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# The expression, activity and localisation of the secretory pathway $Ca^{2+}$-ATPase (SPCA1) in different mammalian tissues

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**Abstract**

The distribution of the secretory pathway $Ca^{2+}$-ATPase (SPCA1) was investigated at both the mRNA and protein level in a variety of tissues. The mRNA and the protein for SPCA1 were relatively abundant in rat brain, testis and testicular derived cells (myoid cells, germ cells, primary Sertoli cells and TM4 cells; a mouse Sertoli cell line) and epididymal fat pads. Lower levels were found in aorta (rat and porcine), heart, liver, lung and kidney.

SPCA activities from a number of tissues were measured and shown to be particularly high in brain, aorta, heart, fat pads and testis. As the proportion of SPCA activity compared to total $Ca^{2+}$ ATPase activity in brain, aorta, fat pads and testis were relatively high, this suggests that SPCA1 plays a major role in $Ca^{2+}$ storage within these tissues. The subcellular localisation of SPCA1 was shown to be predominantly around the Golgi in both human aortic smooth muscle cells and TM4 cells.


*Keywords:* SPCA; SERCA; RT-PCR; Western blotting; Activity; Immunofluorescence

## 1. Introduction

Many hormones are known to mediate their action by increasing the level of intracellular $Ca^{2+}$, via either the opening of plasma membrane $Ca^{2+}$ channels or by agonist-induced production of inositol 1,4,5-trisphosphate ($IP_3$) and release of $Ca^{2+}$ from intracellular stores via the $IP_3$ receptor [1]. Rises in intracellular $Ca^{2+}$ in response to hormones are known to regulate many processes including neurotransmission, fertilisation and transcription [2]. It is essential that this rise in intracellular $Ca^{2+}$ is transient and is brought back to basal levels in order to prevent processes such as apoptosis from occurring [3].

There are several mechanisms whereby intracellular $Ca^{2+}$ levels may be reduced, with the main focus being on the plasma membrane $Ca^{2+}$ ATPase (PMCA) [4] and the sarco/endoplasmic reticulum (SERCA) $Ca^{2+}$ ATPases [5].

More recently there has been interest in another intracellular $Ca^{2+}$ pump (pmr1), first isolated and cloned from the yeast *Saccharomyces cerevisiae* [6–8]. The mammalian homologue of this protein is known as the secretory pathway $Ca^{2+}$ ATPase (SPCA), which, like SERCA and PMCA, is a P-type transporting ATPase [11,18,19], and can transport a single $Ca^{2+}$ or $Mn^{2+}$ for each ATP molecule hydrolysed. To date, two isoforms of the mammalian SPCA have been identified (SPCA1 and SPCA2) which show 60% sequence identity to each other [5].

In yeast and mammalian cells, pmr1 and SPCA have been shown to be located mainly in the Golgi membranes [5,10,11] where their function of transporting $Ca^{2+}$ or $Mn^{2+}$ is believed to be important for the folding and glycosylation of secretory proteins [9,12]. The Golgi apparatus has also been shown to contain $IP_3$ receptors on its surface [13], suggesting that it is a possible agonist-releasable $Ca^{2+}$ store and thus the SPCA $Ca^{2+}$ pump may also play a role in the refilling of this store. Indeed, endogenous SPCA has been shown to be responsible for baseline spiking in response to histamine in HeLa cells [14] and in response to ATP when overexpressed in COS-1 cells [15].

Two alternatively spliced forms of SPCA have been identified in rat [16], each with molecular weights of approximately 100 kDa. The two splice variants differ at the carboxy-terminus, where one variant has an additional 4-

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amino-acid extension as well as a substitution of val 919 for phe [16]. Northern blot analysis of rat tissues has also shown that SPCA is widely expressed, at least at the mRNA level.

The *ATP2C1* gene encodes the human SPCA1 protein, of which four splice variants have so far been identified [17]. These variants also have modifications at the carboxy-terminus and vary in size from 888 to 949 amino acids [17]. *ATP2C1* is mutated in Hailey–Hailey disease (HHD) [18,19], which is an autosomally dominant inherited skin disorder, characterised by suprabasal cell separation of the epidermis, known as ancantholysis.

In order to understand further the role and function of this $Ca^{2+}$ pump within different tissues, we have analysed the expression of the SPCA1 protein in a variety of tissues at the mRNA and protein level. In addition, we have also measured its activity in these tissues as well as its subcellular localisation in some cell types.

## 2. Materials and methods

### 2.1. Materials

Thapsigargin, uridine diphosphate (UDP), *p*-nitrophenyl α-D-mannopyranoside and fluo-3 were all purchased from Sigma. Thapsigargin and *p*-nitrophenyl α-D-mannopyranoside were both dissolved in DMSO, when used in experiments the level of DMSO was never greater than 1% v/v. Secondary antibodies conjugated to either horseradish peroxidase or fluorescein isothiocyanate (FITC) were purchased from Sigma. The Y1F4 an anti-SERCA monoclonal antibody was a gift from Dr. J. M. East, University of Southampton. Wheat germ agglutinin conjugated with Texas Red-X (WGATR) was purchased from Molecular Probes. All other reagents used were of analytical grade.

In association with BioCarta GmbH, we raised and purified a polyclonal antibody against the SPCA1 peptide sequence LTQQQRDVYQQEKA in rabbit. This sequence corresponds to amino acids 503 to 516 of the human SPCA1 sequence and from alignment studies with SERCA1 is likely to correlate with a surface exposed region within the nucleotide binding domain. As 13/14 of the amino acids in this peptide sequence, to which the antibody was raised, is identical for SPCA1 of all known sequenced mammalian species (including rat and mouse), this antibody does therefore cross react with SPCA1 in all animal tissues tested in this study. It should also be noted that since the antibody recognises an epitope in the middle of the protein it will therefore recognise all splice variants forms of SPCA1, as these differ only at the carboxy-terminus. Furthermore this antibody is unlikely to cross-react with SPCA2, as the sequence to which the antibody was raised showed only a 50% identity with SPCA1, with the differences uniformly distributed throughout this sequence. This antibody was specific for SPCA1, rather than other proteins, as pre-incubation of the antibody with the peptide antigen substantially reduced its labelling of the SPCA1 protein band in Western blots.

### 2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

(a) **Preparation of cDNA:** mRNA was extracted from various rat tissues, rat testicular derived cells and mouse TM4 cells (a mouse Sertoli cell line), using QuickPrep Micro mRNA purification kit from Amersham, following the manufacturer's instructions. The mRNA was then converted to cDNA, using a First-Strand cDNA synthesis kit (Amersham).

(b) **Preparation of Testicular cells:** The testicular cells were prepared from Wistar rats by sequential enzymatic digestion as descibed in Ref. [20]. Briefly, the testis were aseptically removed, decapsulated and washed three times in Dulbecoo's PBS media supplemented with 1 mg/ml D-glucose ($DPSS^+$). Interstitial cells were then lysed by incubating the tubules in $DPSS^+$ (pH 7.2) containing 1 mM glycine, 2 mM EDTA and 10 μg/ml deoxyribonuclease for 10 min at room temperature. Lysed cells were removed by three washes in $DPSS^+$. The tubule fragments were then treated with 0.25% w/v trypsin and 10 μg/ml deoxyribonuclease at 32 °C for 35 min. Digestion was stopped by the addition of 0.1% w/v soybean trypsin inhibitor and the tubule fragments collected by centrifugation. These fragments were washed three times in $DPSS^+$ and then treated under constant agitation with 1 mg/ml collagenase at 32 °C for 30 min. Undigested material was allowed to sediment under gravity for 30 min. The Sertoli/germ cell fractions were collected by gentle centrifugation of the post-collagenase fraction ($60 \times g$ for 4 min); these were grown in culture at 32 °C in 5% $CO_2$/95% air in DMEM/Ham's F12 media (1:1 v/v) supplemented with 2% rat serum, 8 mM glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 100 U/ml penicillin and 100 U/ml streptomycin. After 48 h in culture, the nonadherent germ cells were removed by gentle washing of the Sertoli cell monolayer with DMEM/Ham's F12 (supplemented as above) and collected by centrifugation ($400 \times g$ for 10 min). The Sertoli cells were recovered by trypsinisation. The myoid cells were collected from the supernatent of the post-collagenase low speed spin step by centrifugation at $400 \times g$ for 10 min. Following two washes in $DPSS^+$, the myoid cells were plated in DMEM/Ham's F12 (1:1 v/v) supplemented with 10% foetal calf serum, 8 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. The Sertoli, germ and myoid cells obtained by this method were judged to be greater than 90% pure, assessed as described in Refs. [46,47].

(c) **PCR reaction:** A 224-bp fragment of the rat SPCA1 cDNA was amplified using Taq polymerase from 5 ng of cDNA derived from various tissues/cells. The cDNAs

used were determined to be devoid of genomic DNA contamination. The forward primer used was 5′-AAACTGGAACCCTGACGAAG-3′ and the reverse primer used was 5′-GTGAGACTACCCTTTCGGTT-3′; these were as originally used in Ref. [21]. (These primers are unique for SPCA1 and will not bind to the cDNA of SPCA2). The PCR reaction condition was as described in Ref. [21], with the exception of having 2 mM $Mg^{2+}$ and 5% DMSO present. The PCR reaction was then subjected to 35 cycles (which was shown to be in the linear range when related to PCR product). Of each total PCR reaction, 2.5 μl (10 μl of heart and aorta) was resolved on a 2% w/v agarose gel in TBE buffer. Each gel was stained with ethidium bromide before visualisation using a UV transilluminator. Again it should be noted that these primers are able to recognise the cDNA from both splice variant forms of SPCA1 in rat as well as mouse cDNA, due to identical nucleotide sequences where the primers bind.

### 2.3. Preparation of rat microsomal membranes

Brain, lung, liver, testes, epididymal fat pads, heart, spleen and kidney were obtained from male rats and aorta was obtained from pig. Crude microsomal membranes were prepared as described below. Each tissue was added to 5–10 volumes of ice-cold buffer (5 mM HEPES, 0.32 M sucrose, 0.1 mM benzamidine, 10 μM leupeptin and 0.1 mM PMSF, pH 7.2). The tissues were chopped into small pieces before being homogenised using a Polytron homogeniser. The homogenate was centrifuged at 10,000 × *g*, at 4 °C for 20 min. The supernatent obtained was further centrifuged at 100,000 × *g*, at 4 °C for 1 h. The pellet obtained was resuspended in buffer before being aliquoted and snap frozen in liquid nitrogen prior to storage at −70 °C.

## 3. SDS-PAGE gels and Western blotting

Microsomal membranes (15–30 μg) were prepared in sample buffer and heated at 60 °C for 15 min. The membranes were then resolved on 7.5% (w/v) polyacrylamide gels, as in Ref. [22]. The proteins were transferred onto nitrocellulose in transfer buffer (0.1% w/v SDS, 20% v/v methanol, 25 mM Tris and 190 mM glycine), for 1 h at a current of 750 mA, as in Ref. [23]. The nitrocellulose was then blocked overnight in Tween 20–Tris buffered saline (150 mM NaCl, 25 mM Tris–HCl, 0.05% v/v Tween 20, pH 8) (TTBS) with 5% w/v dried skimmed milk powder. The blocking solution was removed and the nitrocellulose was washed three times in TTBS. The nitrocellulose was incubated with primary antibody diluted in TTBS with 1% w/v BSA (1 in 100 dilution for anti-SPCA and 1 in 500 dilution for Y1F4). The primary antibody solution was removed and the blot was washed for 25 min with five changes of TTBS. The nitrocellulose was then incubated for 1 h with secondary antibody conjugated with horseradish peroxidase in TTBS according to the manufacturer's instructions. The nitrocellulose was then washed as before. The nitrocellulose was incubated for 5 min with SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions. The nitrocellulose was used to expose Kodak BioMax film for varying lengths of time, and the film was developed using a XO-graph developing machine.

### 3.1. Quantification of Golgi enzyme markers in the membranes

UDPase activity has been found to be associated with the Golgi apparatus [24–26]. The UDPase activity of the tissue membranes was assayed as follows: 40 mM HEPES pH 7.0, 10 mM $CaCl_2$, 0.1% v/v Triton X-100, 2 mM UDP and 10–20 μg of membranes in a total volume of 0.5 ml were incubated at 37 °C for 10 min. The reaction was stopped by the addition of 0.25 ml of 6.5%w/v TCA, the tubes were then chilled on ice for 10 min before being centrifuged at low speed to remove denatured protein. To measure the amount of phosphate liberated, 0.25 ml of each assay was added to 1.5 ml of copper acetate buffer (11.25% v/v acetic acid, 0.25% copper sulfate and 0.2 M sodium acetate, pH 4.0). Ammonium molybdate solution (0.25 ml; 5% v/v) was then added to this and mixed. ELAN solution (0.25 ml; 2% *p*-methyl-aminophenol sulfate containing 5% sodium sulfite) was added and mixed. The colour was allowed to develop for 10 min before the absorbance was measured at 870 nm. Zero time incubations were carried out for each tissue. A calibration curve was obtained by addition of known amounts of phosphate to the reaction buffer, in the absence of membranes (see Ref. [30]).

The enzyme α-mannosidase is also known to be associated with the Golgi apparatus [27–29]. The activity of this enzyme was measured in a total volume of 1 ml of buffer (40 mM HEPES, pH 7.0, 10 mM $CaCl_2$, 0.1% Triton X-100) and 10 mM *p*-nitrophenyl-α-D-mannoside with 5–20 μg of microsomes. The accumulation of *p*-nitrophenol was followed at 405 nm for 5 min at 25 °C.

### 3.2. Measurements of SPCA activity

Initial rates of ATP-driven $Ca^{2+}$ uptake were measured by monitoring the change in fluorescence of the calcium sensitive dye fluo-3 [30–32]. Fluorescence changes were monitored using a Perkin-Elmer LS50B spectrofluorimeter, where excitation was set at 506 nm and emission was measured at 526 nm. The fluorescence was related to the [$Ca^{2+}$] using the following equation:

$$[Ca^{2+}] = K_d((F - F_{min})/(F - F_{max}))$$

where $K_d$ is the dissociation constant for $Ca^{2+}$ binding to fluo-3 (900 nM at 37 °C, pH 7.2 in the presence of 100 mM

$K^+$ [33]), $F$ is the fluorescence intensity of the sample, $F_{min}$ and $F_{max}$ are the fluorescence intensities in 1.25 mM EGTA and 2 mM $CaCl_2$, respectively. Membranes were added to a stirred cuvette containing 2 ml of 40 mM Tris/phosphate, 100 mM KCl, 5 mM potassium oxalate, 2 mM sodium azide, 2 μM vanadate, 1.25 μM fluo-3, 10 μg/ml creatine kinase and 10 mM phosphocreatine. The uptake of $Ca^{2+}$ was initiated by the addition of 1.5 mM Mg-ATP and uptake rates were measured over a period of 300 s. To distinguish between $Ca^{2+}$ uptake caused by SERCA compared to that caused by SPCA, experiments were repeated in the presence and absence of 2 μM thapsigargin.

### 3.3. Tissue culture

TM4 cells, a mouse Sertoli cell line, were cultured at 37 °C, under 5% $CO_2$/95% air, in DMEM/Ham's F12 (1:1 v/v) media (Gibco), supplemented with 5% v/v heat inactivated foetal calf serum and 2.5% v/v horse serum. The human primary aortic smooth muscle cells (HuASMC) used were typically passaged between 12 and 14, and maintained at 37 °C under 5% $CO_2$/95% air. The cells were grown in smooth muscle cell basal medium supplemented with 12.5 μg/ml gentamycin, 12.5 ng/ml amphoteracin B, 5% foetal bovine serum (FBS), 10 ng/ml epidermal growth factor, 3 ng/ml basal fibroblast growth factor and 0.4 μg/ml dexamethasone (Promocell, Heidelberg, Germany). In order to convert the cells into the contractile phenotype (which was the type used in the study), the cells were then grown for 6 days in the medium containing low FBS (0.25%) and in the absence of epidermal growth factor, basic fibroblast growth factor and dexamethasone [44].

### 3.4. Immunofluorescence

TM4 and human primary aortic smooth muscle cells (HuASMC) were cultured on scratched glass coverslips and gelatine-coated coverslips, respectively. The cells were grown to approximately 80% confluence. The cells were then washed three times in PBS. The TM4 cells were fixed using 4% paraformaldehyde in PBS at room temperature for 15 min. The human primary aortic smooth muscle cells were fixed at room temperature for 15 min with 2% formaldehyde. The coverslips were again washed three times in PBS. The cells were permeabilised for 5 min at room temperature with 0.1% v/v Triton X-100 in PBS. The permeabilisaton media was removed and the coverslips were washed again three times with PBS. The cells were incubated at room temperature for 1 h with primary antibody. The primary anti-SPCA1 antibody was diluted 1/100 in PBS and Y1F4 (anti-SERCA antibody) was also diluted 1/100 in PBS. Following incubation with the primary antibody, the coverslips were washed three times with PBS. The coverslips were then incubated with the appropriate secondary antibody conjugated with FITC. The coverslips were again washed three times with PBS and once with distilled water. The coverslips were dried of any excess liquid and were mounted on glass coverslips using Hydramount (National Diagnostics). The cells were visualised using a Leica DMRB fluorescence microscope equipped with a Hamamatsu ORCA camera.

### 3.5. Lectin affinity immunostaining

Wheat germ agglutinin (WGA) binds sialic acid and *N*-acetyl glucosaminyl residues mostly found to be associated with the Golgi complex [34]. TM4 cells were cultured on scratched glass coverslips, fixed and permeabilised as described above. The cells were incubated at room temperature for 1 h with WGA-Texas Red (WGATR) diluted 2 μg/ml in PBS. The coverslips were then washed, mounted and visualised as described above.

## 4. Results

Fig. 1A shows the results of RT-PCR on a variety of rat tissues, except TM4 cells which are derived from mouse. The primers were designed to amplify a 224-bp fragment of the SPCA1 sequence from the cDNA of both rat and mouse. The mRNA for SPCA1 was easily detected in testis, brain (cerebrum) and cerebellar. Lower levels of detection were observed in heart and aorta and levels were undetectable in spleen and skeletal muscle. Fig. 1B shows the results of RT-PCR from rat testis and testicular derived cell types. A 224-bp fragment was detected in juvenile testis, adult testis, Sertoli cells, TM4 cells, germ (spermatids) and myoid cells, indicating the expression of SPCA1, at least at the mRNA level, is abundant within testicular cells. A note of interest is also that the level of SPCA1 mRNA expression appears to be considerably less in juvenile testis compared to adult testis suggesting that SPCA may be up-regulated during development.

Fig. 2 shows the immunoblots of membranes derived from a number of tissues and cells, probed with the anti-SPCA1 antibody. These blots are from the same immunostaining procedure and are typical of three separate blots. The detection of a band of ~100 kDa was observed in the microsomal membranes from rat brain, rat lung, rat kidney, rat heart, rat epididymal fat pads, rat testis, rat liver, porcine aorta and mouse TM4 cells. The level of expression of SPCA was particularly high in brain, testis, epididymal fat pads and in TM4 cells. The expression was of a lower level in heart, aorta, liver, lung, kidney, and not detectable in spleen. Fig. 3 shows the results of immunoblots of the same membranes with Y1F4 (anti-SERCA antibody) [35]. The level of SERCA is highest in heart, testis, lung and brain. Expression is lower in epididymal fat pads, liver, spleen, aorta (pig) and kidney. There is also a significant level of SERCA protein in TM4 cells. A comparison between the levels of expression of SERCA and SPCA in the different tissues shows that although brain and testis appear to be abundant in both

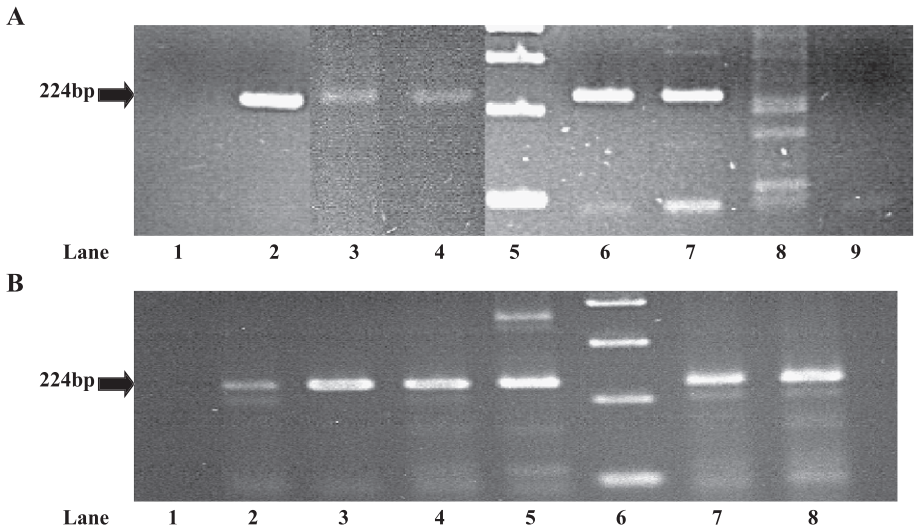


Fig. 1. RT-PCR analysis of SPCA1 in rat tissue microsomes, pig aorta microsomes and the mouse TM4 Sertoli cells. Primers were designed, as originally described in Ref. [21], to amplify over 35 cycles, in the presence of 2 mM $Mg^{2+}$ and 5% DMSO, a 224-bp sequence of the SPCA1 gene from 5 ng of cDNA. In each of the lanes, 2.5 μl (10 μl for heart and aorta) of a 50-μl PCR reaction was resolved a 2% agarose gel, stained with ethidium bromide and visualised using a UV transilluminator. (A) The lanes represent: (1) no cDNA, (2) rat testis, (3) rat heart, (4) rat aorta, (5) 100-bp ladder (NEB), (6) rat brain (cerebrum), (7) rat cerebellar, (8) rat spleen and (9) rat skeletal muscle. (B) The lanes represent: (1) no cDNA, (2) rat juvenile testis, (3) rat adult testis, (4) rat Sertoli, (5) mouse TM4, (6) 100-bp ladder (NEB), (7) rat germ cell and (8) rat myoid cell.

proteins, other tissues such as heart, lung and spleen have distinctly different levels of expression for the two types of $Ca^{2+}$ ATPases.

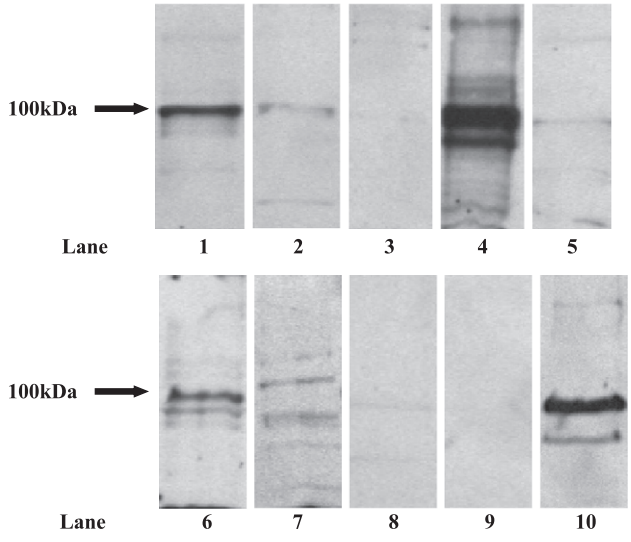


Fig. 2. Western blotting of SPCA1 in a variety of tissue membranes. Tissue membranes (15–30 μg) and TM4 cell homogenate were resolved on a 7.5% SDS-PAGE gel and the proteins were transferred onto nitrocellulose. The nitrocellulose was incubated with anti-SPCA1 antibody diluted 1 in 100 for 1.5 h at room temperature and with secondary anti-rabbit IgG conjugated to horseradish peroxidase for 1 h at room temperature. The blot was then incubated with SuperSignal West Pico substrate (Pierce) according to the manufacturer's instructions and the blot was used to expose Kodak BioMax MR film, which was developed using an XO-graph machine. The lanes represent the following: (1) rat brain (15 μg), (2) rat lung (30 μg), (3) rat liver (30 μg), (4) rat testis (30 μg), (5) rat heart (30 μg), (6) rat fat pads (30 μg), (7) pig aorta (30 μg), (8) rat kidney (30 μg), (9) rat spleen (30 μg) and (10) mouse TM4 cells.

As SPCA is believed to localised to the Golgi apparatus within cells, the amount of Golgi present in the tissue membranes was assessed by measuring the activities of two Golgi-associated enzymes (UDPase and α-mannosi-

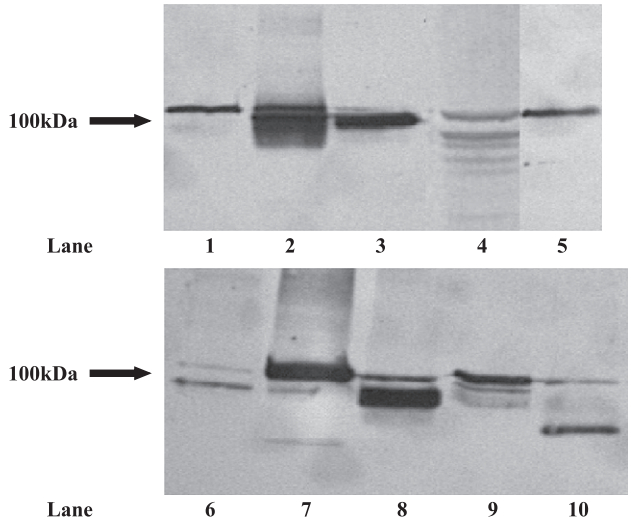


Fig. 3. Western blotting of SERCA in a variety of tissue membranes. Tissue membranes (30 μg) and TM4 cells were resolved on a 7.5% SDS-PAGE before being transferred onto nitrocellulose. The nitrocellulose was incubated with anti-SERCA antibody diluted 1 in 500 for 1.5 h at room temperature and with secondary anti-mouse IgG conjugated to horseradish peroxidase, for 1 h at room temperature, according to the supplier's instructions. The blot was incubated with SuperSignal West Pico (Pierce) according to the manufacturer's instructions and was used to expose Kodak BioMax MR film, which was developed using an XO-graph machine. The lanes represent the following: (1) rat kidney, (2) rat lung, (3) rat brain, (4) pig aorta, (5) mouse TM4 cells, (6) rat fat pads, (7) rat heart, (8) rat testis and (9) rat liver and (10) rat spleen (all microsomal membranes 30 μg).

dase), the activities of which are shown in Table 1. The activity of UDPase in the different membranes is quite variable, with the activity being particularly high in liver and kidney (which showed little expression of SPCA1). The activity of α-mannosidase was again variable between the different tissue membranes, with the highest activity occurring in epididymal fat pads, liver and heart. These results therefore showed little correlation between the amount of SPCA1 and Golgi apparatus present within these tissue membranes.

In order to measure the activity of SPCA in the different tissue membranes, rates of ATP-driven $Ca^{2+}$-uptake were monitored. To minimise the contribution of other known $Ca^{2+}$-ATPases and $Ca^{2+}$-uptake processes which might be present within these membranes, the assays were undertaken in the presence of 2 μM vanadate and 2 mM sodium azide to inhibit plasma membrane $Ca^{2+}$-ATPase activity [48] and mitochondrial $Ca^{2+}$-uptake, respectively. In addition, these activities were measured in the presence and absence of 2 μM thapsigargin (which inhibits SERCA) to determine the contributions of SPCA and SERCA activities within these tissue membranes. It has been previously shown that the Golgi $Ca^{2+}$ ATPase is unaffected by both 2 μM thapsigargin and 2 μM vanadate [9,45]. As the plasma membrane $Ca^{2+}$-ATPase (PMCA) is extremely sensitive to vanadate (i.e. complete inhibition occurs at about 2 μM [48]) this was added to the assay buffer. However, since SERCA is also inhibited by vanadate (albeit at much higher concentrations [49]), we also wanted to make sure that our 'total activity' was not substantially affected by the 2 μM vanadate present in the buffer causing SERCA inhibition. Therefore, in order to assess this possibility, the $Ca^{2+}$ pump uptake activity was measured in a number of tissues membranes which express the main SERCA isoforms: i.e. heart for SERCA 2a isoform, brain for SERCA2b and testis for SERCA2b and SERCA3 [50,51]. The degree of inhibition in the presence of 2 μM vanadate compared to its absence was not greatly affected in all three tissue membranes tested (i.e. heart, brain and testis membranes showed only 3%, 0% and 12% inhibition, respectively).

Table 1
Activity of UDPase and α-mannosidase in tissue membranes

| Tissue | UDPase activity (nmol Pi/min/mg) | α-Mannosidase II activity (nmol pNP/min/mg) |
|---|---|---|
| Testis | 300 ± 1 | 3.45 ± 0.14 |
| Heart | 105 ± 1 | 7.51 ± 0.04 |
| Fat pads | 357 ± 2 | 8.44 ± 0.09 |
| Spleen | 128 ± 1 | 5.41 ± 0.12 |
| Liver | 1440 ± 30 | 5.39 ± 0.44 |
| Kidney | 780 ± 4 | 7.62 ± 0.14 |
| Brain | 90 ± 1 | 6.52 ± 1.03 |
| Lung | 155 ± 1 | 2.20 ± .0.25 |
| Aorta | 78 ± 2 | 0.83 ± 0.04 |

These values presented are the mean ± S.E. of at least three or more determinations. All tissues are derived from rat except aorta which was derived from pig.

Table 2
$Ca^{2+}$ pump activity in the absence and presence of 2 μM thapsigargin

| Tissue | Initial rate of $Ca^{2+}$ uptake (nmol/min/mg) | Initial rate of $Ca^{2+}$ uptake in the presence of 2 μM thapsigargin (nmol/min/mg) | Initial rate of $Ca^{2+}$ uptake due to SERCA (nmol/min/mg) | Ratio of $TG_{Insensitive}$ activity/total activity |
|---|---|---|---|---|
| | 'Total activity' | '$TG_{Insensitive}$ activity' | 'Total − $TG_{Insensitive}$' | |
| Testis | 0.740 ± 0.181 | 0.250 ± 0.005 | 0.49 ± 0.19 | 0.34 |
| Heart | 8.830 ± 0.060 | 0.260 ± 0.017 | 8.57 ± 0.08 | 0.03 |
| Fat Pads | 0.390 ± 0.068 | 0.320 ± 0.034 | 0.07 ± 0.1 | 0.82 |
| Spleen | 0.026 ± 0.006 | 0.021 ± 0.004 | 0.005 ± 0.01 | 0.81 |
| Liver | 0.600 ± 0.070 | 0.023 ± 0.003 | 0.577 ± 0.07 | 0.04 |
| Kidney | 0.110 ± 0.015 | 0.055 ± 0.009 | 0.055 ± 0.02 | 0.50 |
| Brain | 3.085 ± .0513 | 1.420 ± 0.090 | 1.67 ± 0.06 | 0.46 |
| Lung | 0.430 ± 0.101 | 0.067 ± 0.005 | 0.36 ± 0.10 | 0.16 |
| Aorta | 1.480 ± 0.204 | 0.815 ± 0.025 | 0.67 ± 0.22 | 0.55 |

These values are presented as the mean ± S.E. of at least three or more determinations. $TG_{Insensitive}$ stands for thapsigargin-insensitive $Ca^{2+}$ uptake activity. All tissue membranes were derived from rat except aorta which was derived from pig.

Table 2 lists the initial rates of $Ca^{2+}$ uptake for the different rat tissue membranes, in the presence and absence of 2 μM thapsigargin. When measured in the presence of thapsigargin (i.e. to monitor the thapsigargin-insensitive activity presumably due to SPCA), brain and porcine aorta had by far the highest levels, followed by epididymal fat pads, heart and testis. The other tissues tested had relatively very little SPCA activity. Surprisingly, testis membranes had relatively low levels of activity compared to brain considering the relative abundance of SPCA protein from the immunoblots. 'Total' $Ca^{2+}$-ATPase activity (i.e. activities in the absence of thapsigargin, where SERCA would likely have a substantial contribution) or total activity minus the thapsigargin-insensitive activity (which would correspond mainly with the SERCA activity) was highest in heart, brain, porcine aorta and testis, as expected from the SERCA immunoblots results. When comparing the activity of SPCA within tissues that had reasonable amounts of total $Ca^{2+}$ pump activity, it was found that brain, aorta, testis and fat pads, all had considerable levels of thapsigargin-insensitive activity corresponding to 34% or more of the total $Ca^{2+}$ pump activity.

In order to investigate the subcellular localisation of SPCA and compare it to the location of the Golgi complex and SERCA, TM4 cells and HuASMC (contractile phenotype) were used, since they expressed SPCA and SERCA to reasonable levels. These cells were therefore stained with the anti-SERCA antibody (Y1F4), anti-SPCA1 antibody and with WGATR, which binds to and stains the Golgi complex. Fig. 4 shows the results of immunofluorescence staining of TM4 and HuASMC. TM4 cells stained with the anti-SPCA antibody revealed labelling around the nucleus, consistent with localisation in the Golgi, as staining with WGATR of the same cell revealed a very similar distribu-

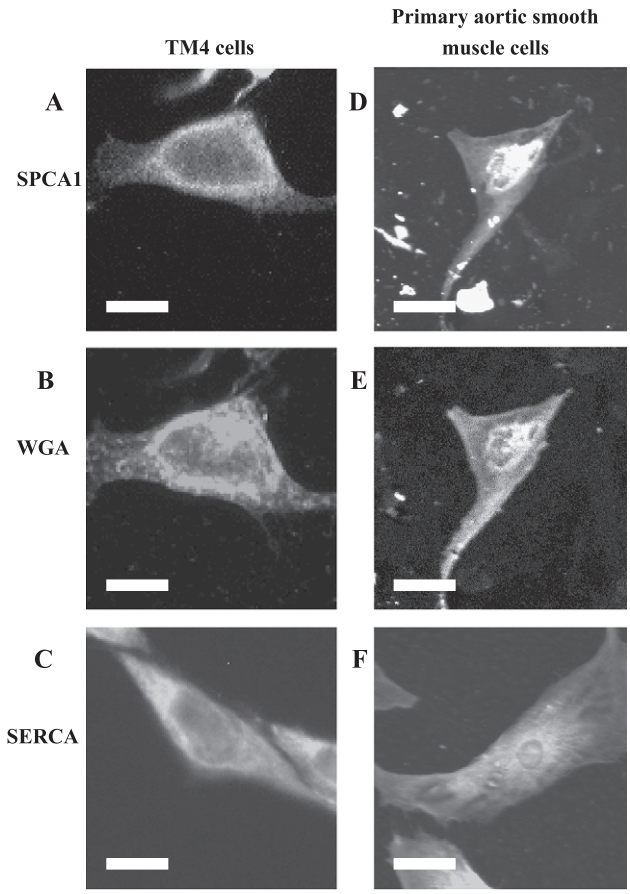


Fig. 4. Immunofluorescence staining of TM4 cells and human primary aortic smooth muscle cells. TM4 cells were cultured on scratched glass coverslips; primary human aortic smooth muscle cells were cultured on gelatine-coated glass cover slips. The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS and 2% formaldehyde in PBS, before being permeabilised for 5 min at room temperature with 0.1% Triton X-100 in PBS. The cells were incubated with either anti-SPCA1 diluted 1 in 100 in PBS or anti-SERCA antibody diluted 1 in 100 for 45 min. The cells were washed with PBS before being incubated with the appropriate secondary FITC-conjugated IgG according to the manufacturer's instructions. As an alternative to antibody, the cells were incubated for 45 min at room temperature with WGATR diluted to a concentration of 2 μg/ml in PBS and mounted on glass slides using Hydramount and visualised as for the immunostained cells. The bar in each of the TM4 cell diagrams represents 50 μm. The bar in each of the human primary aortic smooth muscle cell diagrams represents 100 μm. (A) TM4 cell stained with anti-SPCA1 antibody (1/100 dilution) and anti-rabbit IgG FITC (1/64 dilution). (B) TM4 cell stained with WGATR (2 μg/ml in PBS). (C) TM4 cell stained with Y1F4 anti-SERCA antibody (1/100 dilution) and anti-mouse IgG FITC (1/40 dilution). (D) Primary human aortic smooth muscle cell (HuASMC) stained with anti-Pmr1 (1/100 dilution) and anti7-rabbit IgG FITC (1/64 dilution). (E) HuASMC stained with WGATR (2 μg/ml in PBS). (F) HuASMC stained with Y1F4 anti-SERCA monoclonal antibody (1/100 dilution) and anti-mouse IgG FITC (1/64 dilution).

tion. Such staining is also consistent with previous immunostaining of human SPCA in keratinocytes [17]. Staining of TM4 cells with the Y1F4 anti-SERCA antibody revealed a more diffuse staining pattern of the majority of the cell, with the exception of the nucleus, consistent with localisation throughout the ER. HuASMCs co-labelled with the anti-SPCA antibody and WGATR also stained a distinct area around the nucleus. There was no significant staining observed with either the secondary antibody alone or with pre-immune serum and FITC secondary antibody.

## 5. Discussion

From RT-PCR of rat tissue samples and from Western blotting of rat tissue membranes, it has been shown that the SPCA1 protein is highly expressed in both brain and testis. Analysis of rat poly ($A^+$) RNA from several tissues by Northern blotting by Gunteski-Hamblin et al. [16] also showed that the levels of message for SPCA for brain and testis were higher relative to other tissues. Hu et al. [19] also analysed the levels of poly ($A^+$) RNA from several human tissues by Northern blotting, showing that the level of message for ATP2C1a and ATP2C1b for brain was lower compared with heart, skeletal muscle and kidney, inconsistent with our findings in rat tissues.

The secretory function of both the brain and testis is important. The brain in particular secretes many neurotransmitters from vesicles. It has been shown that SPCA is important for the transportation of $Ca^{2+}$ into secretory vesicles in neuroendocrine cells [36] and that $Ca^{2+}$ is important for the regulation of Golgi transport [37], thus SPCA may be abundant in the brain as it may regulate the secretion of neurotransmitters and secretory proteins.

Many of the testicular cell types have an important secretory role in paracrine signalling; Leydig cells are known to secrete testosterone [38], myoid cells are able to secrete adenosine [39] and Sertoli cells secrete a variety of hormones [40] and other factors to aid the developing sperm. As many testicular cell types have important secretory function, it may be expected that the level of SPCA1 expression may be higher in testis in order to aid the synthesis of secretory proteins.

The highest SPCA activity was observed in brain, the expression of SPCA in testis was at a comparable level to that of brain, yet the activities were much lower in this tissue. The reason for this is unclear, however, one possibility is that SPCA in testis is regulated by a modulatory protein, which decreases its activity, a suggestion previously made about human SPCA1d in keratinocytes to explain a reduction in $V_{max}$ in $Ca^{2+}$ transport [17]. There also appears to be some evidence for the role of SPCA2 in inhibiting the activity of SPCA1 and SERCA, in tissues where both are expressed, while SPCA2 alone appears to have little or no activity (Jo Vanoevelen and Ludwig Missiaen, personal communication).

Furthermore, as the proportion of SPCA activity compared to total $Ca^{2+}$ ATPase activity in brain, aorta, testis and epididymal fat pads was particularly high, this would indicate that SPCA may play a major role in the refilling of a distinct and abundant type of $Ca^{2+}$ store within these tissues.

Immunofluorescence staining of both the TM4 cells and human primary aortic smooth muscle cells has revealed that SPCA1 is localised at or near the Golgi in these cell types. Potentially SPCA may play a fundamental role in fertility as the function of the Sertoli cell is to secrete factors, such as purines [39], growth factors [40] and plasminogen activator [41], and to respond to paracrine signalling in order to nurture immature sperm. Interestingly it has been shown that patients suffering from Klinefelter's syndrome, a condition causing male infertility, have dysfunctional secretory mechanisms within their Sertoli cells [42].

In aortic cells (where our results would indicated that around 50% of the $Ca^{2+}$ stores are loaded by SPCA), SPCA could act as a backup to SERCA $Ca^{2+}$ pumps in controlling the contractile status and therefore regulating vasodilatation/vasoconstriction.

To summarize, in both TM4 cells and aortic smooth muscle cells, SPCA may be important for the filling of an agonist-releasable store of $Ca^{2+}$, a role suggested for SPCA in A7r5 cells [21], HeLa cells [14] and keratinocytes [43]. In these cells the generation of baseline $Ca^{2+}$ spiking, which may be important in modulating spatio-temporal $Ca^{2+}$ patterns and oscillations, has been attributed to SPCA.

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