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# Regulation of subcellular localization of muscle FBPase in cardiomyocytes. The decisive role of calcium ions

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**Glyconeogenesis, the synthesis of glycogen from carbohydrate precursors like lactate, seems to be an important pathway participating in replenishing glycogen in cardiomyocytes. Fructose-1,6-bisphosphatase (FBPase), an indispensible enzyme of glyconeogenesis, has been found in cardiomyocytes on the Z-line, in the nuclei and in the intercalated discs. Glyconeogenesis may proceed only when FBPase accumulates on the Z-line. Searching for the mechanism of a FBPase regulation we investigated the effects of the calcium ionophore A23187, a muscle relaxant dantrolene, glucagon, insulin and medium without glucose on the subcellular localization of this enzyme in primary culture of neonatal rat cardiomyocytes. Immunofluorescence was used for protein localization and the intracellular calcium concentration was measured with Fura. We found that the concentration of calcium ions was the decisive factor determining the localization of muscle FBPase on the Z-line. Calcium ions had no effect on the localization of the enzyme in the intercalated discs or in the nuclei, but accumulation of FBPase in the nuclei was induced by insulin.**

**Keywords:** muscle FBPase, cardiomyocytes, calcium, insulin, Z-line, intercalated disc

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## INTRODUCTION

The muscle and liver isozymes of a FBPase [EC 3.1.3.11] have been found in numerous vertebrate tissues (Benkovic & deMaine, 1982; Tejwani, 1983; Dzugaj, 2006). The kinetic properties of both isozymes are virtually the same. In the presence of divalent metal ions like magnesium or manganese FBPase catalyze the hydrolysis of fructose-1,6-bisphosphate producing fructose-6-phosphate and orthophosphate. Both isozymes are inhibited by fructose-2,6-bisphosphate and AMP, but the muscle isozyme is 10 to 100 times more sensitive toward the latter. It has been hypothesized that the muscle isozyme regulates glyconeogenesis, the synthesis of glycogen from carbohydrate precursors like lactate. Glyconeogenesis may proceed only when FBPase is bound with aldolase which desensitizes the former enzyme to the inhibition by AMP. Muscle FBPase is localized in myocytes on the Z-line, and in cardiomyocytes on the Z-line, in the nuclei and in the intercalated discs. In smooth muscle cells FBPase is localized to the nuclei. Furthemore, we have found that unlike the liver isozyme, muscle FBPase is highly sensitive to inhibition by calcium ions (Gizak *et al.,* 2004). The increase of calcium ion concentration during muscle contraction causes the breakdown of the FBPase-aldolase complex which results in inhibition of glyconeogenesis. Supposedly, in the muscle calcium plays a similar role concerning carbohydrate metabolism to that of fructose-2,6-bisphosphate in the liver, where it is a regulator of gluconeogenesis and glycolysis. The level of fructose-2,6-bisphosphate is hormonally regulated and the effect of hormones on the subcellular localization of FBPase in hepatocytes has been investigated (Yanez *et al.,* 2004). Here, searching for the mechanism of glyconeogenesis regulation we investigated the effects of calcium ionophore, dantrolen, glucagon, insulin and medium without glucose on the subcellular localization of muscle fructose-1,6-bisphosphatase in primary culture of neonatal rat cardiomyocytes. Calcium ions were found to be the decisive factor determining the localization of muscle FBPase on the Z-line.

## MATERIALS AND METHODS

Cell culture media and sera were from Gibco. DMEM without glucose was purchased from the Laboratory of Chemistry (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland). Fura-2AM, used for calcium measurements, was from Invitrogen. All other chemicals, enzymes and antibodies were purchased from Sigma (USA).

**Antibodies**. Rabbit antibodies against rabbit muscle FBPase were used. Their reactivity and specifivity were presented earlier (Gizak & Dzugaj, 2003; Gizak *et al.,* 2003; 2006; 2009). Monoclonal mouse antibodies against sarcomeric α-actinin were purchased from Sigma (clone EA-53).

**Cell culture.** Cell isolation was performed in agreement with the rules of the Scientific Research Ethical Committee. Primary cultures of neonatal rat cardiomyocytes were established according to (Przygodzki *et al.,* 2005). Immediately after the decapitation of 2- to 5-day old rats, hearts were isolated and minced in buffer A (20 mM Hepes, 120 mM NaCl, 1 mM $NaH_2PO_4$, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.4). Then the tissue was digested for 10 min in collagenase (80 units/ml)/pancreatin (0.6 mg/ml) solution in buffer A with rapid repipetting. Cell suspension collected from six digestion cycles was combined and incubated with DNase (0.01 mg/ml, 10 min). After filtration through

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**Abbreviations:** BSA, bovine serum albumin; CCD, charge coupled device; DMEM; Dulbecco's modified Eagle medium; FBPase, fructose-1,6-bisphosphatase; FITC, fluorescein isothiocyanate; FK-2, phosphofructokinase-2; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate

a nylon mesh cells were centrifuged, suspended in buffer A and centrifuged in a discontinuous Percoll gradient in order to obtain a fraction enriched in cardiomyocytes. After counting, cells were seeded (35000/cm$^2$) onto gelatine-coated coverslips placed in culture dishes in culture medium (DMEM high glucose/M199 (4:1, v/v), 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin and 10 μg/ml cytosine-β-D-arabinofuranoside). After 24 h the medium was replaced with fresh medium without the cytostatic. The medium was removed after next 48 h and replaced with sera-free medium containing DMEM high glucose/M199 (4:1, v/v), 1% penicillin/streptomycin, 1% BSA, and 5 μg/ml transferrin. For examination of the effect of insulin on intracellular distribution of FBPase the cells were incubated in the medium free of the cytostatic for 24 h and afterwards the medium was replaced with serum-free medium consisting of DMEM high glucose/M199 (4:1, v/v), 1% penicillin/streptomycin. The viability of the cells (at least 90%) was determined with trypan blue exclusion and the percentage of beating myocytes exceed 80%.

After overnight incubation in sera-free medium the cardiomyocytes were stimulated (10 min) in the following medium: DMEM high glucose/M199 4:1, v/v, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin supplemented with muscle relaxant dantrolene (25 μM). Insulin was added to a final concentration of 0.1 μM to the sera-free medium consist of DMEM high glucose/M199 4:1, v/v, 1% penicillin/streptomycin, and incubation was carried out for 2 h. Control cells were incubated in the respective media without supplements. The effects of glucose removal from culture medium was examined in the medium: DMEM without glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin (glucose-free medium). Cardiomyoctes were incubated in such a medium for 1 h. The control incubation was carried out in medium: DMEM high glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin. Calcium ionophore A23187 (10 μM) or glucagon (1.7 nM) was added to the glucose free medium and cells were incubated for 1 h.

**Immunofluorescence staining.** Immediately after the treatments described above, cells were washed with PBS and fixed in cold methanol at –20 °C for 10 min. After washing with PBS the fixed cells were incubated for 30 min in 5% goat serum in PBS. Overnight incubation with rabbit antibody against FBPase (1:100) and mouse antibody against α-actinin (1:500) diluted in PBS was carried out at 4 °C. The immunostaning for α-actinin was carried out in order to identify two subcellular compartments of interest: the Z-line and the intercalated discs. After washing with PBS the cells were incubated for 1 h at room temperature with FITC-conjugated secondary antibodies against rabbit IgG (1:100) and TRITC-conjugated secondary antibodies against mouse IgG (1:100). The cells were mounted on glass slides with Dako Fluorescent Mounting Medium and examined by confocal microscopy.

**Immunohistochemistry**. Neonatal rat heart muscle tissue samples were fixed in Bouin's fluid for 24 h at room temp. and after dehydratation were embedded in polyester wax. Sections of 5 μm were cut and mounted on slides. After deparaffinization sections were blocked with goat serum (5%) in PBS for 30 min and incubated overnight with the primary antibodies against FBPase (1:200). FITC-conjugated antibodies against rabbit IgG were used as a secondary antibody (1:100). To visualize nuclei samples were treated with RNase (1 mg/ml, 1 h) and propidium iodide (10 μg/ml, 30 min). All incubations were followed by triple washing with PBS. The sections were mounted in Dako Fluorescent Mounting Medium and examined by confocal microscopy.

**Confocal microscopy.** Images were obtained with an Olympus FV500 fluorescent confocal laser scanning microscope. Each image was taken with a 60× oil objective (NA 1.4) and to get a good signal to noise ratio four frames were averaged. Sequential acquisition, with separate scan for each dye, was used in order to avoid the emission cross-talk. Optical slices of 1 μm are presented.

**Measurement of intracellular calcium.** For calcium measurments neonatal rat cardiomyocytes were cultivated on round, 22-mm glass coverslips covered with gelatine. Before the measurements, cells on the coverslips were washed once with PBS and once with fresh culture medium and then incubated at 37 °C for 15 min in culture medium with 1 μM Fura-2, AM. Thereafter, the coverslips were mounted in a chamber at 37 °C on a Nikon Diaphot inverted-stage microscope equipped with a fluo 40× (NA 1.3) oil-immersion objective. Pairs of images, excited with 340 nm and 380 nm light, were collected every second. After minute of acquisition the medium was changed for fresh one with or without glucose as indicated in the results, containing one of the following agents: dantrolene (25 μM), calcium ionophore (10 μM) or glucagon (1.5 μg/ml) and acquisition was continued for another minute. Then the cells were left in the microscopic chamber in the case of dantrolene, or transferred to an incubator for the next 30 or 60 min to provide them with the optimal $CO_2$ and humidity conditions. After that time the cells were mounted again in the microscope chamber and the acquisition was continued for the same field of view. Fura-2 digital fluorescence microscopy was used to determine changes in the intracellular calcium levels ($[Ca^{2+}]_i$) (Grynkiewicz *et al.,* 1985). A Ludl Lep MAC 5000 filter wheel system with a Chroma Inc. Fura-2 filter set was used for specimen illumination. Images were acquired using a Retiga 1300 chilled digital CCD camera (QImaging, Inc.). Data processing was carried out using AQM Advance 6 (Kinetic Imaging Inc.) and MS Excel software. Each experiment was repeated at least three times and graphs show data expressed as means from one experiment of each type. All data is expressed as the changes of the ratio of Fura-2 fluorescence excited at 340 and 380 nm against time (Δ 340/380). Further analysis was performed using MatLab software (Mathworks®). Three parameters of the changes of calcium concentration *versus* time were studied: resting mean, or the concentration of free cytoplasmic calcium in resting cell, peak height, or the rise of calcium concentration during the cell contraction, and frequency of contraction associated with calcium transients. Because the calcium bursts varied between experiments, the graphs show the ratio of peak heights. The mean peak height at the beginning of each type of experiment is taken as 1. Spectral analysis of $Ca^{2+}$ oscillation was performed as described by Uhlen (2004). Bars present the median frequency of the oscillations.

**Statistical analysis.** The nonparametric Mann-Whitney U test was used to discriminate the differences between parameters of calcium responses. Differences with $P < 0.01$ were considered highly significant and marked (**) on the plots, those with $P < 0.001$ were marked (***), a lack of statistically significant difference was marked (–).

## RESULTS

We studied the effects of selected hormones and other compounds of biological activity as well as the withdrawal of glucose from culture medium on the distribution of FBPase and on the calcium level in cultured neonatal rat cardiomyocytes. Additionally, we investigated the effect of insulin on the localization of FBPase in neonatal rat cardiomyocytes.

Neonatal rat cardiomyocytes were cultured for 3 days in the presence of sera and then incubated overnight in serum-free medium supplemented with BSA and transferrin. Such cells revealed a well-defined cross striation pattern with developed intercalated discs. FBPase was distributed throughout the cytosol and accumulated in the intercalated discs. No or a weak fluorescence signal was observed from cell nuclei (Fig. 1A). Spontaneous calcium oscillations of the cells were observed under culture conditions (e.g. Fig. 2).

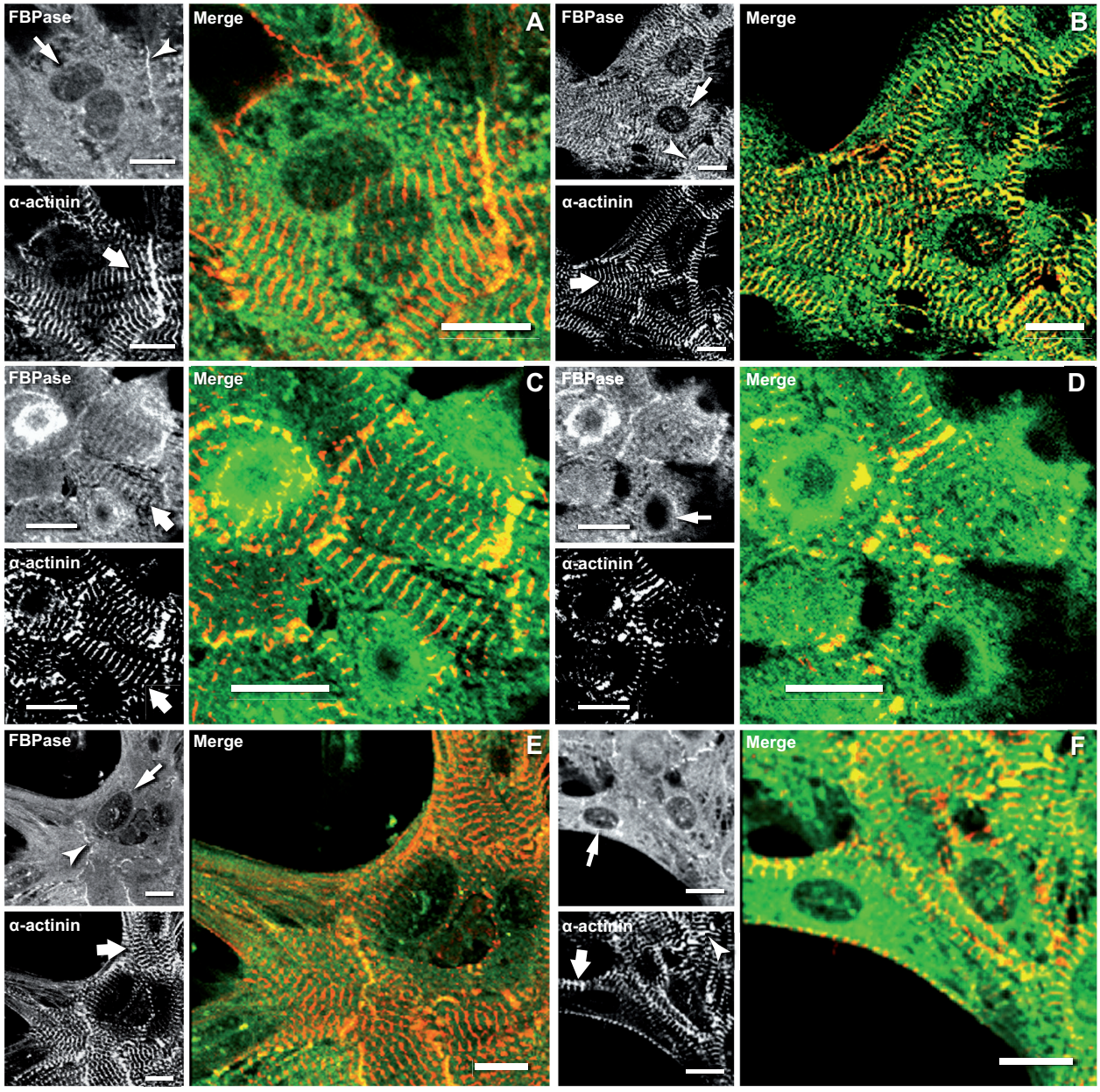


**Figure 1. Subcellular localization of FBPase in rat neonatal cardiomyocytes**
**(A)** In the neonatal rat cardiomyocytes FBPase is present in the cytoplasm and accumulates on the intercalated discs. The thin arrow points to the nuclei, the fat arrow points to the Z-line, the arrow head points to the intercalated disc. The bar represents 20 μm. **(B)** In the neonatal rat cardiomyocytes FBPase is present in the cytoplasm and accumulates on the intercalated discs. The thin arrow points to the nuclei, the fat arrow points to the Z-line, the arrow head points to the intercalated disc. The bar represents 20 μm. In the neonatal rat cardiomyocytes incubated in the presence of dantrolene the FBPase accumulates on the Z-line. Two optical slices of 1 μm are presented (C and D). **(C)** The fat arrows point to Z-line, the arrow head points to the intercalated disc; **(D)** the thin arrow points to the nucleus. The bar represents 15 μm. **(E)** In the neonatal rat cardiomyocytes incubated in the presence of calcium ionophore A23187 FBPase dissociates from the Z-line. The thin arrow points to the nuclei, the fat arrow points to the Z-line, the arrow head points to the intercalated disc. The bar represents 25 μm. **(F)** In the neonatal rat cardiomyocytes incubated in the presence of glucagon the FBPase dissociates from the Z-line. The thin arrow points to the nuclei, the fat arrow points to the Z-line, the arrow head points to the intercalated disc. The bar represents 20 μm.

### Glucose-free medium

Cardiomyocytes incubated in the glucose-free medium revelead enhanced accumulation of FBPase on the Z-line (Fig. 1B) in comparison with cells incubated in the presence of glucose (Fig. 1A). The accumulation of the FBPase on the Z-line in the absence of glucose in culture medium was correlated with a decrease of the average cytosolic calcium peak height (Fig. 2C). While an extended glucose starvation led to the lowering of maximal calcium peak, it did not change the frequency of calcium bursts (Fig. 2D).

### Dantrolene

Cells incubated in the presence of a muscle relaxant, dantrolene revealed accumulation of FBPase on the Z-line (Fig. 1C and D) and slightly increased calcium transients (Fig. 3C), of a significantly decreased frequency (Fig. 3D). We also observed a lower resting mean of

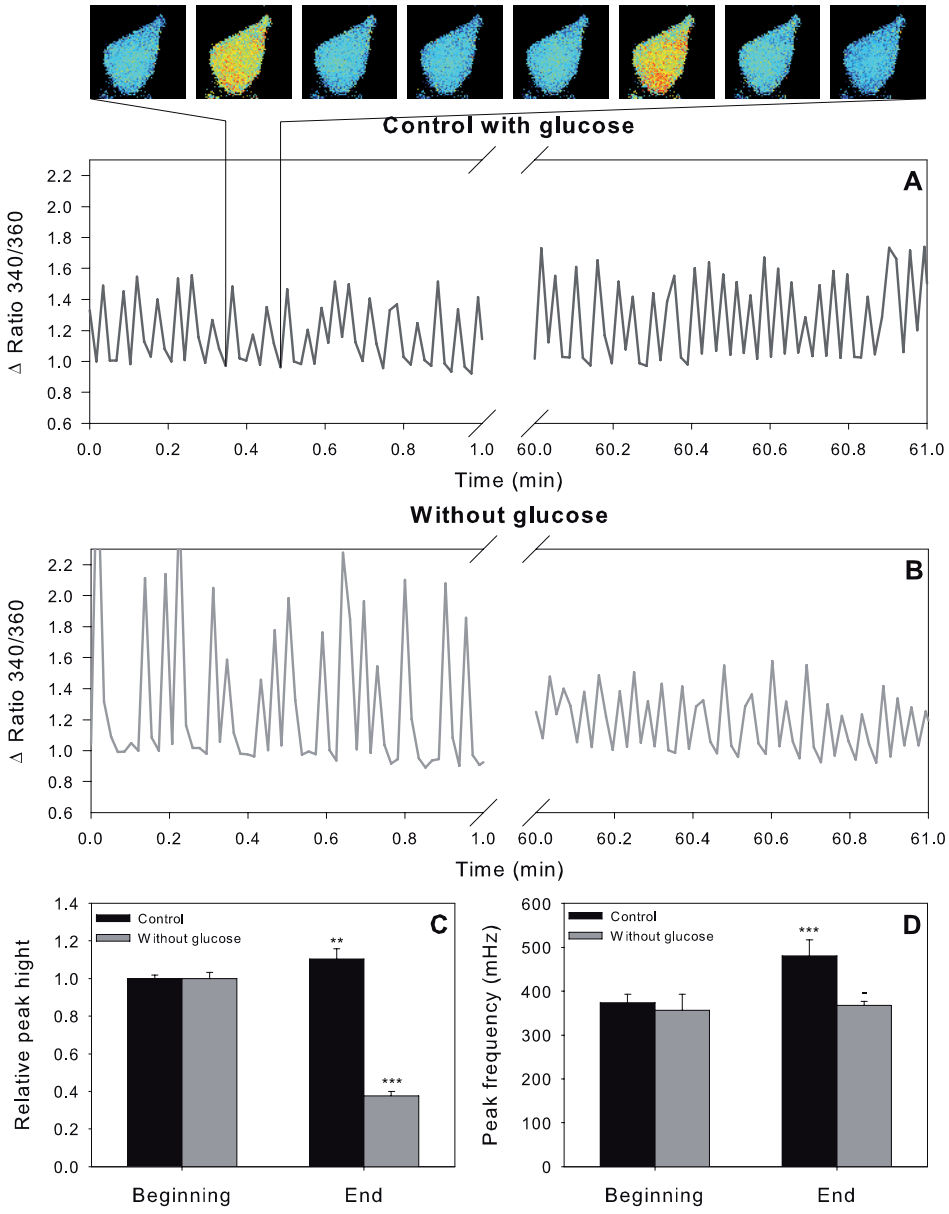


**Figure 2. Effect of glucose removal on calcium signaling in neonatal rat cardiomyocytes**
Cardiomyocytes were cultured for 3 days in the presence of serum, subjected to overnight serum starvation and incubated for 1 h in medium: DMEM high glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin. Afterward the cells were loaded with Fura 2-AM and calcium bursts of individual cells (n) were observed for one minute as described in Materials and Methods (Measurement of intracellular calcium). Then medium was changed for the same fresh medium with high glucose (in control cells) or for DMEM without glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin. Cells were transferred to an incubator for next 60 min. After incubation the measurement of intracellular calcium was continued on the same cells as at the beginning of experiment. **(A)** Calcium oscillations in cells cultured at the beginning and end of experiment in medium with high glucose. Black trace represents mean fluorescence ratio (Δ340/380) value of the responses of individual cells, n = 18. Real images of fluorescence ratio (Δ340/380) from one of the cells showed above. **(B)** Calcium oscillations in cells cultured at beginning of the experiment in medium with high glucose followed by medium without glucose. Dark grey trace represents the mean fluorescence ratio value (Δ340/380) of the responses of individual cells, n=13. **(C)** Relative mean values of peak heights. The mean peak height at the beginning of experiment is taken as 1. Colour of bars as above. Statistically different values marked (***) for $P < 0.001$ and (**) for $P < 0.01$. **(D)** Changes in frequency of $Ca^{2+}$ oscillations. Spectral analysis was performed as described by Uhlen (2004). Bars present the median frequency of oscillations ± S.E. Color of bars as above. Statistically different values marked (***) for $P < 0.001$. The difference between bars marked (–) is statistically insignificant.

cytoplasmic free calcium ions concentration (Fig. 3B). We suggest that the drug decreases of effective cytosolic calcium concentration: the fraction of time when the calcium concentration is high enough to cause the dissociation of FBPase from the Z-line. Here for the first time the reversibility of FBPase binding to the Z-line was showed.

Dantrolene as well as the incubation of the cells in glucose-free medium had no effect on the presence of the enzyme in the nuclei or in the intercalated discs (Fig. 1C and D).

### Glucagon and calcium ionophore in glucose-free medium

Previously we demonstrated that calcium ions caused dissociation of FBPase from the Z-line (Gizak *et al.*, 2004). To determine the effect of the rise in the cytosolic calcium concentration on the FBPase dissociation

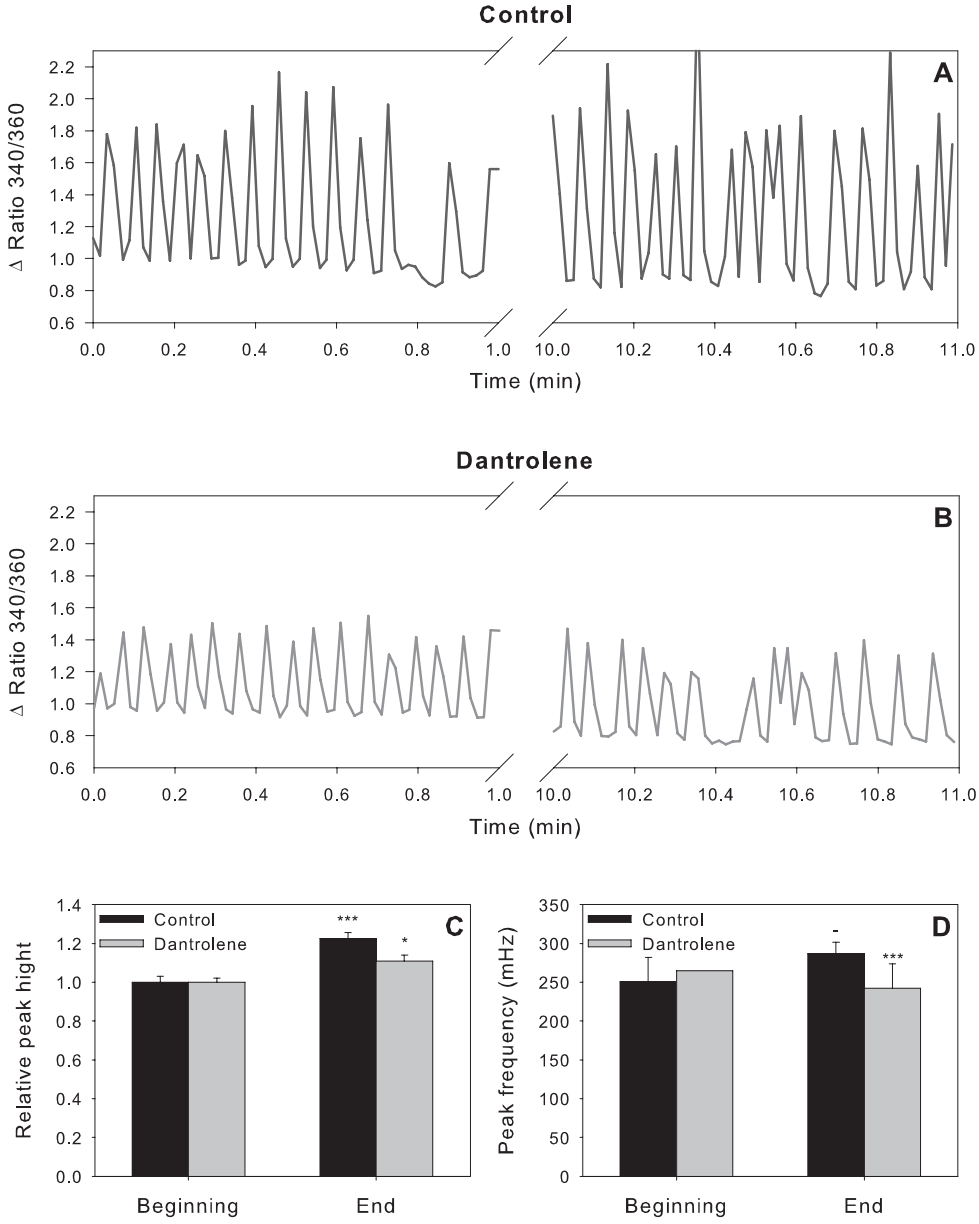


**Figure 3. The effect of dantrolene on the calcium signaling in the neonatal rat cardiomyocytes**
Cardiomyocytes were cultured for 3 days in the presence of serum, subjected to the overnight serum starvation (in the serum free medium) and then incubated for 1 h in the medium: DMEM high glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin. Afterwards the cells were loaded with the Fura-2, AM and calcium bursts of singular cells (n) were observed for one minute as described in Materials and Methods (Measurement of intracellular calcium). At that time the medium was changed for the same fresh medium with the high glucose (in control cells) or for DMEM with high glucose containing 25 μM dantrolene. After 10 min the measurement of intracellular calcium was continued on the same cells as at the beginning of experiment. **(A)** Calcium oscillations in cells cultured at the beginning and the end of the experiment in the medium with high glucose. The black trace represents the mean ratio value of the responses of singular cells, n=9. **(B)** Calcium oscillations in cells cultured at the beginning of the experiment in the medium with high glucose followed by the medium with glucose and 25 μM dantrolene (the end of the experiment). The grey trace represents the mean ratio value of the responses of singular cells, n=8. **(C)** Relative mean values of the peak heights. The mean peak height at the beginning of the experiment is presented as 1. The color of bars as described above. Statistically different values marked (***) for $P<0.001$ and (*) for $P<0.05$. **(D)** Changes in frequency of $Ca^{2+}$ oscillations. The spectral analysis was performed as described (Uhlen, 2004). Bars present the median frequency of the oscillations ± S.E. The color of bars as described above. Statistically different values marked (***) for $P<0.001$. The difference between bars marked (–) is statistically insignificant.

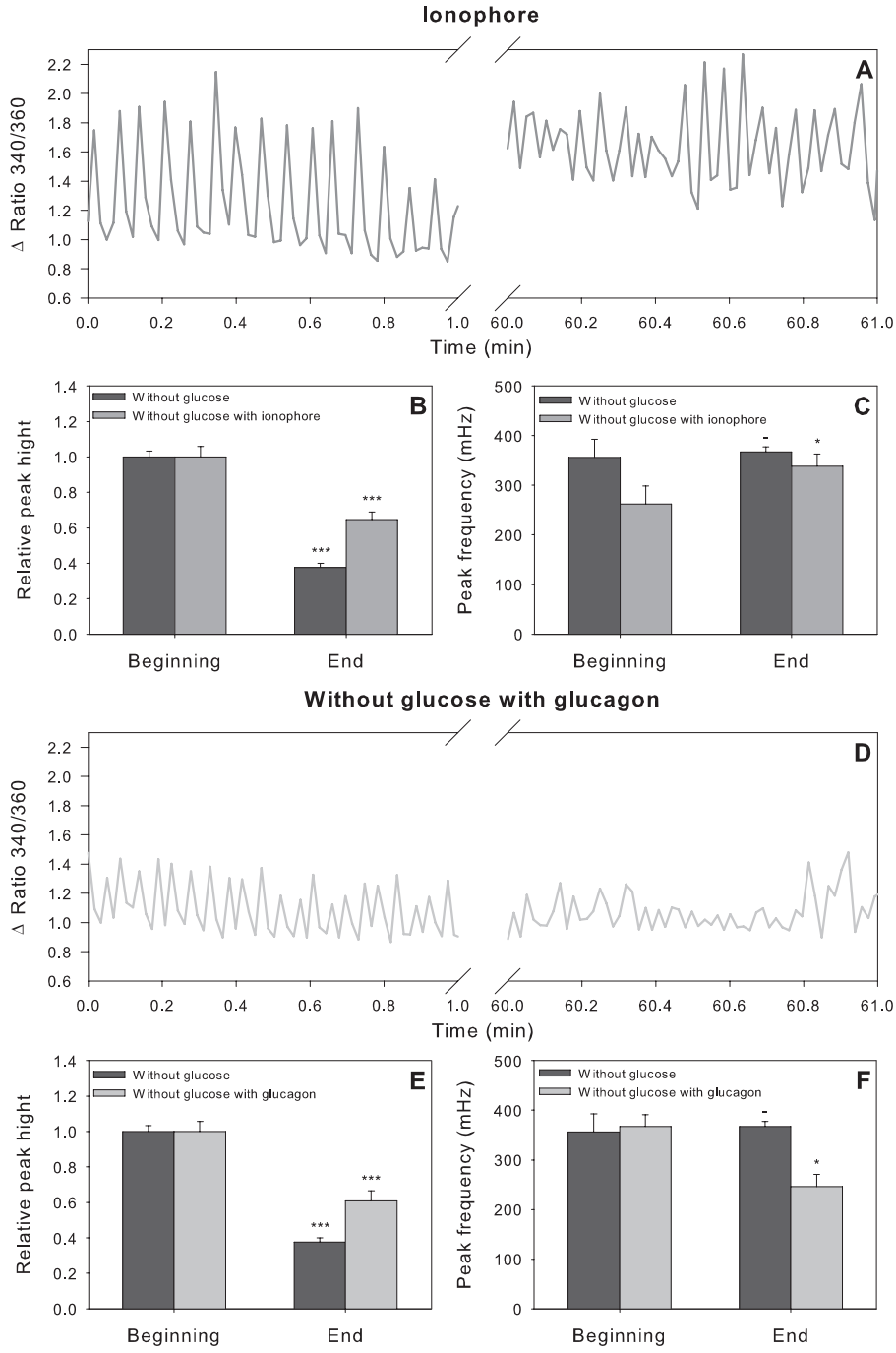


**Figure 4. The effect of ionophore A23187 and glucagon on the calcium signaling in the neonatal rat cardiomyocytes**
Cardiomyocytes were cultured for 3 days in the presence of serum, subjected to the overnight serum starvation (in the serum free medium) and then incubated for 1 h in the medium: DMEM high glucose, 1% penicillin/streptomycin, 1% BSA, 5 μg/ml transferrin. Afterwards the cells were loaded with the Fura-2, AM and calcium bursts of singular cells (n) were observed for one minute as described in Materials and Methods (Measurement of intracellular calcium). At that time the medium was changed for DMEM without glucose, as described under Fig. 10B or the medium without glucose with 10 μM calcium ionophore A23187 (A, B, C) or 1.5 μg/ml glucagon (D, E, F). The cells were transferred to the incubator for next 60 min to provide them with the best $CO_2$ and humidity conditions. After that the measurement of intracellular calcium was continued on the same cells as at the beginning of experiment. **(A)** Calcium oscillations in cells cultured at the beginning of the experiment in the medium with high glucose followed by the medium without glucose with 10 μM calcium ionophore A23187 (the end of the experiment). The grey trace represents the mean ratio value of the responses of singular cells, n=6. **(B)** Relative mean values of the peak heights. The mean peak height at the beginning of the experiment is presented as 1. The dark grey color of bar — cells without glucose (Fig. 2C), the grey color of bar — cells without glucose with ionophore A23187. Statistically different values marked (***) for $P<0.001$. **(C)** Changes in frequency of $Ca^{2+}$ oscillations. The spectral analysis was performed as described previously (Uhlen, 2004). Bars present the median frequency of the oscillations ± S.E. The color of bars as described above. Statistically different values marked (*) for $P<0.05$. The difference between bars marked (-) is statistically insignificant. **(D)** Calcium oscillations in cells cultured at the beginning of the experiment in the medium with the high glucose followed by the medium without glucose with glucagon (the end of the experiment). The grey trace represents the mean ratio value of the responses of singular cells, n=11. **(E)** Relative mean values of the peak heights. The mean peak height at the beginning of the experiment is presented as 1. The dark grey color of bar — cells without glucose (Fig. 2C), the light grey color of bar — cells without glucose with glucagon. Statistically different values marked (***) for $P<0.001$. **(F)** Changes in frequency of $Ca^{2+}$ oscillations. The spectral analysis was performed as described previously (Uhlen, 2004). Bars present the median frequency of the oscillations ± S.E. The color of bars as described above. Statistically different values marked (*) for $P<0.05$. The difference between bars marked (–) is statistically insignificant.

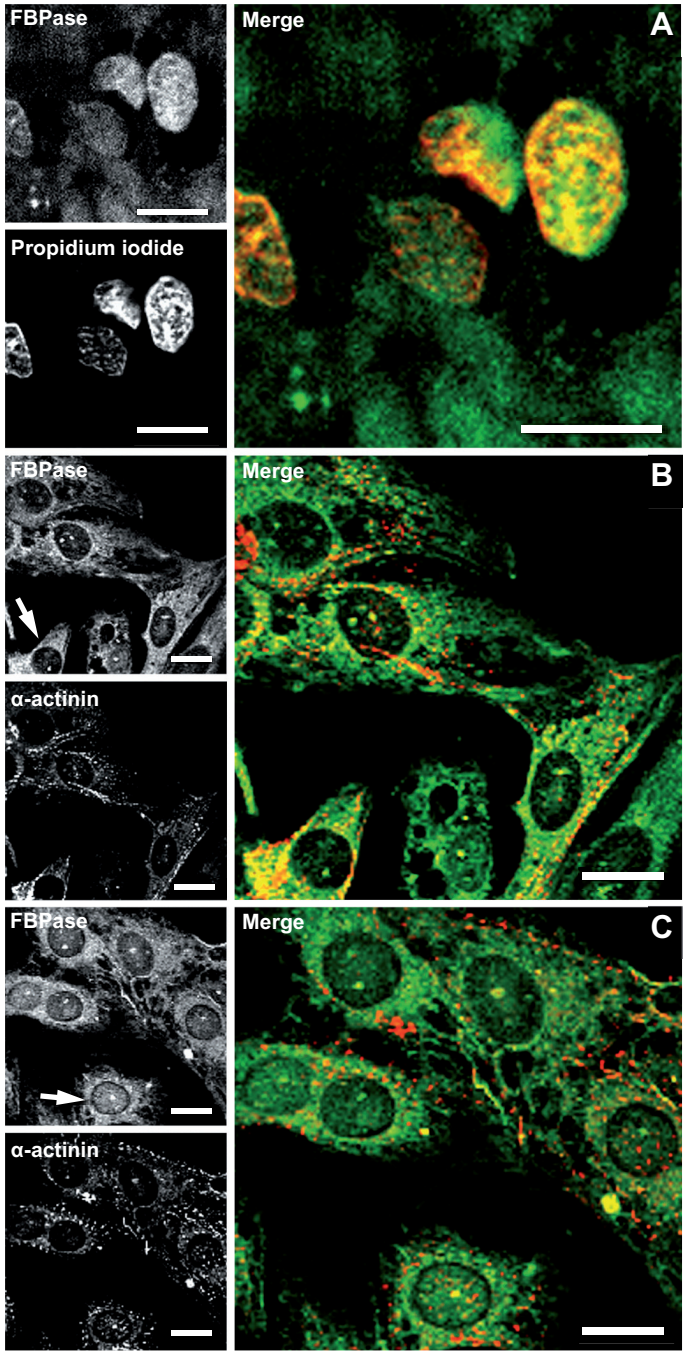


**Figure 5. Nuclear localization of FBPase in rat neonatal cardiomyocytes**
**(A)** In the neonatal rat heart tissue the FBPase is present in the nuclei and the cytoplasm. The bar represents 15 μm (B and C) In the neonatal rat cardiomyocytes incubated in the presence of insulin the FBPase is accumulated in the nuclei. Control cells **(B)**, and the cells incubated in the medium supplemented with insulin **(C)**. The arrows point to the nuclei. The bar represents 20 μm.

from the Z-line, cardiomyocytes were first cultured in conditions which promoted the accumulation of FBPase at the Z-line. This was achieved by incubating the cardiomyocytes in a glucose free-medium. The addition of the calcium ionophore A23187 to such medium resulted in a dramatic increase of cytosolic resting calcium concentration and the dissociation of FBPase from the Z-line (Fig. 4A, Fig. 1E). The pronounced rise of the resting calcium level did not stop the contraction-associated calcium transients, but strongly decreased their height (Fig. 4B).

Also glucagon increased the cytosolic calcium concentration as compared to the glucose free medium (Fig. 4E). This increase was apparently high enough to cause the dissociation of the enzyme from the Z-line (Fig. 1F). Glucagon also decreased the mean frequency of calcium oscillations (Fig. 4F), but observed changes in the ion frequency are caused rather by peak irregularity (Fig. 4D). Such irregularities, namely differentiation of peak height, result in appearance of additional, lower frequencies in Fourier spectrum influence mean frequency but should have no effect on the mean time of FBPase dissociation. Neither the glucagon nor the A23187 had any effect on the FBPase presence in the intercalated discs. All hormones (except insulin) and compounds affecting the intracellular calcium concentration exerted no influence on the intranuclear FBPase concentration.

### Insulin

It has been reported that FBPase is present in the nuclei of cardiomyocytes (heart tissue) (Gizak & Dzugaj, 2003). Here we also found the enzyme in the nuclei of neonatal rat heart tissue, but not in the cultured cardiomyocytes (Figs. 1A and 5A, respectively). Yanez and co-workers reported that insulin increased the intranuclear FBPase transport in rat hepatocytes (Yanez *et al.,* 2004). We found here that the stimulatory effect of the hormone on the nuclear accumulation of FBPase was observed only when neonatal rat cardiomyocytes were cultured for two, but not three or more days in the presence of serum and then stimulated with insulin (Fig. 5B and C; for details see Materials and Methods). Such cells displayed no developed intercalated discs with a much less marked cross striation pattern compared with that in cardiomyocytes cultured for 3 days in the presence of serum (compare Fig. 1A and Fig. 5B and C).

## DISCUSSION

We have shown that muscle FBPase is present inside the nucleus of cardiomyocytes, smooth muscle cells and in dividing muscle satellite cells (Gizak *et al.,* 2006). The results presented here unambiguously demonstrate that in primary culture of neonatal rat cardiomyocytes FBPase is present both on the Z-line and in the intercalated discs, and its binding to the Z-line is regulated by calcium ions.

In considering the physiological role of muscle FBPase we took into account reports on glyconeogenesis, the synthesis of glycogen from carbohydrate precursors like lactate in muscle tissue. It has been reported that in lower vertebrates but also in mammalian muscle tissue, from 13% (Bangsbo *et al.,* 1991) to 75% of lactate (Hermansen & Vaage, 1977) is converted to glycogen. Gluconeogenesis is hormonally regulated *via* fructose-2,6-bisphosphatase-phosphofructokinase-2 (FBPase-2/PFK-2) catalyzing the synthesis and hydrolysis of fructose-2,6-bisphosphate (Fru-2,6-$P_2$), an activator of phosphofructokinase-1 (PFK-1) [EC 2.7.1.11] and inhibitor of FBPase. Different isoforms of

FBPase-2/PFK-2 have been discovered in skeletal muscle (Marsin *et al.,* 2000; Okar *et al.,* 2001) predominantly the muscle isozyme and a small amount of the liver one, both are encoded by the same gene, but as a result of pre-mRNA splicing the muscle isoform is devoid of the domain containing Ser32 and cannot be regulated by reversible phosphorylation (Okar *et al.,* 2001). As an indispensable enzyme of glyconeogenesis the muscle FBPase might play a similar role in this pathway like the liver FBPase does in gluconeogenesis. Searching for the physiological role of muscle FBPase we focused on its subcellular localization and regulation of its activity. *In vitro* experiments revealed that muscle FBPase is not only inhibited by AMP but also by calcium ions, but when in complex with muscle aldolase the enzyme is inhibited by neither. *Ex vivo* experiments revealed that FBPase colocalizes with aldolase on the Z-line in myocytes and cardiomyocytes, and calcium ions cause the breakdown of the complex and FBPase dissociation from the Z-line allowing inhibition of the enzyme. On the basis of these results we postulated a simple mechanism of FBPase regulation in myocytes in which calcium is a regulator of glyconeogenesis and FBPase is a key enzyme of this pathway (Dzugaj, 2006).

Following *ex vivo* experiments, *in vivo* experiments were performed employing neonatal rat cells which are a good model to investigate the effect of hormones on carbohydrate metabolism. Neonatal rat cells were chosen instead of HL-1 cell because of the lack of striation in the latter cells. Glyconeogenesis may proceed only when FBPase colocalizes with aldolase on the Z-line. Therefore this colocalisation may be regarded as a marker of FBPase activity and ongoing glyconeogenesis.

In the first set of experiments neonatal rat cardiomyocytes in primary culture were incubated in a glucose-free medium. The effect of glucose deprivation on the free cytoplasmic calcium level in the cardiac muscle cells was described earlier (Weiss & Venkatesh, 1993; Flagg & Nichols, 2005; Kane *et al.,* 2005). The lack of glucose activates ATP-dependent $K^+$ channels which results in shortening of the action potential. The duration of action potential is tightly connected with the amount of calcium ions entering the cell *via* L-type channels and the lower concentration of these ions evokes the reduced release of calcium from the ER *via* RYR channels by the CICR (calcium induced calcium release) mechanism. The presence of FBPase on the Z-line in cells cultured in the glucose free medium indicates that paradoxically the glyconeogenesis might still proceed replenishing the glycogen level.

The subcellular localization of FBPase in the presence of very low and very high calcium ion concentration were investigated employing dantrolene and a calcium ionophore. Dantrolene decreases the concentration of the intracellular calcium ions, calcium ionophore increases it. The decrease of the calcium ion concentration resulted in the appearance of FBPase on the Z-line, while increasing the calcium concentration by the calcium ionophore resulted in dissociation of FBPase from the Z-line, but the presence of FBPase in the intercalated discs or in the nuclei was unaffected in either condition.

In the liver glucagon stimulates gluconeogenesis, but in the heart cells it stimulates heart rate and glycolysis. The hormone exerts its effects by increasing the cytosolic calcium ion level (Sauvadet *et al.,* 1996). The presence of glucagon in the medium increased the calcium level thus effecting the dissociation of FBPase from the Z-line.

The physiological role of carbohydrate metabolism enzymes in the cell nucleus is still a controversial issue. It has been suggested that some of them like glycogen synthase might play the same role in nuclei as they do in the cytosol. Investigating the localization of liver FBPase, Yanez and co-workers found this isozyme in the nuclei of hepatocytes and postulated that liver FBPase may participate in gluconeogenesis (Yanez *et al.,* 2004). We have found muscle FBPase in the nuclei of cardiomyocytes (Gizak & Dzugaj, 2003), smooth muscle cells (Gizak *et al.,* 2005) and satellite cells (Gizak *et al.,* 2006). We postulated that in the nuclei of satelite cells muscle FBPase may be involved in cell differentiation, but the physiological role of this isozyme in the nuclei of cardiomyocytes is unknown, although its participation in glyconeogenesis cannot be excluded (Mamczur *et al.,* 2007). No effect of calcium ions on the nuclear localization of FBPase in cardiomyocytes were observed here. During the establishing of the cell culture a loss of nuclear FBPase was observed, probably by activation of its nuclear export as in the case of the transformation of satellite cells to myocytes, but the mechanism of this phenomenon is unknown. The action of insulin on the cell culture resulted in accumulation of FBPase in the nuclei of cardiomyocytes, which indicates that FBPase might be somehow involved in the regulation of the cell cycle. Gizak and co-workers employing the HL-1 cell as a model to study FBPase nuclear import presented evidence that norepinephrine induced it (Gizak *et al.,* 2009). Further studie are needed to identify the physiological role of muscle FBPase in the nucleus and the mechanism of its nuclear export and import.

We postulated earlier a simple model of FBPase regulation in myocytes by calcium ions. Myocytes respond instantaneously to an increase or decrease of calcium ion level, by, respectively, accumulation of FBPase on the Z-line or its dissociation to cytosol. In the case of cardiomyocytes the regulation seems to be more complex, with the mean value of calcium concentration maintained for a longer period of time determining whether FBPase accumulates on the Z-line or dissociates from it.

## CONCLUDING REMARKS

In several papers our team have shown *in vitro* ability of FBPase and aldolase to be regulated by calcium ions both on the level of enzymatic activity and protein subcellular localization. This time, we have shown, for the first time, that described previously calcium regulation occur in intact, living cells. The experimental prove this process reversibility shows finally, the physiological relevance of the calcium dependent regulatory pathway dependent on protein complex formation and disruption.

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