Effects of Phospholipase C on the Na⁺-Ca²⁺ Exchange and Ca²⁺ Permeability of Cardiac Sarcolemmal Vesicles*

Kenneth D. Phillipson, Joy S. Frank, and Ann Y. Nishimoto

From the Departments of Medicine and Physiology and the American Heart Association, Greater Los Angeles Affiliate, Cardiovascular Research Laboratories, University of California at Los Angeles, Center for Health Sciences, Los Angeles, California 90024

In this study we investigate the effects of phospholipase C on cardiac sarcolemmal membranes. Like other plasma membranes, cardiac sarcolemma must maintain a high permeability barrier to Ca²⁺. In addition, cardiac sarcolemma possesses at least three Ca²⁺ transport pathways for regulating the Ca²⁺ fluxes which accompany each heartbeat. These pathways are a voltage-sensitive channel (11), an ATP-dependent Ca²⁺ pump (12), and a very active Na⁺-Ca²⁺ exchange mechanism (13). Although the Na⁺-Ca²⁺ exchange of cardiac sarcolemma has been the subject of much recent research (e.g. Refs. 13-16), little is known of the interaction of the Na⁺-Ca²⁺ exchanger with its phospholipid environment. We find that sarcolemmal passive Ca²⁺ permeability increases with mild phospholipase C treatment. Surprisingly, Na⁺-Ca²⁺ exchange activity is markedly stimulated by phospholipid hydrolysis. These effects are analyzed.

MATERIALS AND METHODS

Sarcolemmal Isolation—Sarcolemmal vesicles, with characteristics as described previously (15), were prepared from dog ventricles (courtesy of W. W. Nash, Division of Cardiology, UCLA). The vesicles were about 80-fold enriched in sarcolemmal markers and contained minimal mitochondrial contamination.

Na⁺-Ca²⁺ Exchange—Na⁺-Ca²⁺ exchange was measured as the initial rate of Na⁺-dependent Ca²⁺ uptake as described in detail previously (14-17). Briefly, 0.005 ml of Na⁺-loaded (140 mM NaCl, 5 mM Tris' maleate (pH 7.4, 37 °C)) sarcolemmal vesicles was rapidly diluted into 0.25 ml of Ca²⁺ uptake medium containing 140 mM KCl, variable CaCl₂, 0.4 μCi of ⁵⁵CaCl₂, 0.4 μM valinomycin, 5 mM Tris maleate (pH 7.4, 37 °C). After 1.5 s (unless otherwise noted), the Ca²⁺ uptake was automatically quenched by addition of 0.03 ml of 140 mM KCl, 5 mM LaCl₃. The reaction mixture was then filtered (Millipore, 0.45 μm) and the filter was washed with 2-3 ml aliquots of 140 mM KCl, 1 mM LaCl₃. Blank values were obtained by using an identical protocol except the Ca²⁺ uptake medium contained 140 mM NaCl instead of KCl. The blanks were subtracted for all data points to correct for superficial Ca²⁺ binding and Na⁺ gradient-independent Ca²⁺ fluxes. All procedures were performed at 37 °C.

Phospholipase C Treatment—0.012 ml of Na⁺ (140 mM)-loaded sarcolemmal vesicles (2-5 mg of protein/ml) were mixed with 0.002 ml of 140 mM NaCl, 5 mM Tris maleate (pH 7.4, 37 °C) containing variable concentrations of the phospholipase C. The mixture was incubated for 20 ± 2 min at 37 °C and aliquots were then used immediately in experiments. A different protocol was used in the experiments shown in Fig. 5, as described in the figure legend. The enzymes and their activities were: phospholipase C, Clostridium perfringens (Sigma P-1392, 200 IU/mg of protein); phospholipase C, Bacillus cereus (Boehringer Mannheim 104999, 400 IU/mg of protein).

Phospholipid Analysis—Phospholipids were extracted from the sarcolemma by the method of Bligh and Dyer (18). Thin layer chromatography was performed by the method of Skipski et al. (19); Silica...
passive Ca\(^{2+}\) uptake; we report only Ca\(^{2+}\) transported into the experiment. The Na\(^{+}\)-dependent Ca\(^{2+}\) influx reaction occurred for Standard errors were less than 10% of mean values for all data points.

Electron Microscopy—Sarcolemmal vesicles under control conditions and after phospholipase C treatment were diluted and centrifuged at 160,000 \(\times \) g for 20 min to form a pellet. The pellet was processed for electron microscopy in 2.5% Karnovsky's fixative buffered with 0.2 mM sodium cacodylate. After postfixation in 1% osmium tetroxide, the pellet was passed through a graded series of alcohol rinses and then embedded in Epon 812. The pellets were cut perpendicular to the surface so that the sections encompassed the whole depth of the original pellet. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100 CX electron microscope.

Data are presented as means \(\pm\) standard error of the mean.

RESULTS

The effects of phospholipase C (C. perfringens) pretreatment on the Na\(^{+}\)-dependent Ca\(^{2+}\) influx (Na\(^{+}\)-Ca\(^{2+}\) exchange), (Na\(^{+}\),K\(^{+}\))-ATPase activity, and phospholipid content of highly purified cardiac sarcolemmal vesicles are shown in Fig. 1. Ca\(^{2+}\) influx by Na\(^{+}\)-Ca\(^{2+}\) exchange is markedly stimulated (by \(\sim 50\%\)) when about 20% of the membrane phospholipid has been hydrolyzed. Even more striking is the finding that Na\(^{+}\)-dependent Ca\(^{2+}\) influx is still above or close to control values when 65–75% of the lipid-bound phosphate has been removed. In all experiments, blanks (<10% of experimental values; see under “Materials and Methods”) corrected for passive Ca\(^{2+}\) uptake; we report only Ca\(^{2+}\) transported into sarcolemmal vesicles in exchange for intravesicular Na\(^{+}\).

In contrast, the activity of another sarcolemmal marker, (Na\(^{+}\),K\(^{+}\))-ATPase exhibits a slight stimulation and then inhibition as the result of phospholipase C action. It should be noted that increase in membrane permeability alone would tend to stimulate (Na\(^{+}\),K\(^{+}\))-ATPase by providing greater access for substrates to enzyme-active sites. Thus, inhibition of (Na\(^{+}\),K\(^{+}\))-ATPase activity by phospholipase C treatment as depicted in Fig. 1 could be underestimated if significant masking of (Na\(^{+}\),K\(^{+}\))-ATPase activity occurred simultaneously with enzyme inhibition. In contrast, measurement of Na\(^{+}\)-Ca\(^{2+}\) exchange is dependent upon an intact membrane. Increases in membrane permeability to either Na\(^{+}\) or Ca\(^{2+}\) could only diminish Na\(^{+}\)-dependent Ca\(^{2+}\) influx by dissipating the Na\(^{+}\) gradient or by permitting efflux of transported Ca\(^{2+}\). Valinomycin was present in these experiments to increase electrogenic Na\(^{+}\)-Ca\(^{2+}\) exchange by generating an inside-positive membrane potential (14). Stimulatory effects of phospholipase C on sarcolemmal Na\(^{+}\)-Ca\(^{2+}\) exchange were equally evident in the absence of valinomycin (not shown). Thus, the stimulation of Na\(^{+}\)-Ca\(^{2+}\) exchange by phospholipase C does not appear to be secondary to alterations of vesicle membrane potential.

Phospholipase C (B. cereus) (U/ml)
Time courses of sarcolemmal Na\(^+\)-dependent Ca\(^{2+}\) uptake as a function of phospholipase C (C. perfringens) treatment are shown in Fig. 4. All three curves were linear for at least 1.0 s. The stimulation of Na\(^+\)-Ca\(^{2+}\) exchange by pretreatment with 0.25 unit/ml of phospholipase C was most evident at early times; after 10 s of Ca\(^{2+}\) uptake only a small stimulation was present. With more harsh phospholipase C treatment (1.0 unit/ml), Na\(^+\)-dependent Ca\(^{2+}\) uptake was slightly stimulated at short times and markedly inhibited at longer times. This result suggested that phospholipase C treatment both stimulated Na\(^+\)-dependent Ca\(^{2+}\) uptake and increased passive Ca\(^{2+}\) permeability. As Ca\(^{2+}\) accumulated within the sarcolemmal vesicles due to rapid Na\(^+\)-Ca\(^{2+}\) exchange, passive Ca\(^{2+}\) efflux increased and became more evident after phospholipase C treatment.

We also investigated the effects of phospholipase C (C. perfringens) on the passive Ca\(^{2+}\) permeability of sarcolemmal vesicles in a more direct manner as shown in Fig. 5. Sarcolemmal vesicles were first loaded with Ca\(^{2+}\) by a Na\(^+\)-Ca\(^{2+}\) exchange reaction. An isosmotic medium containing EGTA (to inhibit further Ca\(^{2+}\) uptake) and phospholipase C (C. perfringens) was then added to the vesicles and the passive (Na\(^+\)-independent) loss of Ca\(^{2+}\) from the vesicles was followed. Hydrolysis of only 4% of the sarcolemmal phospholipid with 0.25 unit/ml of phospholipase resulted in a detectable increase in Ca\(^{2+}\) efflux. After 30% of the phospholipids had been hydrolyzed (phospholipase C = 1.0 unit/ml), only 10% of the Ca\(^{2+}\) load remained after a 3-min efflux period. In these experiments, the vesicles were exposed to the phospholipase C only during the 3-min efflux period. In the previous experiments, a 20-min preincubation with the enzyme was used. This explains why the amount of phospholipid hydrolysis with equal concentrations of phospholipase C was greater in the experiments shown in Fig. 1 than in those shown in Fig. 5. The results demonstrate that a small decrease in sarcolemmal phospholipid content is associated with an increase in passive Ca\(^{2+}\) permeability. Qualitatively similar results have been obtained by Chien et al. (21).

Thin layer chromatographic analysis of the effects of phospholipase C (C. perfringens) on canine cardiac sarcolemma is shown in Table I. In control sarcolemma, phosphatidylcholine and phosphatidylethanolamine were the dominant phospholipids. We have previously observed a similar phospholipid distribution pattern in rabbit cardiac sarcolemma (22). Phosphatidylcholine was the phospholipid most readily hydrolyzed by the phospholipase C. Sphingomyelin and phosphatidylethanolamine were also susceptible to the phospholipase. In contrast, the content of the two anionic phospholipids, phosphatidylinerine and phosphatidylinositol, was largely unaffected by exposure to phospholipase C. Similar substrate specificities for phospholipase C (C. perfringens) have been observed by others (5, 7, 10).

Thin section electron micrographs of control and phospholipase C-treated sarcolemmal vesicles are shown in Fig. 6, A and B. The sarcolemmal vesicles in Fig. 6B were pretreated with 0.25 unit/ml of phospholipase C (C. perfringens) which...
resulted in hydrolysis of 55% of the membrane phospholipid and a 45% stimulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity. Several striking differences were evident in the structure of the phospholipase-treated membranes. First, the vesicles were aggregated into clumps. This appeared to result from treatment with the enzymes and was not an artifact of the fixation procedure. Samples of membranes examined with negative staining just after phospholipase C exposure and prior to any fixation showed the same aggregated configuration (not shown). Generally 1 or 2 large vesicles (~0.45 \(\mu\)m) were surrounded by several smaller membrane profiles. On higher magnification (Fig. 7) it could be seen that the vesicles in a clump had actually fused, such that adjacent vesicles shared a common bilayer. Bilayer fusion has been reported in rat liver microsomes after phospholipase C treatment (23). In addition, electron-dense droplets were seen protruding from the bilayer in many of the vesicles. Similar electron-dense droplets have been observed in membranes from other tissues after exposure to phospholipase C (1, 24). They are presumed to be diacylglycerol, a digestion product of phospholipase C.

Because sarcolemmal Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity can be stimulated by protease treatment (16), we examined whether the stimulatory effects of phospholipase C were due to protease contamination of the phospholipase C. We first approached this problem using protease inhibitors. Neither leupeptin (10 pg/ml), aprotinin (0.75 unit/ml), TLCK (100 \(\mu\)M), pepstatin (1 \(\mu\)M), nor phosphoramidon (1 \(\mu\)M) were able to block the phospholipase C-induced stimulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange.

**TABLE I**

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<thead>
<tr>
<th>Phospholipid analysis of cardiac sarcolemma after phospholipase C (C. perfringens) treatment</th>
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<tr>
<td>Control</td>
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<tr>
<td>% of total</td>
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<tr>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
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<tr>
<td>Phosphatidyserine + phosphatidylserine</td>
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<td>Phosphatidylethanolamine</td>
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FIG. 7. High magnification micrograph of phospholipase C-treated membranes. Note that the aggregated vesicles appear to have fused where they share a common bilayer (arrows). The vesicles are from the same preparation as shown in Fig. 6B. Magnification equals 198,000.

FIG. 6. Electron micrographs of control and phospholipase C-treated sarcolemmal vesicles. A, survey field showing homogeneous structure of control sarcolemmal vesicles. B, micrograph showing the striking changes in the configuration of the vesicles after treatment with 0.25 unit/ml of phospholipase C (C. perfringens) for 20 min. Arrows indicate electron-dense droplets. Magnification equals 54,615 in both A and B.
solubilize a fibrin clot. Neither 0.2 unit of phospholipase C (C. perfringens) nor 0.4 unit of phospholipase C (B. cereus) possessed detectable protease activity under conditions where the activities of 0.02 unit of trypsin or 0.01 unit of papain were detectable.

In one experiment, we examined whether phospholipase C (C. perfringens) treatment was solubilizing membrane-bound protein. This was determined as the per cent of the sarcosomal protein which could not be pelleted by high speed centrifugation. Phospholipase C (1.0 unit/ml, 60% phospholipid hydrolysis) solubilized only 1% of the sarcosomal protein.

DISCUSSION

Phospholipase C treatment has two distinct effects on the Ca2+ transport properties of canine cardiac sarcolemmal. Phospholipid hydrolysis and concomitant diacylglycerol production increases the passive Ca2+ permeability of vesicles. This effect is detectable with mild treatment. Hydrolysis of only 4% of the membrane phospholipid appreciably increases passive Ca2+ flux (Fig. 5). Thus, intact phospholipid structure is essential for maintenance of the Ca2+ permeability barrier. A disruptive effect of phospholipase C on the permeability characteristics of intact myocardial cells has previously been observed (26).

Phospholipase C treatment also markedly stimulates the Na+-Ca2+ exchange activity of the cardiac sarcosomal vesicles. In these experiments Na+-Ca2+ exchange is specifically measured as Na+-dependent Ca2+ uptake, and the observed stimulation represents a true increase in the activity of the Na+-Ca2+ exchange mechanism. The effects cannot be explained by altered membrane permeability. Control experiments correct for Ca2+ uptake which is not coupled to the Na+ gradient (<10% of total). Perturbation of the sarcolemma with phospholipase C increases the apparent affinity of the exchanger for Ca2+ (Fig. 3). It is well established that the activities of membrane-bound enzymes are dependent on bilayer phospholipid structure; a variety of other transport enzymes, however, are only inhibited by phospholipase C action (4-10).

Thus, phospholipase C treatment simultaneously increases both the passive Ca2+ permeability and the Na+-Ca2+ exchange activity of cardiac sarcosomal vesicles. The augmented passive Ca2+ leak will tend to attenuate Na+-dependent Ca2+ uptake (Na+-Ca2+ exchange). However, in most cases, the increased Ca2+ leak is not sufficient to mask the stimulated Na+-Ca2+ exchange. This is because the sarcosomal Na+-Ca2+ exchange is highly active and dominates Ca2+ fluxes in this system. The initial rate of Na+-dependent Ca2+ uptake is measured over a 1.5-s period, and this is not enough time for the increased passive Ca2+ leak to have a large effect. As the Na+-dependent Ca2+ uptake proceeds with time, intravesicular Ca2+ accumulates and the increased passive Ca2+ efflux becomes more evident. These opposing effects of phospholipase C action on Ca2+ transport explain the peculiar shapes of the time course curves shown in Fig. 4.

As a consequence of increased passive Ca2+ leak, the true activity of sarcosomal Na+-Ca2+ exchange after phospholipase C treatment may actually be larger than observed in some cases. This would be especially likely after treatment with higher concentrations of phospholipase C. For example, in Fig. 1, pretreatment with 5.0 units/ml of phospholipase C resulted in over 70% phospholipid degradation and an apparent slight inhibition of Na+-Ca2+ exchange. Passive Ca2+ leak is probably exaggerated under these harsh conditions. In fact, the Na+-dependent Ca2+ uptake activity may still be stimulated but the vesicles have lost the ability to retain the transported Ca2+.

It is noteworthy that vesicles displaying severe morphological alterations (Fig. 6) are capable of enhanced transport activity. Phospholipase C preferentially hydrolyzes neutral phospholipids and leaves the negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) intact (Table I). Perhaps Na+-Ca2+ exchange depends on the membrane content of the negatively charged phospholipids. After phospholipase C treatment the relative concentration of these phospholipids is increased and this may underlie the observed stimulation of Na+-Ca2+ exchange.

We have previously reported that the Na+-Ca2+ exchange of sarcosomal vesicles can be markedly stimulated by proteinase pretreatment (16). It was thus essential to ascertain that the stimulatory effects of phospholipase C were not due to proteinase contamination of the phospholipase C. As described above, this was accomplished through the use of a series of proteinase inhibitors and by assessment of the proteinase content of the phospholipases C. Additionally, the quantities of the phospholipases C used in these studies argue against effects due to proteinase contamination. Maximal stimulation of sarcosomal Na+-dependent Ca2+ uptake was obtained with 0.25 and 0.02 unit/ml of the phospholipase C from C. perfringens and B. cereus, respectively (Figs. 1 and 2). This is equivalent to using 1.25 and 0.05 µg/ml of each of these enzymes. In the previous study (16), the maximum amount of trypsin needed to obtain stimulation of Na+-Ca2+ exchange was about 0.5 µg/ml under conditions identical with those used for phospholipase C treatment. Thus an unreasonably large fraction of both phospholipases would need to be contaminated with proteinases for artifacts to arise. Contamination of this magnitude would have been detected by our proteinase assay.

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