Structural and functional relationship between distinct SERCA isoforms

Ph.D. Thesis

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Papers related to the subject of the Thesis

I. Ernő Zádor, **Gerda Szakonyi**, Gábor Rácz, Luca Mendler, Mark Ver Heyen, Jean Lebacq, László Dux, and Frank Wuytack (1998) Expression of the sarco/endoplasmic reticulum Ca^{2+}-transport ATPase protein isoforms during regeneration from notexin induced necrosis of rat soleus muscle. *Acta Histochemica* **100**, 355-369.

II. Luca Mendler, **Gerda Szakonyi**, Ernő Zádor, Anikó Görbe, László Dux, and Frank Wuytack (1998) Expression of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases in the rat extensor digitorum longus (EDL) muscle regenerating from notexin induced necrosis. *Journal of Muscle Research and Cell Motility* **7**, 777-785.

III. Gerda Szakonyi László Dux, and László Horváth (1998) Rearrangement of boundary, stand-by and fluid lipids during the formation of two dimensional crystals of Ca^{2+}-ATPase. *Journal of Molecular Structure* (accepted for publication).

IV. Gerda Szakonyi and László Dux (1998) Structural differences between distinct sarcoplasmic reticulum Ca^{2+}-ATPases. *ACE* **1**, 189-196.

V. Gerda Szakonyi, Györgyi Jakab and László Dux (1999) Effect of thapsigargin and cyclopiazonic acid on the sarcoplasmic reticulum Ca^{2+}-ATPase from carp, sterlet, eel and silure. *Comp. Biochem. Physiol.* (submitted for publication).

Other publications

VI. Béla Török, Károly Felföldi, **Gerda Szakonyi**, and Mihály Bartók (1997) Sonochemical enantioselective hydrogenation of ethyl pyruvate over platinum catalysts. *Ultrasonics Sonochemistry* **4**, 301-304.

VII. Béla Török, Károly Felföldi, **Gerda Szakonyi**, Árpád Molnár, and Mihály Bartók (1998) Ultrasonics in Chiral Metal Catalysis. Effect of Presonication on the Asymmetric Hydrogenation of Ethyl Pyruvate over Platinum Catalysts. Catalysis of Organic Reactions (Ed. F. Herkes), Marcel Dekker, New York, 1998, p. 129.

VIII. Béla Török, Károly Felföldi, **Gerda Szakonyi**, Katalin Balázsik, and Mihály Bartók (1998) Enantiodifferentiation in asymmetric sonochemical hydrogenations. *Catalysis Letters* **52**, 81-84.

IX. György Szöllösi, Béla Török, **Gerda Szakonyi**, István Kun, and Mihály Bartók (1998) Ultrasonic Irradiation as Activity and Selectivity Improving Factor in the Hydrogenation of Cinnamaldehyde over Pt/SiO2 Catalysts. *Applied Catalysis A* **172**, 225-232.

Abbreviations and Textual Footnotes

2D,	two-dimensional;
3D,	three-dimensional;
ATP,	adenosine 5'-triphosphate;
COS,	line of simian cells, obtained by transformation of CV-1 cells with an origin-defective mutant of SV 40;
CPA,	cyclopiazonic acid;
DAB,	3,3 diaminobenzidine;
DMSO,	dimethyl sulfoxide;
DNA,	deoxyribonucleic acid;
EDL,	extensor digitorum longus;
EGTA,	ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid;
EPR,	electron paramagnetic resonance;
FITC,	fluorescein isothiocyanate;
HEPES,	N-(2-hydroxyethyl)piperazine-N'-2-ethansulfonic acid;
MOPS,	3-(N-morpholino)-propanesulfonic acid;
mRNA,	messenger ribonucleic acid;
NADH,	ß-nicotinamide adenine di-nucleotide, reduced form;
PEP,	phospho(enol)pyruvate; 2-(phophonooxyl)-2-propenoic acid;
PAGE,	polyacrylamide gel electrophoresis;
SDS,	sodium dodecyl-sulfate;
SERCA,	sarco/endoplasmic reticulum Ca^{2+}-ATPase;
14-SASL,	14-(4,4-dimethyloxazolidine-N-oxyl)stearic acid;
SR,	sarcoplasmic reticulum;
TG,	thapsigargin;

Contents

1. Summary

Rabbit, carp, sterlet, eel and silure Ca^{2+}-ATPases of sarcoplasmic reticulum from abdominal muscles supposed to represent different SERCA isoforms, were compared in susceptibility to structural studies such as tryptic digestion, two dimensional crystallization and electron paramagnetic resonance measurements of lipid-protein interaction in E_2 form crystalline arrays. Furthermore the function of the Ca^{2+} pump enzyme was analysed using thapsigargin and cyclopiazonic acid as inhibitors. SERCA isoforms of soleus (slow skeletal) and EDL (fast skeletal) muscles from rat were used for regeneration experiments after notexin treatment.

Limited tryptic proteolysis is an effective method to characterize the sarcoplasmic reticulum Ca^{2+}-ATPase structure. SERCA1 isoform (enzime from rabbit fast skeletal muscle) shows different tryptic cleavage pattern in the two conformations. Ca^{2+}-ATPases from sterlet eel and silure were investigated by this method. The tryptic cleavage products were a 57000 and 45000 Da of sterlet, a 45000 Da of eel and a 40000 Da of silure in the presence of EGTA. Additional cleavage product was found in all species in the 23000 molecular weight region when Ca^{2+} was added to the medium.

The sarcoplasmic reticulum Ca^{2+} ATPase exists in two different conformations E_1 and E_2 during its reaction cycle. The E_1 is the high, E_2 is the low calcium affinity conformation. The enzyme can be stabilized in these two conformations while two dimensional crystalline structure develops. E_1 type crystalline array can be developed by Ca^{2+} and lanthanides E_2 type crystals form in Ca^{2+} free medium with decavanadate. None of the crystalline formations were found in the case of Ca^{2+}-ATPases from eel silure and sterlet. SERCA1 (the isoform can be found in fast skeletal muscle) and SERCA3 (mostly detected in spleen, stomach, intestine, lung, uterus, and pancreas cells) isoforms expressed by COS cells were investigated, as well. SERCA1 showed vanadate induced 2D crystals but SERCA3 isoform did not.

The sarcoplasmic reticulum membrane is a lipid-protein system where fluid lipid and motionally restricted boundary lipid sites give multicomponent spin label EPR spectra. The motion of the lipids were followed using by conventional electron paramagnetic method to investigate the changes of sarcoplasmic reticulum from rabbit skeletal and carp abdominal muscle prior to and after vanadate treatment. The formation of two dimensional lattice was

confirmed by electron microscopy. During two dimensional crystal formation of rabbit SR Ca^{2+}-ATPase a third motionally less restricted stand-by lipid shell was found within the boundary site. This change was not observed in the case of carp sarcoplasmic reticulum.

Specific inhibitors occur to be very useful agents in molecular studies of ATPase mechanism. Thapsigargin (TG) and cyclopiazonic acid (CPA) are found to be potent inhibitors of intracellular Ca^{2+} pump proteins of sarcoplasmic reticulum from skeletal muscle. E_2 conformation of SR Ca^{2+}-ATPase can be inhibited by both thapsigargin and cyclopiazonic acid. Sarcoplasmic reticulum Ca^{2+}-ATPases of abdominal muscle from distinct fishes can be inhibited by both TG and CPA, as well. TG seems to be more powerful inhibitor as its complete blocking effect can be observed in nanomolar while in the case of CPA only in micromolar concentration. Among these species the sterlet Ca^{2+} ATPase is the most resistant for both inhibitors. The carp SR enzyme is the most sensitive for cyclopiazonic acid meanwhile the silure enzyme for thapsigargin.

The changes in SERCA1 SERCA2a and SERCA2b isoforms were followed at protein level in EDL (fast) and soleus (slow) skeletal muscles during muscle regeneration by immunoblot technique. The main muscle isoforms (SERCA1 and 2a) decreased dramatically on day 3 after notexin induced necrosis. Their quantity increased continuously until day 21 in some cases then decreased until day 28. The level of non-muscle isoform SERCA2b remained relatively constant throughout the whole regeneration period in both cases. The transcripts and proteins of SERCA isoforms showed similar time-dependent changes in expression pattern during the regeneration of the muscles.

2. Introduction

Numerous cell functions are triggered by signals originating from the plasma membrane. G protein or tyrosine kinase linked receptors are activated through signalling pathways involving intracellular ryanodine or inositol triphosphate receptors and leading to Ca^{2+} release from intracellular stores *(Berridge, 1993)*. The consequent elevation of cytosolic Ca^{2+} occurs with specific spatio temporal patterns which are involved in control and/or regulation of functions such as contractility, sensory perception, neuromodulation, fertility, development, growth and transformation. The availability of intracellular Ca^{2+} in turn, is dependent on the function of Ca^{2+} transport ATPases, which are bound to sarco- and endoplasmic reticulum membranes and transport of cytosolic Ca^{2+} into intracellular compartments actively *(Inesi & Sagara, 1994)*.

Further studies revealed that these Ca^{2+} pump proteins can be expressed by three genes. The SERCA1 is the fast twitch skeletal muscle isoform with its two alternative splice product SERCA1a (adult form) and SERCA1b (neonatal form). SERCA2a is typical for slow skeletal and cardiac muscle and SERCA2b is present in many cell types. SERCA3 can be found in non muscle cell types *(Brandl et al., 1987; Burk et al., 1989; Lytton et al., 1989)*.

The different SERCA family members' common function is to maintain the low intracellular calcium concentration, although they show dissimilarities. An altered pH and temperature optimum *(deToledo et al., 1995)*, different tryptic cleavage pattern *(Dux et al., 1989)*, distinct affinity for Ca^{2+} and for vanadate, varied TG sensitivity *(Papp et al., 1991)* were reported.

All eukaryotic cells appear to express at least one SERCA gene. This is probably the most closely related to the vertebrate SERCA2. However three different genes have been described (SERCA1-3) in higher vertebrates only *(Wuytack et al., 1995)*.

The comparison of sarcoplasmic reticulum Ca^{2+}-ATPases from different species representing distinct SERCA isoforms is very important for the better characterization of their accurate structure and biological function. The Ca^{2+} pump of the sarcoplasmic reticulum has proven to be exceptionally useful for the analysis of structure-function relationships within a transport molecule and for the understanding of the mechanism of cation transport. Most of these studies have paid attention to the conformational changes of this membrane

protein. The study of the function of the SR Ca^{2+}-ATPase above the structural studies plays an important role in the understanding its mode of action. Two chemical compounds thapsigargin and cyclopiazonic acid have been recently discovered to inhibit the SERCA ATPases specifically. These inhibitors are useful agents for the molecular studies of the ATPase mechanism.

Changes in the level of the different SERCA transcripts have been described in the notexin induced necrosis and regeneration of the rat soleus muscle *(Zádor et al., 1996)*. The injection of notexin, the venom of an Australian snake, causes a rapid necrosis and subsequent regeneration in skeletal muscle. The SERCA transcripts showed similar time dependent changes, the neonatal transcript appeared before the adult one, as observed during normal muscle development.

The major aim of this work was to gain a better insight into the structure-function relationship and dissimilarities of sarcoplasmic reticulum Ca^{2+}-ATPases using different biochemical methods. Ca^{2+}-ATPase isolated from lower species (carp, sterlet, eel and silure) were chosen to investigate the evolutionary adaptation of SR Ca^{2+} pump protein. Tryptic cleavage pattern of Ca^{2+}-ATPase from different fishes was revealed in the medium contained Ca^{2+} or EGTA and vanadate. The effect of decavanadate on the native structure of the enzyme was checked by electron microscope after negative staining. The lipid-protein interactions in the E_2 conformation of the molecule was followed by EPR measurement. Investigating the function of Ca^{2+}-ATPase we report the potent inhibition of thapsigargin and cyclopiazonic acid on the Ca^{2+} pump protein from carp, sterlet, eel and silure. Furthermore we describe the changes in the protein level of the SERCA isoforms during the regeneration of EDL and soleus from notexin induced necrosis.

3. Theoretical and Historical Overview

3.1. *The Structure of the Sarcoplasmic Reticulum Ca^{2+}-ATPase*

The Ca^{2+} transport ATPase of sarcoplasmic reticulum is a single subunit integral membrane protein of 110.000 molecular weight. During its reaction cycle the enzyme exists in two main conformation, called E_1 and E_2 *(de Meis & Vianna,1979)*. There are different interactions between ATPase molecules in the E_1 and E_2 crystals *(Martonosi, 1995)* indicating distinct conformations. This conclusion is supported by different effects of membrane potential *(Dux & Martonosi, 1983d)*, high hydrostatic pressure *(Varga et al., 1986)*, tryptic proteolysis *(Dux et al., 1985; Dux & Martonosi, 1983b)* and vanadate catalyzed photocleavage *(Végh et al., 1990; Molnár et al., 1991)* on enzyme in the E_1 and E_2 states, together with differences in tryptophane fluorescence *(Jona & Martonosi, 1986)*, protein dynamics measured by fluorescence energy transfer *(Jona et al., 1990)* and reactivity with monoclonal antibodies *(Molnár et al., 1990; 1992)*.

The limited tryptic proteolysis is suitable to identify the E_1 and E_2 conformations in the case of Ca^{2+}-ATPase (SERCA1 isoform) from rabbit skeletal muscle. The first cleavage at Arg^{505} resulted in two fragments A and B with molecular weight of 57000 and 52000 in the presence of calcium. The second cleavage takes place on the A fragment at Arg^{198} yielding two subfragments called A_1 (34 kD) and A_2 (23 kD) *(Thorley-Lawson & Green, 1977; Shamoo, 1978)*. The accessibility of the second tryptic cleavage site is highly sensitive to the actual conformation of the enzyme *(Dux & Martonosi, 1983b; Dux et al., 1985)*. Antibody labelling experiments detected that the second tryptic cleavage site is buried deep inside the protein mass of the extramembrane domain *(Molnár et al., 1990)*.

Sarcoplasmic reticulum Ca^{2+}-ATPases of lobster *(Ohnoki et al., 1980)*, carp *(Dux et al., 1989)* and trout *(de Toledo et al., 1995)* gave different tryptic cleavage fragments. A large polypeptide of 80 kD mass with smaller amounts of 50, 35 and 23 kD fragments were accumulated during the digestion procedure. The SERCA3 isoform shows different tryptic fragmentation patterns, as well. This is due to the fact that tryptic cleavage site T1 which characterize SERCA1 and SERCA2 is absent from SERCA3 while the Ca^{2+} dependent cleavage site T2 is present. However the SERCA3 isoform forms only an approximately 80 kD fragment after prolonged trypsinization.

The equilibrium between the various enzyme states can be shifted by the ion composition of the medium as predicted by the E_1-E_2 model. Ca^{2+} or lanthanides stabilize the E_1 state and promote crystallization of Ca^{2+}-ATPase in a P1 type space group with ATPase monomers as structural units *(Dux et al., 1985)*. Inorganic phosphate or vanadate in Ca^{2+} free medium, stabilizes the Ca^{2+}-ATPase in the E_2 state and induces the formation of distinct P2 type Ca^{2+}-ATPase crystals with dimers as structural units *(Dux & Martonosi, 1983a; b; c; d; Taylor et al., 1984; 1986a; b;)*. This work led to the first isodensity contour maps of SR membrane crystals but the major part of the intramembrane domain was not revealed.

Carp SR Ca^{2+}-ATPase failed to form E_1 or E_2 2D crystals *(Dux et al., 1989)* but extensive two dimensional crystalline structure was detected in the sarcoplasmic reticulum of scallop even without addition of vanadate *(Castellani & Hardwicke, 1983)*.

Growth of three-dimensional crystals represents a key step in obtaining high-resolution structures of membrane proteins. Therefore the discovery of 3D microcrystals of Ca^{2+}-ATPase *(Dux et al., 1987)* is a significant advance. General experience indicates that there are two types of three-dimensional crystals of membrane proteins *(Michel, 1983)*. On the one hand, „type I" crystals are composed of stacks of two dimensional crystals. On the other hand, „type II" crystals are characterized by a more isotropic arrangement of fully solubilized molecules, similar to crystals of many soluble proteins. All intermolecular contacts are thought to occur between hydrophilic surfaces.

Recently, three dimensional reconstruction of cryo-electron microscopic images can extend the resolution of the structural studies to 1.4 nm for determination of 3D-structures of the crystals from native SR, and to 0.6 nm resolution for the structure of thin, plate like crystals of detergent solubilized Ca^{2+}-ATPase *(Toyoshima et al., 1993; Stokes & Green, 1990)*. The Ca^{2+}-ATPase has not been crystallized in appropriate form for X-ray diffraction and there is a definite lack of experimental data to confirm the various models which have been proposed for the secondary and tertiary folding of the protein *(Mintz & Guillan, 1997)*.

The cryo-electron microscopic experiments extended the resolution of the structural studies on the ATPase *(Taylor et al., 1988)*. The intramembrane part of the Ca^{2+}-ATPase is formed by 10 helices joined by 5 lumenal short loops and 4 cytoplasmic loops. The cytoplasmic part of the molecule is linked to the 10 transmembrane helices by a stalk region

which maintains the hydrophilic globule in the external bulk. The main specific sites of the Ca^{2+}-ATPase, the ATP binding site and phosphorylation site are located in the cytoplasmic part of the molecule whereas the transport sites (the calcium binding site) are thought to be buried in the membrane.

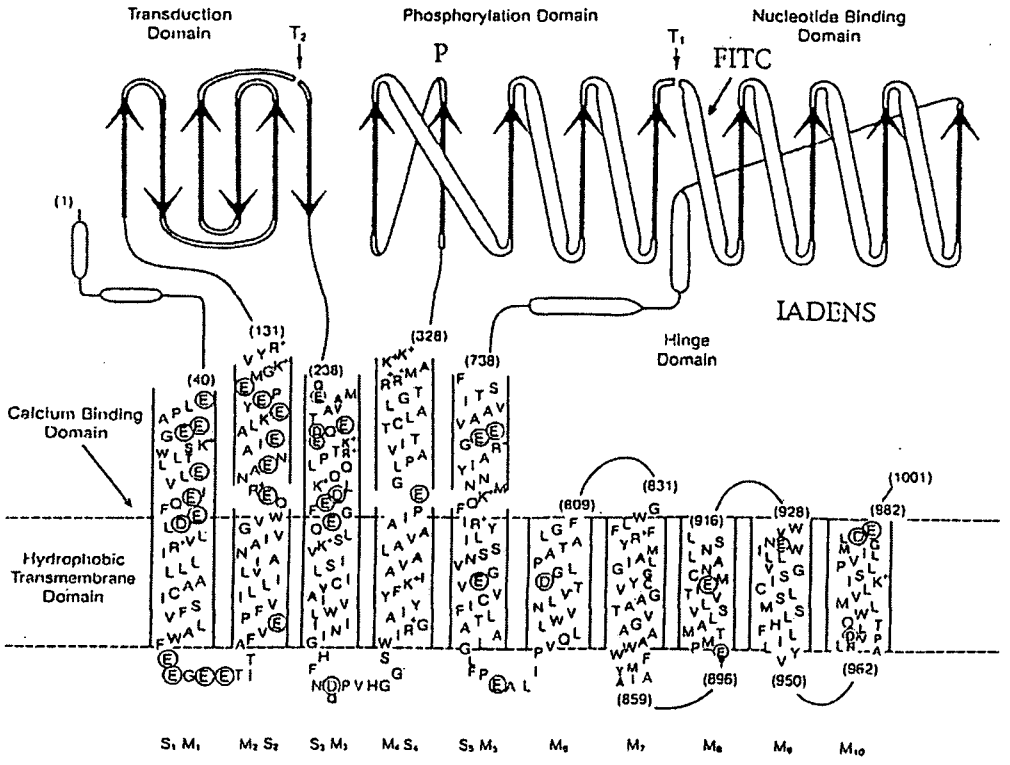

Figure 1. Planar illustration of the structure of SERCA1a in which the amino acid sequence is laid out in accordance with assigned domains and probable secondary structure.

Site directed mutagenesis have been used to deduce the active sites within the Ca^{2+}-ATPase molecule and to relate these sites to function in the construction of a model for the mechanism of Ca^{2+} transport *(MacLennan, 1990)*. For the calcium binding the Glu^{309}, Glu^{771}, Asn^{796}, Thr^{799}, and Asp^{800} seemed to be essential *(Andersen & Vilsen, 1994)*. Glu^{309} and Asn^{796} are associate with one Ca^{2+} binding site, Glu^{771} and Thr^{799} are associated with the other and Asn^{800} is associated both. This suggests that the two sites are distinguishable.

The ATP-binding site of the sarcoplasmic reticulum Ca^{2+}-ATPase enzyme has been studied mainly by chemical modifications by FITC or glutaraldehyde, labelling with ATP analogous by azido derivatives of ATP and site directed mutagenesis. The latter method has

shown that only the Gly^{626} and Asp^{627} are essential for phophoenzyme formation *(Maruyama et al., 1989)*. An interesting conclusion of this work was that most of the amino acids of these highly conserved regions are not essential for the nucleotide binding. Asp^{351} is an absolute requirement for the phosphorylation of the Ca^{2+}-ATPase molecule.

3.2. The Function of the Sarcoplasmic Reticulum Ca^{2+}-ATPase

The sarcoplasmic reticulum Ca^{2+}-ATPase belongs to the P type (E_1-E_2 type) ion transporting family. Its principal function is the regulation of the cytoplasmic Ca^{2+} concentration. This enzyme pumps Ca^{2+} from the cytoplasm of the muscle cells into the sarcoplasmic reticulum, initiating muscle relaxation *(MacLennan et al., 1992)*.

In the E_1 (high calcium affinity) conformation, the enzyme binds two calcium ions on the cytoplasmic side of the membrane. Upon the binding and subsequent hydrolysis of one molecule of ATP, the enzyme forms a phosphorylated intermediate *(Yamamoto & Tonomura, 1967; Makinose, 1969; Martonosi, 1969; Froelich & Taylor, 1976)* In the following step the enzyme undergoes a conformational rearrangement (E_2). The calcium ions become occluded inside the protein and finally reappear at the luminal side of the membrane (Waas *& Hasselbach, 1981)*. The low calcium affinity of the E_2 form helps the release of the translocated calcium ions into the lumen of the SR *(Ikemoto, 1975)*. Following a step of dephosphorylation, the enzyme returns to the E_1 conformation *(Inesi et al., 1970; Kanazawa, 1971)*.

3.2.1. Role of the Lipids in Ca^{2+}-ATPase Enzyme Mechanism

The activity of the sarcoplasmic reticulum Ca^{2+}-ATPase is dependent on the conditions of the phospholipids surrounding it in the membrane *(Michelangeli et al., 1991; Starling et al., 1993)*. This dependence could originate directly form the effects of phospholipids on the conformation of the ATPase either, or indirectly from the changes in some physical properties of the phospholipid bilayer, such as fluidity or thickness.

Phospholipids phosphatidylcholine (65 to 75%), phosphatidylethanolamine (15 to 20%) phosphatidylinositol (8-10%) are the main lipid components of the native SR

membrane *(Meissner et al., 1972; Drabikowski et al., 1966; Fiehn et al., 1971; Waku et al., 1971)*. Cholesterol is present only low concentrations (5% or less).

There are 80 to 100 molecules of phospholipids associated with one molecule of enzyme in the native SR membrane *(Herbette et al., 1985)*. Structural studies indicate that these phospholipids are somewhat asymmetrically distributed between the halves of bilayer with more phospholipid (54%) in the inner monolayer. Removal of substantial fraction of the endogenous lipids present in SR vesicles from the original 80 to 100 lipids per enzyme down to 40 to 30 lipids per enzyme, has no effect on Ca^{2+}-ATPase activity *(Nakamura et al., 1975; Moore et al., 1978)*. Subsequent removal of lipids below 40 to 30 lipids per enzyme causes a drastic inhibition of ATPase activity to almost undetectable levels for preparations containing 10 to 15 lipids per enzyme. The inhibition can be reversed by readdition of the lipids *(Hidalgo et al., 1986)*. Lipid removal does not affect other partial steps of the ATPase reaction cycle such as calcium binding, and nucleotide binding but markedly interferes with the binding of vanadate *(Medda & Hasselbach, 1984; 1985)*.

Physical studies carried out either with the native SR membrane or with the reconstituted enzyme systems indicate that the SR phospholipids are present in two distinguishable motional populations, as probed with fatty acid or phospholipid spin labels *(Thomas et al., 1982)*. The spin label probes report one component with similar motion to that obtained from extracted SR lipids and another less mobile component (boundary lipids) that is presumably due to the contact with the protein. The boundary lipids are probably needed to maintain the enzyme in a configuration dispersed enough, to allow access of ATP either to its binding site or to the catalytic site, or access of calcium to the high affinity binding sites *(Hidalgo, 1987)*.

3.3. *Specific Inhibitors of the Sarcoplasmic Reticulum Ca^{2+}-ATPases*

The inhibitors of intracellular Ca^{2+}-ATPases promise to be very useful agents in molecular studies of ATPase mechanism as well as in the studies of cell functions which are regulated by cytosolic Ca^{2+} *(Inesi & Sagara, 1994)*. It has been recently reported that various chemical compounds inhibit specifically the SERCA ATPases.

3.3.1. Thapsigargin

The most specific and potent of these inhibitors is thapsigargin. It was isolated from the root of the Mediterranean umbelliferous plant Thapsia garganica *(Rasmussen et al., 1978)*. The root extract of Thapsia garganica was used for rheumatic pains, muscle and joint inflammations. The potential medical benefit of the plant is attested by its use in traditional Arabian and European medicine up to its listing in the French Pharmacopoeia of 1937 *(Treiman et al., 1998)*.

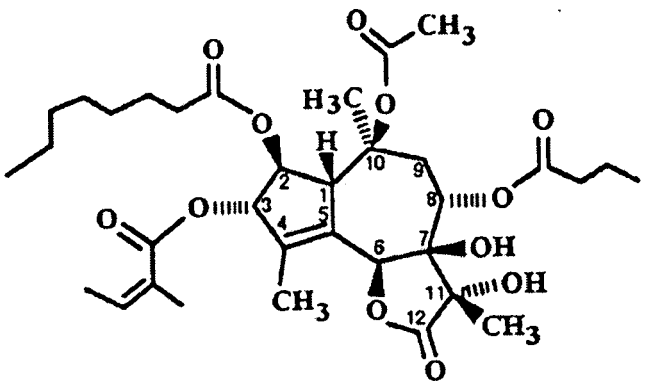

Figure 2. The chemical structure of thapsigargin *(Inesi & Sagara, 1994)*.

Thapsigargin is a sesquiterpene lactone (Fig. 2.). The high lipid solubility of this compound accounts for its excellent penetration into the biological membranes. A specific and interesting aspect of thapsigargin's mode of action, came to light when it was found, that this agent produces elevation of intracellular Ca^{2+} *(Thastrup et al., 1987a; 1987b)*. This effect was related to the inhibition of the endoplasmic reticulum Ca^{2+} sequestering activity *(Thastrup et al., 1990)*. It was established that thapsigargin is a high affinity inhibitor of all intracellular Ca^{2+} transport ATPases tested *(Sagara & Inesi, 1991; Campbell et al., 1991; Lytton et al., 1991; Kijima et al., 1991)*. Nevertheless it was reported that TG inhibited all of the SERCA enzymes including SERCA3 with equal potency. However results on the E-P levels of Ca^{2+}-ATPase in platelets, which coexpress SERCA3 and SERCA2b, suggested that a difference in thapsigargin sensitivity might exist between the two isoforms, with SERCA2b being more sensitive to the inhibitor *(Papp et al., 1991)*.

Further studies showed that TG interacts only with the Ca^{2+} free enzyme state thereby forming dead-end inhibitory complex *(Sagara & Inesi, 1991)*. Both the Ca^{2+} binding and the phosphorylation reactions are inhibited by thapsigargin *(Sagara et al., 1992)*. Interesting feature of the TG inhibition is its global character, involving the entire ATPase molecule *(Sagara et al., 1992)*. Thapsigargin was found to stabilize the transmembrane region of the ATPase *(de Meis & Inesi, 1992)*. TG appears to bind to the M3 transmembrane sequence *(Norregaard et al., 1994)*. It stabilizes the E_2 state of the pump and promotes formation of helical tubes *(Stokes & Lacapere, 1994)*.

3.3.2. Cyclopiazonic Acid

Another SERCA inhibitor is cyclopiazonic acid. The toxic metabolite produced by the molds Pencillium cyclopium and Aspergillus flavus *(Holzapfel, 1968)*. CPA represents a toxicological problem for humans and animals, since it may be found as a contaminant in processed food, grain and poultry *(LeBars, 1979; Landsen & Davidson, 1983; Dorner et al., 1983)*.The first clinical symptoms related to the ingestion of CPA were associated with several neurological and muscular disorders *(Nishie et al., 1985; Norred et al., 1985)*.

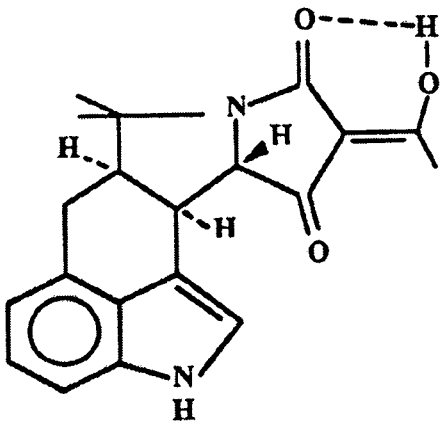

Figure 3. The chemical structure of cyclopiazonic acid *(Inesi & Sagara, 1994)*.

Subsequent biochemical characterization revealed that CPA inhibits the Ca^{2+}-ATPase activity of isolated SR vesicles *(Goeger & Riley, 1989)*. The inhibition occurs at equimolar levels of the toxin and has no effect on other cation transport ATPases *(Seidler et*

al., 1989). Cyclopiazonic acid inhibits the Ca^{2+} binding and ATP dependent phosphorylation of Ca^{2+}-ATPase *(Goeger & Riley, 1989)*. CPA appears to have lower affinity than TG, as it is effective in the micromolar range compared to the nanomolar range of TG.

Covalent labelling of the Ca^{2+}-ATPase with radioactive form of CPA may help in the identification of the region of the molecule where CPA exerts its striking effect on the conformational transitions related to Ca^{2+} transport *(Goeger & Riley, 1989)*.

3.4. Isoforms of the Sarcoplasmic Reticulum Ca^{2+}-ATPase

The primary sequence of the Ca^{2+}-ATPase was established after sequencing the encoding DNA *(MacLennan et al., 1985)*. Further studies of the sarcoplasmic reticulum Ca^{2+} pump gene revealed three main isoforms *(Brandl et al., 1986; 1987; Lytton et al., 1989; Burk et al., 1989)*. The gene expressed in fast skeletal muscle is called SERCA1 *(Burk et al., 1989)*. It has two different products generated by alternative splicing. While the SERCA1a form is present in adult, the SERCA1b form in neonatal fast muscle. The neonatal form contains seven additional amino acids at C-terminal end. The functional role of this difference is unknown *(Brandl et al., 1987; Burk et al., 1989)*.

The SERCA2 gene has two different splice products, as well. The SERCA2a is the slow skeletal and cardiac muscle isoform. The SERCA2b variant, having 41 additional amino acids at the C-terminal end, is present in smooth muscle, and in low quantities in many other cell types *(Burk et al., 1989; Lytton et al., 1989)*.

The third member of the SERCA gene family is SERCA3. SERCA3a isoform was detected in spleen, stomach, intestine, lung, uterus, and pancreas *(Burk et al., 1989; Grover & Khan, 1992)*, moreover in haematopoietic cell lineages and related cells: platelets, lymphoid cells, mast cells *(Wuytack et al., 1994; Bobe et al., 1994)*. SERCA3b and SERCA3c form can be found in mouse pancreatic islets of Langerhans and human kidney.

Most differences were found at the N-terminal region of the molecule. The most conserved regions were the transmembrane helices, the stalk region, and the so-called hinge domain connecting the phosphorylation and ion transporting regions *(Brandl et al., 1986)*. Further structural and functional dissimilarities can be revealed by the investigation of distinct Ca^{2+}-ATPase isoforms of lower species.

4. Materials and Methods

4.1. Chemicals and Animals

Peroxidase conjugated rabbit anti-mouse immunoglobulines and swine anti-rabbit immunoglobulines were from Dako A/S (Glostrup, Denmark). All the other chemicals were of analytical grade and purity, and purchased from Sigma-Aldrich (Munich, Germany).

Sarcoplasmic reticulum from the white abdominal muscle of different fishes: carp, (Cyprinus carpio L.), silure (Ictalurus nebulosus), eel (Anguilla anguilla), sterlet (Acipenser ruthenus); and from the longitudinal back muscle of rabbit were used for limited tryptic digestion analysis, electron microscopic studies, EPR measurements and enzymatic assays. Soleus and EDL muscles of 300-360 g male Wistar rats were used for the notexin treatment and immunoblot analysis.

4.2. Preparation of Sarcoplasmic Reticulum

Sarcoplasmic reticulum was prepared from carp, silure, eel, sterlet white abdominal and rabbit skeletal muscle by the method of Nakamura *(Nakamura et al., 1976)*. Protein concentration of the samples was measured by the method of Lowry *(Lowry et al., 1951)*. Protein composition of the samples was tested on 6-18% SDS polyacrylamid gels according to Laemmli *(Laemmli, 1970)*.

4.3. Limited Tryptic Proteolysis of the Sarcoplasmic Reticulum

The SR samples were incubated in 10 mM imidazole pH 7.4, 0.1 M KCl, 5 mM $MgCl_2$ and 20 μM $CaCl_2$ or 0.5 mM EGTA and 5 mM Na_3VO_4. The distinct Ca^{2+}-ATPase molecules were exposed to tryptic cleavage in a concentration of 3 mg/ml. The protein:trypsin ratio was: 50:1. The digestion was carried out at 25 °C. Aliquots were taken after 3 and 15 min. The digestion was stopped by the addition of 0.5 mg/ml trypsin inhibitor (final concentration) and equal volume of SDS sample buffer. The digested samples were separated on 6-18% gradient polyacrylamide gels. A Bio-Rad standard was used: phosphorilase b 97.4 kD; serum albumin 45.0 kD; carbonic anhydrase 31.0 kD; trypsin inhibitor 21.5 kD; lysosyme 14.4 kD.

4.4. Induction of Two Dimensional Crystals and Negative Staining

E_2 crystals were induced by the method of Dux and Martonosi *(Dux & Martonosi, 1983; b; c; d)*. The SR membranes were incubated in a medium contained 10 mM imidazole pH 7.4, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM EGTA and 5 mM Na_3VO_4 at 4°C for 24 hours. The surface structure of the membrane suspensions was controlled after negative staining with 1% uranyl acetate in Philips CM 10 electron microscope at 100 kV accelerating voltage.

4.5. Spin Labelling and EPR Measurement

14-SASL (14-(4,4-dimethyloxazolidine-N-oxyl)stearic acid) was synthesised as described by Marsh *(Marsh et al., 1982)*. Minute amount (1 mol%) of spin labelled stearic acid was incorporated into the SR membrane, prior to or after the overnight vanadate treatment, from ethanolic solution of the labels, followed by incubation at ambient temperature for 30 minutes. EPR spectra were recorded with a Bruker ECS 106 Series spectrometer equipped with a temperature regulation system, based on pressured air gas flow. Spectral subtractions and lineshape simulations were done as described by Horváth *(Horváth et al., 1988)*. The lipid-protein complexes (for example SR membrane) give two component EPR spectra. The spectra of fluid lipid and native sarcoplasmic reticulum were obtained in order to find the best matching line-width parameters for spectrum of boundary lipids. The component spectra of the fluid and boundary lipids can be obtained by the intersubtraction of two two-component spectra, as well. The ratio of the fluid and boundary lipids are not equal in these spectra. A factor is required for making for example the fluid lipid sites equal. The boundary component can be determined after the intersubtraction of these two component spectra.

4.6. Enzymatic Activity Assays without and with Inhibitors

1 mg/ml final concentration of SR samples were incubated in the following medium: 5 mM HEPES pH , 100 mM KCl, 5 mM $MgCl_2$, 100 μM $CaCl_2$ 60 μM EGTA, 0.4 mM NADH, 1.5 μg/ml A23187 calcium ionophore, 2 mM PEP and 10^{-10}, 10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} $2x10^{-7}$, $5x10^{-7}$ and 10^{-6} M TG or CPA at 25 °C for 5 min. The reaction was started by the addition of ATP to give a final concentration of 1 mM. The reaction was run

for 5 minutes and then EGTA pH 7.0 (final concentration 4 mM) was added to the reaction mixture to obtain the basal ATPase rate.

The oxidation of NADH is continuously monitored by the decreasing absorbance at 340 nm using Hitachi UV-3000 spectrophotometer. The assay is based on the following coupled enzyme reactions:

$$\text{ATP} \xrightarrow{\text{SR CaATPase}} \text{ADP} + \text{pyruvate}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}$$

The amount of NADH is equivalent to the ATP hydrolyzed.

4.7. Immunoblots of EDL and Soleus Muscles after Notexin treatment.

The notexin treatment and preparation of the muscles are described in details in annex: Zádor et al. 1998, and Medler & Szakonyi et al. 1998.

The microsomal fractions of EDL and soleus were resuspended in 300 µl of 0.25 M sucrose. 5 µl of these suspensions were electrophoresed on Laemmli-type 7.5 % SDS PAGE and then electroblotted onto immobilon-P (Millipore). The SERCA1 protein was detected by a 1:5 dilution of culture supernatant of the A3 mouse anti-SERCA1 monoclonal antibody *(Zubrzycka-Gaarn et al., 1984)*. The blots were quenched in 10 mM Tris HCl pH 7.5, 0.9% NaCl, 0.05% TWEEN-20, (TBST) for 1 hour. The antibodies were also dissolved in TBST solution. The SERCA2a protein was detected by a 1:500 dilution of the SERCA2a- and SERCA2b protein by a 1:200 of the SERCA2b-specific antisera *(Wuytack et al., 1989, Eggermont et al., 1990)*. The incubations with the primary antibodies and the secondary antibodies (peroxidase conjugated rabbit anti-mouse immunoglobulines or swine anti-rabbit immunoglobulines) lasted for 1 hour. For visualisation of the immunocomplexes, nickel-enhanced diaminobenzidine precipitation was used *(Wuytack et al., 1994)*. Quantification was performed through densitometer scanning, using ScanPack 10.1 A20 program (Biometra, Göttingen, Germany).

5. Results and Discussion

5.1. Tryptic Cleavage Pattern of the Sarcoplasmic Reticulum from Sterlet, Eel and Silure

The Ca^{2+} transport ATPase is a major protein component of sarcoplasmic reticulum from rabbit, carp, sterlet, eel and silure muscles. In addition to the ATPase other proteins are observed regularly. The relative amount of these proteins is different in sarcoplasmic reticulum membranes from distinct species. The 80000 Da protein of carp and silure and the 90000 Da compound of sterlet microsome fractions represent the largest amount of these additional proteins.

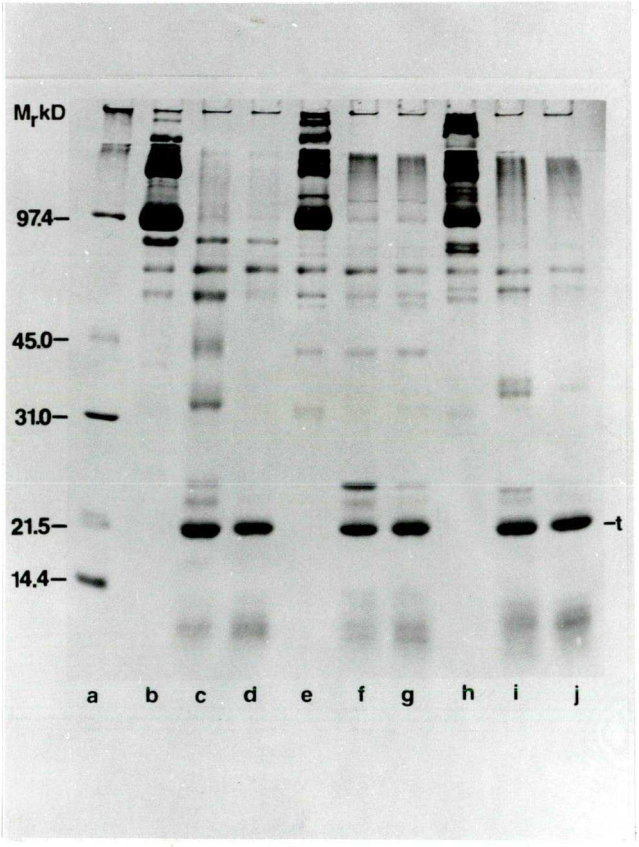

Figure 4. Tryptic cleavage pattern of sterlet, eel, and silure sarcoplasmic reticulum Ca^{2+}-ATPase in the presence of calcium at 25°C on 6-18% gradient SDS-polyacrylamide gel electrophoresis. (a) Molecular mass marker, (b) sterlet undigested sample, (c) sterlet digested for 3 min, (d) sterlet digested for 15 min, (e) eel undigested sample, (f) eel digested for 3 min, (g) eel digested for 15 min, (h) silure undigested sample, (i) silure digested for 3 min, (j) silure digested for 15 min.

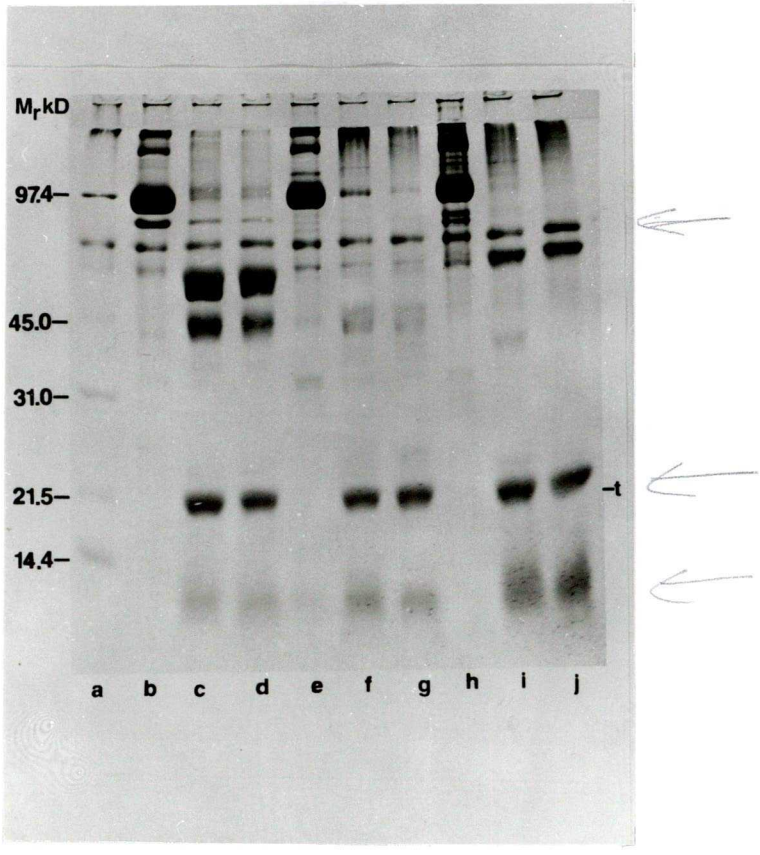

Figure 5. Tryptic cleavage pattern of sterlet, eel, and silure sarcoplasmic reticulum Ca^{2+}-ATPase in the presence of EGTA at 25°C on 6-18% gradient SDS-polyacrylamide gel electrophoresis. (a) Molecular mass marker, (b) sterlet undigested sample, (c) sterlet digested for 3 min, (d) sterlet digested for 15 min, (e) eel undigested sample, (f) eel digested for 3 min, (g) eel digested for 15 min, (h) silure undigested sample, (i) silure digested for 3 min, (j) silure digested for 15 min.

The rabbit and carp sarcoplasmic reticulum showed products of tryptic digestion which were consistent with the previously described cleavage pattern (annex IV. Fig. 1. and 3.) *(Dux et al., 1989; 1985; Dux & Martonosi, 1983b)*.

The sterlet eel and silure sarcoplasmic reticulum Ca^{2+}-ATPase gave different tryptic cleavage pattern from the ones found in rabbit and carp in the presence of calcium (Fig. 4.). Under the same conditions the major products of the partial proteolysis of the sterlet enzyme are polypeptides of 57000 and 45000 dalton mass, in the eel is 45000 and in the silure is

40000. The cleavage product in the 23000 molecular weight region appeared in all of the three species tested.

The sterlet sarcoplasmic reticulum Ca^{2+}-ATPase gave a fragment in the 57000 and an another in the 45000, the eel in the 45000 and the silure in the 40000 molecular weight region in the presence of EGTA (Fig. 5.). None of the SR Ca^{2+}-ATPases accumulated significantly the 23 kDa fragment when EGTA was added to the medium.

These observations indicate that the disposition of the trypsin sensitive regions of the ATPase molecule in rabbit, carp, sterlet, eel and silure sarcoplasmic reticulum Ca^{2+}-ATPase is different.

The carp, eel and silure SR Ca^{2+}-ATPase gave similar tryptic cleavage pattern. Their main protein components were a 40-50 kD and a 23 kD fragment. These protein compounds could be found in lobster *(Ohnoki & Martonosi, 1980)* and in trout *(de Toledo et al., 1995)* suggesting that these Ca^{2+}-ATPase isoforms are very closely related. It is interesting to note that the molecular weight of the tryptic fragments of the sterlet (57000, 45000 and 23000 kD) were very close to the fragments found in rabbit SR.

5.2. Electron Microscopic Examination of Sterlet, Eel and Silure SR after Vanadate Treatment

During its reaction cycle the Ca^{2+}-ATPase of sarcoplasmic reticulum alternates between two distinct conformations. Vanadate ions stabilizes the enzyme in the E_2 conformation *(Pick, 1982; Pick & Karlish, 1982)*. Therefore the SR Ca^{2+} pump crystals were induced by the addition of Na_3VO_4. The other E_1 type two-dimensional crystalline arrays of Ca^{2+}-ATPase molecules develop in sarcoplasmic reticulum vesicles exposed to Ca^{2+} or lanthanide ions.

Addition of vanadate to the sterlet eel and silure sarcoplasmic reticulum microsome fractions failed to induce two-dimensional arrays (Fig 6.). On the other hand extensive 2D array formation was detected in the SR of scallop (Pecten magellanicus) even without addition of vanadate *(Castellani & Hardwicke, 1983)*. The membrane fraction derived from the brain heater organ of the swordfish (Xiphias gladius) gave highly ordered Ca^{2+}-ATPase arrays upon addition of vanadate *(Bloch et al., 1986)*.

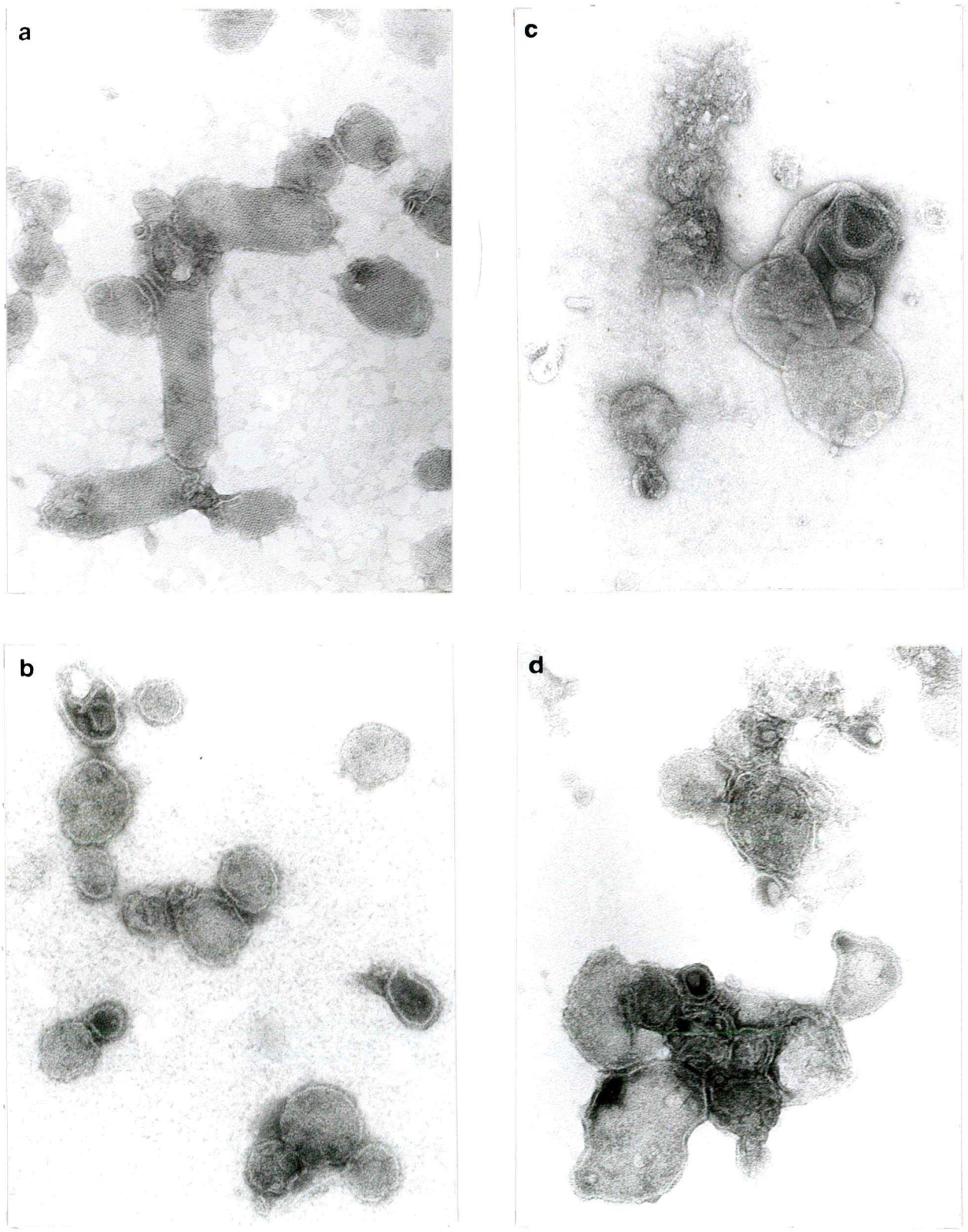

Figure 6. Vanadate induced surface structure on rabbit (a) as a control, sterlet (b), eel (c) and silure (d) microsome fractions after 24 hours incubations at 4°C. Negative staining was done by 1% uranyl acetate. Magnification is 104000 x.

SERCA1 and SERCA3 isoforms expressed by COS cells were exposed to the effect of decavanadate, as well. Vanadate induced two dimensional crystals developed in SERCA1 protein although the sample buffer contained 25 μM $CaCl_2$. 0.5 mM EGTA (final concentration) was added to the crystallization solution to help the formation of E_2 conformation of the enzyme by the binding of Ca^{2+} ions. The SERCA3 isoform did not show similar ordered structure (Fig. 7.).

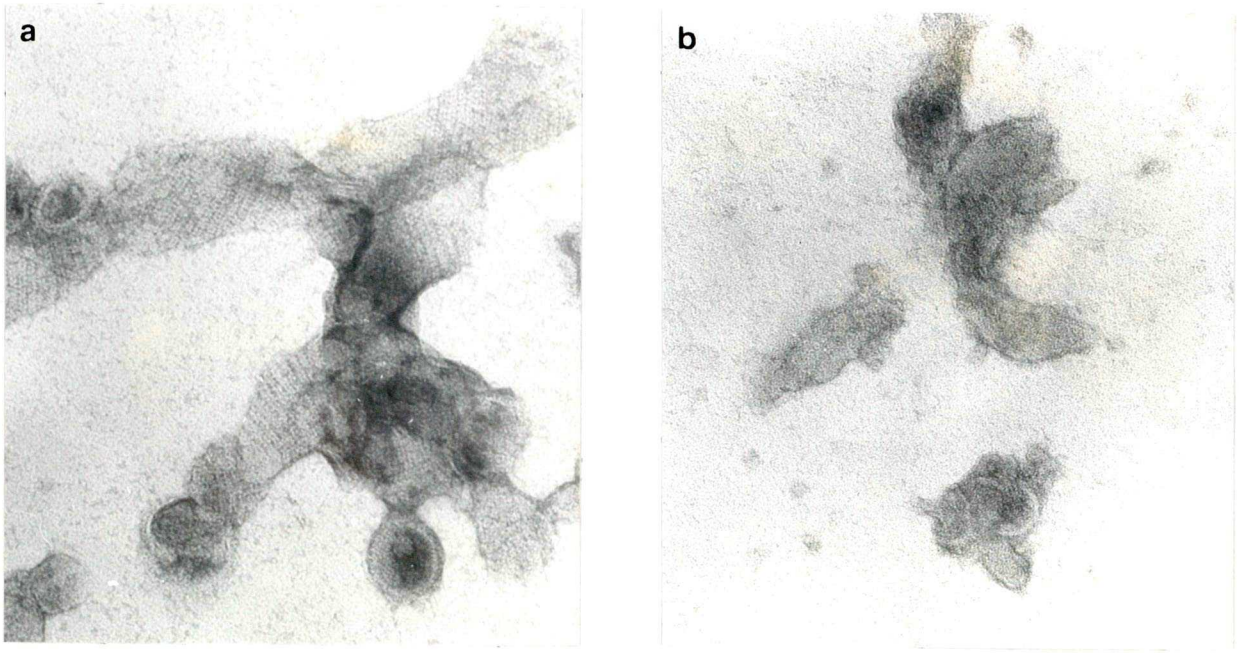

Figure 7. Vanadate induced surface structure on SERCA1 (a) as a control and SERCA3 (b) microsome fractions expressed by COS cells after 24 hours incubations at 4°C. Negative staining was done by 1% uranyl acetate. Magnification is 104000 x.

5.3. Studies of Lipid-Protein Interaction of Vanadate Treated Rabbit and Carp SR by Electron Paramagnetic Resonance

Direct measurement of molecular motions is essential for the understanding of protein-lipid interactions in SR and other membrane systems. In native membranes two kind of lipids can be distinguished: the fluid lipids and the motionally restricted boundary lipids. The sarcoplasmic reticulum Ca^{2+}-ATPase of rabbit skeletal muscle forms 2D crystals in the presence of decavanadate. The motional feature of the lipids has not been studied during the crystallization yet.

On incorporating spin labelled stearic acid into the SR membrane multicomponent EPR spectra were observed (Fig. 8. trace A). There was a great difference between the motional rates of lipid molecules. Typical correlation times in the fluid lipid sites were in the range of ~10^{-9} s and in boundary lipid sites ≥10^{-8} s. Overlapping rigid limit spectra were

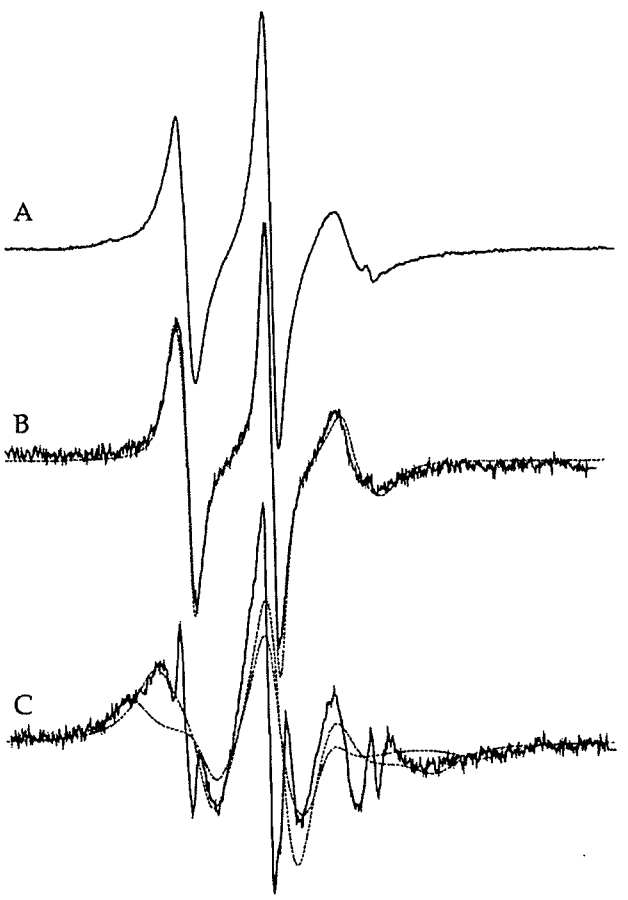

Figure 8. Multicomponent EPR spectrum of 14-SASL in rabbit sarcoplasmic reticulum at 30°C (trace A); fluid lipid lineshape of the 14-SASL in lipid extract of rabbit SR at 25 C (trace B) with simulated fluid lipid component (dashed spectrum); motionally restricted boundary lipid lineshape obtained by subtracting B (simulated spectrum)-A (multicomponent spectrum) and its simulation (dashed line) typical for boundary and stand-by sites (trace C). Total scan width 10 mTesla.

noted representing two classes of lipids in the boundary shell of proteins (Fig. 8. trace C) after subtraction the fluid lipid component (trace B in Fig. 8.). There can be found a motionally less restricted stand-by lipid layer, as well (Fig. 10.).

Lipids in the SR membrane undergo a phase transition at 15-20°C. Below this range, the resolution between EPR components corresponding to boundary, stand-by and fluid sites

was lost. Thus the EPR measurement required ≥25°C. It has to be noted that after raising the temperature of the sample to 30°C the two dimensional crystals was maintained at least for four hours (data not shown).

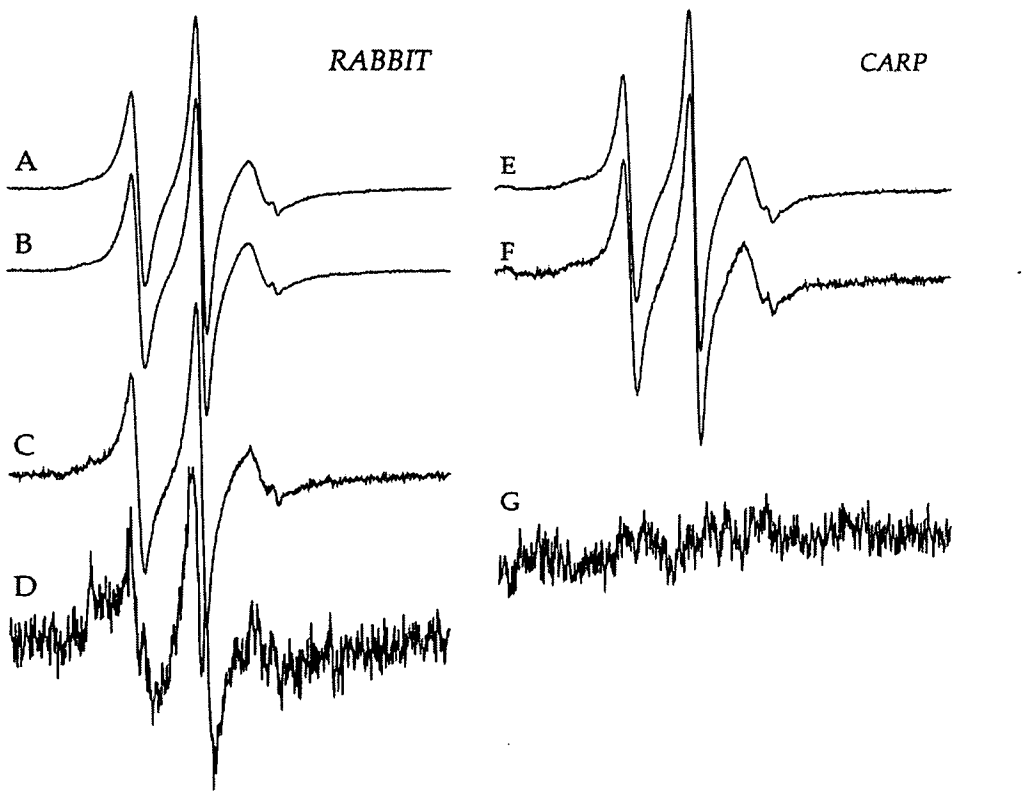

Figure 9. The EPR spectrum of 14-SASL in rabbit skeletal (traces A-D) and carp abdominal (traces E-G) muscle sarcoplasmic reticulum membrane after and prior to the addition of 5 mM decavanadate and the intersubtraction endpoints. Isotropic fluid lipid lineshape (trace C obtained by B-A); motionally restricted boundary lipid lineshape (trace D obtained by A-B). No spectral endpoints were obtained by intersubtractions in the case of carp SR (trace G obtained by E-F or F-E). Total scan width 10 mTesla.

Although decavanadate was bound to Ca^{2+}-ATPase molecules of carp SR, abolishing their enzymatic activity, no two-dimensional crystalline arrays were formed in the SR membrane of carp muscle. No difference lineshape, i. e. spectral endpoints were obtained in the case of SR membrane from carp abdominal muscle (traces E trough G in Fig 9.) in agreement with the lack of two dimensional crystalline structures in electron micrographic pictures even after prolonged cooling.

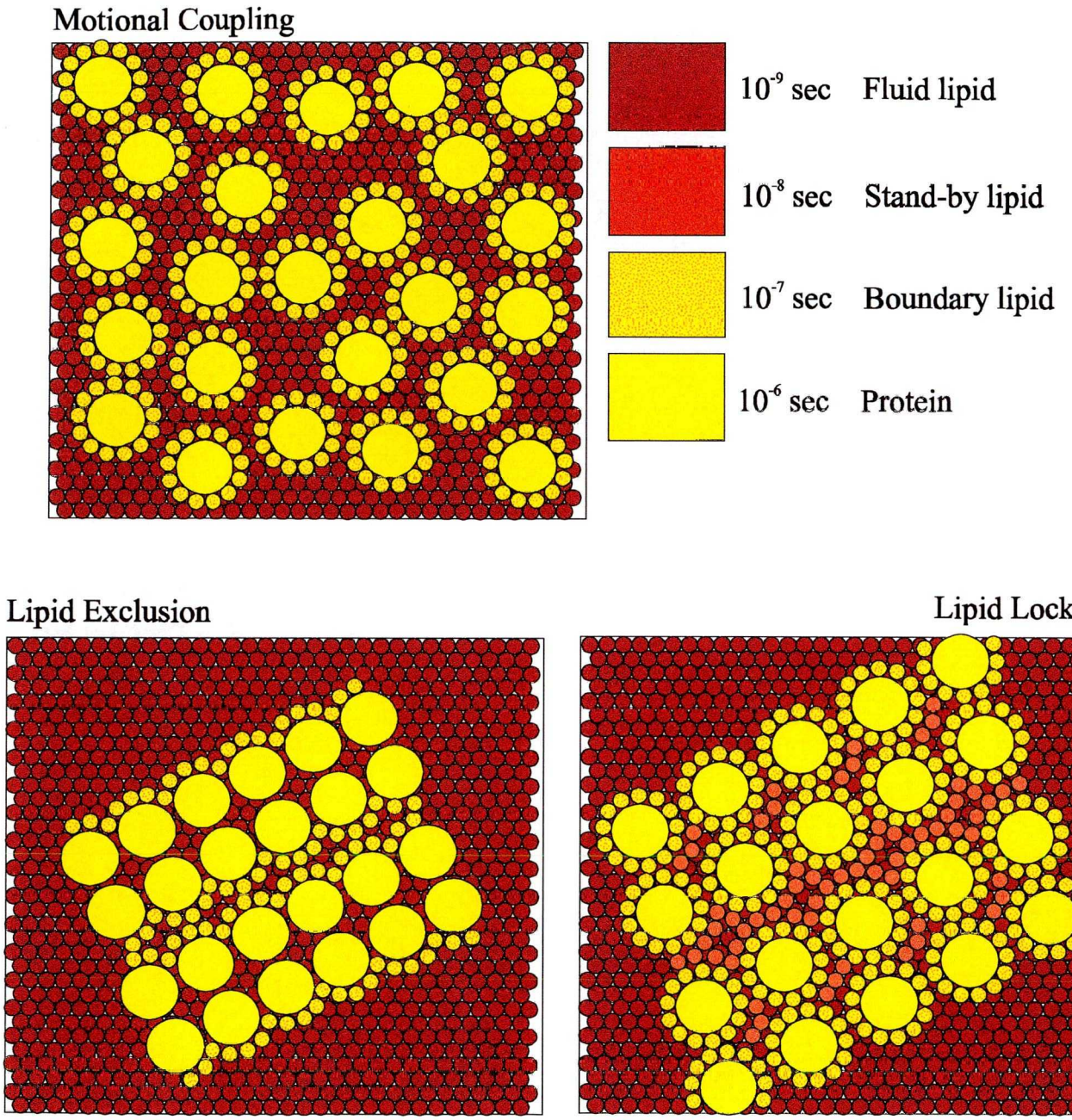

Figure 10. Hypothetical model of two dimensional crystalline structure of rabbit sarcoplasmic reticulum. The native SR membrane can be seen on the upper part of the figure. The „lipid exclusion" hypothesis means that boundary lipids are found between the dimer chains. The „lipid locking" figure shows the boundary lipids around the Ca^{2+}-ATPase molecule and the „stand-by" lipid between the dimers and dimer chains.

5.4. Effect of Specific Inhibitors on SR Ca^{2+}-ATPase from Carp, Sterlet, Eel and Silure

The conformational specificity of thapsigargin and cyclopiazonic acid inhibition may provide an additional tool for studying the membrane calcium transport. We found progressive inhibition of the Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum from carp, sterlet, eel and silure with increasing concentration thapsigargin and cyclopiazonic acid. For each set of experiments the concentration of the inhibitors varied between 10^{-10} and 10^{-6} M.

The different species tested, showed different sensitivities for thapsigargin and

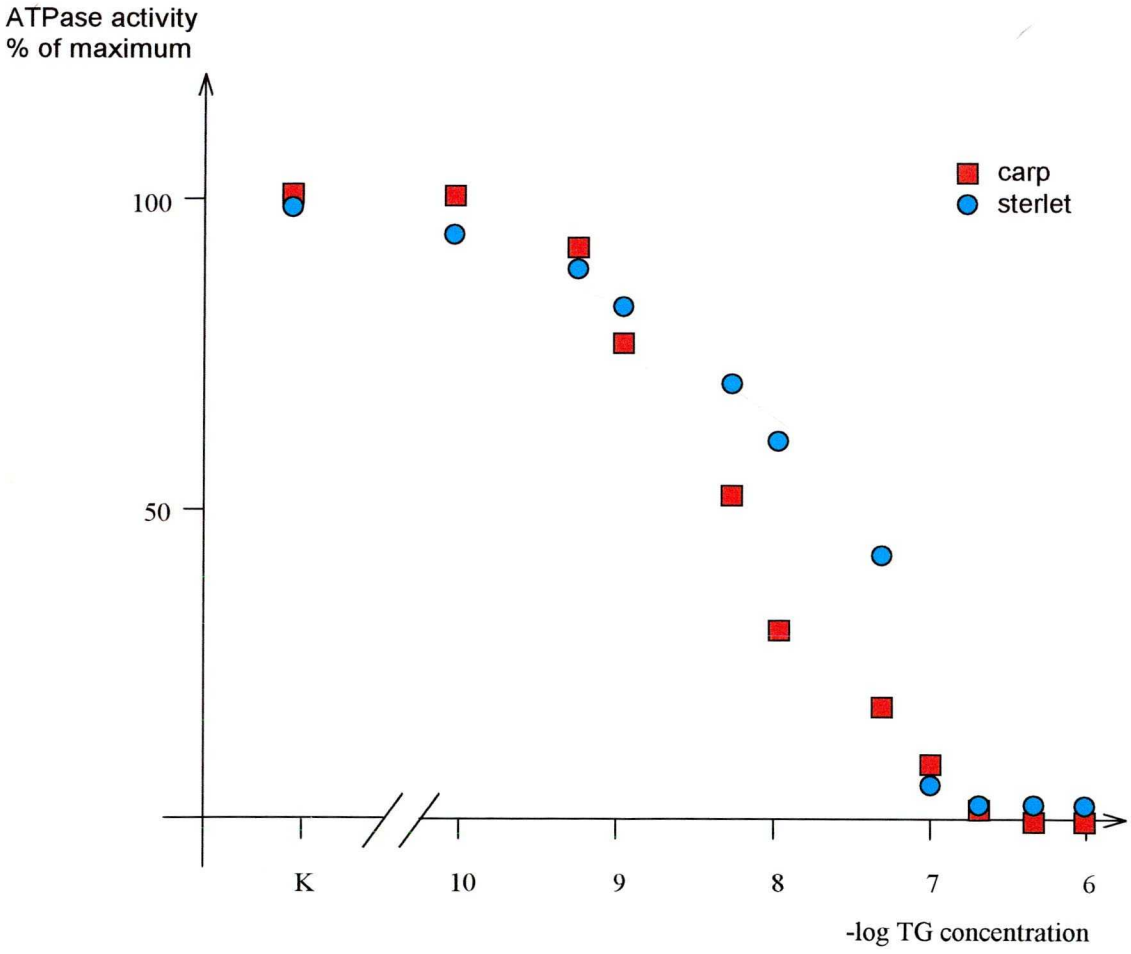

Figure 11. The effect of TG on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum from carp and sterlet. The ATPase activity was measured using the coupled enzyme system in the presence of different concentration of thapsigargin: 10^{-10}, 10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} $2x10^{-7}$, $5x10^{-7}$ and 10^{-6}. The data are given as means of three to five experiments using the same sarcoplasmic reticulum preparation made of the pooled muscles of 2-20 individual fishes, per species.

cyclopiazonic acid. The inhibition of eel SR Ca^{2+}-ATPase is the strongest at low TG concentration (between 10^{-10} and $5x10^{-9}$ M). The sterlet seems to be the most resistant to the inhibitor at this concentration range. The eel, and silure are less sensitive for thapsigargin between 10^{-7} and 10^{-6} M. Their ATPase activity is around 10% of the control enzyme activity. The carp and sterlet Ca^{2+} pumps are blocked completely at $2x10^{-7}$ M. It is interesting that three of four species the carp, the eel and the silure showed 50% inhibition at $5x10^{-9}$ M, while the sterlet at $5x10^{-8}$ M.

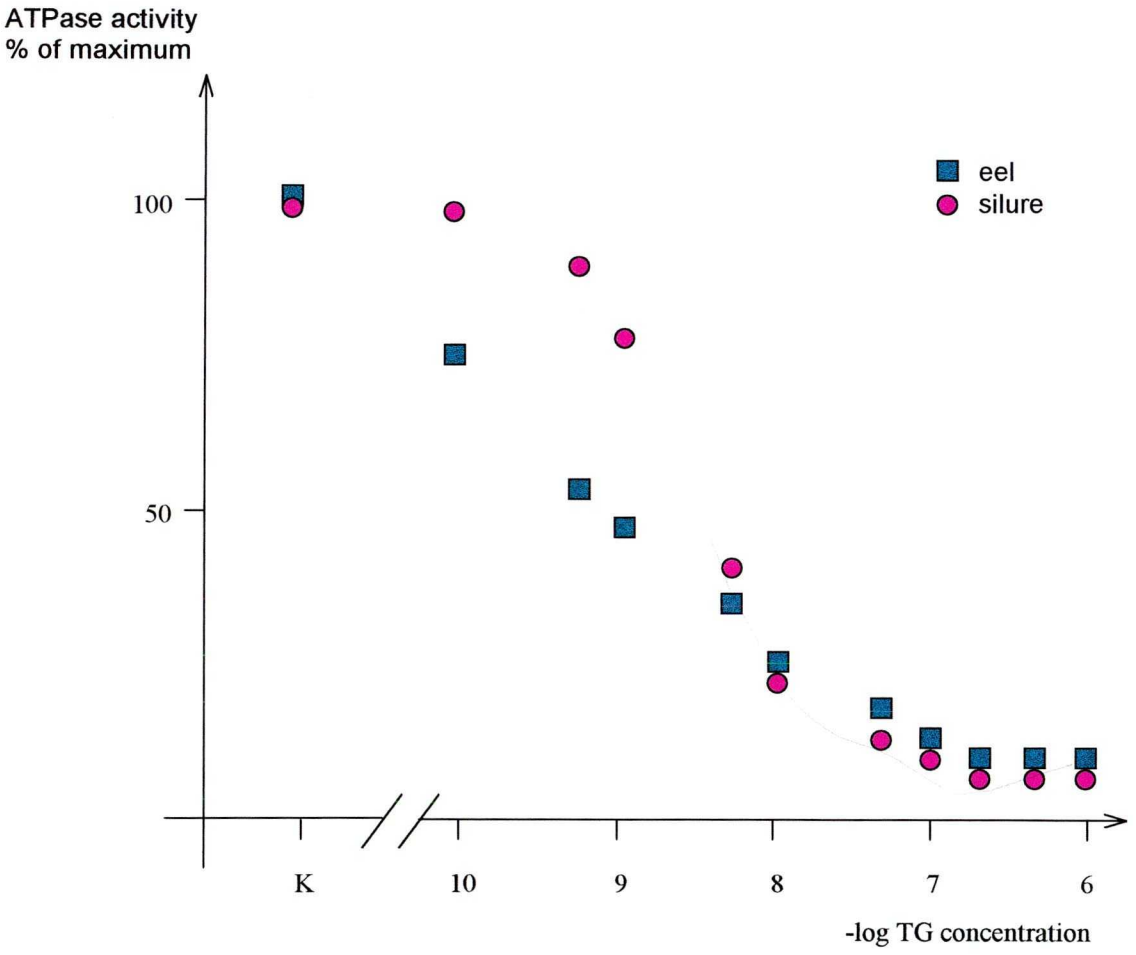

Figure 12. The effect of TG on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum from eel and silure. The ATPase activity was measured using the coupled enzyme system in the presence of different concentration of thapsigargin: 10^{-10}, 10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} $2x10^{-7}$, $5x10^{-7}$ and 10^{-6}. The data are given as means of three to five experiments using the same sarcoplasmic reticulum preparation made of the pooled muscles of 2-20 individual fishes, per species.

The carp enzyme is the most sensitive for cyclopiazonic acid while sterlet is the less sensitive one. The 50% inhibition can not be described at the same concentration. The carp at $5x10^{-9}$, the silure at $5x10^{-8}$, the eel at $5x10^{-8}$ and the sterlet above the concentration of $5x10^{-8}$ reach the 50% inhibition. The total inhibition can be experienced only at 10^{-6} M.

Our results show higher affinity of the Ca^{2+}-ATPase for thapsigargin than for cyclopiazonic acid, as described previously *(Inesi & Sagara, 1994)*. TG is effective at nanomolar range compared to the micromolar range of CPA.

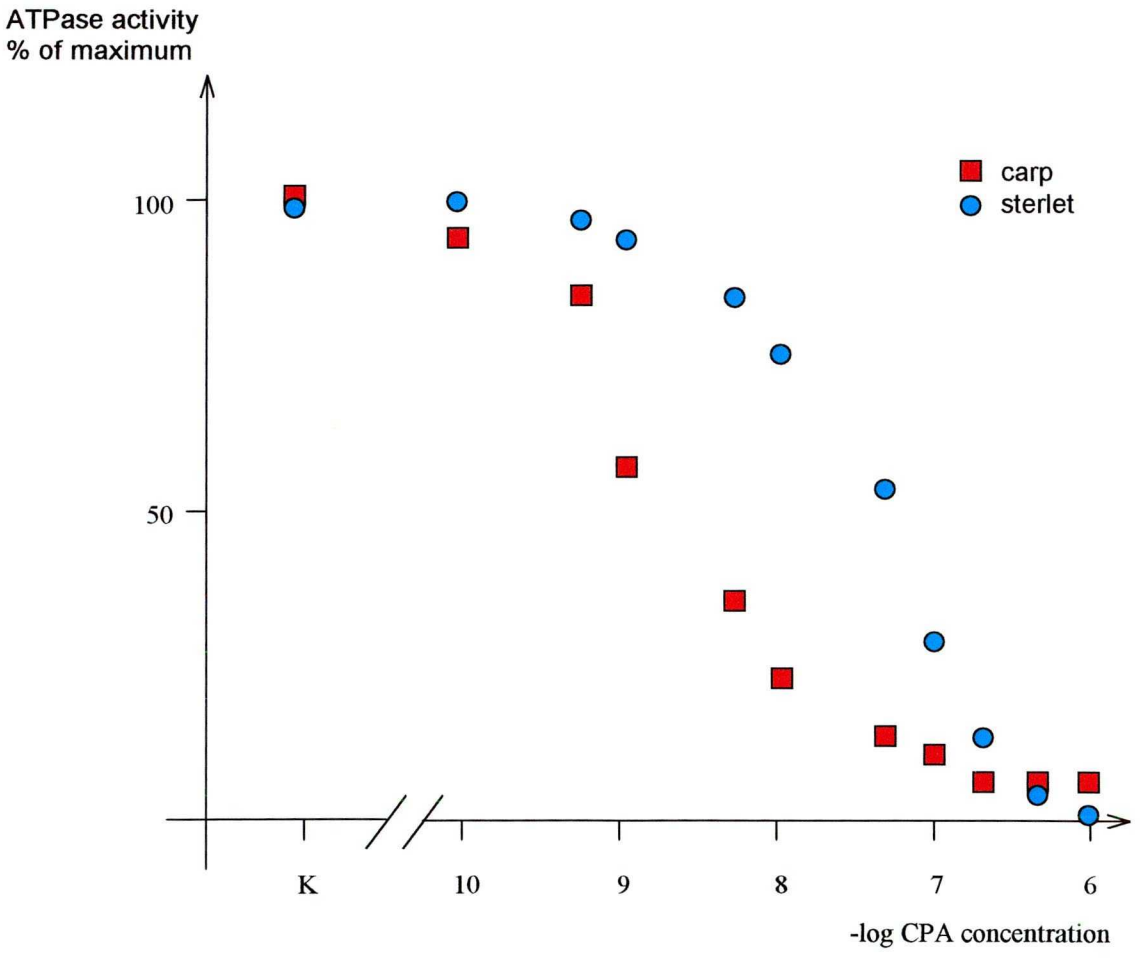

Figure 13. The effect of CPA on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum from carp and sterlet. The ATPase activity was measured using the coupled enzyme system in the presence of different concentration of cyclopiazonic acid: 10^{-10}, 10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} $2x10^{-7}$, $5x10^{-7}$ and 10^{-6}. The data are given as means of three to five experiments using the same sarcoplasmic reticulum preparation made of the pooled muscles of 2-20 individual fishes, per species.

Serious toxic effect of thapsigargin and cyclopiazonic acid can be noticed on carp, sterlet, eel and silure. We demonstrated that these inhibitors may endanger both warm- and cold-blooded animals. They inhibit the Ca^{2+}-ATPase enzyme and raise the intracellular Ca^{2+} level. TG and CPA may prove to be extremely useful in providing a simple pharmacological marker for Ca^{2+} transporting systems.

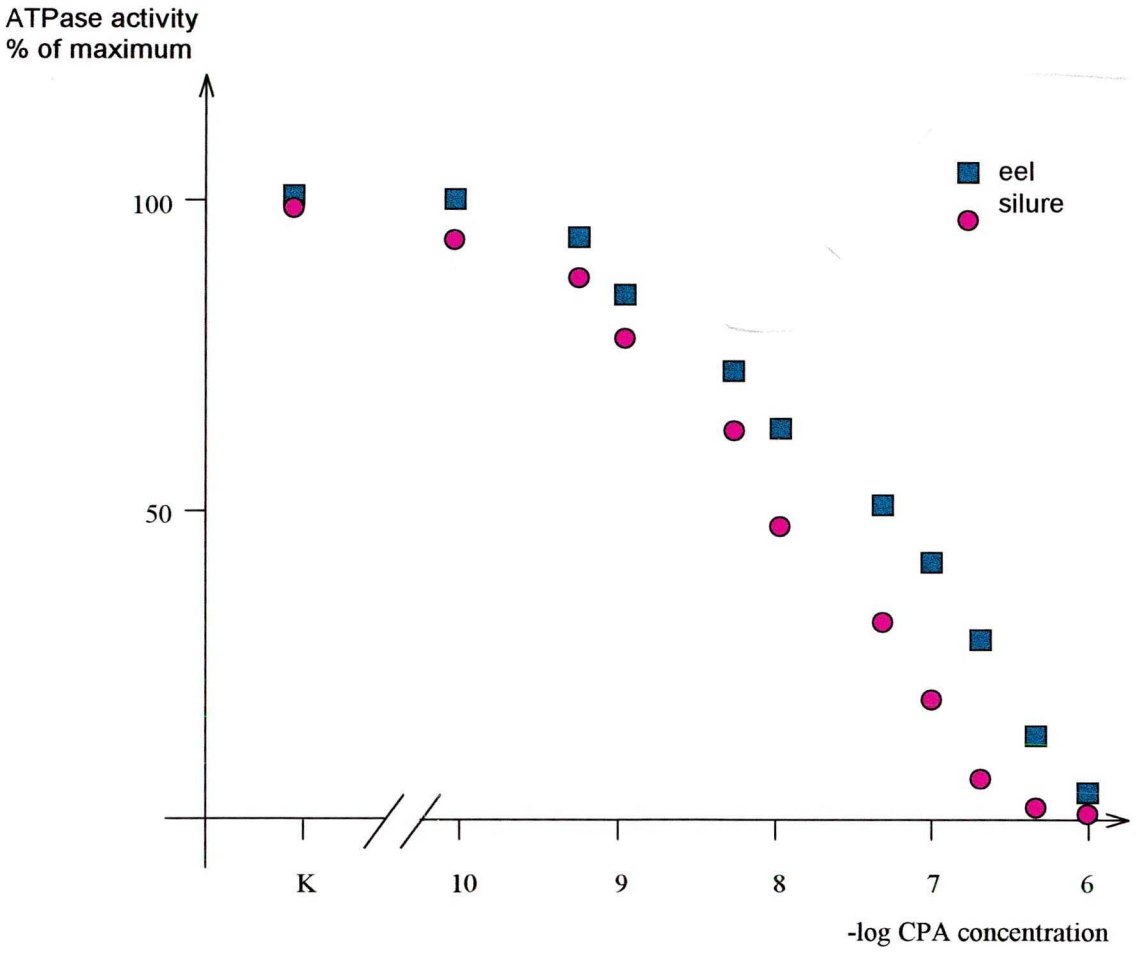

Figure 14. The effect of CPA on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum from eel and silure. The ATPase activity was measured using the coupled enzyme system in the presence of different concentration of cyclopiazonic acid: 10^{-10}, 10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} $2x10^{-7}$, $5x10^{-7}$ and 10^{-6}. The data are given as means of three to five experiments using the same sarcoplasmic reticulum preparation made of the pooled muscles of 2-20 individual fishes, per species.

5.5. Investigations of SERCA Protein Levels after Notexin Administration

Four different isoforms of sarcoplasmic reticulum Ca^{2+}-ATPases have been described in skeletal muscle. They are encoded by two genes *(McLennan et al., 1992)*. SERCA1, the gene expressed in fast skeletal muscles has two products generated by alternative splicing. The SERCA1a is present in adult while the SERCA1b form is neonatal SR membrane. The SERCA2 gene encodes the slow skeletal and cardiac muscle (SERCA2a) isoforms and the smooth muscle (SERCA2b) Ca^{2+} pump protein *(Brandl et al., 1987; Burk et al., 1989)*.

SERCA expression in the EDL representing a fast-twitch and in soleus, which is a slow skeletal muscle, was monitored after notexin induced necrosis by immunoblot. SERCA1, SERCA2a and SERCA2b antibodies were used to follow the protein levels during the regeneration.

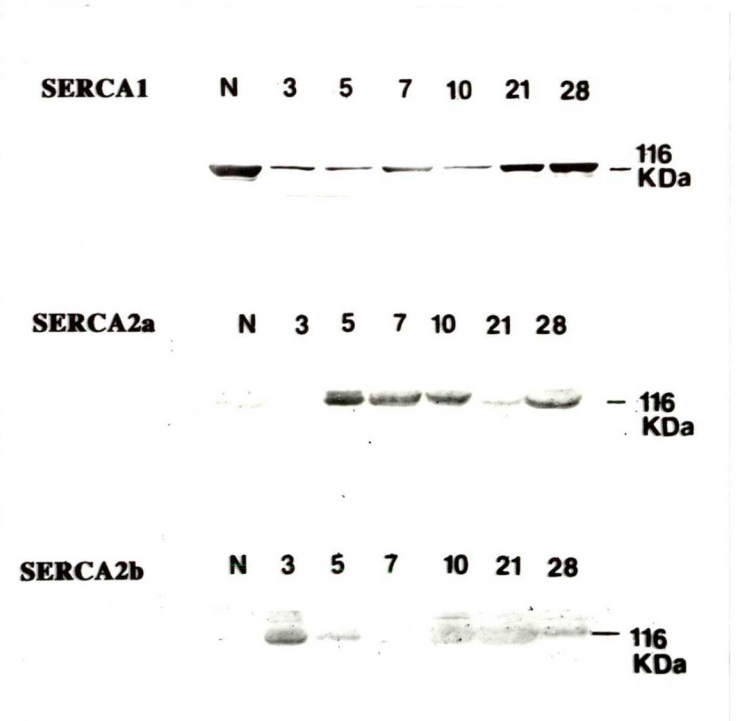

Figure 13. Levels of SERCA1, SERCA2a and SERCA2b proteins in fragmented muscle membranes during the regeneration after notexin induced necrosis. N, normal EDL, 3, 5, 7, 10, 21, 28 days after notexin administration.

In EDL muscle the SERCA1 protein is the main isoform. Its level were decreased significantly on days 3-10, then it increased to the control level on days 21-28. Only very small amount of SERCA2a was found in EDL. It was lower on day 3, but showed a transient increase on days 5 and 10, declined on day 21, and remained at that level until day 28 (Fig. 13.).

The SERCA1 level of soleus muscle was strongly reduced three days after the administration of the toxin (Fig. 14.), but it largely recovered between day 5 and 21. In some animals the SERCA1 declined again on day 28 to a value below the level of normal controls, but the mean levels were not significantly different from them. The level of slow type Ca^{2+} pump protein (SERCA2a) was also extremely decreased on the third day after notexin

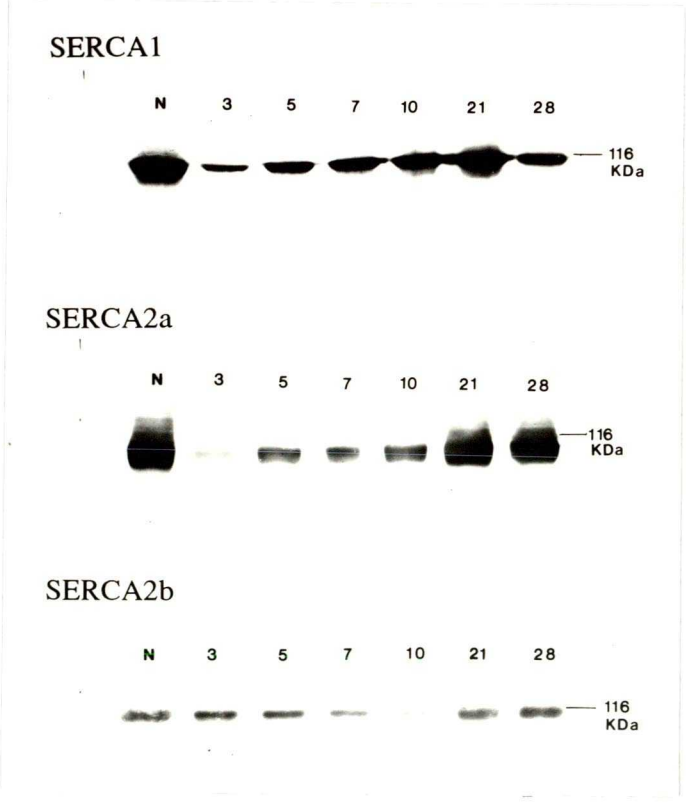

Figure 14. Levels of SERCA1, SERCA2a and SERCA2b proteins in fragmented muscle membranes during the regeneration after notexin induced necrosis. N, normal soleus, 3, 5, 7, 10, 21, 28 days after notexin administration.

treatment. On days 5, 7 and 10 the SERCA2a recovered to a level at approximately 1/3 of the control level. On day 21, a further marked increase was observed in the SERCA2a level to a value that remained constant to day 28.

Previous results show that the fast-twitch SERCA1 increased predominantly between day 3 and 10, whereas the expression of the slow-twitch SERCA2a isoform increased mostly between day 10 and 21. It is of interest, that the reinnervation of the regenerating soleus muscle is nearly complete after 10 days *(Grubb et al., 1991)*. Therefore, the switch between the two isoforms appears to follow the establishment of new reinnervation.

Whereas the amount of muscle specific SERCA1 and SERCA2a Ca^{2+} isoform changed dramatically, the level of the SERCA2b isoform remained relatively constant throughout the whole regeneration period in the case of both EDL and soleus muscles.

6. Conclusions

The structure and the function of different SERCA isoforms were investigated by distinct methods. Moreover the regeneration of different muscles were followed after notexin induced necrosis. On the basis of these results, the major conclusions of our work are as follows.

The native sarcoplasmic reticulum of rabbit, carp, sterlet, eel and silure contain the Ca^{2+}-ATPase enzyme in the largest quantities. Additional protein components were detected in all cases. Different Ca^{2+} binding proteins can be found in the rabbit SR. A 80 kD protein component is in the carp and eel and a 90 kD protein in the sterlet microsome fractions.

The structure of the Ca^{2+}-ATPases from different species showed dissimilarities. The molecular weights of the tryptic cleavage fragments (a 40-50 kD and 23 kD) of carp, eel and silure appeared in very close regions. These protein fragments could be found in lobster *(Ohnoki et al., 1980)* and in trout *(de Toledo et al., 1995)*. So the lobster, trout, carp, eel and silure SR Ca^{2+} pumps supposed to be closely related. The tryptic compounds (57 kD, 45 kD and 23 kD) generated in sterlet Ca^{2+}-ATPase are similar to the ones found in rabbit. It is interesting that the 23 kD fragment (appeared only in the presence of Ca^{2+}) is present all of the studied species, suggesting a highly conserved region of the Ca^{2+}-ATPase enzyme.

Although the carp enzyme cross-reacted with polyclonal antibodies, generated against rat fast Ca^{2+}-ATPase, the lack of the two-dimensional crystal formation was found in the presence of EGTA and vanadate, *(Dux et al., 1989; Hieu et al., 1992;)*. The induction of 2D Ca^{2+}-ATPase crystals failed in the microsome fraction of sterlet, eel, silure and SERCA3a isoform expressed by COS cells. These data show clearly the difference between rabbit and sterlet as well as between SERCA1 and SERCA3a isoform. This phenomenon is due to the distinct protein structures or the effect of additional protein components.

Following the motions of the lipids during the two dimensional crystal formation of the rabbit SR Ca^{2+}-ATPase three different lipid layers could be distinguished: the fluid lipids, the motionally less restricted stand-by lipids and boundary lipids. In the case of carp sarcoplasmic reticulum there were not dissimilarities between the samples prior and after to the addition of vanadate. Two classes of lipids could be detected the fluid and boundary lipid

shells. The presence of stand-by lipid sites is probably simply the result of the 2D crystal formation of the Ca^{2+}-ATPase enzyme, not the other way around.

The SR Ca^{2+}-ATPase function was studied in different fishes using thapsigargin and cyclopiazonic acid. These inhibitors bind to the SR Ca^{2+} pump of rabbit, blocking it. Their effect appears to be fairly specific *(Campbell et al., 1991; Kijima et al., 1991; Lytton et al., 1991; Sagara & Inesi, 1991; Goeger et al., 1988)*. The inhibition of Ca^{2+}-ATPase by TG and CPA does not display the same mechanism, it depends on both the species and the inhibitor. The most resistant was the sterlet enzyme for thapsigargin and cyclopiazonic acid. The most sensitive for TG was the silure, meanwhile for CPA the carp Ca^{2+}-ATPase was. The complete inhibition was experienced in nanomolar range in the case of thapsigargin and in the case of cyclopiazonic acid in the micromolar concentration range. The results do not differ from the ones previously described, in the case of rabbit sarcoplasmic reticulum *(Sagara & Inesi, 1991)*. Thapsigargin and cyclopiazonic acid cause toxicological problems and present potential health risk to cold-blooded animals (different fishes) as well as to the farm animals and humans. Although the inhibitors appear to be specific for SERCA ATPases, other cellular effects as yet undetected cannot be excluded with certainty at this time. The inhibitor is likely very useful in characterization of intracellular Ca^{2+} pools, and of cellular phenomena, which are subject to regulation by Ca^{2+}, ranging from growth- and transformation-related gene expression to muscle contraction.

The expression of SERCA1, SERCA2a and SERCA2b followed different time courses during EDL and soleus muscle regeneration. SERCA1 protein appears on day 3 and its level increases continuously after the notexin administration. Antibodies recognizing specifically SERCA1b are not available. The first increase of the SERCA1 protein is due to the elevation of SERCA1b mRNA according to the transcript levels. The SERCA2a isoform is present in much lower quantity in EDL (fast skeletal muscle). It declined on day 3 and increased from day 5. The SERCA2a protein decreased drastically after notexin injection in soleus muscle. The level of this isoform recovered on day 5, 7 and 10 to approximately 1/3 of the control level. Further increase was noticed on day 21 in SERCA2a protein and remained constant until day 28. Whereas the amount of muscle specific Ca^{2+} pump enzymes changed significantly. The level of the housekeeping SERCA2b isoform was relatively unchanged throughout the whole regeneration period.

The transcripts and proteins of SERCA isoforms show similar time-dependent changes in expression pattern during regeneration of the EDL and soleus from notexin-induced necrosis. They recapitulate changes observed during normal muscle development *(Brandl et al., 1987)*. At the time, when the regenerating fibers regained their normal diameter, they expressed similar levels of SERCA enzymes as in the normal muscles.

Recent efforts in the structural, functional characterization and muscle regeneration of the sarcoplasmic reticulum Ca^{2+}-ATPase isoforms have provided more insights into this area. The unique cleavage pattern of carp, sterlet, eel and silure, the dissimilarities in crystallization together with the different response to the specific inhibitors, indicates interesting discrepancies in the structure and function of the enzyme, making it suitable target for further studies as SR evolution.

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