

# Ca<sup>2+</sup>-ATPase distributes differently in cardiac sarcolemma than dihydropyridine receptor $\alpha$ 1 subunit and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger

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**Abstract** We have investigated the distribution of the sarcolemmal Ca<sup>2+</sup> transporters in hamster and dog ventricular myocytes by immunocytochemical and membrane fractionation techniques. The data suggest that the DHP receptor  $\alpha$ 1 subunit and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger are present in surface sarcolemma as well as T-tubule membranes located at the cardiac dyads. Compared with these Ca<sup>2+</sup> transporters, the sarcolemmal Ca<sup>2+</sup>-ATPase is much less abundant in the latter fraction. Thus the sarcolemmal Ca<sup>2+</sup>-ATPase seems to be located predominantly in surface sarcolemma.

**Key words:** Sarcolemmal Ca<sup>2+</sup>-ATPase; Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; DHP receptor  $\alpha$ 1 subunit; Cardiac myocyte; Hamster heart; Canine heart

## 1. Introduction

In the myocardium, cyclic rise and fall of the cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) give rise to the contraction/relaxation cycle. Delivery of Ca<sup>2+</sup> to and removal from the cytoplasm in cardiomyocytes are mainly effected by Ca<sup>2+</sup> transporters in the SR. However, those in the sarcolemma also contribute significantly to this Ca<sup>2+</sup> handling. During each action potential, extracellular Ca<sup>2+</sup> enters the cells through voltage-dependent Ca<sup>2+</sup> channels in the sarcolemma, which triggers Ca<sup>2+</sup> release from the pool stored in the SR. In addition, Ca<sup>2+</sup> is extruded from cytoplasm via both the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and the sarcolemmal Ca<sup>2+</sup>-ATPase, as in other excitable cells.

The Na<sup>+</sup>/Ca<sup>2+</sup> exchanger is physiologically important, because in cardiomyocytes it is capable not only of rapidly extruding the Ca<sup>2+</sup> entering the cells via the sarcolemmal Ca<sup>2+</sup> channels during each action potential, but also of slowly removing cytoplasmic Ca<sup>2+</sup> during diastole [1,2]. The sarcolemmal Ca<sup>2+</sup>-ATPase, on the other hand, extrudes cytoplasmic Ca<sup>2+</sup> at a slow rate, and thus its role in myocardial Ca<sup>2+</sup> regulation has been obscure [1,2]. In the present study, we investigated the distribution of the sarcolemmal Ca<sup>2+</sup> transporters in cardiomyocytes to obtain insights into their roles in the Ca<sup>2+</sup> homeostasis.

## 2. Materials and methods

### 2.1. Isolation of membrane subfractions

Cardiac microsomes were prepared from hamster and canine ventricles as described previously [3,4]. Microsomes (1 mg/ml) were incubated at 37°C in 50 mM histidine-HCl (pH 7.0), 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 2.0 mM EGTA, 10 mM potassium oxalate, 5 mM NaN<sub>3</sub> and 0.3 mM ryanodine. 10 min later, Na<sub>2</sub>ATP was added to a final concentration of 3 mM and the incubation continued for additional 15 min. Microsomes were then centrifuged at 4°C for 15 min at 200,000 × *g*<sub>max</sub>. We used two different protocols for further subfractionation of microsomes. For hamster ventricles, the microsomes were resuspended in 0.4 ml of 0.25 M sucrose and 100 mM Tris-HCl (pH 7.0) and layered over a discontinuous sucrose gradient of 20, 30, 40, and 50% sucrose containing the same buffer (0.5 ml each). After the centrif-

ugation for 30 min at 200,000 × *g*<sub>max</sub>, protein was recovered from the respective sucrose layer and the pellet, the latter being resuspended in 0.5 ml of 0.25 M sucrose and 100 mM Tris-HCl (pH 7.0). Subfractions were numbered 1 to 6 from top to bottom. For the microsomes prepared from canine ventricles, the density gradient centrifugation was carried out for 60 min at 200,000 × *g*<sub>max</sub> using the buffered sucrose solutions additionally containing 0.3 M KCl and 50 mM sodium pyrophosphate [5].

### 2.2. Immunological quantification of Ca<sup>2+</sup> transporters in isolated subcellular fractions

Membrane protein (40 µg) from each subcellular fraction was subjected to SDS-PAGE on a 7.5% gel [3]. The proteins in the gel were transferred to Immobilon membranes (Millipore) and then treated with a specific antibody and a peroxidase-conjugated secondary antibody as in [3]. The protein recognized by each specific antibody was quantified by using an Amersham ECL immunoblotting detection system also as described in [3]. We used the following antibodies; mouse monoclonal antibodies (1B11, KY-1 [6] and 5F10 [7]) against the plasma membrane Ca<sup>2+</sup>-ATPase purified from human erythrocyte; mouse monoclonal antibody against the canine cardiac ryanodine receptor (RY3) [4]; rabbit polyclonal antibody against the rabbit  $\alpha$ 1 subunit of cardiac DHP receptors (CR2) [8]; and rabbit polyclonal antibody against dog cardiac Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. 1B11 and KY-1 were prepared according to the method described in [6]. To raise antibodies against the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, a MBP fusion protein construct was generated by subcloning a PCR-amplified region (nucleotides 844–2334 encoding amino acids 273–769) of the dog cardiac Na<sup>+</sup>/Ca<sup>2+</sup> exchanger cDNA [9] into the *mal E* gene of the pMAL-c vector (New England Biolabs). Soluble fusion protein was purified from transformed *Escherichia coli* (HB101 strain) according to the manufacturer's protocol. The antiserum was produced by immunizing rabbits with the fusion protein over a period of 2 months. We affinity-purified anti-Na<sup>+</sup>/Ca<sup>2+</sup> exchanger antibody by treating Immobilon membrane carrying the fusion protein overnight with antiserum in the presence of excess MBP, washing the Immobilon membrane with phosphate-buffered saline, and then eluting the antibody with 0.2 M glycine (pH 2). In our immunoblot assay of ventricular homogenates, all these antibodies recognized single proteins of the expected molecular weights for the target proteins (Fig. 1; see also [3]).

### 2.3. Immunofluorescence microscopy

Immunofluorescence staining and visualization of samples were performed as described in [10].

## 3. Results and discussion

Fig. 2 shows the distribution of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, the  $\alpha$ 1 subunit of DHP receptors (L-type Ca<sup>2+</sup> channels) and the sarcolemmal Ca<sup>2+</sup>-ATPase, as revealed by indirect

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**Abbreviations:** SR, sarcoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MBP, maltose binding protein.

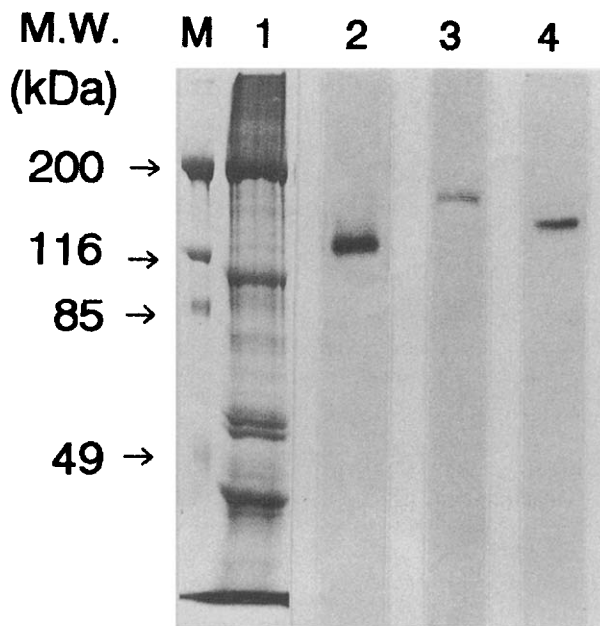


Fig. 1. Immunoblot analysis of hamster ventricular homogenates. Hamster ventricular homogenates were subjected to SDS-PAGE (100  $\mu$ g protein per lane) followed by immunoblot staining with anti- $\text{Na}^+/\text{Ca}^{2+}$  exchanger antibody (lane 2), anti-DHP receptor  $\alpha 1$  subunit antibody (lane 3) or anti-erythrocyte  $\text{Ca}^{2+}$ -ATPase antibody (5F10) (lane 4). For CBB (lane 1), the gel was stained with Coomassie brilliant blue. Molecular mass standards are shown on the left (M).

immunofluorescence microscopy of hamster ventricular myocytes. The antibodies against the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and the DHP receptor  $\alpha 1$  subunit stained both surface sarcolemma and cell interior. The surface sarcolemma was stained less intensely with anti-DHP receptor antibody than with anti- $\text{Na}^+/\text{Ca}^{2+}$  exchanger antibody, whereas the cell interior was stained more intensely with the former than the latter. The intercalated disks were stained with both antibodies. In the cell interior, we observed fluorescent projections from the sarcolemma running toward the center of cells (Fig. 2a and b, cross-section) and a regular striated pattern similar to the sarcomeric Z-line pattern of the myofibril (Fig. 2d and e, longitudinal section). A very similar striated staining pattern was observed when hamster ventricular myocytes were stained with RY3, an anti-ryanodine receptor antibody (data not shown). These immunofluorescence data strongly suggest that antibodies against the DHP receptor  $\alpha 1$  subunit and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger heavily stain T tubule membranes in hamster cardiomyocytes. Intriguingly, however, anti- $\text{Ca}^{2+}$ -ATPase antibody 5F10 stained only the surface sarcolemma under exactly the same staining conditions (Fig. 2c and f). We obtained the same results using other anti- $\text{Ca}^{2+}$ -ATPase antibodies 1B11 and KY1 (data not shown).

We also investigated the distribution of these three sarcolemmal  $\text{Ca}^{2+}$  transporters by subcellular fractionation of cardiac microsomes followed by immunoblot analysis (Figs. 3 and 4). We incubated the microsomes with  $\text{Ca}^{2+}$ , oxalate, and MgATP in the presence of a high concentration of ryanodine to load SR vesicles with the  $\text{Ca}^{2+}$ -oxalate and then centrifuged the microsomes on a discontinuous sucrose gradient. Previous data

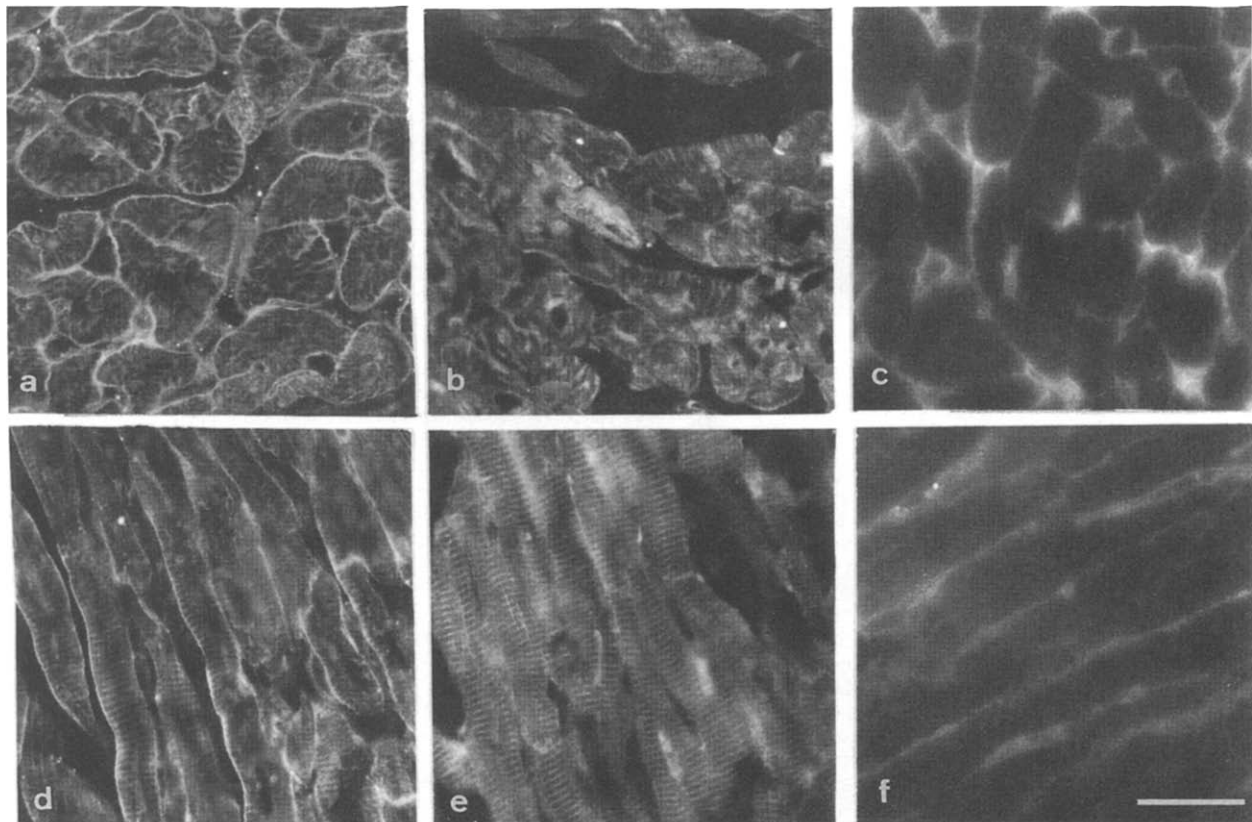


Fig. 2. Immunohistochemical localization of sarcolemmal  $\text{Ca}^{2+}$  transporters in hamster ventricular myocytes. Frozen cross- (a,b,c) or longitudinal- (d,e,f) sections of hamster ventricular muscle were labeled by indirect immunofluorescence. Sections were incubated with anti- $\text{Na}^+/\text{Ca}^{2+}$  exchanger antibody (a,d), anti-DHP receptor  $\alpha 1$  subunit antibody (b,e) or anti-erythrocyte  $\text{Ca}^{2+}$ -ATPase antibody 5F10 (c,f). Bar = 20  $\mu$ m.

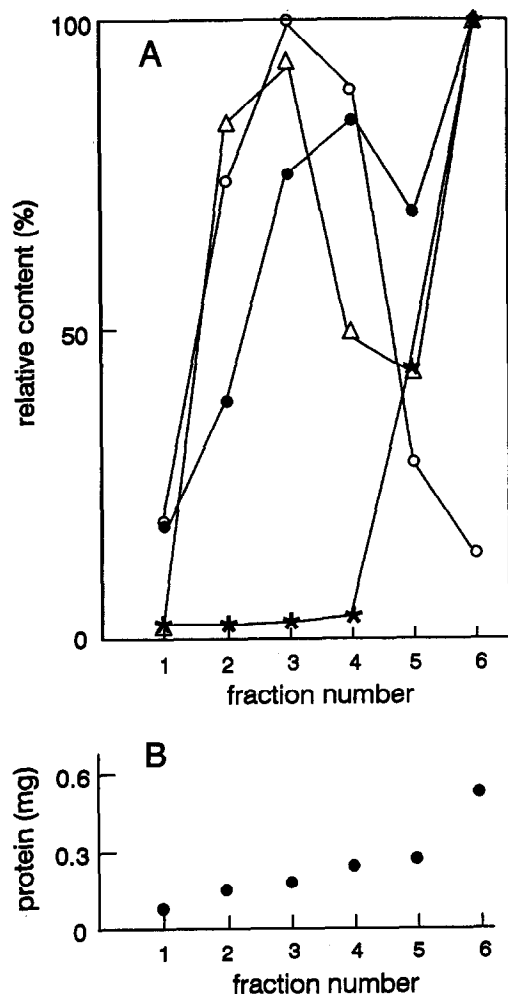


Fig. 3. Separation of subfractions from  $\text{Ca}^{2+}$ -oxalate-loaded cardiac microsomes prepared from hamster ventricles. The  $\text{Ca}^{2+}$ -oxalate-loaded microsomes prepared from hamster ventricles were centrifuged on a discontinuous sucrose gradient. (A) Each fraction (40  $\mu\text{g}$  protein) was subjected to SDS-PAGE followed by immunoblot staining with anti- $\text{Na}^+/\text{Ca}^{2+}$  exchanger antibody (●), anti-DHP receptor  $\alpha 1$  subunit antibody ( $\Delta$ ), anti-erythrocyte  $\text{Ca}^{2+}$ -ATPase antibody (5F10) ( $\circ$ ) and anti-ryanodine receptor antibody (\*). Immunoblots were quantified as described in section 2. The ordinate shows the relative content of a given  $\text{Ca}^{2+}$  transporter in 40  $\mu\text{g}$  protein from each fraction. (B) The amount of protein recovered in each fraction is shown.

obtained in similar subfractionation protocols indicated that surface sarcolemma is enriched in the light fractions and that the  $\text{Ca}^{2+}$ -oxalate-loaded SR vesicles are enriched in the pellet [5,11]. SR vesicles are known to be heterogenous, consisting of ryanodine-sensitive and -insensitive vesicles, which contain or lack the ryanodine receptor  $\text{Ca}^{2+}$  release channels, respectively [5,12]. The ryanodine-sensitive SR vesicles are presumably derived from the junctional SR, which, together with T-tubules, form the cardiac dyads. In the presence of a high concentration of ryanodine, both the ryanodine-sensitive and -insensitive SR vesicles accumulate the  $\text{Ca}^{2+}$ -oxalate [5]. Thus the portions of T-tubules associated with the junctional SR would be fractionated into the pellet, whereas the non-junctional T tubule membranes would be fractionated into the lighter fractions under the conditions used here.

Fig. 3 shows the result of sucrose density gradient centrifugation of the  $\text{Ca}^{2+}$ -oxalate-loaded microsomes from hamster ventricles. The DHP receptor  $\alpha 1$  subunit and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger were enriched in the pellet (fraction 6) as well as in the lighter fractions. In contrast, the sarcolemmal  $\text{Ca}^{2+}$ -ATPase was much less enriched in the pellet, as compared to other two  $\text{Ca}^{2+}$  transporters. The contents of the sarcolemmal  $\text{Ca}^{2+}$ -ATPase and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger relative to that of the DHP receptor  $\alpha 1$  subunit were  $1.17 \pm 0.16$  ( $n = 3$ ) and  $1.00 \pm 0.28$  ( $n = 3$ ) in fraction 3 and  $0.19 \pm 0.04$  ( $n = 3$ ) and  $1.00 \pm 0.01$  ( $n = 3$ ) in fraction 6, respectively. We detected the ryanodine receptor predominantly in the pellet by immunoblot analysis.

Fig. 4 shows the result of a similar experiment in which the  $\text{Ca}^{2+}$ -oxalate-loaded microsomes from canine ventricles were subfractionated using the protocol of Jones and Cala [5], which employed the buffered sucrose solutions containing pyrophosphate and high KCl (see section 2). Under these conditions, the DHP receptor  $\alpha 1$  subunit and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger again distributed in the light (fractions 2 and 3) and

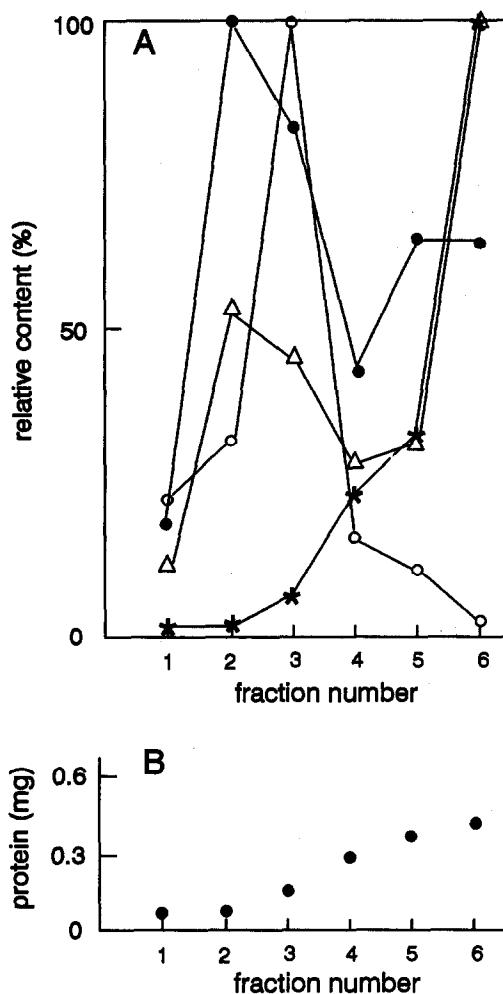


Fig. 4. Separation of subfractions from  $\text{Ca}^{2+}$ -oxalate-loaded cardiac microsomes prepared from canine ventricles. Experiments were performed as described in the legend to Fig. 3, except that microsomes were prepared from canine ventricles and that the sucrose solutions used for density gradient centrifugation contained 0.3 M KCl and 50 mM sodium pyrophosphate. Immunoblot staining was performed with anti- $\text{Na}^+/\text{Ca}^{2+}$  exchanger antibody (●), anti-DHP receptor  $\alpha 1$  subunit antibody ( $\Delta$ ), anti-erythrocyte  $\text{Ca}^{2+}$ -ATPase antibody (5F10) ( $\circ$ ) and anti-ryanodine receptor antibody (\*).

the pellet fractions and the sarcolemmal  $\text{Ca}^{2+}$ -ATPase was enriched only in the former (fraction 3). The ryanodine receptor was present mostly in the pellet.

These immunofluorescence and subfractionation data are consistent with a view that the DHP receptor  $\alpha 1$  subunit and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger are present in surface sarcolemma as well as T-tubule membranes located at the cardiac dyads. T-tubule membranes were successfully isolated from skeletal muscle, and the DHP receptors were shown to be enriched predominantly in these membranes, but not in membranes derived from the SR [12,13]. However, a comparable degree of purification of T-tubule membranes has not been achieved from cardiac muscle. Using these incompletely fractionated cardiac membranes and radiolabeled DHPs, cardiac DHP receptors were suggested to be localized at surface sarcolemma and T-tubules [11,14]. The cardiac  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, on the other hand, has recently been immunohistochemically localized to surface sarcolemma as well as T-tubules in guinea pig and rat heart [15,16].

In this study, we used three anti-erythrocyte  $\text{Ca}^{2+}$ -ATPase monoclonal antibodies (5F10, KY1, and 1B11) to detect the sarcolemmal  $\text{Ca}^{2+}$ -ATPase. The plasma membrane  $\text{Ca}^{2+}$ -ATPase is known to form a multigene family comprising at least four different genes, of which three have been reported to be expressed in rat adult cardiomyocytes [17]. The antibody 5F10 has been shown to react with the  $\text{Ca}^{2+}$ -ATPase in tissues from a wide variety of species [7]. In fact, according to a recent report by Caride et al. [18], all four  $\text{Ca}^{2+}$ -ATPase gene products contain homologous peptide sequences that correspond to the epitope for 5F10. Thus it is highly likely that 5F10 detects the presence of all the isoforms of the plasma membrane  $\text{Ca}^{2+}$ -ATPase in hamster and dog cardiomyocytes. Our findings that 5F10 did not stain intracellular structures (Fig. 2c and f) and that the material reacting with 5F10 was much less abundant in the pellets as compared to those reacting with antibodies against other  $\text{Ca}^{2+}$  transporters (Figs. 3 and 4) indicate that the  $\text{Ca}^{2+}$ -ATPase is located predominantly in the surface sarcolemma in the hamster and dog cardiomyocytes. A similar conclusion was also obtained with the antibody KY1. We previously characterized immunoreactivity of this antibody with the  $\text{Ca}^{2+}$ -ATPase in the sarcolemma purified from bovine ventricles and found that it immunoprecipitated 70–90% of the total sarcolemmal  $\text{Ca}^{2+}$ -ATPase activity [6]. Using KY1 and the  $\text{Ca}^{2+}$ -oxalate-loaded microsomes from bovine ventricles, we found that the distribution of the  $\text{Ca}^{2+}$ -ATPase is similar to that shown in Fig. 3 (Iwata Y. et al., unpublished observation).

All these results indicate that at least in hamster and dog ventricular myocytes the distribution of sarcolemmal  $\text{Ca}^{2+}$  transporters is not the same. These transporters contribute differently to the  $[\text{Ca}^{2+}]_i$  regulation in cardiomyocytes [1,2], as briefly discussed in section 1. During each cardiac cycle, the

entry and extrusion of  $\text{Ca}^{2+}$  across the sarcolemma occur predominantly via the DHP receptor  $\text{Ca}^{2+}$  channels and the  $\text{Na}^+/\text{Ca}^{2+}$  exchangers, respectively. On the other hand, the sarcolemmal  $\text{Ca}^{2+}$ -ATPase has been shown to extrude cytoplasmic  $\text{Ca}^{2+}$  at a rate significantly lower than the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger even under resting conditions (diastole) [2]. Since delivery of  $\text{Ca}^{2+}$  to the cytoplasm occurs predominantly from the junctional SR at the cardiac dyads, extrusion of cytoplasmic  $\text{Ca}^{2+}$  may also occur mainly from T-tubules at the same dyads. In this context, low abundance of the  $\text{Ca}^{2+}$ -ATPase relative to other  $\text{Ca}^{2+}$  transporters in T-tubule membranes at the dyads may at least partly account for the inability of this  $\text{Ca}^{2+}$ -ATPase to extrude cytoplasmic  $\text{Ca}^{2+}$  at a fast rate, though the low  $\text{Ca}^{2+}$  pumping activity may also be due to its low density in the sarcolemma [6].

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