Multifunctional Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaMKII) is a Ser/Thr protein kinase uniformly distributed within the sarcoplasmic reticulum (SR) of skeletal muscle. In fast twitch muscle, no specific subunits of CaMKII have yet been identified in nonjunctional SR. Previous electron microscopy data showed that glycogen particles containing glycogen synthase (GS) associate with SR at the I band level. Furthermore, recent evidence implicates CaMKII in regulation of glucose and glycogen metabolism. Here, we demonstrate that the glycogen- and protein phosphatase 1-targeting subunit, also known as G\(_M\), selectively localizes to the SR membranes of rabbit skeletal muscle and that G\(_M\) and GS co-localize at the level of the I band. We further show that G\(_M\), GS, and PP1c assemble in a structural complex that selectively localizes to nonjunctional SR and that G\(_M\) is phosphorylated by SR-bound CaMKII and dephosphorylated by PP1c. On the other hand, no evidence for a structural interaction between G\(_M\) and CaMKII was obtained. Using His-tagged G\(_M\) recombinant protein corresponding to the aa 77–118 and 219–240 (19). However, thus far, there are no studies showing that G\(_M\) associates with SR, Liu and Brautigan (19) found that transfected G\(_M\) co-immunoprecipitated with endogenous GS. The interaction between G\(_M\) and GS occurs at specific sites of G\(_M\), localized between aa 77–118 and 219–240 (19). However, thus far, there is no experimental evidence supporting a direct, structural association between G\(_M\) and GS in native SR membranes.

In skeletal muscle, a regulatory subunit called G\(_M\) (also known as G, R\(_G\), R\(_3\), and PP1R3A) (1, 2) is responsible for the targeting of glycogen and PP1c (protein phosphatase 1 catalytic subunit) to the sarcoplasmic reticulum (SR) (3–6). G\(_M\) is a striated muscle-specific protein expressed at higher levels in skeletal than in cardiac muscle (7, 8). G\(_M\) (glycogen- and PP1c-targeting subunit) belongs to a family of mammalian glycogen- and PP1c-binding proteins (PPP1R6, PTG, and GL) (9). G\(_M\) rabbit skeletal muscle cDNA sequence (7) predicts a protein of 1109 amino acids with a calculated M\(_r\) of 124,257. However, on SDS-PAGE, G\(_M\) displays an apparent M\(_r\) of ~160,000 (7, 10), likely because of its acidic pI. Binding sites for PP1c (aa 64–69; see Ref. 11) and glycogen (aa 150–159 in the case of rabbit G\(_M\); see Ref. 12) have been identified in the NH\(_2\)-terminal domain of G\(_M\). On the other hand, G\(_M\) is characterized by the unique presence, at the COOH terminus, of a stretch of hydrophobic residues (aa 1063–1097; see Ref. 7). It has been proposed that this amino acid sequence is indicative of a transmembrane helix that mediates its binding to SR membranes (7, 8). Interestingly, this transmembrane region appears to be involved in the interaction of G\(_M\) with the SERCA2-regulatory protein phospholamban (13).

A key role for G\(_M\) phosphorylation in regulation of glycogen synthase (GS) is suggested by a number of observations: (i) cAMP-dependent protein kinase (PKA) phosphorylates G\(_M\) on Ser\(^{48}\) and Ser\(^{67}\) both in vitro (3–5) and in vivo in response to adrenaline catecholamines (10, 14). Notably, both phosphorylation sites are conserved between rabbit and human G\(_M\) (7–8). Ser\(^{67}\) lies within the PP1c-binding motif of G\(_M\) (11). PKA-dependent phosphorylation triggers dissociation of PP1c from G\(_M\), thereby inactivating its phosphatase activity (4, 5). (ii) G\(_M\) is also phosphorylated by GSK3 at Ser\(^{46}\) and Ser\(^{14}\) (15). (iii) G\(_M\) is possibly phosphorylated by insulin-dependent protein kinase at Ser\(^{48}\) (14, 16).

Experiments using G\(_M\) null mice (17, 18) further support the possibility that G\(_M\) may be involved in regulation of GS activity. Recently, using COS7 cells and C\(_5\)C\(_{12}\) myotubes transfected with G\(_M\), Liu and Braunigan (19) found that transfected G\(_M\) co-immunoprecipitated with endogenous GS. The interaction between G\(_M\) and GS occurs at specific sites of G\(_M\), localized between aa 77–118 and 219–240 (19). However, thus far, there is no experimental evidence supporting a direct, structural association between G\(_M\) and GS in native SR membranes.

Here, we provide clear evidence that G\(_M\) associates with nonjunctional SR membranes, where it forms a complex with GS and PP1c. In addition, we demonstrate that G\(_M\) is phosphodephosphorylated by SR-bound CaMKII and PP1c. Using His-tagged G\(_M\) recombinant protein corresponding to the aa 40–338 sequence, we also demonstrate that endogenous, as well as exogenous CaMKII, is able to phosphorylate serine residue(s) in the NH\(_2\)-terminal region of G\(_M\). The finding that CaMKII is unable to phosphorylate both a truncated G\(_M\) (aa 69–338) lacking Ser\(^{48}\) and Ser\(^{67}\) and a point-mutated form in which Ser\(^{48}\) is replaced by Ala identifies this residue as a...
CaMKII target in G\textsubscript{M}. We thus suggest that Ca\textsuperscript{2+}--calmodulin activation of SR-bound CaMKII might play a pivotal role in the regulation of glycolysis metabolism during physical exercise through phosphorylation of G\textsubscript{M}.

**EXPERIMENTAL PROCEDURES**

**Materials**—Molecular mass standards were purchased from BDH Laboratories (Poole, UK; molecular weight range 200,000–43,000 or 77,000–12,000). (\textsuperscript{3}H or \textsuperscript{14}C)-labeled compounds were purchased from PerkinElmer Life Sciences. Exogenous Ca\textsuperscript{2+}--calmodulin-dependent protein kinase (500,000 units/ml) was purchased from New England Biolabs. Uridine-diphospho-\textsuperscript{5}-3\textsuperscript{H}glucose, NH\textsubscript{4} salt (14.1 Ci/mmol) was purchased from Amersham Biosciences. Hog brain calmodulin was purchased from Roche Applied Science. CaMKII inhibitor KN-93 was purchased from Calbiochem (San Diego, CA). Protein kinase A from bovine heart (1–2 units/\mu g of protein kinase), \textit{E. coli} propanol, protein A-Sepharose, and glucose assay kit were purchased from Sigma-Aldrich. Okadaic acid was purchased from Calbiochem-Novabiochem (Bad Soden, Germany). All other chemicals were analytical grade and were purchased from Sigma-Aldrich.

**Animals and Muscles**—New Zealand male adult rabbits were lawfully obtained from a local supplier and housed, fed, and taken care of in accordance with the Annual Colony of the Department of Experimental Biomedical Sciences of the University of Padova in compliance with Italian Law (Decreto Legge, September 27, 1992, no. 116). The adductor muscle was used as a representative fast twitch muscle (20).

**Generation and Purification of His\textsubscript{6}-GM Fusion Proteins**—Total cellular RNA extraction from rabbit adductor and cDNA synthesis were performed (as described in (21)). G\textsubscript{M} cDNA was amplified by RT-PCR to generate two forms of G\textsubscript{M}, G\textsubscript{M} (specific radioactivity, 0.10 Ci/mmol) and G\textsubscript{M} (specific radioactivity, 0.10 Ci/mmol) were used for site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

**EXPERIMENTAL PROCEDURES**

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Phosphodephosphorylation of $G_M$ Protein in Rabbit SR

**RESULTS**

**Localization of $G_M$ to SR**—When longitudinal cryosections from rabbit adductor muscle were probed with a guinea pig anti-$G_M$ antibody, a cross-striated pattern was observed (Fig. 1A). By comparison with phase contrast microscopy, the fluorescent striations were found to correspond to the I band of the sarcomere. Fig. 1B shows that $G_M$ co-localized with GS, which also is resident in the I band (35). The overlapping between the two proteins was virtually complete. It was verified that non-specific antibody or secondary alone did not give this fluorescence pattern (not shown).

The subcellular localization of $G_M$ was further investigated by differential centrifugation of muscle homogenates. The immunoblot analysis shown in Fig. 1C clearly shows that most $G_M$ sedimented mostly in the microsomal fraction, which was devoid of contamination by myofibrillar proteins and highly enriched in SR membranes, as demonstrated by the localization of the SR-specific protein markers, RyR1 and GP$_{53}$. It is noteworthy that $G_M$ and GS co-fractionated throughout the entire preparation.

SDS-solubilizing buffer to samples. $^{33}$P-Labeled protein were detected by autoradiography (Hyperfilm Amersham) or by a Bio-Rad model GS-250 Molecular Imager. Bound radioactivity was quantified by using $^{33}$P-sensitive screen and a model GS-250 Molecular Imager (Bio-Rad). One unit of exogenous CaMKII and PKA is defined as the amount of enzyme that will transfer 1 pmol of phosphate to specific substrate in 1 min at 30 °C.

**Protein Dephosphorylation**—Isolated SR membranes were incubated at 30 °C with 50 μM $[^{33}$P]ATP in assay medium for phosphorylation by endogenous CaMKII. After 30 min of incubation, CaMKII was inhibited by 2 μM staurosporin. As reported in detail in the legends to Fig. 5, protein dephosphorylation by endogenous PP1c was carried out in the same medium for 30 min at 30 °C.

**Phosphoamino acid Analysis**—$G_M$, $^{33}$P-phosphorylated by exogenous CaMKII as described above, was blotted to nitrocellulose membrane, localized by autoradiography, and digested by trypsin. The tryptic peptides were subjected to acidic hydrolysis (6 M HCl for 4 h at 110 °C), and the radiolabeled phosphoamino acids were separated by high voltage paper electrophoresis at pH 1.9, as described by Perich et al. (31).

**Preparation of Antiserum against $G_M$ Fusion Proteins**—Chicken polyclonal antibodies against the purified His-tagged $G_M$(40–338) fusion protein were raised, as previously reported by Damiani et al. (32). Guinea pig polyclonal antibodies were raised against a 11.2-kDa peptide corresponding to an 754–852 of rabbit $G_M$(7). The immunogen was expressed and purified by on-column purification through an IMAC column and was then used to raise the antiserum (Harlan Sera-Lab Ltd., Bicester Oxon, UK).

**Immunoprecipitation**—Immunoprecipitation experiments were carried out as described (21). 250 μg of F4 protein, after solubilization, was incubated for 2 h in cold room with the guinea pig anti-$G_M$ antibody to (1:1000 dilution). Protein A-Sepharose (40 μl) was added to the mixture, incubated for 1 h, and sedimented using an Eppendorf centrifuge. After washing, the pellets were solubilized with SDS solubilization buffer.

**Gel Electrophoresis and Immunoblotting**—SDS-PAGE (33) and immunoblotting were carried out, as described (21). Slab gels were stained with Coomassie Blue and then with Stains All. Apparent Mr values were calculated from a graph of relative mobilities versus log Mr of standard proteins. The blots were probed with: (i) mouse monoclonal antibodies to α-actinin (Sigma), RyR1 (sarcoplasmic reticulum Ca$^{2+}$ release channel, skeletal isoform; BioMol, Plymouth Meeting, PA), GP$_{53}$ (a SR glycoprotein of 53 kDa) (ABR, Golden, CO), GS (Chemicon International, Temecula, CA), and PP1c (Santa Cruz Biotechnology, CA); (ii) guinea pig polyclonal antibodies to $G_M$; or (iii) chicken polyclonal antibodies to $G_M$. Antibody binding was detected by immunoenzymic staining (21). Densimetry of blotted proteins, after immunostaining, was carried out using a Bio-Rad model GS-670 imaging densitometer. GS content was determined by densitometry of proteins immunostained with mouse monoclonal antibody GS, with reference to a calibration curve of purified GS. $G_M$ content was determined by densitometry of blots immunostained with chicken polyclonal antibodies to $G_M$, with reference to a calibration curve obtained with purified $G_M$(40–338).

**Immunofluorescence Microscopy**—Muscle cryosections were fixed with parafomaldehyde and immunostained by incubating with primary antibodies to $G_M$ and GS (dilution 1:100), followed by incubation with the appropriate secondary antibody conjugated with TRITC or fluorescein isothiocyanate (Dako), as described by Saccheto et al. (34). The images were captured by a B/W chilled CCD camera (Hamamatsu, Japan), transmitted to an interactive image analysis system equipped with image memory (High Fish Beta, version 2.0) and image processing software (Image Processing, version 3.4, Casti Imaging, Venice, Italy). The images were printed on a CP-D1E printer (Mitsubishi, Japan).

**Localization of $G_M$ to I band in rabbit skeletal muscle and co-enrichment of $G_M$ and GS in SR membranes. A**, comparison between immunofluorescence (IF) and phase contrast (PC) images of rabbit adductor fibers labeled with guinea pig anti-$G_M$ antibody. **Bar**, 10 μm. **B**, comparison of immunofluorescence localization of $G_M$ and GS. Longitudinal cryosections were double-labeled with antibodies to $G_M$ (red fluorescence) and to GS (green fluorescence), as indicated. Simultaneous visualization by image analysis (see “Experimental Procedures”) of the two fluorochromes (merge) shows an extensive overlapping (yellow signal). **Bar**, 10 μm. **C**, muscle homogenates were subfractionated by differential centrifugation, as described under “Experimental Procedures,” and aliquots of the different fractions subjected to 5–10% SDS-PAGE, followed by Western blot analysis with the organelle-specific antibody RyR1 and GP$_{53}$. Protein loading was 50 μg/lane. Lane 1, total homogenate; lane 2, post-myofibrillar supernatant obtained at 6,600 g; lane 3, post-mitochondrial supernatant obtained at 15,000 × g; lane 4, post-microsomal supernatant obtained at 150,000 × g; lane 5, microsomes.
A fraction enriched in nonjunctional SR membranes was obtained from muscle microsomes by isopycnic sucrose density centrifugation (22). This procedure, at variance with that of Saito et al. (23), which yields a much higher proportion of membranes deriving from the terminal cisternae of junctional SR, yields mainly vesicles derived from nonjunctional SR (22).

The nonjunctional SR origin of this fraction was confirmed by \([^3H]ryanodine binding measurements, carried out at optimal conditions of free Ca\([\text{II}]\) (100 \(\mu\text{M}\)) and ionic strength (1 x KCl). The specific SR illustrated in Fig. 2A was isolated from this fraction (Fig. 2B). Based on this evidence, henceforth all experiments were carried out using the F4 fraction.

**Fig. 2.** \(G_M\) associates to the Ca\([\text{II}]\) pump membrane of nonjunctional SR. A, 5–10% SDS-PAGE analysis, followed by Stains All staining, of nonjunctional SR fraction purified from total microsomes by isopycnic sucrose density centrifugation (see “Experimental Procedures”). Protein loading was 50 \(\mu\text{g/lane}.\) The positions of specific proteins are indicated on the right. The positions of molecular mass markers are indicated on the left. B, immunoblot analysis of F4 membranes for content of \(G_M\), GS, and PP1c. The blots were incubated with the indicated specific antibody. Protein loading was 30 \(\mu\text{g/lane}.\) C, D, and F4 membranes were treated with 0.25% detergent Chaps and centrifuged to separate Ca\([\text{II}]\) pump membrane from JFM vesicles. The samples were analyzed by 5–10% SDS-PAGE. The proteins were either stained with Coomassie Blue (C) or blotted onto nitrocellulose (D). The blots were incubated with the indicated antibodies. Protein loading was 30 \(\mu\text{g/lane}.\) Lanes 1, F4, starting material; lanes 2, Ca\([\text{II}]\) pump membrane; lanes 3, JFM.

**Fig. 3.** \(G_M\) co-immunoprecipitates with GS and PP1c from SR membranes. A, F4 membranes were solubilized and immunoprecipitated (IP) with guinea pig anti-\(G_M\) antibody, as described under “Experimental Procedures.” The immunocomplexes were probed with antibodies to GS. Negative controls were incubated without antibody or with monoclonal antibody to \(\alpha\)-actinin as indicated. Only the molecular weight region between 200,000 and 116,000 is shown. B, solubilized F4 membranes were immunoprecipitated with guinea pig polyclonal anti-GS antibody, as in A. Blots of immunoprecipitates were probed with the indicated specific antibodies.

Co-immunoprecipitation of \(G_M\), GS, and PP1c—F4 membranes were solubilized and immunoprecipitated with a guinea pig polyclonal antibody to \(G_M\) protein. Fig. 3A shows that a prominent \(G_M\) band was specifically immunoprecipitated by this antibody. \(G_M\) protein neither was bound to protein A in the absence of antibody nor was immunoprecipitated by a nonrelated anti-\(\alpha\)-actinin antibody (Fig. 3A). Fig. 3B shows that \(G_M\) co-immunoprecipitated not only with PP1c, as expected (36), but also with GS. The specificity of this result was validated as in Fig. 3A (data not shown).

**Fig. 3.** Phosphorylation-Dephosphorylation of \(G_M\) by SR-bound CaMKII and PP1c—\(G_M\) is phosphorylated by PKA at Ser\(^{46}\) and Ser\(^{67}\) (Refs. 4 and 5; see also Ref. 7). Based on the consensus sequence (37), another potential PKA target site of \(G_M\) is Thr\(^{215}\). The analysis of these potential phosphorylation sites reveals that the amino acid sequence (RXX(S/T)) also corresponds to a consensus sequence for CaMKII (37, 38). Because rabbit SR membranes contain CaMKII (see Ref. 40 for a review), we tested whether \(G_M\) is phosphorylated by SR-bound CaMKII.

To address this point, F4 membranes were incubated with [\(\gamma\)^3P]ATP in the presence of Ca\([\text{II}]\)-calmodulin in the assay.
Phosphodephosphorylation of G\textsubscript{M} Protein in Rabbit SR

**Fig. 4.** G\textsubscript{M} is phosphorylated by endogenous CaMKII. A, F4 vesicles were phosphorylated for 30 min at 30 °C with 50 μM [γ-\textsuperscript{32}P]ATP in the presence of 30 μM Ca\textsuperscript{2+} and 0.2 μM calmodulin without or with 100 μM KN-93. \textsuperscript{32}P-Labeled proteins were resolved by 5–12.5% SDS-PAGE and either identified by autoradiography of dried gels (lanes 1 and 2), or blotted. The blots were then immunostained with antibodies to G\textsubscript{M} (lane 3). Protein loading was 10 μg/lane. The expected mobilities of triadin, 60-kDa CaMKII, and glyceraldehyde-3-phosphate dehydrogenase are indicated. An arrow indicates the 84-kDa phosphoprotein identified as GS. B, F4 proteins phosphorylated 30 min at 30 °C with 50 μM [γ-\textsuperscript{32}P]ATP in the presence of 100 μM Ca\textsuperscript{2+} and 0.2 μM calmodulin were solubilized and immunoprecipitated with the guinea pig anti-G\textsubscript{M} antibody. The immunocomplexes were resolved by 5–12.5% SDS-PAGE and blotted onto nitrocellulose. The blots, after autoradiography (AutoRx) (lane 1), were immunostained with antibody to G\textsubscript{M} (lane 2). C, F4 vesicles were phosphorylated for 30 min at 30 °C with 50 μM [γ-\textsuperscript{32}P]ATP by exogenous PKA and processed as described for B. Lane 1, autoradiography (AutoRx); lane 2, immunoblot (IB) after autoradiography with anti-G\textsubscript{M} antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

medium. As we have already reported (39, 24, 21), the incorporation of radiolabeled phosphate into SR proteins is poor, if it occurs at all, in the absence of exogenously added Ca\textsuperscript{2+}-calmodulin. On the contrary, in the presence of these effectors several proteins were phosphorylated, including a 60-kDa protein corresponding to auto-phosphorylated CaMKII (21, 40). Additional SR proteins phosphorylated by CaMKII were 95-kDa triadin (39) and 36-kDa glyceraldehyde-3-phosphate dehydrogenase (41) (Fig. 4A, lane 1). As already reported (39), a prominent \textsuperscript{32}P-labeled protein was also detected at about 85 kDa. This phosphoprotein proved to be GS, based on Western blot analysis with anti-GS antibody. The addition of 100 μM KN-93, a specific inhibitor of CaMKII that competes for calmodulin binding to the enzyme (42), almost completely abolished the phosphorylation of SR proteins (Fig. 4A, lane 2) (see also Ref. 21). Taken together, these results strongly support the notion that the Ca\textsuperscript{2+}-calmodulin-dependent phosphorylation of SR proteins is mediated by endogenous CaMKII.

Fig. 4A shows that \textsuperscript{32}P-labeled protein bands were also detectable in the M\textsubscript{r} range of 160,000–170,000, including a protein that displayed the same mobility of G\textsubscript{M} as judged by Western blot analysis (Fig. 4A, lane 3). The low extent of the protein phosphorylation is consistent with the low content of G\textsubscript{M} in F4 membranes (about 0.5 μg/mg of SR protein; see “Experimental Procedures”). Because another SR protein, called HRC, with the apparent M\textsubscript{r} of 170,000 has been described to be phosphorylated by endogenous CaMKII (39, 40) to confirm the phosphorylation of G\textsubscript{M}, \textsuperscript{32}P-labeled SR proteins were solubilized and immunoprecipitated with the guinea pig anti-G\textsubscript{M} antibody. Fig. 4B shows that the \textsuperscript{32}P-labeled protein of about 160 kDa was indeed G\textsubscript{M}. As a positive control, we performed parallel experiments in which F4 membranes were phosphorylated with exogenous PKA. Also in this case, the anti-G\textsubscript{M} antibody immunoprecipitated a 160-kDa \textsuperscript{32}P-labeled protein that was immunostained with anti-G\textsubscript{M} antibody (Fig. 4C).

To investigate whether G\textsubscript{M} could be dephosphorylated by endogenous PP1c, F4 proteins were first \textsuperscript{32}P-phosphorylated in the presence of Ca\textsuperscript{2+} and calmodulin (Fig. 5, lane 1). After 30 min of incubation, endogenous CaMKII was inhibited by adding 2 μM staurosporin to the assay medium. In this particular case, we ought to use staurosporin instead of KN-93, because, once autophosphorylated, CaMKII remains active even in the absence of Ca\textsuperscript{2+}-calmodulin (autonomous form). The membranes were then solubilized and immunoprecipitated with anti-G\textsubscript{M} antibody. Fig. 5 clearly demonstrates that radiolabeled G\textsubscript{M} was completely dephosphorylated by endogenous PP1c (lanes 1 and 2). Indeed, the level of G\textsubscript{M} phosphorylation remained constant when the PP1c inhibitor okadaic acid was also present in the incubation medium (Fig. 5, lane 3).

Identification of CaMKII Phosphorylation Sites of G\textsubscript{M}—All of the phosphorylation sites thus far identified in G\textsubscript{M} are clustered within 28 residues (Ser\textsuperscript{40} to Ser\textsuperscript{67}) located at the NH\textsubscript{2} terminus of the molecule. Therefore, we investigated the possibility that CaMKII might phosphorylate G\textsubscript{M} within this phosphoregulatory region. A His-tagged recombinant protein corresponding to aa 40–338 of G\textsubscript{M} (G\textsubscript{M}\textsubscript{40–338}) was expressed in E. coli and purified according to the manufacturer’s instructions. Fig. 6A shows that, when added to SR fraction, G\textsubscript{M}\textsubscript{40–338} was phosphorylated by endogenous CaMKII (lane 5) and that this phosphorylation was almost completely abolished by KN-93 (lane 6). Fig. 6B shows that the G\textsubscript{M}\textsubscript{40–338} fusion protein was also phosphorylated by exogenous, commercially available CaMKII. No phosphorylation of G\textsubscript{M}\textsubscript{40–338} fusion protein occurred in the absence of activated CaMKII (not shown).

Based on the known consensus sequence for CaMKII (37, 38), there are six potential Ser or Thr phosphoacceptor sites in G\textsubscript{M}\textsubscript{40–338} fusion protein: Ser\textsuperscript{48}, Ser\textsuperscript{67}, Ser\textsuperscript{205}, Thr\textsuperscript{215}, Ser\textsuperscript{263}, and Ser\textsuperscript{267}. The analysis of the phosphorylated residues obtained from G\textsubscript{M}\textsubscript{40–338} \textsuperscript{32}P-phosphorylated by the commer-

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\textsuperscript{2} R. Sacchetto, L. Salviati, E. Damiani, and A. Margreth, unpublished data.
obtained after PKA phosphorylation of SR proteins (Fig. 8). Pig anti-GM antibody. Immunoprecipitated GM was detected by autoradiography of blots. The blots were then immunostained with the guinea pig anti-GM antibody. Only the molecular weight region between 200,000 and 116,000 is shown.

Fig. 5. SR-bound PP1c dephosphorylates G_M. F4 proteins were phosphorylated for 30 min at 30 °C with [γ-32P]ATP by endogenous CaMKII, as described in the legend to Fig. 4A. CaMKII activity was then inhibited by adding 2 μM staurosporin to the medium (lane 1), or otherwise the incubation was prolonged for 30 min at 30 °C in the absence (lane 2) or presence (lane 3) of 2 μM okadaic acid. The membranes were then solubilized and immunoprecipitated with the guinea pig anti-GM antibody. Immunoprecipitated G_M was detected by autoradiography of the blots. The blots were then immunostained with the guinea pig anti-GM antibody. Only the molecular weight region between 200,000 and 116,000 is shown.

Firstly, exogenous CaMKII showed that only serine residue(s) were affected by the kinase (results not shown).

Further experiments were carried out to identify the target residues of CaMKII. To this purpose, an additional His-tagged G_M fusion protein lacking aa 40–68 (G_M(69–338)) was generated. Fig. 7A shows that the phosphorylability of G_M(69–338) by both CaMKII and PKA was completely lost upon deletion of the sequence containing Ser48 and Ser67 (lanes 2 and 3). These results suggest that the serine residue(s) phosphorylated by CaMKII are located in the 40–68-amino acid sequence. It is noteworthy that PKA phosphorylation of G_M(40–338), which reached a stoichiometry of 1 mol/mol protein, induced a substantial shift of the Coomassie Blue band toward higher molecular mass (Fig. 7A, lane 1), whereas CaMKII phosphorylation did not affect the protein mobility (lane 3).

To discriminate between the two potential CaMKII phosphorylation sites, a recombinant G_M(40–338) protein was generated in which Ser48 was mutated to alanine (G_M(40–338)S48A). As expected, when G_M(40–338)S48A was incubated with PKA, a substantial decrease of 32P incorporation into the protein was observed, as compared with wild-type G_M(40–338) (Fig. 7B, lanes 3 and 4). The inability of CaMKII to phosphorylate the point-mutated G_M(40–338)S48A (Fig. 7B, lanes 5 and 6) demonstrated that the only target residue affected by CaMKII is Ser48.

Phosphorylation of Ser48 by PKA does not dissociate PP1c from G_M. Therefore, we compared the effect of CaMKII and PKA phosphorylation on the interaction of PP1c with SR membranes. To maximize the phosphorylation by CaMKII, SR membranes were incubated in a medium containing not only Ca2+ and calmodulin but also exogenous CaMKII. Under these experimental conditions and as determined by densitometry of radiolabeled proteins, we observed that the extent of phosphorylation by CaMKII of G_M protein was comparable with that obtained after PKA phosphorylation of SR proteins (Fig. 8A).

As a further indication of the increased efficiency of CaMKII phosphorylation under these experimental conditions, we found that the amount of radioactivity incorporated into GS was more than 2-fold higher (22,050 arbitrary counts) than that observed for PKA-dependent phosphorylation (9859 arbitrary counts). When the association of PP1c to G_M was monitored by Western blot analysis, a substantial amount of PP1c was released from SR membranes into the supernatant, following phosphorylation by PKA (Fig. 8, lanes 3 and 4). On the contrary, CaMKII-mediated phosphorylation of G_M only negligibly affected the association of PP1c to SR (lanes 5 and 6). It was verified by Western blot analysis that association of GS to G_M was unaffected under either condition (not shown).

DISCUSSION

In skeletal muscle CaMKII is targeted to SR by a specific anchoring protein, αCaMKII association protein (43). Even though both the αCaMKII association protein and CaMKII are uniformly distributed within junctional and nonjunctional SR (40), the function of nonjunctional SR CaMKII is still unknown in fast twitch muscle.

Previous electron microscopy studies in resting skeletal muscle (44) showed that glycogen particles associate to SR at the level of the I band, where the highest density of nonjunctional SR tubules is observed. This association is preserved under conditions of sucrose density centrifugation (45), likely because of the presence of the glycogen- and PP1c-targeting subunit G_M. It is also well established that glycogen granules contain enzymes involved in their metabolism, such as GS and glycogen phosphorylase (46). Recent studies implicated CaMKII in regulation of glucose metabolism in skeletal muscle (41, 47). This involvement seems reasonable, because during physical exercise cytosolic [Ca2+] increases, thereby activating Ca2+/calmodulin-dependent enzymes. As a matter of fact, activity of CaMKII is increased by exercise in human skeletal muscle (48).

Based on immunofluorescence and subfractionation studies, here we demonstrated that G_M, one of the key regulatory components of glycogen metabolism, selectively localizes to SR. Furthermore, we show that G_M is a component of the highly specialized Ca2+-pump membrane forming nonjunctional tubules of SR.

Our subcellular localization studies do not support the previous report by Walker et al. (14) suggesting the presence of two pools of G_M in skeletal muscle, one cytosolic and one bound to the SR. Our experiments demonstrate unequivocally that G_M previously detected in the low speed, post-mitochondrial supernatant was due to the contamination by unsedimented SR vesicles. Based on this evidence, we conclude that in skeletal muscle only one form of G_M is present that localizes to the nonjunctional SR.

Our work also shows that a structural complex containing G_M, GS, and PP1c exists in native SR membranes. Using COS7 cells or C2C12 myotubes transiently expressing G_M, Liu and Brautigan (19) reported that transfected G_M immunoprecipitated with endogenous GS. However, our data provide the first direct experimental evidence for the presence of this complex in native nonjunctional SR membranes isolated from fast twitch skeletal muscle.

The main goal of the present study was to investigate whether nonjunctional SR-bound CaMKII might phosphorylate key proteins of glycogen metabolism. We have shown that endogenous CaMKII phosphorylates G_M and that phospho-G_M is dephosphorylated by endogenous PP1c. By using G_M recombinant fragments and site-directed mutagenesis, we demonstrate that CaMKII phosphorylated Ser48 in the NH2 terminus of G_M. This finding is consistent with previous observations (10, 15) reporting high levels of Ser48 phosphorylation in vivo, as a consequence of phosphorylation by muscle contraction-activated protein kinase(s) (14). This serine residue is phosphorylated by PKA in vivo (14) and in vitro (4), as well as, possibly, by an insulin-dependent protein kinase (14, 19, 49). When G_M is phosphorylated at Ser48, PP1c activity toward GS and phosphorylase kinase is much higher than that of unphosphoryl-
Our results support the concept that GM, like GS, is a multisite phosphorylated protein and that the domain of the protein critical for regulation by reversible phosphorylation is the region near the amino terminus, termed the phosphoregulatory domain (49). In this study, we considered the possibility that GM might directly interact with CaMKII, via the transmembrane domain of GM, on the analogy of what was observed for the slow twitch muscle integral SR membrane protein, phospholamban (13). However, co-immunoprecipitation experiments failed to provide evidence supporting a direct protein-protein interaction between CaMKII and the heteromeric GM, GS, and PP1c protein complex (not shown).

The physiological significance of the CaMKII-mediated phosphorylation of GM Ser48 is complex. Because CaMKII is activated during exercise, a role for CaMKII in inhibition of GS should be conceivable. However, it is also possible that phosphorylation of Ser48 of GM by CaMKII plays a role during the recovery period. On this respect, it is interesting to remember the unique regulatory features of CaMKII, which, once autophosphorylated, becomes independent on regulation by Ca2+/calmodulin. Therefore the signal conveyed by this autonomous form of CaMKII might well outlast that of adrenergic
stimulation. CaMKII-dependent phosphorylation of Ser^{48} might provide a potential mechanism for reactivating glycogen resynthesis, by increasing the rate of dephosphorylation of GS during the recovery period after exercise, when adrenergic stimulus is terminated.

Ser^{48} phosphorylation might also provide a mechanism for regulation of autophosphorylated Ca^{2+}-calmodulin-independent CaMKII. In fact, we observed that, in addition to G_m and GS, PP1c also dephosphorylates CaMKII (not shown). As a matter of fact, this finding identifies PP1c as the major CaMKII phosphatase thus far identified in skeletal muscle SR. This observation is even more striking, because PP1c activity seems to be specific for glycogen-associated substrates (49), such as GS, glycogen phosphorylase, and G_m, as also shown here. Therefore, activation of PP1c secondary to CaMKII-dependent phosphorylation of G_m Ser^{48} might result in dephosphorylation of autonomous CaMKII and hence termination of CaMKII signal.

It is well known that direct phosphorylation of GS by several protein kinases, such as PKA, CK1, or CK2, poorly inhibits GS activity (50, 51). On this respect, the most relevant kinase is GSK3 (50, 51). Like PKA, CK1, and CK2, SR-bound CaMKII also phosphorylates GS. In preliminary experiments we found that the inhibition of GS activity by this phosphorylation was minimal (not shown). It is also known that GS activity can be regulated by the synergic action of two protein kinases. This is the case of phosphorylase kinase and CK1 (52), PKA and CK1 (53), and CK2 and GSK3 (50, 51). Therefore, the possibility exists that CaMKII and PKA may act synergistically in the regulation of GS activity in a complex fashion also depending on the intensity and duration of exercise (54). It is well known that levels of circulating catecholamines are directly related to the intensity of the exercise.

Finally, the finding that CaMKII phosphorylates G_m at site 1 suggests a synergy with GSK3 because it is well known that GSK3 can phosphorylate G_m at Ser^{40} and Ser^{44} only following the primary phosphorylation of Ser^{48}. Thus far, this was demonstrated only for PKA (15). Our results provide that CaMKII might substitute for PKA in this priming role for GSK3. It should be remembered that the functional role of GSK3 phosphorylation of G_m is still undefined.

In summary, our data indicate the existence of a functional relationship between SR-bound CaMKII and the heteromeric protein complex formed by G_m, GS, and PP1c. This interaction takes place in the Ca^{2+} pump membrane of nonfunctional SR. The finding that Ser^{48} of G_m is phosphorylated by CaMKII supports the hypothesis that SR-bound CaMKII is involved in the modulation of glycogen metabolism in skeletal muscle during exercise.

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