Phospholipase D Produces Increased Contractile Force in Rabbit Ventricular Muscle

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SUMMARY. Inclusion of phospholipase D (Streptomyces chromofuscus, 5 U/ml) in the medium perfusing rabbit papillary muscles increased peak force development by 78% and peak dF/dt by 89%. The maximal contractile response occurred 40–50 minutes after addition of the enzyme to the perfusate. As peak contractile response developed, aftercontractions appeared in most muscles. The inotropic response was slowly reversible, with disappearance of aftercontractions, upon discontinuation of the enzyme. Phospholipase D produces a specific increase of phosphatidic acid in the sarcolemma and, therefore, an increase in net anionic charge on the membrane. It has been shown previously that phospholipase D induces a large increase in sarcolemmal calcium binding. We propose, on the basis of the present study, that phospholipid-bound calcium may play a significant role in the control of contractile force in mammalian myocardium. (Circ Res 56: 146–149, 1985)

IT has been proposed that calcium (Ca++) bound to sarcolemmal sites of mammalian myocardium plays a major role in control of force development by the heart (Bers and Langer, 1979; Langer and Nudd, 1983). Previous work indicates that, at physiological concentrations of Ca++, 80–85% of Ca++ bound to sarcolemma is bound to phospholipid (Philipson et al., 1980). A major fraction of this binding is presumed to be at anionic sites on the hydrophilic ends of the amphiphilic phospholipid molecules (Langer and Nudd, 1983). Studies on purified cardiac sarcolemma and cultured myocardial cells show Ca++ displacement by the specific cationic probe for anionic phospholipid sites, polymyxin B (PXB), and large increases of Ca++ binding after exposure to phospholipase D (PLD) (Burt and Langer, 1983; Burt et al., 1983; Burt et al., in press). Finally, it has been shown that PLD treatment of purified sarcolemmal vesicles increases Na+-Ca++ exchange by 300–400% (Philipson and Nishimoto, 1984). Therefore, there is considerable evidence that Ca++ binding at the sarcolemma can be predictably manipulated by alteration of anionic phospholipid sites, and that the activity of the Na+-Ca++ exchanger is linked, by an undefined mechanism, to Ca++ at these sites. An important question which remains is whether alteration in phospholipid Ca++ binding produces an inotropic response in whole, functional myocardium, and the present study is directed to that question.

Methods

A total of 24 papillary muscles dissected from the right ventricle of adult New Zealand rabbits was mounted in a Lucite chamber with one end connected to a capacitance transducer in order to measure isometric force and its first derivative (dF/dt). After dissection and mounting in the chamber, the muscles were perfused for 60 minutes with a solution of the following composition (mM): 140 NaCl, 4 KCl, 1.0 CaCl2, 1.0 MgCl2, 22 NaHCO3, 10 glucose with 10 mU/ml insulin. The perfusate was equilibrated to a mixture of 95% O2-5% CO2 by passage of the solution through a gas exchanger prior to its entry into the chamber. The pH of the solution was 7.4. After initial equilibration, 8 μM bovine serum albumin and 0.5% glycerol were added to the perfusate, and an additional 25–30 minutes of equilibration was allowed. The PLD used in these studies was the enzyme purified from Streptomyces chromofuscus (Calbiochem). This enzyme, in contrast to that derived from cabbage, is active at physiological Ca++ concentration and pH (Imamura and Horiiuti, 1979). It is stabilized by albumin and stored in glycerol and was added to the muscle perfusate with these agents present to give a final concentration of 5 U/ml PLD. The albumin/glycerol additive, by itself, caused a slow decline in muscle force over the 25- to 30-minute equilibration period. Experiments were performed between 36 and 37°C with field stimulation at 1.25-1.50 times threshold at 30 beats/minute.

Results

The pattern of the response to PLD is shown in Figure 1. Twenty one of 24 muscles demonstrated a latency period in the force response with a mean delay of 20.2 ± 3.1 (SE) minutes for the entire group. The molecular weight of the enzyme is 50 K daltons. Although the papillary muscles were selected for minimum radius (0.42 ± 0.03 mm), it is expected that time would be required for the large molecule to diffuse through the endocardium and subendocardial connective tissue of the muscles. The peak contractile response usually occurred between 20 and 30 minutes after onset, or between 40 and 50
minutes after first exposure to PLD. The increase in peak force after PLD was 1.78 ± 0.11-fold with a 1.89 ± 0.13-fold increase in dF/dt.

As the peak force response continued to increase, aftercontractions appeared and resting tension began to rise, as shown in Figure 2, which represents the response of another muscle. The aftercontractions and rise in resting force are clear indications of increased Ca ++ loading by the cells (Orchard et al., 1983). Removal of PLD is followed by a decline in resting force and disappearance of the aftercontractions over the course of 30 minutes, at which time active force is little changed and dF/dt has declined by 12%. The inotropic response to PLD was slowly reversible upon removal of the enzyme. In the experiment shown in Figure 2, the increment in force declined by 50% over the course of 100 minutes after the enzyme had been removed (not shown).

All of the activity of the enzyme is lost when heated to 80°C for 15 minutes (Imamura and Horiuti, 1979). Perfusion of papillary muscle which had demonstrated a 111% increase in force after application of active enzyme lost all of its positive inotropy within 30 minutes after substitution of heat-inactivated enzyme in the perfusate.

Finally, it was of interest to explore the possibility that PLD, through creation of additional anionic surface charge, would produce accumulation of cations, such as Ca ++, in the aqueous diffuse double layer (McLaughlin, 1977). Such an accumulation of Ca ++ could conceivably produce the demonstrated positive inotropy without the requirement that additional Ca ++ actually binds to the anionic sites created by PLD treatment. A specific probe has been developed for investigation of the diffuse double layer (McLaughlin et al., 1983). Dimethonium (ethane-bis-trimethylammonium) is a divalent cation of molecular weight 306 that replaces inorganic cations in the diffuse double layer but does not bind to the membrane. Therefore it will compete with Ca ++, as well as other cations in the double layer, but not with Ca ++ bound to the membrane. The effectiveness of dimethonium as a displacer of Ca ++...
in the diffuse double layer has been proven in tissue culture studies (Fintel et al., unpublished data). It has also been shown to produce a small but significant negative inotropic effect on the positive inotropy induced by sucrose-substituted low sodium perfusion in whole tissue. Substitution of neutral sucrose for sodium induces a large increase in diffuse double layer Ca++. The negative inotropic response to dimethonium ion under these conditions indicates that it gains access to the double layer in whole tissue. Application of 10 mM dimethonium ion to a papillary muscle which had developed an increase of 50% in force after PLD exposure resulted in no decrease in the positive inotropy.

**Discussion**

PLD cleaves the nitrogenous base from phospholipids, producing phosphatidic acid, and thereby increasing the net anionic charge on the membrane. Philipson and Nishimoto (1984) found that PLD treatment (0.4 U/ml) of sarcolemmal vesicles increased the phosphatidic acid level from 0.9 to 8.9% of total phospholipid content. This increase in phosphatidic acid should provide additional binding sites for cations, particularly Ca++. Such increased binding after PLD treatment has been demonstrated in sarcolemma prepared from rat cardiac myocyte culture (Langer and Nudd, 1983) where 0.1 U/ml PLD (cabbage) caused a 56% increase in sarcolemma Ca++ binding.

Details of Ca++ movement subsequent to binding have not been defined. Ohsako and Deguchi (1981) found that exogenous as well as endogenous phosphatidic acid stimulated Ca++ influx in neuroblastoma and in cultured heart cells. Salmon and Honeyman (1980) proposed that increased phosphatidate may mediate the increased cellular Ca++ levels produced by cholinergic stimulation and noted that phosphatidate at concentrations as low as 10^-8 M produced contractions in isolated smooth muscle cells. Phosphatidic acid has been proposed as a Ca++ ionophore in the promotion of Ca++ uptake in isolated nerve terminals (Harris et al., 1981) and in liposomes (Serhan et al., 1982). By contrast, an ionophoretic action of phosphatidic acid could not be demonstrated in another liposomal membrane model (Holmes and Yoss, 1983). Pertinent to the possibility of ionophoretic action is the demonstration by Philipson and Nishimoto (1984) that, in sarcolemmal vesicles, phosphatidic acid formation increased Na+-Ca++ exchange activity by 300–400%. In the vesicle system, the enhanced Ca++ uptake is Na+ gradient-dependent and results in an outwardly directed Ca++ gradient. As they point out, this eliminates the possibility, in the vesicle system, that the PLD-induced increase in Ca++ uptake is due to an ionophoretic action of phosphatidic acid.

The present study, in association with the demonstration of increased phosphatidic acid levels in sarcolemmal vesicles (Philipson and Nishimoto, 1984) clearly shows that such increase is associated with a marked positive inotropy, and there is other evidence that the inotropy might be related to Na+-Ca++ exchange. It is possible that augmented Ca++ uptake with increased force development is dependent only on a net increase in anionic sarcolemmal charge and not, specifically, to an increase of phosphatidic acid. Evidence for this possibility comes from previous PXB studies (Burt and Langer, 1983; Burt et al., 1983). PXB is an amphiphilic peptidolipid with a highly charged cationic head group and a lipophilic tail. It has been shown to bind only to artificial membranes which contain negatively charged phospholipids or to zwitterions like phosphatidylethanolamine at pH > 7.2 (Feingold et al., 1974; Imai et al., 1975; Teuber and Miller, 1977). The cationic head of PXB therefore interacts with several anionic phospholipids and is a potent displacer of Ca++ from isolated sarcolemma (Burt and Langer, 1983) and cultured cardiac cells (Burt et al., 1983). We have also demonstrated that it is negatively inotropic (unpublished observation). The conclusion from the PXB studies is that a variety of negatively charged phospholipids may contribute to the supply of Ca++ which crosses the sarcolemma upon excitation. Another effect of increase of anionic surface charge would be to augment movement of cations, including Ca++, into the diffuse double layer. If such movement contributed to increased Ca++ influx, increased force development would be expected. The fact that 10 mM dimethonium ion produced no decrease in the PLD-induced inotropy indicates that double layer Ca++ does not contribute significantly to the response. In addition, the fact that PXB removes most of the PLD-induced increment of Ca++ from cultured cells (Burt et al., 1984) supports the proposal that Ca++ binding is augmented. If such is the case, then the increased anionic charge produced by PLD-induced phosphatidate production would be neutralized by the increased bound Ca++. Therefore, net surface charge would not be altered at a time when post-PLD steady state had been reached, and any effects which might be attributed to a nonspecific increase in surface charge would not apply.

Factors other than changes in charge on phospholipid head groups play a role in cation binding. One of these is fluidity of lipids in the membrane. There is an enhancement in cation affinity with increased lipid fluidity (Puskin and Martin, 1979). Fluidity changes of the membrane usually occur, however, by change in cholesterol content or by modification of the fatty acid chains of the phospholipids. PLD alters neither of these, and it is unlikely that its modification of head group charge would have significant effects on the fluidity of the in vivo membrane. Therefore, we believe the evidence supports the proposal that negatively charged phospholipids at the external sarcolemmal surface bind the Ca++ which may supply the systems responsible for trans-
sarcolemmal movement of Ca++. Philipson and Nishimoto (1984) have already shown stimulation of Na+-Ca++ exchange by elevation of the sarcolemmal phosphatidic acid level. There are, as yet, no experimental data directed to possible effects of phosphatidic acid-mediated or other anion-mediated Ca++ binding on slow inward current. However, it is clear, from the present study, that increase in at least one negatively charged phospholipid, phosphatidic acid, is associated with large reversible increases in myocardial contractile force mediated by increased Ca++ uptake.

It might be expected that phosphatidate once formed by PLD would persist, and the inotropy might be sustained after removal of the enzyme. It has been found, however, that sarcolemmal membrane contains an endogenous phosphatidate hydrolase (Philipson and Nishimoto, 1984). The action of this enzyme may account for the slow return to control levels of force upon removal of PLD.

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