FEBS Letters 355 (1994) 65–68

FEBS 14787

Ca^{2+}-ATPase distributes differently in cardiac sarcolemma than dihydropyridine receptor α1 subunit and Na^+/Ca^{2+} exchanger

Yuko Iwata[a], Hironori Hanada[a], Masami Takahashi[b], Munekazu Shigekawa[a,*]

[a]*Department of Molecular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan*
[b]*Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan*

Received 6 September 1994; revised version received 14 October 1994

Abstract We have investigated the distribution of the sarcolemmal Ca^{2+} transporters in hamster and dog ventricular myocytes by immunocytochemical and membrane fractionation techniques. The data suggest that the DHP receptor α1 subunit and the Na^+/Ca^{2+} exchanger are present in surface sarcolemma as well as T-tubule membranes located at the cardiac dyads. Compared with these Ca^{2+} transporters, the sarcolemmal Ca^{2+}-ATPase is much less abundant in the latter fraction. Thus the sarcolemmal Ca^{2+}-ATPase seems to be located predominantly in surface sarcolemma.

Key words: Sarcolemmal Ca^{2+}-ATPase; Na^+/Ca^{2+} exchanger; DHP receptor α1 subunit; Cardiac myocyte; Hamster heart; Canine heart

1. Introduction

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

The Na^+/Ca^{2+} exchanger is physiologically important, because in cardiomyocytes it is capable not only of rapidly extruding the Ca^{2+} entering the cells via the sarcolemmal Ca^{2+} channels during each action potential, but also of slowly removing cytoplasmic Ca^{2+} during diastole [1,2]. The sarcolemmal Ca^{2+}-ATPase, on the other hand, extrudes cytoplasmic Ca^{2+} at a slow rate, and thus its role in myocardial Ca^{2+} regulation has been obscure [1,2]. In the present study, we investigated the distribution of the sarcolemmal Ca^{2+} transporters in cardiomyocytes to obtain insights into their roles in the Ca^{2+} 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 $MgCl_2$, 1.8 mM $CaCl_2$, 2.0 mM EGTA, 10 mM potassium oxalate, 5 mM NaN_3 and 0.3 mM ryanodine. 10 min later, Na_2ATP 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 $200,000 \times g_{max}$. 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 centrifugation for 30 min at $200,000 \times g_{max}$, 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 $200,000 \times g_{max}$ using the buffered sucrose solutions additionally containing 0.3 M KCl and 50 mM sodium pyrophosphate [5].

2.2. Immunological quantification of Ca^{2+} 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^{2+}-ATPase purified from human erythrocyte; mouse monoclonal antibody against the canine cardiac ryanodine receptor (RY3) [4]; rabbit polyclonal antibody against the rabbit α1 subunit of cardiac DHP receptors (CR2) [8]; and rabbit polyclonal antibody against dog cardiac Na^+/Ca^{2+} exchanger. 1B11 and KY-1 were prepared according to the method described in [6]. To raise antibodies against the Na^+/Ca^{2+} 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^+/Ca^{2+} 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^+/Ca^{2+} 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^+/Ca^{2+} exchanger, the α1 subunit of DHP receptors (L-type Ca^{2+} channels) and the sarcolemmal Ca^{2+}-ATPase, as revealed by indirect

*Corresponding author. Fax: (81) (6) 872-7485.

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

SSDI 0014-5793(94)01174-5

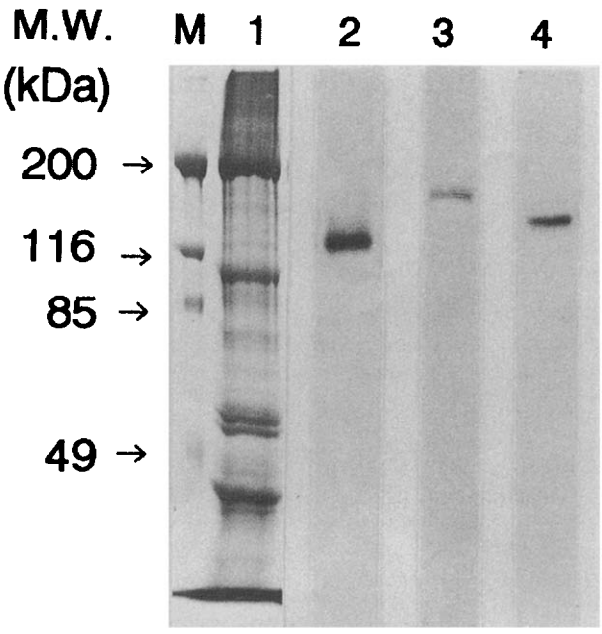

Fig. 1. Immunoblot analysis of hamster ventricular homogenates. Hamster ventricular homogenates were subjected to SDS-PAGE (100 μg protein per lane) followed by immunoblot staining with anti-Na^+/Ca^{2+} exchanger antibody (lane 2), anti-DHP receptor α1 subunit antibody (lane 3) or anti-erythrocyte Ca^{2+}-ATPase 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 Na^+/Ca^{2+} exchanger and the DHP receptor α1 subunit stained both surface sarcolemma and cell interior. The surface sarcolemma was stained less intensely with anti-DHP receptor antibody than with anti-Na^+/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 α1 subunit and the Na^+/Ca^{2+} exchanger heavily stain T tubule membranes in hamster cardiomyocytes. Intriguingly, however, anti-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-Ca^{2+}-ATPase antibodies 1B11 and KY1 (data not shown).

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

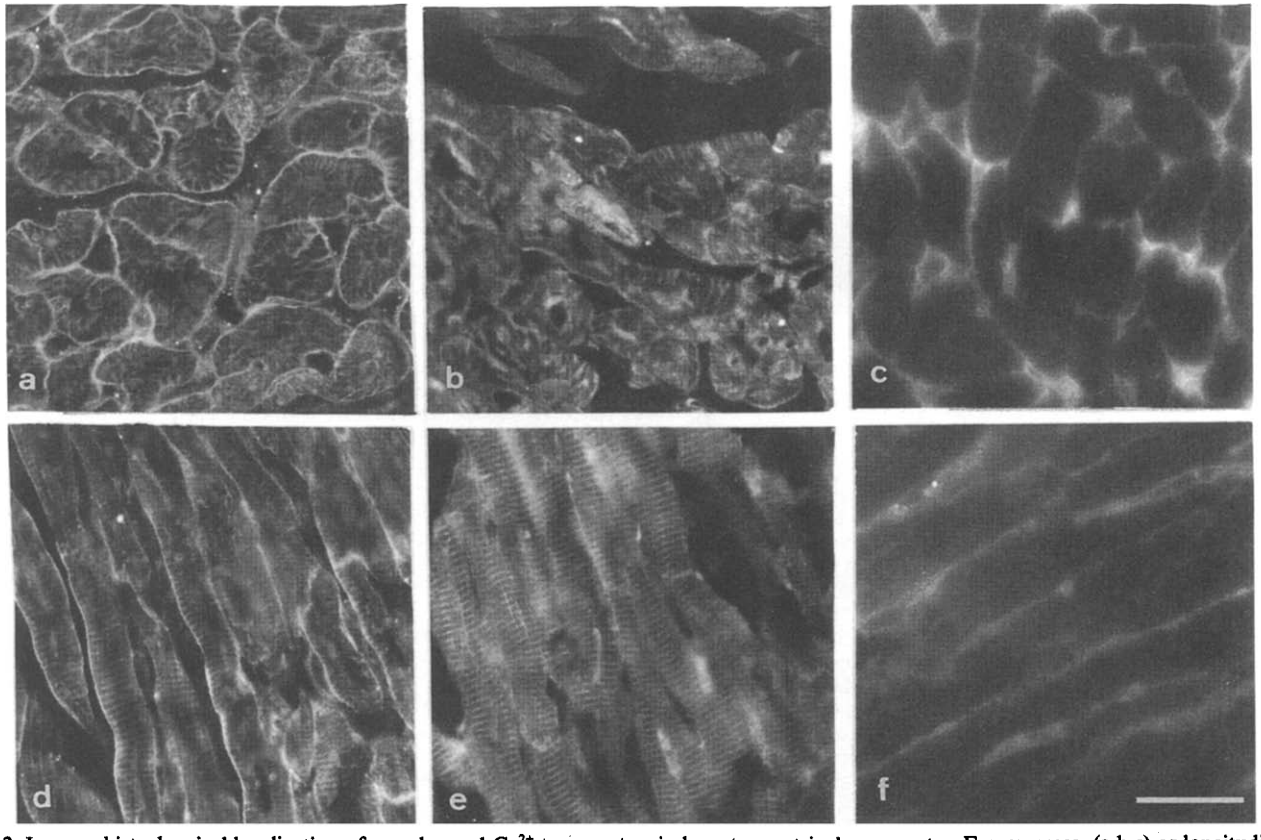

Fig. 2. Immunohistochemical localization of sarcolemmal Ca^{2+} transporters in hamster ventricular myocytes. Frozen cross- (a,b,c) or longitudinal- (d,e,f) sections of hamster ventricular muscle were labeled by indirect immunofluorescence. Sections were incubated with anti-Na^+/Ca^{2+} exchanger antibody (a,d), anti-DHP receptor α1 subunit antibody (b,e) or anti-erythrocyte Ca^{2+}-ATPase antibody 5F10 (c,f). Bar = 20 μm.

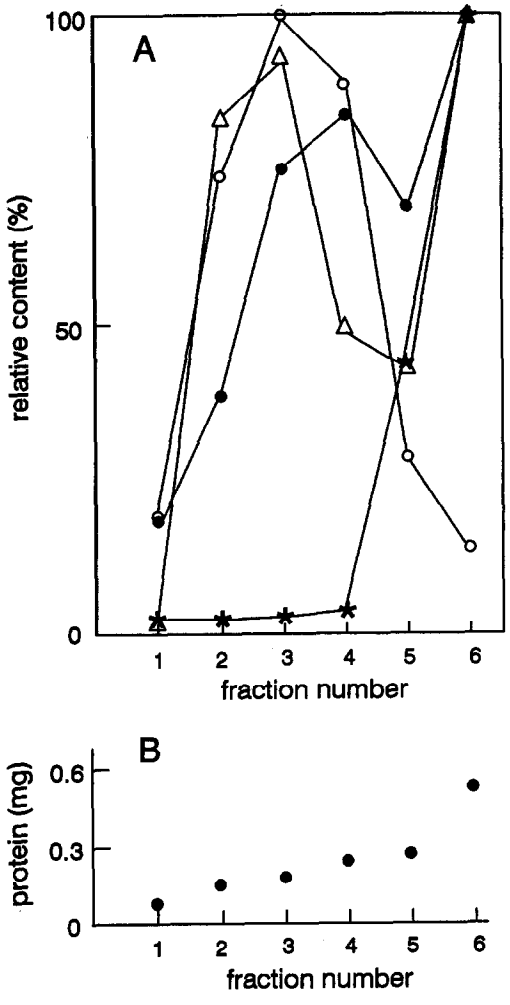

Fig. 3. Separation of subfractions from Ca^{2+}-oxalate-loaded cardiac microsomes prepared from hamster ventricles. The Ca^{2+}-oxalate-loaded microsomes prepared from hamster ventricles were centrifuged on a discontinuous sucrose gradient. (A) Each fraction (40 μg protein) was subjected to SDS-PAGE followed by immunoblot staining with anti-Na^+/Ca^{2+} exchanger antibody (●), anti-DHP receptor α1 subunit antibody (△), anti-erythrocyte Ca^{2+}-ATPase antibody (5F10) (○) and anti-ryanodine receptor antibody (*). Immunoblots were quantified as described in section 2. The ordinate shows the relative content of a given Ca^{2+} transporter in 40 μg 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 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 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 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 Ca^{2+}-oxalate-loaded microsomes from hamster ventricles. The DHP receptor α1 subunit and the Na^+/Ca^{2+} exchanger were enriched in the pellet (fraction 6) as well as in the lighter fractions. In contrast, the sarcolemmal Ca^{2+}-ATPase was much less enriched in the pellet, as compared to other two Ca^{2+} transporters. The contents of the sarcolemmal Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger relative to that of the DHP receptor α1 subunit were 1.17 ± 0.16 ($n = 3$) and 1.00 ± 0.28 ($n = 3$) in fraction 3 and 0.19 ± 0.04 ($n = 3$) and 1.00 ± 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 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 α1 subunit and the Na^+/Ca^{2+} exchanger again distributed in the light (fractions 2 and 3) and

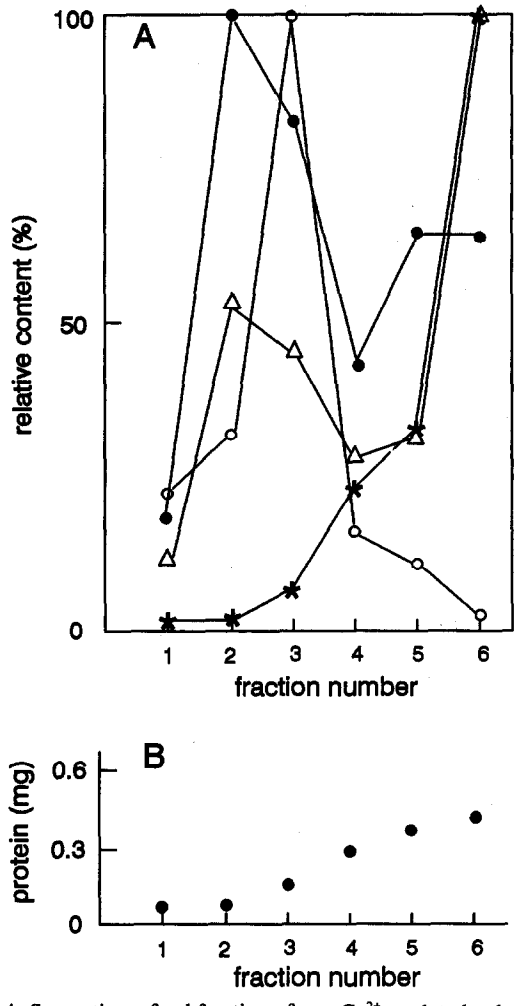

Fig. 4. Separation of subfractions from Ca^{2+}-oxalate-loaded cardiac microsomes prepared from canine ventricles. Experiments were performed as described in the legend to Fig. 3, except that microsomes were prepared from canine ventricles and that the sucrose solutions used for density gradient centrifugation contained 0.3 M KCl and 50 mM sodium pyrophosphate. Immunoblot staining was performed with anti-Na^+/Ca^{2+} exchanger antibody (●), anti-DHP receptor α1 subunit antibody (△), anti-erythrocyte Ca^{2+}-ATPase antibody (5F10) (○) and anti-ryanodine receptor antibody (*).

the pellet fractions and the sarcolemmal 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 α1 subunit and the Na^+/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 Na^+/Ca^{2+} exchanger, on the other hand, has recently been immnohistochemically 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 Ca^{2+}-ATPase monoclonal antibodies (5F10, KY1, and 1B11) to detect the sarcolemmal Ca^{2+}-ATPase. The plasma membrane 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 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 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 Ca^{2+}-ATPase in hamster and dog cardiomyoctes. 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 Ca^{2+} transporters (Figs. 3 and 4) indicate that the 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 Ca^{2+}-ATPase in the sarcolemma purified from bovine ventricles and found that it immunoprecipitated 70–90% of the total sarcolemmal Ca^{2+}-ATPase activity [6]. Using KY1 and the Ca^{2+}-oxalate-loaded microsomes from bovine ventricles, we found that the distribution of the 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 Ca^{2+} transporters is not the same. These transporters contribute differently to the $[Ca^{2+}]_i$ regulation in cardiomyocytes [1,2], as briefly discussed in section 1. During each cardiac cycle, the entry and extrusion of Ca^{2+} across the sarcolemma occur predominantly via the DHP receptor Ca^{2+} channels and the Na^+/Ca^{2+} exchangers, respectively. On the other hand, the sarcolemmal Ca^{2+}-ATPase has been shown to extrude cytoplasmic Ca^{2+} at a rate significantly lower than the Na^+/Ca^{2+} exchanger even under resting conditions (diastole) [2]. Since delivery of Ca^{2+} to the cytoplasm occurs predominantly from the junctional SR at the cardiac dyads, extrusion of cytoplasmic Ca^{2+} may also occur mainly from T-tubules at the same dyads. In this context, low abundance of the Ca^{2+}-ATPase relative to other Ca^{2+} transporters in T-tubule membranes at the dyads may at least partly account for the inability of this Ca^{2+}-ATPase to extrude cytoplasmic Ca^{2+} at a fast rate, though the low Ca^{2+} pumping activity may also be due to its low density in the sarcolemma [6].

Acknowledgements: We thank Dr. J.T. Penniston of the Mayo Clinic/Foundation for generously providing 5F10.

References

[1] Wier, W.G. (1990) Annu. Rev. Physiol. 52, 467–485.
[2] Bers, D.M. (1991) Ann. NY Acad. Sci. 639, 375–385.
[3] Tawada-Iwata, Y., Imagawa. T., Yoshida, A., Takahashi, M., Nakamura, H. and Shigekawa, M. (1993) Am. J. Physiol. 264, H1447–1453.
[4] Imagawa, T., Takasago, T. and Shigekawa, M. (1989) J. Biochem. (Tokyo) 106, 342–348.
[5] Jones, L.R. and Cala, S.E. (1981) J. Biol. Chem. 256, 11809–11818.
[6] Ogurusu, T., Wakabayashi, S., Furukawa, K., Tawada-Iwata, Y., Imagawa, T. and Shigekawa, M. (1990) J. Biochem. (Tokyo) 108, 222–229.
[7] Borke, J.L., Caride, A., Verma, A.K., Penntston, J.T. and Kumar, R. (1989) Am. J. Physiol. 257, F842–F849.
[8] Yoshida, A., Takahashi, M., Nishimura, S., Takeshima, H. and Kokubun, S. (1992) FEBS Lett. 309, 343–349.
[9] Nakasaki, Y., Iwamoto, T., Hanada, H., Imagawa, T. and Shigekawa, M. (1993) J. Biochem. (Tokyo) 114, 528–534.
[10] Iwata, Y., Nakamura, H., Mizuno, Y., Yoshida, M., Ozawa, E. and Shigekawa, M. (1993) FEBS lett. 329, 227–231.
[11] Williams, L.T. and Jones, L.R. (1983) J. Biol. Chem. 258, 5344–5347.
[12] Fleischer, S. and Inui, M. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 333–364.
[13] Fosset, M., Jaimovich, E., Delpont, E. and Lazdunsky, M. (1983) J. Biol. Chem. 258, 6086–6092.
[14] Brandt, N. and Bassett, A. (1986) Arch. Biochem. Biophys. 244, 872–875.
[15] Frank, J.S., Mottino G., Reid, D., Molday, R.S. and Philipson K.D. (1992) J. Cell. Biol. 117, 337–345.
[16] Kieval, R.S., Bloch, R.J., Lindenmayer, G.E., Ambesi, A. and Lederer, W.J. (1992) Am. J. Physiol. 263, C545–C550.
[17] Hammes, A., Oberdorf, S., Strechler, E. E., Stauffer, T., Carafoli, E., Vetter, H. and Neyses, L. (1994) FASEB J. 8, 428–435.
[18] Caride, A.J., Filoteo, A.G., Enyedi, A. and Penniston, J.T. (1994) Biophys. J. 66, A120.