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- Ca2+-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 Ca2+ uptake rate. The ATP regeneration potential of SR-bound CK was similar to the rate of Ca2+-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 Ca2+-ATPase, as well as for regulation of local ATP/ADP ratios in the proximity of the Ca2+ pump and of other ATP-requiring reactions in the excitation-contraction coupling pathway.

The SR of striated muscle, as a specialized form of endom-
MM-CK at SR Membranes Supports Ca\(^{2+}\) Pumping

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 \(\times g_{	ext{av}}\)) 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-polyacrylamide strips (Gelman Sepharose III) was carried out according to the procedure described by Saito et al. (17), which also worked for chicken SR fractions (R2, R3, and R4). The polypeptide bands were visualized by Coomassie Brilliant Blue or with some LSR, respectively (17).

**Immunoblots of SR Fractions**—SR fractions were tested for MM-CK and other proteins, also stained blue by this particular dye as already described earlier (33), were the 130-kDa and the 97-kDa bands (Fig. 2, lane a). The highest content of calsequestrin, with an apparent M, of 55,000, was present in the highest fraction (lane b), corresponding to longitudinal SR tubules. The R4 or TC fraction (lane d), consisting of a polypeptide band with an apparent M, of 97,000, corresponded to the low content of calsequestrin, with an apparent M, of 55,000, as already described earlier (33). The reaction was started by adding 25 \(\mu\)mol 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 \(\mu\)M DNFB was used to inhibit CK activity.

**Ultracytosectioning and Localization of CK at the SR and the M Band of Skeletal Muscle by Immunogold Electron Microscopy**—Intact chicken pectoralis muscle fibers were permeabilized by 50 \(\mu\)g/ml sonnin and washed in relaxing solution as described (9) prior to fixation, processing for ultracryotomy (42), and immunogold labeling with specific anti-MM-CK antibodies followed by appropriately diluted goat anti-rabbit IgG gold conjugate (GarG, 5 or 10 nm of colloidal gold, Pharmacia, Uppsala, Sweden) (42). Alternatively, ultrathin cryosections prepared as described earlier (42) were washed extensively to remove soluble CK before postfixing with 2% paraformaldehyde and 0.5% glutaraldehyde in ice-cold phosphate-buffered saline at pH 7.4 for 1 h and washed in relaxing solution as described (9). The reaction was started by adding 25 \(\mu\)mol 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 \(\mu\)M DNFB was used to inhibit CK activity.

**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 for chicken SR fractions (R1–R4) obtained by the procedure described by Saito et al. (17), also worked for chicken SR fractions (R1–R4). The polypeptide bands were visualized by Coomassie Brilliant Blue or with some LSR, respectively (17). The highest content of calsequestrin, with an apparent M, of 55,000, was present in the highest 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 (11), showed a higher content of calsequestrin, with an apparent M, of 57,000 (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...
glycoprotein enriched in R4 (lane d, black asterisk). High M, 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. 2D), 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- to 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. 2B (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. 2C, 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. 2C, lanes a', c', e', and g'). Conversely, the EDTA/low salt treatment (Fig. 2A), 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,
under both of these extreme conditions, CK still displayed strong associative behavior to SR membranes, indicative for the same preparation.

nation present in all four SR fractions was very low (see small legend of Fig. 2). CK activity (IU/ml) measured both of the extracted pellets, and the corresponding supernatants are expressed as per-
centages of the initial CK activity (IU/ml) of the various SR fractions. The values reported are the mean ± S.D. for nine determinations from several different SR vesicle preparations.

The CK isoenzymes in the SR fractions were identified by cellulose-polyacetate electrophoresis under native conditions (Fig. 3, MM-CK). CK activity was found in the supernatants. This confirmed that CK is rather specifically bound to SR vesicles withstand-
ing 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 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 enzyme assay, would also be contained in the KC1 pellets, as also suggested by the stronger reaction on the immunoblot of some of the SR-bound CK since the lack of any immunoreactive material on the blot (Fig. 2C, lanes b–h). This also suggests that not even inactive enzyme protein is present in the KC1 supernatants. Therefore, we assume that some CK may have gotten trapped inside the SR vesicles during purification or that the interaction of CK bound to the enzyme assay, would also be contained in the KC1 pellets, as also suggested by the stronger reaction on the immunoblot of the CK bands of the KC1 pellets compared with those of the EDTA/low salt pellets (Fig. 2C).

TABLE II
Table: Quantitation of mitochondrial contamination of the various SR fractions

| SR fraction | Succinate-cytochrome c reductase activity (nmol/mg protein/min) |
|-------------|---------------------------------------------------------------|
| R1          | 1.96 ± 0.82                                                   |
| R2          | 3.26 ± 0.95                                                   |
| R3          | 3.93 ± 1.08                                                   |
| R4          | 6.55 ± 0.98                                                   |

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 Lowieryl embedding (30) con-

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Table: 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* | After KC1 extraction | After EDTA extraction |
|-------------|-----------------------|----------------------|----------------------|
|             | IU/mg SR proteins     | Pellet               | Supernatant          | Pellet               | Supernatant |
| R1          | 1200 ± 348            | 73                   | 3                    | 70.2                 | 29.3        |
| R2          | 700 ± 428             | 77.3                 | 4                    | 70                   | 29.8        |
| R3          | 1110 ± 877            | 81.4                 | 7.8                  | 80                   | 16.7        |

*CK activity is expressed in IU corresponding to 1 μmol of CP transphosphorylated/min/mg of protein, as measured by pH-stat assay (36). The values reported are the mean ± S.D. for nine determinations from several different SR vesicle preparations.

**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 ± S.D. of three determinations each from different preparations.

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Fig. 2. 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.

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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.

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TABLE II
Table: 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          | 3.93 ± 1.08                                                   |
| R4          | 6.55 ± 0.98                                                   |
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. 5A, arrowheads). The latter is seen more clearly at higher magnification (Fig. 5B, arrowheads). By contrast, no significant labeling was observed with preimmune antibody (Fig. 5C). 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 membrane 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. 6C). The labeling with anti-Ca\(^{2+}\)-ATPase antibodies appeared very strong and often clustered at the surface of the vesicles (Fig. 6B). 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. 6A). 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 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 \(\mu\)M concentration at the end of the Ca\(^{2+}\) uptake, ATP-supported Ca\(^{2+}\) loading was resumed upon a second addition of Ca\(^{2+}\) (Fig. 7B).

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. 7A 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 \(\mu\)M concentration at the end of the Ca\(^{2+}\) uptake, ATP-supported Ca\(^{2+}\) loading was resumed upon a second addition of Ca\(^{2+}\) (Fig. 7B).

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 pmol of Ca$^{2+}$·min$^{-1}$·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 μmol·min$^{-1}$·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 1,389 μmol of Ca$^{2+}$·min$^{-1}$·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 ± 0.138/2 = 0.8 ATP·min$^{-1}$·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, 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-
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**-ATPase during in vitro contraction of myofibrils (26). In the present experiments with SR, the Ca** 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** 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** 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**-ATPase at maximal speed (1.713 μmol of Ca**/min/mg of R2 protein; see Table III) shows that the two numbers are indeed very close, if an ideal coupling ratio of 2Ca**/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** uptake.

The fact that the ratio of bound CK to Ca**-ATPase activity is significantly higher in R3 and R4 (compare CK activity values in Table I versus Ca**-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** pump in vitro. In addition, the fact that the amount of CK activity is not proportional to the Ca**-ATPase content of the various SR subfractions (R1-R4), which suggests earlier by Baskin and Deamer (15) and Levitsky (16), may have additional physiological functions within the excitation contraction pathway other than simply supporting Ca** 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** 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**-loading experiments, using the coupled in vitro assay, started to reduce the Ca**-loading rate (not shown). A concentration of 0.5 mM ADP is well above the K 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** pump may be guaranteed.

Champeil et al. (39) have suggested that the SR Ca**-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 K (ADP)*/(ATP) (see Ref. 50). As a third possibility, CK may direct the ATP needed

| SR fraction (9 μg protein) | ATP | ADP | CP | Succinate | Rotenone | Exogenous CK added | Ca**-loading rate | ATP (1 mM) |
|--------------------------|-----|-----|----|----------|----------|------------------|-------------------|-----------|
|                          | mM  | μM  | μg |          |          |                  |                   |           |
| R2                       | 1   |     |    |          |          |                  | 1.713 ± 0.188     | 1.430 ± 0.120 |
| R2                       | 1   | 10  |    |          |          |                  | 1.395 ± 0.105     | 0.092 ± 0.010 |
| R2                       | 1   | 10  | 50 |          |          |                  | 0.050 ± 0.011     | 0          |
| R2                       | 1   | 10  | 50 |          |          |                  | 1.363 ± 0.125     | 0          |
| R3                       | 1   | 10  |    |          |          |                  | 1.27 ± 0.114      | 0          |
| R3                       | 1   | 10  |    |          |          |                  | 1.740 ± 0.120     | 0          |
| R3                       | 1   | 10  |    |          |          |                  | 0.351 ± 0.130     | 0          |
| R3                       | 1   | 10  |    |          |          |                  | 0.250 ± 0.115     | 0.288 ± 0.070 |
| R4                       | 1   | 10  |    |          |          |                  | 0.152 ± 0.040     | 0          |

* 0.1 mm ATP was added after inhibition of CK with DNFB.
+ Ca**-loading rate in the presence of 1 mM ATP was measured by adding ethanol (as a control), the solvent in which DNFB was dissolved.

** Values reported are the mean ± S.D. of at least 10 determinations each using different SR vesicle preparations.
to the protein kinases responsible for the phosphorylation of the Ca\textsuperscript{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 subfamily of the brain isoform B-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 \( \alpha \)-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 (40), which is in line with the above findings.

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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 Toku- yama, K. (1983) J. Biol. Chem. 258, 409–410
5. Jacobus, W. E., and Lehninger, A. L. (1973) J. Biol. Chem. 248, 4803–4810
6. Saks, V. A., Rosenstrauch, 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. Biol. Chem. 252, 287–317
10. Groos, R., Sjöblom, E., Kuipio, P., Vainio, K. M., 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 503, 495–501
12. Wallimann, T. (1978) Ph.D. thesis, 5437, Eidgenössische Technische Hochschule, Zurich, Switzerland
13. Beeman, 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, 1945–1947
16. Levitsky, D. O., Lovchenko, T. S., Saks, V. A., Sharov, V. G., and Smirnov, V. D. (1978) Membr. Biochem. 2, 91–96
17. Saito, A., Selier, S., Chu, A., and Fleischer, S. (1984) J. Biol. Chem. 259, 885–887
18. Dungan, P. N., and Martonoci, A. (1970) J. Gen. Physiol. 56, 146
19. Dawson, D. M., and Eppenberger, H. M. (1970) Methods Enzymol. 19, 995–1002
20. Lamml, U. K. (1970) Nature 227, 580–585
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., Hardwick, P. M. J., and Szent-Györgyi, A. G. (1982) J. Mol. Biol. 156, 151–173
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. Biol. Cell. 104, 461–472
26. Wallimann, T., Schlosser, T., and Eppenberger, H. M. (1977) J. Biol. Chem. 252, 5238–5246
27. Fleischer, S., and Kervins, M. (1974) Methods Enzymol. 31, 6–41
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Mitchell, R. D., Volpe, P., Palade, A., and Fleischer, S. (1983) J. Biol. Chem. 258, 9867–9877
30. Hummel, B., and Murer, M. (1986) Science of Biological Specimen Preparation, pp. 170–183, SEM Inc., AMF O'Hare, Chicago
31. Walther, P., and Murer, M. (1986) Science of Biological Specimen Preparation, pp. 195–201, SEM Inc., AMF O'Hare, Chicago
32. Meissner, G. (1981) Biochim. Biophys. Acta 659, 51–65
33. Zorzato, F., and Volpe, P. (1988) Arch. Biochem. Biophys. 263, 324–329
34. Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A., and Fleischer, S. (1986) J. Biol. Cell. 103, 741–753
35. Tomes, 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) FEMS Lett. 32, 293–296
37. Palmer, G., Horgan, D. J., Tisdale, H., Singer, T. P., and Beinert, H. (1986) J. Biol. Chem. 245, 844–847
38. Volpe, P., Gutwenger, H. E., and Montecucco, C. (1987) Arch. Biochem. Biophys. 253, 138–145
39. Champeil, P., Roletto, S., Orloski, S., Guillan, F., Seebergs, C. J., and McIntosh, D. B. (1988) J. Biol. Chem. 263, 10922–10924
40. Quest, A., Eppenberger, H. M., and Wallimann, T. (1987) Experientia (Basel) 43, 676 (abstr.)
41. Barrantes, F. J., Moser, H., and Eppenberger, H. M., and Barrantes, F. (1985) J. Cell Biol. 100, 1063–1072
42. Muller, M., Moser, R., Cheneval, D., and Carafoli, E. (1985) J. Biol. Chem. 260, 3839–3843
43. Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M., and Wallimann, T. (1988) J. Biol. Chem. 263, 16954–16962
44. Schmidt, T., Engell, A., Lustig, A., and Wallimann, T. (1988) J. Biol. Chem. 263, 16954–16962
45. Quest, A., Eppenberger, H. M., and Wallimann, T. (1987) Proceedings of the Ninth International Biophysics Congress, August 23–28, 1987, Jerusalem, Israel
46. 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. 12294–12301, Uninress, Padova, Italy
Muscle-type MM creatine kinase is specifically bound to sarcoplasmic reticulum and can support Ca2+ uptake and regulate local ATP/ADP ratios. A M Rossi, H M Eppenberger, P Volpe, R Cotrufo and T Wallimann

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