LANTHANUM IN HEART CELL CULTURE

Effect on Calcium Exchange Correlated with Its Localization

G. A. LANGER and J. S. FRANK

From the Departments of Physiology and Medicine and the Los Angeles County Cardiovascular Research Laboratory, University of California at Los Angeles, Center for Health Sciences, Los Angeles, California 90024

ABSTRACT

Correlation of the localization of La^{+++} with its effects on Ca^{++} exchange in cultured rat heart cells is examined with the use of a recently developed technique. 75% of cellular Ca^{++} is exchangeable and is completely accounted for by two kinetically defined phases. The rapidly exchangeable phase 1 has a $t\ 1/2 = 1.15$ min and accounts for 1 1 mmoles Ca^{++}/kg wet cells or 43% of the exchangeable Ca^{++} (cells perfused with $[Ca^{++}]_o = 1$ mM) Phase 2 has a $t\ 1/2 = 19.2$ min and accounts for 1.5 mmoles Ca^{++}/kg wet cells or 57% of the exchangeable Ca^{++}. 0.5 mM $[La^{+++}]_o$ displaces 0 52 mmoles Ca^{++}/kg wet cells—all from phase 1—and almost completely abolishes subsequent Ca^{++} influx and efflux The presence of La^{+++} in the washout converts the washout pattern to a single phase system with a $t\ 1/2 = 124$ min. The effects upon Ca^{++} exchange are coincident with abolition of contractile tension but regenerative depolarization of the tissue is maintained Electron microscope localization of the La^{+++} places it exclusively in the external lamina or basement membrane of the cells. The study indicates that negatively charged sites in the basement membrane play a crucial role in the E-C coupling process in heart muscle

Sanborn and Langer (12) recently demonstrated that the trivalent cation lanthanum, La^{+++}, specifically uncoupled excitation from contraction in mammalian cardiac tissue The effects of La^{+++} on calcium (Ca^{++}) exchange were correlated with its uncoupling action, and these results, in association with the well-recognized affinity of La^{+++} for extracellular sites (4, 11), indicated that contractile-dependent Ca^{++} was derived primarily from superficial cellular regions in heart muscle The fact that La^{+++} is electron-opaque raised the possibility that its effects upon Ca^{++} exchange and contractile function could be correlated with its morphological distribution The problem, however, is that La^{+++} is found in capillary membrane, throughout the interstitial space, at intercellular junctions, and on the cellular surface in whole tissue. In addition, the accumula- tion of La^{+++} on capillary structures when perfused through the vasculature of heart tissue results in a filtering effect which reduces the amount distributed to the cellular surface and makes reproducible ultrastructural localization impossible The development of a new technique (10) for analysis of ionic exchange in tissue culture cells obviated these problems.

The technique employed depends upon the growth of a single layer of cells, derived from neonatal rat heart, on both surfaces of a slide composed of glass scintillator material Cellular ionic influx and efflux, free of other tissue components, can be continuously monitored, substances in the perfusing medium have direct access to the cellular surface, and fixation for electron microscope analysis is virtually instantaneous

The study indicates that a significant fraction of

cellular Ca^{++} is surface bound. La^{+++} displaces a portion of this superficially placed Ca^{++} and markedly limits further exchange. La^{+++} is localized at the basement membrane external to the unit membrane.

METHODS

The method has been previously described (10) in detail. The basis of the technique 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 cells directly on the radioisotope detector.

Culture Technique

The cells are prepared essentially according to the method of Harary and Farley (7). 25 ml of growth medium containing the dispersed cells from four to six rat hearts is poured into an 8 cm Petri dish which contains two scintillator slides (Nuclear Enterprises, Ltd., San Carlos, Calif). The slides are supported by a rack which elevates their undersurface 1 mm above the bottom of the Petri dish. After 24 hr the slides are turned over and the procedure is repeated in order that both surfaces of the slide are covered with cells. The growth is observed until both surfaces contain a layer of synchronously beating cells This occurs on either the 3rd or 4th day of incubation, at which time the study is done. This means that, at the time of study, the cells have been incubated for either 2 and 3 days or for 3 and 4 days.

The cell population consists of both myoblasts and fibroblasts. Myoblasts are present to the extent of 60–80% at the time of study. The percentage of myoblasts is improved by permitting the inoculum to stand for 3 hr and, at this time, pouring the inoculum into the dish containing the slides. Fibroblastic cells settle more rapidly and remain in the original dish. Pure fibroblastic cultures are obtained by exposing the slides to the inoculum only during the initial 3 hr period and then removing them to incubate in a cell-free growth medium. After 3–4 days of incubation these slides are covered by a single layer of typical fibroblasts with no contractile cells present.

Perfusion Technique

The slides with cultures attached are maintained in full incubation medium until isotopic labeling is commenced. The standard solution used for the isotope studies has the following millimolar composition: NaCl, 133; KCl, 3.6, $CaCl_2$, 1.0, $MgCl_2$, 0.3; glucose, 16.0. In the studies in which La^{+++} was used, it was added in the form of $LaCl_3$, 0.5 mM.

The solution was buffered with 3 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) to a pH of 7.3–7.4. The cultures remained contractile for at least 3 hr in the solution or for the duration of the experiments. For isotopic labeling the solution contained 1 μCi/ml ^{45}Ca (New England Nuclear Corp., Boston, Mass.). It should be noted that phosphate and bicarbonate buffers cannot be used in the presence of La^{+++}. Their lanthanum salts are extremely insoluble.

The slide is placed in a specially designed flow cell (10) for the purpose of recording the sequence of isotopic labeling and washout. The flow cell is constructed of Lucite plastic. It is provided with intake and outlet orifices at the top which are oriented so that fluid entering the cell is directed against the slide to minimize the development of unstirred layers. The slide bisects the chamber in the longitudinal axis so that the perfusion fluid is directed down one surface, through an aperture in the base of the slide, up the other surface, and to the outlet. The perfusate is supplied at the desired rate from syringes mounted in a Harvard infusion pump. It was previously shown (10) that a flow rate of at least 24 ml/min was not rate limiting for ^{45}Ca exchange. This flow rate was used for all washout studies.

The flow cell is mounted upon a handle which facilitates its insertion into the well of a scintillation spectrometer (Beckman Model LS200B). Care must be taken to avoid exposure of the slide to light of the shorter wavelengths which excites the scintillator and elevates the background emission above the usual 500–1000 cpm for prolonged periods. The slide is equilibrated to the nonisotopic perfusion solution for 30–60 min before introduction of the ^{45}Ca label. The uptake and washout of the cells is monitored continuously by diverting the readout of the spectrometer to a strip chart recorder as well as recording the digital printout each 13.2 sec.

At the completion of isotopic labeling or washout the slide is removed its surface is briefly rinsed to remove unbound isotope, and the cells are scraped onto a 10–15 mg piece of predried, weighed Millipore filter. The cells are dried overnight at 100°C and the dry weight is obtained. The Millipore with the dried cells is then placed in 2 ml of Protosol (New England Nuclear Corp.) tissue solubilizer for 10–12 hr. 0.5 ml glacial acetic acid is added to neutralize the alkaline solution in order to reduce background luminescence, and this is followed by the addition of 15 ml of Aquasol (New England Nuclear Corp.) scintillator solution. Appropriate standards (internal and external) are made and counted with each experiment in order to determine counting efficiency for the solubilized tissue sample. The isotopic activity in the tissue is then compared to the final activity recorded from the tissue on the slide in the

flow cell to derive the counting efficiency of the glass scintillator slide. The efficiency for detecting ^{45}Ca emission of the group of slides used is in the range of 35–40%.

In each experiment, after removal of the cell layer from the slide, the slide is rinsed with ethylenediaminetetraacetate (EDTA) to remove all traces of radioactivity, returned to the flow cell, and exposed to the same experimental sequence applied to the slide when cells were attached. This procedure establishes the response of the "blank". Subtraction of the "blank slide" sequence from the "slide with cells" sequence defines the isotopic activity of the cellular layer alone. At a perfusion rate of 10 ml/min the blank reaches asymptotic counting values within 1.5 min (Figs 2, 3), and the counting level remains fixed for the remainder of the experiment. At the perfusion rate of 24 ml/min used for washout experiments, the chamber and blank slide clear 99% of radioactivity within 1–1.5 min. At this time the counts are usually about 500/min above background and fall slowly (rate constant approximately 0 02/min) for the remainder of the experiment. The total background after the initial 2 min of washout of labeled cells represents less than 5% of the total counts, i e., greater than 95% of the recorded activity is derived from the cellular layers.

Total tissue calcium was measured by titrimetric analysis according to a modification of the method described by Bachra et al. (1). This technique has an accuracy of ± 4% in our hands, as determined by blind titration of a series of standard solutions.

Preparation for Electron Microscopy

The cells were cultured as previously described, but for the ultrastructural studies they were grown directly in plastic Petri dishes All solutions involved in processing the cells for electron microscopy were poured directly onto the cells in the dish. Penetration of the fixative is not a problem in cells grown in monolayer tissue culture, and hence, the cells were well fixed after 5 min of exposure to the fixative.

The primary fixative was glutaraldehyde, in concentrations between 1 and 3 per cent, buffered with 0.1 M cacodylate buffer (pH 7 2). Postfixation in 1% osmium tetroxide was preceded by a brief rinse in 0.1 M cacodylate buffer at pH 7.2.

In the studies involving lanthanum, solutions of 1 mM LaCl$_3$ buffered with 3 mM HEPES (pH 7.2) were added to the culture for 5 min. It was noted that when 1 mM LaCl$_3$ was added to the cells before glutaraldehyde fixation, the cell borders tended to retract. (This can probably be attributed to alterations in interfacial tension characteristics induced by the effects of La^{+++} on Ca^{++} binding) When glutaraldehyde was then added, the cells contracted severely. This obscured much of the cellular ultrastructural

detail. This difficulty was overcome by exposure of the culture to buffered 1% glutaraldehyde solution for 5 min. The cells were then rinsed, and buffered LaCl$_3$ solution (without glutaraldehyde) was added for 5 min. This procedure was followed by postfixation in osmium tetroxide. It should be emphasized that LaCl$_3$ was not present in any of the fixatives and that total exposure to LaCl$_3$ was limited to 5 mm. The cells were dehydrated in ethanol and embedded in Epon 812. Gelatin capsules filled with Epon were inverted over the cells in the Petri dish. After the resin was cured in a 60°C oven, the capsules were simply broken off and cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome.

Sections from the control studies were stained with uranyl acetate followed by lead citrate. Sections from the lanthanum studies were usually studied unstained, except for the section illustrated in Fig. 11 which was briefly stained (1 min) in uranyl acetate.

RESULTS

Preliminary Investigations

Observation of the rhythmically contracting cell layer disclosed that contraction ceased immediately upon the application of standard perfusate to which 0 5 mM LaCl$_3$ had been added. Removal of the LaCl$_3$ solution and flushing with standard perfusate resulted in a return of rhythmic contraction over a period of 30–60 min. The period is consistent with the washout characteristics of La from heart tissue (12), which demonstrates that La is quite strongly bound to the cell

The possibility that La^{+++} produced cessation of contraction through interruption of the excitation process in the neonatal rat heart was checked. A thin strand of ventricular muscle from a 3 day old rat was mounted in a perfusion chamber attached to a transducer for isometric tension recording. Unicellular action potentials were recorded through KCl-filled microelectrodes. Fig 1 illustrates the response to LaCl$_3$ perfusion. Contractile tension fell to 50% of control within 1 min and to 5% within 10 min. The configuration of the action potential changed considerably with loss of the plateau region, but the amplitude of the spike remained unchanged at 100 mv and the total duration remained essentially unchanged at 270 msec. It is clear that the nearly complete elimination of contractile tension cannot be attributed to failure of excitation in the neonatal rat

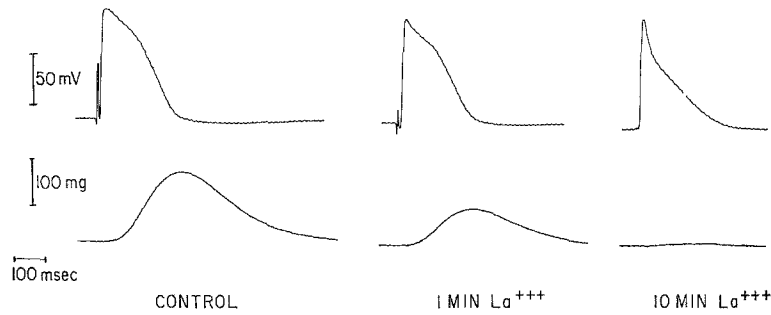Quantitative values for Ca are expressed in terms of content per kilogram dry weight of cells

FIGURE 1   The effect of La^{+++} (0.5 mM) on the action potential (upper traces) and twitch (lower traces) of ventricular muscle from the 3 day old rat.

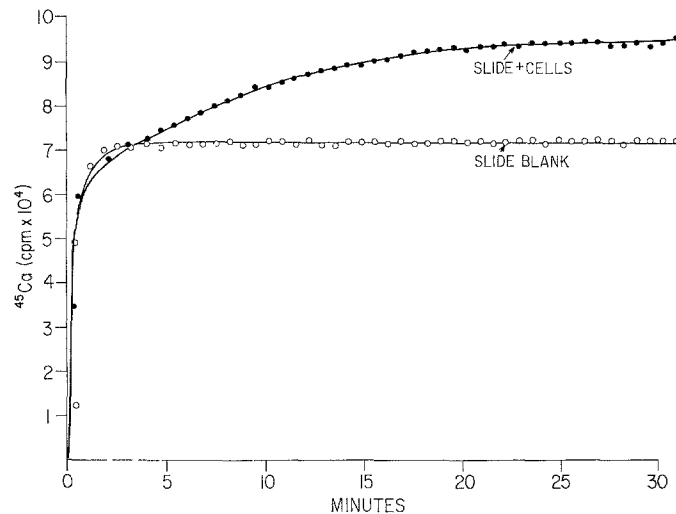

FIGURE 2 ^{45}Ca uptake as recorded from scintillator slide with and without heart cells attached.

in this study This is the most accurate way to normalize for cell mass when dealing with small masses of cells In order, however, to permit comparison with values expressed in terms of cell water, large masses of cells were grown on Petri dishes and wet weight:dry weight ratios were determined. The cell water was 82 9 ± 0 4% in 15 cultures Therefore the wet weight:dry weight ratio is 5 85 in these cultures

^{45}Ca Uptake and Total Ca Content

The pattern of ^{45}Ca labeling of a slide and of the slide blank is illustrated in Fig 2. The course of cellular labeling is clearly apparent, with 2 3 × 10^4 cpm attributable to the cells after 30 min of labeling This represents 14 2 mmoles/kg dry

weight of labeled calcium. ^{45}Ca labeling was carried out for 120 min in 19 cultures The mean labeled content was 15.0 ± 2 2 mmoles/kg dry weight The total calcium content derived from the titrimetric analysis of 3 to 4-day cultures (12 pooled samples) perfused with standard solution for 120 min was 19.7 ± 3 0 mmoles/kg dry weight The standard error is large but this is not unexpected in groups of cells at an early stage of development. Since 120 min of labeling is beyond the point at which the ^{45}Ca uptake is virtually asymptotic, it indicates that approximately 25% of the cellular calcium is very slowly exchangeable or inexchangeable

Fig 3 illustrates the effect of adding 0 5 mM LaCl$_3$ to the perfusing solution during ^{45}Ca uptake This produces a loss of 5000 cpm from the cell

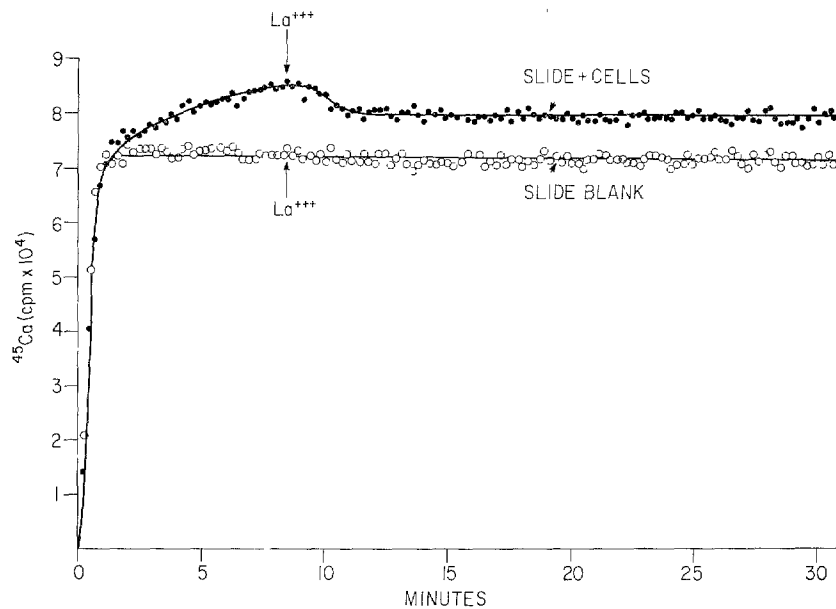FIGURE 3 ^{45}Ca uptake as affected by the addition of 0 5 mM LaCl$_3$ to the perfusate at 8 5 min. LaCl$_3$ remained in the solution for the 30 min course of the uptake.

TABLE I

Components of Cellular Calcium

	No of experiments	mmoles/kg dry wt
Total calcium	12	19 7 ± 3 0*
Exchangeable calcium	19	15 0 ± 2 2
La-Displaceable calcium	18	3 01 ± 0.29

1 SEM.

layer which is complete within 2 min. ^{45}Ca exchange then ceases in the presence of La^{+++} Note that La^{+++} had no effect on the blank slide in this experiment The loss of ^{45}Ca represents 3 9 mmoles/kg dry weight from a total of 10.9 mmoles labeled immediately before the addition of La at 8.5 min

The effect of La^{+++} was invariable, i e displacement of Ca and marked inhibition of further exchange, in 18 experiments The total amount of ^{45}Ca displaced by 0.5 mM LaCl$_3$ after 120 min of labeling was 3 01 ± 0 29 mmoles/kg dry weight. This represents 20% of the total labeled calcium of the cell There was no evidence that ^{45}Ca labeling beyond 8–10 min resulted in the displacement of greater quantities of ^{45}Ca upon the addition

of LaCl$_3$. The components of cellular calcium are summarized in Table I.

^{45}Ca Washout

The pattern of ^{45}Ca washout of cultured cells is shown in Fig 4 The first portion of the washout which contributes the most rapidly exchangeable component (broken line) is attributable to the clearance of the labeling solution in the chamber (10) Otherwise, only the cellular component of the slide-cell combination is shown—the background contribution of the blank has been subtracted The slide had been labeled for 130 min, and the last 10 min of labeling is illustrated in the inset It is noted that the labeling had reached an asymptotic value, with 3.2 × 10^4 cpm attributable to the cell layers ([slide-cells]—slide blank) This represents 18 7 mmoles Ca^{++}/kg dry weight in this experiment

The washout pattern is typical in that it is resolved into two exponential components (solid lines) which are attributable to the cellular layer The rate constants for phase 1 (λ_1 = 0.77 min^{-1}) and phase 2 (λ_2 = 0 023) differ widely The intercept of phase 2 is 1 8 × 10^4 cpm and of phase 1 is 1 4 × 10^4 cpm—their sum of 3 2 × 10^4
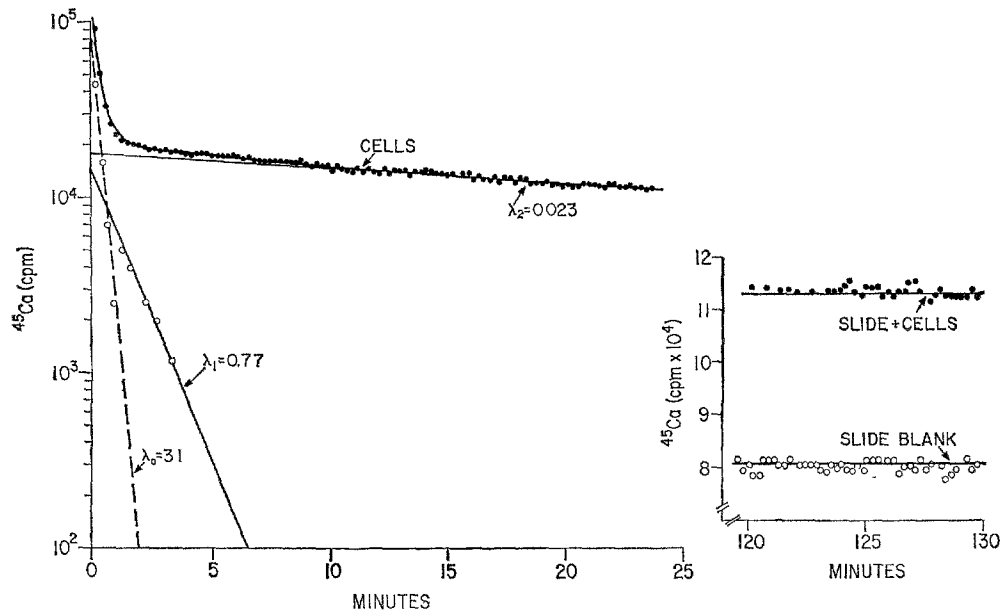
Figure 4 ^{45}Ca washout from the cellular layer. The last 10 min of a 130 min labeling period is indicated in the *inset* at right. Subtraction of the activity of the blank from the slide plus cells indicates 3.2×10^4 cpm attributable to the cellular layer. The contribution of the blank has been subtracted from the washout curve (on left) so that the curve described by the solid circles represents the cells. It is graphically resolved into two exponential components (solid lines). The slow component (phase 2) is defined by a $\lambda_2 = 0.023$ min^{-1} and an ordinate intercept of 1.8×10^4 cpm. The fast component has a $\lambda_1 = 0.77$ min^{-1} and an intercept of 1.4×10^4 cpm. The broken line ($\lambda_0 = 3.1$ min^{-1}) in this and other figures represents the washout of the ^{45}Ca-labeled perfusate from the flow cell chamber.

TABLE II

Exchange Rates and Calcium Content Ratio of Cellular Calcium (120 min Labeling)

	Half time	Rate const. (min^{-1})
Phase 1	1.15	0.60 ± 0.08*
Phase 2	19 2	0.036 ± 0.004

Phase 1 : phase 2 content $= 0.75 \pm 0.14$.
* 1 SEM.

cpm is equal to the counts attributable to the cell layer immediately before the washout. Therefore, 100% of the labeled calcium is accounted for by the two phases. There is no need to consider a correction for the intercepts of the phases as outlined by Huxley (8), even if the compartments were arranged in series. This is because $\lambda_1 \gg \lambda_2$. The exchange rates and fractional contents of phases 1 and 2 are summarized in Table II.

Fig. 5 illustrates the effect of exposure of the cell layer to La^{+++} before and throughout the wash-out. The inset shows the uptake curve and the response to La^{+++} before the onset of washout after 70 min of labeling La^{+++} displaced 1.2×10^4 cpm (effect on blank subtracted), representing 4 3 mmoles Ca^{++}/kg dry weight—a displacement of 22% of the labeled Ca 4.3 $\times 10^4$ cpm remained in the cell layer before washout This represented 15 2 mmoles.

The washout curve illustrates the marked effect of La^{+++} The cellular washout is now described by a single exponential phase. The ^{45}Ca which is attributed to the residual labeling solution is cleared rapidly, and this is indicated by the broken lines The cellular washout no longer demonstrates a rapid phase 1 component (see Fig. 4) but only a very slowly exchangeable single phase ($\lambda = 0.0031$ min^{-1}).

Note that all of the labeled cellular Ca (4.3×10^4 cpm) is accounted for by the single slow phase. The results depicted in Fig. 5 were repeated in 19 experiments. All showed a monophasic cellular washout with a mean rate constant of 0.0056 $\pm$
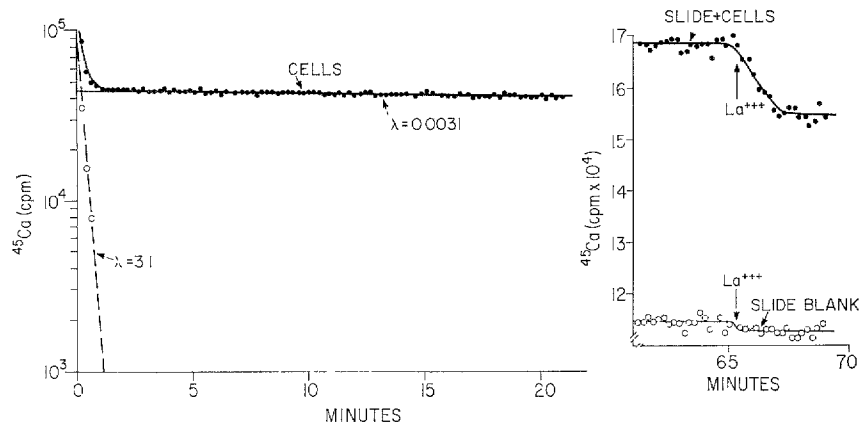
FIGURE 5 ^{45}Ca washout as affected by 0 5 mM LaCl$_3$. LaCl$_3$ was added to the perfusate for the last 5 min of the labeling period (*inset* at right) and throughout the subsequent washout (at left). Note that all of the ^{45}Ca activity attributable to the cellular layer at the end of the labeling period (4.3 × 10^4 cpm) is accounted for in the cellular washout described by a single slow phase (λ = 0.0031 min^{-1}) with an ordinate intercept of 4 3 × 10^4 cpm. Compare with control washout illustrated in Fig. 4.

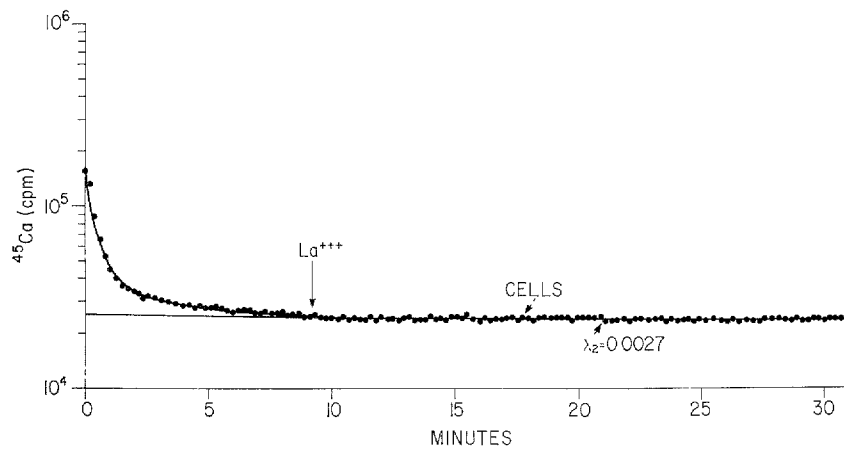FIGURE 6   The effect of 0.5 mM LaCl$_3$ introduced at the 9th min of ^{45}Ca washout. Note that there is no evidence of ^{45}Ca displacement but only a slowing of the subsequent washout (λ_2 = 0.0027 min^{-1}) See text for analysis

0 0005 min^{-1}. This is only 15% the rate of phase 2 in the control studies

The kinetic studies indicate that La^{+++} displaces approximately 20% of exchangeable cellular Ca and then markedly inhibits further influx and efflux

It is of interest to define the kinetic phase from which La^{+++} displaces Ca^{++} This was accomplished by exposure of the cell layer to La^{+++}

9–10 min after ^{45}Ca washout had begun The results are illustrated in Fig. 6 The pattern of the washout is similar to that of control for the initial 9 min. Phase 1 is essentially washed out by the time La is introduced at 9.2 min, but phase 2 with λ_1 in the range of 0 036 min^{-1} (Table II) remains 70–75% labeled. It is clear that La produces no ^{45}Ca displacement but only slows the subsequent washout. Therefore, all of the labeled
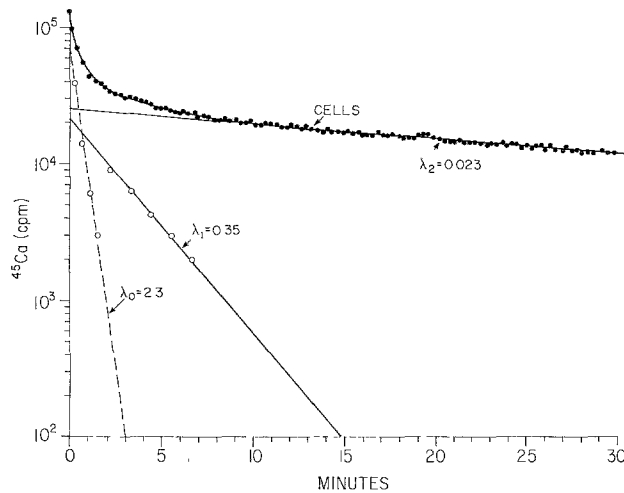
FIGURE 7 ^{45}Ca washout from a layer composed of 100% fibroblasts. Note that the pattern is similar to that obtained from cellular layers which are approximately 80% myoblastic (Fig. 4). See text for further discussion.

displaceable Ca^{++} had washed out before 9 min and is, therefore, attributable to the rapid phase (phase 1)

Exchange Characteristics of Fibroblasts

As indicated in the description of the culture technique, the cell population is mixed and contains 20–40% fibroblastic cells Therefore, it is necessary to define the Ca^{++} exchange characteristics of these cells in order to evaluate their contribution to the Ca^{++} exchange of the mixed, but predominantly myoblastic, cell layers described above. Modification of the culture technique (see Methods) resulted in the culture of cell layers which, upon examination by phase-contrast microscopy, disclosed that greater than 99% of the cells were fibroblastic

The pattern of ^{45}Ca washout of a fibroblastic culture, after a 120 min labeling period, is shown in Fig. 7. The pattern is seen to be similar to that illustrated in Fig 4 and demonstrates two cellular phases. The phase 1 rate constant (0.35) is less than that recorded for the myoblastic cultures (Table II), but it is apparent that the major components of the washout are not markedly different in a pure fibroblastic culture. The total labeled Ca^{++} after the 120 min labeling period in these cells was 20 4 mmoles/kg dry weight

The effect of La^{+++} on ^{45}Ca uptake and washout in pure fibroblastic cells is illustrated in Fig. 8.

As with the experiment illustrated in Fig. 5, the cell layer was exposed to La^{+++} for the last 5 min of the labeling period and throughout the succeeding washout. The inset shows that La displaced 1.1 × 10^4 cpm from the slide and cells and 2 × 10^8 from the blank. Therefore, La displaced 9 × 10^8 cpm from the cells which had contained 2.7 × 10^4 cpm before exposure—a displacement of 33% of the labeled Ca 1 8 × 10^4 cpm remained in the cell layer

The washout curve should be compared with that shown in Fig. 5. As with the mixed culture, the washout is reduced to a single exponential phase The phase 1 component is eliminated and the remaining component is markedly slowed (λ = 0.0020 min^{-1}). Again note that all of the labeled cellular Ca (1.8 × 10^4 cpm) is accounted for by the single phase with its intercept of 1.8 × 10^4 cpm The response illustrated in Fig. 8 was reproduced in a total of 10 fibroblastic cultures The La-displaceable Ca represented 28 5 ± 4 4% of the Ca labeled in a 120 min period in this group.

The point to be emphasized is that the Ca exchange of fibroblasts and the effects of La on this exchange are quite similar to those seen in predominantly myoblastic cultures. This was further documented by preparing a slide which demonstrated more than 90% contractile myoblastic cells. This was done by allowing the fibroblastic
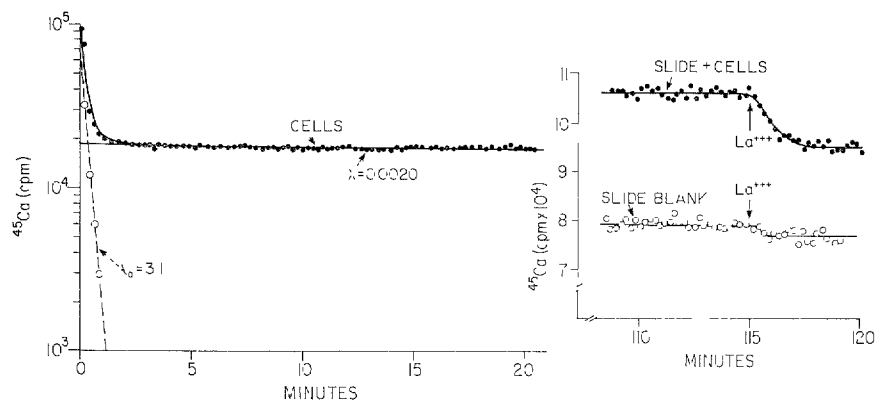
FIGURE 8 ^{45}Ca washout from a 100% fibroblastic culture as affected by 0 5 mM LaCl$_3$. LaCl$_3$ was added to the perfusate for the last 5 min of the labeling period (*inset* at right) and throughout the subsequent washout (at left). Note that the pattern is similar to that of Fig 5—an 80% myoblastic culture. All of the cellular activity (1 8 × 10^4 cpm) is accounted for by a single, slowly exchanging component (λ = 0.0020 min^{-1}, ordinate intercept = 1 8 × 10^4 cpm). Compare with control washout from fibroblastic culture (Fig 7).

components to settle and culturing the supernatant on a single side of the slide which was studied on the third day 0 5 mM LaCl$_3$ displaced 33% of the labeled Ca^{++}. The subsequent washout in the presence of La demonstrated a single slow cellular phase (λ = 0 0024 min^{-1})

Ultrastructural Localization of La

As indicated in Methods, it was necessary to fix the cellular layer before exposure to La^{+++} in order to prevent cellular retraction and obscuration of details of cell membrane ultrastructure It is, then, necessary to demonstrate the effect of glutaraldehyde fixation on cellular Ca^{++} exchange and the response of this exchange to La^{+++} after glutaraldehyde exposure

The effect of glutaraldehyde is illustrated in Fig 9, and these results should be compared to those shown in Figs 3 and 5 After 44 min of ^{45}Ca labeling, 1% glutaraldehyde was added to the standard labeling solution (buffered to pH 7 3) This produced a small change in ^{45}Ca specific activity of the perfusate which is responsible for the alteration in counts recorded from the blank ^{45}Ca uptake of the cells is slightly increased since glutaraldehyde decreases the counts attributable to the blank Exposure to the fixative-containing solution was continued for 7 min, at which time the solution containing 0 5 mM LaCl$_3$ without glutaraldehyde was perfused This is the same

sequence used (except for 1 mM LaCl$_3$) in preparation of the cells for La^{+++} localization by electron microscopy. This produced a displacement of 22% of the counts attributable to the cell layer This is the same percentage displaced by La^{+++} from a nonfixed cell layer (Fig 5) and is comparable to the mean value of 20% in the 18 experiments summarized in Table I Washout was started after 58 min of labeling with 0.5 mM LaCl$_3$ in the washout solution The washout pattern is virtually identical to that illustrated in Fig 5, i e, it demonstrates only a slowly exchanging (λ = 0 0032 min^{-1}) single phase.

In addition, the effect of glutaraldehyde on the response of the cell layer to La^{+++} after 9–10 min of washout had been completed was determined, i e duplication of the experiment illustrated in Fig 6, in the presence of glutaraldehyde 1% glutaraldehyde was introduced 6 min before washout and was continued throughout the washout 0 5 mM LaCl$_3$ was added after 9 min. The washout pattern was virtually identical to that shown in Fig 6. La^{+++} produced no displacement of ^{45}Ca but slowed the subsequent washout It is concluded that the effects of La^{+++} on ^{45}Ca exchange of cells exposed to 1% glutaraldehyde are essentially the same as upon nonfixed cells

Lanthanum was found to bind to the surface of the developing myocardial cells and also to penetrate the interspace of the intercalated disc In
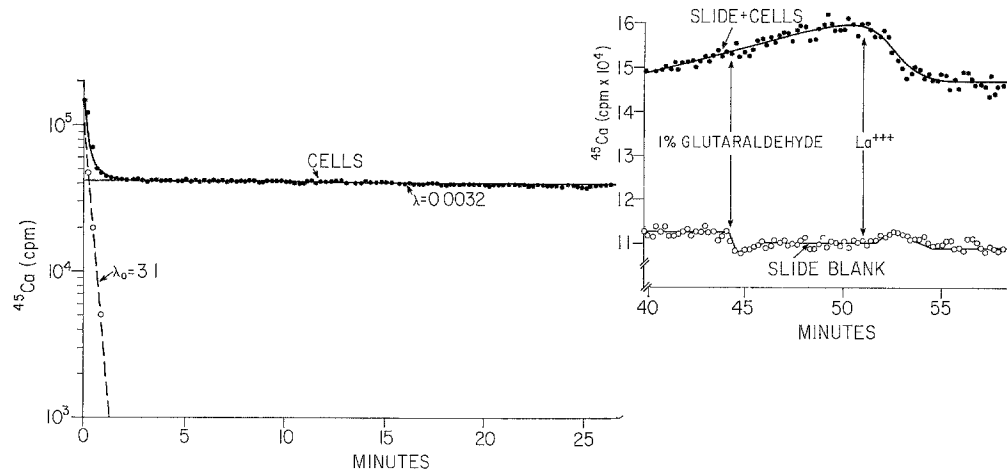
FIGURE 9   The effect of 1% glutaraldehyde on the response of ^{45}Ca exchange to $LaCl_3$. The *inset* at right indicates that 1% glutaraldehyde was added to the labeling solution at 44 min, followed by the addition of 0.5 mM $LaCl_3$ without glutaraldehyde 7 min later. Washout was commenced after 58 min of labeling in the presence of 0.5 mM $LaCl_3$. The blank was treated in an identical manner (the transient alterations in the blank response are attributable to a 2% lower ^{45}Ca specific activity of the glutaraldehyde solution). The pattern is essentially that found in the absence of glutaraldehyde (compare with Fig 5).
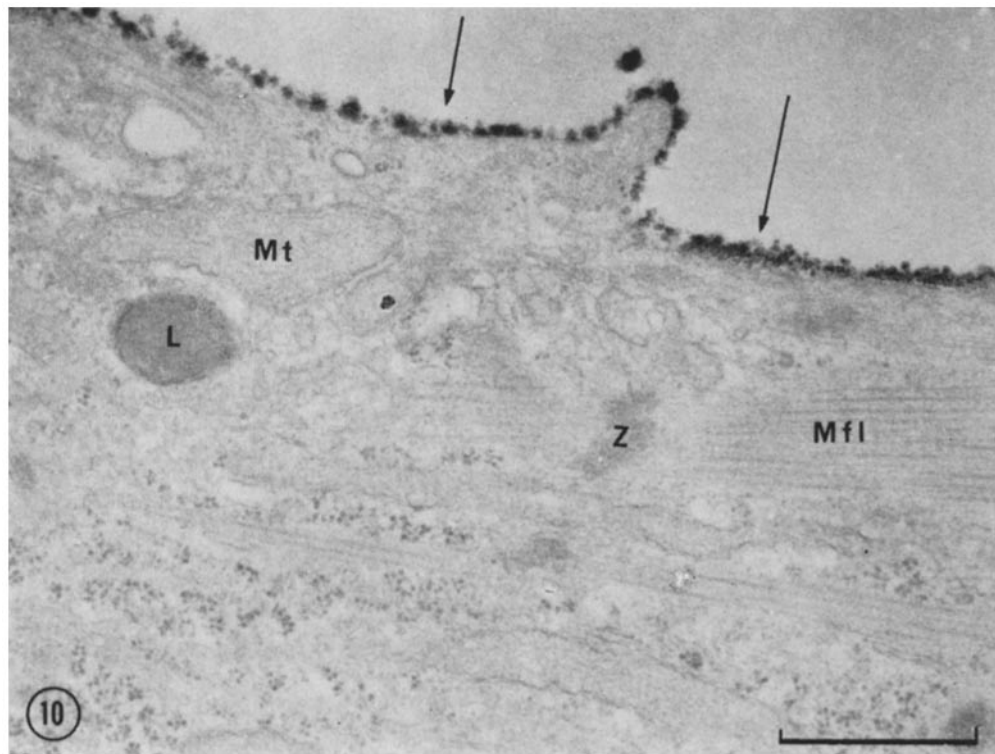

FIGURE 10   A typical myoblast cell from a 2 day tissue culture preparation that was exposed to 1.0 mM $LaCl_3$ for 5 min. This section was unstained Note the accumulation of lanthanum exclusively on the surface of the cell (arrows). Mitochondria (*Mt*), myofilaments (*Mfl*), Z line (*Z*), and lipid droplets (*L*) are evident. Scale, 0.5 μ; $\times$ 50,000.
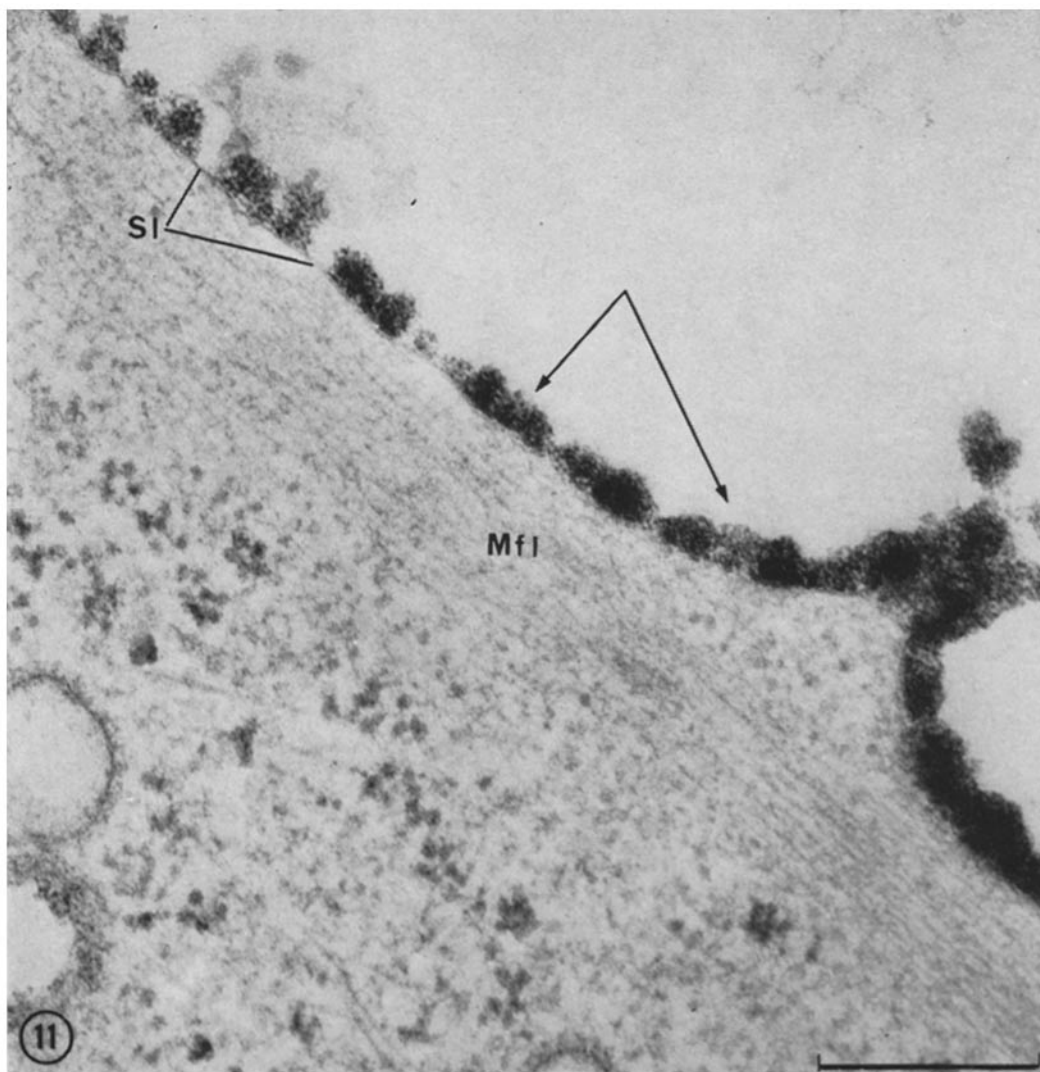
FIGURE 11   High magnification of cellular surface of myoblast exposed to 1 mM LaCl$_3$. Section stained for 1 min in uranyl acetate. The sarcolemma (Sl) is indicated. Note that the lanthanum accumulation on the cellular surface is superficial to the sarcolemmal membrane (arrows). Developing myofilaments are present in subsarcolemmal region (Mfl). Scale, 0.25 μ; $\times$ 112,000.

addition, lanthanum was present on the surface of the fibroblasts present in the tissue culture In over 500 cells examined, lanthanum densities were never found in the interior of the myoblast or fibroblast cell. The densities are limited to the external side of the membrane or plasmalemma structure.

The typical pattern of lanthanum binding to the cellular surface of the myoblast is shown in Figs. 10 and 11. The lanthanum is clearly present on the surface of the cell and outlines its surface configuration. It is clear that lanthanum binds to the surface coating of the cells—the so-called external lamina or basement membrane. Fig. 11 clearly shows the electron-opaque plasmalemma, with the bound lanthanum superficial to this membrane. In these developing myocardial cells the basement membrane is indeed very prominent. It is, in general, thicker than in the adult myocardial cell. Figs. 12 a, b, c are from typical myo-
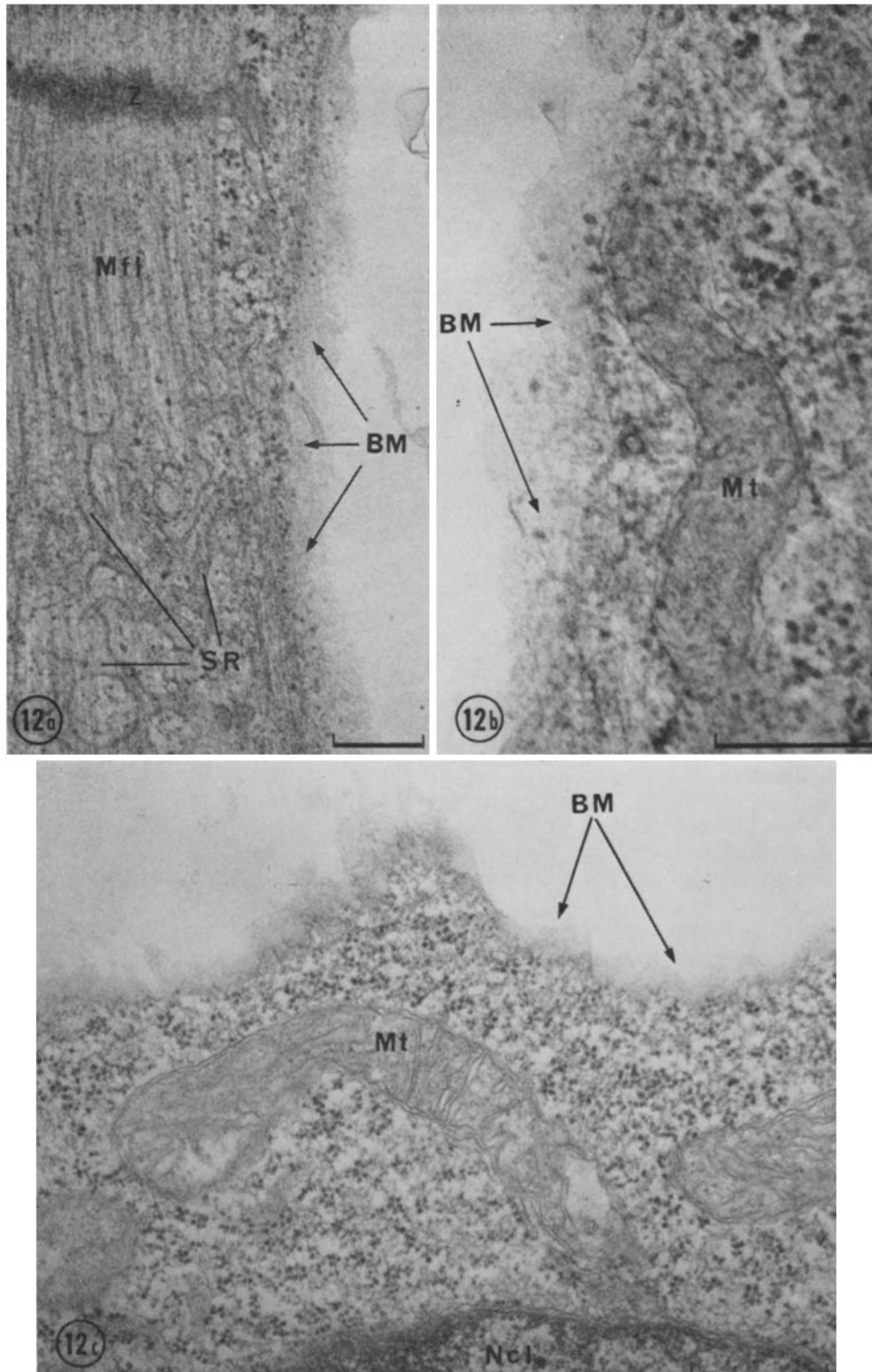
FIGURE 12 a, b, c Control myoblasts from 2-day culture cells. This culture was not exposed to lanthanum. Note the prominent basement membrane present on surface of these cells (BM). The wavy appearance of the basement membrane in c is quite common in developing myoblasts. In a a well-ordered sarcoplasmic reticulum is indicated (SR). Z, Z line, Mfl, myofilaments; Mt, mitochondria, Ncl, nucleus. Fig. 12 a. scale, 0.25 μ; $\times$ 52,500. Fig. 12 b: scale, 0.25 μ; $\times$ 96,250. Fig. 12 c: scale, 0.25 μ; $\times$ 70,000.

cardial cells grown in tissue culture, fixed, and embedded as previously described These cells were not exposed to lanthanum and served as controls. The prominent basement membrane is obvious in these electron micrographs The thickness of this surface coating varies from 0 06 to 0.1 μ and, as Fig. 12 c indicates, this coating often has a wavelike appearance Comparison of non-lanthanum-treated basement membrane configuration with the pattern of lanthanum density clearly emphasizes the predilection of lanthanum for this structure in heart cells

DISCUSSION

Effect of La^{+++} on Neonatal Rat Heart Cells

The ability of La^{+++} to uncouple the excitation-contraction process in 3 day old rat heart cells (Fig. 1) indicates that its action is similar to that found in the adult rabbit heart (12) The action potential spike is preserved in undiminished amplitude at a time when force development has been almost completely abolished. The loss of a portion of the plateau component of the action potential indicates that La^{+++} alters ionic conductances to a considerable degree during this phase but that the current responsible for the spike is little affected It is clear that the abolition of contractile force cannot be attributed to a failure of the excitation process

Characteristics of Ca^{++} Exchange

The cells achieve virtual asymptotic ^{45}Ca activity within a 2 hr labeling period At this time 75% of the total cellular Ca^{++} has been exchanged (Table I) All of this Ca^{++} exchanges as a two-phase or two-component system The fast phase (λ_1 = 0 60 min^{-1}, $t \frac{1}{2}$ = 1.15 min) accounts for 43% of the exchangeable pool, or 6 4 mmoles/kg dry weight The remainder of the exchangeable Ca^{++} exchanges much more slowly (λ_2 = 0 036 min^{-1}, $t \frac{1}{2}$ = 19 2 min) The remaining 25% of the cellular Ca^{++} is virtually inexchangeable, as demonstrated by the fact that none is labeled within a 2 hr period.

Pure fibroblastic cells demonstrate the same pattern with fast and slow components of exchange (Fig 7). This pattern appears to be common to a wide range of cell types The washout pattern of HeLa cells described by Borle (3)

is remarkably similar to that described in the present study Using a different technique, Borle found a fast phase with a $t \frac{1}{2}$ of 1 58 min and a slow phase with a $t \frac{1}{2}$ of 33 0 min. The exchangeable Ca^{++} attributable to the fast component was 40% of that attributable to the slow component The present study indicates a higher percentage attributable to the fast component of heart cells—75% that of the slow component (Table II).

The fast and slow phase fluxes are 0 66 and 0 05 mmoles/kg wet weight per min, respectively, as computed from the rate constants and phase contents. This gives a total flux of 0.71 mmoles/kg wet weight per min in these cells which have been equilibrated to a 1 0 mM Ca^{++} medium. It is not realistic to place this flux in terms of per unit area membrane, since it is not possible to accurately estimate the cellular area across which the flux takes place The cellular flux can, however, be compared to the cellular components of flux as kinetically determined in rabbit ventricle (15). In tissue perfused with 1.0 mM (Ca)$_o$ the minimum cellular flux component is estimated at approximately 0 5 mmoles/kg cells per min This is within the same range as calculated for the culture cells, especially when it is possible that a fraction of flux attributed to the interstitium in rabbit ventricle represents a rapidly exchanging superficial cellular component

The Effects of La^{+++} on Ca^{++} Exchange

Fig 3 demonstrates that 0 5 mM La^{+++} produces a rapid displacement of ^{45}Ca from the cell and then almost completely inhibits further uptake. Observation of the cellular layer indicates that contraction ceases almost instantaneously upon the application of the La^{+++}·La^{+++}, at the concentration used, was capable of displacing 3.01 mmoles Ca^{++}/kg dry weight or 515 μmoles per kg wet weight from the cells The labeling curve in Fig 3 indicates that the La^{+++} displaceable Ca^{++} is all within the rapidly exchangeable component (phase 1) since the ^{45}Ca displacement is maximal after 8 min of labeling. This is confirmed by the washout study illustrated in Fig. 6 La^{+++} introduced at the 9th min of ^{45}Ca washout fails to displace any isotope and produces only a slowing of subsequent exchange At the 9th min, more than 99% of the rapidly exchangeable component has been washed out, whereas over 70%

of the slow phase label remains Phase 1 contains 6.4 mmoles/kg dry weight. This means that 0 5 mm La^{+++} is capable of displacing almost half of the cell's rapidly exchangeable Ca^{++} fraction.

In addition to displacement of Ca^{++} and inhibition of influx, La^{+++} markedly diminishes the efflux rate from both the phase 1 and phase 2 components (Figs 5, 8) The washout pattern is reduced to a single phase which exchanges at a rate only 15% that of phase 2. The total efflux is then reduced from 0 71 to 0 01 mmoles/kg wet cells per min Ca^{++} exchange virtually ceases. Since La^{+++} displaces only about 50% of rapidly exchangeable Ca^{++}, both phases contain ^{45}Ca label at the initiation of the washout with La^{+++} Therefore, both components of efflux are slowed to produce a washout with a single phase.

Exposure of the cell layer to 1% glutaraldehyde produced a small increase in ^{45}Ca uptake but did not significantly alter the response to La^{+++} (Fig 9) The displacement and inhibition of exchange are clearly apparent and are quantitatively similar to that of the cells not exposed to glutaraldehyde.

Localization of La^{+++}

The surface of the cells is covered by a layer of amorphous material varying in thickness from 0.06 to 0.1 μ (Figs 12, a, b, c). This layer has been termed the external lamina or basement membrane. It is a prominent constituent of adult heart cells (5). It is this superficial layer to which La^{+++} binds. It stains this region of the cell exclusively and is never observed to penetrate beyond the unit membrane structure (Figs. 10, 11). La^{+++} binds similarly to the fibroblastic cells. Therefore, the displacement of Ca^{++}, inhibition of Ca^{++} exchange, and abolition of contractile tension occur as La^{+++} is bound to the basement membrane of the heart cell.

The present study discloses that the rapidly exchangeable Ca^{++} (phase 1) is localized to the basement membrane and that about 1.1 mmoles/kg cells are bound to this region when the cells are perfused with 1.0 mm [Ca^{++}]$_o$. Approximately 0.5 mmole is displaceable by 0.5 mm [La^{+++}]$_o$. This is a large quantity of Ca^{++} localized to a superficial and rapidly exchangeable region If similar amounts are "stored" in this region in adult myocardium, the release of 5% upon excitation would be sufficient to saturate the contractile proteins It has been noted that

contractile tension in adult rabbit heart is dependent upon a pool of Ca^{++} (phase 1) which exchanges with a $\lambda_1 = 0\ 5\ \text{min}^{-1}$ ($t\frac{1}{2} = 1\ 4\,\text{min}$)— a rate similar to the rapidly exchangeable component of the cultured cells (15). The phase 1 component of rabbit heart was proposed to represent interstitial Ca^{++} and any additional Ca^{++} located at a superficial region of the cells in rapid equilibrium with interstitial Ca^{++}. The results of the present study are in accord with this proposal In addition, the effects of La^{+++} perfusion upon function and Ca^{++} exchange in rabbit ventricle were attributed to the ability of La^{+++} to effect a release of contractile-dependent Ca^{++} by modifying the normal permselectivity of a superficial membrane for activator Ca^{++} (12). The results of the present study indicate that the superficial membrane is the basement membrane.

The affinity of La^{+++} for surface sites was to be expected from previous work (4, 11). More recent studies (14) strongly support the conclusion that La^{+++} is binding to mucopolysaccharide or to a mucopolysaccharide-protein complex. The basis for binding of cations to this region is assumed to be negatively charged sites of mucopolysaccharides (13) La^{+++} has a marked affinity for these sites and thus consistently induces the displacement of Ca^{++} observed. In addition the occupation of these sites by a cation of high affinity prevents further Ca^{++} exchange This implies that negatively charged sites in the basement membrane play a crucial role in transmembrane Ca^{++} flux in mammalian heart muscle.

Previous results (12) and those of the present study in heart tissue should be considered in the light of the effect of La^{+++} upon skeletal muscle The effect of La on twitch tension of perfused guinea pig semimembranosus muscle is strikingly different from that found in the perfused myocardium Whereas 40 μM La^{+++} completely eliminates force development within 1 5 min in heart. 50 μM La^{+++} fails to reduce twitch tension by more than 20% over the course of 10 min of perfusion in the skeletal muscle (T. L. Rich and G. A Langer, unpublished observation) This indicates a fundamental difference in the E-C coupling process of the two tissues. In heart muscle the process depends upon the presence of negatively charged sites on or in the vicinity of the basement membrane. Elimination or reduction in the number of these sites results in displacement of Ca^{++} and inhibition of transmembrane Ca^{++} flux. These effects are closely correlated with the abolition of contractile tension. The fact that the

skeletal muscle twitch is relatively insensitive to La^{+++} indicates that superficially located negatively charged sites play little role in the E-C coupling process in this tissue.

Recent studies on heart (2) and skeletal (6) muscle are interpreted to suggest that the action potential results in a release of a small amount of Ca^{++} which does not directly couple to contractile protein but functions as a primer for release of larger amounts from internal sites of storage If this sequence is correct, the La^{+++} studies have to be interpreted to indicate that the priming Ca^{++} is derived from entirely different sites in heart and skeletal muscle. Alternatively, if the need for a priming release of Ca^{++} is eliminated, it can be suggested that the major store of that Ca which moves directly to the myofilaments is superficially located on or near the basement membrane in heart tissue The store in skeletal muscle would be located at a deeper site internal to the sarcolemma—a site not accessible to La^{+++}. This would be consistent with the proposal (9) that the sarcotubular system functions predominantly as a Ca^{++}-sequestering system and plays a minor role in release of activator Ca^{++} in mammalian heart muscle In skeletal muscle the sarcotubules would both release (lateral cisternae) and sequester (medial tubular system) and Ca^{++} would circulate internally (16) Present information does not permit a final definition of the coupling sequence but it clearly validates a fundamental difference in the process as it occurs in heart and in skeletal muscle

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