# $Ca^{2+}$ Uptake Coupled to Glycogen Phosphorolysis in the Glycogenolytic-Sarcoplasmic Reticulum Complex from Rat Skeletal Muscle

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The glycogenolytic-sarcoplasmic reticulum complex from rat skeletal muscle accumulates $Ca^{2+}$ upon stimulation of glycogen phosphorolysis in the absence of added ATP. It is shown that an efficient $Ca^{2+}$ uptake involves the sequential action of glycogen phosphorylase, phosphoglucomutase and hexokinase, which generate low concentrations of ATP (approximately 1–2 μM) compartmentalized in the immediate vicinity of the sarcoplasmic reticulum $Ca^{2+}$, $Mg^{2+}$-ATPase (the $Ca^{2+}$ pump). The $Ca^{2+}$ uptake supported by glycogenolysis in this subcellular structure is strongly stimulated by micromolar concentrations of AMP, showing that the glycogen phosphorylase associated with this complex is in the dephosphorylated *b* form. The results point out that the flux through this compartmentalized metabolic pathway should be enhanced in physiological conditions leading to increased AMP concentrations in the sarcoplasm, such as long-lasting contractions and in ischemic muscle.

## Introduction

Glycogen particles are intimatelly associated with sarcoplasmic reticulum (SR)[1] in rabbit skeletal muscle (Wanson and Drochmans, 1972, Entman *et al.*, 1980). Subcellular fragmentation allows for preparation of glycogenolytic sarcoplasmic reticulum complexes (Entman *et al.*, 1980). Moreover, SR membrane preparations from rabbit skeletal muscle contain large amounts of phosphorylase in its dephosphorylated form (**b** form), and also glycogen and several enzymes directly involved in energy metabolism in muscle, such as myokinase, creatine kinase and AMP deaminase (Cuenda *et al.* 1994). Early studies with GL-SR have shown that this association allows to trigger a faster glycogen phosphorolysis at the onset of skeletal muscle contraction (Entman *et al.*, 1980).

Since the phosphorylated phosphorylase (*a* form) has low affinity for binding to SR membranes (Cuenda *et al.*, 1991), GL-SR acts as a phosphorylase *b* reservoir in skeletal muscle cells due to the large SR membrane network of these cells (Cuenda *et al.*, 1995). The activity of phosphorylase *b* is dependent of AMP and, under physiological conditions which raise the AMP levels in skeletal muscle sarcoplasm such as anoxia and during contraction (Busby and Radda, 1976, Newsholme and Leech, 1983) glycogen phosphorolysis in the vicinity of SR membranes should be stimulated.

Using purified SR membranes and purified glycogen phosphorylase *b* from rabbit skeletal muscle we have shown that this association can form *in vitro* a metabolic shuttle by coupling glycogenolysis to support $Ca^{2+}$ uptake through production of ATP in the vicinity of the SR $Ca^{2+}$,$Mg^{2+}$-ATPase (Cuenda *et al.*, 1993). This metabolic pathway can be written as follows,

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*Abbreviations:* $K_{0.5}$(AMP), concentration of AMP that produced 50% of maximum activation; $Ap_5A$, $P^1P^5$-di-(adenosine-5′) pentaphosphate; $Ca^{2+}$,$Mg^{2+}$-ATPase, $Ca^{2+}$ and $Mg^{2+}$ dependent adenosine triphosphatase (EC 3.6.1.38); EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; GL-SR, glycogenolytic-sarcoplasmic reticulum complex; hexokinase, ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1); IU, amount of enzyme that released 1 μmol product per min at saturating substrate concentration; PGM, phosphoglucomutase (α-D-glucose-1,6 phosphomutase, EC 5.4.2.2); phosphorylase, glycogen phosphorylase (EC 2.4.1.1); SR, sarcoplasmic reticulum; Tes, 2-[(2-hydroxy-1,1 bis-(hydroxymethyl) ethylamine]; Tris, tris (hydroxymethyl) aminomethane.

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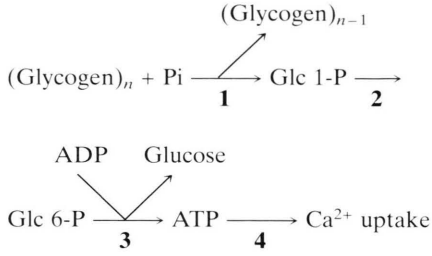


where Glc 1-P and Glc 6-P are glucose-1-phosphate and glucose-6-phosphate, respectively; and 1–4 are the enzymes phosphorylase, PGM, hexokinase and $Ca^{2+}$,$Mg^{2+}$-ATPase, respectively. As noted in (Cuenda *et al.*, 1993), a major bioenergetic advantage of this compartmentalized metabolic pathway is the efficient use in the cell of low concentrations of high energy phosphate in the form of ATP generated from low energy phosphate compounds, such as Glc 6-P. The flux through this metabolic pathway will be blocked upon activation of the glycogen phosphorylase *b* kinase at the onset of muscle contraction, because the phosphorylated *a* form of glycogen phosphorylase has low affinity for binding to SR membranes (Cuenda *et al.*, 1991).

In this communication we report experimental data showing that our previous results in rabbit skeletal muscle can be extended to rat skeletal muscle where a GL-SR subcellular structure is also present. Using the isolated GL-SR from rat skeletal muscle we show that this metabolic shuttle should operate more efficiently in physiological conditions where the AMP concentration raises in the micromolar range, such as ischemic muscle and in tetanic or long lasting contractions.

## Materials and Methods

GL-SR has been prepared from skeletal muscle of male Fischer rats, following the protocol of Entman *et al.* (1980). Phosphorylase of rat skeletal muscle was prepared as described by Gutierrez-Merino *et al.* (1980). The protein concentration was determined using the method of Lowry (Lowry *et al.*, 1951), with bovine serum albumin as standard.

The glycogen content of GL-SR preparations has been measured following the phenol sulfuric method (Dubois *et al.*, 1956), using glycogen as standard, and was found to be on average 25 ± 5 μg glycogen per mg of protein ($n > 10$).

$Ca^{2+}$ uptake by GL-SR membranes was measured with the use of $^{45}Ca^{2+}$ and Millipore HA filters as indicated elsewhere (Cuenda *et al.*, 1993). All the data reported in this paper are the average of triplicate experiments carried out with, at least, two different GL-SR preparations.

### *Enzyme assays*

The ATPase activity was measured using the coupled enzyme system pyruvate kinase/lactate dehydrogenase as indicated in Cuenda *et al.* (1990), with the following assay medium: 100 mM Tes (pH 7.0)/ 100 mM KCl/ 20% v/v glycerol/ 5 mM $MgCl_2$/ 2.5 mM ATP/ 0.1 mM $CaCl_2$/ 0.25 mM NADH/ 0.42 mM phospho*enol*pyruvate/ 7.5 IU pyruvate kinase/ 18 IU lactate dehydrogenase/ 5 mM $NaN_3$/ 0.4 μg/ml calcimycin and 10 μg GL-SR protein · $ml^{-1}$. The $Ca^{2+}$-dependent ATPase activity of different preparations ($n > 10$) of the rat GL-SR complex at 25 °C ranged from 0.85 to 1.45 μmol ATP hydrolyzed per min per mg protein.

Unless stated otherwise, the phosphorylase activity had been measured in the direction of glycogen degradation as indicated in Cuenda *et al.* (1991), with the following assay medium: 50 mM imidazole (pH 6.9)/ 10 mM magnesium acetate/ 12 mM $KH_2PO_4$/ 0.63 mM $NADP^+$/ 0.45 g · $l^{-1}$ glycogen/ 50 μM glucose-1,6-diphosphate/ 5 IU PGM/ 5 IU glucose-6-phosphate dehydrogenase and 100 μg GL-SR protein · $ml^{-1}$. The phosphorylase activity in the presence of 1 mM AMP of different preparations of the rat GL-SR ($n \cdot > 10$) at 30 °C ranged from 0.12 to 0.72 μmol glucose-1-phosphate produced per min per mg protein.

The PGM and hexokinase activities were measured at 35 °C using the coupled enzyme glucose 6-phosphate dehydrogenase with the following assay mixture: 100 mM Tes-Tris (pH 7.0)/ 50 mM KCl/ 10 mM $MgCl_2$/ 20 mM $H_2PO_4K$/ 50 μM $CaCl_2$/ 0.48 mM $NADP^+$/ 1 IU glucose 6-phosphate dehydrogenase, and 10 mM glucose 1-phosphate/ 50 μM glucose 1,6-diphosphate for PGM activity measurements or 3 mM glucose/ 2 mM ATP for the hexokinase activity assay medium. Variations in $NADP^+$ absorbance were recorded at 340 nm.

The AMP content of ADP solutions had been determined using the coupled enzyme AMP-deaminase as indicated in Tovmasian *et al.* (1990), with a value for the differential extinction coefficient ($\in_{AMP} - \in_{IMP}$) at 265 nm of $8.2 \times 10^3$ $M^{-1}$ $cm^{-1}$.

*Chemicals*

Bovine serum albumin, ADP, AMP, ATP, phosphoenolpyruvate, EGTA, glucose-1,6-diphosphate, glucose 6-phosphate, Tris (TRIZMA base) and TES were obtained from Sigma. Glycogen, $Ap_5A$, NADH, $NADP^+$, calcimycin, pyruvate kinase, lactate dehydrogenase, PGM, hexokinase and glucose 6-phosphate dehydrogenase were purchased from Boehringer Mannheim. Hexokinase and PGM were sulfate-free by centrifugation. All the other chemicals used in this study were obtained from Merck.

## Results and Discussion

Our GL-SR preparations from Fischer rats skeletal muscle had a phosphorylase activity which is dependent upon the presence of AMP and it is inhibited by caffeine (Fig. 1A), therefore, indicating that the phosphorylase bound to these complexes is mainly in its *b* (dephosphorylated) form. This is a property shared with the GL-SR complex from rabbit skeletal muscle (Entman *et al.*, 1980). The GL-SR and the SR from rabbit skeletal muscle also had myokinase activity (Entman *et al.*, 1980; Cuenda *et al.*, 1994), which produces ATP from AMP and ADP, and this would interfere with the assays of $Ca^{2+}$ uptake supported by glycogen phosphorolysis in the absence of added ATP. We, thus, confirmed the presence of myokinase activity of the GL-SR complex from rat skeletal muscle, which on average was found to be $0.14 \pm 0.05$ μmol ADP per min per mg GL-SR protein. Because the SR $Ca^{2+}$ pump is highly efficient even in the pres-

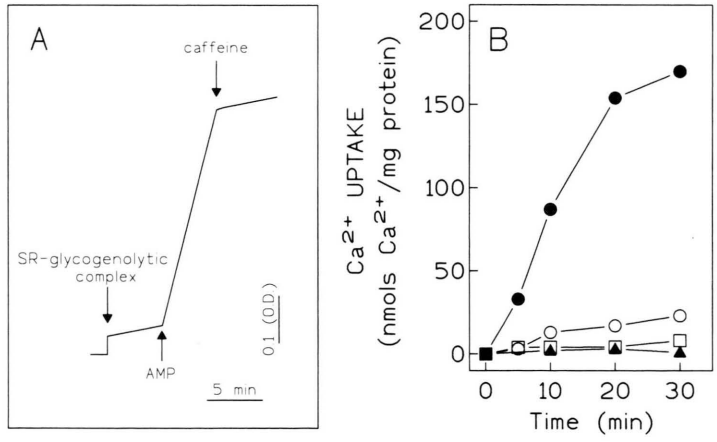


Fig. 1. Phosphorylase activity and $Ca^{2+}$ uptake by rat GL-SR. Panel A. Phosphorylase activity of the rat GL-SR, monitored by the production of NADPH with the coupled enzyme system PGM/glucose-6-phosphate dehydrogenase. The phosphorylase activity was measured at 35 °C in the following assay medium: 50 mM Tes Tris (pH 7.0)/ 10 mM $MgCl_2$/ 20 mM Pi/ 60 mM KCl/ 40 μM $CaCl_2$/ 5 mM $NaN_3$/ 0.5 mM $NADP^+$/ 3.6 $g \cdot^{-1}$ glycogen/ 0.1 mM ADP/ 50 μM $Ap_5A$/ 50 μM glucose-1,6-diphosphate/ 32 IU PGM/ 10 IU glucose-6 phosphate dehydrogenase. The different arrows show the addition of 0.1 mg GL-SR protein/ml, 1 mM AMP and 10 mM caffeine. The absorbance (O.D.) was measured at 340 nm, in quartz cuvettes of 1 cm light-path. The calibration bar of 0.1 O.D. corresponds to 16.08 nmols/ml of glucose-1-phosphate, as one mol of NADPH is produced per mol of glucose-1-phosphate and the standard molar absorption coefficient of NADPH is 6220 $M^{-1} \cdot cm^{-1}$.
Panel B. Effects of caffeine, glucose and glucose-1,6-diphosphate on $Ca^{2+}$ uptake by rat GL-SR in the absence of added ATP. The $Ca^{2+}$ uptake by rat GL-SR was supported by glycogen phosphorolysis and measured in the absence of ATP. The assay medium contained 50 mM Tes-Tris (pH 7.0)/ 10 mM $MgCl_2$/ 20 mM Pi/ 60 mM KCl/ 40 μM $^{45}CaCl_2$ (4000 cpm.nmol$^{-1}$)/ 5 mM $NaN_3$ and 0.1 mg GL-SR protein. $ml^{-1}$, supplemented with 3.6 $g \cdot^{-1}$ glycogen/ 1 mM AMP/ 0.1 mM ADP/ 50 μM $Ap_5A$/ 50 μM glucose-1,6-diphosphate/ 0.14 mg.$ml^{-1}$ (28 IU) PGM, 0.13 mg.$ml^{-1}$ (18 IU) hexokinase. The different symbols correspond to: 1 mM AMP (●), 1 mM AMP in the absence of glucose 1,6-diphosphate (○), 1 mM AMP and 10 mM caffeine (□), and 1 mM AMP and 50 mM glucose (▲).

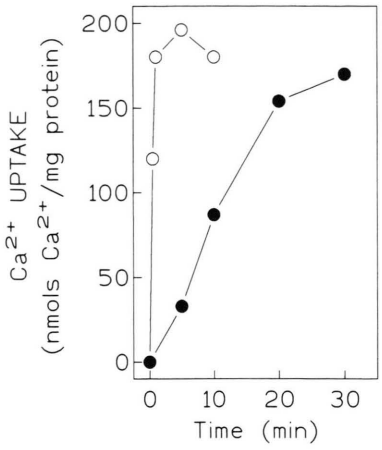


Fig. 2. $Ca^{2+}$ uptake by rat GL-SR in the absence (●) and presence (○) of added ATP. In both experiments the assay medium contained 50 mM Tes-Tris (pH 7.0)/ 10 mM $MgCl_2$/ 20 mM Pi/ 60 mM KCl/ 40 μM $^{45}CaCl_2$ (4000 cpm·nmol$^{-1}$)/ 5 mM $NaN_3$ and 0.1 mg GL-SR protein·ml$^{-1}$. The $Ca^{2+}$ uptake in control experiments was measured in the presence of 2.5 mM ATP (○). In experiments performed in the absence of ATP (●) the assay medium also contained: 3.6 g·l$^{-1}$ glycogen/ 1 mM AMP/ 0.1 mM ADP/ 50 μM $Ap_5A$/ 50 μM glucose-1,6 diphosphate/ 0.14 mg·ml$^{-1}$ (28 IU) PGM, 0.13 mg·ml$^{-1}$ (18 IU) hexokinase.

ence of micromolar ATP concentrations we added to all the assays 50 μM of the myokinase inhibitor $Ap_5A$, a concentration which produced full inhibition of the myokinase activity of the rat GL-SR complex (data not shown).

When the assay medium is supplemented with the enzymes PGM, hexokinase and ADP plus the physiological PGM activator glucose 1,6-diphosphate, glycogen phosphorolysis by the phosphorylase associated with the rat GL-SR complex supports $Ca^{2+}$ uptake in the absence of added ATP (Fig. 1B and 2). The overall activities of hexokinase and PGM added to the assay medium are much lower than those present in the sarcoplasm of rat skeletal muscle (Clark and Lardy, 1984). Inhibition of the phosphorylase activity of the GL-SR by caffeine blocks $Ca^{2+}$ uptake (Fig. 1B). In separate control experiments, the effect of caffeine on PGM and hexokinase activities was measured. Caffeine (up to 10 mM) was found without significant effect on the activity of PGM and of hexokinase, as it produced less than 10% inhibition of their enzymatic activities when assayed under the same experimental conditions with varying concentrations of their substrates, glucose-1-phosphate (up to 10 mM) and glucose (up to 3 mM)/ ATP (up to 2 mM), respectively (results not shown). $Ca^{2+}$ uptake shows an absolute requirement for PGM and hexokinase activities in the assay medium, such that removal of any of these enzymes results in blockade of $Ca^{2+}$ uptake (data not shown). In the absence of the PGM activator glucose-1,6-diphosphate the rate of $Ca^{2+}$ uptake is largely reduced (Fig. 1B). The inhibition of $Ca^{2+}$ uptake by 50 mM glucose, which blocks the production of glucose and ATP from glucose-6-phosphate and ADP, see also Fig. 1B, demonstrated the need of the hexokinase activity to couple glycogen phosphorolysis to $Ca^{2+}$ uptake by GL-SR. It is to be noticed that GL-SR preparations retain low amounts of hexokinase, as indicated by activity measurements (data not shown). However endogenous hexokinase activity is not able to sustain a measurable $Ca^{2+}$ uptake. Similarly, the glycogen content of GL-SR preparations is too low to reliably measure $Ca^{2+}$ uptake in the absence of added exogenous glycogen.

As indicated above, the activity of phosphorylase associated with GL-SR preparations is dependent upon AMP. Fig. 3 shows that $Ca^{2+}$ uptake is stimulated by AMP, in the concentration range that stimulates the phosphorylase activity of the GL-SR (Fig. 3B), and in the range of AMP concentrations present in the cytoplasm of skeletal muscle cells, which has been reported to range between 2 and 10 μM in resting muscle (Newsholme and Leech, 1983; Yamada and Sugi, 1987), raising up to 100–200 μM during long-lasting exercise or in ischemic muscle (Newsholme and Leech, 1983). The results shown in the Fig. 3B also point out that the $K_{0.5}$ of AMP and the shape of the dependence upon AMP of $Ca^{2+}$ uptake is different to that of the phosphorylase activity of GL SR. This result suggests that above a given concentration of AMP (> 0.1 mM) glycogen phosphorolysis is not the only rate-limiting step of this process. This is probably due to enhanced $Ca^{2+}$ release from SR fragments by submillimolar concentrations of AMP (Meissner, 1984), that can counterbalance $Ca^{2+}$ uptake. If this hypothesis is correct, addition of phosphorylase purified from rat skeletal muscle should produce a steeper dependence of $Ca^{2+}$ uptake upon AMP concentration and higher levels of $Ca^{2+}$ uptake at saturation by AMP. The results

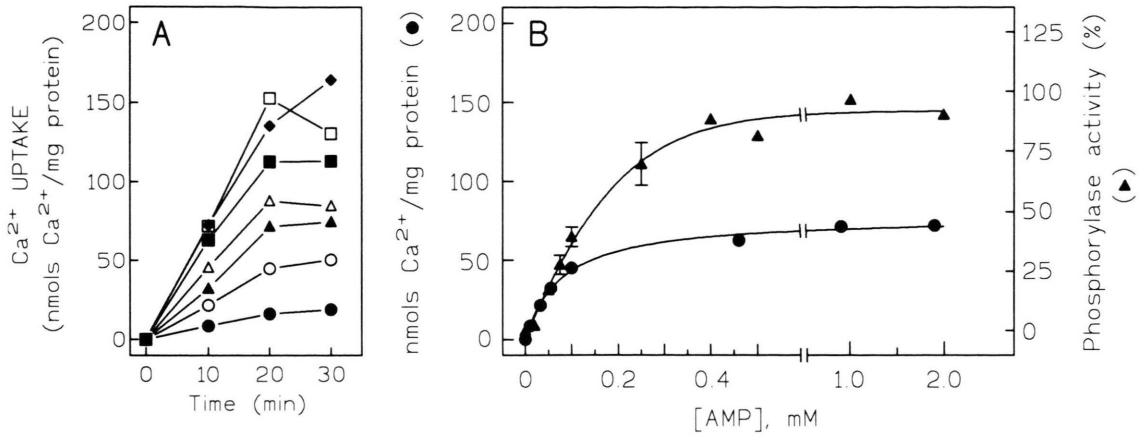


Fig. 3. Dependence upon AMP of $Ca^{2+}$ uptake and phosphorylase activity of rat GL-SR. Panel A. Dependence upon AMP of $Ca^{2+}$ uptake as a function of time. $Ca^{2+}$ uptake was measured in the absence of ATP as indicated in the legend of the Fig. 2. The different concentrations of AMP (in μM) were: 10 (●), 32.5 (○), 55 (▲), 100 (△), 460 (■), 1000 (□) and 2000 (◆).
Panel B. Dependence upon AMP concentration of $Ca^{2+}$ uptake and phosphorylase **b** activity. $Ca^{2+}$ uptake as a function of AMP concentrations with 10 min of reaction (●). The different values of $Ca^{2+}$ uptake were obtained from the data of Panel A. Phosphorylase activity (▲) had been measured as indicated in the Materials and Methods.

shown in the Fig. 4 are in good agreement with this prediction.

In addition, the results of Fig. 4 demonstrated that the higher the phosphorylase concentration, the lower the concentration of AMP required to produce significant $Ca^{2+}$ uptake by GL-SR preparations, e.g. the lower the $K_{0.5}$(AMP) values. This effect is not due to an altered $Ca^{2+}$ dependence of the $Ca^{2+}$ pump of the SR membrane in the presence of phosphorylase, because the $Ca^{2+}$ dependence of the $Ca^{2+}$,$Mg^{2+}$-ATPase of rat SR (devoid of phosphorylase) is not significantly different from that of the rat GL-SR (Fig. 5A). It is to be

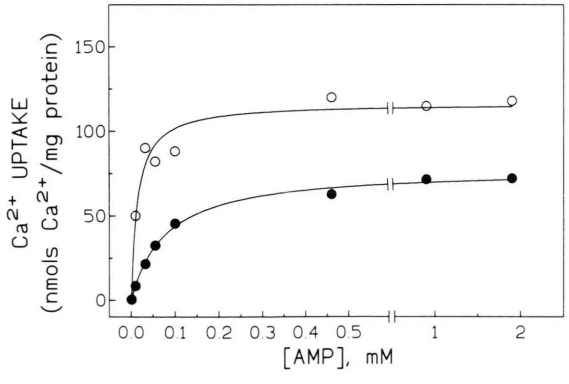


Fig. 4. Dependence upon AMP of $Ca^{2+}$ uptake by rat GL-SR after 10 min reaction in the absence (●) or presence (○) of 0.1 mg·ml$^{-1}$ of phosphorylase *b* purified from rat skeletal muscle. The assay medium is indicated in the legend of Fig. 2 for $Ca^{2+}$ uptake measurements in the absence of added ATP.

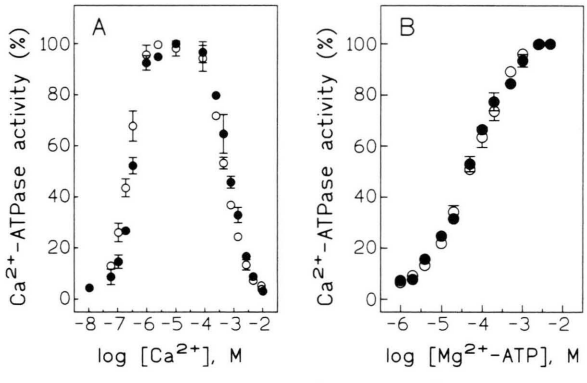


Fig. 5. Dependence upon $Ca^{2+}$ and $Mg^{2+}$-ATP of the $Ca^{2+}$-ATPase activity of rat GL-SR. Panel A. Dependence of the $Ca^{2+}$-ATPase activity of rat GL-SR (●) and SR (○) upon $Ca^{2+}$ concentration. Panel B. Dependence of the $Ca^{2+}$-ATPase activity of rat GL-SR upon $Mg^{2+}$-ATP. The activity measurements were done at 25 °C in the assay medium indicated in the Materials and methods, with varying $Ca^{2+}$ (Panel A) or $Mg^{2+}$-ATP (Panel B) concentrations. The data are the average of $n > 6$ separate measurements at each $Ca^{2+}$ and $Mg^{2+}$ ATP concentrations. The 100% value of the $Ca^{2+}$-ATPase activity were 3.2 and 1.15 μmol ATP per min per mg protein for SR and GL SR, respectively.

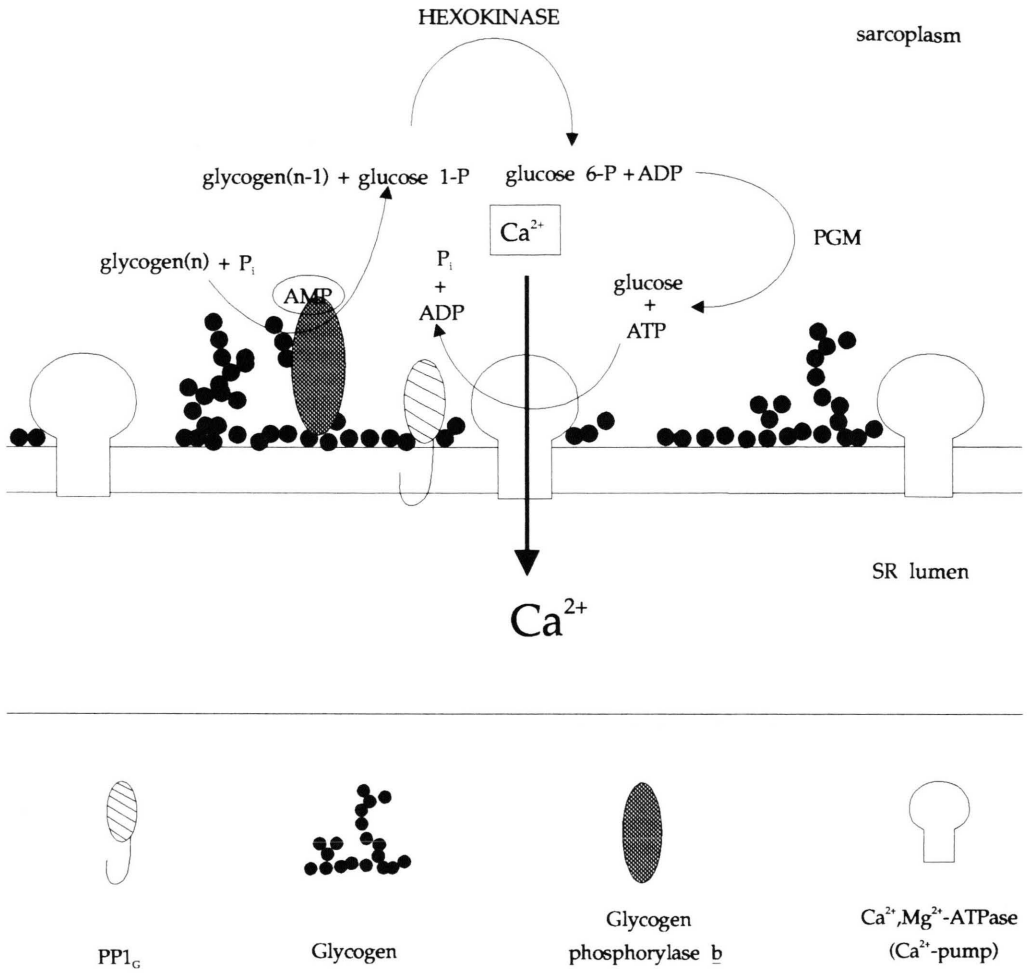


Fig. 6. Schematic view of the proposed metabolic shuttle for ATP production in GL-SR from rat skeletal muscle. Phosphorylase *b* binds to GL-SR through glycogen fragments as non-covalent anchoring sites (Wanson and Drochmans, 1972; Cuenda *et al.*, 1995), which in turn are anchored to SR membranes by the hydrophobic tail of the glycogen-associated form of protein phosphatase 1 ($PP1_G$) (Hubbard and Cohen, 1993). Since the location of phosphorylase **b** kinase of GL-SR relative to the SR membrane components is unknown and also because phosphorylase *b* kinase is not directly involved in this metabolic pathway, this protein has not been included in the diagram.

noted here that previous studies carried out in our laboratory with rabbit SR preparations showed that phosphorylase *b* binding to SR membranes does not alter $Ca^{2+}$ binding to the $Ca^{2+}$,$Mg^{2+}$-ATPase, nor the $Ca^{2+}$ dependence of this activity (Cuenda *et al.*, 1995). Since the concentration of phosphorylase in skeletal muscle sarcoplasm is $\geq 10$ mg/ml (Fischer *et al.*, 1978), i.e. more than 100-fold higher than that used in the experiments reported in this study, these results indicate that this process takes place more efficiently at the concentrations of AMP reached in anoxia or during skeletal muscle contraction, 100–200 μM (Busby and Radda, 1976; Newsholme and Leech, 1983).

The comparison of these data with those obtained in the presence of 2.5 mM ATP points out that the $Ca^{2+}$ uptake saturates at approximately 200 nmol $Ca^{2+}$/mg protein in both sets of data, and that the rate of $Ca^{2+}$ uptake supported by glycogen phosphorolysis is about 5% of that obtained in the presence of 2.5 mM ATP. Attempts to directly measure the steady state concentration of ATP during $Ca^{2+}$ uptake coupled to glycogen phospho-

rolysis failed, indicating that the ATP is kinetically compartmentalized in the GL-SR, i.e., the synthesized ATP has a restricted diffusion to the bulk medium. In several other cellular and subcellular systems, glucose metabolism in membrane associated compartments has been demonstrated to be functionally related to the activity of membrane ion pumps or channels through localized ATP synthesis (Paul *et al.*, 1988; Weiss and Lamp, 1989; de Meis *et al.*, 1992; Montero-Lomeli and de Meis, 1992). Compartimentalized ATP synthesis has also been demonstrated in skeletal muscle triads, which consist of transverse tubules connected to adjacent terminal cisternae of the SR membrane (Han *et al.*, 1992). The local concentration of ATP in the GL-SR can be estimated from the dependence of the $Ca^{2+}$,$Mg^{2+}$-ATPase activity of GL-SR upon $Mg^{2+}$-ATP (Fig. 5B). From the data shown in this Figure we obtain a steady state local concentration of 1–2 μM $Mg^{2+}$-ATP.

In conclusion, in rat skeletal muscle the association of glycogenolysis in GL-SR membranes produces ATP nearby the SR $Ca^{2+}$,$Mg^{2+}$-ATPase which is employed to drive the $Ca^{2+}$ pump of the SR, as schematically drawn in the Fig. 6. This could lead to an efficient and faster decrease of cytosolic $Ca^{2+}$ during the relaxation of the muscle, at a metabolic energy cost clearly lower than production of ATP elsewhere in the cell by glycolysis or by mitochondrial respiration. The compartmentalized production of ATP in GL-SR subcellular structures should increase in physiological situations in which the AMP concentration of the sarcoplasm is raised, such as in ischemia and in long-lasting contractions (Busby and Radda, 1976; Newsholme and Leech, 1983), and might serve to protect muscle cells against the toxic effects of sustained high $Ca^{2+}$ levels in the sarcoplasm.

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