# Calcium inhibits muscle FBPase and affects its intracellular localization in cardiomyocytes

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**Abstract** **As our recent investigation revealed, in mammalian heart muscle, fructose 1,6-bisphosphatase (FBPase) – a key enzyme of glyconeogenesis – is located around the Z-line, inside cells' nuclei and, as we demonstrate here for the first time, it associates with intercalated discs. Since the degree of association of numerous enzymes with subcellular structures depends on the metabolic state of the cell, we studied the effect of elevated $Ca^{2+}$ concentration on localization of FBPase in cardiomyocytes. In such conditions, FBPase dissociated from the Z-line, but no visible effect on FBPase associated with intercalated discs or on the nuclear localization of the enzyme was observed. Additionally, $Ca^{2+}$ appeared to be a strong inhibitor of muscle FBPase.**


*Keywords:* Fructose 1,6-bisphosphatase; Cardiomyocyte; Calcium; Glyconeogenesis

## 1. Introduction

Fructose 1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11, FBPase) catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate in the presence of divalent metal ions like magnesium, manganese, cobalt or zinc [1,2]. Its liver and muscle isozymes have been found in vertebrate tissues [2–4]. Liver FBPase is recognized as a regulatory enzyme of gluconeogenesis. The muscle isozyme participates in the glycogen synthesis from lactate [5–7].

Previously, we have demonstrated that in cardiac muscle FBPase is present in cells' nuclei [8]. We have also found that in striated muscle FBPase accumulates in close proximity to the Z-line, where it binds to α-actinin [9,10], and we have postulated the existence of glyconeogenic metabolon around the Z-line [10] in analogy to the multi-enzyme complex formed by glycolytic enzymes bound to thin filaments [11,12]. Since simultaneous activity of the glyconeogenic and glycolytic pathways would result in the loss of energy via futile cycles [7], structural separation of the two pathways seems to be advantageous.

Optimal energy provision is fundamental for a healthy myocardium. Transition from rest to exercise is accompanied by an increase in the rate of cellular ATP hydrolysis [13]. ATP should therefore be synthesized at a sufficient rate that meets metabolic demands during contractile activity. During rest, metabolic processes designed to restore energy supplies should be activated.

One of the main regulatory mechanisms controlling both muscle contraction and production of energy for the contraction is presumed to arise from changes in the intracellular calcium concentration. Calcium is essential in the cardiac excitation–contraction coupling [14] – the process from electrical excitation of cardiomyocyte to contraction of the heart, achieved through an increase of cytosolic $Ca^{2+}$ level from nanomolar to micromolar values [15]. Changes in the cytosolic calcium concentration also regulate the dissociation of actin and myosin, resulting in sarcomere relaxation. Moreover, elevated $Ca^{2+}$ modulates activities of numerous enzymes, increasing their phosphorylation. Importantly, among the enzymes are those involved in glycogen mobilization and glycolysis (e.g., glycogen phosphorylase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) [16].

In the present paper, we demonstrate the result of our study of the effect of calcium ions on subcellular localization of FBPase in mammalian cardiomyocytes and on the activity of the enzyme. The protein exchange method and the nuclear import assay using fluorescein isothiocyanate (FITC)-labeled FBPase not only confirmed the presence of FBPase on the Z-line and inside the nuclei of cardiomyocytes, but they also revealed that the enzyme accumulates strongly in the intercalated discs. However, the localization experiments were carried out in calcium-free media, therefore the results approximate the state of a resting muscle.

An increase in calcium concentration in the incubating media caused dissociation of FBPase from the Z-line within seconds but had no visible effect on FBPase bound to the intercalated discs. An elevated cytosolic calcium concentration also did not influence the transportation of FBPase into the nuclei. Additionally, $Ca^{2+}$ appeared to be a strong inhibitor of the muscle FBPase.

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*Abbreviations:* EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FBPase, fructose 1,6-bisphosphatase; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin

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Table 1
The result of FBPase modification with FITC

| Enzyme | Specific activity before modification (U/mg) | Specific activity after modification (U/mg) | Stoichiometry (molecule of fluorochrome/ monomer of the enzyme) |
|---|---|---|---|
| FBPase | 30 | 12.6 | 2 |

## 2. Materials and methods

Phosphocellulose P-11 was purchased from Whatman (Maidstone, England). Other reagents were from Sigma (St. Louis, USA). All the chemicals used were of analytical grade.

### 2.1. Enzyme purification and activity determination

Rabbit muscle and liver FBPase were purified according to [15,17], respectively. The concentration of the enzyme was determined spectrophotometrically and the purity of FBPase was checked by 10% SDS–PAGE [18]. The activity of the enzyme was determined as described in [17]. To check the effect of $Ca^{2+}$ on the activity of FBPase, the assay mixture was incubated with various concentrations of $Ca^{2+}$ for 3 min, then the FBPase substrate was used to start the reaction.

Spectrophotometric measurements were performed with an HP 8452A diode array spectrophotometer. All kinetic parameters were calculated with the GraFit 4 program [19].

### 2.2. Fluorescent labeling

Fluorescently labeled FBPase was obtained by modification of the proteins with FITC, as described in [20].

The lack of proteolysis of fluorescently labeled protein was checked by 10% SDS–PAGE [18]. The number of fluorochrome molecules conjugated to the enzyme was estimated spectrophotometrically (Table 1).

### 2.3. Isolation of small bundles of cardiomyocytes from pig and rat heart

Preparation of small bundles of cardiomyocytes from rat and pig heart muscle and chemical permeabilization of the membranes were carried out according to the method described in [11] with slight modifications. Fresh pig heart muscle was provided by local slaughterhouse. The rat was killed by cervical dislocation, heart muscle was excised and used immediately. Small bundles ($0.5 \times 0.5 \times 0.5$ cm) of heart muscle cells were dissected and placed in a skinning solution (0.5% Triton X-100, 5 mM $KH_2PO_4$, 3 mM magnesium acetate, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM ATP, 50 mM creatine phosphate, 5 mM sodium azide, 10 mM glutathione, 2 mM dithiothreitol, and 20 μM leupeptin; pH 7.5) for 30 min at 4 °C. After that, the muscle was transferred to the skinning solution without Triton X-100 and smaller bundles ($0.2 \times 0.2 \times 0.2$ cm) were prepared. The bundles were kept in the skinning solution for 4–5 h before incubation with any of the enzymes.

### 2.4. The protein exchange and the effect of calcium ions on localization of FBPase in cardiomyocytes

Directly before the incubation of the labeled proteins with skinned cardiomyocyte bundles, the proteins were dialyzed for 5 h against a relaxing solution (10 mM imidazole, 2 mM magnesium chloride, 1 mM EGTA, 1 mM ATP, 20 mM creatine phosphate, 2 mM dithiothreitol, and 106 mM potassium propionate; pH 7.5, $T = 4$ °C). The skinned cells were mounted on microscopic slides (in chambers made with PAP Pen, without covering the chambers) and incubated overnight at 4 °C in a drop (100 μL) of the relaxing solution with 0.4 mg/mL of FITC-FBPase added. After the incubations, the cells were washed several times with the relaxing solution to remove the unbound, fluorescently labeled proteins. The cells were then incubated in a drop of A1 solution containing $Ca^{2+}$ ions (final concentration: 0.01 or 0.1 mM $CaCl_2$, 10 mM imidazole, 2 mM magnesium chloride, 5 mM dithiothreitol, 20 mM creatine phosphate, and 106 mM potassium propionate; pH 7.0; $T = 4$ °C). Control cells were incubated in a drop of A1 solution without $Ca^{2+}$ ions and covered with coverslips. All images were obtained immediately after preparing the slides.

### 2.5. The effect of calcium ions on nuclear localization of FBPase

Pig cardiomyocyte nuclei were isolated as described before [8], except that the incubation with Triton X-100 during the isolation was omitted. Immediately after isolation the nuclei were placed on microscopic slides and incubated for 1 h at 37 °C in a complete nuclear import assay [21] containing 20 mM HEPES, pH 7.3, 110 mM potassium propionate, 5 mM sodium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 U/ml creatine kinase, 1 mM MgATP, 0.5 mM EGTA, 5 mM creatine phosphate, 400 nM FBPase-FITC and 1% rabbit reticulocyte lysate. To check the effect of an elevated calcium concentration on nuclear localization of FBPase, $Ca^{2+}$ was added to the buffer to a final concentration of 0.1 mM.

In a control reaction, a portion of isolated nuclei was incubated for 30 min with 1 mg/ml wheat germ agglutinin (WGA) in import buffer [22] prior to addition of FBPase-FITC.

### 2.6. Confocal microscopy

The fluorescent images were obtained with a Bio-Rad MRC 1024ES confocal scanner unit (equipped with a krypton/argon laser) with 488 laser line and a Zeiss Axiovert S100 inverted microscope (with 63× Plan Apo Oil objective, NA = 1.4).

## 3. Results

The protein exchange experiment, with the use of FITC-labeled FBPase and confocal microscopy, resulted in accumulation of the enzyme on the Z-line of cardiomyocytes (Fig. 1). The same striated pattern was achieved previously using the immunogold method and electron microscopy [8]. Additionally, the protein exchange method revealed a strong fluorescent signal from the intercalated disc. Since one of the well known proteins of the structure is α-actinin [23], and we have previously demonstrated a strong interaction between α-actinin and FBPase [9,10], such location of FBPase within intercalated discs might have been expected. The lack of positive FBPase staining of the intercalated discs in our previous observation (electron microscopy) resulted presumably from high dilutions of antibodies used in the reaction.

In control experiments, where cardiomyocytes were incubated with fluorochrome alone, no staining was observed (data not shown).

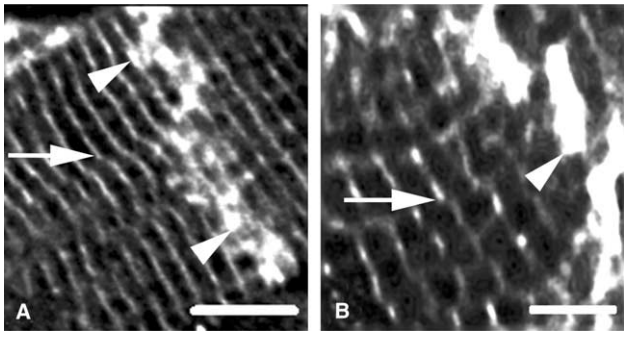


Fig. 1. Accumulation of FBPase-FITC on the Z-line and the intercalated discs of rat (A, bar = 10 μm) and pig (B, bar = 5 μm) cardiomyocytes. Arrows point to the Z-line, arrowheads to the intercalated discs.

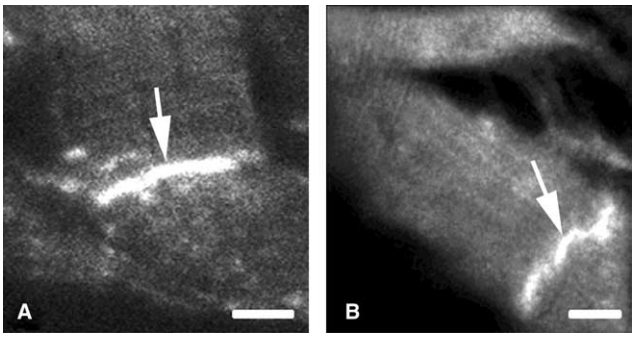


Fig. 2. Influence of $Ca^{2+}$ on the localization of FBPase on the Z-line and intercalated discs of rat (A) and pig (B) cardiomyocytes. Arrows point to the intercalated discs. Bar = 10 μm.

Incubation of the cardiomyocytes with 0.01 or 0.1 mM $Ca^{2+}$ resulted in dissociation of FBPase from the Z-lines within seconds, but not from the intercalated discs (Fig. 2).

Incubation of isolated cardiac nuclei in import assay with added FBPase-FITC resulted in accumulation of green fluorescence inside the nuclei (Fig. 3A). Removal of cytosol from the import assay resulted in a lack of nuclear staining (data not shown). Accumulation of fluorescence in the nuclei was also blocked in the presence of WGA (Fig. 3C) [22], indicating that envelopes of the nuclei used in the experiment were intact.

An elevated cytoplasmic $Ca^{2+}$ concentration had no effect on nuclear localization of FBPase, as one might have expected (Fig. 3B). The experiment did, however, demonstrate that penetration of FBPase into the nuclei proceeds through the nuclear pore complex and that the transport is dependent on cytosolic factors.

When purified muscle FBPase was incubated with different concentrations of $Ca^{2+}$ (0.1 μM–1 mM), a decrease in the enzyme activity was observed. The calculated $I_{0.5}$ for $Ca^{2+}$ for purified muscle FBPase was 0.59 μM (Fig. 4). This result differs from the findings presented by Vaughan et al. [24] showing that the activity of FBPase from muscle homogenates is modified by $Ca^{2+}$ concentrations only higher than 0.1 mM. In our experiment, purified muscle FBPase exposed to 0.1 mM $Ca^{2+}$ revealed only about 10% of its initial activity (Fig. 4). On the other hand, purified liver FBPase under the same conditions retained over 80% of its activity (data not shown), and the activity decreased significantly only when the $Ca^{2+}$ concentration approached 1 mM.

The inhibitory effect of calcium on muscle FBPase was previously mentioned also by Horecker and his team in 1972 [25]. According to the results of their experiment, at alkaline pH, $Ca^{2+}$ appeared to be a competitive muscle FBPase inhibitor with regard to $Mg^{2+}$, but $I_{0.5}$ for calcium for the enzyme was still high (0.21 mM).

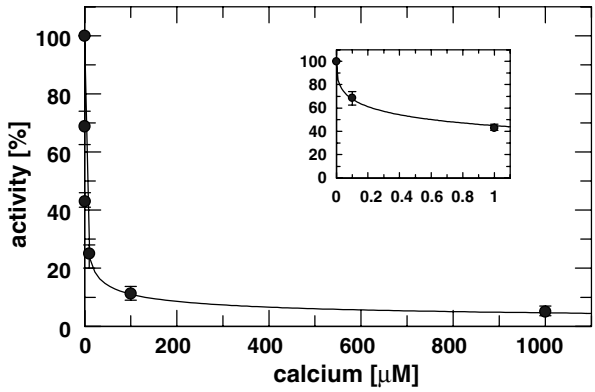


Fig. 4. Inhibition of purified muscle FBPase by calcium ions. $I_{0.5} = 0.59$ μM. The inset shows the FBPase inhibition within the range 0–1 μM $Ca^{2+}$.

## 4. Discussion

FBPase reaction proceeds in the presence of divalent cations like Mg, Mn or Zn, and the effect of the ions on liver FBPase has been broadly investigated. For many years, the physiological role of muscle FBPase was rather an enigma. Therefore, the report by Horecker and his team, that high calcium concentration inhibits muscle FBPase, was not appreciated. In recent years, evidence has accumulated indicating that muscle FBPase is a regulatory enzyme of glyconeogenesis. From this point of view, inhibition of FBPase by calcium as well as its effect on subcellular localization of the enzyme seem to be of importance.

In the present work, we demonstrate that dissociation of FBPase from the Z-lines of cardiac muscle is promoted by micromolar calcium concentrations, typical of contracting fibers only, and that $Ca^{2+}$ is a potent inhibitor of purified muscle FBPase.

Muscle tissue is probably one of the most tightly packed and yet highly ordered structures. In the tissue, as frequently demonstrated, actin filaments are presumed to provide a structural basis for the glycolytic complex [26,27]. Recently, evidence has come to light supporting the hypothesis that striated muscle glyconeogenesis is separated from glycolysis

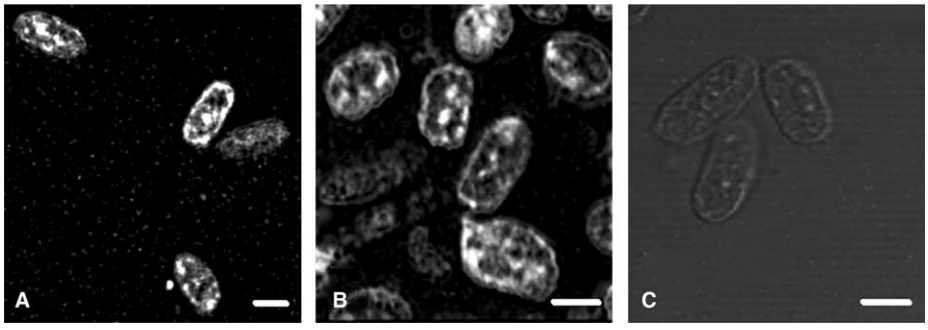


Fig. 3. Nuclear localization of FBPase-FITC in the presence (A) and absence (B) of 0.1 mM $Ca^{2+}$ in the import assay. WGA blocked import of FBPase to the nuclei (C). Bar = 5 μm.

and that enzymes of the glyconeogenic pathway are organized as a multienzymatic complex located on α-actinin of the Z-line [10,28,29]. Structural separation of the two pathways may protect muscle cell, specialized in the conversion of energy from chemical to mechanical, against the loss of the energy via futile cycles, thus contributing to cardiac and skeletal muscle efficiency.

Assuming that formation of multienzymatic complexes results in an increased effectiveness of pathways (e.g., due to substrate channeling), changes in the metabolic state of the fiber associated with muscle contraction are likely to promote formation of the glycolytic complex and disintegration of the glyconeogenic metabolon.

Muscle contraction is triggered by a rapid release of $Ca^{2+}$ from sarcoplasmic reticulum. An elevated cytoplasmic $Ca^{2+}$ concentration should, therefore, stimulate activities of regulatory enzymes of glycolysis and inhibit activities of the key enzymes of glyconeogenesis. Relaxation of muscle – preceded by a reuptake of $Ca^{2+}$ to the reticulum – should be followed by inhibition of glycolysis and activation of glycogen synthesis. An increase in the activity of glycolytic enzymes in response to high $Ca^{2+}$ has recently been demonstrated by Singh et al. [16] and the possible effect of calcium on local ATP generation has been discussed.

Therefore, in the light of our current findings, the dissociation of FBPase from the Z-lines promoted by an elevated calcium concentration, together with a strong inhibitory effect of the cation on muscle FBPase activity, might suggest the presence of a calcium-dependent mechanism of glyconeogenic metabolon destruction and of glycogen synthesis inhibition during muscle contraction.

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