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PHOSPHATIDATE RELEASES CALCIUM FROM CARDIAC SARCOPLASMIC RETICULUM

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SUMMARY: Phosphatidate (PA) inhibits calcium accumulation by cardiac sarcoplasmic reticulum (SR) and enhances its Ca^{++} ATPase activity. These effects seem to be related to a phosphatidate-induced increase in the calcium permeability of the SR membrane with resultant calcium release. The amount of calcium released by phosphatidate is dependent both on the calcium concentration outside the SR vesicles and the internal calcium concentration. The ionophoric effects of phosphatidate on the sarcoplasmic membrane provide a novel pathway for controlling Ca^{++} transport in the cardiac cell.

INTRODUCTION: The activity of the SR calcium transport ATPase depends, to a large extent, on its microenvironment in the membrane particularly on its lipid composition (1,2). This dependency may reflect mobility constraints by the degree of membrane fluidity or result from direct effects of membrane constituents on Ca^{++} transport. Although phospholipids are known to have ionophoric properties with artificial or natural membranes (3,4), the constraints imposed by the bilayer structure of the membrane limit the physiologic relevance of these properties. Glycerolipid intermediates, like phosphatidate, which are less subject to this limitation are more attractive candidates for control of Ca^{++} translocation across the SR membrane. The key position of phosphatidate in the metabolic pathways of glycerolipids links Ca^{++} fluxes to neurohumoral influences on the cell membrane. Indeed, an association between phospholipid turnover, transient phosphatidate accumulation and Ca^{++} influx has been demonstrated in a variety of cell systems (5-7). The effect of PA on the function of

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SR has not been studied and, yet, it would represent an important mechanism for modulating calcium fluxes upon which muscle relaxation and tension development are critically dependent.

MATERIALS AND METHODS: Experiments were carried out on adult male Sprague-Dawley rats.

Isolation of sarcoplasmic reticulum: The animals were killed by cervical dislocation and their hearts were rapidly excised and chilled in crushed ice. The myocardial tissue was washed twice with a solution containing 10 mM $NaHCO_3$-5 mM NaN_3 and was then homogenized in the same solution with a Polytron PT-20 homogenizer three times at a rheostat setting of 3 for 5 sec with 15 second rest intervals. The homogenates were processed for sarcoplasmic reticulum isolation as described by Sumida et al (8).

Ca^{2+} uptake: Microsomes were suspended in 1 ml of a reaction mixture consisting of 40 mM histidine-HCl buffer (pH 6.8), 5 mM $MgCl_2$ 0.1 M KCl, 0.005 M sodium oxalate, 0.05 μCi ^{45}Ca (Ca^{2+}-EGTA buffer concentration 390 μM EGTA and 125 μM $CaCl_2$, giving a free Ca^{2+} concentration of 1 μM by the use (9) of 4.4×10^5 M^{-1} as the association constant for the Ca^{2+}-EGTA complex), 5 mM ATP, and variable amounts of microsomal protein. The mixture was incubated for various lengths of time at 25°C, and the reaction was stopped by filtration through a Millipore filter (HA 0.45 μM). In some experiments, calcium accumulation in the absence of oxalate (calcium binding) was measured as previously described (10).

Ca^{2+}-activated ATPase: ATPase activity was determined at 25°C in standard reaction mixtures containing 40 mM histidine-HCl buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM (γ-^{32}P) ATP (Amersham/Searle, Arlington Heights, Illinois, sp. act. 28.5 Ci/nmol), 0.1 M KCL, 5 mM NaN_3, 5 mM sodium oxalate, calcium-EGTA buffer containing 1 μM free Ca^{2+} and 50-80 μg/ml of microsomal protein in a total volume of 0.5 ml. Reaction mixtures were pre-incubated at 25°C for 5 minutes after which the reaction was started by the addition of microsomal protien. To determine "basal" (Mg^{2+}-ATPase) activity, reactions were carried out in the presence of 0.2 mM EGTA instead of the Ca^{2+}-EGTA buffer. At various intervals after the start of the reaction, 100 μl aliquots were added to tubes (5x50 mm) containing 25 ml of 25% (w/v) trichloroacetic acid (TCA) and 125 μM inorganic phosphate (P_i). After centrifugation at 3,000 xg for 10 minutes inorganic phosphate was determined (12) in the supernatants.

Calcium release was studied as described by Kirchberger and Wong (12). Cardiac microsomes (25 μg/ml) were first loaded with calcium in a medium containing 40 mM histidine-HCl (pH 6.8), 0.17 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 30 mM potassium phosphate, 4 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase and 10-200 μM $CaCl_2$. Calcium loading was carried out at 25°C for 30 min at which time calcium accumulation had reached a steady-state (as determined by a parallel set of experiments with $^{45}CaCl_2$). A small amount of tracer $^{45}CaCl_2$ (0.1 μCi) was added to estimate the unidirectional influx of calcium into the microsomes. Samples were removed at 30 sec intervals for 2 min thereafter; calcium influx was linear during that time. Calcium efflux was calculated from the measured influx of ^{45}Ca under the steady-state conditions during which efflux equals influx and internal calcium remains constant.

RESULTS AND DISCUSSION: Ca++ uptake by cardiac sarcoplasmic reticulum is inhibited by micromolar amounts of phosphatidate in a concentration-dependent manner (Figure 1). Ca++ binding (accumulation in the absence of calcium-precipitating anions) is also significantly inhibited (54 ± 7 nmol/mg/min in the presence of 5 μM

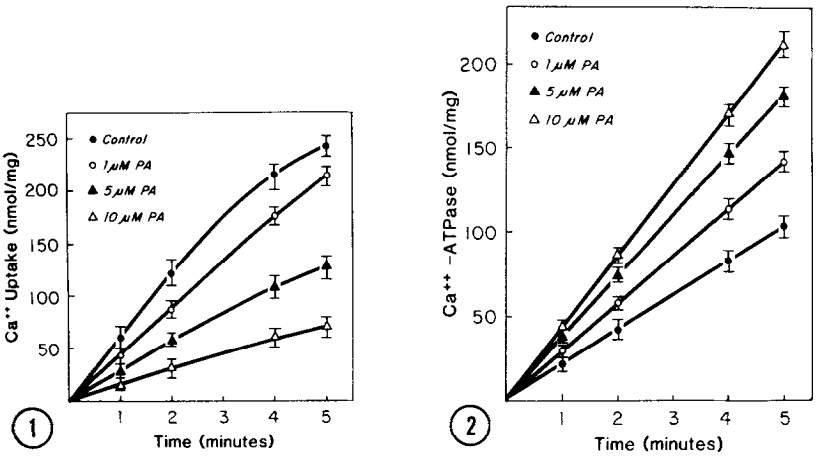


Figure 1: Time course of Ca^{++} uptake in the absence (•) and presence (o,Δ,▲) of varying concentrations of phosphatidate (PA). Results represent mean ± SE for seven experiments.

Figure 2: Time course of Ca^{++}-ATPase activity in the absence (•) and presence (o,Δ,▲) of varying concentrations of phosphatidate (PA). Results represent mean± SE for seven experiments.

phosphatidate vs 89 ± 6 nmol/mg/min in controls, $p<0.01$, $n = 7$). In contrast, the rate of ATP hydrolysis is strongly enhanced by phosphatidate (Figure 2). In order to exclude direct effects of PA on the membrane of indirect influence resulting from cation complexation, sarcoplasmic reticulum vesicles were pre-incubated with 10 μM PA for 5 min at 25°C, centrifuged at 100,000 g for 15 min and the pellets washed in 50 mM KCl, 20 mM tris-HCl (pH 6.8), As shown in Table I, the degree of inhibition of calcium accumulation was unaltered by such treatment.

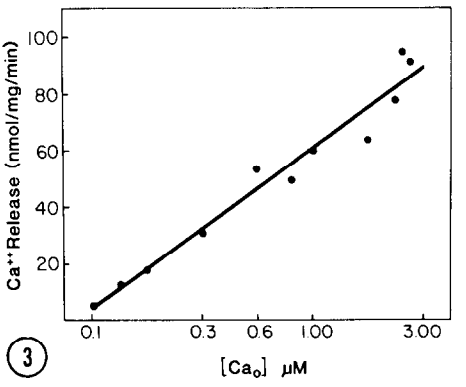
The possibility was examined that phosphatidate increases the permeability of the sarcoplasmic reticulum membrane to calcium. As shown in Figure 3, PA induces calcium release from pre-loaded vesicles which is directly proportional to the Ca++ concentration (Ca_o) outside the vesicles at the time of PA addition. Calcium release induced by phosphatidate is not due to the reversal of the calcium pump since it is still evident when calcium uptake is initiated in the presence of acetyl phosphate, rather than ATP, which

Table 1:

	Calcium Accumulation (nmol/mg)	
	Oxalate Absent	Oxalate Present
Control	91± 6.2	117± 7
+ 10 μM PA (unwashed)	50± 4.1*	46± 3*
+ 10 μM PA (washed)	52± 4.0*†	49± 4*†

Effect of prior exposure of cardiac SR to phosphatidate (PA) followed by washing on subsequent calcium transport. SR (0.5 mg/ml) was incubated with 10 μM PA for 5 min at 25°C followed by centrifugation at 100,000 g for 15 min. The washed pellets were used for calcium transport in the presence or absence of 5 mM sodium oxalate and were compared to controls without PA or SR incubated with 10 μM PA without subsequent washing. Results represent mean ± SE for 5 experiments. *$p<0.01$ compared to controls, †no statistically significant difference compared to unwashed SR.

does not support reversal of the pump (data not shown). In addition, the amount of calcium released by PA is strongly dependent on the concentration of Ca++ inside the SR vesicles (Ca_i), as shown by the effects of increasing the amounts of potassium phosphate in the assay medium (Figure 4).



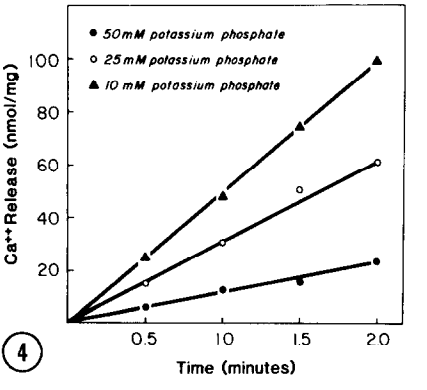


Figure 3: Dependence of Ca^{++} release by phosphatidate on the concentration of calcium (Ca_o) outside the SR vesicles. Cardiac SR (25 μg) was filled with nonradioactive calcium in the presence of 50 mM potassium phosphate. Tracer $^{45}CaCl_2$ and 15 μM PA were added 30 min. later and influx was measured at 30 sec intervals.

Figure 4: Dependence of phosphatidate-induced Ca^{++} release on the amount of potassium phosphate in the assay medium. Ca^{++} uptake was initiated with nonradioactive calcium in the presence of various concentrations of inorganic phosphate. Tracer $^{45}CaCl_2$ and 15 μM PA were added 30 min. later and influx was measured at 30 sec intervals for 2 min.

The effects of phosphatidate on calcium transport by the cardiac sarcoplasmic reticulum are those expected of an ionophore (13,14). Increased permeability of the membrane to calcium may explain the uncoupling between Ca++ uptake and ATP hydrolysis since intravesicular Ca++ does not readily reach levels high enough to turn off the ATPase. The dependency of PA-induced Ca++ release on Ca_o reflects the constraints imposed by a fixed Ca_i on the pump-leak properties of the SR vesicles containing a large amount of an insoluble calcium precipitate where most of the calcium is within the vesicles and Ca_i is maintained at a relatively low level. These constraints restrict calcium loss after membrane permeability is increased because calcium release stimulates the calcium pump so that a new steady-state equilibrium is reached (13). The amount of release Ca++ needed to achieve this new equilibrium varies directly with the $[Ca_o]$ at the time of the permeability change. The dependence of Ca++ release on Ca_i (at any given level of Ca_o) is explained by the fact that the apparent K_{Ca} for stimulation of initial calcium uptake velocity decreases at low levels of calcium precipitating anions (15). Under these conditions, the ability of the calcium released by any change in membrane permeability to stimulate calcium influx would be enhanced at high Ca_i. The behavior of phosphatidate is in accord with observations on the effects of ionophore X537A and caffeine on the sarcoplasmic reticulum (13, 15).

Although several phospholipids are capable of acting as ionophores in a Pressman cell, phosphatidate is (along with cardiolipin) the most effective (3). It has, moreover, the advantage over other zwitterionic phospholipds that it is not subject to the mobility constratins imposed by the bilary structure of the membrane. Numerous reports support the concept that phosphatidate form electronegative domains,particles, or inverted micelles in membranes,

especially in the presence of Ca++ and basic proteins (4, 17, 18). Thus, the association between Ca++ influx and changes in phosphatidate metabolism may be partly explained by the generation of phosphatidate domains functioning as Ca++ channels. An attractive alternative would be the potentiation by phosphatidate of the ionophoric activity of the Ca++ ATPase in a manner analogous to its interaction with cytochrome(19). It has been reported (20) that Ca++ release is enhanced when purified ATPase is incorporated into liposomes. It is likely that the association of the enzyme with specific lipid moieties leads to more than additive ionophoric capacity. Whatever the mechanisms involved, the influence of PA on cardiac SR provides a novel pathway for modulating calcium transport through synthesis and/or degradation of PA.

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