

EFFECTS OF PHOSPHOLIPASE C ON ACTION POTENTIALS AND INTRACELLULAR Ca^{2+} CONCENTRATION IN GUINEA PIG HEART

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To clarify the role of phospholipase C (PLC) in arrhythmias and cell injury during myocardial ischemia/reperfusion, we studied its effects on electrophysiology and $[\text{Ca}^{2+}]_i$ in guinea pig hearts. After exposure to PLC (1 and 2 U/ml), the action potential durations of right ventricular papillary muscles were decreased. Delayed afterdepolarizations were observed in all of the preparations, and some developed into triggered activities. Developed tension decreased after an initial increase for the first 5 min, while resting tension increased consistently. The effects of PLC (0.02, 0.1, and 0.2 U/ml) on $[\text{Ca}^{2+}]_i$ of ventricular myocytes were measured using fura-2 fluorescence. The ratio of rod-shaped cells to all cells decreased in a time- and a concentration-dependent manner. Perfusion with 0.1 U/ml PLC elevated $[\text{Ca}^{2+}]_i$ from 56 ± 5 nM to 245 ± 47 nM before cell rounding, and to 1167 ± 172 nM after cell rounding, suggesting that PLC causes Ca^{2+} overload. In conclusion, activation of PLC may play a role in arrhythmias and cell injury during ischemia/reperfusion. The increase in $[\text{Ca}^{2+}]_i$ during ischemia/reperfusion may activate phospholipase, which would further increase $[\text{Ca}^{2+}]_i$ to form a vicious cycle. (*Jpn Circ J* 1993; 57: 344–352)

IRREVERSIBLE injury during myocardial ischemia/reperfusion may be associated with a sarcolemmal permeability defect^{1,2} which is manifested by the leakage of intracellular components into the extracellular space and disturbance of ion homeostasis, especially an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)^{3–5}. Furthermore, a significant depletion of membrane phospholipids occurs several hours after myocardial ischemia^{6,7} whereas that induced by reperfusion can be seen within an hour⁸. This cell membrane defect may be due to

accelerated phospholipid degradation or an imbalance between deacylation and reacylation of myocardial membrane phospholipids.⁹

phospholipase activity has been found in myocardial tissue in association with the plasma membrane¹⁰ as well as with mitochondria, microsomes and lysosomes^{11,12}. These endogenous phospholipases are stimulated to varying degrees by increased $[\text{Ca}^{2+}]_i$ ^{12,13} and may become active during ischemia¹⁴. Since the increase in $[\text{Ca}^{2+}]_i$ is the essential factor in the activation of phospholipases, Ca^{2+} influx associated with myocardial reperfusion¹⁵ might trigger the activation of phospholipase.

Since it has been reported that all detected free fatty acids, both saturated and unsatu-

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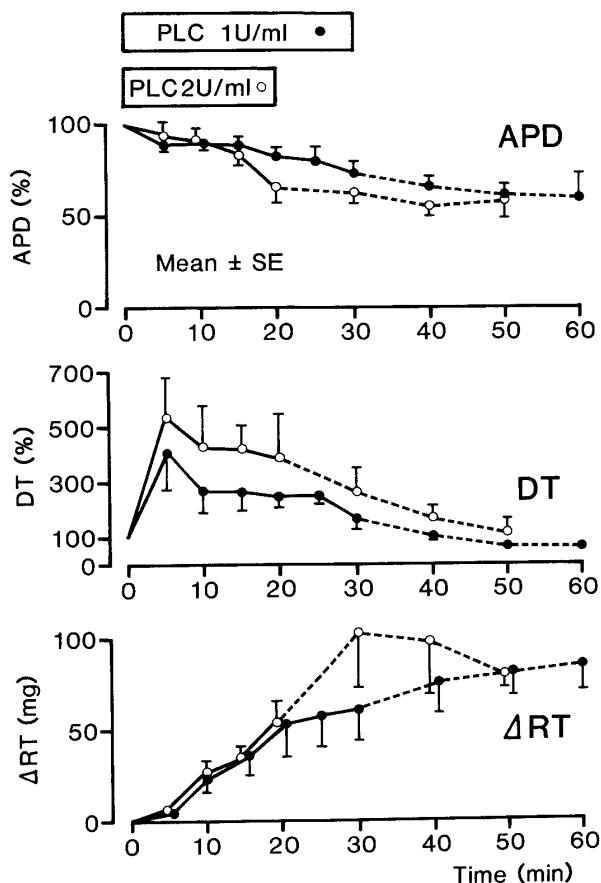


Fig.1. Effects of PLC on electrophysiological and contractile variables in guinea pig papillary muscles. The upper panel shows action potential duration measured at 75% repolarization (APD). The middle panel shows developed tension expressed as a percentage of the control value (DT), and the lower panel shows the increase in resting tension above the initial resting tension (Δ RT). Closed circles are the values with 1 U/ml PLC, and open circles are those with 2 U/ml PLC. Dotted lines indicate the washout of PLC. Values are mean \pm SE. $n=6$ with each concentration.

rated, were elevated in dogs with reperfusion arrhythmias, the initially activated phospholipase might be phospholipase C (PLC),¹⁶ although both phospholipase A₂(PLA₂)⁸ and lysophospholipase¹⁷ may also be activated. Langer et al¹⁸ have reported that PLA₂ produced no change in ionic permeability in cultured rat heart, while PLC did. Furthermore, phospholipase A₂ did not cause lysis of cultured cardiac myocyte membranes, but phospholipase C did!¹⁹

The functional consequence of phospholipid degradation is a marked impairment of membrane structure^{3,14} and function,¹⁸ even-

tually leading to irreversible cell injury. Accumulation of free fatty acids and lysophospholipids generated as a result of membrane phospholipid degradation, is also known to alter a variety of membrane-bound or membrane-associated enzymes by detergent action.^{20,21} Arrhythmia may result from the degradation of membrane function brought about by the disordered activation of phospholipases, since electrophysiologic properties are influenced by the functional integrity of the sarcolemmal membrane. Activation of phospholipase might also cause an increase in $[Ca^{2+}]_i$, since the membrane phospholipid bilayer constitutes an ionic permeability barrier which maintains a 10,000-fold Ca^{2+} gradient across the plasma membrane.

Very little is known about the functional consequences that may accompany activation of endogenous, inactive phospholipases. This study was undertaken to evaluate cellular electrophysiological changes produced by PLC in isolated guinea pig papillary muscles. The effects of PLC on cell morphology and $[Ca^{2+}]_i$ were also investigated using isolated guinea pig ventricular myocytes.

METHODS

Electrophysiological study

Papillary muscles were obtained from the right ventricles of guinea pig hearts. Animals weighing 250–300 g were sacrificed by cervical dislocation, and their hearts were removed and placed in modified Krebs solution (mM): NaCl 113.1, KCl 4.6, CaCl₂ 2.45, MgCl₂ 1.2, NaH₂PO₄ 3.5, NaHCO₃ 21.9 and glucose 5, equilibrated with 95% O₂-5% CO₂ (pH 7.4). Papillary muscles (about 3 mm long, 0.5 mm wide) were excised and mounted in a Perspex bath (1 ml) perfused with solution at $37 \pm 0.5^\circ\text{C}$. Mural ends of muscles were secured in a clamp and tendinous ends were tied by a silk thread to a stainless steel rod extending from the head of a force transducer. Stimulating pulses (1 Hz, 2 msec duration, $2 \times$ threshold intensity) were applied to muscles through a bipolar Ag-AgCl electrode. Muscle length was adjusted until the resting tension was 50–100 mg (sufficient for about 75% maximum twitch tension in control experiments). A 60 min equilibration period in oxygenated

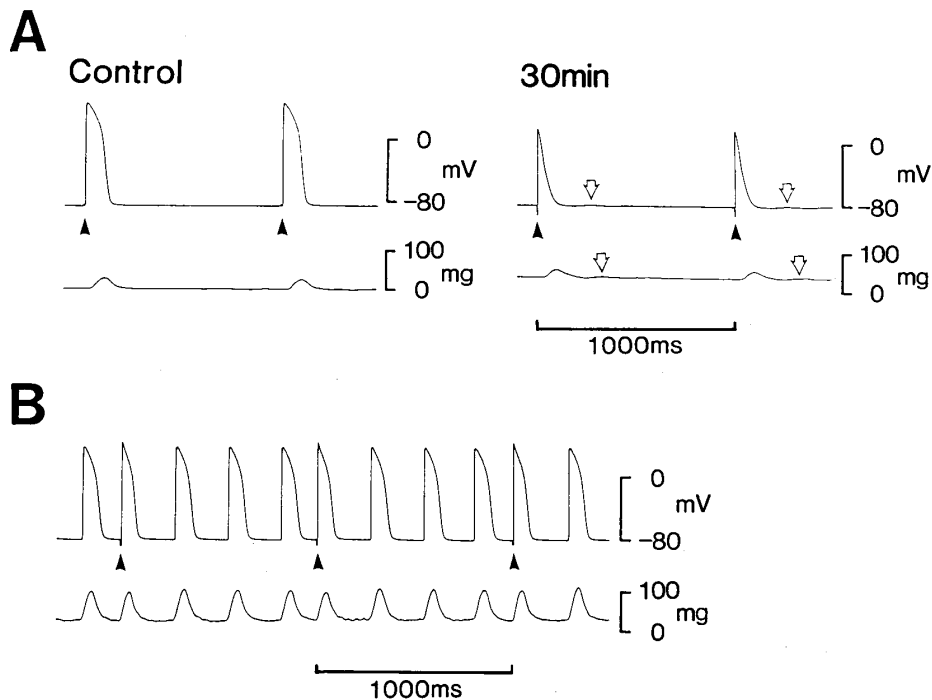


Fig.2. Effects of PLC on action potential and tension. (A) The records during the control perfusion (left), and after 30 min of perfusion with 1 U/ml PLC (right). Preparations were stimulated at a basic cycle length (BCL) of 1,000 ms, as shown by small arrowheads beneath the action potentials. Delayed afterdepolarizations and aftercontractions were observed after perfusion with PLC, as shown by the white arrows. (B) Triggered activity which developed in a muscle after 15 min of perfusion with 2 U/ml PLC. Stimulations at a BCL of 1,000 msec are indicated as small arrowheads beneath the action potentials.

Krebs solution preceded all experimental procedures.

Action potentials were recorded with a 3 M KCl-filled microelectrode (8–10 M Ω) connected to a high input impedance amplifier via an Ag-AgCl pellet. Action potentials and tension were displayed on a Tektronix oscilloscope.

Isolated ventricular cells

Ventricular myocytes were isolated from male guinea pigs (250–300 g) following a previously described method²². The hearts were excised, attached to the bottom of a Langendorff column (60 cm height), and perfused with solutions gassed with 95% O₂-5% CO₂ and maintained at 37 \pm 0.5 $^{\circ}$ C and pH 7.4. The first perfusate was calcium-free Krebs solution to wash out the blood remaining in the heart cavities and coronary arteries. After 3–4 min of the initial perfusion, 50 ml of low CaCl₂ (50 μ M) Krebs solution containing enzymes (100 U/ml collagenase type V and 500 U/ml trypsin

type III, Sigma Chemical Co.) were added to the column and perfused for 3–5 min. Calcium-free Krebs solution was then introduced to wash out the residual enzyme solution in the heart. The ventricles were cut into small fragments with iris scissors, and myocytes were dispersed by gentle agitation in oxygenated calcium-free Krebs solution. Finally, the calcium concentration in the cell suspension was raised to 2.45 mM by the addition of CaCl₂.

After isolation, cells were loaded with fura-2 by exposure to the acetoxymethyl ester, fura-2 AM, at a concentration of 5 μ M, at 37 $^{\circ}$ C for 40 min. Some of the fura-2-loaded cells were placed in an experimental chamber which was mounted on the stage of a Nikon TMD inverted microscope. Myocytes were perfused with modified Krebs solution equilibrated with 95% O₂-5% CO₂ (pH 7.4) and maintained at 37 \pm 0.5 $^{\circ}$ C. The cells could be illuminated by transmitted illuminator or ultraviolet (UV), light via an epifluorescence illumina-

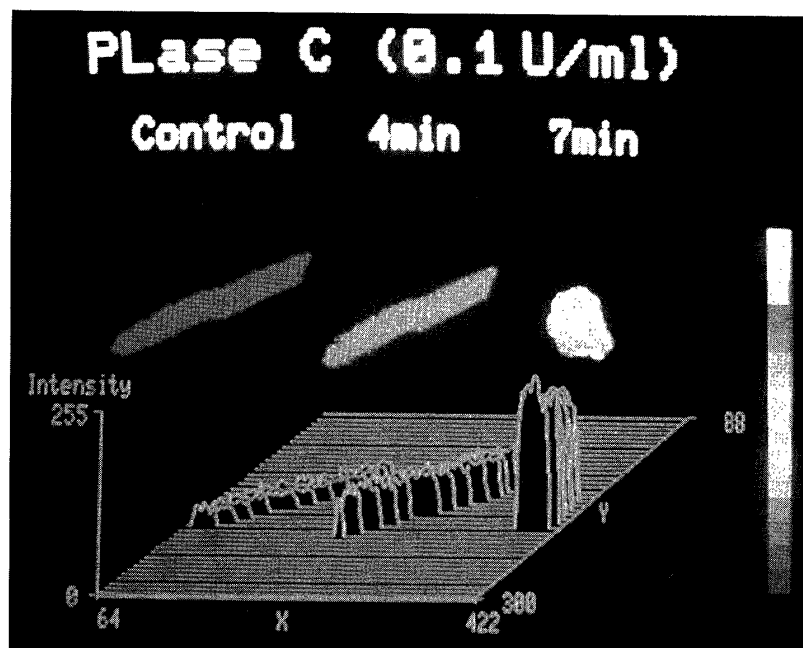


Fig. 3. Changes in cell shape and $[Ca^{2+}]_i$ in a cell perfused with 0.1 U/ml PLC. Pseudo-colour images of $[Ca^{2+}]_i$ (upper) and three-dimensional presentations of ratios (lower) in a control rod-shaped cell (left), a shortened cell (middle) after 4 min, and a rounded cell (right) after 7 min of perfusion. A higher $[Ca^{2+}]_i$ is indicated with a higher colour from the colour bar on the right and with a higher pixel.

tor from a 300 W Xenon lamp equipped with an interference filter. Fluorescence of fura-2 loaded cells was imaged using a Zeiss ultrafluor 33x, objective. Video images were obtained using a silicon-intensified target camera (Hamamatsu Photonics K. K.), with the output digitized to a resolution of 512×512 pixels by ARGUS (Hamamatsu Photonics K. K.). Images of fura-2 fluorescence at 510 nm were obtained using excitation wavelengths of 340 and 380 nm. Images of fluorescence ratios were then obtained by dividing, pixel by pixel, the 340 nm image after background subtraction by the 380 nm image after background subtraction.

The relationship between $[Ca^{2+}]_i$ and fura-2 fluorescence ratios was determined by adding fura-2 pentapotassium salt ($50 \mu M$) to small volumes of buffer (mM: K-MOPS 10, K_2H_2 -EGTA+Ca-EGTA 10, NaCl 20, KCl 130; pH 7.05, temperature $37^\circ C$; dissociation constant (K_d) of EGTA, $214 nM$)²³ and sealing the mixture in a microslide.

Phospholipase C (type I from *Clostridium perfringens*; Sigma Chemical Co.) was dissolved in modified Krebs solution immediately before use. One unit of phospholipase C will liberate $1.0 \mu M$ of water soluble organic phosphorus from L- α -phos-

phatidylcholine per min at pH 7.3 and $37^\circ C$ (Sigma Chemical Co.).

Results are expressed as mean \pm SE. Statistical analyses were made by the non-paired t test; p values < 0.05 were considered significant.

RESULTS

Electrophysiological effects

Fig. 1 shows the changes in the action potential duration measured at 75% repolarization, the developed tension, and the increase in resting tension during perfusion with 1 and 2 U/ml PLC in 6 separate preparations. The perfusions with 1 and 2 U/ml PLC were limited to 30 and 20 min, respectively, since the muscles became inexcitable when the preparations were perfused further. With both concentrations, the action potential duration gradually decreased and did not recover within 30 min after the washout of PLC. The developed tension increased for the first 5 min of the perfusion, then decreased for the remaining 15 or 25 min. The developed tension continued to decrease even after the washout of PLC, and reached $71 \pm 14\%$ and $115 \pm 53\%$ of the control at 30 min after the washout of 1 and

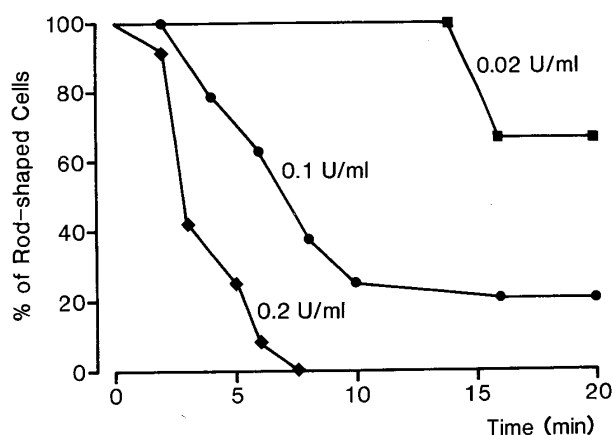


Fig. 4. Effects of PLC (0.02, 0.1, and 0.2 U/ml) on the percentage of rod-shaped myocytes. $n=6$ (0.02 U/ml), $n=24$ (0.1 U/ml), and $n=12$ (0.2 U/ml).

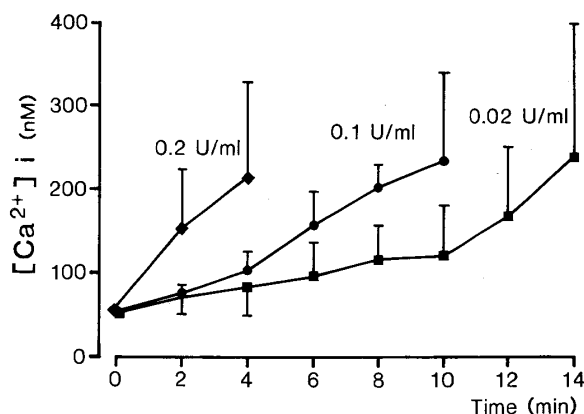


Fig. 5. Effects of PLC on the time course of the changes in $[Ca^{2+}]_i$ of rod-shaped cells. Quiescent myocytes were superfused with 0.02, 0.1, and 0.2 U/ml PLC. The values are the mean \pm SE of 6–24 cells.

2 U/ml PLC, respectively. Resting tension began to increase after 5 min of the perfusion. The resting membrane potential decreased from -85 mV to -60 – -80 mV after 30 and 20 min of perfusion.

Fig. 2A shows typical action potentials and tensions during the control perfusion and after 30 min of perfusion with 1 U/ml PLC. There was decrease in the duration of the action potential and a slight depolarization. Delayed afterdepolarizations (DADs) and aftercontractions were observed in all of the preparations, as shown by the white arrows in Fig. 2A. Triggered activities due to DADs were observed in 1 of 6 preparations during perfusion with 1 U/ml PLC, and in 3 of 6 preparations during perfusion with 2

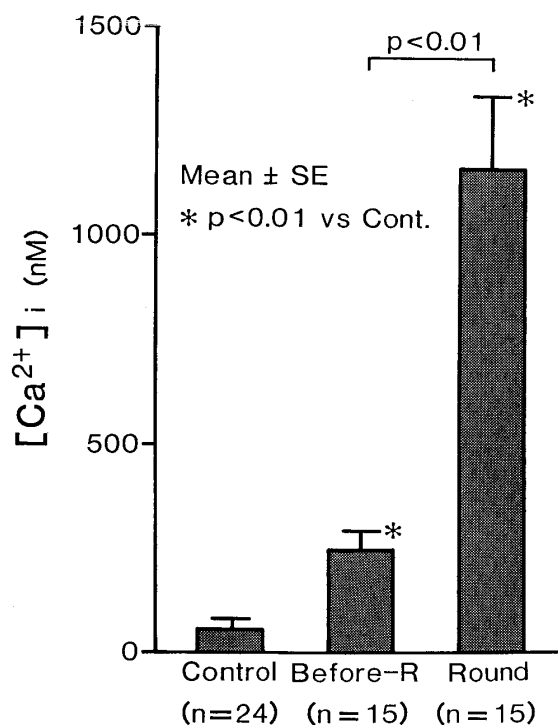


Fig. 6. Relationship between $[Ca^{2+}]_i$ and cell shape during perfusion with 0.1 U/ml PLC. Columns are mean \pm SE of $[Ca^{2+}]_i$ in cells of different morphology. $n=24$ (Control), $n=15$ (Before-R: before cell rounding), $n=15$ (Round: after cell rounding). Asterisks show $p < 0.01$ vs control.

U/ml PLC. Fig. 2B shows triggered activity which developed 15 min after perfusion with 2 U/ml PLC.

Intracellular Ca^{2+} concentration in isolated cells

The isolated ventricular myocytes were perfused with solutions which contained 0.02, 0.1 and 0.2 U/ml PLC. Fig. 3 shows a typical perfusion with 0.1 U/ml PLC. The cell shapes are shown on the upper column, and the color expresses the 340/380 nm ratio. The lower column shows a three-dimensional presentation of the ratio. Cells were shortened at 4 min after perfusion, and became rounded at 7 min after perfusion with PLC. $[Ca^{2+}]_i$ increased as the cell shapes changed.

The percentage of rod-shaped cells to all cells decreased as the concentration of PLC, and as the time of exposure to a given concentration of PLC, increased, as shown in Fig. 4. The changes in $[Ca^{2+}]_i$ of rod-shaped cells are shown in Fig. 5. $[Ca^{2+}]_i$ increased gradually at each concentration of PLC, and

the increase of $[Ca^{2+}]_i$ was faster as the concentration of PLC increased. Fifteen of 24 cells were rounded during the perfusion with 0.1 U/ml PLC. Nine of these 15 cells were rounded immediately from rod-shaped cells. Six of the 15 rounded cells were shortened (between 70% and 100% of their original longitudinal length) before becoming round. The $[Ca^{2+}]_i$ was 468 ± 78 nM ($p < 0.01$ vs control) when cells were shortened. Fig. 6 shows the mean $[Ca^{2+}]_i$ of the controls, the values before cell rounding, and the values after cell rounding during perfusion with 0.1 U/ml PLC. The $[Ca^{2+}]_i$ increased from 56 ± 5 nM ($n=24$), to 245 ± 47 nM ($n=15$; $p < 0.01$ vs control) before cell rounding, and to 1167 ± 172 nM ($n=15$; $p < 0.01$ vs control) after cell rounding.

DISCUSSION

The electrophysiological changes during perfusion with PLC in this study include the decrease in the action potential duration, the decrease in resting membrane potential, and the appearance of delayed afterdepolarizations (DADs) and aftercontractions. Triggered activities were also observed in some preparations. The appearance of DADs and aftercontractions suggests that PLC causes intracellular Ca^{2+} overload. The decrease in the APD may be due to the decrease in slow inward Ca^{2+} current or Na^{2+} window current, or the increase in outward K^{+} current. The decrease in resting membrane potential may result from the decrease in the inward rectifier K^{+} current. An increase in K^{+} current caused by increased intracellular Ca^{2+} may be another mechanism by which action potential duration is decreased.²⁴ These electrophysiological changes are similar to those produced by amphiphiles, such as lysophosphoglyceride²⁵ and long chain acyl carnitine.²⁶

Although the exact nature of the injuries associated with myocardial ischemia/reperfusion is not yet clear, disturbances in membrane structure and function have been reported to characterize the loss of reversibility in cell injury associated with ischemia/reperfusion.^{1,2} In the present study, the finding that the action potential duration did not recover after the washout of PLC suggests that perfusion with PLC produces

irreversible injury. A significant loss of membrane has been shown to occur during a 3 h ischemic insult,⁷ or after 60 min of reperfusion following 60 min of ischemia.⁸

Phospholipases have several important functions: i.e., as the initial enzyme of an arachidonate cascade reaction⁷ or phosphatidylinositol response.²⁷ However, an abnormal activation of phospholipases may disturb various cellular functions. Phospholipase A_2 activity results in the production of lysophosphoglycerides which alter sarcolemmal membrane molecular dynamics and produce profound electrophysiologic changes.²⁰ Hydrolysis of sarcolemmal glycerophospholipid by PLC also results in dramatic electrophysiologic changes in cardiac myocardium,¹⁶ as shown in this study. Therefore, arrhythmia may be due to the degradation of membrane function caused by the disordered activation of phospholipases.

$[Ca^{2+}]_i$ and cell morphology were monitored simultaneously using fura-2 and a digital imaging fluorescence microscope.^{22,23} PLC decreased the number of rod-shaped cells of isolated ventricular myocytes in a time- and a concentration-dependent manner. $[Ca^{2+}]_i$ increased gradually, and a relationship between cell morphology and $[Ca^{2+}]_i$ was observed. The transient increase and the subsequent decrease in developed tension, and the increase in resting tension in papillary muscle preparations seen in this study, can be explained by the increase in $[Ca^{2+}]_i$ and the resulting Ca^{2+} overload.

However, the concentration of PLC which caused changes in $[Ca^{2+}]_i$ and cell shape in myocytes was significantly lower than that in papillary muscle preparations. This could be due to a much greater fragility of isolated myocytes. Myocytes may be more fragile than papillary muscles for two reasons: (1) The energy status of the cell may determine the function and stability of the cell membrane. Higgins et al²⁸ have shown that a relationship exists between intracellular ATP content and the resistance of cardiac cell membranes to attack by phospholipase. However, this explanation is unlikely, since the ATP content of viable rat myocytes has been shown to be comparable to that of intact myocardial tissue.²⁹ (2) The sarcolemma of isolated myocytes may be more suscepti-

ble to attack by phospholipase than that of intact tissue. Ultrastructural studies have shown that isolated myocytes prepared using enzyme, separated along the line of the intercalated discs with tearing of the plasma membrane in the region of the gap junction.³⁰ We have already reported that hydrogen peroxide, which has been suggested to act on cell membrane, causes cell injury at a lower concentration in myocytes than in papillary muscles.³¹

In the present study, the magnitude of the increase in $[Ca^{2+}]_i$ could have been underestimated because we used an *in vitro* calibration curve to determine the absolute values of $[Ca^{2+}]_i$ from the ratios. However, there was no significant difference between the values of $[Ca^{2+}]_i$ calculated by *in vivo* calibration and those calculated by *in vitro* calibration, when the $[Ca^{2+}]_i$ by *in vitro* calibration was under 800 nM.³² Several other phenomena may have distorted the present $[Ca^{2+}]_i$ measurements, including (1) Compartment of fura-2³³ (2) Photobleaching of fura-2³⁴ (3) Incomplete deesterification of the acetoxymethyl form, leaving Ca^{2+} -insensitive fluorescent species³⁵ and (4) Buffering of intracellular Ca^{2+} by intracellular fura-2³⁶. The absolute levels of $[Ca^{2+}]_i$ calculated from the ratios may have to be revised, especially when the effect of intracellular environment on fura-2 becomes clear.

Recent evidence indicates that the accumulation of Ca^{2+} is involved in the process of lipase activation. The increase in $[Ca^{2+}]_i$ during ischemia has been shown to activate membrane phospholipase, resulting in degradation of membrane phospholipids.^{3,4,14} Myocardial reperfusion is known to increase Ca^{2+} influx into the cardiac cell,¹⁵ which may then stimulate phospholipase activity. Phospholipase may increase $[Ca^{2+}]_i$ by acting on subcellular organelles which regulate intracellular Ca^{2+} homeostasis.^{37,38} However, it is likely that stimulation of phospholipase activity results in disruption of the membrane phospholipid bilayer, which would further increase Ca^{2+} influx.^{18,39} A vicious cycle may exist in which increased $[Ca^{2+}]_i$ during ischemia/reperfusion activates phospholipase activity which further increases $[Ca^{2+}]_i$.

We have already reported that $[Ca^{2+}]_i$ has

a primary role in mediating irreversible injury in Ca^{2+} -overloaded myocytes during perfusion with strophanthidin,²² since there was a close correlation between $[Ca^{2+}]_i$ and the degree of cell shortening. We have also reported that strophanthidin (0.1 mM) increased the $[Ca^{2+}]_i$ from 66 ± 5 to 579 ± 112 nM before cell rounding.²² The $[Ca^{2+}]_i$ before cell rounding during perfusion with PLC (i.e. 245 ± 47 nM) was significantly lower ($p < 0.01$) than that during perfusion with strophanthidin. These findings suggest that Ca^{2+} overload is not likely to be the sole, or even primary, mechanism of cell injury during perfusion with PLC. Some other mechanism(s), such as membrane degradation, are probably involved in cell injury.

Ischemia/reperfusion-induced injury and arrhythmia have been shown to be due, at least in part, to Ca^{2+} overload.^{5,15} In the present study, PLC induced DADs and arrhythmia, decreased the number of rod-shaped cells, and increased $[Ca^{2+}]_i$. Therefore, activation of PLC may play a role in arrhythmia and cell injury during myocardial ischemia/reperfusion. An increase in $[Ca^{2+}]_i$ during ischemia/reperfusion may activate phospholipase which further increases $[Ca^{2+}]_i$ to form a vicious cycle. However, further studies are required before we can relate arrhythmias and cell injury to the activation of phospholipase because the mechanism and consequences of the activation of endogenous phospholipase are presently unknown.

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