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Ca-ATPase Activity and Protein Composition of Sarcoplasmic Reticulum Membranes Isolated from Skeletal Muscles of Typical Hibernator, the Ground Squirrel *Spermophilus undulatus*

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Ca-ATPase activity in sarcoplasmic reticulum (SR) membranes isolated from skeletal muscles of the typical hibernator, the ground squirrel *Spermophilus undulatus*, is about 2-fold lower than that in SR membranes of rats and rabbits and is further decreased 2-fold during hibernation. The use of carbocyanine anionic dye Stains-All has revealed that Ca-binding proteins of SR membranes, histidine-rich Ca-binding protein and sarcalumenin, in ground squirrel, rat, and rabbit SR have different electrophoretic mobility corresponding to apparent molecular masses 165, 155, and 170 kDa and 130, 145, and 160 kDa, respectively; the electrophoretic mobility of calsequestrin (63 kDa) is the same in all preparations. The content of these Ca-binding proteins in SR membranes of the ground squirrels is decreased 3–4 fold and the content of 55, 30, and 22 kDa proteins is significantly increased during hibernation.

KEY WORDS: Sarcoplasmic reticulum; Ca-ATPase; calsequestrin; sarcalumenin; histidine-rich Ca-binding protein; ground squirrel *Spermophilus undulatus*; hibernation

INTRODUCTION

For many small mammals winter survival can be assured by a special physiological state of organism, the hibernation (Lyman *et al.*, 1982; Kalabukhov, 1985). In response to cold temperatures, shortening of day light, and food restriction animals enter into deep torpor state which is accompanied by the strong metabolic rate depression (often to only 1–5% of normal values) and a reduction in body temperature to near ambient (0–5°C) (Panteleev, 1983; Wang, 1985). Hibernation is not a discontinuous process but consists of a number of periods of deep torpor lasting 2–4 weeks (so-called hibernation bouts) with short (20–30 hr) intervals (bouts) of awakening between them. During these awakening bouts the contractile activity of

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skeletal muscles is shifted from totally inactive state to practically normal functioning. Therefore, all enzyme systems responsible for muscle contraction should be adapted for very rapid changes of their activity during short-time intervals when body temperature is increased from 0 to 37°C in 4–6 hr during arousals.

Sarcoplasmic reticulum (SR), a highly developed intracellular membrane network, is one of these systems. Ca-release channels (ryanodine receptors) located in the junction facing membranes of SR terminal cisterns in close proximity to T-tubules provide fast efflux from the SR lumen of the Ca^{2+} necessary for muscle contraction, whereas Ca-ATPase located in SR longitudinal tubules removes Ca^{2+} from cytoplasm using the energy of ATP hydrolysis that leads to the relaxation of muscles (MacLennan *et al*., 1997; Ogawa *et al*., 1999). Functional activities of SR Ca-release channels and Ca-ATPase are regulated, in particular, via interaction with various cytoplasmic and SR membrane proteins (Mackrill, 1999). Since SR plays a key role in excitation-contraction coupling in different types of muscles its protein composition and functional characteristics in hibernators are, probably, different from those of nonhibernating species.

For instance, in the heart of two hibernators, the ground squirrels *Spermophilus richardsonii* and *Spermophilus columbianus*, the new unique isoform of SR Ca-binding protein calsequestrin was found (Milner *et al*., 1999). This isoform is different from all known cardiac and skeletal muscle calsequestrin isophorms by its electrophoretic mobility, *N*-terminal sequence, and glycosylation pattern. Moreover, cardiac SR membranes of these hibernators contain abnormally high number of Ca-release channel molecules with unusual Ca-sensitivity. An increased Ca-accumulating ability of cardiac SR during hibernation period was suggested for heart of chipmunk (Kondo, 1988) and was experimentally shown for heart of the ground squirrel *S. richardsonii* (Belke *et al*., 1987). This directly demonstrates that cardiac SR functional activity is changed in dependence of the physiological state of hibernators.

In contrast to contractile activity of heart that is decreased during hibernation bouts to a few beating per minute, practically all skeletal muscles are totally inactive during torpor state. Therefore, it could be suggested that the functional activity of skeletal muscle SR is also changed during hibernation, probably, even in a deeper extent than that of cardiac SR.

Until now the information about possible adaptations in the properties of skeletal muscle SR for hibernation is practically absent. Therefore, the main goal of the present study was to analyze the Ca-ATPase activity and protein composition of SR membranes obtained from the skeletal muscles of summer active and winter hibernating ground squirrels *Spermophilus undulatus* and to compare these parameters with those of nonhibernators—rats and rabbits.

METHODS

Adult ground squirrels *S. undulatus* were collected by live trapping in Yakutiya and were maintained in the Animal Facility of the Institute of Cell Biophysics (Pushchino, Moscow Region) in individual cages at 20–25°C, in natural daylight. The animals were supplied with satisfactory food, water, and nest material. In November, animals were put into the dark room at temperature 2–4°C. For experiments,

summer active (body temperature 37°C) and winter hibernating (body temperature 2–5°C) animals were killed by decapitation in June–July and in January–February in the middle of hibernation bout, respectively. Hind leg skeletal muscles were immediately cut off and plunged into liquid nitrogen for transportation. For long storage (for 2–4 weeks), tissues were transferred into a deep freezer (below −70°C). SR fragments from hind leg skeletal muscles of ground squirrels, rats, and rabbits were obtained by differential centrifugation (Ritov *et al*., 1977) with minor modifications described earlier (Shutova *et al*., 1999). In control experiments, skeletal muscles from rat and rabbit were similarly frozen and kept for 1–4 weeks at −70°C. Comparative analysis of SR preparations obtained from frozen and fresh muscles has shown that this treatment does not affect the general SR parameters analyzed in the present study. The final SR preparations were frozen in liquid nitrogen and stored at −70°C.

Protein concentration was measured according to Lowry *et al*. (1951) using BSA as a standard. Ca-ATPase activity was measured using a coupled enzyme system (pyruvate kinase + lactate dehydrogenase) as described in full details earlier (Shorina *et al*., 1997). SDS-PAGE was carried out according to Laemmli (1970) using 3% stacking and 3–20% gradient running gels (Shorina *et al*., 1997). Myosin heavy chains (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (67 kDa), ovalbumin (45 kDa), and carboanhydrase (29 kDa) were used as protein markers for measurement of molecular masses of SR proteins. After electrophoresis the gels were fixed and washed three times in 25% ethanol and stained with the cationic carbocyanine dye, Stains-All, in a solution containing 25% ethanol, 7.5% formamide, 0.0025% Stains-All, and 30 mM Tris, pH 8.8, for 36–48 hr in the dark. The gels were briefly destained in 25% ethanol in the dark and scanned on an UltroScan XL laser densitometer at 595 nm (LKB, Sweden). The gels were finally destained in 25% ethanol in the light. Subsequently, the gels were stained with Coomassie Brilliant Blue R-250 and destained by a standard procedure. Then they were scanned on an UltroScan XL laser densitometer at the same wavelength. The protein peak areas were calculated using the GelScanXL program (LKB, Sweden).

Each parameter for every individual SR preparation was measured at least three times and the mean value for this parameter was calculated for further statistical calculations. In the tables, the mean values for a number of individual SR preparations isolated from different animals are presented ± standard deviation (mean ± SD). The number of preparations used is indicated in parenthesis. Statistical calculations were made using Student's *t*-criterion.

RESULTS AND DISCUSSION

The properties of SR membrane preparations isolated from skeletal muscles of summer active ground squirrels were compared with those of well-characterized SR preparations isolated from skeletal muscles of rats and rabbits. It was found that specific activity of Ca-ATPase, the main protein component of SR membranes, calculated with the correction on the content of Ca-ATPase protein in SR membranes

Table 1. The Content of the Main Protein Components in SR Membranes of Rats, Rabbits, Summer Active (SA), and Winter Hibernating (WH) Ground Squirrels (mean ± SD).

SR proteins	SA ($n = 6$)	Rats ($n = 4$)	Rabbits ($n = 4$)	WH ($n = 8$)
Stains-All staining				
Histidine-rich Ca-binding protein	100.0 ± 9.1	89.5 ± 5.1	115.8 ± 10.5	39.8 ± 6.7***
Sarcalumenin	100.0 ± 6.4	133.3 ± 14.1**	211.1 ± 55.5***	39.9 ± 4.8***
Calsequestrin	100.0 ± 5.0	70.1 ± 4.5**	258.4 ± 8.2***	28.7 ± 2.8***
Coomassie R-250 staining				
Ryanodine receptor	2.0 ± 0.4	1.3 ± 0.7	2.9 ± 0.5	1.0 ± 0.4**
Histidine-rich Ca-binding protein	1.6 ± 0.1	1.3 ± 0.1	1.0 ± 0.1**	0.6 ± 0.1***
Sarcalumenin	2.0 ± 0.1	2.0 ± 0.3	4.0 ± 0.4***	0.6 ± 0.1***
205 kDa protein	4.1 ± 1.0	0.8 ± 0.4**	< 0.1***	4.7 ± 2.2
Ca-ATPase	42.0 ± 3.1	57.2 ± 2.5**	55.7 ± 0.8**	29.4 ± 2.8**
Calsequestrin	10.3 ± 1.1	7.4 ± 0.7	16.7 ± 0.4***	3.3 ± 0.5***
55 kDA protein	5.7 ± 0.8	3.7 ± 0.6	4.7 ± 1.1	8.7 ± 0.9**
30 kDA protein	11.0 ± 1.9	7.0 ± 0.7	2.1 ± 0.2***	17.0 ± 0.9**
22 kDa protein	4.7 ± 1.6	2.0 ± 0.5*	4.5 ± 2.0	10.6 ± 2.7**

Note: Data obtained from scanning gels stained by the carbocyanine dye Stains-All are expressed relative to the mean intensity for the peak area of each protein in SR preparations of summer active ground squirrels. Data obtained after staining gels with Coomassie Brilliant Blue R-250 are expressed as percentages of the total SR protein peak areas. The number of SR preparations used for analysis is shown in parenthesis. Asterisks indicate values that are significantly different from the corresponding value for SR preparations of summer active ground squirrels.

*$p < 0.1$.
**$p < 0.05$.
***$p < 0.01$.

(see Table 1), was about 2-fold lower in SR preparations of the ground squirrels than that in SR preparations of rats and rabbits (14.2 ± 0.4, 20.3 ± 2.5, and 24.3 ± 2.2 μmole/min per mg of Ca-ATPase protein, respectively).

The protein composition of SR membranes isolated from different species of animals was significantly different (Table 1). For instance, unusually high content of protein with molecular mass 205 kDa was found in SR preparations of the ground squirrels. An electrophoretic mobility of this protein was identical to that of myosin heavy chains. In SR preparations of rats and rabbits this protein was absent. In general, protein composition of SR membranes of rats and ground squirrels was similar. SR preparations of rabbits were characterized by high content of Ca-binding proteins, calsequestrin, sarcalumenin, and histidine-rich Ca-binding protein, and low content of unidentified protein with molecular mass 30 kDa (Table 1).

Calsequestrin, sarcalumenin, and histidine-rich Ca-binding protein were identified in SR preparations with the use of carbicyanine anionic dye Stains-All (Campbell *et al.*, 1983; Orr and Shoshan-Barmatz, 1996). In SR preparations only these three proteins were stained with this dye in dark blue color whereas all other proteins were stained in red color. All these proteins are glycoproteins, are localized in SR lumen, bind Ca^{2+} with relatively low affinity ($K_d \sim 1$ mM) and probably are Ca-buffers lowering free Ca^{2+} concentration in SR lumen. Sarcalumenin and histidine-rich Ca-binding protein are also involved into regulation of SR Ca-release

channels: their phosphorylation by endogenous protein kinase leads to channel inhibition (Orr and Shoshan-Barmatz, 1996; Shoshan-Barmatz *et al.*, 1996).

As shown in Fig. 1, on densitograms of ground squirrel, rat, and rabbit SR preparations stained by Stains-All only three protein bands are stained in dark blue color. These are calsequestrin with molecular mass 63 kDa the content of which is slightly lower in rat SR and 2–2.5-fold higher in rabbit SR than that in ground squirrel SR and two high-molecular weight proteins, sarcalumenin and histidine-rich Ca-binding protein, the electrophoretic mobility of which is different in preparations from these three animal species. These two proteins are, probably, presented in SR

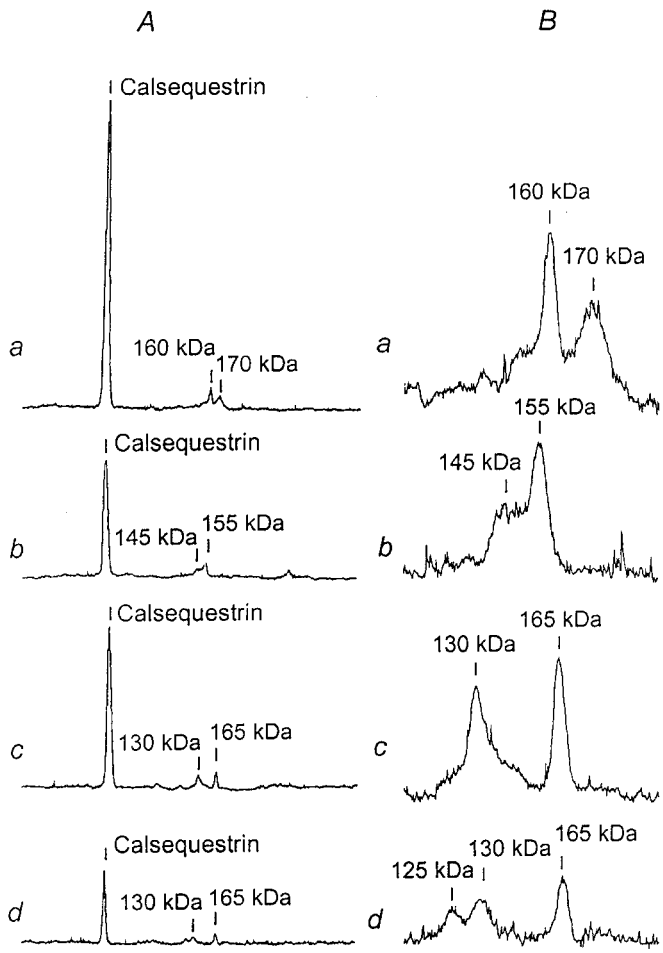

Fig. 1. (A) Densitograms of SR preparations from skeletal muscle of rabbits (a), rats (b), summer active (c), and winter-hibernating (d) ground squirrels, separated by SDS-PAGE and stained by Stains-All. (B) An enhanced view of the region containing proteins of 100–200 kDa. In each case 30 μg of the SR protein was loaded.

membranes of ground squirrels, rats, and rabbits by different isoforms with apparent molecular masses 130, 145, and 160 kDa and 165, 155, and 170 kDa, respectively, for sarcaluymenin and histidine-rich Ca-binding protein (Fig. 1, B). The content of histidine-rich Ca-binding protein is practically the same in SR preparations of different animal species, and the content of sarcalumenin is slightly higher in SR of rats and significantly higher in SR of rabbits in comparison with SR of the ground squirrels (Table 1).

Therefore, an electrophoretic analysis shows that the protein composition of SR membranes from skeletal muscles of two species from order *Rodentia*, ground squirrels and rats, is very similar in general (Table 1; Fig. 1), but specific activity of Ca-ATPase in these preparations is significantly different. Protein composition of SR preparations of rabbits (order *Lagomorpha*) is significantly different from that of ground squirrels and rats, but Ca-ATPase specific activity is practically the same in SR membranes of rabbits and rats. It could be suggested that low specific Ca-ATPase activity in SR preparations of the ground squirrels is connected with the properties of particular enzyme isoform that is present in skeletal muscles of these animals. However, some other factors, in particular, different minor proteins of SR membranes, could affect the enzyme activity.

It was found also that specific Ca-ATPase activity in SR membranes of winter hibernating ground squirrels is about 2-fold lower than that in SR of summer active animals (7.7 ± 1.1 vs. 14.2 ± 0.4 μmole/min per mg Ca-ATPase protein, respectively). In addition, the protein composition of SR membranes of winter hibernating ground squirrels is significantly different from that of summer active ones. The content of 450 kDa protein, calsequestrin, sarcalumenin, and histidine-rich Ca-binding protein is significantly lower and the content of proteins with molecular masses 55, 30, and 22 kDa is significantly higher in SR of winter-hibernating animals (Table 1; Fig. 2).

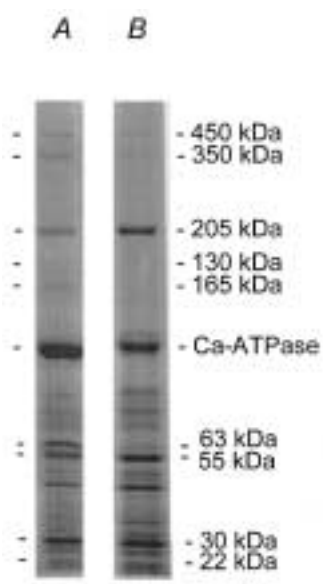

Fig. 2. SDS-PAGE of SR preparations from skeletal muscle of summer active (A) and winter hibernating (B) ground squirrels stained by Coomassie R-250. In each case 30 μm of the SR protein was loaded.

Calsequestrin, sarcalumenin, and histidine-rich Ca-binding protein which are stained in dark blue color by Stains-all in SR preparations from summer active and winter hibernating ground squirrels have the same electrophoretic mobility but their content is 3–4-fold lower in SR preparations of winter hibernating animals (Table 1). In addition, the new protein band with apparent molecular mass 125 kDa that is also stained in dark blue color appears in SR preparations of winter hibernating ground squirrels (Fig. 1). Probably, this is proteolytic fragment of sarcalumenin or its modifications with altered carbohydrate parts.

An unequivocal identification of other proteins, the content of which in SR preparations of summer active and winter hibernating ground squirrels is significantly different, is rather complicated. The 55 kDa protein is, the most probably, Ca-binding protein calreticulin which is stained in red by Stains-All (Krause and Michalak, 1997). It should be noted that the ratio of two main SR Ca-binding proteins, calsequestrin and calreticulin, is changed during skeletal muscle development. If on the early stages of embryonic development calreticulin is dominant Ca-binding protein of SR membranes, in mature skeletal muscle fibers it is replaced by calsequestrin (Koyabu *et al.*, 1994). It can suggest that the opposite process occurs during hibernation: the decrease of calsequestrin content in SR membranes during skeletal muscle disuse is accompanied by an increase of the content of calreticulin (Table 1).

The protein with molecular mass 450 kDa is, probably, ryanodine receptor, protein with molecular mass 30 kDa—calsequestrin-binding protein involved into regulation of SR, Ca-release channel (Kagari *et al.*, 1996). Because the operation of SR Ca-release channels is controlled via interaction with different proteins (Mackrill, 1999), the content of many of which is changed in SR membranes of winter hibernating ground squirrels (calsequestrin, sarcalumenin, histidine-rich Ca-binding protein, and, probably, 30 kDa calsequestrin-binding protein) it can suggest that the functional activity of SR Ca-release channels should be altered in hibernating animals. However, this question needs special investigation. Activity of Ca-ATPase in SR membranes is also controlled via interaction with a number of proteins, such as phospholamban (Simmerman and Jones, 1998), sarcolipin (Odermatt *et al.*, 1998), and calreticulin (Krause and Michalak, 1998). Therefore, it can suggest that the low level of Ca-ATPase specific activity in SR membranes of winter hibernating ground squirrels is connected, at least in part, with the changes in protein composition of SR membranes.

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REFERENCES

Belke, D. D., Pehowich, D. J., and Wang, L. C. H. (1987). *J. Therm. Biol.* **12:**53–56.
Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1983) *J. Biol. Chem.* **258:**11267–11273.

Kagari, T., Yamaguchi, N., and Kasai, M. (1996) *Biochem. Biophys. Res. Commun.* **227:**700–706.
Kalabukhov, N. I. (1985) *Hibernation of Mammals*, Nauka, Moscow.
Kondo, N. (1988) *Br. J. Pharmacol.* **95:**1287–1291.
Koyabu, S., Imanaka-Yoshida, K., Ioshi, S. O., Nakano, T., and Yoshida, T. (1994) *Cell Motility & Cytoskeleton* **29:**259–270.
Krause, K.-H. and Michalak, M. (1997) *Cell* **88:**439–443.
Laemmli, U. K. (1970) *Nature* **227:**680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193:**265–275.
Lyman, C. P., Willis, J. S., Malan, A., and Wang, L. C. H. (1982) *Hibernation and Torpor in Mammals and Birds*, Academic Press, New York.
Mackrill, J. J. (1999) *Biochem. J.* **337:**349–361.
MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) *J. Biol. Chem.* **246:**2702–2710.
Milner, R. E., Michalak, M., and Wang, L. C. H. (1991) *Biochim. Biophys. Acta* **1063:**120–128.
Odermatt, A. *et al.* (1998) *J. Biochem. Chem.* **273:**12360–12369.
Ogawa, Y., Kurebayashi, N., and Murayama, T. (1999) *Adv. Biophys.* **36:**27–64.
Orr, I., and Shoshan-Barmatz, V. (1996) *Biochim. Biophys. Acta* **1283:**80–88.
Penteleev, P. A. (1983) *Bioenergetics of Small-Sized Mammals*, Nauka, Moscow.
Ritov, V. B., Melgunov, V. I., Komarov, P. G., Alekseeva, O. M., and Akimova, E. I. (1977) *Dokl. Akad. Nauk SSSR* **233:**727–733.
Shorina, E. A., Mast, N. V., Lopina, O. D., and Rubtsov, A. M. (1997) *Biochemistry* **36:**13455–13460.
Shoshan-Barmatz, V., Orr, I., Wells, S., Meyer, H., Versanyi, M., and Heilmeyer, L. M. (1996) *Biochim. Biophys. Acta* **1283:**89–100.
Shutova, A. N., Storey, K. B., Lopina, O. D., and Rubtsov, A. M. (1999) *Biochemistry* (Moscow) **64:**1480–1488.
Simmerman, H. K., and Jones, L. R. (1998) *Physiol. Res.* **78:**921–947.
Wang, L. C. H. (1985) *Cryo-Lett.* **6:**257–274.