THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 9, Issue of March 25, pp. 5258-5266, 1990
Printed in U.S.A.

Muscle-type MM Creatine Kinase Is Specifically Bound to Sarcoplasmic Reticulum and Can Support Ca^{2+} Uptake and Regulate Local ATP/ADP Ratios*

(Received for publication, September 5, 1989)

Anna Maria Rossi‡§¶, Hans M. Eppenberger‡, Pompeo Volpe‖, Roberto Cotrufo§, and Theo Wallimann‡**

From the ‡Institute for Cell Biology, ETH-Hönggerberg, Zürich CH-8093, Switzerland, the §Second Division of Neurology and Chair of Neuropathology and Psychopathology, First School of Medicine, University of Naples, Italy, and the ‖Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

Highly purified fractions of sarcoplasmic reticulum (SR) were prepared from chicken pectoralis muscles (Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol.* 99, 875–885) and analyzed for the presence of creatine kinase (CK). Vesicles derived from longitudinal SR contained 0.703 ± 0.428 IU of CK/mg of (SR) protein.

Immunogold localization of muscle-type MM-CK on ultrathin cryosections of muscle, after removal of soluble CK, revealed relatively strong *in situ* labeling of M-CK remaining bound to the M band as well as to the SR membranes. In addition, purified SR vesicles were also labeled by anti-M-CK antibodies, and the peripheral labeling was similar to that observed with anti-Ca^{2+}-ATPase antibodies.

Only some particulate CK enzyme was released from isolated SR membranes by EDTA/low salt buffer, and CK was resistant to extraction by 0.6 M KCl. Thus, some of the MM-CK present in muscle displays strong associative behavior to the SR membranes.

The SR-bound CK was sufficient to support, in the presence of phosphocreatine plus ADP, a significant portion of the maximal *in vitro* Ca^{2+} uptake rate. The ATP regeneration potential of SR-bound CK was similar to the rate of Ca^{2+}-stimulated ATP hydrolysis of isolated SR vesicles. Thus, CK bound to SR may be physiologically relevant *in vivo* for regeneration of ATP used by the Ca^{2+}-ATPase, as well as for regulation of local ATP/ADP ratios in the proximity of the Ca^{2+} pump and of other ATP-requiring reactions in the excitation-contraction coupling pathway.

The SR[1] of striated muscle, as a specialized form of endoplasmic reticulum (1), plays a key role in the contraction-relaxation cycle by controlling the myoplasmic free Ca^{2+} concentration. It is composed of two continuous yet heterogeneous compartments, namely the longitudinal sarcoplasmic reticulum (LSR) surrounding the myofibrils, and the terminal cisternae (TC), junctionally associated with the transverse T tubule membrane by bridging "feet" structures (2).

The major protein component of skeletal muscle SR is the Ca^{2+}/Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase, EC 3.6.1.38). This transmembrane enzyme, which plays a crucial role in Ca^{2+} homeostasis, is responsible for the ATP-dependent Ca^{2+} uptake into the lumen of the SR, enabling muscle to relax (3). The Ca^{2+} pump, requiring a considerable amount of ATP for muscle relaxation, is uniformly distributed within the SR membranes except for the junctional face membrane of the TC, which is essentially free of the enzyme (2, 4).

In muscle it has been shown that small amounts of total CK are bound in an isoenzyme-specific fashion to subcellular structures such as the inner mitochondrial membrane (5, 6), the M band of myofibrils (7–9), and the plasma membrane (10, 11). These observations have lent support to the "CP shuttle" (6, 12–14) or "phosphocreatine circuit" models (48), which suggest that communication and transfer of energy between ATP-generating (mitochondria and cytosolic glycolysis) and ATP-utilizing sites (myofibrillar actin-activated Mg^{2+}-ATPase, (Na^+/K^+)-ATPase) are facilitated via the CP/CK system.

Since in muscle a significant amount of energy is spent for Ca^{2+} sequestration by the SR Ca^{2+}-ATPase, and the local ATP/ADP ratio may be a critical regulatory parameter for the Ca^{2+} pump, one might predict from the recent phosphocreatine circuit model (48) that CK forms a functionally coupled compartment with the Ca^{2+}-ATPase at the SR, as was suggested earlier (15, 16; see below), and thus would be an *in situ* ATP regenerator in supporting Ca^{2+} uptake and an *in situ* regulator of local ATP/ADP ratios.

The present work describes experimental evidence for the presence of creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) bound to highly purified SR fractions and for a direct functional coupling of some of the SR-associated CK with the Ca^{2+} pump. The presence of CK attached to SR has been suggested by Baskin and Deamer in 1970 (15), and some experimental evidence for such an association was provided by Levitsky *et al.* in 1978 (16). However, since the bulk of CK in muscle is soluble, it was important to exclude adventitious binding of the enzyme to SR membranes and to characterize the specificity of CK binding to the SR with highly purified SR vesicles exposed to various ionic condi-

* This work was supported by Swiss National Foundation Grant 3.376.086 and by the grant from the Swiss Foundation for Muscle Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Parts of this work have been presented at the Conference of the European Society for Muscle and Cell Motility (47).

¶ Present address: Dept. of Pharmacology Second School of Medicine, University of Naples, Via S. Pansini 5, Naples 80131, Italy.

** To whom correspondence should be sent.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; LSR, longitudinal tubules of SR; TC, terminal cisternae; R1, LSR with some transverse tubules and plasma membrane; R2, LSR; R3, LSR with some TC; R4, TC with some LSR; Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-dependent ATPase; CK, creatine kinase; M- and B-CK refer to the subunits of the dimeric muscle-type MM-CK and brain-type BB-CK isoforms, respectively; Mi-CK refers to the general term of mitochondrial CK; CP, phosphocreatine; DNFB, dinitrofluorobenzene.

tions. Furthermore, in order to test a possible direct involvement of CK in the ATP-dependent Ca^{2+} sequestration by SR vesicles, Ca^{2+} uptake experiments had to be done with homogeneous well characterized SR fractions in the presence of either ATP or CP plus ADP.

In this study we present evidence that MM-CK is specifically bound to the SR membrane *in situ* and on isolated SR vesicles and that some of the bound enzyme does indeed support Ca^{2+} pumping by locally regenerating ATP in the proximity of the Ca^{2+} pump. Thus, our results indicate a direct functional coupling of CK with the Ca^{2+}-ATPase, both as an *in situ* regenerator of ATP and as a regulator of local ATP/ADP ratios.

EXPERIMENTAL PROCEDURES

Isolation of SR Fractions—SR was isolated from breast muscle (white and mixed portion of the pectoralis major) excised from adult chickens immediately after killing. SR was fractionated into LSR (fraction R2) and TC (fraction R4) by isopycnic gradient centrifugation, as described by Saito *et al.* (17). The purification was done in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (2 mg/liter) and 2 mM 2-mercaptoethanol. By such a procedure the SR vesicles nicely separate from the myofibrils. As far as rabbit muscle is concerned, the fractions R1, R2, R3, and R4 obtained by this method contained LSR with some transverse tubules and plasma membrane, LSR, LSR with some TC contaminants, and TC with some LSR, respectively (17).

SR fractions from chicken muscle were resuspended in 0.3 M sucrose, 5 mM imidazole, 2 mM 2-mercaptoethanol, pH 7.4 (buffer A). In some cases, SR fractions were extracted with 50–100 volumes of either 0.6 M KCl in buffer A for 1 h (referred to as high salt extraction) or 10 mM Tris, 1 mM EDTA, pH 8.0, for 30 min (18) (referred to as low salt extraction) and centrifuged at 42,000 rpm (130,000 × g_{max}) in a Beckman Ti-75 rotor. The extracted pellets were then resuspended in 1 ml of buffer A, and the corresponding supernatants were lyophilized in order to get the same volume (1 ml) as the resuspended pellets. KCl supernatants were dialyzed against buffer A before lyophilization. SR fractions were either used right away or quickly frozen in liquid nitrogen and stored at −70 °C until used.

Analytical Procedures—Electrophoresis of SR fractions on cellulose-polyacetate strips (Gelman Sepraphore III) was carried out under native conditions for 2 h at 250 V in 0.06 M Veronal buffer, pH 8.6, containing 1 mM 2-mercaptoethanol. CK activity was revealed by the coupled enzyme agar overlay gel technique (19). Omission of CP from the overlay gel or addition of diadenosine pentaphosphate was used to identify adenylate kinase (myokinase) activity.

Sodium dodecyl sulfate-slab gel electrophoresis was performed in 5–15% polyacrylamide linear gradient gels according to Laemmli (20). Slab gels were stained either with Coomassie Brilliant Blue or with Stains-All (21). Bio-Rad low molecular mass protein standards (98-kDa muscle phosphorylase *b*, 66-kDa bovine serum albumin, 42.6-kDa ovalbumin, 31-kDa bovine carbonic anhydrase, 21.5-kDa soybean trypsin inhibitor, 14.4-kDa lysozyme) were used.

Immunoblots of SR fractions were carried out according to the method of Towbin *et al.* (22) as modified by Wallimann *et al.* (23) using specific rabbit antibodies elicited either against chicken muscle M-CK (8), mitochondrial CK (24), skeletal myosin (gift of Dr. Susan Lowey, Brandeis University, Waltham, MA), or SR Ca^{2+}/Mg^{2+}-ATPase (25), followed by peroxidase-labeled goat anti-rabbit IgG (Cappel Dynatech, Kloten, Switzerland).

CK activity was measured by a pH-stat assay as described in detail in Ref. 26. Specific CK activity is expressed in μmol of CP transphosphorylated $\cdot min^{-1} \cdot mg^{-1}$ of SR protein at 25 °C. Mitochondrial contamination was estimated by measuring the specific succinate-cytochrome *c* reductase activity (27). Protein determinations were done according to Bradford (28) using the Bio-Rad procedure and bovine serum albumin (Pierce Chemical Co.) as a standard.

Ca^{2+} Loading—Ca^{2+} loading by SR fractions was measured spectrophotometrically at 37 °C according to Mitchell *et al.* (29). The decrease in absorbance (710–790 nm) of the metallochromic Ca^{2+} indicator antipyrylazo III was continuously monitored in a Hewlett-Packard 8451A diode array spectrophotometer. The reaction medium contained, in a final volume of 1 ml, 92 mM potassium phosphate, pH 7.0, 5 mM $MgSO_4$, 1 mM MgATP, 250 μM antipyrylazo III, 50 μg of SR protein at pH 7.0. The reaction was started by adding 25 μmol of $CaCl_2$. In a second set of experiments, ATP was substituted by 10 mM CP and various concentrations of ADP; and in a third type of experiments, 25 μM DNFB was used to inhibit CK activity.

Ultracryosectioning and Localization of CK at the SR and the M Band of Skeletal Muscle by Immunogold Electron Microscopic Histochemistry—Intact chicken pectoralis muscle fibers were permeabilized by 50 μg/ml saponin and washed in relaxing solution as described (8) prior to fixation, processing for ultracryotomy (42), and immunogold labeling with specific anti-M-CK antibodies followed by appropriately diluted goat anti-rabbit IgG gold conjugate (GarG, 5 or 10 nm of colloidal gold, Pharmaceutica, Beerse, Belgium) (42). Alternatively, ultrathin cryosections prepared as described earlier (42) were washed extensively to remove soluble CK before postfixing with 2% paraformaldehyde and 0.2% glutaraldehyde in ice-cold phosphate-buffered saline at pH 7.4 for 15 min and immunolabeling of bound CK by anti-M-CK IgG (1–10 μg/ml) followed by GarG (42). This method also allowed the removal of most of the soluble CK which, due to its prevalence, would have otherwise interfered with the visualization of SR-bound CK by the immunogold method.

Immunoelectron Microscopy of Isolated SR Vesicles—SR-containing fractions were fixed for 2 h with 2% paraformaldehyde and 0.2% glutaraldehyde in ice-cold buffer A. The fixed SR vesicles were centrifuged at 42,000 rpm (130,000 × g_{max}) in a Beckman Ti-75 rotor in order to remove the fixative, were resuspended in a small volume of buffer A containing 0.2% paraformaldehyde and 0.02% glutaraldehyde, and were then either quickly frozen in liquid nitrogen for storage at −70 °C or used at once. SR vesicles were diluted with buffer A to a final protein concentration of 0.1 mg/ml and adsorbed onto carbon-coated glow-discharged copper grids for 1 min. All immunolabeling steps were performed on 100-μl drops placed on sheets of Parafilm. Grids with adsorbed vesicles were first refixed by floating onto 2% paraformaldehyde and 0.2% glutaraldehyde in buffer A for 1 min and then washed three times with phosphate-buffered saline supplemented with 50 mM glycine and three times with phosphate-buffered saline supplemented with 0.2% gelatin and 0.5% bovine serum albumin, pH 7.4 (referred to as PBG) in order to saturate nonspecific protein-binding sites. After incubation for 2 h at 20 °C with either rabbit anti-chicken M-CK, rabbit anti-chicken Ca^{2+}-ATPase antibodies, or the corresponding preimmune IgG, each diluted to 1:100 (to less than 10 μg of protein/ml) with PBG, the grids were transferred through three drops of PBG in order to wash away unbound antibodies. The grids were then incubated for 1 h with 1:50 diluted goat anti-rabbit IgG-gold complex (GarG; 10 nm colloidal gold). After washing three times with PBG, three times with 50 mM glycine in phosphate-buffered saline and three times with water, the grids were negatively stained for 15 s with 1% acidic uranyl acetate in H_2O. The vesicles were observed in a Jeol Jem 100C electron microscope at 100 kV. Photographs were taken on Agfa-Gevaert Scientia film. Freeze substitution, embedding in Lowicryl KM4, and observations of SR vesicles by scanning electronmicroscopy were done as described (30, 31).

RESULTS

Evidence for MM-CK Specifically Bound to SR Membranes—Analysis of the protein composition of purified chicken skeletal muscle SR fractions (R1–R4) obtained by the procedure described by Saito *et al.* (17), which also worked very well for chicken muscle, revealed the presence of a major polypeptide band with an apparent M_r of 97,000, corresponding to the Ca^{2+}-ATPase subunit (that is the main constituent of the SR membranes) in all four SR fractions (Fig. 1, *lanes a–d*). However, the relative amount of Ca^{2+}-ATPase was highest in the R2 fraction (*lane b*), corresponding to longitudinal SR tubules. The R4 or TC fraction (*lane d*), consisting of two different types of SR membranes, namely the junctional face membrane and the Ca^{2+} pump membrane, which, in the rabbit, represent 15–20% and 80% of the total membranes present in R4 fractions, respectively (17), showed a higher content of calsequestrin, with an apparent M_r of 55,000 (25), than R1, R2, and R3 fractions. Calsequestrin, known to be specifically localized in the lumen of TCs (32), stained blue after staining with Stains-All (Fig. 1, *CS, black asterisk*). Other proteins, also stained blue by this particular dye as already described earlier (33), were the 130-kDa and the 120-kDa

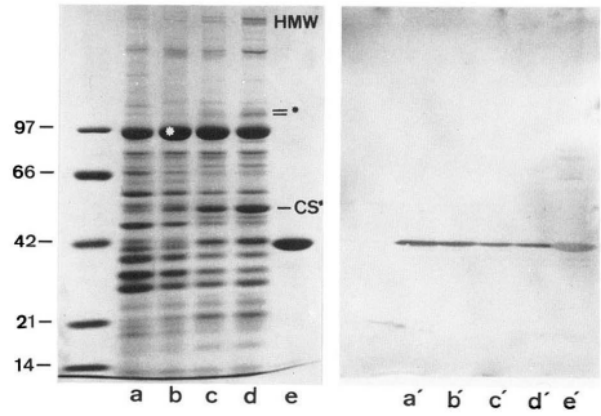

FIG. 1. **Protein composition of various SR fractions and identification of SR-associated M-CK by immunoblotting.** *Left panel* (*a–e*), linear 5–15% polyacrylamide gradient gel according to Laemmli (20) showing Coomassie Blue-stained polypeptides of SR fractions (60 μg of protein each) R1 (*lane a*), R2 (*lane b*), R3 (*lane c*), R4 (*lane d*), M-CK standard (Sigma) (*lane e*). Low molecular mass protein standard indicated is in kDa on the *far left*. Protein bands stained blue by Stains-All are indicated by *black asterisks*, and Ca^{2+}-ATPase protein is indicated by a *white star* in *lane b*. *CS*, calsequestrin. *Right panel* (*a′–e′*), immunoblot corresponding to *left gel panel* after electrophoretic transfer of protein bands to nitrocellulose followed by staining with specific rabbit anti-chicken M-CK antibodies (diluted 1:1000) and second peroxidase-labeled goat anti-rabbit IgG (diluted 1:2500). Note the presence of M-CK in all SR fractions, as indicated by the single antibody-stained protein bands with an apparent M_r of about 42,000.

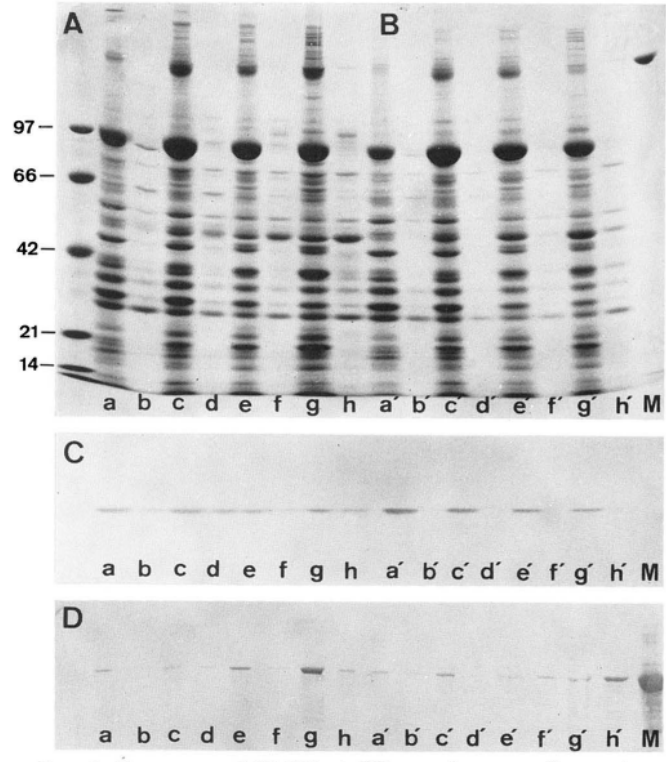

FIG. 2. **Presence of M-CK at SR membranes after extraction of SR fractions with high and low salt buffers.** Heavily loaded Coomassie Blue-stained gel of SR fractions after extraction with 50 volumes of either 10 mM Tris, 2 mM EDTA, pH 8.0 (*panel A*), or 0.6 M KCl in buffer A (*panel B*) and the corresponding supernatants thereof to test for released M-CK. On both *panels*, R1, R2, R3, and R4 fractions were loaded in *lanes a*, *c*, *e*, and *g*, respectively, and the corresponding supernatants in *lanes b*, *d*, *f*, and *h*. *Lane M* contains a myosin standard. Low molecular mass protein standards are indicated in kDa on the *far left* on each *panel*. The *lower panels* show the region of interest of the blot corresponding to the *upper gel*, after electrophoretic transfer of protein bands to nitrocellulose followed by staining with specific rabbit anti-chicken M-CK antibodies (diluted 1:1000) (*panel C*) and anti-chicken skeletal myosin antibodies (diluted 1:500) (*panel D*), both followed by second peroxidase-labeled goat anti-rabbit IgG (diluted 1:2500) and staining for peroxidase activity. The extracted pellets were resuspended in 1 ml of buffer A, and the corresponding supernatants were lyophilized in order to get the same volume (1 ml) as the resuspended pellets. KCl supernatants were dialyzed against buffer A before lyophilization. The same volume of each fraction was applied/*lane*. EDTA/low salt buffer opens the vesicles and releases their content; note the calsequestrin band (55 kDa) enriched in the supernatant of R4 (*lane h*). M-CK is still bound to the SR membranes of all SR fractions after both high and low salt treatment (*panel C*). No CK is released by KCl extraction (*panel C*, *lanes b′*, *d′*, *f′*, and *h′*), but by contrast, some CK, however not exceeding 30% of the total, as determined by activity measurements (Table I), was set free by alkaline low salt/EDTA extraction (*panel C*, *lanes b*, *d*, *f*, and *h*). Note the very small amounts of myosin contamination as judged by the *small* and *faint anti-myosin-stained bands* present in R1–R3, with R4 showing more myosin than R1–R3 (*panel D*). Only the *upper fine band* of the prominent band at 200-kDa seen on the Coomassie Blue-stained gel (*panel A*) corresponds to myosin contamination (*panel D*). (Compare *lanes g′* and *h′* of *panel A* with the *same lanes* of *panel D*.)

glycoprotein enriched in R4 (*lane d*, *black asterisk*). High M_r proteins, presumably feet proteins of junctional face membranes, are seen mainly in fraction R4 (Fig. 1, *lane d*). Besides these proteins, even in highly purified SR preparations, a number of other more or less prominent proteins, some of them still unidentified, can be seen on heavily overloaded sodium dodecyl sulfate gels, as also pointed out by other investigators (17, 38).

Immunoblotting with anti-M-CK antibodies of the SR fractions, obtained by sucrose gradient centrifugation and seen as four distinctly layered bands (R1–R4), demonstrated the presence of significant amounts of M-CK in all four of these SR fractions (Fig. 1, *lanes a′–d′*). None of the four SR fractions contained intact myofibrils or significant amounts of mitochondria, as shown by electron microscopical analysis of embedded SR vesicles (see Fig. 4). In addition, only small amounts of myosin contamination were detected by immunoblotting in all four SR membrane fractions (Fig. 2*D*), and the same was true for mitochondria (see below).

In order to verify that the CK bound to these SR fractions was not simply due to contamination or adventitious binding from bulk soluble CK or due to small amounts of myosin-bound CK (7, 14), aliquots of all four SR fractions were extracted with a 50–100-fold excess of either high salt (0.6 M KCl) or low salt solution (10 mM Tris, 1 mM EDTA, pH 8.0). Extraction by 0.6 M KCl released various proteins from the SR fractions, which are recovered in the supernatants as seen in Fig. 2*B* (*lanes b′*, *d′*, *f′*, and *h′*), on Coomassie-stained 5–15% polyacrylamide linear gradient gel. However, among such proteins there were no significant amounts of CK, as demonstrated by immunostaining of the corresponding blot with anti-M-CK antibodies (Fig. 2*C*, *lanes b′*, *d′*, *f′*, and *h′*); instead, most of the M-CK remained bound to the SR vesicles and appeared on the immunoblot in the salt-extracted SR pellet fractions (Fig. 2*C*, *lanes a′*, *c′*, *e′*, and *g′*). Conversely, the EDTA/low salt treatment (Fig. 2*A*), known to open SR vesicles and to release their contents (34), seems to set free some CK into the supernatants. However, the activity directly measured by the enzyme assay in such supernatants (see Table I) amounts only to 15–30% of the initial CK activity. Thus, the MM-CK bound to the various SR membranes at the end of the rather lengthy purification procedure, involving many washing and centrifugation steps (17), is resistant to extensive extraction by high and low salt buffers. Therefore,

TABLE I

Quantitation of CK activity in various SR fractions before and after high and low salt extraction; evidence for an associative behavior of CK to SR

SR fraction	Specific CK activity[a]	CK activity recovered[b]			
		After KCl extraction		After EDTA extraction	
		Pellet	Supernatant	Pellet	Supernatant
	IU/mg SR proteins	*%*			
R1	1.200 ± 0.348	73	3	70.2	29.3
R2	0.703 ± 0.428	77.3	4	70	29.8
R3	1.110 ± 0.577	81.4	7.8	80	16.7
R4	1.450 ± 0.654	82	8	91.7	14.8

[a] CK activity is expressed in IU corresponding to 1 μmol of CP transphosphorylated/min/mg of protein, as measured by pH-stat assay (26). The values reported are the mean ± S.D. for nine determinations from several different SR vesicle preparations.

[b] SR fractions were extracted either for 1 h with 0.6 M KCl in buffer A or for 30 min with 10 mM Tris, 1 mM EDTA, pH 8.0 (see legend of Fig. 2). CK activity (IU/ml) measured both of the extracted pellets, and the corresponding supernatants are expressed as percentages of the initial CK activity (IU/ml) of the various SR fractions. The values reported are the mean of three determinations, each from the same preparation.

under both of these extreme conditions, CK still displayed strong associative behavior to SR membranes, indicative for a specific interaction of CK with SR vesicles. Under these conditions, CK bound to myofibrils at a molar ratio of 1 CK molecule/20 myosins (9, 12, 14) is completely solubilized by both high or low salt buffers (9, 14). For this reason, the CK activity determined in our SR preparations is unlikely due to myofibrillar or myosin-bound CK, for the myosin contamination present in all four SR fractions was very low (see *small* and *faint bands* in Fig. 2*D* after staining with excess of antimyosin antibody), as indicated by the fact that during low and high salt extraction only small amounts of myosin were either precipitated (Fig. 2*D*, *lanes a–h*) or released into the supernatants (Fig. 2*D*, *lanes a′–h′*). This also indicates that only one of the *faint upper bands* and not the *prominent band* itself seen around 200 kDa in Fig. 2, *A* and *B*, is myosin (this is seen best by comparing *lanes g′* and *h′* of Fig. 2*A* with the *same lanes* in Fig. 2*D*).

The CK isoenzymes in the SR fractions were identified by cellulose-polyacetate electrophoresis under native conditions followed by staining for CK activity. All four SR fractions contained MM-CK as the most prominent isoform, as judged by the strong CK activity staining (Fig. 3, *MM-CK*), whereas the heavier SR fractions (especially R3 and R4) showed in addition a faint band corresponding to mitochondrial CK (Fig. 3, *lanes c*, *d*, and *e*, *Mi-CK*). However, direct measurements of the specific succinate-cytochrome *c* reductase activity (Table II) showed that mitochondrial contamination was less than 1% in all cases (for calculations, see legend of Table II). The low contamination by mitochondria of the SR preparations was also confirmed by electron microscopy (see below). An additional faintly stained band moving toward the anode was most probably due to some MB-CK isoenzyme seen in R2–R4 (Fig. 3, *lanes b–d*). Omission of CP from the overlay gel resulted in a very weak band only at the MM-CK position within the R1 fraction (not shown), indicating the presence of trace amounts of adenylate kinase.

Amount of CK Activity Bound to the SR—All four SR fractions contained considerable specific CK activities ranging from 0.7 to 1.4 IU/mg of protein (Table I). Treatment with 0.6 M KCl released into the supernatants only 3–8% of

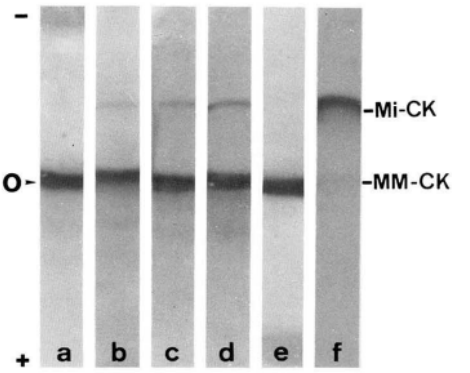

FIG. 3. **Identification of SR-bound CK as the muscle isoenzyme (MM-CK) by zymograms after electrophoresis under native conditions.** R1 (*lane a*), R2 (*lane b*), R3 (*lane c*), and R4 (*lane d*) after electrophoresis on cellulose-polyacetate strips and staining for CK activity. *Lanes e* and *f* contain purified MM-CK and Mi-CK, respectively. *O* is the origin of sample application. Samples containing 3 IU/ml were applied/lane. *Faint bands* due to MB-CK activity are visible in R3 (*lane c*) and R4 (*lane d*) fractions. Weak myokinase activity near the MM-CK band (not shown) was detected in R1 after omission of CP from the staining medium.

TABLE II

Quantitation of mitochondrial contamination of the various SR fractions

Mitochondrial contamination of SR fractions was evaluated by measuring succinate-cytochrome *c* reductase activity as described by Fleischer and Kervina (27). Since native beef heart mitochondria display a specific activity of 0.8–0.9 μmol of cytochrome *c* reductase/min/mg of protein, the values shown in this table correspond to mitochondrial contaminations of 0.23, 0.38, 0.4, and 0.8% for the R1, R2, R3, and R4 fractions, respectively. The values reported are the mean ± S.D. of three determinations each from different preparations.

SR fraction	Succinate-cytochrome *c* reductase activity
	nmol/mg protein/min
R1	1.96 ± 0.82
R2	3.26 ± 0.95
R3	3.33 ± 1.08
R4	6.85 ± 0.98

the initial CK activity present on the SR vesicles before extraction, whereas after low salt treatment, 15–30% of the CK activity was found in the supernatants. This confirmed that CK is rather specifically bound to SR vesicles withstanding high salt extraction. The release of a certain amount of CK by EDTA/low salt treatment may indicate either that some CK may have gotten trapped inside the SR vesicles during purification or that the interaction of CK bound to the outside of SR vesicles is rather sensitive to alkaline pH. The incomplete recovery of the initial CK activity after 0.6 M KCl extraction may be explained by inhibition and/or denaturation of some of the SR-bound CK since the lack of any immunoreactive material on the blot (Fig. 2*C*, *lanes b′*, *d′*, *f′*, and *h′*) suggests that not even inactive enzyme protein is present in the KCl supernatants. Therefore, we assume that an additional 10–15% of inactivated CK, not detectable by the enzyme assay, would also be contained in the KCl pellets, as also suggested by the stronger reaction on the immunoblot of the CK bands of the KCl pellets compared with those of the EDTA/low salt pellets (Fig. 2*C*).

Characterization of SR Vesicles by Electron Microscopy—Fig. 4 shows a representative electron micrograph from thin sections of isolated, densely packed R2 SR vesicle fractions, after freeze substitution and Lowicryl embedding (30) con-

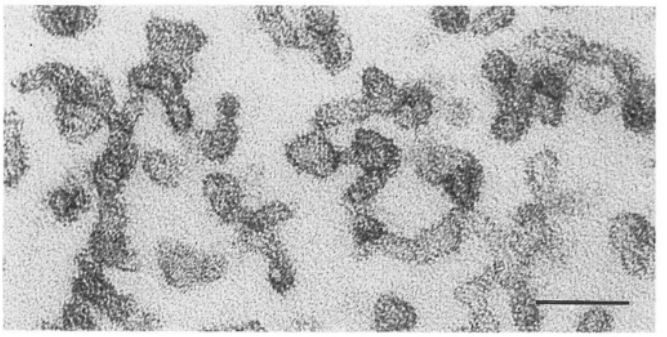

FIG. 4. **Characterization of longitudinal tubular SR (fraction R2) by electron microscopy.** Representative thin section through frozen, cryosubstituted, and Lowicryl-embedded (Ref. 30) SR pellets enriched in longitudinal tubular SR (R2) shown at a magnification of ×140,000 with a *bar* corresponding to 100 nm. Note the rather homogeneous appearance of the R2 SR vesicle population and the absence of myofibrillar and mitochondrial contaminants.

taining mostly longitudinal SR as judged from the rather homogeneous population of vesicles devoid of electron-opaque content and from the absence of myofibrils with no or only very few mitochondria (Fig. 4). Mitochondria were routinely seen only in R4. The size of the LSR vesicles ranged from 70 to 300 nm, which is in agreement with published data (17). Scanning electron microscopy of the R2 fraction showed the rather smooth surface of the vesicles (not shown).

In Situ Localization of CK at the SR in Skeletal Muscle—Since skeletal and cardiac muscle contains large amounts of CK (14), and only fractions of the total enzyme activity are tightly associated with subcellular structures, it was necessary to remove the soluble part of the enzyme for *in situ* localization of CK bound to SR. Therefore, muscle tissue was either permeabilized and washed with relaxing solution prior to chemical fixation, ultracryotomy, and immunolabeling, or ultrathin cryosections of mildly fixed muscle were incubated extensively in relaxing solution prior to postfixation and immunostaining. Immunogold labeling of these cryosections with specific anti-M-CK antibodies revealed bound CK at the M line (Fig. 5) of the sarcomer, a well established location of M-CK (7, 8, 14), and additionally at those places near the Z band and A-I band junctions, where T tubules and SR are prominent (Fig. 5*A*, *arrowheads*). The latter is seen more clearly at higher magnification (Fig. 5*B*, *arrowheads*). By contrast, no significant labeling was observed with preimmune antibody (Fig. 5*C*). Even though in negatively stained ultrathin cryosections SR membranes are difficult to visualize directly (Ref. 4), the immunolabeling shown in Fig. 5, *A* and *B*, is topologically consistent with the location of SR where membranous structures near the A-I junctions and along the myofibrils were often seen to be stained by gold antibodies (Fig. 5, *A* and *B*; see *arrowheads*). The *in situ* immunolocalization of CK at the SR agrees well with the immunogold labeling of isolated SR vesicles with anti-M-CK antibodies (Fig. 6) and argues against adventitious binding of CK to SR vesicles during their isolation. Similar results have been obtained earlier by *in situ* histochemical staining of muscle for CK enzyme activity (16). However, the diffusibility of the various reaction products generated by the coupled enzyme-staining technique for CK activity makes the interpretation of these results very difficult. A direct *in situ* immunolocalization of the M-CK protein with anti-M-CK antibodies, as shown here, seems to be far superior.

Localization of M-CK and Ca^{2+}-ATPase on Purified SR Vesicles by Immunoelectron Microscopy—LSR vesicles were adsorbed to glow-discharged carbon film, incubated with primary antibodies either against Ca^{2+}-ATPase or M-CK, and then incubated again with secondary gold-conjugated antibodies. After negative staining, LSR vesicles showed specific labeling by both anti Ca^{2+}-ATPase and anti-M-CK antibodies (Fig. 6, *A* and *B*). On the other hand, incubation with identical concentrations of preimmune IgG showed only low background labeling (Fig. 6*C*). The labeling with anti-Ca^{2+}-ATPase antibodies appeared very strong and often clustered at the surface of the vesicles (Fig. 6*B*). Considering the fact that M-CK, relative to the Ca^{2+}-ATPase, is only a minor protein of the SR (Fig. 1), the extent of labeling with anti-M-CK was rather high (Fig. 6*A*). The distribution of the gold clusters was similar in both cases, indicating that some epitopes of both enzymes are indeed exposed on the outside of the SR vesicles and thus are accessible to immunogold staining.

Extent of CK-supported ATP-dependent Ca^{2+} Uptake by SR Vesicles—In order to investigate the functional role of SR-bound CK, the Ca^{2+}-loading rate by SR vesicles was measured by the *in vitro* assay described under "Experimental Procedures," using mainly the homogeneous well characterized R2 SR fraction where Ca^{2+}-ATPase was highly enriched. In a first set of experiments, the Ca^{2+} loading of LSR vesicles in the presence of excess ATP (1 mM) was determined (Fig. 7*B* and Table III) and taken as a control value corresponding to 100%. Under these conditions, a maximal Ca^{2+}-loading rate was observed since increasing the ATP concentration above 1 mM did not enhance Ca^{2+}-pumping activity. The reaction was always started by the addition of 25 nmol of Ca^{2+}. When DNFB, a specific inhibitor of CK if used at low concentration (35), was added at 25 μM concentration at the end of the Ca^{2+} uptake, ATP-supported Ca^{2+} loading was resumed upon a second addition of Ca^{2+} (Fig. 7*B*).

In a second set of experiments, ATP was substituted by 1 mM ADP plus 10 mM CP, both substrates of the CK reaction, and then the CK-supported Ca^{2+}-loading rate was measured again (Fig. 7*A* and Table III). The relative Ca^{2+}-loading rate, supported by R2-bound CK and CP plus ADP, was approximately 24 ± 5% compared with that measured in the presence of ATP (control). When DNFB was added to the vesicles (Fig. 7 and Table III), the Ca^{2+} uptake slowly ceased as a consequence of the inhibition of CK and did not recover after a second addition of Ca^{2+} (Fig. 7*A*) due to irreversible inactivation of CK by DNFB. However, upon addition of ATP to the same preparation, Ca^{2+} loading was resumed, albeit at a somewhat lower absolute rate than the control (Fig. 7*A*, *end of tracing*), indicating that the Ca^{2+}-ATPase itself was only slightly affected by DNFB. In fact, the slight inhibition exerted by DNFB (approximately 17%) on the Ca^{2+}-ATPase in the presence of an excess of ATP was actually due to the ethanol used as a solvent for the DNFB (Table III). Taken together, these findings suggest that endogenous SR-bound CK of highly purified SR vesicles is indeed capable of supporting Ca^{2+} uptake to a considerable extent by local *in situ* regeneration of ATP.

When the assay was performed in the presence of CP and ADP plus an excess of exogenously added CK, the Ca^{2+}-loading rate measured was similar to the control value. In a further set of controls, Ca^{2+} uptake was measured in the presence of ADP alone to evaluate a contribution to ATP regeneration by the adenylate kinase or myokinase reaction. However, myokinase-supported Ca^{2+} loading was negligible (Table III). In order to exclude that the mitochondrial ATP production significantly affected the above results, Ca^{2+} uptake measurements were made in the presence of either succinate plus ATP or succinate plus ADP as potential energy sources for oxidative phosphorylation. In the first case, as

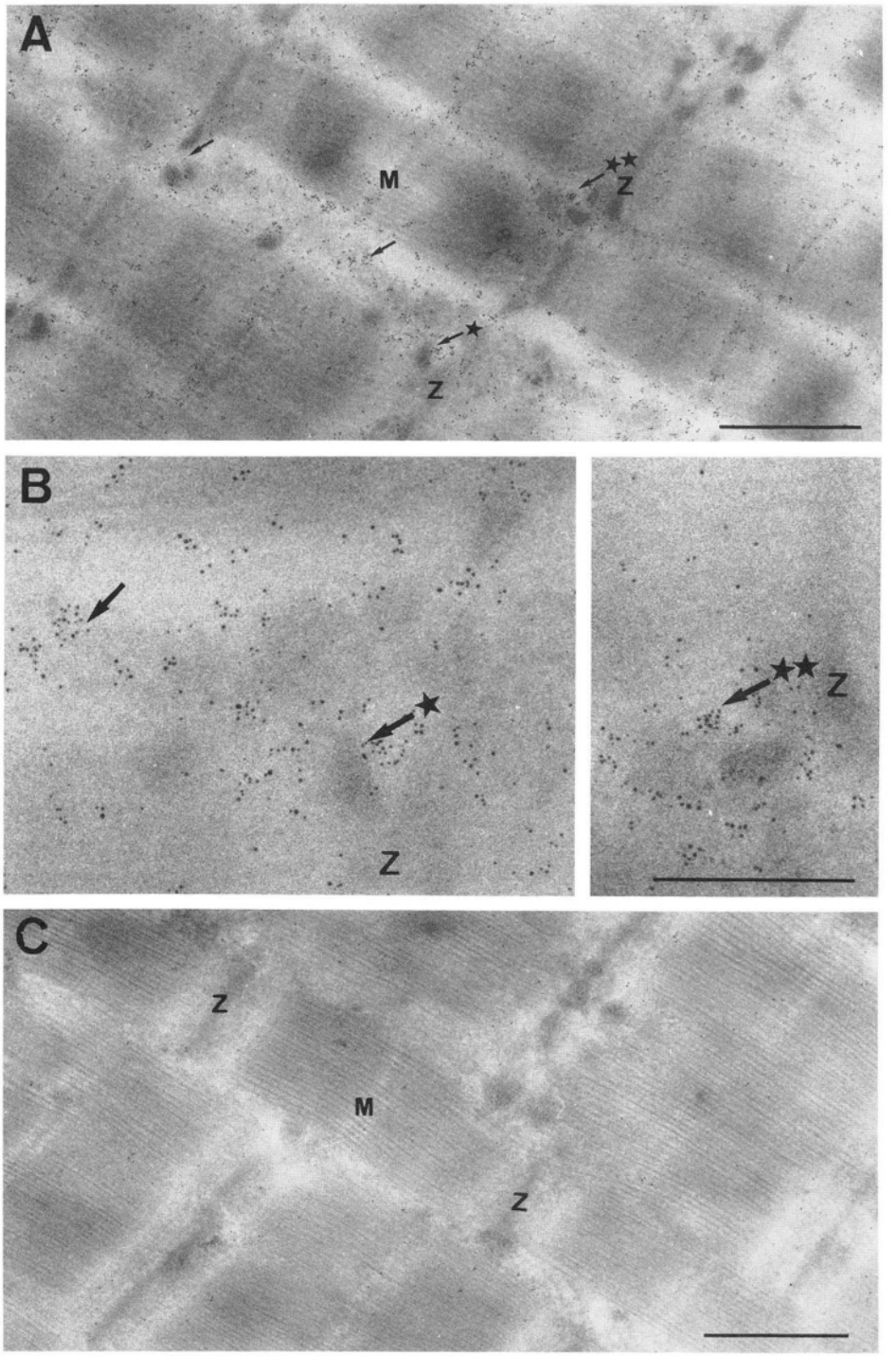

FIG. 5. **Direct *in situ* localization by immunogold labeling of CK bound to the SR system and the M band of skeletal muscle.** *A*, ultrathin cryosection of chicken pectoralis muscle, from which soluble CK has been removed by washing under physiological conditions (see "Experimental Procedures"), shown after immunogold staining with anti-M-CK antibodies and gold-conjugated second antibody. CK remaining bound to the T tubule/SR system (*arrowheads*) and the myofibrillar M band (*M*) is stained (see Refs. 7, 8, and 14). Note specific staining of membranous material (*arrowheads*) reminiscent in its appearance and localization of the T tubule/SR system as it is seen after cryoultramicroscopy and negative staining of skeletal muscle (Ref. 4) Note also prominent staining of the myofibrillar M band region where a small but significant fraction of CK is specifically bound (for review, see Ref. 14). *Bar* = 1.0 μm. *B*, same as in *panel A* but at higher magnification (for orientation, see corresponding *arrowheads* with a *single* and a *double star* in *A* and *B* as well as the Z bands (*Z*). *Bar* = 0.5 μm. *C*, control stained, under identical conditions, with preimmune IgG, followed by gold-conjugated second antibody. Note the very low nonspecific background labeling. *Magnification bar* represents 1.0 μm.

expected, the Ca^{2+} loading was similar to the control value; whereas in the latter case, no Ca^{2+} uptake was measured (Table III). The presence of rotenone, a known inhibitor of mitochondrial electron transport chain (37), did not decrease or shorten the ATP-dependent Ca^{2+} loading by the LSR vesicles (Table III), indicating that under our conditions mitochondria or mitochondrial CK were not involved in supporting Ca^{2+} uptake.

This confirmed that the very low contamination of the SR fractions, especially of R2, by mitochondria (as shown in Table II) had a negligible effect on the Ca^{2+} uptake measurements. The assay was also performed in the presence of CP alone (data not shown), but no Ca^{2+} pumping was observed.

Furthermore, rates of Ca^{2+} loading and CK-supported Ca^{2+} loading were also determined using the R3 fraction, representing a mixture of LSR plus TC. The CK-supported Ca^{2+}-loading rate was found to be 42% of the control value (Table III). As expected, the absolute Ca^{2+}-loading rate of R3 was slower due to the fact that this fraction contained less Ca^{2+}-ATPase. However, since it contained a higher CK content compared with the R2 fraction (Table I), the percentage of CK-supported Ca^{2+} pumping went up to over 40% (Table III).

ATP Regeneration Potential by SR-bound CK—The amounts of CK bound to the different SR vesicle fractions (Table 1) and the observed *in vitro* Ca^{2+} uptake rate of the same fractions (Table III) are theoretically sufficient to support fully the Ca^{2+} pump, *e.g.* 0.703 ± 0.428 μmol of ATP may be regenerated/min/mg of R2 by the CK bound to R2 longi-

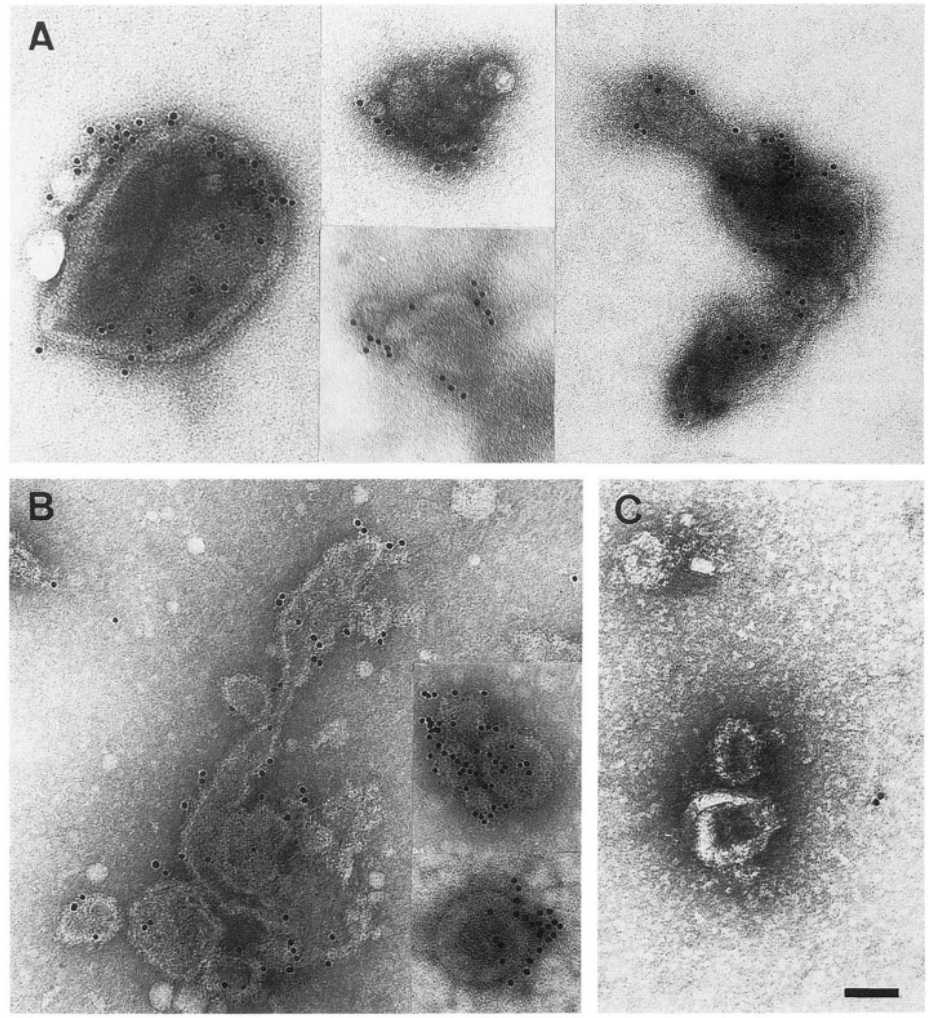

FIG. 6. **Localization of M-CK at the periphery of longitudinal SR vesicles.** Immunogold labeling of longitudinal R2 SR vesicles adsorbed to glow-discharged carbon films after incubation with rabbit anti-chicken M-CK (*panel A*), rabbit anti-Ca^{2+}-ATPase antibodies (*panel B*), or preimmune IgG (*panel C*). As second antibody, goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles was used. Note specific labeling of R2 vesicles by both anti-M-CK and anti-Ca^{2+}-ATPase antibodies and low background staining of the control in *panel C*. Magnification, ×100,000 with a *bar* corresponding to 60 nm.

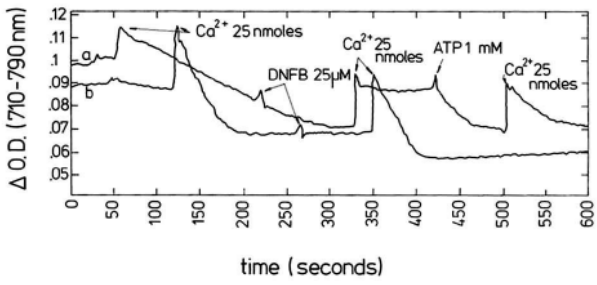

FIG. 7. **Ca^{2+} accumulation rate into SR vesicles directly supported by SR-bound CK.** The Ca^{2+}-loading rate into R2 vesicles was measured under various conditions (see also Table III). Representative tracings are shown. The assay was performed with 50 μg of SR proteins. *Tracing a*, Ca^{2+} loading in the presence of 10 mM CP plus 1 mM ADP initiated by the addition of 25 μmol of Ca^{2+}. The Ca^{2+}-loading rate supported by SR-bound CK was 0.576 μmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ of SR protein. After the addition of 25 μM DNFB, Ca^{2+} loading was abolished due to blockade of CK but recovered upon addition of 1 mM ATP as in the control and was stimulated again by adding Ca^{2+} (Ca^{2+}-loading rate 0.547 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of SR protein). *Tracing b*, Ca^{2+} loading in the presence of 1 mM ATP as a control was initiated by the addition of 25 μmol of Ca^{2+}. The Ca^{2+}-loading rate was 1.369 μmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ of SR protein. The addition of 25 μM DNFB followed by readdition of Ca^{2+} only slightly inhibited Ca^{2+} loading.

tudinal SR vesicles (Table I), which display a maximal ATPase rate of approximately $1{,}713 \pm 0.138/2 = 0.8$ $ATP \cdot min^{-1} \cdot mg^{-1}$ of R2 (Table III) if a loading efficiency of $2Ca^{2+}/ATP$ is taken as the correct value (49). In the heavier SR fractions (R3 and R4) where the ratio of SR-associated CK to Ca^{2+}-ATPase is higher, the ATP regeneration potential by the bound CK exceeds the ATP required for Ca^{2+} loading, indicating that CK may, in addition, be involved in processes other than simply regenerating the ATP required for the Ca^{2+} pump. Thus, even though the direct support of Ca^{2+} loading in SR vesicles by CK is not sufficient in our *in vitro* assay, the ATP regeneration potential of SR-bound CK should be sufficient to support fully the *in vivo* Ca^{2+} loading (see "Discussion").

DISCUSSION

In a previous study, Volpe *et al.* (38), using photoactivatable phospholipid analogues, noticed the presence of a 41-kDa integral membrane protein in highly purified SR preparations, which, at that time, was not identified. We were therefore curious to see whether this protein, showing an apparent M_r very similar to that of the M-CK monomer, was membrane-bound CK. We have demonstrated by biochemical experiments and by immunoelectron microscopy using anti-M-CK antibodies that CK is indeed bound specifically to all four SR fractions obtained from chicken skeletal muscle and that significant amounts of CK were still bound to the SR mem-

TABLE III

Ca^{2+}-loading rate of LSR supported either by ATP or SR-bound CK and CP plus ADP

Ca^{2+}-loading rate was measured at 37 °C in the presence of the metallochromic Ca^{2+} indicator antipyrylazo III as described under "Experimental Procedures." The reaction was started by the addition of 25 nmol of $CaCl_2$. The values reported are the mean ± S.D. of at least 10 determinations each using different SR vesicle preparations.

SR fraction (50 μg protein)	ATP	ADP	CP	Succinate	Rotenone	Exogenous CK added	Ca^{2+}-loading rate		
							−DNFB	+DNFB (25 μM)	+ATP (1 mM)[a]
	mM				*μM*	*μg*	*μmol Ca^{2+}/mg protein/min*		
R2	1						1.713 ± 0.138	1.430 ± 0.120	
	1						1.395 ± 0.105[b]		
R2		1	10				0.399 ± 0.182	0.034 ± 0.010	0.642 ± 0.102
R2		1					0.050 ± 0.010	0	
R2		1	10			20	1.363 ± 0.125	0	
R2	1			50			1.327 ± 0.114		
R2		1		50			0		
R2	1				2		1.740 ± 0.120		
R2		1	10		2		0.351 ± 0.130	0	
R3	1						0.360 ± 0.154	0.288 ± 0.070	
R3		1	10				0.152 ± 0.040	0	0.147 ± 0.020

[a] 1 mM ATP was added after inhibition of CK with DNFB.

[b] Ca^{2+}-loading rate in the presence of 1 mM ATP was measured by adding ethanol (as a control), the solvent in which DNFB was dissolved.

branes after both low and high salt treatments, indicating a rather tight and specific binding of CK to the SR membranes. After EDTA treatment, only 15–30% of the initial CK activity was released into the supernatant. This relatively small fraction could be derived either from soluble CK that may have gotten trapped inside SR vesicles during their purification or from CK bound to the outside of the SR vesicles, which was dissociated by alkaline pH, low salt, or EDTA. However, the fact that SR-bound CK completely resisted extensive extraction by 0.6 M KCl suggests a strong associative behavior of this enzyme to SR membranes and argues against a significant contamination of the SR preparation by myofibrillar or myosin-bound CK, which is known to be released into the supernatant by high and low salt buffers (12, 14).

The presence of CK bound to SR membranes had been suggested earlier by Baskin and Deamer (15) and Levitsky (16); however, these authors neither characterized the SR vesicles to exclude adventitious binding of CK to these vesicles, nor did they specify the CK isoform involved.

In previous studies concerning myofibrils, the M line-bound CK was shown by a similar coupled *in vitro* assay to suffice completely for the regeneration of ATP hydrolyzed by the actin-activated Mg^{2+}-ATPase during *in vitro* contraction of myofibrils (26). In the present experiments with SR, the Ca^{2+} uptake rate was slower if solely supported by endogenous SR-bound CK, as compared with the control rate measured in the presence of excess ATP. However, the contribution of the *in situ* ATP regeneration by the SR-bound CK in the presence of excess CP plus ADP was significant. *In vitro*, SR-bound CK supported 24–40% of the maximal Ca^{2+} uptake rate, depending on which of the SR vesicle fractions were taken. This could mean that some of the CK originally bound to the SR may have been lost during fractionation and extensive washing of the SR vesicles or that in this *in vitro* assay, working at very low concentrations of vesicles, some of the ATP regenerated *in situ* on the surface of the SR vesicles may continuously diffuse away and thus may be lost for immediate utilization by the Ca^{2+} pump. The latter explanation also seems reasonable since a comparison of the ATP regeneration potential of CK on the R2 SR vesicles (0.703 μmol of ATP regenerated/min/mg of R2 protein; see Table I) with the ATP hydrolysis rate of the Ca^{2+}-ATPase at maximal speed (1.713 μmol of Ca^{2+}/min/mg of R2 protein; see Table III) shows that the two numbers are indeed very close, if an ideal coupling ratio of $2Ca^{2+}$/ATP is assumed (49). Thus, under ideal conditions and even more so *in vivo* where diffusion of nucleotides is severely limited, the SR-bound CK may suffice for supporting fully the Ca^{2+} uptake.

The fact that the ratio of bound CK to Ca^{2+}-ATPase activity is significantly higher in R3 and R4 (compare CK activity values in Table I *versus* Ca^{2+}-loading rate divided by 2 in Table III) shows that in this region of the SR the ATP regeneration potential of bound CK exceeds the ATP consumption by the Ca^{2+} pump *in vitro*. In addition, the fact that the amount of CK activity is not proportional to the Ca^{2+}-ATPase content of the various SR subfractions (R1–R4) indicates that CK, especially at the junctional face membrane (R4), may have additional physiological functions within the excitation contraction pathway other than simply supporting Ca^{2+} sequestration (see below).

Thus, it seems that even if we assume that the overall ATP concentration in a cell remains constant, the CK present at the SR may be physiologically important for local regeneration of ATP directly in the vicinity of the Ca^{2+} pump, where, under extremely heavy work load, metabolic stress, or ischemia, the ATP/ADP ratio is bound to be quite different from that in the rest of the myoplasm. The same explanation may hold true for the fact that lowering the ADP concentration to less than 0.5 mM in the CK- and CP-supported Ca^{2+}-loading experiments, using the coupled *in vitro* assay, started to reduce the Ca^{2+}-loading rate (not shown). A concentration of 0.5 mM ADP is well above the K_m of M-CK for ADP (36) and is also higher than the cellular overall ADP concentration found *in vivo*, except under heavy work load. However, under the latter conditions, the ADP concentration may increase locally very quickly to such a level, and thus a more efficient functional *in situ* coupling of CK with the Ca^{2+} pump may be guaranteed.

Champeil *et al.* (39) have suggested that the SR Ca^{2+}-ATPase itself is regulated by local ATP levels, *e.g.* that dephosphorylation is regulated by [ATP] and that the catalytic site is the locus of the "regulatory" ATP-binding site. Thus, the membrane-bound CK may not only be important for replenishment of ATP, but also for fine-tuning of local ATP levels and more important for regulation of local ATP/ADP ratios. The maintenance of high local ATP/ADP ratios increases the thermodynamic efficiency of ATP hydrolysis, which depends critically on the ln $(ADP)\cdot(P_i)/(ATP)$ (see Ref. 50). As a third possibility, CK may direct the ATP needed

to the protein kinases responsible for the phosphorylation of the Ca^{2+}-ATPase (49).

The finding that some CK remained attached firmly and specifically to purified SR vesicles indicates a rather strong association of some of the enzyme to the SR membrane with a possible involvement of a post-translational modification, *e.g.* a membrane anchor, for specific association of CK with the SR membrane. Such a hypothesis is supported by the fact that recently an acidic subspecies of the brain isoform B_a-CK has been found, which was blocked at the N terminus (40), possibly resulting from an acylated N terminal amino acid. A similar tight binding of CK has been illustrated with acetylcholine receptor-rich vesicles of *Torpedo* where a fraction of CK, identified as the so-called v_2-proteins (41), has been shown to be tightly associated with the postsynaptic membranes of electrocytes and with synaptic vesicles (42). In cardiac muscle, the cardiolipin moiety of the inner mitochondrial membrane has been inferred to be important as a membrane receptor for the mitochondrial Mi-CK isoform (43), although a direct functional and physiological interaction of Mi-CK octamers with the ATP/ADP translocator protein is also likely to exist (5, 6, 44, 45). In addition, recently a membrane-associated B-CK has also been found in photoreceptor cell rod outer segments (46), which is in line with the above findings.

Acknowledgements—We wish to thank Dr. Martin Müller and Rene Hermann for having performed the Lowicryl embedding of SR vesicles and the observations by scanning microscope, respectively. We thank Dr. Gabi Wegmann for help and suggestions with the immunolabeling of ultrathin cryosections. We also thank Prof. Alfredo Margreth for making available to us the diode-array spectrophotometer in Padua, Dr. Paolo Gazzotti for lending equipment, Dr. Michele Chiesi for advice, Thomas Schnyder for help with the electron microscopy, Dr. Andrew Quest for helpful discussions, Dr. Yukisato Ishida for careful reading of the manuscript, Inka Riesinger for valuable comments concerning immunogold labeling, and Else Zanolla for excellent technical assistance.

REFERENCES

1. Porter, K. R., and Palade, G. E. (1957) *Biophys. Biochem. Cytol.* **3,** 269–300
2. Franzini-Armstrong, C. (1980) *Fed. Proc.* **39,** 2403–2409
3. Inesi, G. (1985) *Annu. Rev. Physiol.* **47,** 573–601
4. Jorgensen, A. O., Shen, A. C. Y., MacLennan, D. H., and Tokuyasu, K. T. (1982) *J. Cell Biol.* **92,** 409–416
5. Jacobus, W. E., and Lehninger, A. L. (1973) *J. Biol. Chem.* **248,** 4803–4810
6. Saks, V. A., Rosenstraukh, L. V., Smirnov, V. N., and Chazov, E. I. (1978) *Can. J. Physiol. Pharmacol.* **56,** 691–706
7. Turner, D. C., Wallimann, T., and Eppenberger, H. M. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 702–705
8. Wallimann, T., Moser, H., and Eppenberger, H. M. (1983) *J. Muscle Res. Cell Motil.* **4,** 429–441
9. Wallimann, T., Turner, D. C., and Eppenberger, H. M. (1977) *J. Cell Biol.* **75,** 297–317
10. Grosse, R., Spitzer, E., Kupriyanov, V. V., Saks, V. A., and Repke, K. R. H. (1980) *Biochim. Biophys. Acta* **603,** 142–156
11. Sharov, V. G., Saks, V. A., Smirnov, V. N., and Chazov, E. I. (1978) *Biochim. Biophys. Acta* **468,** 495–501
12. Wallimann, T. (1975) Ph.D. thesis 5437, Eidgenössische Technische Hochschule, Zürich, Switzerland
13. Bessman, S. P., and Geiger, P. J. (1981) *Science (N.Y.)* **211,** 448–452
14. Wallimann, T., and Eppenberger, H. M. (1985) in *Cell and Muscle Motility* (Shay, J. W., ed) Vol. 6., pp. 239–285, Plenum Publishing Corp., New York
15. Baskin, R. J., and Deamer, D. W. (1970) *J. Biol. Chem.* **245,** 1345–1347
16. Levitsky, D. O., Levchenko, T. S., Saks, V. A., Sharov, V. G., and Smirnov, V. D. (1978) *Memb. Biochem.* **2,** 81–96
17. Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol.* **99,** 875–885
18. Duggan, P. F., and Martonosi, A. (1970) *J. Gen. Physiol.* **56,** 146–167
19. Dawson, D. M., and Eppenberger, H. M. (1970) *Methods Enzymol.* **17A,** 995–1002
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685
21. Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1983) *J. Biol. Chem.* **258,** 11267–11273
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
23. Wallimann, T., Hardwicke, P. M. D., and Szent-Györgyi, A. G. (1982) *J. Mol. Biol.* **156,** 153–173
24. Wallimann, T., Moser, H., Zurbriggen, B., Wegmann, G., and Eppenberger, H. M. (1986) *J. Muscle Res. Cell Motil.* **7,** 25–34
25. Damiani, E., Margreth, A., Furlan, A., Stephen Dahms, A., Arnn, J., and Sabbadini, R. A. (1987) *J. Cell Biol.* **104,** 461–472
26. Wallimann, T., Schlösser, T., and Eppenberger, H. M. (1977) *J. Biol. Chem.* **259,** 5238–5246
27. Fleischer, S., and Kervina, M. (1974) *Methods Enzymol.* **31,** 6–41
28. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
29. Mitchell, R. D., Volpe, P., Palade, P., and Fleischer, S. (1983) *J. Biol. Chem.* **258,** 9867–9877
30. Humbel, B., and Müller, M. (1986) *Science of Biological Specimen Preparation,* pp. 175–183, SEM Inc., AMF O'Hare, Chicago
31. Walther, P., and Müller, M. (1986) *Science of Biological Specimen Preparation,* pp. 195–201, SEM Inc., AMF O'Hare, Chicago
32. Meissner, G. (1975) *Biochim. Biophys. Acta* **389,** 51–68
33. Zorzato, F., and Volpe, P. (1988) *Arch. Biochem. Biophys.* **261,** 324–329
34. Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A., and Fleischer, S. (1986) *J. Cell Biol.* **103,** 741–753
35. Tombes, R. M., and Shapiro, B. M. (1985) *Cell* **41,** 325–334
36. Saks, V. A., Chernousova, G. B., Vetter, R., Smirnov, V. N., and Chazov, E. I. (1976) *FEBS Lett.* **62,** 293–296
37. Palmer, G., Horgan, D. J., Tisdale, H., Singer, T. P., and Beinert, H. (1968) *J. Biol. Chem.* **243,** 844–847
38. Volpe, P., Gutweniger, H. E., and Montecucco, C. (1987) *Arch. Biochem. Biophys.* **253,** 138–145
39. Champeil, P., Riollet, S., Orlowski, S., Guillan, F., Seebregts, C. J., and McIntosh, D. B. (1988) *J. Biol. Chem.* **263,** 12288–12294
40. Quest, A., Eppenberger, H. M., and Wallimann, T. (1987) *Experientia (Basel)* **43,** 676 (abstr.)
41. Barrantes, F. J., Mieskes, G., and Wallimann, T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5440–5444
42. Wallimann, T., Waltzthöny, D., Wegmann, G., Moser, H., Eppenberger, H. M., and Barrantes, F. (1985) *J. Cell Biol.* **100,** 1063–1072
43. Müller, M., Moser, R., Cheneval, D., and Carafoli, E. (1985) *J. Biol. Chem.* **260,** 3839–3843
44. Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M., and Wallimann, T. (1988) *J. Biol. Chem.* **263,** 16942–16953
45. Schnyder, T., Engel, A., Lustig, A., and Wallimann, T. (1988) *J. Biol. Chem.* **263,** 16954–16962
46. Quest, A., Wegmann, G., Capt, A., Eppenberger, H. M., and Wallimann, T. (1987) *Proceedings of the Ninth International Biophysics Congress,* August 23–28, 1987, Jerusalem, Israel
47. Rossi, A. M., Volpe, P., Eppenberger, H. M., and Wallimann, T. (1988) *In Sarcomeric and Non Sarcomeric Muscles: Basic and Applied Research Prospects for the 90's* (Carraro, U., ed) pp. 595–600, Unipress, Padova, Italy
48. Wallimann, T., Schnyder, T., Schlegel, J., Wyss, M., Wegmann, G., Rossi, A. M., Hemmer, W., Eppenberger, H. M., and Quest, A. (1989) in *Progress in Clinical and Biological Research,* Vol. 315: *Muscle Energetics* (Paul, R. J., Elzinga, G., and Yamada, K., eds) pp. 159–176, Alan R. Liss Inc., New York
49. Fleischer, S., and Inui, M. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18,** 333–364
50. Kammermeier, H. (1987) *J. Mol. Cell. Cardiol.* **19,** 115–118