The Modulation of the Calcium Transport by Skeletal Muscle Sarcoplasmic Reticulum in the Hibernating European Hamster

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Calcium transport of skeletal muscle sarcoplasmic reticulum was comparatively studied in hibernating and summer active European hamsters (Cricetus cricetus L.). Crude homogenates from psoas, soleus and mixed skeletal muscles were used. Protein yield was strongly reduced in the muscle homogenates of hibernating hamsters. The calcium concentration in the muscle of hibernating hamsters was increased to a much higher content than in the serum. In the same animals the maximal rate of calcium uptake and the calcium storing capacity of sarcoplasmic reticulum were augmented by 43% and respectively 17%. Kinetic experiments with various concentrations of free calcium revealed in the hibernating animals higher uptake rates and a lower apparent calcium affinity than in the summer active hamsters. Some shift of calcium uptake rate and calcium affinity similar to that of a fast-twitch muscle was also observed in winter active animals kept at 22 °C under natural photoperiod. By contrast, the activity of the calcium dependent ATPase was not increased, suggesting a tighter coupling during hibernation between calcium dependent ATP-hydrolysis and calcium transport. No seasonal difference was observed in the calcium release by KCl-caffeine from calcium loaded vesicles of sarcoplasmic reticulum.

Proportion and size of fibre types were studied with cold cross sections from psoas and soleus muscles. An average atrophy of about 25% was found during hibernation in both muscles. Cytchemistry revealed, however, a different reduction of cross area between type-I- and type-II-fibres, which reaches values up to 46% in the type-II-fast-fibres of the slow soleus muscle. Electron microscopy did not show any definite change in the distribution and amount of sarcoplasmic reticulum.

The results suggest that during hibernation a modulation in the properties of calcium transport ATPase of sarcoplasmic reticulum occurs to better support the calcium transport function at low temperatures, which in turn warrants the restoration of ion homeostasis in the course of the arousal.

Introduction

Hibernation or torpor is a natural process occurring in response to cold and food shortage characterized by a profound reduction in metabolism up to 1% and body temperature to near 0 °C. Under the conditions an energy saving up to 90% may be realized as compared to active eutherian animals (cf. [1–4]). The precise central mechanisms in the hibernators are considered a fundamental requisite for the maintenance of the intracellular ion concentration in a range compatible with cell function also during the period of reduced body temperature [3, 5, 9]. This in turn would be of extreme importance for the restoration in the course of the arousal of ion homeostasis, possibly dissipated during hibernation, to the normal values of the active state [2, 3, 7, 12, 13]. So far a superior capability of hibernators as compared to the norm al values of the active state [2, 3, 7, 12, 13]. So far a superior capability of hibernators as compared to the norm al values of the active state [2, 3, 7, 12, 13].

Abbreviations: Au-Ha, autumn active European hamsters; Hi-Ha, hibernating European hamsters; Su-Ha, summer active European hamsters; Wi-Ha, winter active European hamsters; Ps, psoas muscle; Sol, soleus muscle; Hi, hibernation; Su, summer; Wi, winter; Ps-Hi, psoas muscle-hibernation; Sol-Hi, soleus muscle-hibernation; Ps-Su, psoas muscle-summer; Sol-Su, soleus muscle-summer; PMSF, phenylmethylsulfonylfluoride; MOPS, morpholinepropane sulfonic acid; TRIS, tris(hydroxymethyl)-aminomethane.

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pared to obligate homeotherms to retain Na\(^+\) and K\(^+\) gradients at low temperature is well documented by several studies done with various tissues and cells [6, 14–18]. Furthermore, it is well known that both the Na\(^+\)–K\(^+\)-ATPase [15, 19, 22] and the Ca\(^{2+}\)-transport ATPase [23, 27] of the red cell are at low temperature more active in the hibernator than in obligate homeotherms. Yet, the response to cold acclimation and hibernation of the Ca\(^{2+}\)-transport ATPase of sarcoplasmic reticulum has received only little attention [13, 28–30], although a remarkable increase of calcium level in serum [31–33] and within the skeletal [32] and cardiac [32, 34] muscle in cold acclimated and even more in torpid animals has been known for several years.

We recently reported [30] a significant increase of the calcium uptake rate and a decrease of the calcium affinity in the isolated vesicles of skeletal muscle sarcoplasmic reticulum from golden hamster (Syrian hamster, Mesocricetus auratus L.) in winter as compared to the summer animal. We also found that the cold acclimation in winter leads to a further small reduction of calcium affinity. Since the golden hamster is a facultative hibernator [35] and never entered into hibernation in our laboratory, we therefore extended our study to the calcium transport function of skeletal muscle sarcoplasmic reticulum in the active and torpid European hamster (Cricetus cricetus L.), an obligate hibernator, now easy to raise in laboratory [36]. This animal hibernates in its natural habitat from the middle of October to the end of March and usually enters into hibernation when exposed to cold experimental conditions [1, 31].

In this contribution we show that during hibernation the calcium transporting system of skeletal muscle sarcoplasmic reticulum undergoes a marked increase in activity which is connected with considerable qualitative changes in the properties of the Ca\(^{2+}\)-transport ATPase. Preliminary reports of part of these results were already presented [29, 37].

**Materials and Methods**

**Animals and hibernation**

The experiments were carried out through four years with seventy adult male European hamsters (Cricetus cricetus L., strain MHH: EPH) obtained from the Institut für Versuchstierkunde und Zentralen Tierlaboratorium, Medizinische Hochschule Hannover (Hannover, F.R.G.). The animals were individually housed in Makrolon cages at 22±2 °C, relative humidity 55±5% under natural light-dark conditions, and had free access to tap water and food (“Altromin 1234”, Dry Food, Altromin International, Lage, F.R.G.). As nesting material wheatstraw and hay were used. Thirty-one hamsters, kept for two months at least under the above laboratory conditions, were killed between June 3rd and August 15th at the age of 240±43 days. We call these animals, which in this study are considered to be the experimental control, summer active hamsters (Su-Ha).

In the beginning of November, twenty-eight prehibernating hamsters aged 262±34 days were induced to hibernate by transferring the cages into a darkened, well-aired cold chamber at +2±0.5 °C, relative humidity 90±5%. These conditions were gradually reached within one week. We call hibernating hamsters (Hi-Ha) the animals which had been hibernating for four weeks or more. They were killed between December 10th and February 19th at the age of 325±42 days. We always took care to kill the animals of this group during a well established hibernation bout.

For comparison we extended our study to active animals in autumn and in winter. To avoid hibernation during the cold season, eleven hamsters were kept at 22±2 °C, relative humidity 55±5% on a 12 h light:12 h dark photoperiod, starting by the end of August. Six of them were killed in November and five in January. We call these animals autumn active (Au-Ha) and winter active (Wi-Ha) hamsters, respectively. These terms, which are operational and descriptive of the experimental conditions chosen, only partially reflect the intrinsic physiological state of animals free in nature, which during the cold season would ordinarily enter into the state of hibernation.

All animals were killed between 8 and 9 p.m. by a blow on the head, and the blood, with or without addition of heparin, was collected after decapitation.

**Determination of ions in serum**

The determination of calcium, sodium and potassium were done flamephotometrically in an an-
analytical run using a Flame photometer AFM 5051. Magnesium was determined with a Perkin-Elmer 100 atomic absorption photometer.

**Histochemistry, morphometry and electron microscopy**

The water content of muscle tissue was measured as the difference between wet weight and weight after drying of muscle samples at 90 °C for 24 h (cf. [38]).

Psosas and soleus muscles from three summer active animals (Su-Ha), killed in July, and from the hibernating animals (Hi-Ha), killed in February, were removed in a slight stretched position and frozen in melting methylbutane at −160 °C as a composite block. Serial 5 and 15 μm unfixed cryostate microtome sections were usually stained with haematoxylin-eosin or assayed for the succinic dehydrogenase, as reported by Lojda et al. [39]. Other sections were tested for the standard myosin ATPase reaction at pH 9.4 after formaldehyde fixation and alkaline preincubation at pH 10.4, according to Guth and Samaha [40]. Fibre subtypes were identified by the same reaction on unfixed sections preincubated at various pH’s − 9.4, 4.6, 4.3, and 3.9 − using the method of Brooke and Kaiser [41].

For the morphometric evaluation of the muscle fibres measurements were done with cross muscle sections in a Leitz Orthoplan Microscope connected to the Leitz Semiautomatic Image Analyzer ASM 68 K (Leitz, Wetzlar, F.R.G.).

For preparation of ultrathin sections samples of musculus rectus abdominis, psosas and soleus removed in stretched position by a cold clamp were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer − pH 7.3 − for 2 h. Small muscle fragments were postfixed in 1% OsO₄ in distilled water for 90 min, dehydrated in alcohol and embedded in Epon 812. Ultrathin sections were cut by a diamond knife in a LKB Ultratome IV ultramicrotome, stained with aqueous uranyl acetate and/or lead citrate according to standard procedures, and observed in a Siemens Elmiskop 101 electron microscope.

**Preparation of muscle homogenates**

The technique introduced by Briggs et al. [42] was applied. Samples from the central part of psoas and soleus muscles, as well as mixed skeletal muscles were quickly dissected and immediately immersed in an ice-cold solution containing 300 mM sucrose, 100 mM KCl and 40 mM imidazole sulfate, pH 6.9. After removal of connective tissue, nerves and blood vessels, muscle tissue was weighed, minced in very small fragments, and homogenized in 10 vol of the above solution for two periods of 10 s with a Polytron Homogenizer at step 7 of velocity. The homogenate was filtered through cheese-cloth, complemented with 1 mM PMSF (phenylmethylsulfonylfluoride) to inhibit protease activity, and used without delay after determination of protein concentration by the biuret method. For the experiments on calcium release homogenates of mixed skeletal muscle were prepared as above in the absence of KCl.

**Determination of calcium in muscle homogenates**

One ml homogenate from mixed skeletal muscles was added with 0.5 ml 1.5% HNO₃ to denature proteins and boiled for 5–10 min at 100 °C. Thereafter the mixture was centrifuged for 20 min at 10,000 rpm in a Beckmann Ultracentrifuge L 8-55 M. 40 μl supernatant were transferred to a graphite tube and ashed in a Perkin-Elmer 4000 atomic absorption spectrophotometer. Standards were run with various concentrations of CaCl₂ (10−100 μM) in 0.5 ml bidistilled water added with 1.5% HNO₃ to prepare a standard curve. Three determinations for each of seven summer active and, respectively, for five hibernating hamsters were done.

**Assays of calcium transport function**

For the estimation of the rate of calcium uptake and calcium storing capacity, crude homogenate − final concentration 1 mg of protein/ml − was incubated at room temperature (20–22 °C) in a standard solution containing 5 mM NaH₂PO₄, − to inhibit mitochondrial activity −, 100 mM KCl, 40 mM imidazole buffer − pH 6.9 −, 5 mM MgCl₂, 5 mM potassium oxalate, 0.6 mM EGTA, 5 mM ATP and 0.5 mM ⁴⁵CaCl₂. Under the conditions the free calcium concentration was 1.5 μM.

The dependence on free C²⁺ concentration of the rate of calcium uptake of sarcoplasmic reticulum was studied at different free calcium concentrations, adjusted with EGTA 0.1, 0.22, 0.3, 0.4,
0.8, and 1.2 mM in an incubation medium as above which contained 0.2 mM $^{45}$CaCl$_2$; the corresponding free Ca$^{2+}$ concentrations are reported in the legend of Fig. 6 and in Table VI and VII. A free calcium concentration of 10 ± 1 μM in the uptake medium described above containing 0.2 mM calcium and 0.1 mM EGTA was determined with a calcium sensitive electrode.

Calcium uptake was started by adding 20 mg homogenate protein to 20 ml of the assay solution. Aliquots were taken 1, 2, 3, 5, and 20 min after the addition of the protein and filtered through a glass nitrocellulose 0.45 μm filter combination (GF 9 glass filter and BA 85 nitrocellulose filter, Schleicher & Schuell, Dassel, F.R.G.) protected from clotting by large components of the homogenate by a Whatman GF/C glass filter. The radioactivity remaining in the filtrate was determined by liquid scintillation counting.

The maximal uptake capacity as well as the maximal uptake velocity were measured at a free calcium concentration of 10 μM in the medium containing 0.2 mM calcium and 0.1 mM EGTA. The maximal velocities and dissociation constants of calcium were calculated using a data analyzing program (Enzfitter, Elsevier Biosoft, Amsterdam, Holland) assuming Michaelis-Menten kinetics.

**Assay of ATPase activity**

The assay medium for determining the ATPase activity of sarcoplasmic reticulum contained 100 mM KCl, 40 mM imidazole sulfate – pH 6.9 –, 5 mM MgCl$_2$, 5 mM potassium oxalate, 0.1 mM EGTA, 5 mM ATP and 5 mM NaN$_3$ – to inhibit mitochondrial activity. At first the Mg$^{2+}$ dependent ATPase activity was measured. The reaction was started by addition at room temperature (20–22 °C) of 1 mg/ml homogenate protein. Aliquots of the assay mixture were taken immediately after addition of the protein and after 6 min incubation. At this time of the experiment 0.2 mM $^{45}$CaCl$_2$ was added to the incubation medium for assaying the Ca$^{2+}$ dependent ATPase activity. This was determined with aliquots of the assay mixture taken 1, 5, 8 and 12 min after the addition of calcium. All aliquots taken were mixed with an equal volume of 6% trichloroacetic acid. The mixture was filtered and Pi was determined in the supernatant according to Rockstein and Herron [43].

**Calcium release experiments**

Calcium uptake and release experiments were performed at room temperature (20–22 °C) essentially as described by Su and Hasselbach [44] with small modifications. The homogenate prepared from mixed skeletal muscles in the absence of KCl was used. The medium contained 5 mM magnesium gluconate, 100 mM potassium gluconate, 100 mM sucrose, 5 mM phosphoenolpyruvate, 2 mM ATP, 0.04 mg/ml phosphoenolpyruvate kinase, 50 mM MOPS (morpholinepropane sulfonic acid) pH 7.0 with tris buffer (tris-hydroxymethyl-aminomethane) and 10 μM $^{45}$CaCl$_2$.

Calcium uptake was started by addition of 2 mg/ml homogenate protein to 20 ml of the assay solution and determined 1, 3, 5, 10 and 15 min incubation as described above. At this time final concentrations of 100 mM KCl, or 10 mM caffeine, or 100 mM KCl and 10 mM caffeine were added to the assay mixture to test the calcium release from the loaded vesicles. Aliquots of the assay suspensions were filtered as described above 15 and 60 s after release initiation – i.e. 15 min 15 s and respectively 16 min after starting the experiments – and the amount of released $^{45}$Ca$^{2+}$ was determined by liquid scintillation counting of the radioactivity in the filtrate.

**Results**

**Animals**

All hamsters kept at +2 ± 0.5 °C in the dark entered hibernation. Their body weight decreased remarkably from 385 ± 92 g at the beginning of November to 287 ± 50 g during the average hibernation time of 62 ± 22 days, and reached a loss about 40% in the animals killed in February. On the opposite the body weight increased about 12% from November until January in the winter active hamsters living under standard laboratory conditions. These animals showed only occasionally some degree of torpor.

**Identification and morphometry of fibre types of soleus and psoas muscle**

As shown in Fig. 1 (a and c) and in Table I, both soleus and psoas muscles of the summer active European hamsters display a fibre type pattern of a mixed muscle. The slow-twitch soleus muscle con-
Fig. 1. Fibre types of soleus and psoas muscle in summer active (a, Sol-Su; c, Ps-Su) and in hibernating European hamster (b, Sol-Hi; d, Ps-Hi) as revealed by the cytochemical reaction for the myosin ATPase after formaldehyde fixation and alkaline preincubation (cf. [40]). While the slow-twitch soleus muscle (a) consists for about 70% of weak-stained type-I-fibres, the fast-twitch psoas muscle (c) contains about 80% of strong stained type-II-fibres. Similar proportion of fibre types is seen in the soleus (b) and respectively psoas (d) muscle of the hibernating animals. Muscle fibres of hibernating hamsters display a clear caliber reduction which is more pronounced in the soleus than in the psoas muscle, especially in the type-II-fibres. x 160. Bar in d corresponds to 100 μm.
Table I. Distribution of fibre types in the soleus and psoas muscle of summer active and hibernating European hamsters. Fibre were counted on cross muscle sections stained for the myosin ATPase like those represented in Fig. 1.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ATPase stain</th>
<th>Animals</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Summer active</td>
<td>Hibernating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$n = 3$</td>
<td>$n = 3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. Soleus</td>
<td>$f = 4.127$</td>
<td>$f = 3.199$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-I-fibres</td>
<td>weak</td>
<td>$70 \pm 5%$</td>
<td>$69 \pm 6%$</td>
<td>$-1%$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-II-fibres</td>
<td>strong</td>
<td>$30 \pm 5%$</td>
<td>$31 \pm 6%$</td>
<td>$+1%$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. Psoas</td>
<td>$f = 4.494$</td>
<td>$f = 3.264$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-I-fibres</td>
<td>weak</td>
<td>$20 \pm 4%$</td>
<td>$24 \pm 2%$</td>
<td>$+4%$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-II-fibres</td>
<td>strong</td>
<td>$80 \pm 4%$</td>
<td>$76 \pm 2%$</td>
<td>$-4%$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in $\bar{x} \pm$ SD. $n =$ number of animals. $f =$ number of fibres counted. 
$\Delta =$ Change of fibre pattern in hibernating animals in %.

contains a large amount of type-II-fast-fibres ($30 \pm 5\%$). Conversely the fast fibres of the fast-twitch psoas muscle are mixed with a high proportion of type-I-slow fibres ($20 \pm 4\%$). Intermediate fibres were only occasionally found in both muscles. Fig. 1 also shows that the above muscle fibre pattern is not remarkably altered in the corresponding muscles of the hibernating animal (cf. a with b and c with d). Values reported in Table I confirm that no change occurs in the fibre type pattern of soleus muscle and reveals a moderate increase from 20 to 24% in the proportion of type-I-fibres in the psoas muscle, which even if salient, representing 15% of all type-I-fibres, is not significant.

Correspondingly to the dramatic loss of body weight skeletal muscles undergo a striking mass reduction during hibernation. The muscle of hibernating animals did not display, however, any decrease in the relative water content, which reaches values around 28% of wet weight in the animals of various groups. As reported in Table II, an average reduction of cross area by 27.40% and 25.80% was found in the fibres of soleus and psoas muscle respectively of three hamsters killed in February after a long hibernation time of about hundred days. These average values obtained with unfixed sections after haematoxylin-eosin stain may directly reflect the cross area size of the muscle fibres since no fixation shrinkage occurred. They do not provide, however, any information whether various fibre types undergo a different degree of atrophy, as the conventional stain applied is not suitable to distinguish different types of muscles fibres. This question is answered when muscle fibre types are identified by the cytochemical stain of myofibrillar myosin ATPase after formaldehyde fixation, as represented in Fig. 1a–d.

Comparison of $a$ with $b$ and, respectively, of $c$ with $d$ reveals that in the hibernating hamster various muscle fibres display a reduced cross area, the atrophy being, however, more severe in those of soleus ($b$) than in those of psoas ($d$) muscle, especially in the type-II-fast-fibres. These differences are substantiated by the morphometric values reported in Table III. While in the soleus muscle the cross area reduction of type-II-fibres ($-46.60\%$) is almost double than that of type-I-fibres.
Table III. Fibre types area in µm² of soleus and psoas muscle from summer active and hibernating European hamster. Measurement were done on the same cross muscle sections stained for the myosin ATPase used for determining the fibre types pattern (cf. Fig. 1 and Table I).

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ATPase</th>
<th>Animals</th>
<th>Summer active</th>
<th>Hibernating</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n = 3</td>
<td>n = 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. Soleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-I-fibres</td>
<td>weak</td>
<td>3.311 ± 1.028</td>
<td>2.166 ± 271</td>
<td>-27.30%</td>
<td></td>
</tr>
<tr>
<td>Typ-II-fibres</td>
<td>strong</td>
<td>2.423 ± 734</td>
<td>1.130 ± 493</td>
<td>-46.60%</td>
<td></td>
</tr>
<tr>
<td>M. Psoas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typ-I-fibres</td>
<td>weak</td>
<td>1.812 ± 437</td>
<td>1.408 ± 211</td>
<td>-13.60%</td>
<td></td>
</tr>
<tr>
<td>Typ-II-fibres</td>
<td>strong</td>
<td>1.842 ± 518</td>
<td>1.396 ± 312</td>
<td>-8.00%</td>
<td></td>
</tr>
</tbody>
</table>

Values in ± SD. n = number of animals. f = number of fibres measured. Δ = Reduction of fibre types are during hibernation in %.

(-27.30%), the moderate difference in atrophy extent between the two corresponding fibre types of psoas muscle (-8.00% for the type-II- and -13% for the type-I-fibres) is not significant.

Electron microscopy

No definite changes, which could be referred to hibernation, were observed by conventional electron microscopy in the fibres of soleus, psoas and rectus abdominis muscles. This is illustrated for the musculus rectus abdominis in Fig. 2 and 3. As one could expect (Fig. 2), a quite identical pattern is observed in the muscle fibres of the rectus abdominis from winter active (a, Wi) and respectively from summer active (b, Su) animals. The corresponding micrograph of a hibernating hamster (Fig. 3) shows that no remarkable alteration occurs during the state of torpor in the disposition and amount of sarcoplasmic reticulum, and proportion of mitochondrial granules.

Concentration of ions in serum

Values of sodium and potassium in serum of autumn and winter active hamsters, and of hibernating animals as well, did not remarkably differ from those of summer active hamsters -\(149.4 ± 4.97\) mM Na/l and -\(7.11 ± 1.03\) mM K/l, n = 10. As reported in Table IV, no seasonal difference of calcium and magnesium levels was found in the serum of the various groups of active animals. By contrast, a significant increase of calcium - by 14%, \(P < 0.02\) - and of magnesium concentration - by 26%, \(P < 0.01\) - occurs in the serum of the hibernating hamsters.

Table IV. Seasonal variations of levels of calcium and magnesium in the serum of European hamster. Values - mmol/l serum - are given in ± SD. n = number of animals. For each animals two determinations were done.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Summer active</th>
<th>Autumn active</th>
<th>Winter active</th>
<th>Hibernating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 12</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.66 ± 0.16</td>
<td>2.48 ± 0.16</td>
<td>2.70 ± 0.04</td>
<td>3.10 ± 0.15</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.93 ± 0.08</td>
<td>0.80 ± 0.05</td>
<td>0.90 ± 0.03</td>
<td>1.26 ± 0.10</td>
</tr>
</tbody>
</table>
Fig. 2 and 3. Electron micrographs of longitudinal sections through the musculus rectus abdominis of the European hamster under different seasonal conditions. Uranyl acetate and lead citrate stain. x 30,000. Bars correspond to 1 μm.

Fig. 2. Muscle fibre from a winter active (a, Wi) and from a summer active (b, Su) European hamster. The same pattern is observed. Mitochondria (M) with well outlined cristae and characteristic granules (arrows), triads (Tr) and longitudinal tubuli (L) of sarcoplasmic reticulum, and glycogen particles (Gl) are identifiable within the contracted myofibrils. Z, Z-lines.
Fig. 3. Muscle fibres from the rectus abdominis of a hibernating European hamster (Hi). No remarkable change in the amount and disposition of sarcoplasmic reticulum, and proportion of mitochondrial granules (arrows) can be seen as compared with the muscle fibres of the active animals represented in Fig. 2. Due to the hypercontraction state the Z-lines (Z) appear widened and the sarcoplasmic components densely packed each other.

Protein content of muscles homogenates

As shown in Table V, values of protein yield in the various muscle homogenates of hibernating hamsters are remarkably lower than those of summer active animals. Nevertheless the protein loss by 23.9% of soleus muscle from hibernating hamsters is higher than that of psoas muscle, being significant at P<0.05. By contrast the reduction of protein content by 18.3% in the psoas muscle is only marginally significant. The severe protein de-
Calcium content in homogenate from mixed muscles

Values of 72 ± 16 and 118.2 ± 24.2 μM Ca²⁺/g muscle (x ± SD) were found for the homogenate from mixed skeletal muscles of summer and respectively hibernating hamsters. This increase by 39% is significant at P < 0.0025.

Calcium transport of sarcoplasmic reticulum from soleus and psoas muscles of summer active European hamsters

Fig. 4 shows the time course of calcium uptake of sarcoplasmic reticulum from psoas (▲) and soleus (▼) muscle of summer active European hamster. Calcium uptake rate and calcium storing are much higher in the fast-twitch psoas than in the slow-twitch soleus muscle. Calcium uptake was measured with crude homogenate in the presence of oxalate and 1.5 μM free calcium as described in Materials and Methods. The results are given in x. n = 6. The differences of values at various incubation times explored are significant at P < 0.005.

Calcium transport of sarcoplasmic reticulum from mixed skeletal muscles of summer active, hibernating and winter active European hamsters

Comparison of Fig. 5 with Fig. 4 reveals – as one could expect – that in summer active European hamsters values of calcium uptake rate and maximal storing capacity by sarcoplasmic reticulum of mixed skeletal muscles (▲) are higher than those of the slow-twitch soleus muscle (▼), ap-
Fig. 5. Comparison of the time course of calcium uptake by skeletal muscle sarcoplasmic reticulum from summer active (●) and hibernating (■) European hamsters. In the hibernating animals calcium uptake rate and calcium storing capacity are remarkably higher than in the summer active ones. Uptake conditions as in Fig. 4. The results are given in $\bar{x} \pm$ SD as indicated by the bars. Summer active animals: $n = 9$. Hibernating animals: $n = 11$. The differences of values found after various times of incubation are significant at $P < 0.005$.

As represented in Fig. 5, in the hibernating animals (■) the calcium uptake function of skeletal muscle sarcoplasmic reticulum is remarkably augmented. The values of the maximal uptake rate – $75 \pm 1.0$ nmol Ca$^{2+}$/mg protein$^{-1}$ min$^{-1}$ – and calcium storing capacity – $189 \pm 2.0$ nmol Ca$^{2+}$/mg homogenate protein – are during hibernation 43% and respectively 17% higher than those of the summer active animals (●).

Comparison of Fig. 5 with Fig. 4 also shows that all values obtained with mixed muscles from hibernating hamsters (■) are higher than those of summer psoas muscle (▲).

As compared with summer active hamsters the calcium uptake function of the winter active animals was noticeable augmented, the values of uptake rate – not reported in Fig. 5 – being, however, not as high as those of the hibernating animals.

Dependence on free calcium concentration of the calcium uptake by sarcoplasmic reticulum of summer active and hibernating animals

Fig. 6 shows the time course of calcium uptake by sarcoplasmic reticulum of summer active (A) and hibernating (B) European hamsters in the presence of six different concentrations of free calcium varying from 0.06 up to 10.0 $\mu$M. It is easy to recognize that in both groups of animals the calcium uptake rate and calcium storing capacity directly depend on the concentration of free calcium applied. All values registered during hibernation (Fig. 6B) are, however, definitely higher than appearing more close to the corresponding values of the fast-twitch psoas muscle (▲).

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those found with summer active animals (Fig. 6 A).

Fig. 6 reveals that the lowest threshold for the activation of the calcium transport system lies in the range of a free calcium concentration of about 0.08 µM. In the presence of such a small concentration of calcium the measurements of the threshold activities are affected by high errors, up to 40–60% of the real values. It is evident in the figure that the calcium transport activity saturates when the concentration of free calcium is higher than 3 µM. Under the conditions the calcium transport activity can be measured with high precision, the relative error being only about 10%. As represented in Table VI and VII, these differences are confirmed when the experimental values (second column) are compared with the corresponding values computed using a data analyzing program (third column) assuming that the dependence of calcium transport activity on the free calcium concentration follows Michaelis-Menten kinetics. This is illustrated e.g. in Fig. 7. All calculated values are in the range of the standard deviations. Comparison of Table VI with Table VII reveals that the difference of the maximal calcium uptake velocity between summer active and hibernating European hamsters –43 ± 1.4 and respectively 78 ± 3.0 nmol·mg⁻¹ homogenate protein·min⁻¹ – is significant at

Table VI: Dependence on free calcium concentration of the rate of calcium uptake by sarcoplasmic reticulum in the skeletal muscle homogenate of summer active hamsters.

<table>
<thead>
<tr>
<th>Free calcium concentration [µM]</th>
<th>Calcium uptake velocity Experimental values [nmol·mg⁻¹·min⁻¹]</th>
<th>Calculated values [nmol·mg⁻¹·min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>3 ± 2</td>
<td>5.9</td>
</tr>
<tr>
<td>0.5</td>
<td>17 ± 7</td>
<td>14.0</td>
</tr>
<tr>
<td>0.6</td>
<td>27 ± 5</td>
<td>21.3</td>
</tr>
<tr>
<td>3.0</td>
<td>38 ± 6</td>
<td>36.7</td>
</tr>
<tr>
<td>10.0</td>
<td>42 ± 1</td>
<td>42.1</td>
</tr>
</tbody>
</table>

Maximal velocity 43 ± 1.4* nmol·mg⁻¹·min⁻¹. Dissociation constant 380 ± 48* nm. *x ± SE.

The calculated values were computed from the experimental values (mean ± SD, n = 9) using a data analyzing program (Enzfitter, Elsevier Biosoft, Amsterdam, Holland) assuming Michaelis-Menten kinetics.

Table VII: Dependence on free calcium concentration of the rate of calcium uptake by sarcoplasmic reticulum in the skeletal muscle homogenate of hibernating hamsters.

<table>
<thead>
<tr>
<th>Free calcium concentration [µM]</th>
<th>Calcium uptake velocity Experimental values [nmol·mg⁻¹·min⁻¹]</th>
<th>Calculated values [nmol·mg⁻¹·min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>7 ± 3</td>
<td>10.0</td>
</tr>
<tr>
<td>0.3</td>
<td>26 ± 4</td>
<td>24.0</td>
</tr>
<tr>
<td>0.6</td>
<td>44 ± 13</td>
<td>36.9</td>
</tr>
<tr>
<td>3.0</td>
<td>61 ± 10</td>
<td>64.6</td>
</tr>
<tr>
<td>10.0</td>
<td>75 ± 8</td>
<td>74.4</td>
</tr>
</tbody>
</table>

Maximal velocity 78 ± 3.0* nmol·mg⁻¹·min⁻¹. Dissociation constant 590 ± 55* nm. *x ± SE.

The calculated values were computed from the experimental values (mean ± SD, n = 11) as reported in Table VI (cf. Fig. 7).
Fig. 7. Dependence of the rate of calcium uptake on the concentration of free calcium in the hibernating European hamster. Data fitting was performed assuming Michaelis-Menten kinetics as reported in Table VII.

P < 0.001. Besides the values of the maximal velocity of calcium uptake, the fitting program used provides also values for the calcium dissociation. The difference between the corresponding values — 590 ± 55 nm and 380 ± 48 nm for hibernating and non-hibernating animals — is also significant at P < 0.001.

**ATPase activity of sarcoplasmic reticulum**

As represented in Table VIII, no definite change of the magnesium dependent (basic) ATPase activity occurs in the sarcoplasmic reticulum of hibernating hamsters. Likewise the average values of the calcium dependent (extra) ATPase activity does not differ significantly. Hence, the enhancement of calcium transport activity is not accompanied by a corresponding activity of the calcium transport ATPase.

**Calcium release from calcium loaded vesicles of sarcoplasmic reticulum**

The time course of calcium release from calcium loaded vesicles of mixed skeletal muscle sarcoplasmic reticulum for summer active (○) and hibernating (■) hamsters is represented in Fig. 8.

### Table VIII. ATPase activity of sarcoplasmic reticulum from mixed skeletal muscles of summer active and hibernating European hamsters.

<table>
<thead>
<tr>
<th>Animals</th>
<th>ATPase activity</th>
<th>Mg²⁺ dependent</th>
<th>Ca²⁺ dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol Pi · mg prot⁻¹ min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer active (3)</td>
<td>12.2 ± 1.7</td>
<td>31.6 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>Hibernating (3)</td>
<td>13.7 ± 1.2</td>
<td>34.8 ± 3.2</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 8. Calcium release by KCl and caffeine from calcium loaded vesicles of sarcoplasmic reticulum from summer active (○) and hibernating (■) European hamsters. Sarcoplasmic reticulum vesicles of muscle homogenate were first loaded with calcium using an appropriate medium for the study of calcium release (cf. [44]) as described in Materials and Methods. After 15 min incubation (arrow) KCl and caffeine were added at the final concentration of 100 and respectively 10 mM to induce calcium release. No remarkable difference is seen in the time course of calcium release between hibernating and summer active animals. The results are given in x ± SD. Summer active animals: n = 9. Hibernating animals: n = 11.
Under the conditions chosen for calcium loading more than 90% of the calcium present in the incubation medium is taken up into the vesicles of both groups of animals within 15 min. Values of uptake rates are, however, significantly higher - P < 0.05 - at min 5 and 10 in the hibernating animals. By contrast, the time course of the KCl-caffeine-induced calcium release in the preparations from hibernating hamsters is very similar to that of summer active animals. The difference between the amounts of calcium released within 1 min after KCl-caffeine application - 15.4% and 18.9% for the summer active and, respectively, for the hibernating hamsters - is not significant. The amounts of calcium released by application of either only 100 mM KCl or 10 mM caffeine were negligible and are not reported in Fig. 8.

Discussion

In the past hibernation has mainly been studied to understand the ability of the animal to cope with the problem of thermoregulation and adaptation to food shortage. Recently biochemical methods were applied to approach the molecular mechanisms which underly hibernation. In this respect the analysis of membrane transport processes also gained new impetus (cf. [3, 4, 7–9, 12, 26]).

This contribution shows that during hibernation remarkable alterations occur in the calcium transport of skeletal muscle sarcoplasmic reticulum. Our kinetic studies with homogenate obtained from mixed skeletal muscle of hibernating European hamsters have revealed that during the torpid state the calcium transport function of sarcoplasmic reticulum undergoes a marked increase in activity. The alteration is connected with considerable qualitative changes in the properties of calcium transport ATPase which becomes similar to that of a fast-twitch muscle.

By contrast, no corresponding shift in fibre-type composition - i.e. an increase proportion of type-II-fast-fibres - could be ascertained during the torpid state by histochemical study using the soleus and the psoas muscle as a model of slow- and respectively fast-twitch muscle. Furthermore, the fast-type-I-fibres appear to contribute to a higher extent and degree than the slow-type-II-fibres to the average fibre atrophy of about 25% observed on unfixed, non-dehydrated sections from both muscles of hibernating animals. The atrophy by 8% in the type-II-fibres of psoas muscle is remarkable, if one takes into account that this muscle contains about 80% of such fibres, and even reaches values up to 46% in the fast-type-II-fibres of the soleus muscle. This indicates that various fibre types react differently during hibernation.

The striking reduction of muscle masses by 25% during the prolonged torpid state cannot depend on alteration of muscle hydration since the muscle of hibernating animal did not display any relative decrease in the water content. On the other hand the reduction of protein content in the muscle homogenates of hibernating hamsters indicates that remarkable alterations in chemical composition of muscle fibres occur during the torpid state. Muscle atrophy cannot even be due to inactivity during the torpid state as in the case of muscle immobilization. The atrophied skeletal muscle of the hibernating golden hamster displays increased mass-specific maximum activities of certain oxidative enzymes [45] which on the opposite dramatically decrease in the inactive muscle of a non-hibernating rat [46]. Li and Steffen [47] recently reported a less pronounced atrophy in muscle soleus, gastrocnemius and plantaris of the hibernating ground squirrel, an obligate hibernator, as compared to the winter active animal. Translatable actin mRNA levels in gastrocnemius muscle were not different between summer active and hibernating animals, but exhibited a significant two-fold decrease in the winter active animals.

The mechanism of the increase of calcium concentrations by 14% in serum and even the higher rise by 39% of the calcium content in skeletal muscle is not known. The lack of any definite calcium increase in the serum of the animals kept awake in autumn and in winter - autumn and, respectively, winter hamsters - under standard laboratory conditions indicates that the increase of serum calcium during hibernation is related to the development of the torpid state. Rath [31] found that in the European hamster the serum calcium level increases in a linear manner when body temperature drops from 30 to 5 °C. Evidently the alteration of serum calcium concentration occurs concomitantly with the reduction of body temperature, which in turn is necessary for the transition into the torpid state,
and it is not a consequence of the latter. On the other hand it is well established that the average cytosolic (Ca^{2+}) increases significantly when isolated ventricular myocytes are exposed to low temperature [48]. With regard to the origin of the high amounts of calcium in serum and in the muscle fibres, we do not know how the turnover of parathyroid hormone and calcitonin, the dominant hormones regulating body calcium homeostasis (cf. [49]), is regulated throughout the annual cycle (cf. [50]). A purely passive rise of muscle calcium as the result of the observed elevated calcium level in plasma is unlikely, if the rise in muscle calcium is compared with the much smaller increase in plasma calcium. The question arises in which organelles of the muscle fibre the high amount of calcium, we assured, is stored or to which proteins it is bound.

The significant increase of the maximal rate of calcium uptake and that of calcium storing capacity of sarcoplasmic reticulum, first ascertained by us in the homogenate of skeletal muscle, points out that the sarcoplasmic reticulum is an important structure in handling excess of calcium in the skeletal muscle fibres during hibernation. This fits very well into the current views, recognizing that the endoplasmic reticulum, rather than the mitochondria, is the main organelle regulating the cytoplasmic calcium concentration [51, 52]. The rise of calcium storing capacity and of maximal uptake rate may result from an increase of the sarcoplasmic reticulum leading to a higher yield of calcium transporting vesicles in the homogenate. In our study neither a proliferation of sarcoplasmic reticulum membrane nor a relative increase of it in the atrophic fibres, which would explain the augmented calcium transport activity, could be ascertained by electron microscopy. The similar aspect of sarcoplasmic reticulum observed in active and torpid animals is not surprising, since electron microscopy merely reveals the configuration the membrane assumes upon cellular death during fixation without discerning different physiological states of the membrane enzymes like those we describe in this contribution. Alternatively, the enhanced calcium transport activity of hibernating animals could be mediated via an increase of calcium transport ATPase molecules/unit sarcoplasmic reticulum area as well as through a modulation of the enzyme molecule. Until the calcium uptake rates can be related to the amounts of the transport protein present in the preparations, the question will remain open (cf. [13, 53]). As already shown [30, 54, 55], only the study of activity parameters of the calcium transporting enzyme which do not depend on the protein yield of the preparation and rather reflect its qualitative changes can shed some light on the problem (see below).

The increase in calcium transport activity we found in the skeletal muscle sarcoplasmic reticulum of hibernating European hamsters is comparable to that observed by Belke et al. [13] for the cardiac muscle sarcoplasmic reticulum of the hibernating Richardson's ground squirrel. Yet, the reported uptake rates of the two preparations are difficult to be directly compared. Taking into account that the vesicular fraction protein only amounts to 5% of the protein concentration of the homogenate (cf. [56, 57]), the activity of the sarcoplasmic reticulum vesicles in the skeletal muscle homogenate would exceed the activity of the isolated cardiac vesicles by a factor of approximate 6–7. This difference is comparable to that reported for isolated sarcoplasmic reticulum preparations from both muscles [58]. The fact that the procedures for separating the sarcoplasmic reticulum vesicles from the homogenate can interfere with their activity makes it difficult to ascertain activity differences between different preparations.

We have therefore focused on the attainment of activity parameters that do neither depend on the protein yield nor on accidental activity losses. In this respect the apparent affinity of the transport enzyme for calcium must be considered to be a most significant parameter. It is defined as the calcium concentration at which the enzyme is half maximally activated and was thus obtained by measuring the rate of calcium uptake at different free calcium concentrations. In the following we use instead of the term “apparent calcium affinity” the more generally used of “calcium sensitivity” (cf. for bibliography [59]). We have always compared under identical conditions the activities of preparations from hibernating and non-hibernating animals. We could assure that hibernation is going along with a highly significant decrease of calcium sensitivity. The concentrations for half maximal activation differ by a factor of 1.73 for the calculated values obtained from the experi-
mental values as shown in Table VI and VII. The absolute activity at saturating calcium concentrations is not measurably affected. Hence we must assume that calcium sensitivity is a more stable characteristic feature of the calcium metabolism in hibernation than activity itself.

It is not known if the contractile proteins also undergo changes in their calcium sensitivity during hibernation as one might infer. Such a change could be revealed from the results of our morphological studies on fibre composition of the respective muscles. It is well established that type-II-fibres are less sensitive to calcium than type-I-fibres [60–62]. A considerable atrophy was found to occur, but no significant change in fibre type composition could be ascertained. This is evidently in contrast to cardiac muscle. Its myosin heavy chains change during hibernation from AB to AA composition which is connected with a reduction of calcium sensitivity [63]. This difference between skeletal and cardiac muscle is another example for the notion that adaptation in different muscles follows different routes.

As a result of a reduced calcium sensitivity the muscle fibre could tolerate somewhat higher free calcium concentration. This could lead to a higher degree of occupancy of the various calcium binding protein and as a consequence to an elevation of the muscle total calcium. Yet, it is not very likely that the considerable rise in total calcium assured by us in muscle homogenates can be explained by this mechanism. Whether part of the calcium is stored in the mitochondria, remains to be shown. Nevertheless, the involvement of mitochondria as the last high-capacity low-affinity calcium store to protect cells from abnormal increases of (Ca^{2+}) could be assumed also for the muscle fibres of hibernating animals (cf. [64]). Evidence has been presented indeed by Pehovich and Wang [65] suggesting that in the hibernating Richardson’s ground squirrel liver mitochondria retain the capacity to take up calcium even during long periods of torpor. However, it remains to be determined whether the depressed rates of mitochondrial calcium uptake observed during hibernation are rapid enough to buffer the perturbation in cytosolic free (Ca^{2+}).

Another parameter of muscle calcium metabolism which does not depend on protein yield is the quotient relating the rate of calcium transport to the activity of the calcium transport ATPase, the calcium transport ratio. Its maximal value of two was first measured by Hasselbach and Makinose [66] in reaction media containing 5 mM oxalate as calcium precipitating agent. The oxalate concentration has to be chosen in such a way that calcium is only precipitated inside the vesicles as the result of calcium accumulation. As a consequence the concentration of soluble calcium in the vesicular lumen remains low keeping passive calcium efflux small. Thus the measured calcium uptake becomes equal to calcium influx. In the absence of oxalate the transport ratio reaches value of two only in the early beginning of calcium uptake, as long as the luminal calcium concentration has not yet reached its threshold for efflux carried by leaks or the pump itself. Therefore only initial uptake rates must be considered. Yet, also in the presence of oxalate transport ratios lower than two have been reported repeatedly. The reason for this deviation, which often impedes the results of comparative studies, is not always clear. It most often occurs when the mean calcium load remains under a critical level of 0.3 μmol Ca^{2+}/mg protein [67]. This is not the case in our experiments with control as well as with hibernating animals, where the mean load related to vesicular protein exceeds 1.0 μmol Ca^{2+}/mg protein. We must therefore assume that the low transport ratio observed in experiments with homogenates from control animals is due to the presence of a low threshold calcium efflux pathway or a calcium leak. The notion is supported by the fact that the significant improvement of the calcium transport ratio is not due to a reduction of the calcium dependent ATPase activity, but to an activation of calcium uptake. That means that hibernation affects not the influx related reaction steps, but rather efflux related reactions. Their sensible dependence on reaction conditions has recently been demonstrated [68]. An involvement of the channel which mediates the physiological calcium release in calcium permeation under uptake conditions is not very likely. We found that the release induced by caffeine, which is a specific channel modulator, in the presence of appropriate concentrations of KCl (cf. [44]) is identical in extent and time course for sarcoplasmic reticulum from control and hibernating animals.

It is interesting to compare the results here presented with those we recently obtained with golden hamsters [30]. In contrast to the European hamster
the golden hamster did not hibernate under the same experimental conditions. This is in agreement with the reports describing golden hamsters as not obligate hibernators [35]. Nevertheless, we found [30] that the muscle calcium metabolism of the winter and even more of the cold adapted golden hamsters reflects some properties characteristic for the hibernating species, like the reduced calcium sensitivity of the calcium pump and the increase of the calcium uptake rate. In this respect the golden hamster behaves like the European hamster kept awake in winter under standard laboratory conditions — winter hamster.

In conclusion: it has been shown by our studies that in the obligate hibernator — European hamster — in winter and during the torpid state as well as in facultative hibernating animal — golden hamster — in winter and after cold acclimation the calcium transport system of skeletal muscle sarcoplasmic reticulum is affected. We could establish that the calcium sensitivity of the sarcoplasmic reticulum calcium pump is significantly reduced in the skeletal muscle of the hibernating European hamster as well as in that of the winter and cold acclimated golden hamster. For the European hamster we could further show that the efficiency of its sarcoplasmic reticulum calcium pump is markedly improved during hibernation. One might assume that both changes in the properties of the calcium pump are adaptive mechanisms by which the muscle capability to eliminate more rapidly during arousal the calcium accumulated in the low temperature state of hibernation is warranted. Yet, the mechanism responsible for the sustained calcium transport function by sarcoplasmic reticulum membrane during the torpid state is not clear.

The reported modulation of calcium transport ATPase of skeletal muscle sarcoplasmic reticulum is correlable to the increase of the myosin isoforms characteristic of fast-twitch-fibres observed in the cardiac muscle of the same animal which is also an expression of cold adaption (cf. [63]). This is another example indicating that correlate calcium transport ATPase and myosin heavy chain isoforms expressed during normal development are also coordinately regulated under pathophysiological conditions (cf. also for bibliography [69–71]).

Acknowledgements

The diligent and expert technical assistance of Andrea Döbler in many experiments and the skilled and careful cooperation of Elke Velten in the morphometric study are gratefully acknowledged.

We also thank Professor Dr. Walter Fiehn, Zentrallabor der Medizinischen Klinik, University of Heidelberg, F.R.G., for the determination of ions in serum.

Notes

1. Studies on hibernation were done according to the guidelines on animal protection with the approval of the Regierungspärisidium Karlsruhe, Karlsruhe, F.R.G.
2. Luisa De Martino was a research fellow of the Max-Planck-Gesellschaft, München, F.R.G., on leave from Istituto di Microbiologia, 1. Facoltà di Medicina e Chirurgia, Università degli Studi Federico II di Napoli, Italy.
3. Contribution of Barbara Soltau to this work is part of her M.D. Thesis at the University of Heidelberg, F.R.G.