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Ca^{2+} TRANSPORT BY THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase IN SEA CUCUMBER (*LUDWIGOTHUREA GRISEA*) MUSCLE

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Summary

In muscle cells, the excitation–contraction cycle is triggered by an increase in the concentration of free cytoplasmic Ca^{2+}. The Ca^{2+}-ATPase present in the membrane of the sarcoplasmic reticulum (SR) pumps Ca^{2+} from the cytosol into this intracellular compartment, thus promoting muscle relaxation. The microsomal fraction derived from the longitudinal smooth muscle of the body wall from the sea cucumber *Ludwigothurea grisea* retains a membrane-bound Ca^{2+}-ATPase that is able to transport Ca^{2+} mediated by ATP hydrolysis. Immunological analyses reveal that monoclonal antibodies against sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA1 and SERCA2a) cross-react with a 110 kDa band, indicating that the sea cucumber Ca^{2+}-ATPase is a SERCA-type ATPase. Like the mammalian Ca^{2+}-ATPase isoforms so far described, the enzyme also shows a high affinity for Ca^{2+} and ATP, has an optimum pH of approximately 7.0 and is sensitive to thapsigargin and cyclopiazonic acid, specific inhibitors of the SERCA pumps. However, unlike the mammalian SERCA isoforms, concentrations of ATP above 2 mmol l^{-1} inhibit Ca^{2+} transport, but not ATP hydrolysis, in sea cucumber vesicles, suggesting that high ATP concentrations uncouple the Ca^{2+}-ATPase. Another unique feature observed with the sea cucumber Ca^{2+}-ATPase is the high dependence of maximal activity on K^+ or Na^+. Similar activation promoted by these cations was observed with various mammalian Ca^{2+}-ATPase preparations when they were incubated in the presence of low concentrations of sulphated polysaccharides. In control experiments, K^+ and Na^+ have almost no effect on Ca^{2+} transport, but in the presence of heparin or fucosylated chondroitin sulphate, the activity of the different mammalian Ca^{2+}-ATPases is inhibited and they are activated by either K^+ or Na^+ in a manner similar to the native sea cucumber ATPase. These results led us to investigate the possible occurrence of a highly sulphated polysaccharide on vesicles from the SR of sea cucumber smooth muscle that could act as an 'endogenous' Ca^{2+}-ATPase inhibitor. In fact, SR vesicles derived from the sea cucumber, but not from rabbit muscle, contain a highly sulphated polysaccharide. After extraction and purification of these polysaccharide molecules, their effect was tested on vesicles obtained from rabbit muscle. This compound inhibited Ca^{2+} uptake in rabbit SR vesicles, at concentrations lower than heparin, and restored the dependence on monovalent cations. These results strongly suggest that the sea cucumber Ca^{2+}-ATPase is activated by monovalent cations because of the presence of endogenous sulphated polysaccharides.

Key words: Ca^{2+} transport, sea cucumber, smooth muscle, sulphate polysaccharides, K^+ dependence, SERCA-ATPase, bioenergetics, marine invertebrate.

Introduction

Holothuroids are an abundant and diverse group of marine invertebrates. Most of the approximately 1400 species described, comprising 200 genera, occur in benthic environments (Smiley, 1994). The sea cucumber *Ludwigothurea grisea* (Echinodermata: Holothuroidea) is an ectothermic animal that buries itself in the sand at the bottom of the sea and moves at a very slow pace with accompanying marked changes in body length (Motokawa, 1982; Motokawa, 1984; Eylers, 1982). The body wall is rigid and firm and is composed of a thin pigmented outer layer, often with spicules, and a dense white inner layer of connective tissue. A layer of circular muscle and five pairs of longitudinal smooth muscle are attached to the body wall. Each longitudinal muscle is composed of two strips situated along the longitudinal median juncture (Prosser and Mackie, 1980; Chen, 1986). When the animal is stressed, the five longitudinal muscles contract in order to eject its viscera out of the body (evisceration). These muscles are also involved in gas exchange by circulating oxygenated water through the body. As in other muscles, contraction is triggered by an increase in cytosolic free Ca^{2+} concentration (Lehman et al., 1973; Hill et al., 1978; Devlin, 1993).

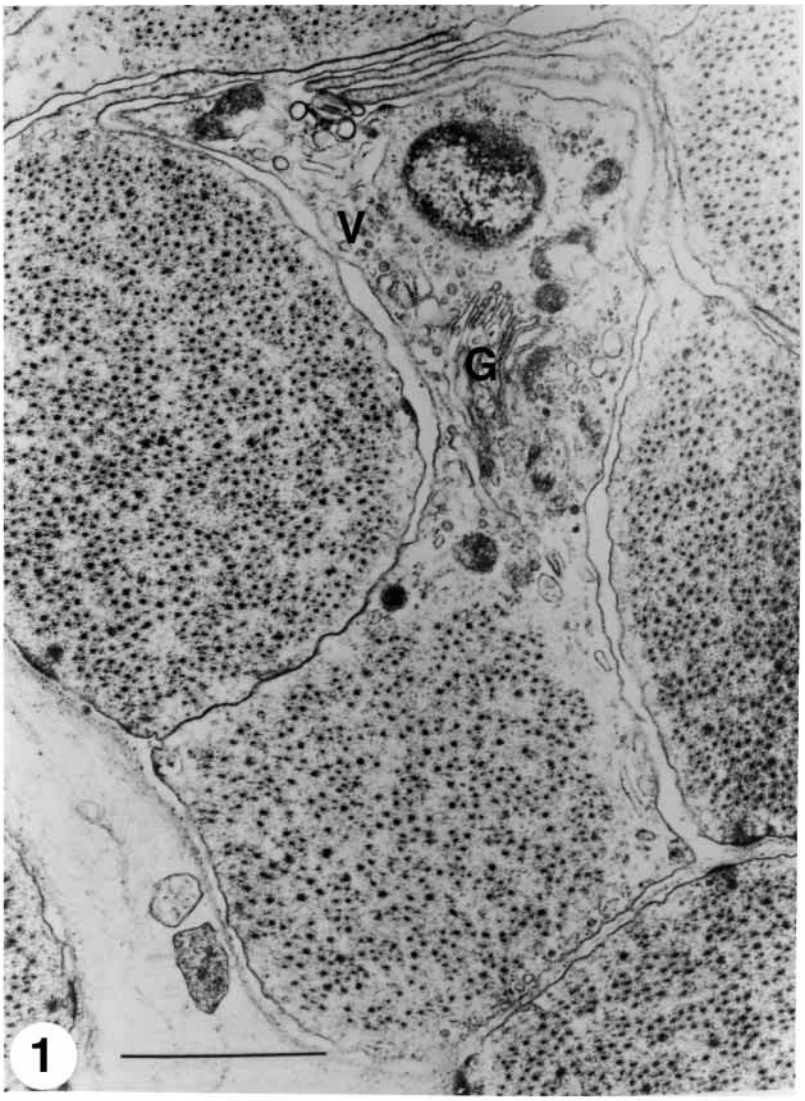

Fig. 1. A transverse section illustrating the presence of Golgi body (G) and vesicles (V) associated with the cell surface. Some of these vesicles may be sarcoplasmic reticulum. Scale bar, 1 µm. (For details, see Hill et al., 1978; figure reproduced with permission.)

It is well established that, in vertebrate skeletal muscle, the contraction–relaxation cycle is regulated by the release and uptake of Ca^{2+} from the sarcoplasmic reticulum (SR) (de Meis, 1981; Inesi, 1985); however, for different types of smooth muscle, the sources of Ca^{2+} that activate the contractile mechanism remain unclear. Electron micrographs of the longitudinal muscle of the body wall (LMBW) of the sea cucumber *Isostichopus badionotus* reveal small myocytes connected in small bundles with extensive areas of tight surface-contact between the cells and projections at the centre of the bundles (Hill et al., 1978). The myocytes contain only a few small mitochondria and negligible amounts of SR (Fig. 1), suggesting that the contraction of the LMBW fibres is caused mainly by the inward movement of extracellular Ca^{2+}. However, in the same study, it was shown that, to abolish muscle contractility, the isolated muscle must be incubated for up to 10 h in Ca^{2+}-free solutions (containing chelating agents). Only after such treatment does the contraction become directly dependent on added external Ca^{2+}. Additional evidence for the presence of an intracellular Ca^{2+} pool in sea cucumber LMBW was obtained from experiments using caffeine. This substance induces the release of Ca^{2+} from mammalian SR through the ryanodine-sensitive Ca^{2+} channel and, when applied to the sea cucumber LMBW, causes contraction even in Ca^{2+}-free solutions (Hill et al., 1978). Suzuki (Suzuki, 1982) observed, using electron microscopy, the presence of Ca^{2+} as a pyroantimonate precipitate, localised in resting fibres, in intracellular Ca^{2+} stores at both the subsarcolemmal vesicles and at the inner surface of the plasma membrane (Fig. 2). In contrast, in fibres fixed during contraction, the precipitate concentration is greatly decreased at the subsarcolemma vesicles and at the plasma membrane, and is observed diffusely distributed in the myoplasm (Fig. 3). Electron-probe X-ray microanalysis showed that the precipitate contained significant amounts of Ca^{2+} (Suzuki, 1982). These observations clearly indicate that the contraction–relaxation cycle of the longitudinal muscles involves not only the movement of Ca^{2+} between the extracellular medium and the cytosol of the muscle cells but also the release and accumulation of Ca^{2+} from intracellular stores.

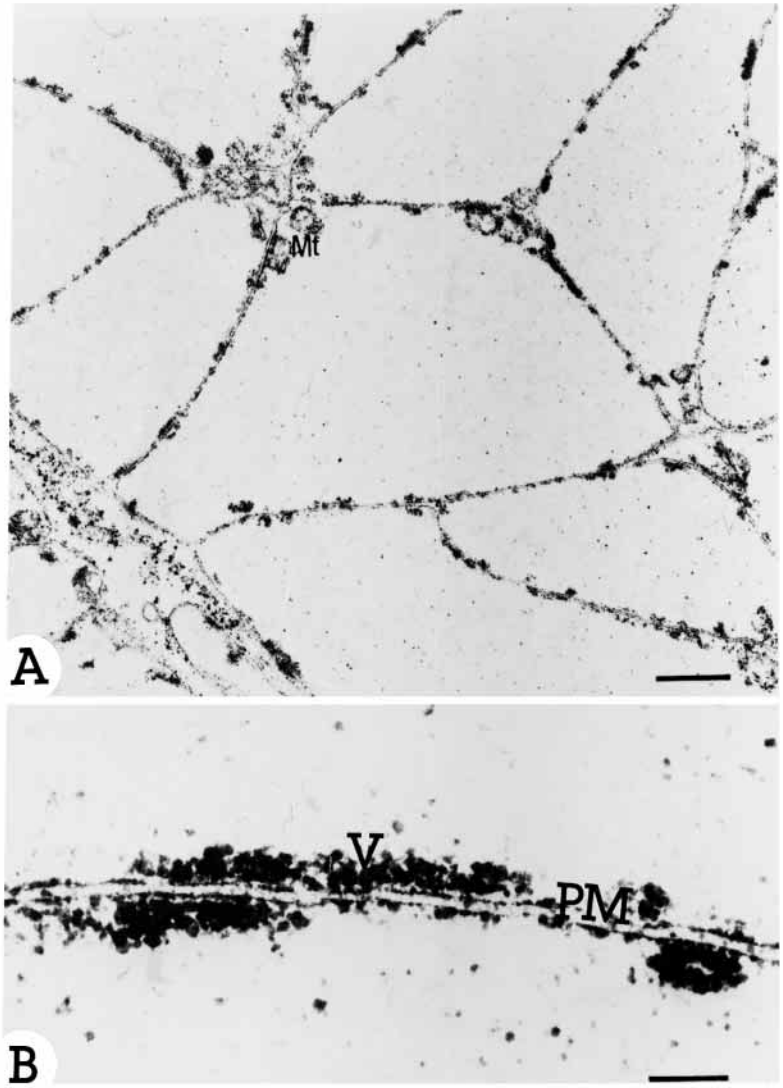

Fig. 2. (A) Cytochemical electron micrograph of a transverse section of the resting longitudinal muscle fibres showing the localisation of the electron-opaque precipitate, mainly at the peripheral region of the fibres. The precipitate is also localised to the mitochondria (Mt). Lightly stained with uranyl acetate. Scale bar, 1 μm. (B) High-magnification view of the subsarcolemmal vesicles (V) that are closely apposed to the plasma membrane (PM). Double stained with uranyl acetate and lead citrate. Scale bar, 0.1 μm. (For details, see Suzuki, 1982; figure reproduced with permission.)

Ca^{2+}-ATPases are proteins bound in the SR membrane that pump Ca^{2+} into this intracellular compartment against an electrochemical Ca^{2+} gradient using the energy derived from ATP hydrolysis. These pumps maintain a low cytoplasmic Ca^{2+} concentration ($<10^{-7}$ mol l^{-1}) and promote muscle relaxation. The SR Ca^{2+}-ATPase (SERCA) of rabbit skeletal muscle has been extensively studied (de Meis, 1981; Inesi, 1985; Mintz and Guillain, 1997; Wolosker et al., 1998). However, little is known about the SERCA in the muscle of marine invertebrates.

Echinoderms occupy an interesting phylogenetic position in animal evolution. Although they are invertebrates, the echinoderms are deuterostomes like the vertebrates, and some chordate-like structures present in this group indicate their close relationship to the chordates (Morris, 1993). Thus, comparative studies between invertebrate and vertebrate Ca^{2+}-ATPase isoforms may lead us to a better understanding of the evolution of the SERCA molecules and of how this pump acts during muscle contraction. This review focuses mainly on the study of the mechanism of Ca^{2+} transport and energy transduction catalysed by the SERCA found in the LMBW from a sea cucumber (*Ludwigothurea grisea*). Functional comparisons between the echinoderm enzyme and the well-studied vertebrate SERCAs are also made throughout this review.

The Ca^{2+} pump from sea cucumber muscle is a member of the SERCA family

The various sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoforms found in mammalian tissues are cation-transporting ATPases that belongs to the family of P-type or E1/E2 ATPases. During the catalytic cycle of these enzymes, aspartyl phosphate is formed, and the protein undergoes a transition between two major conformations designed E1 and E2. Other members of this family include the Na^+/K^+-, Ca^{2+}- and H^+/K^+-ATPases of animal cells and the H^+-ATPase found in the plasma membrane of fungi and plants (de Meis and Vianna, 1979; Pick, 1982; Pedersen and Carafoli, 1987a; Pedersen and Carafoli, 1987b). They are made up of a single

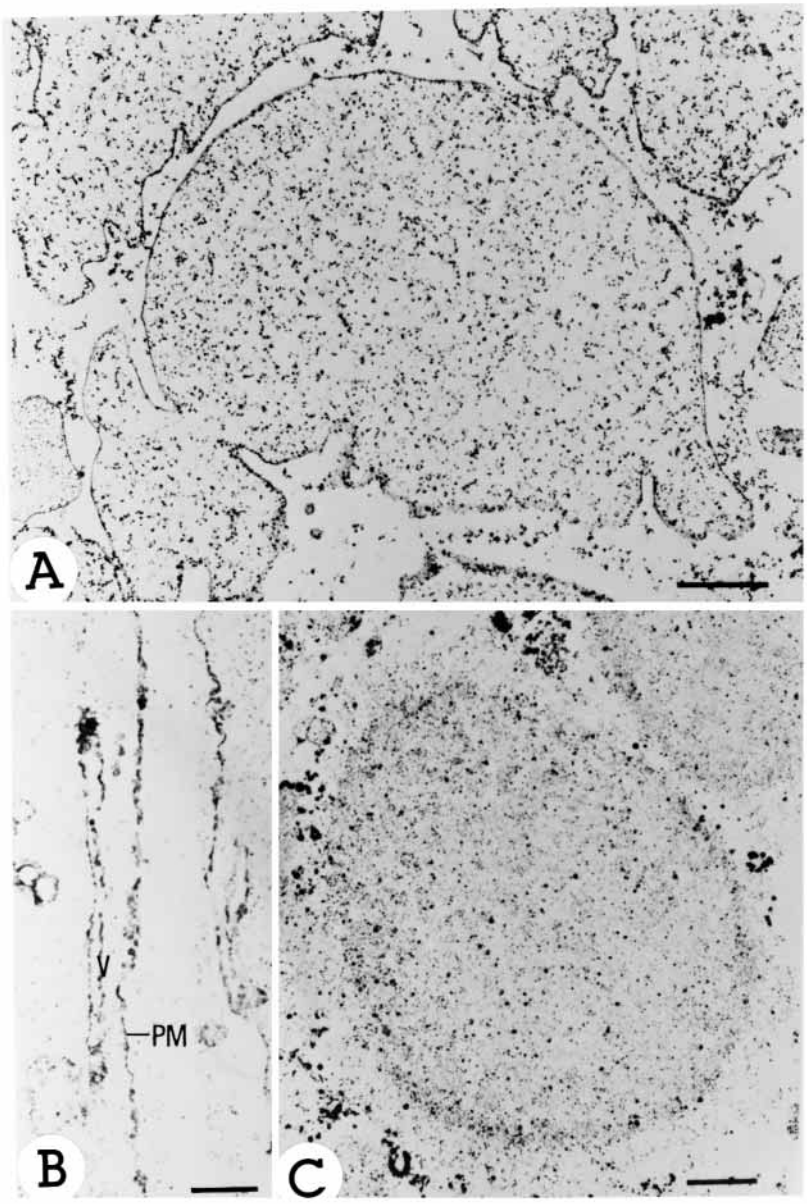

Fig. 3. (A) Cytochemical electron micrograph of a transverse section of the longitudinal muscle fibres fixed during mechanical response to 10^{-3} mol l^{-1} acetylcholine. Note the diffuse distribution of the precipitate in the myoplasm. Lightly stained with uranyl acetate. Scale bar, 1 μm. (B) High-magnification view around the plasma membrane of the fibres fixed during mechanical response to 10^{-3} mol l^{-1} acetylcholine. Note the marked decrease in the amount of precipitate at the subsarcolemma vesicles (V) and the plasma membrane (PM). Lightly stained with uranyl acetate. Scale bar, 0.5 μm. (C) Cytochemical electron micrograph of transverse section of the longitudinal muscle fibres fixed during mechanical response to 200 mmol l^{-1} K^+. Note the diffuse distribution of the precipitate in the myoplasm. Lightly stained with uranyl acetate. Scale bar, 1 μm. (For details, see Suzuki, 1982; figure reproduced with permission.)

polypeptide chain with a molecular mass of approximately 110 kDa. The SERCA isoforms are expressed in several tissues and are encoded at least by three different genes. The SERCA1 gene is expressed exclusively in fast skeletal muscle (MacLennan, 1985; Brandl et al., 1987). The SERCA2 gene gives rise to the SERCA2a and 2b isoforms by alternative splicing (Lytton and MacLennan, 1988). The SERCA2a isoform is muscle-specific, being expressed in cardiac and slow-twitch skeletal muscles, whereas SERCA2b is ubiquitous and is the isoform predominant in the cerebellum (Lytton et al., 1989). SERCA3 is expressed in non-muscle tissues such as blood platelets and lymphoid tissue (Burk et al., 1989). All the SERCA isoforms encode a cytoplasmic region that contains the catalytic site for ATP hydrolysis and a transmembrane domain that forms a channel-like structure that allows Ca^{2+} permeation through the membrane (Inesi, 1985). The protein contents of different preparations of microsome vesicles derived from sea cucumber smooth muscle, rabbit skeletal muscle and dog cardiac muscle were analysed by SDS–PAGE and silver staining and revealed a major band at 110 kDa (Landeira-Fernandez et al., 2000b). When western blots of these preparations were probed with either anti-SERCA1 or anti-SERCA2a monoclonal antibodies, only the 110 kDa band of sea cucumber SR vesicles showed cross-reactivity with both anti-SERCA1 and anti-SERCA2a antibodies, implying that sea cucumber vesicles express a SERCA-type ATPase (Landeira-Fernandez et al., 2000b). Vesicles derived from sea cucumber smooth muscle are able to accumulate Ca^{2+} at the expense of ATP hydrolysis (Landeira-Fernandez et al., 2000b). Ca^{2+} uptake into these vesicles was not inhibited by ouabain (a specific inhibitor of the Na^+/K^+-ATPase), sodium azide (a classical inhibitor of the mitochondrial ATP synthase) or the proton ionophor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Table 1). In

Table 1. *Effect of different inhibitors on Ca^{2+} transport in smooth muscle vesicles from an echinoderm*

Ca^{2+} transport system	Inhibitor	Activity (%)
None	–	100
Proton gradient	FCCP (5 μmol l^{-1})	98
Mitochondrial ATP synthase	Sodium azide (5 mmol l^{-1})	100
Na^+/K^+-ATPase	Ouabain (2 mmol l^{-1})	98
E1/E2-type ATPases	Vanadate (10 mmol l^{-1})	3
E1/E2-type ATPases	FITC (0.15 mmol l^{-1})	3
SERCA isoforms	Thapsigargin (1 μmol l^{-1})	2
SERCA isoforms	Cyclopiazonic acid (20 μmol l^{-1})	3

100 % activity is taken as 400 nmol Ca^{2+} transported mg^{-1} protein 40 min^{-1}.

For further details, see Landeira-Fernandez et al., 2000b.

SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; FITC, fluorescein isothiocyanate.

contrast, the sea cucumber Ca^{2+}-ATPase was inhibited by general inhibitors of the E1/E2-type ATPases, vanadate and fluorescein isothiocyanate (FITC), and by the specific inhibitors of the SERCA isoforms, thapsigargin and cyclopiazonic acid (Table 1), suggesting that the Ca^{2+}-ATPase found in sea cucumber microsomes is related to the SERCA isoforms and not to the Ca^{2+}-ATPase found in the plasma membrane of muscles. A comparison between the mammalian Ca^{2+}-ATPase isoforms and the sea cucumber isoform shows that the kinetics and the sensitivity to inhibitors are very similar between the different isoforms (Table 2). The sea cucumber Ca^{2+}-ATPase shows close similarities with both SERCA1 and SERCA2 isoforms, the exception being that the slightly alkaline optimum pH is similar to that of the SERCA3 isoform (Table 2).

Most of the E1/E2-type transport ATPases have two apparent K_m values for ATP, one of high affinity in the range 1–10 μmol l^{-1} (K_{m1}) and other of low affinity in the range 0.05–0.4 mmol l^{-1} (K_{m2}). In these enzymes, the binding of high ATP concentrations leads to a three- to sixfold increase in the V_{max} (de Meis, 1981; Engelender and de Meis, 1996). The high-affinity K_{m1} reflects the binding of ATP to the catalytic site of the enzyme, while the low-affinity K_{m2} reflects the binding of ATP to a regulatory site on the enzyme that accelerates the rate of conversion of the form E2 into the form E1. This conversion is the rate-limiting step in the catalytic cycle of the ATPase (de Meis, 1981; de Meis and Sorenson, 1989; Engelender and de Meis, 1996). In the presence of oxalate, a condition that leads to a lower intravesicular free Ca^{2+} concentration, and measuring the initial rate of Ca^{2+} uptake, the sea cucumber ATPase showed a high affinity for Mg.ATP with a K_m value similar to those of other E1/E2-type ATPases (Table 2). No second K_m for ATP was detected and, instead of activating the enzyme, high concentrations of ATP inhibit Ca^{2+} transport both in the presence and absence of K^+ (Fig. 4A). In fact, high concentrations of ATP promote Ca^{2+} leakage from the vesicles (Fig. 4B). Curiously, the inhibition is limited to Ca^{2+} uptake and is not detected for the measurements of the Ca^{2+}-dependent ATPase activity (Landeira-Fernandez et al., 2000b). This result suggests that high Mg.ATP concentrations uncouple Ca^{2+} transport from ATP hydrolysis by the sea cucumber Ca^{2+}-ATPase (Fig. 4).

Rabbit skeletal muscle heavy SR contains the ryanodine Ca^{2+} release channel that possesses a low-affinity regulatory

Table 2. *Comparison of kinetic features between different SERCA isoforms from various deuterostome tissue preparations*

	Tissue					
	Fast skeletal muscle	Slow-twitch muscle[a]	All tissues[b]	Non-muscle tissue[c]	References	Sea cucumber[d]
Dominant isoform	SERCA 1	SERCA 2a	SERCA 2b	SERCA 3	MacLennan, 1985; Lytton and MacLennan, 1988; Burk et al., 1989	?
Thapsigargin, K_i (nmol l^{-1})	4	4	4	4	Lytton et al., 1991	4
Vanadate, K_i (μmol l^{-1})	200	40	100	10	Lytton et al., 1992	200
Optimum pH	6.8–7.0	6.8–7.0	6.8–7.0	7.2–7.4	Lytton et al., 1992	7.0–7.2
$K_{0.5}$ Ca^{2+} (μmol l^{-1}) (*n**)	0.44 (2.1)	0.38 (2.2)	0.27 (1.7)	1.1 (1.8)	Lytton et al., 1992	0.35 (1.5)
K_m ATP, 0.3 mmol l^{-1} free Mg^{2+} (μmol l^{-1})	K_{m1} 1 K_{m2} 315	K_{m1} 4 K_{m2} 192	K_{m1} 4.9 K_{m2} 47	–	Engelender and de Meis, 1996	K_m 1.6
Effect of free Mg^{2+} (>0.3 mmol l^{-1})	Activates	Inhibits	Inhibits	–	Engelender and de Meis, 1996	Inhibits

[a]Muscle-specific slow-twitch skeletal, cardiac and smooth muscle.
[b]Ubiquitously expressed in all tissues such as brain and platelets.
[c]Non-muscle tissue such as platelets and lymphoid tissue.
[d]Sea cucumber smooth muscle.
**n*, Hill coefficient.
SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

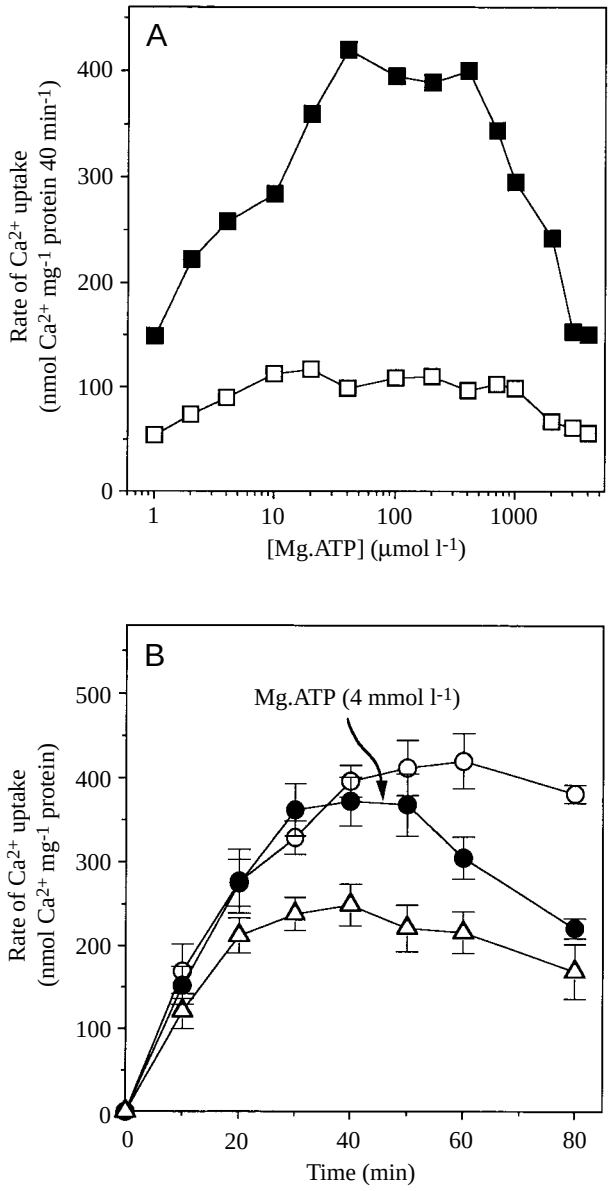

Fig. 4. Ca^{2+} transport in vesicles derived from the longitudinal smooth muscle of the body wall (LMBW) from the sea cucumber *Ludwigothurea grisea*: ATP dependence (A) and ATP-induced Ca^{2+} efflux (B). In A and B, the assay medium contained 20 µg ml^{-1} microsomes, 50 mmol l^{-1} Mops-Tris buffer (pH 7.0), 20 µmol l^{-1} $CaCl_2$, 5 mmol l^{-1} oxalate-Tris. In A, the assay medium was supplemented with 2 mmol l^{-1} phosphoenolpyruvate, 0.05 mg ml^{-1} pyruvate kinase and various concentrations of ATP and $MgCl_2$ to yield the Mg.ATP concentrations shown. At all ATP concentrations, the free Mg^{2+} concentrations varied between 0.1 and 0.2 mmol l^{-1}. □, without KCl; ■, with 100 mmol l^{-1} KCl. In B, the assay medium was supplemented with 100 mmol l^{-1} KCl and either 0.1 mmol l^{-1} Mg.ATP (○, ●) or 4.0 mmol l^{-1} Mg.ATP (△). The arrow indicates the addition of 4.0 mmol l^{-1} Mg.ATP to the medium containing 0.1 mmol l^{-1} ATP (●). Values are means ± S.E.M. of four experiments. (For details, see Landeira-Fernandez et al., 2000b; figure reproduced with permission.)

ATP binding site (Meissner, 1994; Xu et al., 1996). Because of the heterogeneous nature of this preparation, this channel could be present and could account for the inhibition of Ca^{2+} uptake observed at high ATP.Mg concentrations (Fig. 4B). Thus, the uptake measurements presented in Fig. 4B were made in the presence and absence of ryanodine and Ruthenium Red, which are known to block the Ca^{2+} release channel (Xu et al., 1999). Neither ryanodine nor Ruthenium Red was able to prevent the release of Ca^{2+} induced by the addition of 4 mmol l^{-1} ATP.Mg, and they also failed to antagonise the inhibition of Ca^{2+} uptake observed in the presence of 4 mmol l^{-1} ATP.Mg (data not shown). In a different set of experiments, 10 mmol l^{-1} caffeine, which is known to open the Ca^{2+} release channel (Meissner, 1994; Ozawa, 1999), was added to medium containing 0.1 mmol l^{-1} ATP.Mg after the vesicles had been loaded and a steady state had been reached. In this experiment, no efflux of Ca^{2+} was observed after the addition of caffeine (data not shown). These data indicate that the effect of high ATP concentrations is not related to opening of the Ca^{2+} release channel.

In addition, the sea cucumber Ca^{2+}-ATPase can use also ITP, GTP and CTP as substrates, but with a lower affinity than ATP (Landeira-Fernandez et al., 2000b).

During catalysis, an aspartyl residue located in the catalytic site of the different E1/E2-type ATPases is phosphorylated by either ATP or inorganic phosphate (P_i). For the SERCA isoforms, Ca^{2+} transport and ATP hydrolysis are initiated by phosphorylation of the enzyme by ATP; in the reverse process, the synthesis of ATP is initiated by phosphorylation of the ATPase by P_i (de Meis and Vianna, 1979; de Meis, 1981). We were not able to measure phosphorylation of the sea cucumber ATPase by either ATP or P_i. High levels of enzyme phosphorylation by either ATP or P_i are only detected with the SERCA1 isoform. For all other isoforms, the steady-state level of phosphoenzyme is very low and difficult to measure (Engelender et al., 1995). The steady-state level of phosphoenzyme depends on the rate of phosphoenzyme formation and phosphoenzyme hydrolysis and, in SERCA1, the rate of formation has been shown to be at least one order of magnitude faster than the rate of cleavage (de Meis, 1981). The ability to transport Ca^{2+} and the inhibitors used indicate that the sea cucumber belongs to the the E1/E2 family of transport enzymes and should be phosphorylated by both ATP and P_i. Therefore, the fact that we were not able to measure the intermediate formation of phosphoenzyme suggests that, as for isoforms SERCA2 and 3, the rate of phosphoenzyme cleavage of the sea cucumber enzyme is faster than the rate of phosphoenzyme formation.

These kinetic data and immunological approaches indicate that the sea cucumber enzyme has some unique properties as well as some similarities with the various mammalian SERCA isoforms, suggesting that the sea cucumber Ca^{2+}-ATPase consists of a distinct but related SERCA isoform.

Activation by monovalent cations

In contrast to the vertebrate SERCA isoforms, Ca^{2+} transport

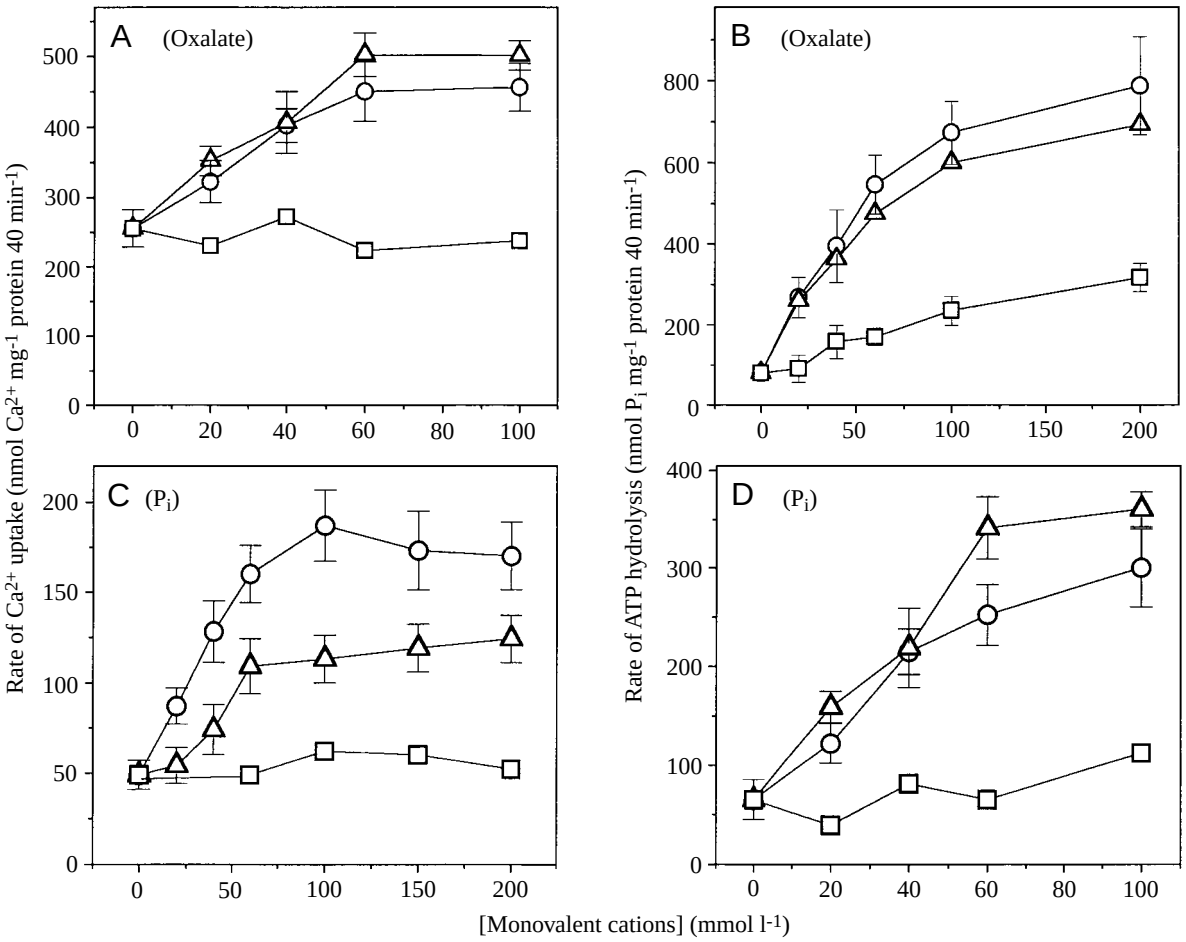

Fig. 5. Activation of Ca^{2+}-uptake (A,C) or Ca^{2+}-dependent ATP hydrolysis (B,D) by monovalent cations in sea cucumber vesicles. The assay medium contained 50 mmol l^{-1} Mops-Tris buffer (pH 7.0), 1 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} ATP and 5 mmol l^{-1} oxalate-Tris + 20 mmol l^{-1} $CaCl_2$ (A,B) or 50 mmol l^{-1} $CaCl_2$ + 10 mmol l^{-1} inorganic phosphate (P_i) (C,D). Increasing concentrations of KCl (○), NaCl (△) or LiCl (□) were added. The medium was divided into two samples (for each condition: oxalate or P_i) and trace amounts of either $^{45}CaCl_2$ or [γ-^{32}P]dATP were added to each sample for measurements of Ca^{2+} uptake (A,C) or ATP hydrolysis (B,D). In B and D, Ca^{2+}-dependent ATP hydrolysis was calculated by subtracting the values measured in the presence of 2 mmol l^{-1} EGTA (Mg^{2+}-dependent ATPase activity) from the values measured in the presence of $CaCl_2$ (total ATPase activity). In the presence of P_i, the differences between the values of Ca^{2+} uptake measured in the presence of 100 mmol l^{-1} KCl and 100 mmol l^{-1} NaCl were statistically significant (P<0.01). Values are means ± S.E.M. of either 6–8 experiments or (for LiCl) the average of two experiments performed with three different vesicle preparations. (For details, see Landeira-Fernandez et al., 2000b; figure reproduced with permission.)

and ATP hydrolysis catalysed by the sea cucumber Ca^{2+}-ATPase are highly activated by monovalent cations (Fig. 5). As in microsomes derived from some vertebrate tissues (Wolosker et al., 1997; Mitidieri and de Meis, 1999), two different ATPase activities could be distinguished in the sea cucumber microsomes: (i) a Mg^{2+}-dependent activity measured in the absence of Ca^{2+}, and (ii) a total ATPase activity measured in the presence of both Mg^{2+} and Ca^{2+}. The difference between the two activities is referred to as Ca^{2+}-dependent ATPase and is responsible for the translocation of Ca^{2+} through the microsomal membrane. All three cations tested, K^+, Na^+ and Li^+, inhibit the Mg^{2+}-dependent activity of the sea cucumber microsomes, while only Na^+ and K^+ increase of the total activity (Landeira-Fernandez et al., 2000b). The activation of Ca^{2+} uptake by the sea cucumber vesicles promoted by K^+ and Na^+ varies depending on whether oxalate or P_i is used as the Ca^{2+}-precipitating agent (Fig. 5). These two anions are known to increase the Ca^{2+}-loading capacity of microsomes isolated from a variety of tissues, including skeletal muscle, blood platelets and brain (de Meis et al., 1974; de Meis, 1981; Wolosker et al., 1997). During transport, they diffuse through the membrane and form calcium phosphate and calcium oxalate crystals in the vesicle lumen (de Meis et al., 1974). The rate of Ca^{2+} uptake is faster (Landeira-Fernandez et al., 2000b) and the amount of Ca^{2+} retained by the vesicles greater during oxalate treatment than in the presence of P_i (Fig. 5). Also, K^+ and Na^+ activate both Ca^{2+}-dependent ATP hydrolysis and Ca^{2+} transport to the same extent during oxalate treatment (Fig. 5A,B). However, when P_i is used, K^+ activates Ca^{2+} transport more effectively than Na^+ (Fig. 5C). This is not

Table 3. *Effects of heparin and activation promoted by KCl on different Ca^{2+}-ATPase isoforms from various deuterostome tissue preparations*

Vesicle preparation	References	Incubation time (min)	Addition	Ca^{2+} uptake (nmol mg^{-1} protein) Without KCl	With 100 mmol l^{-1} KCl	% activation
Rabbit skeletal muscle	de Meis and Suzano, 1994	20	None	3200±400 (5)	3400±500 (4)	6.3
			Heparin (3 μg ml^{-1})	626±64 (5)	3100±300 (5)	395
Rat brain	Rocha et al., 1996	45	None	213±17 (5)	228±18 (5)	7.0
			Heparin (100 μg ml^{-1})	76±5 (5)	199±11 (5)	162
Human blood platelets	de Meis and Suzano, 1994	60	None	170±20 (4)	180±20 (4)	5.9
			Heparin (10 μg ml^{-1})	18±4 (4)	77±9 (4)	327
Sea cucumber smooth muscle	Landeira-Fernandez et al., 2000b	60	None	50±8 (7)	187±20 (7)	274.0

Values are means ± S.E.M. (*N*).

The temperatures of the assay media were: 35 °C for rabbit skeletal muscle, rat brain and human blood platelets; 25 °C for sea cucumber Ca^{2+}-ATPase.

observed for ATP hydrolysis, where the degree of activation by Na^+ and K^+ is the same in the presence of 5 mmol l^{-1} oxalate or 10 mmol l^{-1} P_i (Fig. 5B,D). One explanation for the distinct effect of K^+ and Na^+ may be a variable free Ca^{2+} concentration inside the vesicles, which is determined by the solubility of the calcium oxalate (approximately 0.1 mmol l^{-1}) and calcium phosphate (approximately 5 mmol l^{-1}) precipitates formed inside the vesicles (de Meis et al., 1974). The degree of activation promoted by monovalent cations is greater for ATP hydrolysis than for Ca^{2+} uptake, being fourfold and twofold higher, respectively (Fig. 5). However, the concentrations needed for half-maximal activation of Ca^{2+} uptake and of ATP hydrolysis are almost the same for K^+ and Na^+, ranging in both cases from 30 to 40 mmol l^{-1} (Fig. 5).

The effect of monovalent cations on vesicles derived from the SR of rabbit skeletal muscle has been extensively studied. K^+ and Na^+ modify different intermediate steps of the catalytic cycle of the enzyme (Chaloub and de Meis, 1980; Moutin and Dupont, 1991; Champeil et al., 1997). The overall effect, however, varies depending on the conditions used. For instance, in presence of a low concentration of ATP (2 μmol l^{-1}) and Ca^{2+} (less than 1 μmol l^{-1}), monovalent cations inhibit Ca^{2+} transport and ATP hydrolysis, with Na^+ being more effective than K^+ (de Meis and Hasselbach, 1971; de Meis, 1971; Shigekawa and Pearl, 1976; Duggan, 1977; Ribeiro and Vianna, 1978). In the presence of saturating Ca^{2+} concentrations (10–50 μmol l^{-1}), monovalent cations increase the rate of Ca^{2+} uptake, with an order of effectiveness of $K^+ > Na^+ \gg Li^+$. Activation of Ca^{2+} uptake by K^+ is also dependent on temperature. A two- to threefold activation of Ca^{2+} transport by 100 mmol l^{-1} KCl in preparations at 10 °C has been reported (Duggan, 1977; Shigekawa and Pearl, 1976). However, in rabbit skeletal muscle, rat brain and human blood platelets, a much smaller activation of both Ca^{2+} uptake and ATP hydrolysis by K^+ is observed in preparations at their physiological temperatures (de Meis and Suzano, 1994; Rocha et al., 1996; Engelender et al., 1995). The finding that the sea cucumber ATPase is activated by K^+ under all experimental conditions tested (Landeira-Fernandez et al., 2000b) indicates that this enzyme has an absolute dependence on K^+ to reach maximal transport efficiency, a feature different from the other SERCA so far studied. A similar activation by monovalent cations such as K^+, and to a lesser extent Na^+, on ATP hydrolysis has been described in microsomes derived from the striated adductor muscle of deep sea scallops (Mollusca) (Castellani et al., 1989; Kalabokis et al., 1991). Although the scallop microsomes were able to transport Ca^{2+}, the possible effect of these monovalent cations on Ca^{2+} transport, as far as we know, was not measured.

We have previously reported that heparin and other glycosaminoglycans can, *in vitro*, inhibit the activity of a variety of ATPases of the E1/E2-type family, including the various SERCA isoforms and the plant plasma membrane H^+-ATPase (de Meis and Suzano, 1994; Landeira-Fernandez et al., 1996; Rocha et al., 1996; Rocha et al., 1998). These results are summarised in Table 3, in which the values obtained with sea cucumber Ca^{2+}-ATPase are compared with the values for several different mammalian SERCA isoforms. In control experiments, K^+ has almost no effect or promotes minimal activation of Ca^{2+} uptake. However, in the presence of heparin, the activity is inhibited, and K^+ is able to activate Ca^{2+} transport in preparations from a variety of animals. Thus, rabbit SERCA is not dependent on KCl, but in the presence of heparin the ATPase becomes highly sensitive to K^+ in a manner similar

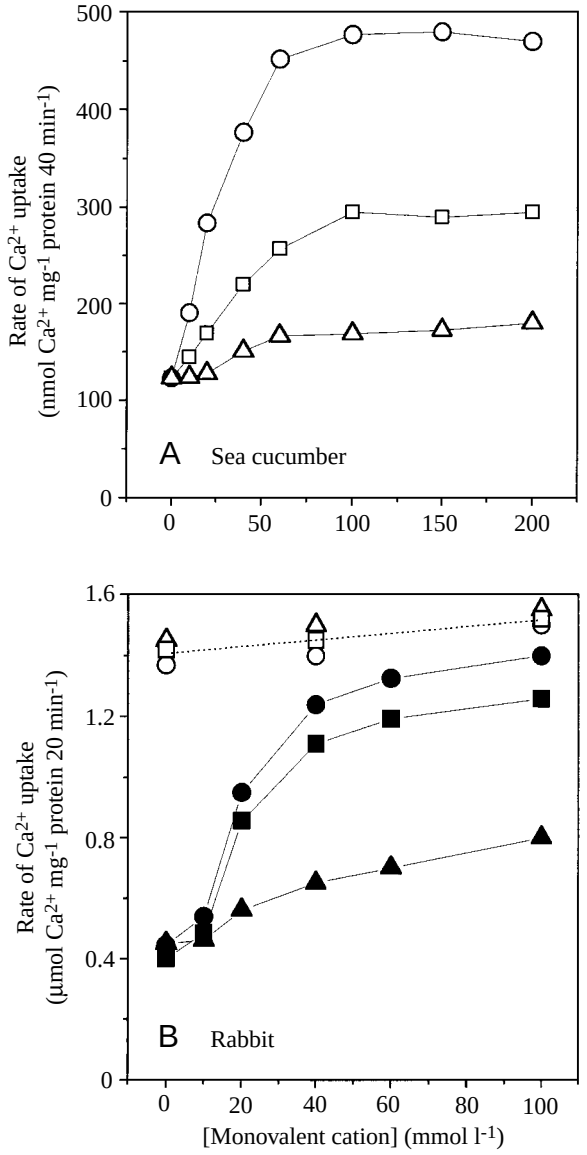

Fig. 6. Effects of monovalent cations on Ca^{2+} uptake by sea cucumber (A) and rabbit (B) microsomes. The assay medium contained 20 mg ml^{-1} microsomes, 50 mmol l^{-1} Mops-Tris buffer (pH 7.0), 1 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} oxalate-Tris, 1 mmol l^{-1} ATP and increasing concentrations of KCl (○), NaCl (△) or LiCl (□). After incubation for 40 min at 25 °C for sea cucumber vesicles (A) or for 20 min at 35 °C for rabbit vesicles (B), Ca^{2+} uptake was measured by filtration on Millipore filters. Filled symbols in B are results in the presence of 10 mg ml^{-1} heparin. The values are the average of three experiments. (For details, see Landeira-Fernandez et al., 2000a; figure reproduced with permission.)

to that of the sea cucumber Ca^{2+}-ATPase (Fig. 6). The blockade of the inhibition does not appear to be due to neutralisation of the negative charges of the sulphate residues because Li^+, the most electropositive ion among the alkali ions tested, has no effect. It is unlikely that binding of Ca^{2+} to the sulphate polysaccharides plays an important role in the effects of these compounds on the Ca^{2+}-ATPase. The Ca^{2+} affinity constant for fucosylated chondroitin sulphate (Ruggiero et al., 1994) and heparin (Mattai and Kwak, 1981) is approximately 50 μmol l^{-1}, while the Ca^{2+} affinity of the ATPase is approximately 0.1 μmol l^{-1} (de Meis, 1981). In addition, the concentration of sulphate polysaccharide used in these experiments was very low (approximately 10 μg ml^{-1}) to decrease the amount of free Ca^{2+} in the medium.

The possible presence of glycogen granules contaminating the SR sea cucumber vesicles was evaluated enzymatically (Arlington, 1990). It was found that the vesicles were contaminated with 1.3 mg glycogen mg^{-1} vesicle protein. We then digested the contaminating glycogen using the enzyme amiloglucosidase for 3 h at room temperature (25 °C) and at pH 7.0, conditions that completely digested the contaminant glycogen. It was found that, similar to the vesicles incubated in the absence of amiloglucosidase, KCl activated the enzyme two- to threefold, indicating that the KCl-dependence of sea cucumber vesicles is not related to the presence of these glycogen granules. In addition, the effect of high concentrations of glycogen (up to 40 mg ml^{-1}) was measured using rabbit SR vesicles. In this experiment, glycogen did not modify the rate of Ca^{2+} uptake either in the presence or in the absence of heparin.

A possible physiological role of the sulphated polysaccharides

A unique fucosylated chondroitin sulphate isolated from sea cucumber connective tissue can mimic the effects of heparin (de Meis and Suzano, 1994; Landeira-Fernandez et al., 1996; Rocha et al., 1996). However, the possible physiological implication of these results can be questioned since these highly sulphated polysaccharides (such as heparin and fucosylated chondroitin sulphate) are not found in plants or mammalian tissues where these ATPases are present.

The activation by K^+ of the native sea cucumber ATPase suggested that the smooth muscle may contain endogenous polysaccharides. To test this hypothesis, we attempted to isolate sulphated polysaccharides from the entire muscle of the sea cucumber and from the vesicles derived from its SR. The longitudinal muscles of the sea cucumber body wall revealed a high content of sulphated polysaccharides. Protease digestion and purification by anion exchange chromatography, followed by molecular mass and chemical composition determination, showed that the predominant polysaccharide present in the echinoderm tissue is a fucosylated chondroitin sulphate (Landeira-Fernandez et al., 2000a). However, when the sea cucumber muscle was homogenised and the vesicles derived from its SR purified, the fucosylated chondroitin sulphate was no longer detected. Instead, a sulphated polysaccharide was eluted from the anion-exchange column at higher salt concentrations; this polysaccharide had a pattern of molecular mass distribution unusual for sulphated polysaccharides. In contrast, vesicles obtained from the SR of rabbit muscles contained no detectable sulphated polysaccharide (Landeira-Fernandez et al., 2000a).

A nuclear magnetic resonance ^{1}H (NMR) spectrum of the

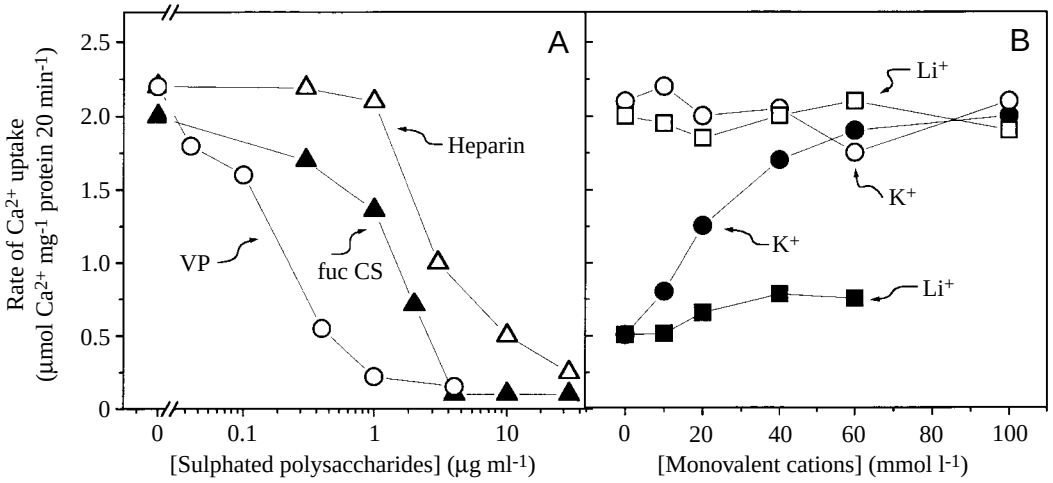

Fig. 7. The effects of different sulphated polysaccharides (A) and of K^+ or Li^+ (B) on Ca^{2+} uptake by vesicles from rabbit muscle. In A, increasing concentrations of heparin (△), fucosylated chondroitin sulphate (▲) or the sulphated polysaccharide extracted from the vesicles of sea cucumber muscle (○) were incubated with microsomes from rabbit muscles, and Ca^{2+} uptake was determined as described in the legend to Fig. 6. In B, increasing concentrations of KCl (●, ○) or LiCl (■, □) were incubated with rabbit microsomes in the absence (open symbols) or in the presence (filled symbols) of 2 mg ml^{-1} of the sulphated polysaccharide extracted from the sea cucumber vesicles. The values are the average of three experiments. (For details, see Landeira-Fernandez et al., 2000a; figure reproduced with permission.)

polysaccharide isolated from the sea cucumber vesicles shows a broader and poorly resolved signal, indicating a heterogeneous chemical structure. This polysaccharide differs from mammalian glycosaminoglycans since it is resistant to chondroitin lyase digestion and to nitrous acid deamination. Determination of its structure with chemical methods has not been possible because of the limited amounts of material available, but NMR and methylation analyses did not show a simple repetitive structure. Partial acid hydrolysis (150 mmol l^{-1} H_2SO_4, 100 °C for 30 min) removes most of the sulphate fucose from the polymer, but not galactose and hexosamines. Thus, the sulphate fucose residues are apparently branched units. Other attempts to prepare small fragments using partial hydrolysis procedures were unsuccessful.

The colocalization of this unique polysaccharide along with the sea cucumber Ca^{2+}-ATPase led us to speculate about a possible physiological relationship. The effect of the sulphated polysaccharides extracted from sea cucumber vesicles was tested on rabbit Ca^{2+}-ATPase. Surprisingly, the sulphated polysaccharide inhibits Ca^{2+} uptake by rabbit muscle-derived vesicles at an even lower concentration than heparin and restores the dependence on monovalent cations (Fig. 7). These results suggest that the sulphated polysaccharide present in the sea cucumber vesicles can act as an 'endogenous' Ca^{2+}-ATPase inhibitor.

Purified vesicles from the sea cucumber muscle were incubated with papain or solutions containing either a high salt concentration (1.0 mol l^{-1} KCl) or detergent (5 % $C_{12}E_8$). The extracted polysaccharides were analysed by anion-exchange chromatography and polyacrylamide gel electrophoresis. Most of the sulphated polysaccharide (>90 %) was extracted exclusively by protease. A small proportion (approximately 30 %) was also removed by concentrated salt solution, whereas detergent was ineffective. Electrophoresis on a polyacrylamide gel reveals some differences in the proportions of the various bands of sulphated polysaccharide extracted by papain and by high salt concentration. These procedures may preferentially remove different populations of the sulphated polysaccharides. Curiously, the detergent solution removes two different groups of sulphated polysaccharides; one is a high-molecular-mass component that remains at the origin of the gel, and the other is a low-molecular-mass component that migrates as a broad band. The sulphated polysaccharides extracted from the sea cucumber vesicles by papain inhibited rabbit Ca^{2+}-ATPase, while the KCl-soluble sample had a less inhibitory effect and the detergent-extracted material was inactive (data not shown; Landeira-Fernandez et al., 2000a). The sulphated polysaccharide is preferentially extracted from the muscle-derived vesicles by protease, suggesting a direct interaction with proteins present in the vesicles and intracellular localisation of these molecules. Thus, it is conceivable that the polysaccharides may be located in the lumen of the SR; however, the nature of this interaction requires further investigations.

The sea cucumber muscle contains two different pools of sulphated polysaccharides. Fucosylated chondroitin sulphate is present at very high concentrations (equivalent to those found in the connective tissue of the invertebrate) and is located around the muscle fibres. Another unique sulphated polysaccharide (almost undetectable when extraction is performed using the entire muscle) is found in vesicles derived from the SR. The physiological action of these molecules remains unclear, but some hypotheses have been proposed. The body wall of the sea cucumber can rapidly and reversibly alter its mechanical properties. These alterations, which are thought to be neuronally controlled, allow the tissue to change its

length by more than 200 % (Trotter and Koob, 1989; Trotter et al., 1995). The connective tissue of the sea cucumber *Cucumaria frondosa* contains parallel collagen fibrils that are able to slide past one another during length changes but are inhibited from sliding when the tissue is in 'catch'. It has been suggested that sulphated polysaccharides (specifically the fucosylated chondroitin sulphate) are an important component of the stress-transfer matrix in echinoderms (Trotter et al., 1995; Motokawa, 1982; Motokawa, 1984). The function of this polysaccharide in the echinoderm muscle is still not known. One explanation is that it has a high negative charge density (Mourão et al., 1996), so its capacity to retain water in the extracellular matrix produces a 'space' among muscle fibres allowing the intense change in length, a phenomenon that may occur in echinoderm muscle but is not observed in mammalian muscles.

Conclusions and perspectives

Most of the studies involving Ca^{2+} transport were made using the skeletal muscle Ca^{2+}-ATPase as a model, but other invertebrate Ca^{2+}-ATPases isoforms have been described in the literature. Cario et al. (Cario et al., 1996) demonstrated, using cytochemical analyses, the presence of Ca^{2+}-ATPase activity both in SR and plasma membrane of the smooth muscle of the marine invertebrate *Beroe ovata* (ctenophore) and a Ca^{2+}-loading capacity of the SR using oxalate as a precipitating agent. Coelenterates (cnidarians and ctenophores) are more primitive organisms than echinoderms, but evidence indicates that, like the sea cucumber, they are in the same evolutionary line leading to deuterostomes (Morris, 1993; Cario et al., 1996). The amino acid sequence of the Ca^{2+}-ATPase present in the invertebrate *Artemia franciscana* (Crustacea) reveals a high homology with rabbit SR (71 %) and low similarities with either Na^+/K^+-ATPase and plasma membrane Ca^{2+}-ATPase (24 % and 25 %, respectively) (Palmero and Sastre, 1989). After overexpression of a Ca^{2+}-ATPase of the endoplasmic reticulum of the protozoon *Trypanosoma brucei*, kinetic data showed properties similar to the mammalian SERCA isoforms so far described, including a high affinity for Ca^{2+} and sensitivity to the general E1/E2-type ATPases inhibitor vanadate and to the specific SERCA inhibitor thapsigargin (Nolan et al., 1994). Thus, these data suggest that the mechanism of energy transduction catalysed by the Ca^{2+}-ATPase is highly conserved through evolution. The main differences between the SERCA isoforms seem to be related to both tissue-specific expression and physiological regulation. For the Ca^{2+}-ATPase found in sea cucumber vesicles, the high dependence on monovalent cations for maximal activity and the presence of unique sulphate polysaccharides seem to be specific features of this enzyme and are not found in other isoforms so far described. After extraction and purification of these sulphate polysaccharides from sea cucumber vesicles, their effect was tested on rabbit SR Ca^{2+}-ATPase. In these vesicles, the activity of the Ca^{2+} pump is inhibited and K^+ is able to activate the enzyme several-fold. These results suggest that it is possible that the K^+-dependency of the sea cucumber Ca^{2+}-ATPase is due to the presence of sulphate polysaccharides interacting with the enzyme.

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