Calcium Exchange in a Single Layer of Rat Cardiac Cells Studied by Direct Counting of Cellular Activity of Labeled Calcium

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ABSTRACT

Calcium exchange was studied in rat cardiac cells grown in tissue culture. A new technique was employed which depended on the growth of a single layer of cells on the surface of a slide composed of glass scintillator material. The cells remained actively contractile on the glass surface. The pattern of $^{45}$Ca uptake and washout from the preparation was defined and found to be reproducible. Low Na$^+$ perfusion rapidly augmented $^{45}$Ca uptake in the cultured cells. An increment in $^{45}$Ca uptake (65 μmoles Ca$^{2+}$/kg cells) occurred within 6 seconds of the start of low Na$^+$ perfusion.

ADDITIONAL KEY WORDS: cardiac tissue culture, glass scintillator, Ca$^{2+}$ exchange

A current problem in physiological research is the correlation and reconciliation of results obtained from functioning tissue with those obtained from fractionated cellular components. This problem certainly is found in the area of muscle physiology. Knowledge in the field of skeletal muscle physiology has been greatly advanced by the ability to study preparations of intact single cells, and such studies have provided information useful in bridging the gap between functional tissue and fractionated cell. Unfortunately, because of the much smaller size, viable preparations of single myocardial cells have not been achieved.

The present study describes a preparation of a single layer of contractile myocardial cells and a method for analysis of Ca$^{2+}$ exchange in these cells by direct, continuous counting of cellular radioactivity. The method was developed primarily to analyze ionic movements during a single contractile cycle. This goal is presently being pursued. However, since initial studies have proved the usefulness of the technique and since the method is applicable to a large range of study in cultured cells, it was thought appropriate to present this initial work. It describes certain basic characteristics of Ca$^{2+}$ exchange in the preparation.

Method

The basis of the method is the attachment and growth of cardiac cells from newborn rats on a slide composed of glass scintillator material. This places a single layer of contractile cells directly on
the radioisotopic detector. The glass scintillator slide with viable cells attached is contained within a specially designed flow cell, which is placed in the well of a scintillation spectrometer. Uptake and washout of radioisotope are continuously monitored.

The cells were prepared according to the method described by Harary and Farley (1). The glass scintillator slides were placed on silicone rubber supports elevated about 2 mm from the bottom of a plastic Petri dish. Heart cells derived from 4 or 5 rats were cultured for 24 hours, the glass slides turned over and heart cells from another 4 or 5 rats added to the other side. At 4 days a single cell layer covered between 50% and 90% of the slide surfaces. The cells were usually beating spontaneously and, when in contact, synchronously. Cellular protein from selected slides was determined according to the method of Lowry et al. (2).

The glass scintillator slides were made by Nuclear Enterprises, Ltd. (San Carlos, California) and were composed of their NE 901 glass. The slides were rectangular (50 X 20 X 1.5 mm) with two "legs" 4 mm long at one end as illustrated in Figure 1.

The cultures were maintained in full incubation medium until isotopic labeling and washout were commenced. The isotope studies were done with the cells bathed in a solution with the following millimolar composition: NaCl, 121; KCl, 3.6; CaCl\(_2\), 2H\(_2\)O, 1.0; NaHCO\(_3\), 12.0; Na\(_2\)HPO\(_4\).H\(_2\)O, 1.1; KH\(_2\)PO\(_4\), 0.6; MgCl\(_2\).6H\(_2\)O, 0.3; glucose, 16.0. All studies were done at 23 to 24°C and a pH of 7.3 to 7.4. The "low Na\(^+\)" solution used in some experiments was identical except for the substitution of 90 mM choline chloride for 90 mM NaCl. For isotopic labeling, the solution contained 20 \(\mu\)c/ml \(^{45}\)Ca (Nuclear Science and Engineering, Pittsburgh, Pa.).

To monitor isotopic activity of the cell layer during uptake or washout the slide was placed in the flow cell as illustrated in Figure 1. The flow cell chamber was constructed of Lucite plastic with polished inner and outer surfaces. The fluid capacity of the chamber with slide in place was 5.4 ml. The chamber was provided with intake and outlet orifices at either side at the top and oriented so that fluid entering the cell was directed against the slide in the center of the chamber to provide turbulence within the chamber and prevent unstirred areas as much as possible. The glass scintillator slide bisected the chamber longitudinally. Fluid entering the chamber was directed against the slide, down its surface, through the space between the legs of the slide, up the other surface of the slide and thence to the outlet. Flow during washout studies was maintained at a constant rate from a syringe driven by a Harvard infusion pump.

After the slide was inserted in the cell, the top of the cell, which had a soft rubber gasket, was screwed in place. The entire cell was then attached to the cylindrical plunger (Fig. 1) and was ready for insertion into the well of the scintillation spectrometer. O-rings on the plunger made contact with the walls of the spectrometer well to assure that no light entered the well when the cell was in place. A light coating of silicone grease was applied to the O-rings before insertion.

The tubing which conducted fluid to and from the cell was opaque, black vinyl and passed through the cylindrical plunger. The flow cell and scintillator slide. The slide is shown during insertion into the flow cell. Following insertion, the cap (with four screws) is tightened in place, the tubular fittings on the plunger are inserted in the flow cell tubes and the plunger is attached to metal strap-fittings on each side of the cell. The flow cell is then ready for insertion into the spectrometer well.
through the center of the handle. The time required for placement of the slide in the cell, closing the cell, attachment of the cell to the handle and insertion into the spectrometer well was approximately 1 minute. The cell layer was in contact with air during this period but there was no evidence that this brief exposure was deleterious to the preparation.

The spectrometer used was a Beckman Model LS200B. The minimum period for counting was 6 seconds. Print-out of count required 7.2 seconds. The maximum frequency at which counts could be recorded was therefore every 13.2 seconds in this study. (The spectrometer can, of course, be adapted for continuous recording of count rate. This method will be used in future studies.)

Counting Efficiency for $^{45}$Ca—Care must be taken to avoid exposure of the glass scintillator slide to ultraviolet light immediately before insertion in the well. Light of this wavelength excites the scintillator and will markedly increase the background during the initial stages of counting. Therefore, exposure to daylight and fluorescent lighting should be avoided; when it is, background is approximately 300 counts/min.

The counting efficiency of glass scintillator NE 901 for $^{45}$Ca placed on the surface of the glass was between 22% and 28% for the slides used in the present study. This is in contrast to the counting efficiency for $^{45}$Ca in solution in the chamber but not applied directly to the slide surface. This was approximately 3%. There is, therefore, marked quenching as $^{45}$Ca leaves the surface and diffuses into the fluid in the chamber, and thus extremely rapid turnover of flow in the chamber is not required when measuring $^{45}$Ca washout from myocardial cells attached to the slide surface.

The quenching effect of nonradioactive fluid in the chamber for activity on the slide surface was evaluated as follows. A measured volume of $^{45}$Ca solution was placed on the surface of the slide and dried. The slide was placed in the cell (without fluid) and the counting rate was measured. The dried isotope on the slide was then covered with transparent Scotch tape and recounted. The counting rate was not significantly changed. Lastly, the cell which contained the tape-covered slide was filled with water, and counting efficiency increased by 1.7%. This was a consistent finding and might be attributed to the fact that water is usually found to have a less quenching effect than air.

Results

Cellular Content of Slides.—The total protein content of slides on both sides of which cells had been cultured for 4 days averaged 1.14 mg for 4 slides (range 0.99 to

[FIGURE 2]

Light micrograph of cardiac cells as they appear on glass surface in tissue culture. Note fine strands connecting cells in many places. Central grid lines are 50μ apart.
1.37 mg). The protein content of fetal rat hearts is approximately 7.5 mg/100 mg wet tissue (M. Seraydarian, unpublished results). The total weight of cells adhering to the slides at the time of the isotope studies is therefore approximately 15 mg. It should be emphasized that this value is approximate and would be expected to vary from slide to slide, depending on the density of growth.

**Structure of Cells on the Slide.**—The cells are oriented on the surface of the slide as shown in Figure 2. When the cells are in contact or connected by fine strands, they tend to beat in unison. Though there are areas where the cells are in double layers, most of the surface is only one cell thick. Cross section indicates that the perinuclear region is 10 to 16 μ thick. The thickness diminishes toward the periphery to as little as 0.04 μ at the termination of the cellular extensions. This amounts to little more than apposed membranes.

**45Ca Washout.**—It was first necessary to determine the flow rate which was not limiting for wash of the surface of the slide. Flow rates above 24 ml/min through the cell did not further increase the 45Ca washout rate. Therefore, a flow of 24 ml/min was used for all washout studies. The characteristics of 45Ca washout from a slide without attached cells (blank) is illustrated in Figure 3. There is nonspecific binding of 45Ca to the glass, and the washout curve can be resolved into two exponential phases (3): phase 1 with a rate constant (λ) of 2.3 min⁻¹ and phase 2 with a rate constant of 0.024 min⁻¹. This pattern, with an approximate hundred-fold difference in rate between the phases, was demonstrable whether the slide was labeled for 30 minutes or for 4 hours.

The contribution of cells on the slide to the washout pattern is shown in Figure 4. The slide, with cells attached, was placed in standard solution containing 45Ca for 30

![Figure 3](http://circres.ahajournals.org/)

**FIGURE 3**

45Ca washout (plotted semilogarithmically) from cell-free scintillator slide. Washout curve (solid circles) is resolved into two exponential components with rate constants (λ) of 2.3 and 0.024 min⁻¹.
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**FIGURE 4**

$^{45}$Ca washout (plotted semilogarithmically) from scintillator slide with and without cells. The slide, with cells attached, was labeled with $^{45}$Ca for 30 minutes, then washed out for 20 minutes (solid circles). Cells were then removed, and the slide was rinsed with EDTA and then relabeled with $^{45}$Ca for 30 minutes. It was then washed out for 20 minutes (×). The second washout curve (×) was then subtracted from the first (solid circles) to obtain the washout curve for the cells alone (solid line). This is resolved into three exponential components with rate constants (λ) of 2.3, 0.45 and 0.021 min⁻¹.

**FIGURE 5**

$^{45}$Ca uptake by scintillator slide with and without cells. $^{45}$Ca activity (left ordinate) is plotted against time for the slide with cells (solid circles) and for the slide without cells (×). The cellular component (solid line) was obtained by subtraction (right ordinate). Low Na⁺ solution was substituted at the twenty-fourth minute in both labeling procedures as described in the text. The rapid and marked increase in counts attributable to the cellular component is clearly evident.

minutes before washout. The washout pattern was then determined. The cells were then removed, and the slide was washed with EDTA to remove all traces of radioactivity and again incubated for 30 minutes in the $^{45}$Ca labeled standard solution. The washout...
Subtraction of this washout curve from the first resulted in a curve representative of the $^{45}$Ca washout from the cellular layer. The cellular washout curve could be consistently resolved into three exponential phases. The rates of two of the phases were not significantly different from the two phases resolved for washout of the blank (Fig. 3). The presence of the cells, however, contributed an intermediate phase with $\lambda = 0.45$ min$^{-1}$ (Fig. 4). This phase was consistently present when cells were attached to the slide surface and consistently absent when they were not. The average rate constants ($\text{min}^{-1}$) for the three phases of washout from slides with cells attached were $3.1 \pm 0.2$ (1 SEM), $0.48 \pm 0.05$, and $0.020 \pm 0.003$.

$^{45}$Ca Uptake.—The pattern of $^{45}$Ca uptake by slide alone and by slide with cells attached is shown in Figure 5. The difference between the two curves is attributable to the cell layers. The background due to the labeling solution in the chamber is obviously high—over $10^6$ counts/min. The contribution of the cells of $5$ to $6 \times 10^4$ counts/min is, however, clearly evident. Three uptake experiments demonstrated the same pattern. Uptake was quite rapid for the initial 8 to 10 minutes, after which increase in uptake was very slow.

Effect of Low Na$^+$ Perfusion.—After the uptake rate had slowed, the effect of low Na$^+$ perfusion on $^{45}$Ca uptake was evaluated. Solution in which $75\%$ of NaCl had been replaced by choline chloride was added at the twenty-fourth minute. The change of solutions was accomplished within the time required (7.2 seconds) to print out between counting periods. By the time the next counting period (6 seconds) was completed, the cell count had increased by 22% (+12,500 counts/min). The uptake then proceeded at its former slow rate. The effect of reduced Na$^+$ on the rate of $^{45}$Ca uptake was, then, completed within a few seconds at most. The limitation imposed by intermittent counting prevented further definition of the rate of the augmented uptake. It is of interest that the scintillator glass also showed the effect of low Na$^+$ on $^{45}$Ca uptake, but to a much lesser extent.

In the experiment illustrated in Figure 5, the increment in Ca$^{2+}$ represented by the augmented $^{45}$Ca uptake was estimated. The total protein content analyzed for two slides cultured in the same dish as the slide used for the isotope study illustrated in Figure 4 was 1.19 and 1.37 mg (mean, 1.28 mg). With a protein content of 7.5 mg/100 mg wet tissue, each slide contained approximately 17 mg cells. The counting efficiency of the slide was 27.4% and therefore the 12,500 count/min increment was corrected to 44,600 counts/min. This represents $1.1 \times 10^{-9}$ moles Ca$^{2+}$, or 65 $\mu$moles/kg cells. The total amount of labeled
Ca\textsuperscript{2+} after 40 minutes of perfusion was \(6.3 \times 10^{-9}\) moles or \(370 \mu\text{moles/kg cells}\).

The ability to remove the extra \(43\text{Ca}\) bound following low Na\textsuperscript{+} perfusion was evaluated with washout studies. It was first ascertained that washouts following two 30-minute labeling periods with perfusate of normal Na\textsuperscript{+} concentration were virtually superimposable. A slide was then labeled in normal Na\textsuperscript{+} solution, washed out, relabeled in low Na\textsuperscript{+} solution and washed out again. Both washout solutions were with solution of normal Na\textsuperscript{+} content. Two experiments indicated that the increment in \(45\text{Ca}\) was maintained for at least 20 minutes of normal Na\textsuperscript{+} washout. This was confirmed by labeling a slide in low Na\textsuperscript{+} solution, instituting washout with low Na\textsuperscript{+} but abruptly switching to normal Na\textsuperscript{+} during the washout. As indicated in Figure 6 there is no evidence of an increased rate of \(45\text{Ca}\) loss. Therefore, the additional Ca\textsuperscript{2+} bound after low Na\textsuperscript{+} perfusion has a strong affinity for the cell.

**Discussion**

The technique has the advantage of nearly continuous analysis of the isotopic content of a single layer of functionally intact myocardial cells. The cell layer remains contractile during exposure to the serum-free labeling and washout solutions. This is not surprising, since Levinson and Green (4) found that cultured chick heart cells in a balanced saline medium containing Na\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and K\textsuperscript{+} retained phosphate and nucleotides as well as did cells in a complete culture medium for a period of at least 2 hours. This indicates that the integrity of the membrane is not significantly compromised.

The ultrastructural studies of Cedergren and Harary (5) of cultured cells of hearts from newborn rats at the same stage of development as those used in the present study demonstrate that all the major components of adult myocardial cells are present. Myofilaments, mitochondria, sarcotubules, and intercalated discs are found. These vary in proportion from cell to cell but all are represented in the cell layer. As indicated by Mark et al. (6) two different cell populations are present—muscle cells and endothelioid cells. The latter are believed to be derived from capillaries. After 72 hours of growth, the ratio of muscle cells to endothelioid cells is approximately 2:1. The cell population is therefore heterogeneous.

As seen in Figure 2, the cells grow in a flat, thin sheet. Cross sections indicate that they are tightly adherent to the glass surface. Burrows and Lamb (7) suggested that significant exchange with the perfusing solution occurs at the surface apposed to the glass through small-diameter (500 Å) channels or tunnels formed by unattached regions. It seems to us that the exchanging area contributed by these tunnels can be estimated only by serial cross-sectioning of each cell. In addition, it would certainly vary from cell to cell. We therefore hesitate to estimate the total cellular area which functions in exchange in this preparation. The minimum area, however, is that of the unattached upper surface. The upper cell surface accounts for at least 50% of both slide surfaces, or 10 cm\textsuperscript{2}. The total cell weight was approximately 15 mg, which, at a cellular sp gr of 1.0, would indicate a total cellular volume of 0.015 cm\textsuperscript{3}. This gives a cellular area-to-volume ratio of over 650/cm\textsuperscript{2} and indicates a flat, platelike configuration of the cells. Electron micrographs confirm this configuration.

The washout pattern from a slide with cells attached (Fig. 4) is graphically resolved into three phases. The most rapid phase is also demonstrable for the slide without cells (Fig. 3). This component almost certainly represents residual labeling solution, which simply adheres to the slide, cell surface, or both following removal of the slide from the labeling solution.

The next component (mean \(\lambda = 0.48\ \text{min}^{-1}\)) of the tissue culture washout is never demonstrable in the washout pattern from a blank and is attributed to a portion of cellular Ca\textsuperscript{2+}. The last component (mean \(\lambda = 0.020\ \text{min}^{-1}\)) from the cell layer exchanges at a rate very similar to that of the blank slide. There is, then, evidence for two components which can be added as parallel contributions to
define the washout curve of $^{45}$Ca from the cellular layer:

\[
\text{cellular activity} = Ae^{-0.48t} + Be^{-0.020t},
\]

where $A$ and $B$ represent the zero time intercepts of the fast and slow components respectively. It is to be emphasized that this is only a convenient way to describe the washout pattern at this juncture.

The resolution of the washout pattern of $^{45}$Ca from the layer of cells in the present study is of the same three-component form as found for $^{24}$Na and $^{42}$K from whole, vascularly perfused myocardium. The most rapid component ($\text{mean } \lambda = 3.1 \text{ min}^{-1}$) is considered to represent surface washing and clearance of the flow cell in the present study. A flow rate of 24 ml/min or above was demonstrated not to be limiting for cellular washout. The most rapid component might be considered to be equivalent to the vascular component of ionic exchange in whole tissue. The elimination of the interstitial space in tissue culture makes it possible to consider tissue Ca$^{2+}$ as a two-compartment system. Studies in progress indicate that $^{45}$Ca-labeling of the cellular layer in the presence of dinitrophenol eliminates much of the slow compartment of $^{45}$Ca uptake. The ability to specifically alter the flux of one of the compartments will permit formulation of a realistic model for the ionic exchange of the plated cells.

A comparison of $^{45}$Ca washout from cell layer and whole tissue is premature at this time. However, a comparison of K$^{+}$ exchange from cultured chick embryo heart muscle by Burrows and Lamb (7) with K$^{+}$ exchange from perfused dog papillary muscle (8) indicates that this may be reasonable. Burrows and Lamb found that washing the culture at a rate of 30 ml/min was not limiting for cellular exchange of $^{42}$K. (This is similar to the rate (24 ml/min), which was found to be nonlimiting for $^{45}$Ca in the present study.) The cellular K$^{+}$ flux from the cultured chick cells was calculated to be between 7.1 and 10.1 pmole/cm$^2$/sec. The cellular flux in arterially perfused (1 ml/g/min) dog papillary was 9.6 pmole/cm$^2$/sec. This indicates that physiological rates of perfusion are not limiting for cellular K$^{+}$ exchange. Since K$^{+}$ has the lowest fast compartment-slow compartment flux ratio of the three cations (Na$^{+}$, K$^{+}$, Ca$^{2+}$) it is unlikely that the exchange of any of these cations is limited at the physiological perfusion rates used in the series of experiments of Langer and Brady recently reviewed (9). Page et al. (10) have suggested that a perfusion rate over 10 times the physiological rate is required in order that cellular K$^{+}$ exchange may not be perfusion limited. This is clearly not the case for the perfused dog papillary muscle or rabbit interventricular septum (9). This does not deny that the rate of cellular K$^{+}$ exchange in the rat heart beating at 225 to 300/min at 37°C is high, as was measured by Page et al. (10). Under these conditions it would be expected to be so (9).

The studies involving low Na$^{+}$ perfusion indicated that the cultured cells respond with an increment in $^{45}$Ca uptake. This increment was to be expected from the studies done on whole tissue (11). The additional $^{45}$Ca uptake which was coincident with low Na$^{+}$ perfusion represented approximately 65 pmole Ca$^{2+}$/kg cells (Fig. 5). This increment was apparent within 6 seconds, or as rapidly as could be resolved with the present counting technique. Examination of Figure 5 indicates that an increment in cellular $^{45}$Ca activity, 30% of that which occurred in that experiment, could be recognized (a 20-µmole/kg increment). This provides a sensitivity and time resolution which has heretofore not been possible in functioning, contractile myocardial tissue.

If the cellular area-to-volume ratio is 650/cm, the exposed surface area of the approximately 17 mg of cells on the slide (Fig. 5) would be 11 cm$^2$. This indicates that low Na$^{+}$ perfusion resulted in the addition of approximately $1 \times 10^{-10}$ moles Ca$^{2+}$/cm$^2$ if the entire additional uptake were bound to the membrane.

The washout studies indicate that the additional Ca$^{2+}$ bound coincident with low Na$^{+}$ perfusion is tightly bound (Fig. 6) to some portion of the cell. Return to normal Na$^{+}$ perfusate during $^{45}$Ca uptake (following a
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period of low Na⁺ perfusion) indicated a probable rapid small loss of Ca²⁺. This could not be defined on the washout studies, however. Studies on whole tissue also demonstrated that the major fraction of Ca²⁺ gained during low Na⁺ perfusion was irreversibly bound (11). Ultrastructural studies (12) gave evidence that much of this fraction was in the mitochondria.

Acknowledgments

The authors acknowledge the fine technical assistance of Mr. Charles Dubkin in construction of the flow cell and the contributions of Mrs. Brenda Eisenberg for electron micrographs of the cultured cells.

References


Circulation Research, Vol. XXIV, May 1969
Calcium Exchange in a Single Layer of Rat Cardiac Cells Studied by Direct Counting of Cellular Activity of Labeled Calcium
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Circ. Res. 1969;24:589-597
doi: 10.1161/01.RES.24.5.589

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

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