Correlation of Alterations in Cation Exchange and Sarcolemmal Ultrastructure Produced by Neuraminidase and Phospholipases in Cardiac Cell Tissue Culture

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SUMMARY Myoblasts and fibroblasts in cultures derived from neonatal rat hearts were exposed to neuraminidase and phospholipases C (PLC) and A1 (PLA1). Calcium (Ca) and potassium (K) exchange of the cells was measured before and after enzymatic exposure. The exchange characteristics of control and treated cells were correlated with cellular ultrastructure including assessment of intramembrane particle (IMP) density and aggregation by freeze-fracture. Neuraminidase exposure (removal of sialic acid) known to produce marked increase in calcium permeability without change in potassium permeability in myoblasts produced no change in IMP configuration of these cells. PLC, however, produced marked increase in both Ca and K permeabilities, permitted entry of La, and was associated with IMP aggregation in myoblastic cells. PLA1 produced no change in ionic permeability and no alteration in intramembrane particle configuration in myoblasts. Exposure of fibroblasts to PLC caused no change in either Ca or K permeability and no change in IMP distribution. These results, coupled with those of previous studies of permeability changes induced by sialic acid removal, indicate that control of cellular Ca permeability resides in at least two separate sites at the cellular surface: (1) the glycocalyx and (2) the lipid bilayer. By contrast, K permeability control is based within the bilayer. Ultrastructural correlations suggest that IMP aggregation may be associated with changes in bilayer permeability. Circ Res 49:1289-1299, 1981

THERE is increasing evidence which indicates that surface structure of the myocardial cell is damaged by ischemia and anoxia (Jennings et al., 1975; Hearse et al., 1975; Frank et al., 1980) and that this damage leads to marked changes in cellular permeability to calcium (Ca) (Burton et al., 1977; Shen and Jennings, 1972). In addition, it is well known that exposure of the myocardium to solutions that contain no Ca produces major changes in membrane permeability upon reintroduction of Ca—the "Ca paradox" (Zimmerman and Hulsmann, 1966; Crevey et al., 1978). All of these interventions (anoxia, ischemia, zero Ca perfusion) produce various ultrastructural changes in the cell membrane, and it has been implied that the structural changes are related to the effects on permeability. It is difficult, however, to draw further conclusions until more is known about the molecular aspects of permeability control at cell membrane sites.

A first approach to molecular dissection of permeability control in myocardium involved consideration of sialic acid, a major constituent of the glycocalyx of cardiac cells (Langer et al., 1976; Frank et al., 1977). It was found in cultured heart cells that enzymatic removal of sialic acid produced a marked increase in cellular Ca permeability but no change in K permeability. This study indicated that a component of the glycocalyx, apart from the lipid bilayer per se, was important in maintenance of selective Ca permeability. That the role of sialic acid may vary with preparation, species, or age is suggested by a recent study (Harding and Halliday, 1980) in which 79% removal of sialic acid had no effect on contractile function of guinea pig atria. In contrast, however, Nathan and Bhattacharyya (1981) found marked electrophysiological effects of sialic acid removal in dog Purkinje fibers.

The present study attempts to provide more information with respect to the relationship among molecular constituents, membrane ultrastructure, and ionic permeability in the living myocardial cell. We are cognizant of the dangers of conclusions that depend upon ultrastructural and functional correlations but believe it important to make observations in a carefully monitored and controlled living system. The results can then be compared with those from artificial systems with the hope that, albeit slowly, a realistic concept of permeability control can be developed.
Methods

Technique for On-Line Measurement of Isotopic Exchange

This methodology has been described recently in detail (Frank et al., 1977). The technique depends upon the growth of a heart cell monolayer on one surface of each of two disks composed of polystyrene combined with a scintillant (Nuclear Enterprises). The disks, 45 mm in diameter by 1 mm thick, are placed on the bottom of a 50-mm culture dish to receive the trypsinized cells from 2- to 4-day-old neonatal rats. The culture technique is standard (Harary and Farley, 1963) with the modification of Blondel et al. (1971) to ensure a high standard of myoblasts. After 2-3 days of culture, the cells are 80-90% myoblastic as defined by electron microscopic examination, are confluent, and beating synchronously.

After the cells have established a synchronously beating monolayer, the disks are mounted in a specially designed flow cell (see Frank et al. (1977) for illustration of flow cell) such that the disks form a portion of each side of the flow cell chamber. The surfaces to which the cells are attached are directed inward so as to be perfused by the fluid flowing through the chamber of the flow cell. The flow cell, with disks in place, is inserted into the well of a modified Beta-Mate II spectrometer (Beckman Instruments) so that the disks are within 4.5 mm of the spectrometer’s opposed photomultiplier tubes.

With the flow cell in place, perfusate enters through four ports and is directed over the surface of the monolayer of cells attached to the inner surface of the scintillator disks. Effluent exists through a single port at the top of the flow cell. The standard perfusate for both kinetic and ultrastructural studies had the following composition (IDH):

\[
\begin{align*}
\text{NaCl, 133; KCl, 3.6; CaCl}_2, 1.0; \text{MgCl}_2, 0.3; \text{glucose, 16.0;} \\
N_2\text{-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer, 3.0 (pH 7.1).}
\end{align*}
\]

For isotopic studies, \(^{45}\text{Ca}\) (ICN Corp.) was added at 1.0 \(\mu\text{Ci/ml}\) and \(^{42}\text{K}\) (New England Nuclear) at 0.25-1.0 \(\mu\text{Ci/ml}\). The cells were perfused with solution at 24°C.

The phospholipases used were obtained from Boehringer Mannheim. Phospholipase C (PLC) from Bacillus cereus was used at concentrations of 0.0025-0.01 mg/ml (1-4 U/ml) and cellular incubation times from 5 to 15 minutes (37°C, pH 7.1). Phospholipase \(A_2\) (PLA\(_2\)) from Crotalus adamanteus venom was used at a concentration of 0.02-0.1 mg/ml (4-20 U/ml) and a cellular incubation of 15 minutes (37°C, pH 7.5). Both enzymes were assayed with extracted lipid from whole cells as a substrate. Neuraminidase (Worthington Biochemicals) was a highly purified preparation (Hatton, Regeczi, 1973). It was used at 0.25 U/ml concentration and cells were exposed for 15-30 minutes. The enzyme had no detectable proteolytic activity and the equivalent of 0.0025 U/ml phospholipase activity. This level of phospholipase had no effect on the cells (Frank et al., 1977).

As stated above, the myoblastic cultures contained 80-90% muscle cells. Pure fibroblastic cultures were obtained for study by culturing the cells which settled onto the discs during the first 1-2 hours of incubation (Blondel et al., 1971). These cultures formed monolayers consisting of 100% fibroblasts within 2-3 days.

With the disks in place in the flow cell and the flow cell inserted into the spectrometer, the monolayers are perfused for 30 minutes with non-isotopic standard perfusate. When \(^{45}\text{Ca}\) is used, both cellular uptake and washout of the isotope can be followed continuously. This is because the relatively low-energy \(\beta\) emission of \(^{45}\text{Ca}\) is largely self-absorbed in the perfusion solution while the \(^{45}\text{Ca}\) uptake by the cellular monolayer counts with an efficiency of greater than 70%. At the completion of isotopic labeling or washout, the disks are removed from the flow cell, the surfaces briefly rinsed to remove unbound isotope, and the cells scraped onto a 10- to 15-mg piece of predried, weighed Millipore filter. The cells are then dried overnight at 100°C and the dry weight obtained. Previously (Langer and Frank, 1972; Frank et al., 1977), after removal of the monolayer from the disks, they were rinsed, returned to the flow cell, and exposed to the same labeling solution used in the experiment. This procedure established a “blank” recording. It has been found that the monolayer screens a fraction of the \(^{45}\text{Ca}\) emission from the perfusate during the labeling period such that the “blank” recording is artificially high relative to the actual level operative during the labeling of the cells. This is because, during measurement of the “blank,” the cellular screening layer is no longer present. This results in a consistent underestimation of the \(^{45}\text{Ca}\) content as obtained from labeling curves. This problem is largely circumvented by the addition of 1 mm lanthanum (La) to the \(^{45}\text{Ca}\)-labeling solution at the end of uptake experiments. It has been ascertained that this consistently removes 90-100% of the exchangeable Ca from normal cultured cells, since virtually all of the exchangeable Ca is La-accessible. In those experiments where Ca is introduced into other compartments and may not be La-accessible, the labeled Ca values as derived by the subtraction of the “blank” will be somewhat underestimated. The “screening effect” is, of course, not operative during isotopic washout studies when there is no isotopic activity in the perfusate.

\(^{45}\text{Ca}\) labeling cannot be followed in the flow cell because of the high energy of emission which prevents separation of cellular uptake from total activ-
ity in the flow cell. Therefore, the cells on the disks were labeled and then inserted in the flow cell for measurement of the $^{42}$K washout pattern.

The unique aspect of the technique is the growth of the cells directly on the isotopic detector (the scintillator plastic disks). This permits continuous counting of $^{44}$Ca uptake and $^{44}$Ca and $^{42}$K washout of the cellular layer. In many cases, the responses of isotopic exchange under control conditions and the response to an intervention can be evaluated in the same culture.

**Electron Microscopy**

**Thin Sections of Cell Cultures**

All cell cultures were fixed and processed for thin-section electron microscopy on culture dishes as previously described (Langer and Frank, 1972). The embedded cell monolayers were removed from the plastic Petri dishes for sectioning or re-embedded in the cross-sectional plane. The sections were cut with diamond knives on a Porter-Blum MT-2 ultramicrotome (Sorvall Operations, DuPont Inst.). The sections were placed on uncoated 300 mesh grids and post-stained with uranyl-acetate and lead citrate. All observations were made with a Siemens 1A electron microscope at 80 kV.

**Freeze-Fracture of Cell Cultures**

Cell populations were fixed on culture dishes with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10–15 minutes at room temperature. After a buffer rinse, the cells were exposed to 10 minutes of 12% glycerol in 0.1 M sodium cacodylate buffer followed by 1 hour in a 25% glycerol-buffered solution. After 45 minutes in 25% glycerol, the cells were removed from the dishes by gentle lifting with a plastic coverslip and small sheets of cells were folded onto 3-mm gold-alloy hats (Balzers Corp.).

In addition, some cultured cells were grown on plastic coverslips placed in a Petri dish. Fixation and glycination was the same as for cells grown directly in culture dishes. Circles, 2 mm in diameter, rich in cells, were scored and cut from the coverslip and inverted over a drop of Vinol 205 S (Air Products and Chemicals) that was placed on the gold-alloy hat (Pauli et al., 1977). With this method, the cultured cells could be fractured without removal from their substrate, and this was done to verify that lifting of the monolayer from their substrate did not affect the experimental results.

Methods were applied for freezing the cultured cells that would exclude the formation of ice crystals. While some tissue was rapidly quenched in liquid Freon 22 cooled to its freezing point by liquid N$_2$, the bulk of the tissue was done using the equipment described by Sjostrand and Kretzer (1975). The gold hats with cells were frozen in liquid propane at $-180^\circ$C using liquid N$_2$ to cool the propane. The temperature of the propane was kept uniform by rapid stirring.

For freeze-fracturing, a Balzers model BA 301 (Balzers High Vacuum Corp.) freeze-etch unit was used. The transfer of the specimen to the pre-cooled specimen table ($-170^\circ$C) was accomplished with a specially designed cover that fit the specimen carrier providing a large cold mass over the cells at all times in the transfer and allowed the specimen to be inserted into the chamber in 1 second or less. Fracturing was performed at $-120^\circ$C at a vacuum of 2 x 10$^{-7}$ torr. Shadowing (45° angle) immediately followed with 20 A platinum-carbon layer. The replicas were cleaned in bleach in distilled water before being mounted on 300 mesh grids. All electron microscope observations were made with a Siemens 1A.

**Quantification of Intramembrane Particle Density and Aggregation**

Particle densities per $\mu m^2$ were determined on control myoblasts and fibroblasts, and on myoblasts exposed to neuraminidase and PLA$_2$. In all instances, care was taken to avoid areas where excessive curvature of the membrane exaggerated the shadowing of the particles, and our counting was limited to areas that contained particles with shadows and diameters of equal length. Particle densities were determined by projecting and magnifying (to final magnification of 300,000) the negative directly on a viewing screen using a Nikon Profile Projector as previously described (Frank et al., 1980). In the experiments that caused aggregation of the integral particles, no selection was made on the basis of the degrees of aggregation and each aggregated membrane fracture was simply quantified by visual comparison as mild, medium, or severe aggregation.

**Results**

**Sialic Acid Removal**

It has been previously demonstrated (Langer et al., 1978, Frank et al., 1977) that removal of sialic acid from the myoblast cell surface markedly increased cellular Ca permeability without affecting K permeability. Before proceeding to examination of the effects of phospholipid alteration, the effect of sialic acid on intramembranous structure was examined by freeze-fracture electron microscopy.

The cultured cells were fractured in two ways: (1) still attached to their substrate (Pauli et al., 1977) and (2) removed from substrate by gentle lifting of the monolayers onto specimen carriers. There were no differences in fractured cells done by these two methods and, as a result, removal of the cells from their substrate was found to be the easier preparation and was the predominant method used. Figure 1, b and c, shows the characteristic fracture faces E (adjacent to the extracellular space) and P (adjacent to cytoplasm) for control myoblasts. As is typical for most cells so far examined, the P face
has the higher density of 85–100 Å particles (McNutt, 1977). Particle density for the P face of control cultured myoblasts was 1773 ± 59 per µm² (SEM) whereas the E face had 295 ± 35 per µm².

Freeze fracture EM of membranes from cells treated with concentrations of neuraminidase that consistently produced significant increase in Ca permeability (0.25 U/ml for 15–30 minutes) produced no discernible change in intramembranous particle distribution or density. P face counts of 1712 ± 81 µm² were recorded with neuraminidase compared to 1773 ± 59/µm² for control.

Therefore, sialic acid removal from glycocalyx produces marked increase in Ca permeability, no increase in K permeability, and no ultrastructural evidence of change in intramembrane particles. It
now remained to alter the bilayer directly and correlate ultrastructural and permeability changes. We elected to alter phospholipid structure using PLC and PLÅ2.

Phospholipase Treatment

Because the enzymes contained small (<0.002 U)—but measurable—protease activity, cultures were exposed to 0.002 U protease (Sigma, type VI) and Ca and K exchange was measured. In five cultures, exchangeable Ca was 3.16 mmol ± 0.47 mmol/kg dry weight, not significantly different from control. K loss after 5 minutes of exposure to protease was 29.4 ± 2.9% above control in four cultures. The lowest concentrations of PLC, containing less than 25% the protease activity tested demonstrated 2.5 times the K loss (see below). These results make it unlikely that permeability alterations could be ascribed to protease contamination. It could be argued that the protease contaminant is different from that in the Sigma enzyme used for the test. The fact that fibroblasts exposed to the highest concentration of PLC for 3 times as long showed no permeability changes (see below) makes a protease effect unlikely.

Calcium Exchange

The cultures used for evaluation of the effect of the phospholipases on myoblasts were 80-90% myoblastic; the remainder of the cells were fibroblastic. Given the lack of effect of the enzymes on fibroblasts (see below), it was important to use highly myoblastic cultures for this series. In our evaluation of the enzymatic effect on fibroblasts, 100% fibroblastic cultures were used.

Figure 2 summarizes the effects of PLC and PLÅ2 on exchangeable Ca of myoblasts and fibroblasts. The inset illustrates the typical pattern of 45Ca uptake in all experiments for both cell types. For 45Ca labeling experiments flow rate was 10 ml/min in order to conserve isotope. At this perfusion rate, the rate of 45Ca uptake by the cells is perfusion-
limited (Langer et al., 1979) and, thus, particular
significance cannot be attached to the early time
course of the uptakes. It should be noted (in the
inset) that asymptotic labeling is reached in less
than 10 minutes. In all experiments, this level re-
mained absolutely stable for at least 40 minutes,
and it is this level from which exchangeable Ca is
calculated.

The total exchangeable Ca in the control cultures
was 2.72 ± 0.19 (se) mmol/kg dry weight cells. As
indicated in Figure 2, exposure of the cells to a high
concentration of PLA₂ produced no increase in ex-
changeable Ca in the cells. A total of six cultures
exposed to 0.02 mg/ml PLA₂ for 15 minutes dem-
onstrated an exchangeable Ca of 2.32 ± 0.34
mmoles/kg dry weight. A 5-fold increase in PLA₂
concentration (0.1 mg/ml) was also without effect
on Ca uptake. Five cultures exposed to 0.1 mg/ml
gave a mean value of 2.33 ± 0.18 mmol/kg dry
weight. On the basis of comparative enzyme activ-
ities, this concentration of PLA₂ was 5 times that of
PLC. Thus PLA₂ had no effect on Ca exchange.
That the PLA₂ was acting on the myoblasts was
confirmed qualitatively by thin layer chromatogra-
phy. A small spot corresponding to lyso phosphati-
dycholine was observable after PLA₂ treatment,
whereas this phospholipid was not detectable in
untreated controls. Exposure of fibroblastic cultures
to PLA₂ was also without effect on the level of
exchangeable Ca (not shown in Fig. 2).

In contrast to the lack of effect of PLA₂, PLC had
a significant effect on myoblastic cultures, as indi-
cated in Figure 2. Application of 0.01 mg/ml PLC
for 15 minutes increased exchangeable Ca to 12.03
mmol/kg dry weight—a highly significant increase
(P < 0.001). This dose and exposure produced re-
gions of cellular damage as determined by electron
microscopy which could, secondarily, augment Ca
uptake. Therefore a group of six muscles was ex-
posed for one-third the time to either one-half or
one-quarter the PLC concentration. As indicated in
Figure 2, the mean uptake for this group was 6.56
± 0.57 mmol/kg dry weight, which is significantly
above control (P < 0.001).

Ca uptake in six pure fibroblastic cultures indi-
cated an exchangeable Ca of 3.11 ± 0.59 mmol Ca/
kg dry weight (Fig. 2). Exposure of six cultures to
0.01 mg/ml PLC for 45 minutes (3 times the period
for the myoblasts) resulted in an uptake indicating
an exchangeable Ca of 2.83 ± 0.79 mmol—not sig-
nificantly different from control. Analysis of total
phospholipid content of a fibroblastic culture un-
treated with enzyme indicated 0.23 μmol phospho-
lipid per mg protein. A paired PLC-treated fib-
roblastic culture gave a value of 0.22 μmol, i.e., no
change. A paired, untreated myoblast culture gave
0.21 μmol and a culture treated with the same
enzyme used on the fibroblasts gave 0.07 μmol—a
loss of two-thirds of the phospholipid from the
myoblasts. The loss of phospholipid correlates with
the effect of PLC on Ca uptake in the two types of
cell.

In another group of experiments, myoblastic cells
exposed to high concentration (0.01 mg/ml) of PLC
were allowed to take up ⁴⁶Ca until asymptote was
reached. They were then exposed to 0.5-1.0 mM
lanthanum (La) in order to determine the quantity of
La-displaceable Ca after enzyme treatment. In six
experiments, 74.0 ± 2.9% of the exchangeable Ca
was La displaceable. After exposure to lower con-
centrations of enzyme (0.005 or 0.0025 mg/ml), La
displaced 79.2 ± 4.3%. An example of this pattern is
illustrated in Figure 3. Pretreatment of a culture
with 0.0025 mg/ml PLC for 5 minutes was followed
by the ⁴⁶Ca uptake indicated. The asymptotic level
(10,400 cpm) represented 8.07 mmol Ca/kg dry wt.
Application of 1 mM La produced a rapid displace-
ment of 78.8% of the exchangeable Ca. The pattern
of ⁴⁶Ca uptake and La displacement in control cul-
tures is illustrated for comparison. The La displace-
ments after PLC are comparable to the 83% dis-
placement recorded after neuraminidase treatment of
the cells (Frank et al., 1977). These kinetic data
indicate that PLC treatment alters the normally La
impermeable membrane to permit cellular La entry
and subsequent displacement of a component of
acellular Ca. This was confirmed ultrastructurally.

Since it had been shown previously (Langer et
al., 1976; Frank et al., 1977) that specific removal of
sialic acid from the glycosalyx produced a large
increase in Ca permeability, it was necessary to
measure the effect of PLC on the sialic acid content
of these cells. A series of five cultures was exposed
to the highest concentration of PLC used (0.01 mg/

\[ \text{Figure 3} \quad \text{⁴⁶Ca uptake (counts/min) in a myoblastic culture previously exposed to 0.0025 mg/ml PLC for 5 minutes. The asymptote represents 8.07 mmol Ca/kg dry weight. At 40 minutes, 1 mM La was added to the} \quad \text{⁴⁶Ca-labeled perfusate. This produced displacement of 78.8\% of the label within 10 minutes. The pattern of} \quad \text{⁴⁶Ca uptake and displacement by La in cultures not exposed to PLC is illustrated for comparison.} \]
ml for 15 minutes), the cells washed and sialic acid content measured according to the method of Warren (1959). The content of the PLC-treated cells was $18.2 \pm 0.94 \times 10^{-9}$ mol per mg cellular protein as compared to the content of five matched controls of $15.4 \pm 1.29 \times 10^{-9}$ mol. Clearly, high dose PLC did not produce a loss of sialic acid from the cells.

**Potassium Exchange**

The procedure for the determination of the effect of the enzymes on K exchange in myoblasts is indicated in Figure 4, which illustrates $^{42}$K washouts from three cultures. All cultures were labeled with K at a specific activity of 0.25-1.0 μCi/ml for 60 minutes prior to the start of the washout. The curve at the top is a control washout in which perfusion rate was at 24 ml/min used for all washouts. The initial few minutes of washout is not plotted, since this represents clearance of the flow cell $^{42}$K activity. The plot then follows a single exponential course with a rate constant ($\lambda$) of 0.016/min which represents cellular washout. After 19-20 minutes, the flow cell was quickly removed from the spectrometer, the discs removed from the flow cell, incubated at 37°C in perfusion medium for 15 minutes, and returned to the flow cell for continuation of the washout. This 18-minute period is indicated by the broken line extrapolation of the initial washout period. Note that, upon resumption of washout, the cells had lost 10% more K activity while out of the flow cell (indicated by arrow) than was predicted for the 10-minute period. The same pattern was confirmed with another control sequence in which the loss of activity was 15% below predicted after an 18-minute period. These small losses may be explained by the 15-minute incubation at 37°C as compared to the 24°C flow cell temperature and by some elevation of extracellular K as the disks incubate in a dish with no flow. Both these factors would be expected to increase K exchange.

Incubation of the cells with 0.01 mg/ml PLC for 15 minutes produced a total loss of $^{42}$K upon return to the flow cell. This was consistent with the extensive cellular damage noted. The effect of a lower concentration of PLC and shorter exposure is shown in the lower curve in Figure 4. In this experiment, an exponential washout ($\lambda = 0.012$) was established for 19 minutes at which time the disks were removed and exposed to 0.0025 mg/ml PLC for just 5 minutes at 37°C. The disks then were replaced in

![Figure 4](http://circres.ahajournals.org/)

**Figure 4** $^{42}$K washout pattern in three myoblastic cultures. After a 1-hour period of labeling, each culture was washed out for 19-20 minutes. At this point, the cultures were removed from the flow cell and incubated for varying periods (broken lines). Replacement of the cultures in the flow cell and resumption of perfusion is indicated by the arrows. Note that the control culture in which the incubation solution contained no enzyme and the culture exposed to 0.02 mg/ml PL A for 15 minutes lost the same small amount of $^{42}$K during the incubation. By contrast, note that the culture incubated with 0.0025 mg/ml PLC for only 5 minutes lost nearly two-thirds of its $^{42}$K label.
the flow cell (removal and replacement required 3 minutes) and ⁴²K washout reinstituted after the total 8-minute period (broken line extrapolation). Note, as indicated by the arrow, that the counts fell 64% below predicted. Exposure to low concentrations of PLC (0.0025-0.005 mg/ml) for 5 minutes in five cultures caused a mean loss of 71.8 ± 8.6% or 4-5 times that measured in the controls. Note that, upon reperfusion, washout of the remaining K then proceeded at a slowed rate (λ = 0.007), and this occurred in all PLC-exposed cultures.

The middle curve in Figure 4 shows the response to a 15-minute incubation with 0.02 mg/ml PLA₂. At reperfusion, the counts were 13% below predicted (as indicated by the arrow). In six cultures exposed to 0.1 mg/ml PLA₂ for 15 minutes, there

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**Figure 5** Thin section electron micrograph which illustrates portions of several myoblasts exposed to phospholipase C (0.0025 mg/ml) for 5 minutes. The myoblasts are intact but contain several small vesicles just under the sarcolemma in the cytoplasm (arrows) and what appear to be a few blebs coming off the sarcolemma (arrowheads) 85,000× Bar = 2.0 μm.
was a loss of counts 13.0 ± 6.3% below predicted. As noted previously, this concentration of PLA2 was 5 times that of PLC but still showed no significant effect on K exchange.

A series of fibroblastic cultures was subjected to the same sequence as were the myoblasts in order to determine the possible effect of PLC on K exchange in these cells. Four fibroblastic cultures exposed to 0.005 mg/ml PLC for 5 minutes demonstrated a net loss of $^{42}$K activity 32.3 ± 2.4% below that predicted for the incubation period. Three control cultures removed and incubated without PLC exposure showed a net loss of $^{42}$K activity 32.5 ± 4.0% over the 5-minute period. There was, therefore, no significant effect of PLC on K permeability under conditions in which the myoblasts showed a highly significant effect.

**Ultrastructure**

At PLC concentrations between 0.005 and 0.0025 mg/ml and incubation time of 5 minutes, which produced significant increases in Ca and K permeability (Figs. 2 and 4), the cells showed no major intracellular abnormalities by thin section electron microscopic analysis (Fig. 5). There were, however, numbers of vesicles under the cell surfaces (arrows), whereas numerous blebs appeared in the cell mem-

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**Figure 6** Freeze-fracture electron micrograph illustrating the degree of aggregation of intramembrane particles seen in the P face of the sarcolemma of myoblasts exposed to 0.005 mg/ml PLC for 5 minutes before fixation. A: slight clustering of particles. 61,240x. B and C: moderate clustering of particles. 64,400x. D: severe clustering. 61,390x. Bars = 0.2 μm.
brane (arrowheads). Other structural components of the cells were not affected.

Ultrastructural evidence of increased sarcotubular permeability was found after incubation of myoblasts for 5 minutes with 0.0025 mg/ml PLC followed by exposure to 1 mM La for 5 minutes. La was deposited throughout the cytoplasm. In intact cells, La is confined to the sarcolemma and cannot be detected intracellularly (Langer and Frank, 1972). Perturbations to the membrane affecting ionic permeability have been shown to permit La entry into the cytoplasm (Langer et al., 1976; Frank et al., 1977).

After exposure of the cultured cells to PLC for 5 minutes at concentrations of 0.0025-0.005 mg/ml, freeze-fracture demonstrated that IMP's were aggregated and no longer randomly distributed in the P fracture face (Fig. 6). All myoblasts examined had IMP aggregation after treatment with PLC but the degree of aggregation varied from cell to cell. Most cells (>60%) had IMP aggregation that was graded as moderate as in Figure 6, b and c; i.e., there were definitely irregularly distributed particle clusters with large smooth fracture faces in the membrane. Approximately 10% of the cells had a slight degree of aggregation (Fig. 6a) and approximately 25% had severe aggregation, as seen in Figure 6d. The particle density was not determined in PLC cells because of the degree of aggregation made particle counting unsatisfactory.

The IMP aggregation seen with PLC is not a non-specific effect of phospholipase in general, because exposure of the cultured cells to PLA2(0.02 mg/ml for 15 minutes at pH 7.5) showed no morphological change in IMP distribution or density. Particle density with PLA2 tested cells was similar to control at 1756 ± 95 per μm².

Fibroblasts were exposed to PLC at a higher concentration (0.01 mg/ml) for 15 minutes. This exposure produced marked alteration of myoblastic structure. Little structural change was evident in the fibroblasts and no IMP aggregation was discernible in the P face. IMP densities for P face of control fibroblasts were generally 30% lower than control myoblasts but there was a wide variation. This variation may be related to rapid cell division which occurs in cultured fibroblasts.

Discussion

Previous work (Langer et al., 1976; Frank et al., 1977) demonstrated that modification of glycocalyx molecular structure (removal of sialic acid) in cultured myocardial cells produced a specific increase in Ca permeability. The present study extends this work to modification of the bilayer with isotopic exchange and structural correlations.

One conclusion can be drawn with considerable certainty: calcium permeability control by the cultured cell resides in at least two separate sites at the cell surface. The fact that sialic acid removal is associated with marked increase in Ca permeability, no change in K permeability (Langer et al., 1976; Frank et al., 1977), and no alteration in IMP distribution or number (present study) indicates that disruption of glycocalyx structure without bilayer disruption alters Ca permeability. These responses apply to cells in culture and may be modified in other preparations (Harding and Halliday, 1980; Nathan and Bhattacharyya, 1981) as discussed on page 1289. Another control site is within the lipid bilayer in that PLC applied to the cell in small concentration and for a brief time is associated with marked changes in both Ca, La, and K permeability (Figs. 2-4) no loss of sialic acid, appearance of subsarcolemmal vesicles and sarcolemma blebs (Fig. 5) and marked aggregation of IMP in the P fracture face (Fig. 6). Disruption of the bilayer is less specific than sialic acid removal as evidenced by the increase in both Ca and K permeability.

Further conclusions about molecular relations at the bilayer are more speculative and less certain but should be mentioned. PLC cleaves the ester linkage at the 3 position of the phospholipids (between the carbon and the phosphoryl group (Abbott et al., 1972). It is possible that such cleavage is, per se, responsible for the permeability and structural changes observed. It is, however, possible that the products of the cleavage (1,2 diacylglycerol and the phosphorylated amines) could influence bilayer alterations (Deuticke et al., 1981) and induce the permeability and ultrastructural changes observed. PLA2 which cleaves the acyl group from the 2 position of the phospholipids (leaving a lysophosphoglyceride and a fatty acid anion) produced no effect on Ca and K permeability nor did it cause any structural change. Our qualitative measure of PLA2 action (by thin layer chromatography) did indicate that the enzyme was active at the cell surface but did not tell us the percentage of the sarcolemmal phospholipid actually affected. Verkleij et al. (1973) indicate that not all cell membranes have the same phospholipid orientation. Thompson (1978), in reviewing phospholipid distribution in membrane, found in five membrane systems examined that none showed a symmetrical distribution of lipid. The pattern of asymmetry was very different in the different membranes with, for example, phosphatidylserine being predominantly on the membrane's outer surface in one system and predominantly on the inner surface in another. Therefore the lack of effect of PLA2 on myoblasts and PLC on fibroblasts may be due to inaccessibility of molecules to the enzymes. If the substrates were accessible, their disruption might produce permeability and structural changes.

Finally, it is difficult to assess the significance of IMP aggregation because it is not yet known how aggregates of integral proteins are formed. However, aggregation of IMP's as observed with PLC treatment is not artificial. It was never seen in control cultured cells and, of greater significance, it
occurred only on treatment with PLC where permeability to K, Ca, and La was increased. It did not occur on treatment with PLA₂ or neuraminidase, or in fibroblasts treated with the highest concentration of PLC. The lack of any aggregation of the IMP's in fibroblast cultures exposed to PLC makes unlikely the possibility that protease contamination contributed to the membrane structural changes.

It is not difficult to imagine mechanisms whereby aggregation of integral proteins might induce an increase in ionic permeability. It may be that by bringing integral membrane proteins into close apposition, transmembrane pathways are introduced in the membrane between adjacent proteins as the direct result of the altered lipid moieties. Current theories of membrane structure (Haydon, 1975; Griffith and Jost, 1978) propose that the hydrophobic portion of the integral protein is surrounded by a layer of phospholipid which may immobilize the protein and play a role in its orientation. Disruption of the associated lipid might well alter the orientation of the integral proteins and thereby the structure of the associated lipid moieties. Such alteration of structure might be expected to affect the permeability of both Ca and K.

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