Compartmentalized ATP Synthesis in Skeletal Muscle Triads†

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ABSTRACT: Isolated skeletal muscle triads contain a compartmentalized glycolytic reaction sequence catalyzed by aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. These enzymes express activity in the structure-associated state leading to synthesis of ATP in the triadic junction upon supply of glyceraldehyde 3-phosphate or fructose 1,6-bisphosphate. ATP formation occurs transiently and appears to be kinetically compartmentalized, i.e., the synthesized ATP is not in equilibrium with the bulk ATP. The apparent rate constants of the aldolase and the glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase reaction are significantly increased when fructose 1,6-bisphosphate instead of glyceraldehyde 3-phosphate is employed as substrate. These observations suggest that fructose 1,6-bisphosphate is especially effectively channelled into the junctional gap. The amplitude of the ATP transient is decreasing with increasing free [Ca^{2+}] in the range of 1 nM to 30 μM. In the presence of fluoride, the ATP transient is significantly enhanced and its declining phase is substantially retarded. This observation suggests utilization of endogenously synthesized ATP in part by structure associated protein kinases and phosphatases which is confirmed by the detection of phosphorylated triadic proteins after gel electrophoresis and autoradiography. Endogenous protein kinases phosphorylate proteins of apparent M_r 450000, 180000, 160000, 145000, 135000, 90000, 54000, 51000, and 20000, respectively. Some of these phosphorylated polypeptides are in the M_r range of known phosphoproteins involved in excitation–contraction coupling of skeletal muscle, which might give a first hint at the functional importance of the sequential glycolytic reactions compartmentalized in triads.

There are some indications for a membrane-localized microcompartmentalized ATP synthesis in skeletal muscle: Incubation of excised frog muscles in [^{32}P]phosphate containing Ringer's solution results in ^{32}P labeling of phosphoinositides with significantly higher specific activities than that of total intracellular ATP (Lagos & Vergara, 1990). It is concluded that the [γ-^{32}P]ATP required for these lipid phosphorylations probably does not derive from the major myoplasmic ATP pool. Furthermore, it has been shown in fatigued frog skeletal muscle fibres that the level of myoplasmic ATP remains sufficiently high to support contraction, whereas excitation–contraction (EC) coupling fails under these conditions (Grabowski et al., 1972). The authors proposed that the decline of an activator of EC coupling, potentially an energy-rich compound participating in the corresponding mechanism of signal transduction, might be responsible for this phenomenon.

The site of EC coupling can be isolated from rabbit skeletal muscle in form of triads which consist of transverse (T) tubules connected to adjacent terminal cisternae (TC) of the sarcoplasmic reticulum (SR) by junctional foot proteins (JFP). Depolarization of the T-tubular membrane, presumably sensed by the dihydropyridine (DHP) receptor (Rios & Brum, 1987), is transformed into a Ca^{2+}-releasing signal and transmitted to the SR Ca^{2+}-release channels inherent in the JFP [for a review, see Lai and Meissner (1989)]. It has been shown that two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Caswell & Corbett, 1985; Corbett et al., 1985; Thieleczek et al., 1989; Brandt et al., 1990) and aldolase (Thieleczek et al., 1989; Brandt et al., 1990), are tightly bound to the JFP. GAPDH facilitates the reformation of triadic junctions from isolated T tubules and TC (Corbett et al., 1985) and was reported to be inhibited upon association with these structures (Caswell & Corbett, 1985). However, GAPDH can be released from triads by high NaCl concentrations without impairing triad integrity (Brandt et al., 1990). In preference to GAPDH, aldolase can be dissociated from triads by micromolar concentrations of inositol 1,4,5-trisphosphate and fructose 1,6-bisphosphate (FBP) (Thieleczek et al., 1989).

In several other cellular systems, glucose metabolism in membrane-associated compartments has been demonstrated which is related functionally to the activity of membrane ion pumps or channels [for a review, see Lynch and Paul (1988)]. In vascular smooth muscle, ATP seems to be synthesized by a plasma membrane associated glycolytic cascade which is employed specifically to drive the Na^+/K^+ ATPase (Paul et al., 1979). Similarly, in erythrocytes, high-energy phosphates generated by a membrane-localized glycolytic pathway may also be used preferentially to support specific membrane functions (Parker & Hoffman, 1967). In support of this view, it has been shown that phosphofructokinase (Karadsheh & Uyeda, 1977), aldolase (Strapazon & Steck, 1977), and GAPDH (Kliman & Steck, 1980) are specifically associated with the band 3 protein of erythrocytes, the anion transporter of the plasma membrane. Furthermore, plasma membrane localized glycolytic reactions are a preferential source of ATP for cardiac ATP-sensitive K^+ channels (Weiss & Lamp, 1989), whereas oxidatively derived ATP mainly supports contraction (Weiss & Hiltbrand, 1985).

In this publication, we demonstrate that sequential glycolytic reactions catalyzed by aldolase, triosephosphate isomerase (TIM), GAPDH, and phosphoglycerate kinase (PGK) are

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compartmentalized at skeletal muscle triads. This microcompartmentalized reaction system will be described quantitatively, and the simultaneous ATP synthesis as well as a preferential ATP consumption in the triadic gap will be characterized.

Materials and Methods

Membrane Preparation. Triads/TC were isolated from rabbit back muscle as described by Caswell et al. (1976) and suspended in 250 mM sucrose and 3 mM histidine, pH 7.3. Protein concentration was determined according to Bradford (1976) employing bovine serum albumin as standard. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli (1970).

Assay of [γ-^{32}P]ATP Formation. [γ-^{32}P]ATP formation by triadic membranes due to the oxidation of glycolytic substrates was assayed at 22 °C in 20 mM imidazole, 20 mM Na acetate, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithioerythritol, 2 mM NAD^+, 1 mM Mg^{2+}, 5 mM ADP, 5 mM NaH_2PO_4, 50 μM P^1,P^5-di(adenosine-5′)pentaphosphate (AP_5A), and 1 μCi[^{32}P]H_3PO_4/mL, pH 7.1 (final volume 0.6 mL). As a glycolytic substrate, either D-glyceraldehyde 3-phosphate (GAP) or FBP was employed as indicated. Reactions were initiated by the addition of 1 mg/mL of triads/TC and terminated at various times by mixing 75 μL of the assay medium with the same volume of 3% ammonium molybdate, 0.66 M HCl, 0.1 M triethylamine, and 1% bromine as described by Sugino and Miyoshi (1964). The triethylammonium salt causes a selective and quantitative precipitation of phosphomolybdate. After centrifugation at 15000*g* for 10 min, the radioactivity in the supernatant representing formed [γ-^{32}P]ATP was measured by scintillation counting and quantified using the specific radioactivity of phosphate in the assay. The free Ca^{2+} concentration in the assay was calculated to be in the order of 10^{-9} M. If required, appropriate amounts of $CaCl_2$ were added to the assay establishing free Ca^{2+} concentrations in the range of 0.3–30 μM. In these studies, 200 μM vanadate was also included in order to inhibit the Ca^{2+}-dependent Ca^{2+}-transport ATPase of the SR (Medda & Hasselbach, 1983).

Enzymatic Determinations of Glycolytic Metabolites. An assay volume of 3 mL was employed to determine the concentrations of FBP, GAP, dihydroxyacetone phosphate (DAP), 3-phosphoglycerate (3PG), and ATP in the same assay medium (see above). The reaction was terminated at various times by addition of ice-cold $HClO_4$ at a final concentration of 0.5 M (Pullman, 1967). After 10 min on ice, the denatured protein was removed by centrifugation for 10 min at 15000*g*. The supernatant was adjusted to pH 7.6 by addition of K_2CO_3, and the formed $KClO_4$ was removed by centrifugation as before. An aliquot of 40 μL of the supernatant was used for the enzymatic determination of FBP, GAP, and DAP according to Bergmeyer (1974). For the determination of 3PG (via lactate formation) and ATP, sample volumes of 80 and 100 μL were used, respectively.

Determination of Adenosine Phosphates by High Performance Liquid Chromatography (HPLC). Adenosine phosphates of the assay media which were either endogenously produced or added (controls), were determined in the absence or presence of an ATP-consuming system consisting of agarose-coupled hexokinase (5 units/mL) and 2-deoxyglucose (5 mM). After a 5-min incubation, the assay was quenched with $HClO_4$ as described above, and 2.4 μL of the supernatant was diluted with 1.2 mL of HPLC buffer (2.5 mM Na acetate, 1 mM NaF, pH 5.0). This solution was applied on Mono Q (Pharmacia HR 5/5) by means of a HPLC system (LKB), and the eluted adenosine phosphates were quantified by metal-dye detection as described by Mayr (1990). The efficiency of the ATP-consuming system was determined without triads/TC employing 200 μM ATP under otherwise identical conditions.

Determination of the Activities of Glycolytic Enzymes in Triads. The activities of aldolase, GAPDH, and TIM associated with triads/TC were determined according to Bergmeyer (1974) by coupling the corresponding enzyme reactions with glycerol-3-phosphate dehydrogenase. The activities of pyruvate kinase, enolase, and phosphoglyceromutase were assayed by coupling their reactions to lactate dehydrogenase (LDH) (Bergmeyer, 1974). Depending on the enzyme activity to be assayed, the concentrations of triads/TC in the assay were 1 μg/mL for GAPDH and pyruvate kinase, 5 μg/mL for LDH, 10 μg/mL for aldolase and TIM, and 50 μg/mL for enolase and phosphoglyceromutase. Adenylate kinase activity of triads/TC was assayed by coupling ATP production to the hexokinase/glucose-6-phosphate dehydrogenase reactions (Bergmeyer, 1974). 50 μg/mL triads/TC were added to the assay containing 72 mM triethanolamine/HCl, pH 7.6, 10 mM cysteine, 0.33 mM $NADP^+$, 462 milliunits/mL glucose-6-phosphate dehydrogenase, 50 mM glucose, 1.8 units/mL hexokinase, and 2 mM ADP. The amounts of the glycolytic enzymes associated with triads/TC were estimated by assuming specific activities of the isolated enzymes of skeletal muscle given by Scopes and Stoter (1982).

Determination of the Basal Ca^{2+}-Independent ATPase Activity of Triads. ATP hydrolyzing activity of triads/TC at about 10^{-9} M free Ca^{2+} was determined in presence of 0.1–5 mM ATP in an assay medium containing 20 mM imidazole, 20 mM sodium acetate, 1 mM EGTA, 5 mM NaH_2PO_4, 1 mM Mg^{2+}, 50 μM AP_5A, and 1 μCi[γ-^{32}P]ATP/mL, pH 7.1. The reaction was initiated by addition of 1 mg/mL triads/TC, and the remaining [γ-^{32}P]ATP was determined as described above.

Data Analysis. The reaction scheme that has been taken into consideration in order to simulate the observed metabolite concentration changes is described by a system of differential rate equations. They contain apparent rate constants (see text) as variable parameters. The numerical software MATLAB (The MathWorks Inc., Natick, MA) was used to approximate the measured data by time courses predicted by the model. The apparent equilibrium constants of the involved reactions were chosen (Table II), and an initial estimate for one of the apparent rate constants of each reaction (k_{12}, k_{22}, k_{32}) was made. The differential equations were solved numerically using an automatic step-size forth- and fifth-order Runge–Kutta–Fehlberg integration method. For each time point for which experimental data were available, the differences between the measured and the predicted data were calculated. The sum of the squares of these residuals was evaluated and minimized using the Nelder–Mead simplex algorithm for multidimensional minimalization. At the encountered (at least local) minimum, the corresponding optimized rate constants returned by the algorithm were used to produce the best fit data set by simulating the system. The reliability of the obtained optimized rate constants were estimated following the outline given by Kuchel (1985). Gaussian noise with a standard deviation of 7% for each datum (estimated from repeated measurements) was added to the data set, and the optimization was carried out again using the optimized rate constants as starting values. This procedure was repeated with 30 synthetic data sets from which as many estimates for each of the three

Table I: Activities of Glycolytic Enzymes Compartmentalized at Skeletal Muscle Triads[a]

enzyme	spec act. (units/mg of triads)	enzyme (μg)/triads (mg)
aldolase	0.57	31.7
GAPDH	12.56	104.7
TIM	0.72	0.12
phosphoglyceromutase	0.02	0.02
enolase	0.11	1.4
pyruvate kinase	3.85	11.3
LDH	1.97	3.3
adenylate kinase	0.18	0.12

[a] Enzyme activities associated with triads/TC were determined as described under Materials and Methods. Enzyme contents in triads/TC were calculated employing specific activities of the purified enzymes of rabbit skeletal muscle (Scopes & Stoter, 1982).

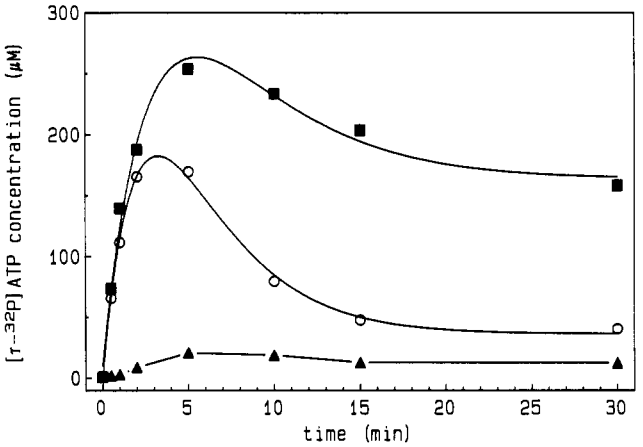

FIGURE 1: Formation of [γ-^{32}P]ATP in triads by oxidation of GAP. 1.9 mM GAP was employed as substrate. The experiments were carried out in absence (○) and presence (■) of 20 mM KF or 10 mM iodoacetamide (▲), respectively. The assay conditions are described under Materials and Methods.

rate constants were obtained. The means of the parameter sets, which were identical with the optimized rate constants obtained with the experimental data, are given in Table II together with the obtained standard deviations.

Materials. [^{32}P]H_3PO_4 was purchased from Du Pont–New England Nuclear and [γ-^{32}P]ATP was prepared according to Walseth and Johnson (1979) using [^{32}P]H_3PO_4. Na_2ATP, iodoacetamide, AP_5A, FBP, and insoluble hexokinase attached to beaded agarose were obtained from Sigma. GAP, ADP, NAD^+, NADH, $NADP^+$, and the employed enzymes were purchased from Boehringer. 2-Deoxyglucose was obtained from Merck. All other chemicals were of at least reagent grade.

Results

Approximately 14% of the total protein of isolated triads/TC consists of aldolase and GAPDH (Table I), indicating a potential for ATP synthesis if supplied with the required substrates and cofactors. Compartmentalized substrate level ATP synthesis could occur also further downstream, since pyruvate kinase still makes up about 1% of the total amount of triadic protein (Table I). However, the phosphoglyceromutase activity is very low, limiting the flux through this glycolytic sequence. ATP is formed transiently if GAP is supplied to triads/TC in presence of saturating concentrations of necessary cofactors, indicating oxidation of this glycolytic intermediate by GAPDH (Figure 1). Furthermore, it documents that PGK is compartmentalized together with

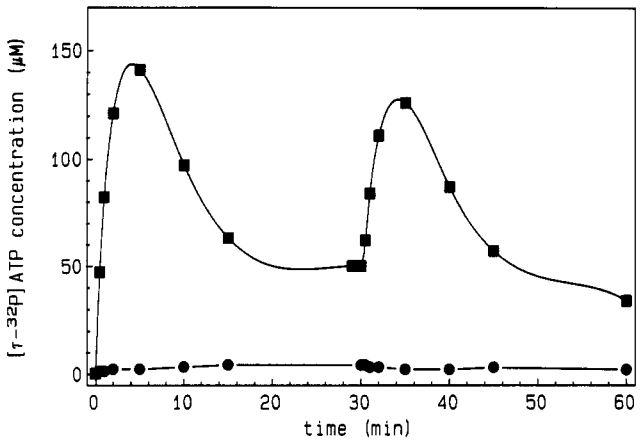

FIGURE 2: Repetitive [γ-^{32}P]ATP transients by readdition of substrate. 1.9 mM GAP was employed as substrate in presence (■) and absence of ADP (●), respectively, and the experiment was carried out as described under Materials and Methods. After 30 min, 1.9 mM GAP was readded to the media.

GAPDH in triads/TC, establishing a complete substrate-level phosphorylation step. Employing 1.9 mM GAP, a maximal ATP concentration of about 180 μM is reached within 3–4 min. Iodoacetamide, an inhibitor of GAPDH, leads to nearly complete suppression of the [γ-^{32}P]ATP transient, which indicates the participation of this enzyme in this process. [γ-^{32}P]ATP formation due to reversal of the SR Ca^{2+}-transport ATPase (Sande-Lemos & De Meis, 1988) can be excluded since there was no [γ-^{32}P]ATP production in absence of glycolytic substrates. The biphasic time course of [γ-^{32}P]ATP formation indicates a coupling of the substrate level phosphorylation to membrane-associated ATP-consuming processes. At the free [Ca^{2+}] of the assay of about 1 nM, ATP utilization by the SR Ca^{2+}-transport ATPase is unlikely. Furthermore, neither 200 μM vanadate, which completely inhibits the SR Ca^{2+}-transport ATPase (Medda & Hasselbach, 1983), nor 100 μM strophanthidin, an inhibitor of the sarcolemmal Na^+/K^+ATPase (Mercer & Dunham, 1981), affect the ATP transient (not shown). In contrast, 20 mM fluoride increases the [γ-^{32}P]ATP concentration reached in the peak by about 30% and significantly retards the declining phase (Figure 1). This declining phase of the ATP transient could be due to interaction of GAPDH with the JFP of triads/TC, which has been reported to lead to inactivation of the enzyme (Caswell & Corbett, 1985). However, Figure 2 shows that readdition of GAP after 30 min, when endogenous ATP synthesis has nearly ceased, produces a second burst of ATP similar to the first one. It argues against an inactivation of GAPDH by interaction with the JFP. In the absence of ADP, no [γ-^{32}P]ATP is synthesized (Figure 2). In the presence of ADP, only ATP was significantly labeled with ^{32}P as detected by thin layer chromatography or high voltage electrophoresis followed by autoradiography (not shown). Analysis of assay media by HPLC with metal dye detection (see below) did not reveal the formation of other phosphocompounds besides ATP. As a control, ATP synthesis was monitored enzymatically (Figure 3). The transient time course of the total amount of ATP synthesized is similar to that of the ^{32}P-labeled nucleotide. However, the amount of total ATP formed is significantly higher than that of the radioactively labeled nucleotide (Figure 3). The additional amount of nonradioactively labeled ATP is synthesized by adenylate kinase despite the presence of the specific inhibitor AP_5A (Lienhard & Secemski, 1973). The time course of its formation approximately explains the apparent difference

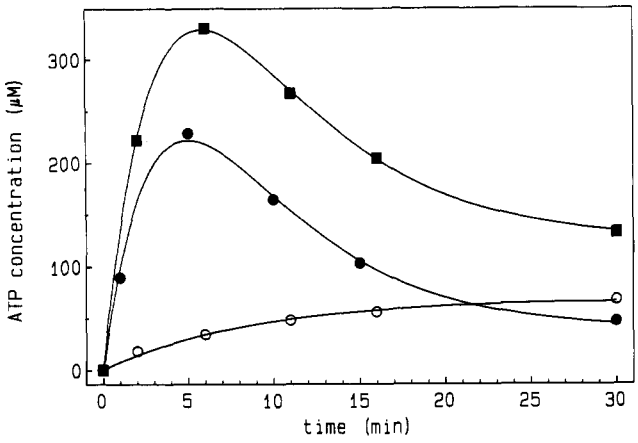

FIGURE 3: Enzymatic and radioisotopic determination of ATP formed in triads. ATP production in assay media containing 1.9 mM GAP as substrate was monitored either by measuring [γ-^{32}P]ATP (●) or by enzymatic determination (■) (see Materials and Methods). ATP formation due to adenylate kinase (○) was measured enzymatically in presence of 50 μM AP_5A.

between the two determinations. The nonradioactively labeled ATP, however, does not interfere with the detection of [γ-^{32}P]ATP produced by the oxidation of GAP, which was the method of choice for studying the compartmentalized ATP formation.

Assuming complete oxidation of 1.9 mM GAP, equimolar formation of [γ-^{32}P]ATP would be expected. Since about 10% of this concentration is reached in the peak of the ATP transient, a rapid degradation or consumption of the synthesized [γ-^{32}P]ATP is taking place in the triadic junction. The localized ATP synthesis appears to be suited to drive energy-dependent reactions especially in the junctional gap as can be concluded from the following experiment (Figure 4). ATP added to the assay medium can be separated from other low molecular weight phosphocompounds by HPLC (Figure 4a). In presence of a solid phase coupled ATP-consuming system consisting of agarose-bound hexokinase and 2-deoxyglucose, the added ATP is used up quantitatively within 5 min of incubation (Figure 4b). In contrast, ATP formed endogenously in the triadic gap apparently can not be used by the solid phase coupled ATP consuming system (compare Figure 4; panels c and d). Contrary to agarose-bound hexokinase which is excluded from the junctional space, soluble hexokinase appears to enter the gap and utilize the synthesized ATP (compare Figure 4; panels e and f). It can be concluded from these observations that diffusion of synthesized ATP out of the gap is somehow limited and thus appears kinetically compartmentalized compared to the ATP in the solute space.

Employing FBP as substrate at concentrations of 10–250 μM yields [γ-^{32}P]ATP transients similar to those induced by GAP (Figure 5). However, the amplitude of these transients reaches relatively higher levels (about 12–40% of the concentration maximally synthesizable from the added FBP), and the declining phase seems to be slower than that observed with GAP. These observations suggest a more effect ATP synthesis from FBP than from GAP. A quantitative description of the [γ-^{32}P]ATP transients produced by GAP or FBP requires the determination of all intermediates, especially those produced by TIM, which expresses an activity approximately as high as that of aldolase (Table I). Due to the very low activity of the triadic phosphoglyceromutase (Table I), the flux of intermediates from 3PG to lactate is very slow. The following model calculations which were carried out to simulate the changes of the glycolytic intermediates were corrected for the

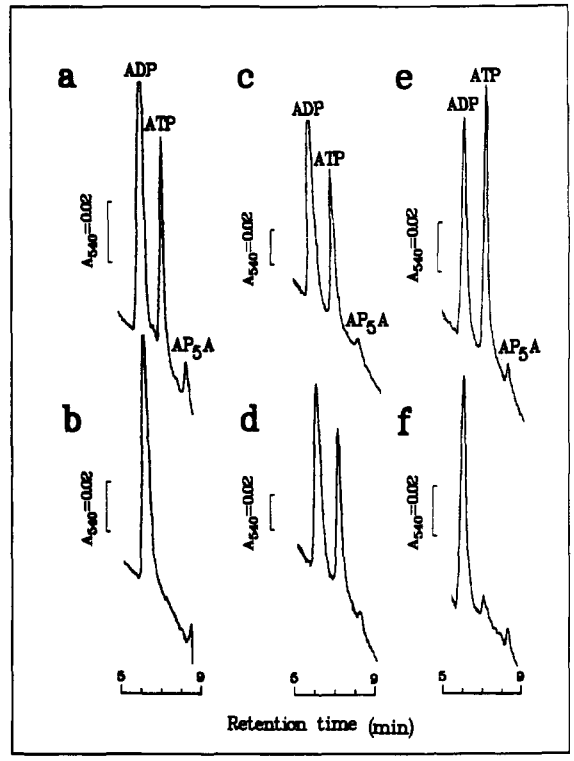

FIGURE 4: Effect of an added ATP-consuming system on endogenously synthesized and exogenously added ATP. The ATP content of assay media after 5 min of incubation was determined from aliquots by HPLC as described under Materials and Methods. The chromatograms in panels a and b were obtained from assays without triads/TC to which 200 μM ATP was added in absence (a) or presence (b) of an ATP-consuming system composed of agarose-coupled hexokinase and 2-deoxyglucose. Chromatograms in panels c to f were derived from assays containing triads/TC (1 mg/mL) and 1.9 mM GAP as substrate for endogenous ATP synthesis. Chromatograms in panels c and e result from samples without an added ATP-consuming system, revealing endogenously produced ATP. Chromatograms in panels d and f were obtained from assays carried out in presence of either agarose-coupled (d) or soluble hexokinase (f); in both cases, the same total hexokinase activity was employed. The ATP peak areas correspond to the following ATP concentrations in the assay media (μM): 200 (a), 0 (b), 193 (c), 151 (d), 194 (e), and 16 (f).

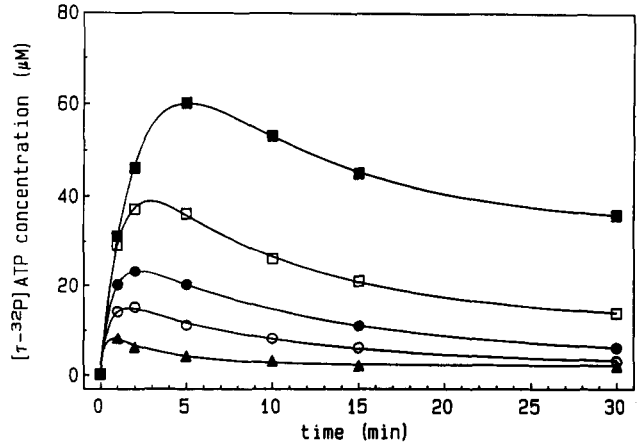

FIGURE 5: Transients of [γ-^{32}P]ATP induced by oxidation of FBP in triads. The assay media contained as substrate 10 (▲), 25 (○), 50 (●), 100 (□), and 250 μM (■) FBP, respectively. Experiments were carried out as described under Materials and Methods.

low amount of lactate formed during the experiments. The reactions depicted below catalyzed by aldolase (eq 1), TIM (eq 2), and GADPH/PGK (eq 3), which do not consider potential allosteric regulations, have been taken into consideration:

$$\mathrm{FBP} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{DAP} + \mathrm{GAP} \quad (1)$$

$$\mathrm{DAP} \underset{k_{21}}{\overset{k_{22}}{\rightleftharpoons}} \mathrm{GAP} \quad (2)$$

$$\mathrm{GAP} + \mathrm{ADP} + \mathrm{P_i} + \mathrm{NAD} \underset{k_{32}}{\overset{k_{31}}{\rightleftharpoons}} 3\mathrm{PG} + \mathrm{ATP_m} + \mathrm{NADH} \quad (3)$$

A conservation equation, $3PG(t) = ATP_m(t) + ATP_c(t)$, was included to account for the mentioned deviation of the actually measurable ATP, $ATP_m(t)$, from the theoretically expected ATP (1 mol per mol of 3PG) due to ATP consumption, $ATP_c(t)$, under the employed experimental conditions. The above reactions are described by the following system of differential rate equations containing the corresponding apparent rate constants, k_{11}, k_{12}, k_{21}, k_{22}, k_{31}, k_{32}, as variable parameters:

$$d[FBP]/dt = k_{12}[DAP][GAP] - k_{11}[FBP]$$

$$d[DAP]/dt = -d[FBP]/dt + k_{21}[GAP] - k_{22}[DAP]$$

$$d[3PG]/dt = k_{31}[GAP][NAD][ADP][P_i] - k_{32}[ATP_m][NADH][3PG]$$

$$d[GAP]/dt = -2(d[FBP]/dt) - d[DAP]/dt - d[3PG]/dt$$

$$d[ATP_m]/dt = \alpha A \exp(-\alpha t) - \beta B \exp(-\beta t)$$

$$d[ATP_c]/dt = d[3PG]/dt - d[ATP_m]/dt$$

$$d[NAD]/dt = -d[3PG]/dt$$

$$d[ADP]/dt = -d[ATP_m]/dt$$

$$d[P_i]/dt = -d[ATP_m]/dt$$

$$d[NADH]/dt = -d[NAD]/dt$$

The measured [γ-^{32}P]ATP transient was approximated by a double-exponential function, $ATP_m(t) = A[1 - \exp(-\alpha t)] - B[1 - \exp(-\beta t)]$; parameters were obtained by a nonlinear least-squares fit of the experimental data. The change of the ATP consumption with time ($d[ATP_c]/dt$) results from the conservation equation. On the basis of assumptions for the apparent equilibrium constants of the considered reactions shown in Table II, which are approximately in the range given by Newsholm and Start (1973), the concentration changes of the metabolites were numerically calculated and approximated to the experimental data by optimizing the apparent rate constants (see Materials and Methods for details). Figure 6 shows that the substrate FBP is continuously decreasing and the products DAP and 3PG are continuously increasing with increasing time whereas GAP changes with a transient time course. The experimental data could fairly well be approximated by the time courses predicted by the described model with the optimized apparent rate constants given in Table II. The approximated rate constants for the aldolase and the GAPDH/PGK reactions are significantly ($p < 0.01$) higher employing FBP instead of GAP as substrate. The good agreement between the experimental data and the model is consistent with the hypothesis that ATP formation at triads is essentially due to the reactions in eqs 1–3 mentioned above. Triads/TC exhibit a basic Ca^{2+}-independent ATP hydrolysis (not shown) which follows Michaelis–Menten kinetics [K_m-(ATP) = 320 μM, V_{max} = 216 nmol min^{-1} mg^{-1}]. If this ATP hydrolysis instead of the ATP transient is explicitly formulated and included in the above model ($d[ATP_c]/dt$), the resulting ATP transient would be much faster than observed experimentally. This observation also indicates kinetic compartmentation of the synthesized ATP in triads as it has been concluded from the hexokinase experiments, i.e., ATP produced in the junctional gap is not directly accessible to the overall ATPase of triads/TC expressed under assay conditions. [γ-^{32}P]ATP transients together with the simultaneously determined glycolytic metabolites can be described well by the above model for the FBP concentrations shown in Figure 5 using the same set of parameters given in Table II.

The amplitude of the [γ-^{32}P]ATP transient obtained by FBP oxidation decreases with increasing free Ca^{2+} concentrations in the assay (Figure 7). It is described by a sigmoidal Hill curve with a Hill coefficient of 1.4. The half-maximal effect is obtained at approximately 1 μM Ca^{2+}, and a maximum decrease of approximately 50% is reached at about 30 μM Ca^{2+}. This Ca^{2+}-dependent ATP consumption cannot be due to Ca^{2+} uptake into the SR because the Ca^{2+}-transport ATPase of the SR was blocked by 200 μM vanadate (Medda & Hasselbach, 1983).

ATP produced in the junctional gap can be utilized to phosphorylate triadic proteins (Figure 8). Autoradiography following SDS–PAGE reveals that polypeptides of apparent M_r 450000, 180000, 160000, 145000, 135000, 90000, 54000, 51000, and 20000, respectively, are phosphorylated by protein kinase(s) endogenously present in triads/TC. Two polypeptides of M_r 145000 and 135000 are very likely peripheral membrane proteins because they can be removed by washes with 0.6 M KCl (not shown). The other phosphoproteins are of unknown identity.

Discussion

The site of EC coupling in skeletal muscle is commonly accepted to be the junctional foot structure of the triad, where essentially two protein complexes, the T-tubular DHP receptor and the ryanodine receptor of the TC, are involved in the signal transduction process. The direct interaction of a DHP receptor domain with a domain in the JFP seems to be critical for skeletal muscle type EC coupling (Tanabe et al., 1990). A secondary linkage between these two proteins might be formed by GAPDH (Corbett et al., 1985; Brandt et al., 1990) which together with aldolase (Thieleczek et al., 1989; Brandt et al., 1990) is found at the triadic junction. As shown in Table I, several further glycolytic enzymes are located at triads together with GAPDH and aldolase which both might form a real structurally bound core (Strapazon & Steck, 1977; Kliman & Steck, 1980; Brandt et al., 1990). These obviously strongly interacting enzymes establish a glycolytic reaction sequence linked to the JFP which can lead to ATP synthesis by substrate level phosphorylation. However, such an ATP synthesis was not envisaged since binding of GAPDH and aldolase to membrane structures was thought to inactivate these enzymes (Tsai et al., 1982; Strapazon & Steck, 1977). For GAPDH isolated from triads, a complete inactivation within 20 min after readdition of triads/TC has been described (Caswell & Corbett, 1985). However, here we demonstrate that GAPDH as well as aldolase expresses activity in the structure-associated state. Furthermore, PGK is also active, establishing a substrate-level phosphorylation step that gives rise to ATP synthesis in the triadic gap due to oxidation of the glycolytic substrates GAP or FBP. The "inactivation" of GAPDH observed previously (Caswell & Corbett, 1985) is very likely due to removal of substrate by conversion of GAP into DAP or by adjustment of a low steady-state level of GAP after supply with FBP as shown in Figure 6. From the initial rate of decrease of FBP supplied to the glycolytic reaction sequence, a specific activity of structure-associated aldolase of about 16 units/mg can be estimated using the amount of triadic aldolase given in Table I. This value is in good agreement with the specific activity reported for the purified skeletal muscle en-

Table II: Rate Constants of the Glycolytic Reactions Associated with Triads[a]

enzyme	K_{appi}	apparent rate constants obtained with GAP	apparent rate constants obtained with FBP
aldolase	5×10^{-5} M	$k_{12} = (2.07 \pm 0.14) \times 10^3$ M^{-1} min^{-1}	$k_{12} = (7.11 \pm 0.96) \times 10^3$ M^{-1} min^{-1}
TIM	22	$k_{22} = (1.40 \pm 0.16) \times 10^{-2}$ min^{-1}	$k_{22} = (1.27 \pm 0.15) \times 10^{-2}$ min^{-1}
GAPDH/PGK	100 M^{-1}	$k_{32} = (5.38 \pm 0.12) \times 10^4$ M^{-2} min^{-1}	$k_{32} = (1.25 \pm 0.06) \times 10^5$ M^{-2} min^{-1}

[a] The apparent rate constants were obtained by numerical analysis (see Materials and Methods) of the metabolite concentration changes measured with the substrates FBP (see Figure 6) and GAP. They refer to the enzymatic reactions in eqs 1–3 described in the text. $K_{appi} = k_{i1}/k_{i2}$ represent the assumed apparent equilibrium constants of the corresponding enzyme reactions (i = 1, 2, 3).

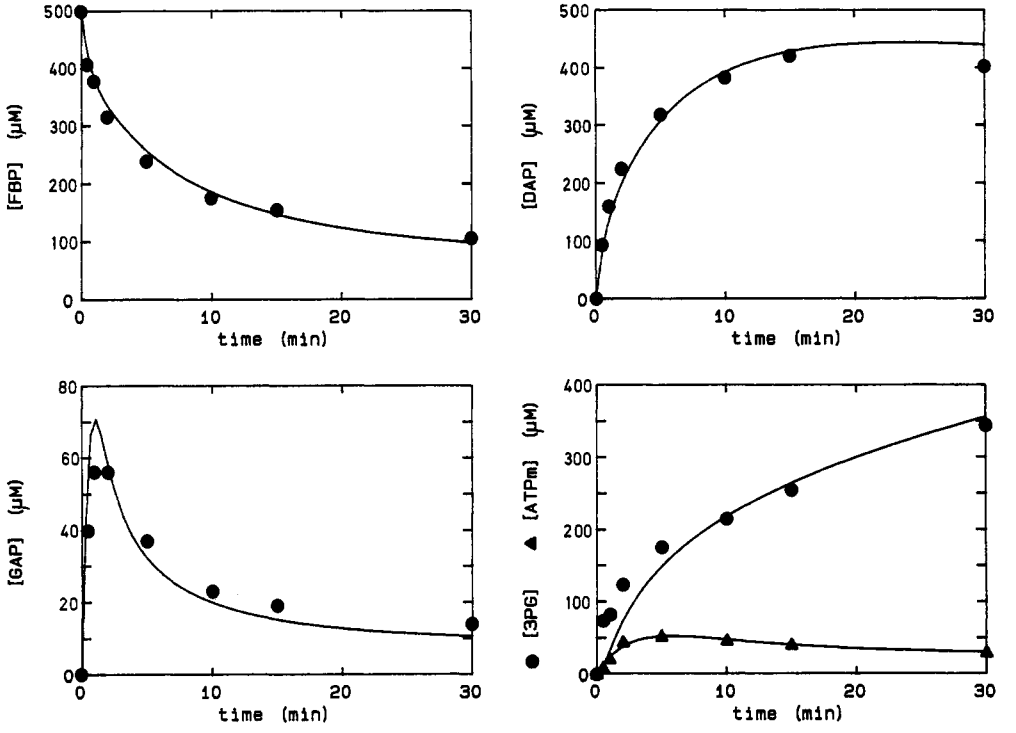

FIGURE 6: Concentration changes of FBP, GAP, DAP, 3PG, and [γ-^{32}P]ATP due to oxidation of 0.5 mM FBP in triads. Aliquots were removed from the incubation mixture at time points as indicated, and glycolytic metabolites and [γ-^{32}P]ATP were determined as described under Materials and Methods. The curves represent the time courses of these compounds predicted by the reaction model (see text).

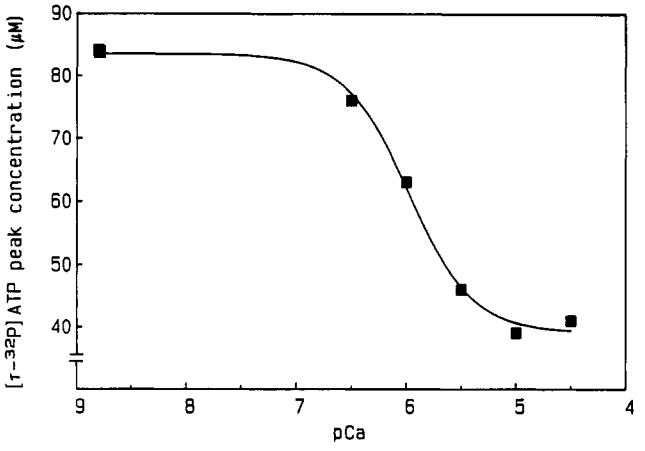

FIGURE 7: Effect of Ca^{2+} on the amplitude of [γ-^{32}P]ATP transients induced by oxidation of 0.5 mM FBP in triads. [γ-^{32}P]ATP transients were determined in presence of 200 μM vanadate (see Materials and Methods) at the indicated free Ca^{2+} concentrations. The changes in peak [γ-^{32}P]ATP concentrations were fitted by a Hill function with a Hill coefficient of 1.4.

zyme (Scopes & Stoter, 1982). An analogue estimate for the structure-associated GAPDH is not very reasonable because employed GAP can be converted via different routes.

The conclusion that ATP synthesized in triads is kinetically compartmentalized, i.e., out of diffusion equilibrium with bulk ATP, can be made from three observations. First, the endogenously produced ATP cannot be effectively used by an ATP-consuming system consisting of agarose-coupled hexokinase and 2-deoxyglucose (Figure 4). Since the hexokinase bound to agarose beads is excluded from the triadic gap, it can utilize only ATP present in the solute for phosphorylation of 2-deoxyglucose. Second, the endogenously synthesized ATP is preferentially utilized in the triadic gap as evidenced by the transient presence of [γ-^{32}P]ATP as well as by the substantial difference between the measured 3PG and [γ-^{32}P]ATP. Apparently, a much higher amount of ATP than can be determined must have been synthesized and partially consumed in the system. Third, the measured basal Ca^{2+}-independent ATPase of triads would be expected to cause a much faster decline of [γ-^{32}P]ATP than has been observed. Thus, kinetic compartmentation of ATP synthesized in triads might be due to effective utilization of ATP at the place of production as well as to limited diffusion of ATP due to binding reactions in this space that will be discussed later. There are several hints for a compartmentation of ATP at skeletal muscle membranes. It has been shown that the specific radioactivity of [^{32}P]phosphate incorporated in phosphorylase kinase, a partly membrane-associated enzyme (Gröschel-Stewart et al., 1978; Thieleczek et al., 1987), is significantly higher than that of the γ-phosphate of ATP (Mayer & Krebs, 1970). The same has been reported recently for phosphoinositides in frog skeletal muscle which are definitely membrane constituents (Lagos & Vergara, 1990). These observations strengthen the argument of a membrane-compartmentalized ATP pool which is not in equilibrium with the major myoplasmic ATP pool.

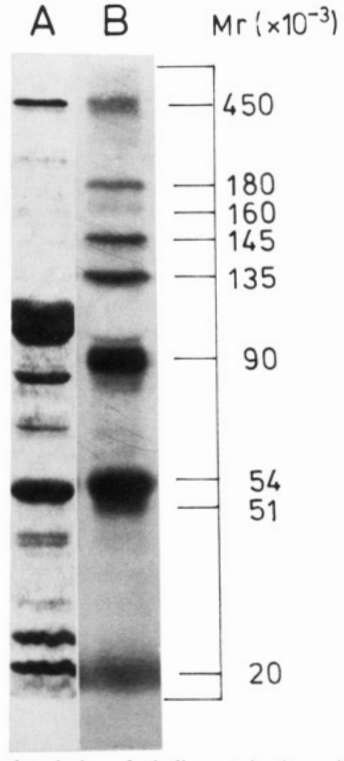

FIGURE 8: Phosphorylation of triadic proteins by endogenous kinases utilizing endogenously synthesized [γ-^{32}P]ATP. The reaction was started by the addition of 1 mM FBP to an assay medium containing 1 mM orthophosphate (580 Ci/mol). After incubation at 30 °C for 30 min, the assay was quenched by PAGE sample buffer (Laemmli, 1970), and 52 μg of protein per lane was separated by SDS–PAGE using 7.5% polyacrylamide. Lane A shows polypeptides stained with Coomassie Blue and lane B the corresponding autoradiogram.

The apparent rate constants of the reaction catalyzed by aldolase was about 3.4-fold and that catalyzed by GAPDH/PGK about 2.3-fold higher when FBP instead of GAP was employed as substrate (Table II). Part of this effect might be due to the ability of FBP to release bound aldolase from triadic structures (Thieleczek et al., 1989). The kinetic properties of aldolase are known to be significantly modified on binding to cellular structures (Walsh et al., 1977). In triads, substrate channelling, which has been described for sequential glycolytic reactions also in other cells [for a review see Srere (1987)], might operate especially effective with the key metabolite of glycolysis, FBP. Channelling of substrate into the triadic junction would determine the level of an ATP pool within the triadic gap separate from the cytosolic ATP pool. It has been shown previously that aldolase but not GAPDH can be selectively released from triads by micromolar concentrations of inositol 1,4,5-trisphosphate (Thieleczek et al., 1989). It points to a role of aldolase in determining the flux of metabolites into the triadic gap. On the other hand, aldolase bound to the triadic junction might establish a membrane-localized sink for inositol 1,4,5-trisphosphate (Thieleczek et al., 1989) due to its high affinity to this second messenger (Koppitz et al., 1986). In addition, the complex interplay of inositol phosphates and FBP on membrane-associated aldolase (Thieleczek et al., 1989; Mayr & Thieleczek, 1991) appears to be well suited to affect the non-steady-state glycolytic flux in a submembranous compartment during muscle activation (Mayr & Thieleczek, 1991).

In the presence of the known protein phosphatase inhibitor fluoride, the amplitude of the ATP transient was significantly increased and its declining phase was substantially retarded. These observations suggest the participation of protein phosphorylation/dephosphorylation reactions in the utilization of synthesized ATP in the triadic gap. Phosphorylation of special triadic proteins might influence the signal transduction pathway from T tubule depolarization to SR Ca^{2+} release. The potential for modulations of the primary EC-coupling event is evident from physiological phenomena like muscle fatigue or posttetanic twitch potentiation. Indeed, a set of distinct triadic proteins of apparent M_r 450 000, 180 000, 160 000, 145 000, 135 000, 90 000, 54 000, 51 000, and 20 000, respectively, are phosphorylated by endogenous kinases under assay conditions. The protein of M_r 450 000 could be the ryanodine receptor which has been shown to be Ca^{2+}/calmodulin dependently phosphorylated (Chu et al., 1990). The phosphoproteins in the range of M_r 180 000 and 50 000 we tentatively assign to the α and β subunits of the DHP receptor, respectively, which both can be phosphorylated (Imagawa et al., 1987; Jahn et al., 1988). The phosphoproteins of apparent M_r 145 000 and 135 000 are very likely peripheral membrane proteins since they can be removed by washes with 0.6 M KCl. Quite a variety of skeletal muscle phosphoproteins in the range of M_r 50 000 are known, like calsequestrin (Varsanyi & Heilmeyer, 1980) and the regulatory subunit of the cyclic-AMP-dependent protein kinase (Salvatori et al., 1990). Minor phosphorylated proteins in the range of M_r 25 000–40 000 might include GAPDH which has been shown to be phosphorylated (Kawamoto & Caswell, 1986). We are currently attempting to isolate phosphopeptides of triadic proteins in order to identify the corresponding proteins and to clarify what type of kinases are involved in these endogenous phosphorylations.

It has been shown by Meissner et al. (1986) that ATP influences the characteristics of the Ca^{2+}-release channel. Thus, the ATP level within the triadic gap generated by the localized substrate coupled ATP synthesis might modulate the Ca^{2+}release properties by binding directly to the ryanodine receptor. A nucleotide-binding consensus sequence has been found at sites of the ryanodine receptor facing the junctional gap (Takeshima et al., 1989). Furthermore, Ca^{2+} release from the SR has been shown to be modulated by Mg^{2+} (Meissner et al., 1986), which is chelated more efficiently by ATP than by ADP.

Acknowledgments

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Registry No. ATP, 56-65-5; GAPDH, 9001-50-7; TIM, 9023-78-3; LDH, 9001-60-9; GAP, 142-10-9; ATPase, 9000-83-3; Ca, 7440-70-2; aldolase, 9024-52-6; phosphoglyceromutase, 9023-91-0; enolase, 9014-08-8; pyruvate kinase, 9001-59-6; adenylate kinase, 9013-02-9; phosphoglycerate kinase, 9001-83-6; dihydroxyacetone phosphate, 57-04-5; phosphoglyceric acid, 820-11-1; fructose 1,6–bisphosphate, 488-69-7.

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