Effects of Drugs and Stimulation on ^{45}Ca Movements in Frog Ventricle (36790)

J. F. DELAHAYES AND E. BOZLER
(Introduced by Dr. R. C. Little)
Department of Physiology, The Ohio State University, Columbus, Ohio 43210

Control of the strength of contraction of cardiac muscle can be explained by assuming changes in calcium influx but evidence for such an assumption is indirect. There is general agreement that beating of the heart increases Ca exchange, but the magnitude of this exchange is uncertain (15, 19, 24, 25, 27), and no simple relation has been found between Ca uptake and strength of contraction in conditions such as the staircase effect and paired stimulation (15, 30). Conflicting results have also been reported on the action of epinephrine on Ca movements: several authors found that the drug increases exchanges (14, 24, 28) while others have reported the opposite result (2, 19).

A difficulty in studies on Ca uptake by active muscles is that contraction increases the mixing of the extracellular space with the medium (6, 33) and thereby influences exchanges with the cells. The method described below insures rapid mixing between the interstitial fluid and the medium at all times and permits continuous determination of cellular ^{45}Ca uptake and mechanical activity. We studied the effect of electrical stimulation and autonomic drugs on Ca movement and tested whether the effects of the drugs on the contracture produced in Ca-rich solutions are due to changes in permeability to Ca (4, 5). A preliminary report on some of the results has been published (8).

Methods. The heart of the bull frog (*Rana catesbiena*) was used. After cannulation, the preparation was washed thoroughly with Ringer solution for at least 1 hr to remove all traces of blood and plasma protein. For continuous determination of ^{45}Ca uptake, the heart was then perfused in a chamber as illustrated in Fig. 1. A small volume of solution (2 ml for a heart weighing 500 mg) was recirculated with a peristaltic pump at a rate of 6.5 ml/min. Cellular uptake of ^{45}Ca was calculated from the change in the radioactivity of the perfusion fluid, which was determined at frequent intervals with a precision of 0.1%. The same principle has been used by Niedergerke and Page (26), but our method differs from theirs in two important respects: (i) The ventricle was enclosed in a chamber and was expanded briefly 48 times/min by a pump which lowered the pressure around the preparation. In this way the whole surface of the endocardium was exposed to the perfusion fluid. (ii) Not only the inside, but also the outside of the preparation was perfused. This was found to be necessary because of a slow transudation across the ventricular wall. To eliminate pressure differences a tube was provided to connect the two perfusion systems.

Rapid exchange between the extracellular fluid and the medium is particularly important because without rhythmic movements of the heart, mixing of extracellular fluids is delayed by diffusion through the whole thickness of the ventricle and through the connection between the ventricular cavity and the reservoir. Initiation or increase in rhythmic activity then can change the composition of the perfusion fluid rapidly by improved mixing. The frog ventricle is favorable for the studies reported here because no part of the myocardium is more than 25 μ from the inner surface of the myocardium (34), but this advantage is utilized only by providing for rhythmic movements.

Except in early experiments, mechanical activity was measured with a Grass FT 03 transducer attaching to the apex of the ventricle by a rod. For electrical stimulation, two platinum electrodes, one in the cannula and

the other in the chamber were used. Stimulation at a frequency of 24 shocks/min, was synchronized with the rhythmic expansion of the heart so that electrically induced contractions occurred just after ventricular expansion. To prevent spontaneous activity in both Ringer and high Ca solution, tetrodotoxin (TTX, 10^{-7} g/ml) was added to the perfusion fluid. The use of this drug is justified because it has been shown that it does not basically change the effect of epinephrine (1, 27). Experiments in which expansion induced activity without electric stimulation in spite of the presence of TTX, were discarded.

At regular intervals, usually 5 min, 200 μl samples were taken and the volume was immediately replaced with fresh solution. The two Hamilton syringes used for this purpose delivered with an accuracy of $\pm$ 0.1%. The samples were added to 10 ml of a scintillation fluid described by Kennedy (18) and counted in a liquid scintillation counter. Each sample was counted for a total of about 10^7 counts. Quenching was checked by a dual channel method. Addition of drugs did not alter counting efficiency. Cellular Ca uptake was calculated from the equation [Niedergerke and Page (26)]:

$$^{45}\text{Ca uptake} = V_s\,(C_0 - C_n) + V_r\,(C_{n-1} - C_n)$$

in which V_s is the volume of samples (200 μl), C_0 is the specific activity of the solution added to the perfusion fluid after each sample, C_n is the specific activity of sample n, C_{n-1} is the activity of sample $n-1$, and V_r is the residual volume of perfusion fluid after a sample has been removed.

To determine the total volume of perfusate ($V_{tot} = V_s + V_r$) the following procedure was used: After equilibration of the cannulated preparation in Tris–Ringer solution, the heart cavity was emptied by suction of the fluid followed by gentle internal blotting with a plunger of filter paper dipped through the cannula; the outside of the heart was also gently blotted. Then a known amount of solution (V_{add}) was added to the preparation mounted in the perfusion chamber with a high precision syringe so that the total volume was $V_{tot} = V_{add} + V_{heart}$, with V_{heart} being the volume of fluid left in the cannulated heart after blotting, extracellular space included. In 15 experiments, the last sample was replaced by an equal amount of solution containing the same concentration of ^{45}Ca plus a known concentration of sulfate ^{35}S or mannitol ^{14}C used as extracellular fluid marker. After an equilibration period of 15 min (7) three samples were taken for analysis, and the ventricle was untied from the cannula, cut open, blotted and weighed. The concentrations of ^{35}S or ^{14}C were determined mainly by dual channel analysis using a gain of the counter which allowed an optimal separation of the spectra of ^{35}S or ^{14}C from the ^{45}Ca spectrum. Control experiments showed a recovery of, respectively, 96.60 $\pm$ 2.20% (SE, $n = 8$) and 100.90 $\pm$ 0.85% (SE, $n = 10$) for ^{35}S and ^{14}C counts in mixtures containing ^{45}Ca. In some experiments the activities of ^{35}S or ^{14}C were obtained by simply subtracting the extrapolated ^{45}Ca counts from the total counts; both methods gave essentially the same results. Experimental counts were always compared to counts of standards in which the dual tagged solution was added to the ^{45}Ca solution in proportions chosen as close as possible to the expected experimental proportions. From the measured total volume V_{heart} was deduced and averaged, respectively, 45.1 ml/100 g wet tissue with sulfate (10 experiments) and 40.3 ml/100 g with mannitol (5 experiments). These values, however, were not statistically different and the overall average value of 43 $\pm$ 2.1 ml/100 g wet tissue (SE) was used in the evaluation of the total volume in later experiments.

The main source of error in the determination of ^{45}Ca uptake is the estimate of the total volume of perfusate; while the relative error on each point of a given uptake curve does not exceed 0.1% at the measured (or evaluated) total volume of fluid, the error in this volume (V_{tot}) affects every point of the curve in the same proportions. Assuming an error on V_{tot} of twice the SE on V_{heart}, the error on ^{45}Ca uptake as well as on the rate of uptake is $\pm$ 15%.

The cannula and the chamber, made of Lucite, were periodically treated with silicone to minimize Ca absorption on surfaces. This treatment appeared to be satisfactory since in a control experiment without heart preparation the recovery of ^{45}Ca counts added to the perfusion system averaged 99.99 ± 0.06%.

Ca-rich solutions contained 30 m*M* Ca. To make up for a dilution by the extracellular space, the perfusion solution was changed several times at the beginning an experiment. Perfusion with the ^{45}Ca solution was started 5 to 7 min after the first exposure of the muscle to high Ca solution, that is shortly after the peak of the Ca contracture.

The solution in which freshly dissected muscles were first brought, and which will be called Ringer solution, contained (m*M*): NaCl, 115; KCl, 2; $CaCl_2$, 1.5; Mg Cl_2, 0.5; tris(hydroxymethyl)aminomethane (Tris) chloride 2 (pH 7.2-7.3). Ca-rich solutions were made up by mixing this solution with modified Ringer solution in which all Na was replaced by 83 m*M* Ca.

Results. Ca uptake in Ringer solution. The initial rate and magnitude of ^{45}Ca uptake varied widely in different preparations. In some cases, the rate of uptake became rather constant after 35 min of perfusion, but usually the rate diminished continuously for 1 hr and no clear separation into two phases as described by Niedergerke, Page and Talbot (27) could be made. Cellular uptake of ^{45}Ca after 60 min of perfusion was 1.057 ± 0.064 mmole/kg wet tissue (SE, $n = 10$). The cellular uptake of ^{45}Ca is expressed per kilogram wet weight since only the total volume of perfusate and the weight of the preparations are known. However, assuming an extracellular space of 25% and a surface:volume ratio of 10^{-4} cm^{-1} (26), the rate of ^{45}Ca uptake at this time, as measured by the slope of the uptake curves, was 10.50 ± 1.50 × 10^{-15} $M/cm^2/sec$, 2.5 times higher than the value reported by Niedergerke, Page

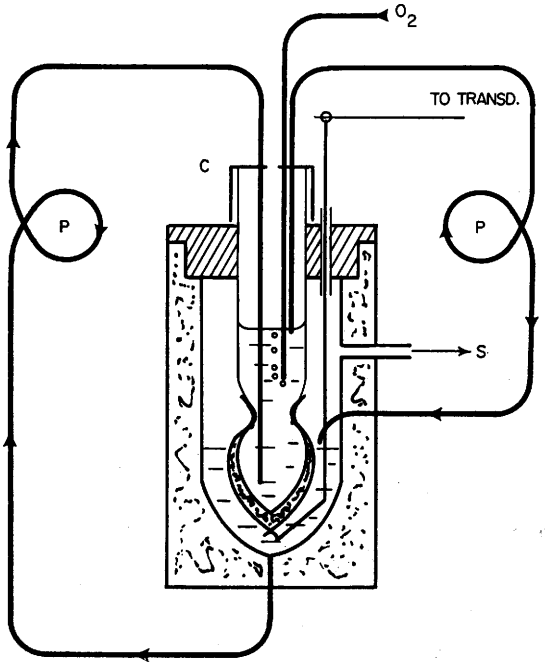

FIG. 1. Perfusion apparatus. Cannulated bull frog ventricle is mounted in a chamber to which suction (S) can be applied rhythmically. Perfusion fluid is circulated by means of a peristaltic pump (P) from inside the heart cavity into the outer chamber (right circuit) and from the outer chamber back into the heart cavity (left circuit). The apex of the preparation is connected to a force transducer (Transd.).

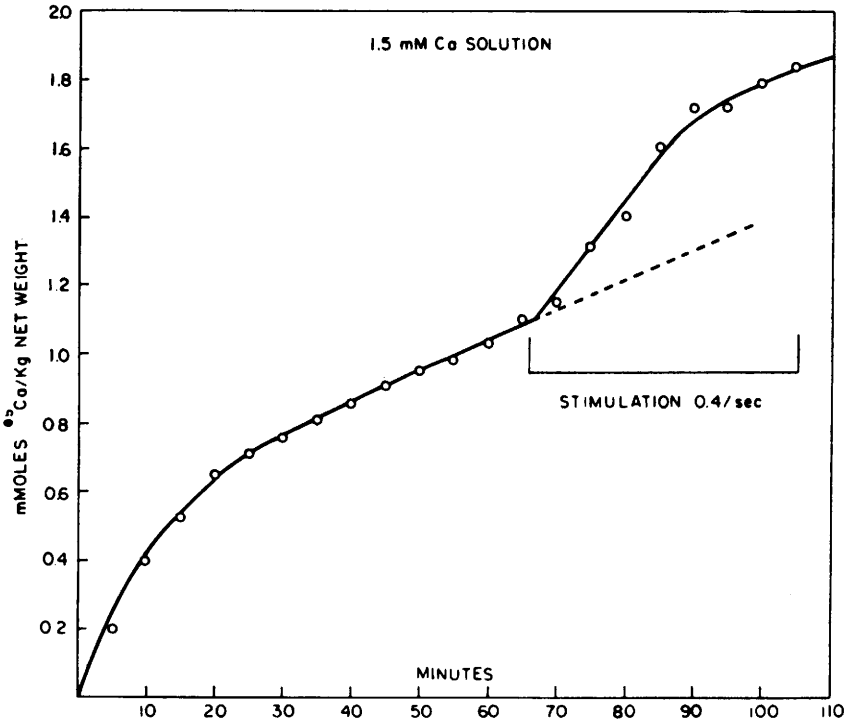

FIG. 2. Effect of electrical stimulation on ^{45}Ca uptake by a ventricle in Ringer solution (1.5 m*M* Ca). Extrapolation of ^{45}Ca uptake in absence of stimulation (- - -).

and Talbot (27). This difference may be due to a better mixing in our experiments. If we assume that the Ca total content in the ventricle is 1.90 ± 0.25 mmole/kg (SD) as recently published by one of us (3) then 50 to 60% of the cellular Ca was exchanged after 1 hr of perfusion, less if other values for frog ventricular Ca (35) are used.

Electrical stimulation was carried out after samples were replaced several times by a solution without TTX. As mentioned above, expansion of the heart, even in the presence of TTX, sometimes induced beating. The results of three experiments in which responses were produced only upon stimulation were as follows: During stimulation, the rate of ^{45}Ca uptake increased at first then leveled off (Fig. 2). In experiments of Niedergerke, Page and Talbot (27) the curve of ^{45}Ca uptake shows a much steeper and longer lasting rise than in our experiments; this difference may be due to a sudden improvment in mixing of the perfusion fluid in the beating heart, while our method provides for constant mixing. The magnitude of the extra uptake of ^{45}C due to activity was determined during the first 15 min of stimulation when the increase was maximum and was calculated from the difference between the actual uptake during activity and the extrapolated uptake at rest. Using the surface:volume ratio previously mentioned, values for the extra uptake were 110×10^{-15}, 143×10^{-15} and 148×10^{-15} M/cm^2 per beat. This raises total intracellular Ca on the average by 1.3×10^{-6} M/kg cell per beat.

Actions of drugs in Ringer solution. Addition of acetylcholine (5×10^{-6} M) to the perfusion fluid did not change the rate of ^{45}Ca uptake of the quiescent heart in 4 experiments. After application of epinephrine (5×10^{-6} M) however, ^{45}Ca uptake of the quiescent preparation increased at first, then returned to its original rate after 15 min. The extra uptake, determined as the difference between the actual uptake after 15 min of drug action and the extrapolation of the uptake before the application of the drug, aver-

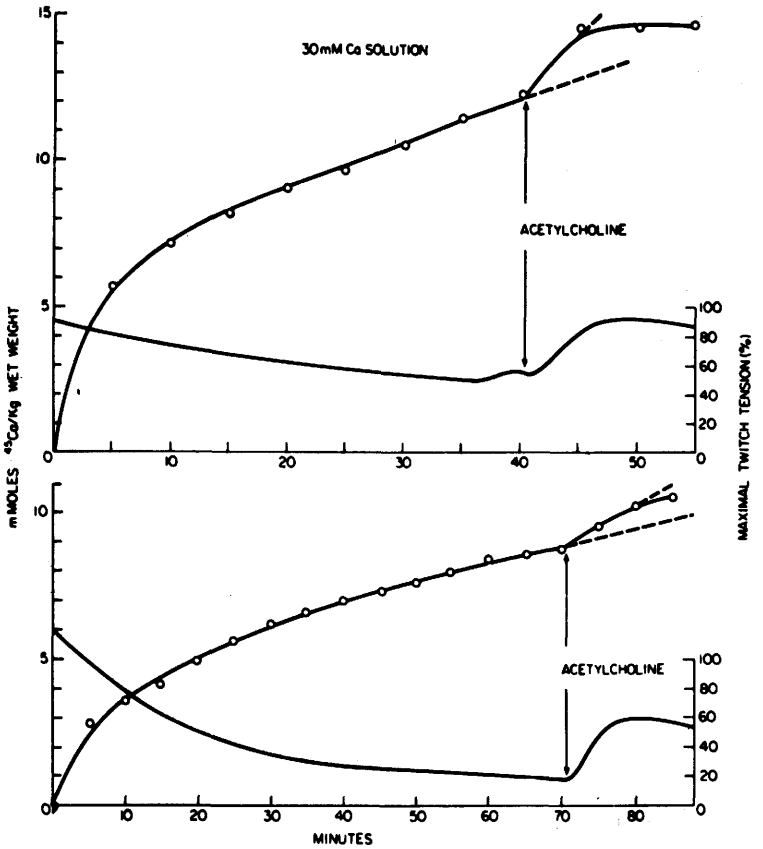

FIG. 3. Effect of acetylcholine 5×10^{-6} *M* on tension (lower curve) and ^{45}Ca uptake (upper curve) in 30 m*M* Ca solution. Muscle is partially relaxed in upper frame, almost completely relaxed in lower frame. Tension is expressed in percentage of the maximal twitch tension in Ringer solution determined before perfusion in Ca-rich solution. (- - -) the slopes of ^{45}Ca uptake before and during the action of the drug.

aged 10.6 ± 3.6% of the extrapolated uptake (SE, $n = 6$). Grossman and Furchgott (14) found only a small, statistically insignificant increase in ^{45}Ca uptake, but this may be caused by a lag due to diffusion into the muscle and the shortness of exposure, 5 min, to radioactive solution and drug.

Ca uptake in Ca-rich solution. This solution induced a contracture which reached a maximum in about 5 min, then diminished slowly and nearly disappeared in about 1 hr. Cellular uptake of ^{45}Ca after 45 min, that is prior to drug action (see below), averaged 10.0 ± 0.3 mmoles/kg wet weight (SE, $n = 17$), about 10 times the value in normal Ringer solution, excluding two extreme values of 5.0 and 17.0 mmoles/kg wet weight.

Action of drugs in high Ca solution. To determine the effect of acetylcholine and epinephrine on Ca uptake the drugs were applied after a loading period of 30 to 45 min (exceptionally later when acetylcholine was used). Acetylcholine increased the rate of ^{45}Ca uptake as measured by the slopes before and after drug application (Fig. 3) on the average by 94 ± 25% (SE, $n = 10$). The rate of uptake often reached a maximum, then dropped, so that after 25 min the total uptake of ^{45}Ca occasionally reached the same level as the extrapolated curve before the drug was applied (Fig. 3 upper curve). Acetylcholine also induced a contracture which reached a peak after 5 to 10 min and passed off again after 40 min.

Epinephrine always increased ^{45}Ca uptake (Fig. 4), on the average by 132 ± 32% (SE,

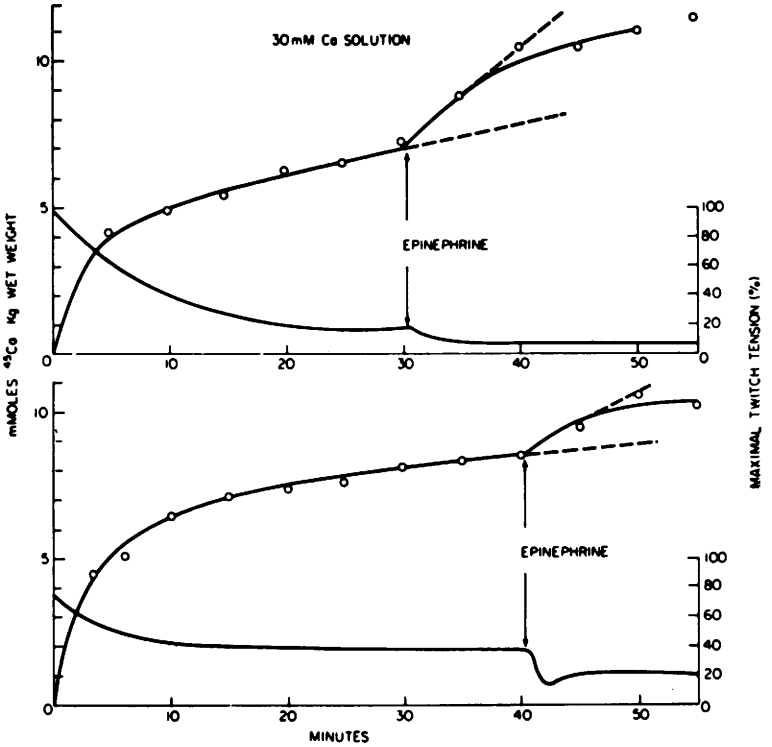

FIG. 4. Effect of epinephrine 5×10^{-6} *M* on tension (lower curve) and ^{45}Ca uptake (upper curve) in 30 m*M* Ca solution. The upper frame shows the effect of epinephrine on an almost completely relaxed muscle, the lower frame on a partially relaxed muscle. Tension is expressed as in Fig. 3.

$n = 10$). The increase was larger than that produced by acetylcholine and was generally greater when the initial ^{45}Ca uptake was low. After a period of 15 to 20 min, however, the total uptake reached nearly the same value in most preparations. This large increase in Ca uptake is surprising because the drug generally caused relaxation. The drop in tension was small (Fig. 4 lower curve) or absent when the drug was applied after the Ca contracture had nearly passed off, but ^{45}Ca uptake was increased also under these conditions. In one unusual case, ^{45}Ca uptake continued at a very high rate for more than 15 min; in this preparation the drug induced a contracture.

Discussion. 1. Ca exchange in Ringer solution. To determine the role of the extra Ca taken up during rhythmic activity, it is important to know the amount of Ca taken up per beat. Estimates of previous investigators are based on different methods of calculation and cannot be compared accurately. Niedergerke, Page and Talbot (27) calculated the uptake from the sudden change in the slopes of the uptake curves. As mentioned above, it seems likely that the observed change upon stimulation is partially due to a sudden increase in mixing of the extracellular fluid with the medium. In the experiments of Haacke, Lullmann and Van Zweiten (15), a single determination of Ca uptake was made after 30 min of stimulation. In our experiments the slope of the extra uptake of ^{45}Ca diminished slowly. This may be expected because the efflux of ^{45}Ca becomes an increasingly important factor during stimulation. Therefore, averages calculated from the net increase in ^{45}Ca during 30 min give too low an estimate of Ca influx.

In spite of the difficulty of calculating Ca uptake during activity, it appears that the

value is too low to produce a significant contractile effect. The uptake per beat in our experiments was 1.3×10^{-6} M/kg during the first 10 min. Several times smaller values were found by Haacke, Lullmann and Van Zweiten (15) using the heart of guinea pig. The threshold concentration for activation of the contractile mechanism is about 10^{-6} M Ca (10), but, because of the binding of Ca, the amount of Ca which must be released is much larger. Based on studies with actomyosin, full activation requires the release of 10^{-4} M Ca/kg muscle (16), somewhat more if a revised value for the constant used in the calculation is used (10). It must be assumed, therefore, that most of the Ca necessary for inducing twitches is derived from an internal store. That the frog myocardium, in spite of a poor development of the reticulum (34), contains an internal store of Ca mobilized during twitches has been shown previously very convincingly by the fact that the frog cardiac muscle contracts in a Ca-free solution if stimulated by a stimulus lasting at least 100 msec (21). The small amount of Ca probably taken up during the action potential may, however, have an important regulatory function.

The increase in ^{45}Ca uptake produced by epinephrine in resting preparations indicates that the drug induces a change in membrane P_{Ca}. A similar effect of epinephrine was found in Ca-rich solution and is discussed below.

2. *Ca exchange in Ca-rich solution.* In a Ca-rich solution, intracellular Ca uptake was increased several times, confirming results for the frog atrium by chemical analysis (3). This finding is significant because such a solution generally produces only a transient contracture, and sometimes none at all. It seems likely, therefore, that most of the Ca taken up is bound (3).

These experiments were carried out mainly to determine the mechanism of the relaxing action of epinephrine and the initiation of contractures by acetylcholine in Ca-rich solution. Because these effects are not associated with significant changes in membrane potential, it has been suggested that the drugs, respectively, decrease and increase Ca influx (5). Contrary to this hypothesis epinephrine produced an even greater rise in ^{45}Ca uptake than acetylcholine, as shown above. Therefore, another explanation, the possibility that the inhibitory effect is due to increased intracellular storage of Ca may be considered. This assumption has previously been made to explain other effects of epinephrine, the acceleration of relaxation during a twitch (17) and the diminution of the K-induced contracture (12, 13, 22). All these effects of epinephrine may be due to an increased synthesis of cyclic adenosine monophosphate (AMP) (21, 23, 24). In agreement with this assumption, two groups of investigators found that the substance mentioned increases the uptake of Ca by microsomal fractions (11, 31, 32), but no such effect was obtained in another investigation (9).

The rise in tension produced by acetylcholine may be caused by the rise in Ca uptake demonstrated in the experiments reported here. However, in analogy with the action of epinephrine, it is possible that the drug has an internal action on the storage of Ca opposite to that of epinephrine. This explanation is feasible in view of recent findings that acetylcholine depresses synthesis of cyclic AMP in the atrium of mammalian heart (20). If it is further assumed that the intracellular Ca store can be reached by electrical activity and thus raises the intracellular Ca^{2+} concentration, the relaxing action of epinephrine during Ca contracture and the opposite effect of acetylcholine can be harmonized with the inotropic effects on twitches under the same conditions (4, 5).

Summary. The relation between contractile activity and Ca movements was studied in the frog heart by a method which permitted the continuous determination of cellular ^{45}C uptake and of tension. In Ringer solution, stimulation increased ^{45}Ca uptake but the extra uptake per beat was too small to explain the initiation of the contraction by itself, indicating the presence of an intracellular Ca store. In Ca-rich solution (30 mM) acetylcholine increased ^{45}Ca uptake in quiescent preparations and at the same time induced or increased a contracture. Epineph-

rine also increased ^{45}Ca uptake under these conditions, but caused a rapid drop in tension. These results suggest that both drugs increase the permeability of the membrane for Ca (P_{Ca}); the inhibitory action of epinephrine may be due to increased intracellular storage of Ca.

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