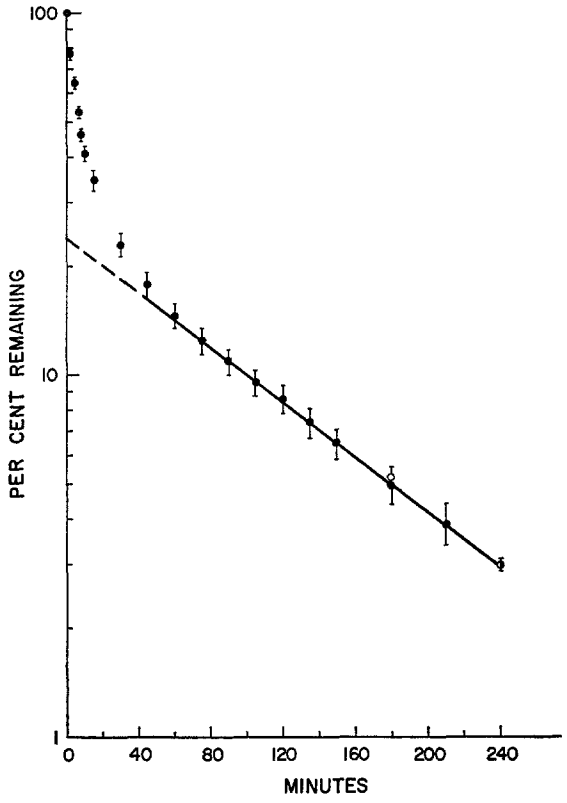

FIGURE 2. The average desaturation curve for atria with $t_{1/2}$ = 86 minutes. The means of thirteen experiments are plotted with variabilities designated by ± 1 standard error of the mean. The two open circles (at 180 and 240 minutes) represent the average residual Ca^{45} space of the undamaged portion of ten atria soaked for identical periods in isotopic solution and washed out for either 3 or 4 hours. The mean of five of the atria washed out for 4 hours has been arbitrarily equated to the 4 hour residual activity of the entire tissue and the mean of the five atria washed out for 3 hours expressed relative to the 4 hour value.

first-order kinetics (*i.e.*, an exponential decline), and a second, and possibly more complex one, that may be the resultant of several processes. The former will be referred to as the slow component and the latter as the fast component.

In earlier experiments performed during the winter of 1959–60, the half-time of exchange ($t_{1/2}$) of the slow component was 168 ± 10 minutes. In all subsequent experiments, performed from the early summer of 1960, a value

TABLE I

THE RATE OF EXCHANGE AND SIZE OF THE SLOW COMPONENT

A	B		C	D	E	F
	Stimulation period in Ca^{45}					
Rest period in Ca^{45}	Duration	Rate	$t_{1/2}$	Duration washout	Space at end of soak	Corrected space*
hrs.	*min.*	*min.*$^{-1}$	*min.*	*min.*	*ml/gm*	*ml/gm*
Group A						
3	0	0	210	240		
3	0	0	125	240		
3	0	0	155	240		
3	0	0	140	240		
3	0	0	180	240		
12	0	0	205	240		
12	0	0	170	240		
1.5	0	0	150	300		
1.5	0	0	180	300		
Average			168±10			
Group B						
3	0	0	100	240	0.018	0.14
3	0	0	100	210	0.016	0.09
3	0	0	85	240		
3	0	0	80	240		
10	0	0	110	230	0.010	0.04
10	0	0	92	260	0.014	0.10
10	0	0	72	240	0.014	0.14
10	5	30	110	246	0.013	0.06
10	5	30	70	240	0.011	0.12
0	120 10	15 30	68	246		
0	120 10	15 30	80	258	0.012	0.11
0.08	10	30	85	263		
0.08	10	30	65	246		
Average			86±4			0.10±0.01

Variability is given as ±1 standard error of the mean.

* Corrected by use of $t_{1/2}$ for loss during washout and incompleteness of exchange during Ca^{45} soak.

of 86 ± 4 minutes has been found (Table I, column C). No explanation can be offered for this difference between the group A and group B preparations shown in Table I. These values were the same whether the muscle was at rest, contracting part of the time, or contracting continually during the soak in Ca^{45} solution. The half-time of the fast component ($T_{1/2}$) was 4.5 ± 0.2 minutes in both groups of muscles.

Since the washout studies were of necessity done on tissues containing damaged cells (the cut edges), it was important to be sure that the rate of Ca^{45} loss from the entire tissue during the linear portion of the desaturation curve is an accurate measure of the rate of loss of the isotope from the undamaged portion. To ascertain this, a comparison was made of the residual Ca^{45} in the undamaged part of atria washed out for 3 hours and of another set of atria washed out for 4 hours after identical exposures to Ca^{45} (shown as open circles in Fig. 2). These times were selected because, after 3 hours of

TABLE II

CA^{45} UPTAKE AT REST AND DURING ACTIVITY IN ATRIA WITH SLOW COMPONENT $T_{1/2}$ OF 86 MINUTES

Row	Rest period in Ca^{45}	Stimulation period in Ca^{45}		Duration washout	Total Ca^{++}	Ca^{45} space		Specific activity	No. of experiments
		Duration	Rate						
	hrs.	*min.*	*min.*$^{-1}$	*min.*	*μmol/gm*	*ml/gm*	*μmol/gm*		
A	1	0	0	0	1.65±0.09	0.38±0.027	0.95±0.68	0.59±0.38	6
B	1	0	0	15	1.72±0.10	0.06±0.009	0.15±0.023		6
C	10	0	0	0	1.78±0.12	0.50±0.03	1.25±0.08	0.70±0.05	4
Average for unstimulated atria:					1.69±0.06				16
D	0	50 10	15 30	0	1.59±0.04	0.48±0.05	1.20±0.13	0.77±0.10	5
E	0	50 10	15 30	15	1.61±0.14	0.23±0.04	0.58±0.09		6
F	9	50 10	15 30	0	1.48±0.22	0.54±0.04	1.35±0.09	0.95±0.07	4

Variability is given as ±1 standard error of the mean.

washout, the decline of tissue Ca^{45} is exponential and more likely to reflect the loss of the isotope from cellular elements. These values fall on the desaturation curve based on washout of the entire tissue, as would be expected if the Ca^{45} in the undamaged tissue and in the whole tissue were exchanging at the same rate.

COMPARTMENT SIZE AND IDENTIFICATION If the slow exchange of Ca^{45} occurs with a $t_{1/2}$ of 86 minutes, then exposure of the tissue to Ca^{45} K-H solution for 10 hours should result in essentially complete exchange of the isotope by the tissue. However, the average of the specific activities of several atria exposed to radioactive solution for 10 hours at rest and then analyzed without washout was 0.70 ± 0.05 (Table II, row C). This indicated that a substantial portion of the total tissue calcium, approximately 30 per cent or 0.5 μmol/gm, was inexchangeable at rest over the duration of the experiment. Stimulation during the last hour of a 10 hour soak in Ca^{45} did not significantly increase

the uptake of the isotope, although the specific activities found were higher (Table II, row C *vs.* F). The latter appears to be at least partly the result of net loss, without exchange, of Ca^{++} from the inexchangeable component. While the variability of the total Ca^{++} content does not permit an assured conclusion, these findings do dictate caution in accepting increases in specific activity as necessary indications of increased exchange.

Thus, from kinetic studies, the calcium of the atrium is divisible into three components: (*a*) a rapidly exchanging fraction, $T_{1/2}$ = 4.5 minutes; (*b*) a slowly exchanging fraction, $t_{1/2}$ = 86 minutes (or 168 minutes in early experiments); and (*c*) an inexchangeable fraction.

The presence of damaged tissue, with its increased calcium-binding capacity, prevents an accurate estimate of the size of the slow component from the zero intercept of the linear portion of the total desaturation curve. The slow component, free from the effects of the cut edges, was therefore estimated in the following way. Atria were soaked in Ca^{45} solution for a long enough time to saturate or almost saturate the tissue and then washed out for 210 to 260 minutes. The residual Ca^{45}, measured after the removal of the cut edges, was extrapolated to zero time and corrected for incompleteness of exchange during the soak in radioactive solution by use of the $t_{1/2}$ value. The results are shown in Table I, columns E and F; the average corrected size of the slow component space is 0.1 ml/gm or 0.25 μmol/gm. The rest of the exchangeable calcium, obtained by subtraction from the total (row C, Table II), is 0.4 ml/gm or 1.0 μmol/gm. This, by inference, composes the rapid component that exchanges with an average $T_{1/2}$ of 4.5 minutes.

The average sucrose space of the atria in six experiments was 0.25 ± 0.01 ml/gm. The washout curve of sucrose was studied in six additional experiments and found to be the sum of two exponentials with half-times of 2.9 ± 0.1 and 54 ± 8 minutes. The faster component included approximately 80 per cent of the total tissue sucrose. The time required for washout of half of the total tissue sucrose was 3.7 ± 0.1 minutes. These data indicate that at least 0.25 ml/gm of the fast component represents calcium in the extracellular water and that the remaining 0.15 ml/gm represents a calcium pool in series or in parallel with the extracellular water.

Four desaturation experiments performed on guinea pig Achilles tendons also demonstrated two components of Ca^{45} loss. These were 0.80 ml/gm and 0.30 ml/gm, with half-time values of approximately 10 minutes and 150 minutes respectively. Chemical assay of the collagen content of the tendon and of atrium by analyses of hydroxyproline (13) showed the tendon collagen content to be 98 per cent of the dry weight and twenty times more concentrated than in the atrium. If the tendon is a suitable model for atrial connective tissue, then 0.04 ml/gm of the fast component and 0.02 ml/gm of the slow component of the atrium are due to connective tissue.

A summary of the distribution of atrial calcium inferred from these data is given in Table III. The slowly exchanging muscle fraction is assumed to be intracellular.

RESTING EFFLUX From the size and the time constant for exchange of the slow component—assuming that the slow component is intracellular calcium and neglecting the possible role of connective tissue in the slow exchange of Ca^{45}—the resting outflux can be estimated to be 2.2×10^{-9} mol/gm-minute or 0.037 $\mu\mu$mol/cm^2-second for muscles with $t_{1/2}$ of 86 minutes. Correction for connective tissue reduces the estimated efflux to

TABLE III
CALCIUM DISTRIBUTION IN GUINEA PIG ATRIUM

Component	Region	Space	Ca^{++}
		ml/gm	*μmol/gm*
Inexchangeable calcium		0.18	0.44
Rapidly exchangeable calcium	Extracellular water	0.25	0.63
	Muscle cell and/or damaged cells	0.11	0.28
	Connective tissue	0.04	0.10
Slowly exchangeable calcium	Muscle cell	0.08	0.20
	Connective tissue	0.02	0.05
Total		0.68	1.70

0.030 $\mu\mu$mol/cm^2-second. Since data on the size of the space of the slow component in the muscles with a $t_{1/2}$ of 168 minutes were incomplete, no estimate of resting outflux for these muscles can be made.

It may be inferred from $T_{1/2}$ of 4.5 minutes, that the extracellular space exchanges with a half-time of no more than 4.5 minutes (see Discussion) compared to the 86 and 168 minutes of the intracellular space. Therefore, the delay in emptying of the extracellular space causes an error of no more than 10 per cent in the extrapolation to zero time used to estimate the outflux (14, 15, 31).

II. *Uptake Studies*

UPTAKE AT REST The results of Ca^{45} uptake experiments in resting atria are shown in Table IV. Atria of both groups exposed to 2.5 mM Ca^{45} K-H solution for 15 minutes and then washed out for 3 hours contained an amount of Ca^{45} per gram wet weight of tissue equal to the Ca^{45} content of 0.0025 ± 0.0001 ml of the radioactive solution. This figure, then, is the residual Ca^{45} space. As a first approximation, the $t_{1/2}$ of 168 and 86 minutes

gives correction factors of 2.1 and 4.2 for converting the Ca^{45} content of the fibers at 3 hours to that at the beginning of the washout. This corresponds to intracellular Ca^{45} spaces, after 15 minutes of Ca^{45} exposure, of 0.0053 ± 0.0002 and 0.0105 ± 0.004 ml/gm tissue and is equivalent to an average influx of 0.013 ± 0.001 and 0.029 ± 0.001 $\mu\mu$mol/cm²-second. This value does not include any correction for connective tissue.

In view of the approximations involved, reasonable agreement appears to exist between resting influx and outflux, suggesting that no net movement of

TABLE IV

RESTING INFLUX

Ca^{45} spaces, measured at the end of 3 hour washout, obtained with 15 minutes' exposure of guinea pig atria to Ca^{45} K-H solutions with different calcium concentrations.

External Ca^{++} concentration	Ca^{45} space after 3 hr. washout	Ca^{45} space corrected to t_o*	Influx
mM	*ml/gm*	*ml/gm*	*$\mu\mu$mol/cm² sec.*
	A. Atria with slow component $t_{1/2}$ of 168 min.		
2.50 (6)	0.0025±0.0001	0.0053±0.0002	0.013±0.001
	B. Atria with slow component $t_{1/2}$ of 86 min.		
1.25 (7)	0.0027±0.0005	0.0113±0.0021	0.016±0.003
2.50 to 1.38 (4)	0.0025±0.0004		
2.50 (10)	0.0025±0.0001	0.0105±0.0004	0.029±0.001
2.50 to 3.63 (1)	0.0025		
3.75 (7)	0.0027±0.0005	0.0113±0.0021	0.048±0.009
Total (29)	0.0026±0.0001		

The numbers in the parentheses in the first column represent the number of experiments performed. Variability is given as ±1 standard error of the mean.

* t_o = time at end of exposure to Ca^{45} solution (*i.e.*, just prior to the beginning of Ca^{45} washout).

calcium occurs at rest. This inference was confirmed by measuring total calcium content of muscles after 1 hour and after 10 hours of soaking in the bathing solution. The figures, given in rows A and C in Table II, show the absence of a significant difference.

When the external calcium concentration is 1.25 mM, the resting influx is 0.016 ± 0.003 $\mu\mu$mol/cm²-second; in 3.75 mM calcium K-H solution, resting influx is 0.048 ± 0.009 $\mu\mu$mol/cm²-second (Table IV). A plot of resting calcium influx against external calcium concentration (Fig. 3) is a straight line with the extrapolation passing near but not through the origin; however, the deviation from the origin is not statistically significant.

To test further the validity of assuming from three points that resting influx is a linear function of external calcium concentration, the following experiments were performed. Instead of equilibrating the tissue with 1.25 mM or 3.75 mM calcium before exposure to the isotopic solution, the tissue was placed

in 1.25 mM Ca^{45} K-H solution or 3.75 mM Ca^{45} K-H solution immediately following the 2 hour post-dissection equilibration in 2.5 mM calcium non-isotopic solution. The specific activities of these solutions were the same as those used in experiments with prior equilibration. During the 15 minute soak in the 1.25 mM Ca^{45} solution the extracellular calcium concentration was calculated (by use of $T_{1/2}$ = 4.5 minutes) to change from 2.50 mM to approximately 1.38 mM and, in 3.75 mM, from 2.50 mM to 3.63 mM. The uptake of the isotope (see Table IV) was not different in these experiments

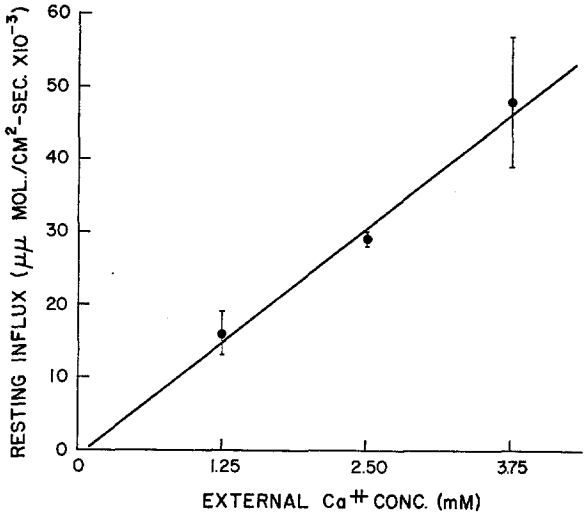

FIGURE 3. The dependence of resting calcium influx on external calcium concentration. Each point is the mean of at least seven experiments. Vertical bars indicate ±1 standard error of mean.

from those with prior equilibration to a constant extracellular calcium concentration. It can be inferred from these data that the influx is a simple linear function of calcium concentration over the range of concentrations studied. The standard error of the mean value for resting influx at 3.75 mM Ca^{++}, however, is so large that a deviation from a linear dependence of resting influx on extracellular concentration cannot be ruled out completely.

INFLUX DURING CONTRACTION One hour of stimulation may have increased the total Ca^{45} exchange of atria from an equivalent space of 0.38 ml/gm to 0.48 ml/gm (Table II, rows A and D). Comparison of the Ca^{45} remaining after a 15 minute washout in muscles which were at rest and in those which were active during a 1 hour soak in Ca^{45} (Table II, rows B and E) demonstrates a greater residual Ca^{45} in the previously active muscles even more strikingly.

It is apparent that, to quantitate the increased uptake of Ca^{45} per beat

more accurately, it is necessary to lengthen the washout period to completely remove the fast component. Moreover, it is necessary to shorten the time of exposure to the isotope to minimize the backflux, which would tend to limit the uptake of Ca^{45}. The uptake of muscles exposed to Ca^{45} for only 15 minutes and washed out in inactive solution for 3 hours is shown in Table V. Influx

TABLE V

THE COMPARISON OF CONTRACTION AND CALCIUM INFLUX AT DIFFERENT FREQUENCIES AND CALCIUM CONCENTRATIONS

Ca^{++} concentration	Rate	3 hr. space*	Influx/beat	Relative uptake per beat‡	Relative tension per beat‡
mM	*min.*$^{-1}$	*ml/gm*	*μμmol/cm²*	*per cent*	*per cent*
A. Atria with slow component $t_{1/2}$ of 168 min.					
2.50 (6)	6	0.0042±0.0005	0.15±0.043	27±9	38±5 (6)
2.50 (9)	15	0.0164±0.0038	0.49±0.13	89±24	84±7 (6)
2.50 (6)	30	0.0335±0.0076	0.55±0.14	100±25	100 (6)
2.50 (2)	30§	0.0047±0.0001	0.38±0.02	69±4	54±3 (6)
2.50 (3)	60	0.0551±0.0053	0.46±0.043	84±8	119±5 (6)‖ 112±5 (3)¶
B. Atria with slow component $t_{1/2}$ of 86 min.					
1.25 (5)	15	0.0063±0.0016	0.13±0.055	38±16	36±7 (6)
1.25 (3)	30	0.0137±0.0012	0.19±0.020	56±6	63±6 (6)
2.50 (5)	15	0.0069±0.0008	0.30±0.055	89±16	84±7 (6)
2.50 (6)	30	0.0124±0.0008	0.34±0.029	100±9	100 (6)
2.50 (3)	30§	0.0032±0.0002	0.21±0.070	62±21	54±3 (6)
3.75 (4)	6	0.0036±0.00036	0.26±0.009	76±26	63±4 (6)
3.75 (11)	15	0.0068±0.0004	0.44±0.042	129±12	106±2 (6)

Variability is given as ± one standard error of the mean.
Figures in parentheses in first and last columns indicate the number of experiments.
The relative tensions refer to the ratios of the steady state twitch tensions reached during the 10 minutes of stimulation. Correction for the submaximal twitch tension developed during the first 1 to 2 minutes of treppe does not significantly alter the relative values.
* Uncorrected for the uptake without stimulation given in corresponding rows in Table IV.
‡ Normalized relative to the value in 2.5 mM Ca^{++} with stimulation for 10 minutes at 30/minute.
§ For 1 minute of stimulation only. All other muscles stimulated for 10 minutes.
‖ 119 equals maximum tension achieved during 10 minutes of stimulation.
¶ 112 equals average tension per beat during 10 minutes of stimulation at 60/minute.

figures were obtained after correction for washout (by the usual extrapolation to zero time) and for resting uptake (*i.e.*, by subtraction of the influx of unstimulated controls).

It may be seen that in the series of experiments performed with group A atria contracting at a rate of 30/minute in 2.5 mM calcium, an average additional calcium influx of 0.55 ± 0.14 $\mu\mu$mol/cm² was associated with each beat. Data not tabulated showed there was no significant increase in Ca^{45}

uptake by the cut edges of the atrium; this part of the tissue could be seen not to contract when stimulated electrically.

Experiments were then selected to determine whether a consistent relationship exists between the strength of contraction and the Ca^{45} uptake during the contraction. Two fundamental properties of heart muscle, the increase in contractility associated with an increase in frequency of contraction and the increase in contractility produced by an increase in external calcium concentration, were employed for this purpose.

The results of these studies are recorded in Table V. *It should be emphasized that although the absolute uptake figures may include errors due to tissue geometry, it can be assumed that relative changes are free from these errors since tissue geometry was essentially the same in all atria used.* The tension and uptake of atria stimulated at 30/minute in 2.5 mM Ca^{++} have been arbitrarily assigned values of 100 per cent and the values under other conditions expressed relative to them.

In series A the muscles were stimulated in normal calcium concentration at a low frequency to produce weak beats and at several higher rates at which the twitch tension is large and further changes in tension with increased frequency are small. At all rates except 60/minute, the relative tension and relative Ca^{45} uptake increase proportionally. An increase in rate of 9/minute from 6 to 15/minute causes a large increase in tension and in uptake; an increase of 15/minute from 15 to 30/minute causes only a small increase in both tension and calcium uptake. The experiments performed at 60/minute must be considered separately because the atria were unable to maintain maximal tension for 10 minutes at this rate. In this case uptake per beat decreased with an increase in frequency, and a close correlation between relative tension and relative calcium uptake does not exist. The discrepancy remains even when a correction is made for the decrease in strength of the twitch that occurred during the 10 minutes of stimulation.

At higher rates of contraction, where the amount of Ca^{45} taken up by the muscle is large with respect to the size of the slow component space and appears to be approaching saturation, backflux may become significant and produce a lower apparent movement of Ca^{45} per contraction. This phenomenon would be particularly applicable to group A muscles stimulated at 30/minute and 60/minute. It is possible, therefore, that the figures given for uptake per beat at these rates underestimate the true calcium movement. Because of the smaller total uptake in the other 10 minute stimulation experiments, the error introduced into these studies by limited size of the slow component would be small. Moreover, the possibility exists that the size of the intracellular compartment has been underestimated (see Discussion) and that except at a frequency of 60/minute the effect of saturation on Ca^{45} uptake has not been large.

When a previously resting tissue is stimulated, twitch tension gradually

rises over a few minutes (the time varies inversely with the frequency of stimulation) and then reaches a "steady state." The average twitch tension during the first minute of activity at a frequency of 30/minute in 2.5 mM Ca^{++} is 54 ± 3 per cent of the maximum twitch tension at this frequency. Five experiments were performed to determine whether Ca^{45} uptake per beat is less during this early phase of the treppe, when the force of contraction is less, than during the steady state (Table V). In order to eliminate possible effects of difference in average specific activity of the extracellular space

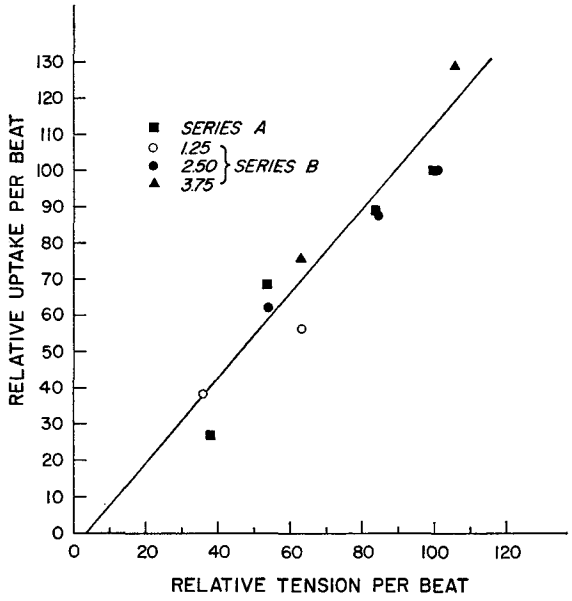

FIGURE 4. Correlation of calcium influx per beat with twitch tension of guinea pig atria. Data from Table V. The line through the points was determined by the method of least squares.

during 1 minute of activity *versus* 10 minutes, the normal procedure was altered somewhat. Instead of initiating contraction after 5 minutes in Ca^{45}, these five atria were stimulated for 1 minute during the 10th minute of a 15 minute soak and then washed out for the usual time. It was assumed that the specific activity of the extracellular space during the 10th minute approximated the average specific activity of the last 10 minutes of a 15 minute exposure. Again a correlation between uptake and twitch tension appears. In addition, the results argue against any large effect of improved mixing, resulting from mechanical activity *per se*, on Ca^{45} uptake into the slow component, for if this were occurring one would expect the first beats to be more efficacious in facilitating the movement than the later ones.

In group B of Table V calcium concentration and frequency were varied.

Concentrations above 3.75 mM were not used in order to avoid the decrease in activity coefficient of calcium which occurs at higher calcium concentrations in the presence of HCO_3^- and PO_4^{-3}. The relative Ca^{45} uptake per beat in the atria contracting in these three calcium concentrations parallels the relative twitch tensions at each calcium concentration.

In Fig. 4 relative Ca^{45} uptake per beat is plotted against relative twitch tension for the sixty experiments reported in Table V (results for atria stimulated at 60/minute are not included). The relation can be approximated by a straight line. It the line is extrapolated toward zero, it has a positive intercept on the tension axis, but the deviation from the origin is not statistically significant. This curve shows not only the correlation between calcium influx and tension, but implies the interchangeability of heart rates and calcium content of the medium as variables controlling flux and mechanical force.

III. *Efflux during Contraction*

In spite of an increased rate of calcium uptake during contraction, stimulation for as long as 1 hour, during which muscles contracted 1050 times, did not change the calcium content of the tissues (Table II, rows A and D, and B and E). This was true even though muscles stimulated for 1 hour and washed out for 15 minutes had an average Ca^{45} space 0.17 ml/gm or 0.43 μmol/gm larger than their resting controls. An equivalent increase in tissue calcium would have been too large to be obscured by the normal variability of the calcium content of the atria ($p = < 0.025$). The possibility has not been eliminated that the entire difference in residual Ca^{45} seen after 15 minutes of washout was not due to difference in uptake, but was the result in part of a transient decrease in the rate of exchange of previously rapidly exchanging calcium following stimulation. The observations of Henrotte *et al.* (21) in turtle ventricle (see Discussion), however, do not support this possibility. Therefore, during these periods of mechanical activity an increase in efflux equivalent to the increase in influx was almost certainly occurring, as observed in skeletal muscle by Shanes and Bianchi (36) with radiocalcium.

Nevertheless, two additional kinds of study failed to demonstrate this increase in the outflux of Ca^{45} during contraction; this failure appeared in atria which were stimulated after they had been washed out for 90 to 150 minutes following the exposure to isotopic solution to minimize interstitial Ca^{45}. The experiments were performed on muscles in which the initial Ca^{45} loading had been accelerated by stimulation during the soak in the radioactive solution as well as on tissues which were loaded at rest.

In the first study, collections were made every 2 minutes, and the tissues remained in the washout solutions during both rest and stimulation. A maximum increase of only 60 per cent in the rate of loss of Ca^{45} was observed

during a 10 minute period of stimulation at 30/minute. This increase, which appeared in the first 2 minute collection after the onset of activity, was not sustained despite continued activity, and by the fifth 2 minute collection the increment had fallen to 20 per cent above the resting value. If an acceleration of Ca^{45} loss during contraction equal to that of uptake had occurred (the possible influence of damaged tissue being neglected), a 400 per cent increase in Ca^{45} loss would have been seen. Furthermore, analogous studies of the

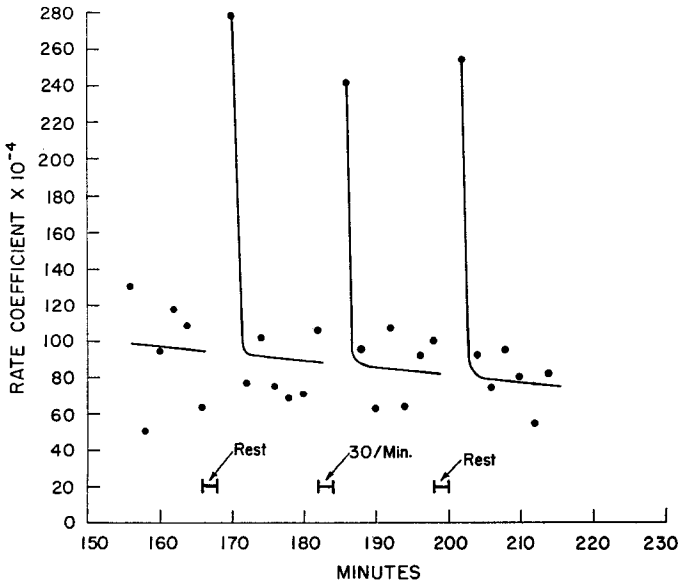

FIGURE 5. The time course of the rate coefficient for the loss of Ca^{45} from an atrium washed out for 150 minutes following Ca^{45} loading. The values are based on 2 minute washout collections. During the periods from 166 to 168, 182 to 184, and 198 to 200 minutes, the tissue was raised into a moist chamber. The atrium was at rest except for stimulation at 30/minute during the interval between 182 and 184 minutes. Ordinate is (per cent/minute) $\times 10^{-4}$.

effect of contraction on the rate of emergence of C^{14}-sucrose from atria showed a similar unsustained increase in the rate of loss of the tracer. It therefore appeared likely that the observed increase in Ca^{45} loss during contraction was due to the mechanical squeezing out of tracer from the intercellular space.

In a second series of studies, designed to minimize such mechanical effects, several baseline 2 minute collections were taken with the muscle at rest in non-radioactive K-H solution. Then the muscle was elevated from the solution into a moist chamber for a 2 minute period of stimulation at 30/minute and subsequently reimmersed at rest in K-H solution for additional 2 minute collections. For controls, the muscles were elevated into the chamber for

2 minutes without stimulation 16 minutes before and 14 minutes after each period of stimulation in the moist chamber; several 2 minute collections preceded and followed this elevation. The elevation into the moist chamber even at rest was expected to give an increased release of Ca^{45} during at least the subsequent collection since the latter would represent the amount of Ca^{45} obtained for a 4 minute period in comparison to the preceding 2 minute collections. Stimulation was across the two steel clips suspending the muscle.

The results from such an experiment are shown in Fig. 5. When the muscle was in the moist chamber during the period of stimulation and then reimmersed for a post-stimulation washout, no loss of the isotope in excess of that observed without stimulation was seen. In sixteen experiments on resting muscles and eleven on contracting muscles, the rate coefficients during the first collection after the return of the muscle to the solution averaged respectively 2.4 ± 0.3 and 2.3 ± 0.2 times the basal levels. This approximate doubling of the baseline value is consistent with a constant efflux of Ca^{45} collected over twice the period of time.

DISCUSSION

As in earlier calcium studies (39), the calcium of the guinea pig atrium has been divided into three different components by the kinetics of Ca^{45} washout: (*a*) rapidly exchangeable; (*b*) slowly exchangeable; and (*c*) non-exchangeable. In addition to calcium in the extracellular fluid and probably calcium bound to connective tissue, it is not unlikely that the rapidly exchanging fraction includes some calcium loosely bound to the cell surface. The half-time for washout of free calcium from the extracellular fluid, to be expected from multiplying the longest half-time for the sucrose washout (3.7 minutes) by the ratio of the diffusion coefficients of calcium and sucrose in water (equal to $1/(2.7)$) (25), is approximately 31 per cent of the actual half-time of the fast component of the Ca^{45} washout (4.5 minutes). Moreover, preliminary experiments show that this estimate is about 23 per cent of the half-time for the change in twitch tension when the calcium concentration in the bathing solution is changed within the range in which twitch tension and external calcium concentration are linearly related. The substantially longer half-time actually obtained for Ca^{45} suggests that an additional step besides diffusion into the extracellular space (*e.g.* binding to surface sites) is necessary for the action of the ion on the contractile process. Niedergerke has inferred this from studies on frog ventricular strips (5). The similarity of half-times of the Ca^{45} washout and of response of the heart to changes in calcium concentration argues against the possibility that the apparently long $T_{1/2}$ for Ca^{45} is primarily a function of the loss of isotope from cut edges.

I. *Fluxes during Contraction*

When the atria are stimulated electrically the Ca^{45} uptake is considerably increased. If the entire increment in the transfer of the isotope associated with contraction occurs during the action potential (duration at 30/minute was 150 msec.)[1]—evidence cited below supports this assumption—then the uptake during depolarization increases to as much as 250 times the resting value. Moreover, at several different levels of contractility produced by different extracellular calcium concentrations and different frequencies of stimulation, the magnitude of the increment in Ca^{45} uptake maintained an approximately constant relation to the strength of contraction.

These results are consistent with the findings of Bianchi and Shanes (11) in frog sartorius muscle. In their experiments the rate of calcium uptake by muscle fibers increased at least 100-fold during contraction. When 75 per cent of the Cl^- of the bathing solution was replaced by NO_3^-, twitch tension increased about 60 per cent and calcium uptake per twitch increased by an approximately equal amount.

In three different types of muscle, depolarization has now been shown to be accompanied by an increase in calcium transfer. In the frog rectus abdominis (24) and sartorius (11) muscles a temporal correlation exists between potassium contracture and the increment in Ca^{45} uptake. Moreover, in the frog sartorius, and now even more strikingly in the guinea pig atrium, a quantitative correlation has been demonstrated between the magnitude of the increase in Ca^{45} movement and the strength of the contraction. There is evidence too that in smooth muscle calcium entry is correlated with contraction in the absence of a change in polarization (40). The data are therefore consistent with the hypothesis that calcium movement into the cell couples excitation with contraction. In addition, they suggest that such a link is an important factor in determining the amount of tension developed.

The apparent lack of an increased rate of loss of Ca^{45} from the heart during contraction has also been noted by Niedergerke (5) and Sekul and Holland (18). Our total calcium measurements, however, demonstrate that no change in tissue calcium content is associated with activity. The two observations may therefore indicate that an increased rate of loss of Ca^{++} does occur during contraction, but that the source of the additional Ca^{++} is a cellular pool unlabeled at the time of the mechanical activity. Such a pool might either not have been labeled with the isotope during the prior exposure to Ca^{45} or have lost its tracer during washout of the extracellular space prior to stimulation. If the first possibility obtains, then two separate intracellular pools of

[1] The authors wish to express their indebtedness to Dr. Walter Freygang for measuring the duration of the action potential.

calcium are involved in the movements associated with contraction. A consequence of this hypothesis would be the absence of any saturation of the cellular pool receiving Ca^{45} at an accelerated rate during contraction. The insignificant increase in uptake in muscles stimulated for the last hour of a 10 hour soak over those at rest for the entire 10 hours (Table II) clearly shows that saturation does occur. More likely, then, is the explanation in terms of the loss of the tracer from a critical region during the washout of the extracellular space prior to stimulation. Such a decline in specific activity is possible if diffusion of calcium within the cell, by virtue of its bound state (41, 42), is one of the rate-limiting steps in the outflux of calcium. Under these conditions Ca^{++} in the cortical regions of the cell would diffuse out faster than calcium closer to the center of the cell. This would result in a specific activity in the cell cortex much lower than in the center after 90 minutes of washout. The fact that after a 15 minute washout the difference in residual Ca^{45} of muscles that were at rest and those which were beating during the exposure to the isotope is greater than the estimated size of the slow component is consistent with the possibility that part of the intracellular calcium involved in contraction exchanges more rapidly than the kinetics of the slow component would predict on the basis of a membrane-limited diffusion process. In a diffusion model in which muscle fibers behave like homogeneous cylinders without a specialized diffusion barrier at their surface, one would expect the initial intracellular Ca^{45} content of a cell saturated with Ca^{45} to be 43 per cent greater than that estimated from extrapolation of the slow component to zero time (31). It is possible that the atrial cell combines some of the features of each of these models, that is, diffusion limitation from intracellular as well as membrane processes.

That no net change in total cell calcium occurs during a contractile cycle does not eliminate the possibility that accelerated calcium influx is crucial in normal contraction. The influx and outflux may be separated in time or in space or may involve calcium in different forms. A transient increase in intracellular calcium in a specific location or in a specific form may still be occurring during contraction.

II. *Source of Calcium for Influx*

Henrotte *et al.* (21) were able to show an increase in Ca^{45} exchange during contraction of turtle ventricles which displayed a Bowditch staircase, but not in those which failed to show the staircase phenomenon. The increment in calcium exchange seen by these workers involves a rapidly exchanging fraction of the tissue and may represent an increase in the amount of superficial, loosely bound calcium. If this is an accurate interpretation, then in turtle ventricles the strength of the contraction can be correlated with the amount of loosely

bound surface calcium. Niedergerke and Harris (6) have shown that changes in the ionic composition of bathing solutions which produce an increase in contractility are associated with an increase in the amount of rapidly exchangeable calcium bound by the tissue. It is not unlikely, then, that at least one source of calcium entering the cell during the contraction is calcium interacting with the cell surface. Bianchi and Shanes concluded that this was true for the frog sartorius. In experiments in NO_3^--Ringer, they noted that twitch tension and calcium uptake per twitch are augmented (11) and that the degree of binding of superficial calcium which is normally easily exchanged appears to be increased (22).

Weidmann (23) has shown that an increase in extracellular calcium concentration during the action potential increases the maximum tension developed during the accompanying contraction. It would appear from this that free calcium in the extracellular space is also a source of calcium entering the cell during the contraction. However, the work of Lüttgau and Niedergerke (7) makes it probable that the calcium of the extracellular space participates in the contraction by first interacting with the membrane, even when the latter is in a depolarized state. It becomes tempting to propose, then, that the calcium which enters the cell during a contraction enters during the action potential but first interacts with membrane sites.

III. *Intracellular Effects of Increased Influx*

Possible relations of calcium influx to contraction become apparent from certain quantitative considerations. Thus, if the molecular weight of myosin is 250,000 (43) and its concentration in heart muscle equals that in skeletal muscle (taken to be 7.6 per cent of wet weight) (26), then each gram of heart contains 3×10^{-4} mmoles of the protein. The maximum increment in calcium influx per beat measured in these experiments was 0.6×10^{-6} mmoles per gram. An underestimation due to possibly more rapid loss of Ca^{45} from the cortical layers of the cell will for convenience be assumed small. For a simple homogeneous cylinder the deficit would be about 40 per cent. The ratio of the number of calcium ions entering the cell during contraction to the number of myosin molecules already present is therefore of the order of 1/500. A similar ratio results if the calcium influx is compared with the concentration of actin and of tropomyosin (again assuming the same concentration in heart muscle as in skeletal muscle). A similar calculation from the results of Bianchi and Shanes (11) on twitches reveals an even smaller ratio of calcium to myosin, but here allowance would have to be made for the twitch to tetanus tension ratio. It is unlikely that only 0.2 per cent of the contractile protein is shortening in a near maximal contraction of the isolated atrium. Therefore, if calcium does initiate the contraction, each ion must ultimately

have an effect on many contractile units. One of the reactions in the contractile process, though not necessarily the one involving calcium, must involve either a chain reaction or the interaction of one molecule or ion with as many as 500 molecules or units. The latter could occur by an enzymatic reaction or by a specific type of molecular alignment in which one molecule is in close association with many other molecules.

In respect to this quantitative relationship between calcium taken up during contraction and the amount of actomyosin, it is interesting to note the calculation of H. E. Huxley (26) that each thick filament in skeletal muscle contains about 400 myosin molecules. If a similar condition exists in the heart, the available data indicate that the ratio of calcium ions taken up in a near maximal contraction to the number of thick filaments is almost 1:1.

If the calcium that enters the cell with contraction were to achieve immediate uniform distribution in the cell water, the concentration would be 1.2×10^{-6} M. Any non-uniform distribution, which appears more likely, would produce regions within the cell of higher "calcium concentration." This level of ionized calcium has been found to inhibit relaxing factor activity *in vitro* (28). Ebashi has further shown that the relaxing factor system secondarily binds calcium tightly and that procedures which decrease this binding of calcium proportionally decrease relaxation activity. He has demonstrated that numerous calcium-chelating agents have relaxation activity proportionate to their ability to chelate calcium (27). It is possible, therefore, that the calcium that enters the cell during excitation inhibits the relaxing system and thereby initiates contraction; the return to the relaxed state could then be produced by a subsequent binding of this calcium by other sites in the relaxing system or by other components within the cell.

The authors wish to thank Dr. E. Muscholl and Dr. H. Lüllmann of the Department of Pharmacology, Mainz University, for calling attention to the viability and quiescence of the guinea pig atrial preparation and to express their appreciation to Dr. D. Prokop for doing the hydroxyproline determinations and to Dr. E. Sonnenblick for the use of his laboratory to make the isotonic measurements.

Part of this research was done during Dr. Winegrad's tenure of a Post-doctoral Research Fellowship of the Public Health Service. Part of the expense in preparation of the manuscript was met by Public Health Service grant B3321.

Received for publication, May 2, 1961.

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