Myotonic Muscular Dystrophy

DEFECTIVE PHOSPHOLIPID METABOLISM IN THE ERYTHROCYTE PLASMA MEMBRANE

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ABSTRACT Myotonic muscular dystrophy (MyD) is a systemic genetic disorder that is thought to result from a generalized cellular membrane defect although the exact nature of this defect is unknown. This study examines two calcium-dependent membrane processes that have been observed in erythrocytes from healthy individuals: calcium-stimulated phosphatidic acid accumulation and calcium-induced potassium leak. We find that erythrocytes from MyD patients, in contrast to controls, have markedly impaired phosphatidic acid accumulations while maintaining normal potassium leaks. The calcium uptakes and ATP contents of MyD erythrocytes are not different from controls. We conclude that phospholipid metabolism is altered in MyD erythrocytes. The specificity of this abnormality and its relationship to altered muscular function are not known.

INTRODUCTION

Myotonic muscular dystrophy (MyD)[1] is a systemic genetic disorder inherited as an autosomal dominant trait. It is characterized by variable penetrance and expression (1). The involvement of multiple organ systems has led to a suspicion that the disease results from a generalized defect in cellular membrane function (2, 3). This hypothesis has been supported by observations in muscle from MyD patients indicating a decreased membrane protein phosphorylation associated with no differences in protein content or other membrane marker enzymes (4). Erythrocytes from MyD patients show an altered [^{32}P]phosphorylation of membrane band 3, an altered stoichiometry of the sodium-potassium pump and differences in membrane properties measured by electron spin resonance spectroscopy (2, 5–8). When erythrocytes from MyD patients are depleted of ATP by exposure to iodoacetic acid and adenosine (9, 10), a diminished calcium-induced potassium efflux is observed. Because the plasma membrane of MyD erythrocytes has an enhanced permeability to calcium (11), this diminished effectiveness of calcium is paradoxical and provided the impetus for our current studies.

This study examines the relative magnitudes and calcium sensitivities of calcium-induced potassium leak and calcium-induced phosphatidic acid accumulation, a measure of phosphoinositide phosphodiesterase activity in normal ATP-replete erythrocytes (12). A uniform impairment in calcium-sensitive phosphatidic acid accumulation has been observed in erythrocytes obtained from MyD patients.

METHODS

Erythrocytes from MyD patients and from sex-, age-matched healthy individuals were obtained by antecubital venipuncture into heparinized (10 U/ml) syringes and placed on ice. Both patient and control samples were obtained at the same time and tested in identical fashion. For incubation, the blood was washed as described previously (13, 14) to remove plasma, buffy coat, and microclots. All incubations were at 20°C in a gently shaking water bath (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) for 1–2 h. The erythrocytes were suspended in either isotonic or hypertonic solutions of tetramethylammonium chloride (TMA-Cl) containing 0 or 40 mM $CaCl_2$ (13, 14). In experiments using the calcium ionophore A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), calcium buffers were used to maintain constant ionic concentrations of calcium. The buffers were prepared with *N*-hydroxyethylenediaminetriacetic acid (HEDTA) using standardized solutions of calcium and

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[1] *Abbreviations used in this paper*: HEDTA, N-hydroxyethylenediaminetriacetic acid; MyD, myotonic muscular dystrophy; TMA-Cl, tetramethylammonium chloride.

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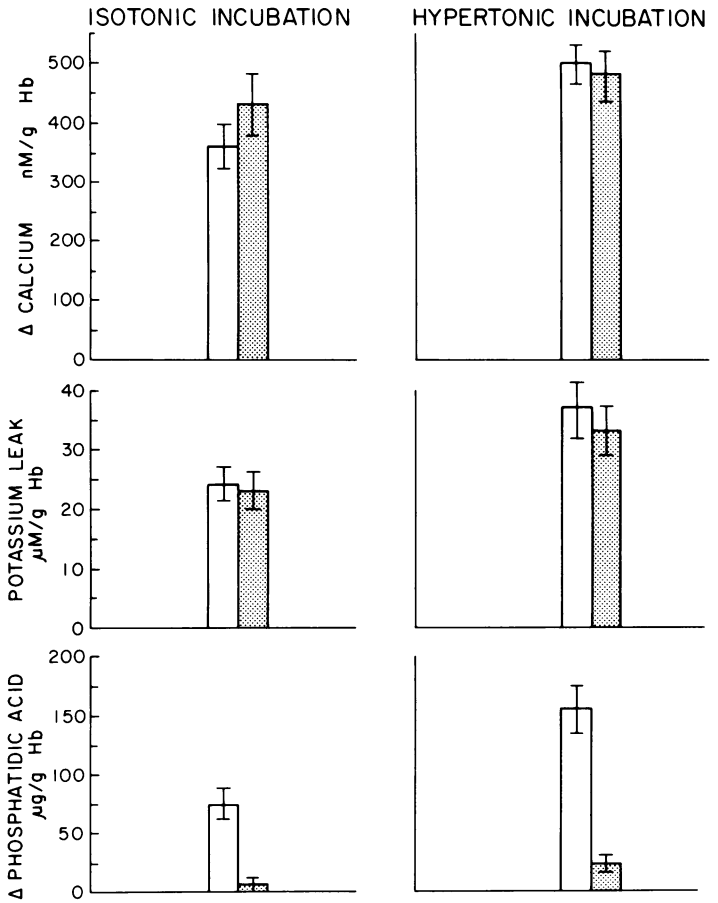

FIGURE 1 Calcium accumulations, potassium leaks, and phosphatidic acid accumulations. Erythrocytes (Hb) from control individuals (□, n = 11) and patients with MyD (▨, n = 9) were incubated for 2 h at 20°C in isotonic (left column) and hypertonic (right column) solutions containing in millimolars per liter: 1 KCl, 10 NaCl, 20 Hepes-Tris (pH 7.8), 40 $CaCl_2$, and TMA-Cl to 300 or 400 mosmol, respectively. The top panel shows the change in calcium contents of the cells. The middle panel shows the Ca^{++}-induced K^+ leaks from the cells. The bottom panel shows the change in phosphatidic acid content of the cells. Calcium accumulations and potassium leaks are not significantly different in the two groups. However, phosphatidic acid accumulation in MyD erythrocytes is significantly impaired ($P < 0.0005$) in both studies.

HEDTA to produce different ratios of Ca HEDTA to HEDTA. The total concentration of HEDTA was maintained at 1 mM. The ionic calcium concentrations were calculated using an apparent pK' of 6.1 for pH 7.80 (15). The Ca HEDTA/HEDTA ratios and respective ionic calcium concentrations were 0:1.0–0 μM; 0.25:0.75–0.264 μM; 0.6:0.4–1.19 μM; 0.75:0.25–2.35 μM.

Cells were prepared for calcium determination by an initial 1:2 dilution of erythrocyte suspension with 300 mosmol/kg TMA-Cl solution containing 10 mM ethylene glycol bis (2 amino ethylether) tetraacetate, pH 7. After centrifugation the cells were resuspended in a 300-mosmol/kg TMA-Cl solution containing 5 mM ethylenediaminetetraacetate, pH 7, and then washed five times with a 300-mosmol/kg TMA-Cl solution containing 5 mM Tris, pH 7.4. Calcium measurements were made with a model 403 atomic spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) equipped with a heated graphite atomizer (16). Potassium concentrations were measured on an Instrumentation Laboratory, Inc., Lexington, Mass. flame photometer. Hemoglobin content was determined by a semiautomated modification of the cyanmethhemoglobin procedure (17). ATP was measured by an automated luciferin-luciferase technique (18).

Erythrocyte lipids were extracted in chloroform/methanol/2 M KCl (1:1:0.5) with a 0.005% butylated hydroxytoluene (12, 19). The lower phase was evaporated under reduced pressure and resuspended in a small volume of chloroform for separation on silica gel H, 0.001 M Na_2CO_3 impregnated, thin-layer chromatography plates (Analtech, Inc., Newark, Del.) (20, 21). Reference lipids were from Avanti Biochemicals, Inc., Birmingham, Ala. and Sigma Chemical Co., St. Louis, Mo. Thin layer plates were run in ether/formic acid (99:1) to move neutral lipids to the solvent front, dried, and developed in chloroform/methanol/acetic acid/water (70:25:6:1) which cleanly separates phosphatidic acid from other erythrocyte phospholipids. The phosphatidic acid zone, determined by running authentic phosphatidic acid (Avanti Biochemicals, Inc.) with the sample was visualized in iodine vapor and extracted five

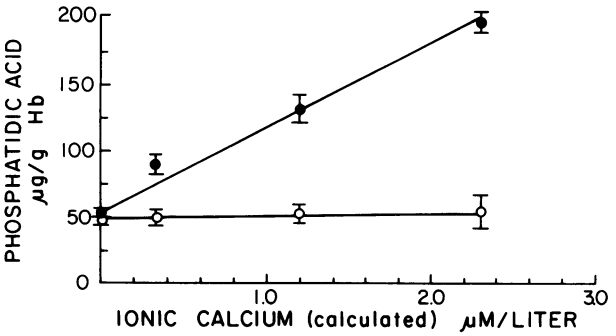

FIGURE 2 Phosphatidic acid accumulation in response to buffered ionic calcium concentrations. Erythrocytes (Hb) from control individuals (●, n = 5) and patients with MyD (○, n = 6) were incubated for 1 h at 20°C in isotonic solutions containing in millimolars per liter: 1 KCL, 10 NaCl, 20 Hepes-Tris (pH 7.8), TMA-Cl:300 mOsmol, 1 μM A23187 and Ca HEDTA/HEDTA buffers to give the ionic calcium concentrations shown. Phosphatidic acid contents are similar in control and MyD erythrocytes in the absence of calcium exposure. However, upon exposure to ionic calcium buffers, MyD erythrocytes have depressed phosphatidic acid accumulation.

times from the silica gel with chloroform/methanol/acetic acid (1:1:0.12) (19). These extracts were pooled, filtered, and evaporated under reduced pressure, then resuspended in 1 ml cyclohexane with 0.005% butylated hydroxytoluene immediately before analysis. Phosphatidic acid contents of these extracts were determined by a semiautomated modification of a rhodamine-G fluorescence assay (22). Recovery values for phosphatidic acid added to erythrocytes before extraction range from 90 to 96%.

RESULTS

Fig. 1 illustrates the calcium accumulations, potassium leaks, and phosphatidic acid accumulations in erythrocytes incubated for 2 h at 20°C in isotonic and hypertonic solutions containing 40 mM. In this study, the calcium accumulations (top panel) and potassium leaks (middle panel) observed in MyD erythrocytes in both isotonic and hypertonic media are not significantly different from those observed in the control cells. However, the accumulation of phosphatidic acid in erythrocytes obtained from MyD patients is markedly impaired in both isotonic and hypertonic incubations (bottom panel).

The calcium ionophore A23187, was used in subsequent experiments to augment calcium accumulation. Ionic calcium buffers were also used so that the effects of calcium accumulation on potassium leak and phosphatidic acid accumulation could be related to ionic calcium exposure rather than the intracellular accumulation of calcium. Fig. 2 shows that the phosphatidic acid contents of control and MyD erythrocytes are similar in the absence of calcium exposure. However, MyD erythrocytes show an impairment in phosphatidic acid accumulation after exposure to calcium. The potassium leaks of both MyD and control erythrocytes were linearly related to ionic calcium and not different from one another (data not shown).

In control erythrocytes, calcium entry leads to the accumulation of phosphatidic acid by hydrolysis of phosphatidylinositol phosphate to diacylglycerol (23) which, in ATP-replete erythrocytes, is further metabolized to phosphatidic acid (24). Because ATP is required for the conversion of diacylglycerol to phosphatidic acid, it was possible that the lack of phosphatidic acid accumulation was related to an excessive loss of ATP in erythrocytes from patients with MyD. We examined this possibility by measuring the ATP contents of control and MyD erythrocytes during both types of calcium loading procedures. The results displayed in Fig. 3 show that the ATP levels of both control and MyD cells were similar throughout all experimental protocols.

DISCUSSION

These data show that intact erythrocytes from MyD patients have an impairment of phosphatidic acid accumulations in response to calcium exposure. We believe these observations are notable in two respects: (*a*) the difference between MyD and control data appears to be more marked than in any other abnormality heretofore described in this disorder; (*b*) the impairment of phosphatidic acid accumulation is evident in patients with mild as well as severe clinical involvement. Hence, it is possible that affected individuals may be discriminated by these measurements. We would caution, however, that we do not know whether the impairment in calcium-induced phosphatidic acid

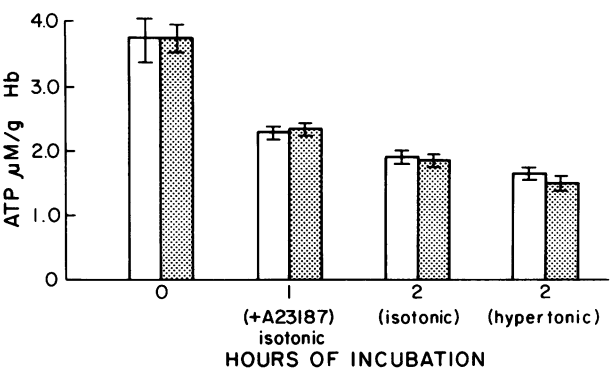

FIGURE 3 ATP levels of control and MyD erythrocytes (Hb). Values are shown for fresh cells (0 time), isotonic incubation for 1 h at 20°C with A23187 described in Fig. 2, isotonic and hypertonic incubation for 2 h at 20°C described in Fig. 1. There is no difference in ATP levels between control and MyD erythrocytes in any of these incubation conditions. □, control subjects; ▨, MyD patients. n = 4 and 5, respectively.

accumulation is unique for MyD nor, as yet, have we elucidated the specific metabolic alterations in phospholipid metabolism that account for this defect. Further work on the specificity and pathogenesis of the biochemical alteration is in progress.

Several possible mechanisms for impaired phosphatidic acid accumulation in response to calcium can be excluded. A decreased calcium uptake in MyD erythrocytes could not be demonstrated in a previous study (11) or in experiments presented in Fig. 1. An explanation involving the excessive loss of ATP in MyD erythrocytes with a consequent inability to produce phosphatidic acid can be excluded on the basis of data in Fig. 3, that show similar contents of ATP in control and MyD erythrocytes. An explanation based upon an altered distribution of calcium within MyD erythrocytes seems unlikely in view of the normal calcium sensitivity of the potassium leak observed under our incubation conditions. Furthermore, phosphatidic acid accumulation is impaired in the absence (Fig. 1) as well as the presence (Fig. 2) of calcium ionophore A23187. Because we performed the ionophore experiments in the presence of ionic calcium buffers, an explanation based on the lack of availability of calcium at an intracellular site seems unlikely.

Mechanisms that we cannot evaluate by current data involve alterations in phospholipid metabolism in plasma membrane. These possibilities include: (*a*) Reduced membrane content of phosphatidylinositol phosphate, the substrate for the calcium-sensitive phosphodiesterase (23). We believe this to be an unlikely possibility on the basis of a previous observation which showed no differences between control and MyD erythrocytes in the incorporation of ^{32}P from [γ-^{32}P] ATP into phosphatidylinositol phosphate.[2] (*b*) A decrease in the amount or calcium sensitivity of the calcium-sensitive phosphodiesterase enzyme. (*c*) A reduction of diacylglycerol kinase activity necessary for the conversion of diacylglycerol to phosphatidic acid. Additional experimental data will be necessary to decide among these possibilities as well as to determine the relationship of these data to altered band 3 phosphorylation (2, 4–6) or the other disturbances in membrane function that have been previously described in this disorder (7–11).

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[2] Roses, A. D., and S. H. Appel. Unpublished observation.

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