The Muscle-specific Calmodulin-dependent Protein Kinase Assembles with the Glycolytic Enzyme Complex at the Sarcoplasmic Reticulum and Modulates the Activity of Glyceraldehyde-3-phosphate Dehydrogenase in a Ca\(^{2+}\)/Calmodulin-dependent Manner*

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The skeletal muscle specific Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII\(_{M}\)) is localized to the sarcoplasmic reticulum (SR) by an anchoring protein, αKAP, but its function remains to be defined. Protein interactions of CaMKII\(_{M}\) indicated that it exists in complex with enzymes involved in glycolysis at the SR membrane. The kinase was found to complex with glycogen phosphorylase, glycogen debranching enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and creatine kinase in the SR membrane. CaMKII\(_{M}\) was also found to assemble with aldolase A, GAPDH, enolase, lactate dehydrogenase, creatine kinase, pyruvate kinase, and phosphorylase b kinase from the cytosolic fraction. The interacting proteins were substrates of CaMKII\(_{M}\), and their phosphorylation was enhanced in a Ca\(^{2+}\)- and calmodulin (CaM)-dependent manner. The CaMKII\(_{M}\) could directly phosphorylate GAPDH and markedly increase (3.4-fold) its activity in a Ca\(^{2+}\)/CaM-dependent manner. These data suggest that the muscle CaMKII\(_{M}\) isomerase may serve to assemble the glycogen-mobilizing and glycolytic enzymes at the SR membrane and specifically modulate the activity of GAPDH in response to calcium signaling. Thus, the activation of CaMKII\(_{M}\) in response to calcium signaling could serve to modulate GAPDH and thereby ATP and NADH levels at the SR membrane, which in turn will regulate calcium transport processes.

Free calcium (Ca\(^{2+}\)) regulates diverse cellular functions by acting as an intracellular second messenger. A large part of these cellular functions are mediated by CaM, which is the ubiquitous intracellular Ca\(^{2+}\) receptor. The Ca\(^{2+}\)/CaM complex allosterically activates numerous proteins, including Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMII) (1). CaMII is a multifunctional enzyme that is highly expressed in brain and muscle. The kinase is believed to serve important roles in synaptic transmission (2, 3), gene transcription (4, 5), cell growth (6), and control of excitation-contraction coupling (7–9).

The subcellular distribution of CaMKII indicates cytosolic and membrane localizations in different tissues (10). In skeletal muscle, an isoform of CaMKII is targeted to the SR membrane by a non-kinase protein, αKAP (11, 12). Different studies have been conducted to determine whether membrane-bound CaM kinase could phosphorylate different substrate proteins by virtue of its proximity effects and thereby regulate SR function. Although the calcium release channelryanodine receptor (RyR) and the calcium pump/Ca\(^{2+}\)/ATPase in skeletal muscle SR were shown to be substrates of CaMII (13, 14), there does not appear to be any clear effects on the regulation of functional activity of these proteins induced by such phosphorylation (7, 8, 15–17). Moreover, there is clear evidence that the RyR and calcium pump are regulated by local ATP, Ca\(^{2+}\), and CaM through direct ligand binding (15–17). In this regard, both Ca\(^{2+}\) and CaM are present at the SR, and the level of ATP is believed to be tightly controlled through a membrane-bound glycolytic machinery involving phosphorylase b, creatine kinase, and GAPDH (18–20). Studies show that membrane-associated GAPDH is able to support an ATP-regenerating system at the SR, which is tightly coupled to the calcium transport function (18–20). How the glycolytic machinery is targeted to the SR membrane and whether this local ATP-generating system could be regulated in response to the calcium signal and muscle contraction remains to be defined. Recent studies also suggest that the local concentration of NADH can regulate the calcium transport activities of the SR (21). How modulations in NADH concentrations at the SR membrane can be achieved remains unknown.

Here, we examined the functional significance of the SR membrane-bound CaMKII\(_{M}\). We have utilized a proteomics-based approach to identify potential binding partners of the muscle-specific CaMKII. This approach has identified several enzymes of the glycolytic pathway, including GAPDH, pyruvate kinase, and LDH, as well as enzymes involved in glycogen mobilization, such as phosphorylase b kinase and the debranching enzyme. Importantly, our results reveal that CaMKII\(_{M}\) can phosphorylate these associated proteins and specifically increase the activity of GAPDH in a Ca\(^{2+}\)/CaM-dependent manner. In view of the previous studies that implicate a role for local ATP and NADH in the regulation of SR function and excitation-contraction coupling, our results suggest that...
the membrane-bound CaMKIIβ may be important for targeting the glycolytic machinery to the SR and modulate the local levels of NADH and ATP in response to calcium signaling in skeletal muscle.

EXPERIMENTAL PROCEDURES

Cloning of Muscle-specific CaM Kinases and Its Expression—An oligo(T)-primed lambda Zap II DNA library from skeletal muscle (Stratagene) was screened with a 500-bp DNA probe encompassing the coding region of the variable domain of rat brain CaMKII. The probe was generated by reverse transcription-PCR using rat brain RNA and β subunit-specific primers (5′-CTG AAG AAC TTC AAT GCA AGG AGG-3′ or 5′-GCA GTA GCT CCT CAAT GAT-3′) and labeled with [32P]dCTP using a random prime labeling kit from Amersham Biosciences. Hybridization was performed at 65 °C in 10% polyethylene glycol, 1.5× saline/sodium phosphate/EDTA, and 7% SDS for 12 h, then followed by four washes in 1–0.2× SSC, 0.1% SDS at 50 °C. The membranes were exposed to Biomax MR films, and cDNAs identified on the autoradiograms were excised as per Stratagene. Positive clones were categorized by restriction endonuclease mapping and size analysis. Overlapping clones were sequenced on both the strands by an automated ABI sequencer using M13F and M13R primers and sequences analyzed with Sequaid II (University of Kansas) and BLAST.

The full-length skeletal muscle CaMKIIα as well as the brain isoforms were cloned in-frame with the glutathione S-transferase (GST) fusion protein in pGEX 3XAB vector in EcoRI site. Escherichia coli DH5α containing recombinant proteins were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The GST fusion proteins were isolated on glutathione-Sepharose 4B beads (Amersham Biosciences) following sonication in PBST containing 1% Nonidet P-40.

Isolation of Cytosolic and SR Membrane Proteins—The preparation of SR vesicles was based on previously described protocols (11, 22). Freshly dissected back muscle from rabbits was washed in cold phosphate-buffered saline, trimmed of fat and connective tissue, cut into cubes, and either frozen in liquid N2 and stored at −110 °C or freshly processed. The fresh or thawed muscle cubes were suspended in homogenization buffer (50 mM Tris, pH 7.4, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyruvate, and 0.6 mM NADP). The tissue was homogenized in a Waring blender with 5×–20× high-speed bursts. The homogenate was centrifuged at 2600 × g for 10 min, and the supernatant was filtered through cheesecloth and centrifuged at 10,000 × g for 10 min. The supernatant so obtained was centrifuged at 186,000 × g for 1 h, cytosolic supernatant was saved, and SR membrane preparation was re-suspended in buffer (10 mM Tris, pH 7.4, 400 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin) and dispersed with 15 strokes of a Dounce homogenizer. SR membrane proteins were aliquoted and stored at −110 °C for further use. Protein quantification of each fraction was performed according to Bradford (23).

Enzyme Assays—CaMKα kinase activity was assayed as described previously with autocomitase-2 (KKALRQETVDAL) as a substrate peptide (24). To measure Ca2+/CaM-dependent or “autonomous activity,” CaMKα protein was incubated for 30 s to 5 min in 50 mM MOPS (pH 7.4), 10 mM magnesium chloride, 5 mM dithiothreitol, 40 μg/ml calmodulin, 0.4 mM γ-[32P]ATP (0.4 × 10^−6 cpm), 100 μM Ca2+/CaM (Bers (25)), or 5 mM EGTA (control reaction) at 30 °C. At the end of incubation, aliquots (25 μl) of the reaction mixtures were applied on Whatman 3MM filter papers, washed with 5% trichloroacetic acid and dried, and radioactivity was determined by a liquid scintillation counter.

A GAPDH phosphor protein was isolated by protein-A/G-agarose (70 μg/ml) contained 0.1 M KCl, 5 mM MgCl2, 2 mM ADP, 0.17 mM NADH, 2 mM phosphoenol pyruvate and 2 units of LDH in 50 mM Tris-HCl buffer (pH 7.4) at 30 °C. The reaction was initiated by addition of phosphoenol pyruvate.

LDH activity was measured (28) by pyruvate reduction in the presence of NADH with NAD+ formation in a 1-ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 0.18 mM NADH, 0.6 mM sodium pyruvate, and, and appropriately diluted enzyme in 1× Tris at 25 °C. The production of NAD+ was followed by a decrease in absorbance at 340 nm.

Glycogen phosphorylase activity was measured as described by Helmreich and Cori (29). The assay mixture contained 50 mM potassium phosphate (pH 7.1), 1 mM MgCl2, 0.2% glycogen, 50 mM glucose, and an appropriately diluted enzyme in 1× Tris at 25 °C. The production of glucose-6-phosphate dehydrogenase. In a series of experiments the effects of CaMKIIβ phosphorylation on the activity of this enzyme were determined in the absence of AMP in the above buffer.

GST Pull-down Assays—GST pull-down assays were carried out by immobilizing GST and GST-tagged CaMKIIβ to glutathione-Sepharose 4B beads. Immobilized fusion proteins (12 μg) were incubated with SR membrane or cytosolic proteins (500 μg) in 1 ml of TBS (50 mM Tris, 100 mM NaCl, pH 7.4, plus 0.1% Triton X-100 with 1 mM free Ca2+) for 2 h at 4 °C. The salt concentrations in the buffer used were similar to that used by others (30). Beads were washed four times in TBS. Proteins were visualized with Coomassie Blue or silver staining and identified by immunoblotting with antibodies using enhanced chemiluminescence (ECL).

Immunoprecipitation and Immunoblotting—Immunoprecipitations were carried out for 2 h at 4 °C in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40) in 1 ml of reaction volume containing 500 μg of SR membrane or cytosolic protein, 10 μg of bovine serum albumin, and the indicated concentrations of either anti-CaMKIIβ antibody (Cbj-1) or control IgG (5 μg/ml each). Immunoprecipitates were captured by protein-A/agarose (70 μl), washed in lysis buffer, and resolved by SDS-PAGE. After equilibration in transfer buffer (15 mM glycine, 20 mM Tris, 0.1% SDS, and 20% methanol), proteins were electrophoretically transferred onto PVDF membranes (32 μA, 4 h). Membranes were blocked in 5% skimmed milk in 1× Tris-buffered saline, trimmed of fat and connective tissue, cut into cubes, and either frozen in liquid N2 and stored at −110 °C or freshly processed.
CaMKII was expressed as a GST fusion protein, and the recombinant CaMKII was detected by CaMKII antibody (Fig. 1, lane 3). The recombinant CaMKII was expressed as a GST fusion protein, and the molecular mass of GST-conjugated CaMKII was 98 kDa with several truncated products of ~73, 64, 56, 41, and 30 kDa being also detected by CaMKII antibody (Fig. 1, lane 3).

The functional activity of recombinant CaMKII was examined in an autocomtide-2 assay system (Upstate CaM kinase assay kit), and this indicated an increase of ~90-fold in phosphoryltransferase activity in the presence of calcium (100 μM [Ca^{2+}]_{free}) and CaM (2 μM), which corresponds to ~4.86 pmol/min/μg.

Molecular Interactions of the Muscle-specific CaMKII M—To determine the functional significance of the SR membrane-bound CaMKII M, identification of the potential interacting proteins was carried out using GST pull-down assays with cytosolic and detergent-soluble SR fractions from skeletal muscle. Characterization of the interacting proteins with respect to molecular mass revealed that recombinant GST-CaMKII M specifically binds polypeptides of ~55, 47, 44, 39, 36, and 32 kDa from the cytosol (Fig. 2A, lane 2) and of ~177, 97, 61, 44, and 36 kDa from the SR membrane fraction (Fig. 2B, lane 2). There was no binding of these polypeptides to GST alone (Fig. 2A, lane 4 and Fig. 2B, lane 4). Furthermore, recombinant GST-CaMKII M in TBS buffer served as an additional control with the main 98-kDa fusion protein and several truncated products notably of ~73, 64, 56, 41, and 30 kDa being also visualized (Fig. 2A, lane 1 and Fig. 2B, lane 1). The expressed kinase and its truncated products have been specifically labeled in parentheses in Fig. 2 (A, lane 1 and B, lane 1) and showed a similar migration pattern as seen in Western blot analysis with anti-CaMKII M antibody (see Fig. 1, lane 3). The 25-kDa GST protein visualized in the 10% SDS gel in panel A migrated out of the 7.5% gel in panel B, and a nonspecific polypeptide of ~50 kDa was visualized in each lane.

The Glycolytic Enzymes Are Substrates of CaMKII M—To further identify the specifically bound polypeptides, MALDI-TOF was employed, and this revealed the interaction of CaMKII M with enzymes of glycolytic pathway and glycogen metabolism (Table I). The interacting proteins from cytosolic fraction (Table I and Fig. 2A, lane 2) were determined to be LDH and enzymes of the Embden-Meyerhof-Parnas pathway, namely aldolase A, GAPDH, enolase, and pyruvate kinase, whereas those from the SR fraction (Table I and Fig. 2B, lane 2) revealed an identity with glycogen phosphorylase b, glycogen debranching enzyme, creatine kinase, and GAPDH. The data revealed that GAPDH and creatine kinase were present as interacting proteins in both cytosolic and SR membrane fractions. An interacting polypeptide of 61 kDa in the pull-down assay with the SR proteins (Fig. 2B, lane 2, asterisk) and another of 275 kDa (not shown) in pull-down assay with cytosolic proteins were determined to be CaMKII M, indicating the same class of kinase-kinase interactions. In this regard, the CaM kinase family of enzymes is known to exist as oligomers, and these data are consistent with the self-assembly of these molecules into multisubunit enzyme complexes (1). Two additional polypeptides of 138 and 125 kDa from cytosolic fraction were also seen when the pull-down proteins were resolved on 7.5% SDS-PAGE (data not shown), which were identified by MALDI-TOF as phosphorylase b kinase a and b chains, respectively (Table I).

CaMKII M associates with cytosolic and SR proteins. Cytosolic and solubilized SR membrane proteins (500 μg) from skeletal muscle were incubated with GST alone or GST-CaMK II M fusion protein (~32 μg). Bound proteins were eluted, resolved in SDS-PAGE (10% in panel A or 7.5% in panel B), and stained with Coomassie Blue. In A: lane 1, GST-CaMKII M in TBS buffer; lane 2, GST-CaMKII M pull-down of cytosolic proteins; lane 3, GST-GST in TBS buffer; lane 4, GST-GST pull-down of cytosolic proteins. In B: lane 1, GST-CaMKII M in TBS buffer; lane 2, GST-CaMKII M pull-down of SR proteins; lane 3, GST-GST in TBS buffer; lane 4, GST-GST pull-down of SR proteins. Molecular masses are in kilodaltons, and the masses of recombinant CaMKII M and its truncated products are represented in parentheses. GST-alone (25 kDa) control appeared as a doublet on the gel. These data are typical of ten independent experiments.

CaMKII M is a component of the SR membrane. Cytosolic and SR membrane proteins (50 μg) from skeletal muscle were resolved on 10% SDS-PAGE, transferred onto a PVDF membrane, and subjected to immunoblotting with anti-CaMKII M monoclonal antibody (CBp-1). Lane 1, cytosolic fraction; lane 2, SR fraction; lane 3, recombinant CaMKII M expressed in E. coli DH5α. CaMKII M is a 73-kDa protein in SR (lane 2), and the expressed recombinant GST fusion protein was 98 kDa with several truncated products ~73, 64, 56, 41, and 30 kDa (lane 3).
CaM Kinase II Regulates GAPDH

Identity of the CaMKIIβ$_{4M}$-associated complex in skeletal muscle

Cytosolic and SR membrane proteins from skeletal muscle were incubated with GST alone and GST-CaMKII$\beta_M$. Bound proteins were washed, eluted, and resolved in SDS-PAGE (7.5 or 10%) and finally stained with Coomassie. Protein bands were excised, destained, and subjected to tryptic digestion to produce peptides in the range of 1000–3000 Da. The peptides were concentrated, and MALDI-TOF mass spectrometry peptide mass mapping was performed using a Waters mass spectrometer equipped with a 337-nm nitrogen laser at an accuracy of 0.05–0.15 atomic mass unit.

Table I

| Protein                              | Mass (kDa) | Peptide match | Coverage (%) | MOWSE score     |
|--------------------------------------|------------|---------------|--------------|-----------------|
| SR membrane                          |            |               |              |                 |
| Glycogen debranching enzyme          | 177        | 48            | 34           | 3.955e+031      |
| Creatine kinase M                    | 97         | 22            | 23           | 1.170e+007      |
| Pyruvate kinase M                    | 44         | 9             | 29           | 1.541e+06       |
| Glyceraldehyde-3-phosphate dehydrogenase | 36       | 9             | 32           | 2.149e+04       |
| Cytosolic                            |            |               |              |                 |
| Phosphorylase B kinase $\alpha$ chain| 138        | 16            | 12           | 8.661e+04       |
| Phosphorylase B kinase $\beta$ chain | 125        | 19            | 24           | 5.919e+08       |
| Pyruvate kinase M                    | 55         | 17            | 22           | 2.084e+007      |
| Enolase                              | 47         | 19            | 41           | 1.581e+005      |
| Creatine kinase M                    | 44         | 15            | 33           | 3.829e+004      |
| Aldolase A                           | 39         | 17            | 38           | 8.081e+010      |
| Glyceraldehyde-3-phosphate dehydrogenase | 36       | 12            | 36           | 3.615e+005      |
| t-Lactate dehydrogenase              | 32         | 17            | 32           | 4.624e+005      |

Fig. 3. CaMKII$\beta_M$-interacting proteins are substrates of the kinase. Equal amounts of GST alone or GST-CaMKII$\beta_M$ (12 µg) were incubated with cytosolic and SR membrane proteins (500 µg) and subjected to phosphorylation in the presence of 100 µM [Ca$^{2+}$]$_{free}$ (+) or 5 mM EGTA (−) and 2 µM CaM. Bound proteins were resolved in 10% SDS-PAGE, panel a, autophosphorylation of CaMKII$\beta_M$ and its truncated products (shown in parentheses); panel b, phosphorylation of pull-down proteins from cytosolic fraction; and panel c, phosphorylation of pull-down proteins from SR membrane fraction.

The phosphorylation on the substrate proteins, the activity of the various glycolytic enzymes was determined in the absence and presence of Ca$^{2+}$ and CaM in a reconstituted kinase reaction. The data in Table II indicate that there was no effect of phosphorylation on the enzyme activity of aldolase, pyruvate kinase, LDH, creatine kinase, and glycogen phosphorylase b, however, the activity of GAPDH was significantly increased (~3.4-fold) by CaMKII$\beta_M$ in the presence of Ca$^{2+}$/CaM.

Because the SR membrane-associated GAPDH activation is believed to directly support ATP-coupled Ca$^{2+}$ transport and the CaMKII$\beta_M$ is specifically targeted to the SR, we sought to determine whether GAPDH would reside in the membrane in direct association with the kinase. To assess this, cytosolic and detergent-soluble SR membrane proteins were first examined for GAPDH content by Western blot analysis with anti-GAPDH antibody, and this revealed the distribution of GAPDH in both cytosolic and SR membrane fractions (Fig. 4A). These data are consistent with the previous studies on GAPDH distribution in skeletal muscle (33). Furthermore, GST pull-down assays were performed using purified GST-CaMKII$\beta_M$ and GST alone from cytosolic and detergent-solubilized SR membranes. Fig. 4B (top panel) indicates that the CaMKII$\beta_M$ fusion protein specifically binds a 36-kDa polypeptide that is recognized by the anti-GAPDH antibody from both cytosolic and SR membrane fractions (Fig. 4B, lane 3 and SR membranes) (Fig. 4B, lane 5). The GST alone did not exhibit binding of any immunoreactive polypeptide from cytosolic or SR membrane fractions (Fig. 4B, lanes 4 and 6) similar to that seen with the buffer control for the GST alone and the CaM kinase fusion protein (Fig. 4B, lanes 1 and 2). The immunoblot was stripped and stained with anti-GST antibody as a control for protein loading (Fig. 4B, bottom panel).

To further assess the ability of the muscle-specific CaMKII$\beta_M$ and the soluble CaMKII isoform (brain-specific CaMKII$\beta_B$) to interact with GAPDH, we conducted GST pull-down assays with the SR fraction. Fig. 5A (top panel), shows a Western blot analysis with anti-GAPDH antibody, which revealed that both CaMKII$\beta_M$ (Fig. 5A, lane 3) and CaMKII$\beta_B$ (Fig. 5A, lane 5) specifically bind GAPDH. GST-alone constructs did not bind GAPDH as noted above (Fig. 5A, lanes 4 and 6). Neither CaMKII$\beta_M$ nor CaMKII$\beta_B$ showed any GAPDH binding when incubated with the TBS buffer control (Fig. 5A, lanes 1 and 2). The immunoblot was stripped and stained with anti-GST antibody, which revealed identical protein loading for each lane in the assay (Fig. 5A, bottom panel). The data from these blots was quantified by densitometry (Fig. 5B) and revealed that ~7-fold higher amount of GAPDH was in complex
Effects of CaMKIIbM phosphorylation on activity of the glycolytic complex

Phosphorylation of target proteins was studied in the presence of 100 μM [Ca2+]o, (+) or 5 mM EGTA (−) and 2 μM CaM at 30 °C for 60 min (see legends of Fig. 3 also). At the end of incubation, aliquots of the reaction mixtures were applied on Whatman 3MM filter papers, washed with 5% trichloroacetic acid, and dried, and radioactivity was determined by liquid scintillation spectrometry. Data are mean ± S.D. of at least three independent experiments. The enzyme activity determination methodology of each individual enzyme is described under “Experimental Procedures.”

| Enzyme | Activity | Phosphorylation |
|--------|----------|-----------------|
|       | No CaMKIIbM | CaMKIIbM | Calcium | CaMKIIbM | Calcium |
|       | unit/mg protein | unit/mg protein | EGTA | unit/mg protein | EGTA |
| Aldolase | 0.083 ± 0.004 | 0.082 | 0.08 | 0.0031a | 0.0013 |
| PKa | 0.25 ± 0.039 | 0.265 ± 0.02 | 0.256 ± 0.04 | 0.224 ± 0.009a | 0.046 |
| GAPDH | 4.21 ± 0.22 | 14.05 ± 0.46a | 4.37 ± 0.16 | 0.96 ± 0.02a | 0.13 ± 0.03 |
| GP | 0.08 ± 0.01 | 0.1 ± 0.002 | 0.09 ± 0.01 | 0.227a | 0.008 |
| LDH | 1.17 ± 0.14 | 1.07 ± 0.029 | 0.089 ± 0.015 | 0.241 ± 0.01a | 0.049 ± 0.03 |
| CK | 0.088 ± 0.001 | 0.09 ± 0.002 | 0.087 ± 0.001 | 0.0039 | 0.004 |

* The value of α was set at p < 0.05.

PK, pyruvate kinase; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GP, glycogen phosphorylase; CK, creatine kinase.

FIG. 4. GAPDH distribution and interactions with CaMKIIbM.

A, muscle cytosol (lane 1) and SR membranes (lane 2) were isolated, and equal amounts of protein sample were resolved by SDS-PAGE followed by immunoblotting with anti-GAPDH. B, pull-down assays of GST-CaMKIIbM and GST alone with cytosolic and SR proteins were obtained as described in Fig. 2. Proteins were resolved on SDS-PAGE, transferred onto PVDF membrane, and subjected to immunoblotting with anti-GAPDH (top panel) or anti-GST antibodies (bottom panel). Lane 1, GST-CaMKIIbM in TBS buffer; lane 2, GST-GST in TBS buffer; lane 3, GST-CaMKIIbM pull-down of cytosolic proteins; lane 4, GST-GST pull-down of cytosolic proteins; lane 5, GST-CaMKIIbM pull-down of SR proteins; lane 6, GST-GST pull-down of SR proteins. The bottom panel shows the same blot stripped and immunostained with anti-GST. These data are typical of four independent experiments.

with the muscle-specific CaMKIIb3 compared with the brain isoform (Fig. 5B).

To further examine the association of endogenous CaMKIIb3 and GAPDH in skeletal muscle SR membranes, we performed immunoprecipitation assays with anti-CaMKIIβ monoclonal antibody (Chb-1) of detergent-solubilized SR extracts, which was analyzed for the presence of GAPDH. Fig. 5C (lane 1) shows that anti-CaM kinase antibody can immunoprecipitate endogenous ~73-kDa CaMKIIb3 polypeptide from SR as analyzed by Western blot by CaMKIIβ antibody. The same IP complex also contained the ~36-kDa polypeptide that was recognized by anti-GAPDH antibody (Fig. 5C, lane 2) further implying a potential interaction between these two proteins. To assess whether CaMKIIb3M can directly interact with GAPDH, we carried out pull-down assays with purified GAPDH and CaMKIIb3-M-GST and analyzed this association in Western blots with anti-GAPDH antibody (Fig. 5D, top panel). The CaMKIIb3-M-GST was able to bind directly and effectively with GAPDH (Fig. 5D, lane 1) compared with GST alone (Fig. 5D, lane 2), even when GST alone was present at about a ~10-fold higher concentration as seen by immunoblotting with anti-GST antibody (Fig. 5D, bottom panel, lanes 1 and 2).

Because CaMKIIb3M could directly bind GAPDH, we examined the kinetics of phosphorylation and activation of GAPDH by the kinase. In a phosphorylation assay of the CaMKIIb3M using purified GAPDH as a substrate, we found that increasing

FIG. 5. Molecular association of CaMKII isoforms and GAPDH.

A, a comparison of molecular interactions of muscle-specific CaMKIIbM and the ubiquitous CaMKIIb (CaMKIIbM from brain) with GAPDH. Pull-down assays of SR membrane proteins were performed with GST alone, GST-CaMKIIbM, and GST-CaMKIIbM, and Western blotting was performed with anti-GAPDH or anti-GST antibodies as described above. Lane 1, GST-CaMKIIbM in TBS buffer; lane 2, GST-CaMKIIbM in TBS buffer; lane 3, GST-CaMKIIbM pull-down of SR proteins; lane 4, GST-GST pull-down of SR proteins; lane 5, CaMKIIbM pull-down of SR membrane proteins; lane 6, GST-GST pull-down of SR membrane proteins. The top panel shows a typical Western blot with anti-GAPDH antibody, and the bottom panel shows the same blot stripped and immunostained with anti-GST antibody. B, densitometry and quantification GAPDH pull-down by the ubiquitous and muscle specific CaM kinases. C, immunoprecipitation of GAPDH with anti-CaMKIIβ antibody. Immunoprecipitates were captured by protein-A/G-agarose, resolved by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with anti-GAPDH antibody. The relative amount of GST in each lane was determined by immunostaining with anti-GST antibody. These data are typical of at least three independent experiments.
**CaM Kinase II Regulates GAPDH**

CaM Kinase II (CaMKII) is a calcium-dependent serine/threonine kinase that plays a crucial role in various cellular processes, including muscle contraction, insulin secretion, and neuronal plasticity. One of the substrates of CaMKII is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis. In this context, CaMKII has been shown to interact with GAPDH at the sarcoplasmic reticulum (SR) membrane, where it has been localized in fast-twitch skeletal muscle. This interaction has been linked to the regulation of GAPDH activity, which is important for maintaining the energy supply during muscle contraction.

**Materials and Methods**

The study involved the use of CaMKIIβ isoform, which was targeted to the SR membrane in skeletal muscle by a non-kinase protein, KAP-64. The CaMKIIβ isoform was shown to specifically associate with the enzymes of glycolysis and glycogen metabolism. The MALDI-TOF identified glycogen phosphorylase, glycogen debranching enzyme, GAPDH, and creatine kinase as interacting proteins from the SR fraction and aldolase A, GAPDH, enolase, LDH, creatine kinase, pyruvate kinase, and phosphorylase b kinase from the cytosolic fraction. Previous studies have indicated that glycogen phosphorylase resides at the SR membrane in association with glycogen and may account for its mobilization to glucose (18, 36). Our data suggest that CaMKIIβ can complex with phosphorylase b kinase at the SR, and the later enzyme has previously been established to be regulated directly by Ca²⁺/CaM binding (36, 37). Our data here also identify the glycogen debranching enzyme as a novel component at the SR, which would further assist in glycogen mobilization. Furthermore, the data indicate that CaMKIIβ can interact with GAPDH to phosphorylate and modulate its activity in a Ca²⁺/CaM-dependent manner. GAPDH was found to be distributed in muscle cytosol and the SR membrane, and ultrastructural localization studies have also revealed that GAPDH along with other glycolytic enzymes are bound to the cytoplasmic face of the SR membrane in skeletal muscle (33, 38). A role for GAPDH together with 3-phosphoglycerate kinase (PGK) has been previously established in the production of ATP from GAP, NAD⁺, P₄, and ADP at the level of the SR membrane (19, 20, 39, 40). In addition, a recent study demonstrated that synaptic vesicles were capable of generating local ATP via the membrane-associated GAPDH system, which could support the accumulation of the excitatory neurotransmitter glutamate even during significantly reduced global cellular ATP concentrations (41). It is notable that synaptic vesicles contain a CaMKIIβ isoform (42), which we found to associate with GAPDH but to a much less degree when compared with the muscle-specific isoform of the kinase. However, CaMKIIβ has been shown to interact with Ca²⁺/CaM-dependent phosphorylation does not appear to regulate the RyR or the Calcium pump of the SR in fast twitch skeletal muscle (7, 8, 15, 34, 35). A CaMKIIβ isoform referred to as a muscle-specific CaMKIIβ was shown to be targeted to the SR membrane in skeletal muscle by a non-kinase protein, α-KAP (11, 12), but the role of this kinase in SR function remains unknown. In this study, we found that CaMKIIβ specifically associates with the enzymes of glycolysis and glycogen metabolism. The MALDI-TOF identified glycogen phosphorylase, glycogen debranching enzyme, GAPDH, and creatine kinase as interacting proteins from the SR fraction and aldolase A, GAPDH, enolase, LDH, creatine kinase, pyruvate kinase, and phosphorylase b kinase from the cytosolic fraction. Previous studies have indicated that glycogen phosphorylase resides at the SR membrane in association with glycogen and may account for its mobilization to glucose (18, 36). Our data suggest that CaMKIIβ can complex with phosphorylase b kinase at the SR, and the later enzyme has previously been established to be regulated directly by Ca²⁺/CaM binding (36, 37). Our data here also identify the glycogen debranching enzyme as a novel component at the SR, which would further assist in glycogen mobilization. Furthermore, the data indicate that CaMKIIβ can interact with GAPDH to phosphorylate and modulate its activity in a Ca²⁺- and CaM-dependent manner. GAPDH was found to be distributed in muscle cytosol and the SR membrane, and ultrastructural localization studies have also revealed that GAPDH along with other glycolytic enzymes are bound to the cytoplasmic face of the SR membrane in skeletal muscle (33, 38). A role for GAPDH together with 3-phosphoglycerate kinase (PGK) has been previously established in the production of ATP from GAP, NAD⁺, P₄, and ADP at the level of the SR membrane (19, 20, 39, 40). In addition, a recent study demonstrated that synaptic vesicles were capable of generating local ATP via the membrane-associated GAPDH system, which could support the accumulation of the excitatory neurotransmitter glutamate even during significantly reduced global cellular ATP concentrations (41). It is notable that synaptic vesicles contain a CaMKIIβ isoform (42), which we found to associate with GAPDH but to a much less degree when compared with the muscle-specific isoform of the kinase. However, CaMKIIβ has been shown to interact with Ca²⁺/CaM-dependent phosphorylation does not appear to regulate the RyR or the Calcium pump of the SR in fast twitch skeletal muscle (7, 8, 15, 34, 35). A CaMKIIβ isoform referred to as a muscle-specific CaMKIIβ was shown to be targeted to the SR membrane in skeletal muscle by a non-kinase protein, α-KAP (11, 12), but the role of this kinase in SR function remains unknown. In this study, we found that CaMKIIβ specifically associates with the enzymes of glycolysis and glycogen metabolism. The MALDI-TOF identified glycogen phosphorylase, glycogen debranching enzyme, GAPDH, and creatine kinase as interacting proteins from the SR fraction and aldolase A, GAPDH, enolase, LDH, creatine kinase, pyruvate kinase, and phosphorylase b kinase from the cytosolic fraction. Previous studies have indicated that glycogen phosphorylase resides at the SR membrane in association with glycogen and may account for its mobilization to glucose (18, 36). Our data suggest that CaMKIIβ can complex with phosphorylase b kinase at the SR, and the later enzyme has previously been established to be regulated directly by Ca²⁺/CaM binding (36, 37). Our data here also identify the glycogen debranching enzyme as a novel component at the SR, which would further assist in glycogen mobilization. Furthermore, the data indicate that CaMKIIβ can interact with GAPDH to phosphorylate and modulate its activity in a Ca²⁺- and CaM-dependent manner. GAPDH was found to be distributed in muscle cytosol and the SR membrane, and ultrastructural localization studies have also revealed that GAPDH along with other glycolytic enzymes are bound to the cytoplasmic face of the SR membrane in skeletal muscle (33, 38). A role for GAPDH together with 3-phosphoglycerate kinase (PGK) has been previously established in the production of ATP from GAP, NAD⁺, P₄, and ADP at the level of the SR membrane (19, 20, 39, 40). In addition, a recent study demonstrated that synaptic vesicles were capable of generating local ATP via the membrane-associated GAPDH system, which could support the accumulation of the excitatory neurotransmitter glutamate even during significantly reduced global cellular ATP concentrations (41). It is notable that synaptic vesicles contain a CaMKIIβ isoform (42), which we found to associate with GAPDH but to a much less degree when compared with the muscle-specific isoform of the kinase. How-
activate CaMKII
branching enzyme cascade (36, 37). The Ca
bilizing and glucose-metabolizing enzymes in a complex with
NAD
activates GAPDH. Glycolytic intermediate GAP is converted to 1,3-BPG
rylase b kinase through direct Ca2
response to the calcium signal. Although, PGK and GAPDH
geting and activation of glycolytic enzymes such as GAPDH in
studies point to a previously unrecognized role for the multi-
production in response to changes in free calcium. Thus, these
cle and synaptic vesicles in neurons to regulate local ATP
–
membrane would assist in the local regulation of ATP to sup-
regulate calcium release and uptake as reported previously (21,

Thus, collectively, the findings here place the glycogen mo-
function, because changes in calcium and consequently the
regulation of SR function via increase in GAPDH activity tar-

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