Original Paper

Changes in expression and activity of the secretory pathway Ca^{2+} ATPase 1 (SPCA1) in A7r5 vascular smooth muscle cells cultured at different glucose concentrations

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Synopsis

Diabetes mellitus-related vascular disease is often associated with both a dysregulation of Ca^{2+} homoeostasis and enhanced secretory activity in VSMCs (vascular smooth muscle cells). Here, we employ a commonly used rat cell line for VSMCs (A7r5 cells) to investigate the effects of glucose on the expression and activity of the SPCA1 (secretory pathway Ca^{2+}-ATPase 1; also known as ATP2C1), which is a P-type Ca^{2+} pump located in the Golgi apparatus that plays a key role in the secretory pathway. Our results show that mRNA expression levels of SPCA1 are significantly increased in A7r5 cells cultured in high glucose (25.0 mM)-supplemented medium compared with normal glucose (5.55 mM)-supplemented medium. SPCA1 protein expression levels and thapsigargin-insensitive Ca^{2+}-dependent ATPase activity were also consistent with a higher than normal expression level of SPCA1 in high-glucose-cultured A7r5 cells. Analysis of AVP (arginine-vasopressin)-induced cytosolic Ca^{2+} transients in A7r5 cells (after pre-treatment with thapsigargin) showed faster rise and decay phases in cells grown in high glucose medium compared with cells grown in normal glucose medium, supporting the observation of increased SPCA expression/activity. The significant levels of both Ca^{2+}-ATPase activity and AVP-induced Ca^{2+} transients, in the presence of thapsigargin, indicate that SPCA must play a significant role in Ca^{2+} uptake within VSMCs. We therefore propose that, if such increases in SPCA expression and activity also occur in primary VSMCs, this may play a substantial role in the aetiology of diabetes mellitus-associated vascular disease, due to alterations in Ca^{2+} homoeostasis within the Golgi apparatus.

Key words: calcium homoeostasis, diabetes, Golgi apparatus, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), secretory pathway Ca^{2+}-ATPase 1 (SPCA1), vascular smooth muscle cell

INTRODUCTION

Cardiovascular diseases, including those associated with diabetes mellitus, have long been associated with the dysregulation of Ca^{2+} homoeostasis in vascular cells [1]. Comparisons between healthy and diabetic subjects in the past have shown that the distribution and use of Ca^{2+} ions differ between the two conditions. For example, diabetes has been associated with higher than normal basal $[Ca^{2+}]_i$ in rat-derived cardiac myocytes [2] and VSMCs (vascular smooth-muscle cells) [3], as demonstrated from their culture in high-glucose medium. Altered hormone-induced Ca^{2+} signalling has also been noted in human VSMCs from diabetic patients [4].

Abnormal expression and/or activity of specific proteins that participate in Ca^{2+} signalling and homoeostasis have been linked to diabetes. These include Ca^{2+}-dependent enzymes, such as increased activity of both calpain in rat endothelial cells [5] and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) in rat whole carotid arteries [6]. In addition, it has been shown that Ca^{2+} transporters can be influenced by the diabetic state in vascular cells. Examples of these include the inhibition of store-operated Ca^{2+} channels in rat microvascular smooth-muscle cells [7] and increased activity of T-type Ca^{2+} channels in rat cardiomyocytes

[8]. The SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) has also been shown to differ between healthy and diabetic subjects, both in the form of decreased expression levels in heart from streptozotocin-induced diabetic rats [9] and increased activity in rat primary VSMCs cultured in high-glucose medium [10].

The SPCA1 (secretory pathway Ca^{2+}-ATPase 1; also known as ATP2C1), a Golgi-apparatus-localized Ca^{2+} ion pump that is relatively highly expressed in VSMCs [11], has not been investigated in relation to diabetes-related cardiovascular pathology. It is unlikely, however, that the other isoform, SPCA2, plays a substantial role in cardiovascular pathology, since it is not significantly expressed in vascular tissue [12]. However, previous studies have linked SPCA1 expression to diabetes in pancreatic cells [13]. Furthermore, alterations in the Golgi apparatus have also been observed in VSMCs treated with kinase inhibitors to inhibit proliferation [14]. SPCA1 is important for establishing a store of Ca^{2+} ions in the Golgi apparatus, which is required by Ca^{2+}-dependent enzymes essential for post-translational modification of proteins in transit through the secretory pathway [15].

In the present study, we utilize one of the most commonly employed cell lines for VSMCs (A7r5 cells) to determine whether culturing them in either NG [glucose (5.55 mM) culture medium] or HG [high glucose (25 mM) culture medium], to mimic the 'normal' and 'hyperglycaemic' glucose homoeostatic states, specifically alters the expression and activity of SPCA1.

MATERIALS AND METHODS

A7r5 cell culture and treatment

A7r5 (rat embryonic thoracic aortic smooth muscle) cells [a gift from Professor C.W. Taylor (University of Cambridge, Cambridge, U.K.)] were cultured in DMEM (Dulbecco's modified Eagle's medium; Lonza), which was supplemented with 10% FBS (foetal bovine serum), 4 mM L-glutamine, 1% non-essential amino acids and 1% 1:1 penicillin/streptomycin (PAA Laboratories). Growth was maintained under conditions of 37°C and 5% CO_2. Culture medium contained 25 mM D-glucose when cells were maintained for growth or for obtaining cells in the 'hyperglycaemic' HG-cultured state. Subculturing of cells was done every 3–4 days by trypsinization using 0.05% trypsin/EDTA solution (Invitrogen). Cells in the NG-cultured state were initially obtained by seeding a new flask of subcultured cells from HG medium into NG (5.55 mM D-glucose) medium. Both NG and HG cells were subcultured for at least a further two passages in their respective medium before being used for experimentation.

PCR

mRNA was extracted from cells and converted into cDNA copies via RT–PCR (reverse transcription–PCR), using a Superscript III CellsDirect™ cDNA synthesis kit (Invitrogen). Cells were harvested by trypsinization, washed with cell culture PBS (phosphate-buffered saline) and resuspended in 0.1% (v/v) DEPC (diethyl pyrocarbonate)-treated PBS. To prepare each cDNA sample, 2×10^4 cells were used.

BIOTAQ™ Taq DNA polymerase and dNTP mixture were purchased from Bioline. All the primers were made by Alta Bioscience (University of Birmingham, Birmingham, U.K.). Primer sequences and their specific PCR conditions were obtained from the following associated references: SPCA1 [16], SERCA [17], GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [18], β-actin [17] and RPL19 (ribosomal protein L19) [19]. The SPCA1 primers only recognized mRNA corresponding to the SPCA1 isoform (all splice variants). The SERCA primers recognized mRNA corresponding to all SERCA isoforms. Each 50 μl PCR reaction mixture contained 2.5 units of BIOTAQ™, 2 mM dNTP mix and 1 μl of cDNA sample. SPCA1 and SERCA PCRs required 35 cycles. GAPDH, β-actin and RPL19 required 28 cycles. All PCR products were separated by agarose-gel electrophoresis, stained with SYBR® Safe DNA gel stain (Invitrogen) and visualized using a UV transluminator. PCR product band sizes were checked using a 100 bp DNA ladder (New England Biolabs).

Images of stained gels were taken using an UVP ImageStore 5000 system. Analysis was undertaken using ImageJ (version 1.41e; National Institutes of Health). Mean pixel intensity values were measured for the product bands, each of which was corrected for background and made relative to those for RPL19 (a commonly used internal control for PCRs [19]) from the same sample to correct for differences in total cDNA quantity. The expression of RPL19, a housekeeping protein, was unlikely to be affected by different glucose culture conditions in A7r5 cells when compared with other control markers, such as GAPDH (a glycolytic enzyme) and β-actin (a contractility marker [20]). Values for HG samples were made relative to their counterpart NG samples.

SDS/PAGE and Western blotting

Cell lysate samples were prepared using a proportion of the same cells used for cDNA preparation. For each sample, $(1–1.25) \times 10^6$ cells were incubated on ice for 2 h in lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM PMSF). The cell suspension was then homogenized by passing it through a 21-gauge needle via a syringe; it was centrifuged at 2400 g for 5 min, and the supernatant was collected.

Proteins in all cell lysate samples were separated by SDS/PAGE, transferred on to nitrocellulose sheets by Western blotting and probed with antibodies for SPCA1, SERCA and α-actin. The methodology was the same as described previously in [11] with the following modifications: 20 μg of protein was loaded for each sample for SDS/PAGE; anti-SPCA1 (custom antibody from BioCarta; [11]), anti-SERCA (YIF4) (a gift from Dr J.M. East, Southampton University, Southampton, U.K.) and anti-α-actin (clone 1A4; Sigma) antibodies were diluted in TTBS (Tween/Tris-buffered saline) at ratios of 1:75, 1:800 and 1:800 respectively and incubated with the blots for 90 min; after incubation with HRP (horseradish peroxidase)-conjugated secondary

antibodies (1:3000 dilution), Immobilon™ Western chemiluminescent HRP substrate (Millipore) was used to visualize antibody-bound protein bands; HRP-substrate-treated blots were viewed and images were captured using a Bio-Rad Fluor-S Max MultiImager. Images were analysed with ImageJ software to determine pixel intensity values for each product band. These were corrected for background and values for the HG samples were made relative to their counterpart NG samples.

Microsomal membrane preparation

The method used was as previously described [11] with the following modifications: cells were harvested by trypsinization, washed with PBS and centrifuged at 900 g for 10 min at 4°C; the pellet was homogenized with both Polytron and Potter–Elvehjem-type homogenizers after resuspension in membrane preparation buffer; the homogenate was centrifuged at 20000 g for 15 min at 4°C, the pellet was re-homogenized and centrifuged again, and the two supernatants were combined and centrifuged at 100000 g for 50 min at 4°C. The final pellet, which contained the microsomal membranes, was resuspended in fresh buffer, divided into aliquots, snap-frozen in liquid nitrogen and stored at –80°C.

Ca^{2+}-dependent ATPase activity

Ca^{2+} dependent ATPase activity was measured using the phosphate liberation method, as previously described [21], with minor modifications. The assay buffer included sodium azide (2 mM) and vanadate (2 μM) to inhibit mitochondrial Ca^{2+} uptake and PMCA (plasma-membrane Ca^{2+}-ATPase) respectively. The reaction period was 90 min and each reaction mixture contained 20 μg of microsomal membrane proteins. In order to distinguish between Ca^{2+}-ATPase activity from SERCA and SPCA, experiments were repeated in the presence and absence of 1 μM thapsigargin (Sigma) as this would completely inhibit SERCA and have minimal effects on SPCA activity [21,22].

Intracellular Ca^{2+} imaging

The method used was the same as described previously in [23], but with minor modifications. The cells were grown at a density of 1×10^4 cells per coverslip and loaded with Fluo-3/AM (acetoxymethyl ester) (Sigma) at a final concentration of 6 μM in HBSS (Hanks balanced salt solution). After 45 min of incubation, the cells were incubated in HBSS containing sulfinpyrazone (200 μM) for 10 min. Fluo-3-loaded cells were then viewed using an inverted epifluorescence microscope (Nikon TS-100F) with filters specifically designed to monitor Fluo-3 fluorescence and a $\times 10$ objective. The video images were recorded using a StellaCam Astrovid³ camera connected to a computer using a Hauppage USB TV live video-capture device and software (set at 1 frame/s). After initiation of the recordings, the cells were treated with 1 μM thapsigargin for 5 min prior to the addition of 10 nM AVP (arginine-vasopressin; Bachem Biosciences). After the latter treatment, recordings were collected for a further 5 min.

Changes in Fluo-3 fluorescence intensity induced by AVP were measured using ImageJ software, and corrected for both background and photobleaching as previously described [23]. Values for each cell were made relative to their basal fluorescence intensity value at the time point of AVP application. These relative values were used to produce Ca^{2+} transient plots, from which the kinetics of the rise and decay phases could be calculated.

Immunohistochemistry and lectin staining

The method used was the same as described previously in [11], but with the following modifications: cells were seeded at a density of $(3–4.5) \times 10^4$ cells per coverslip; prior to antibody treatment, they were fixed with 2% (v/v) formaldehyde in PBS; anti-SPCA1 and anti-SERCA working dilutions were 1:30 and 1:100 respectively; TMR (tetramethylrhodamine)-conjugated WGA (wheat-germ agglutinin; Invitrogen) (for Golgi apparatus labelling) was used at 10 μg/ml per coverslip. The labelled cells were viewed using fluorescence microscopy, with appropriate filters. Digital photographs of the cells were collected using the same imaging system as described above, except that the images were recorded at a 640×480 pixel resolution.

Statistical analysis

S.E.M. were calculated for results from PCRs, Western blots, Ca^{2+}-ATPase assays and kinetic measurements from Fluo-3 fluorescence Ca^{2+} imaging experiments. A two-tailed equal variance Student's t test and ANOVA (Minitab) were used to test for statistical significance (P).

RESULTS

Figure 1 shows the PCR products, which relate to mRNA expression levels, for SPCA1, SERCA, GAPDH and β-actin. The results were obtained from triplicate paired cultures, where the PCR products of the transcripts in HG-cultured cells were compared with their corresponding paired NG-cultured cells and then expressed as a percentage. The results showed that the relative expression of SPCA1 and SERCA, in HG-cultured cells compared with NG-cultured cells, were found to have increased by $60 \pm 13\%$ and $19 \pm 5\%$ respectively. Furthermore, these changes in expression were statistically significant between the two conditions (i.e. $P < 0.01$ for SPCA1 and $P < 0.05$ for SERCA). In contrast, the mRNA expression levels for GAPDH and β-actin were not substantially affected (i.e. $7 \pm 4\%$ and $-4 \pm 3\%$, for GAPDH and β-actin respectively). Figure 1 also shows representative gels of the PCR products for the different mRNA transcripts isolated from cells grown in HG- and NG-cultured media.

Figure 2 shows the mean differences in protein levels determined using total cell lysate samples from triplicate paired cultures and expressed as a percentage of the HG cells compared with NG cells. The results confirmed the PCR study by showing that
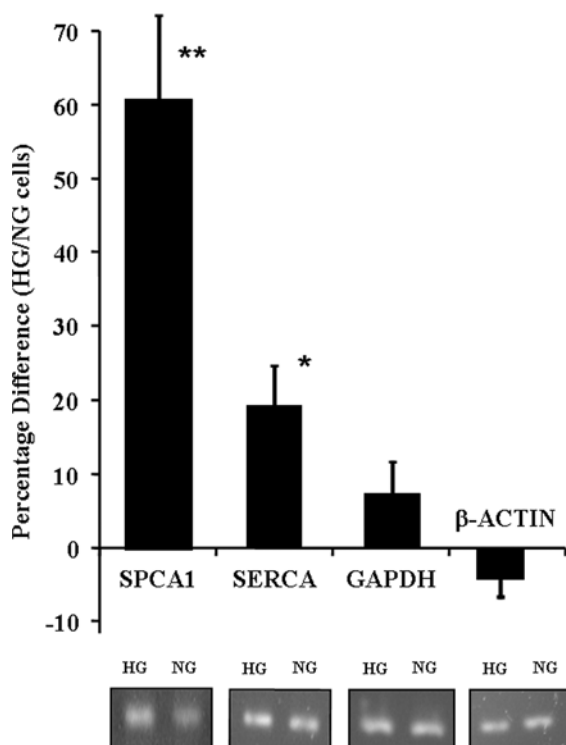
Figure 1 Differences in mRNA content for SPCA1, SERCA, GAPDH and β-actin in hyperglycaemic compared with normal glucose-cultured A7r5 cells

PCRs were repeated in triplicate using samples from three replicate cell cultures for each primer pair and glucose culture condition. A final mean relative band intensity value was calculated from the three PCR replicates for each primer pair and glucose culture condition. Band intensity values from each sample were corrected for background and made relative to RPL19 values from matching samples. Relative percentage changes were calculated from values obtained from HG relative to NG cells (HG/NG). Error bars represent S.E.M. values. Statistically significant P-values are indicated by * (for P values <0.05) and ** (for P values <0.01). The images under each bar show the associated typical PCR product band intensities observed for each of the primer pairs, whereby HG and NG represent the proteins derived from cells grown under hyperglycaemic and NG culture conditions respectively. The PCR product band sizes for each mRNA of interest were, from left to right, 224 bp (SPCA1), 288 bp (SERCA), 400 bp (GAPDH) and 255 bp (β-actin).
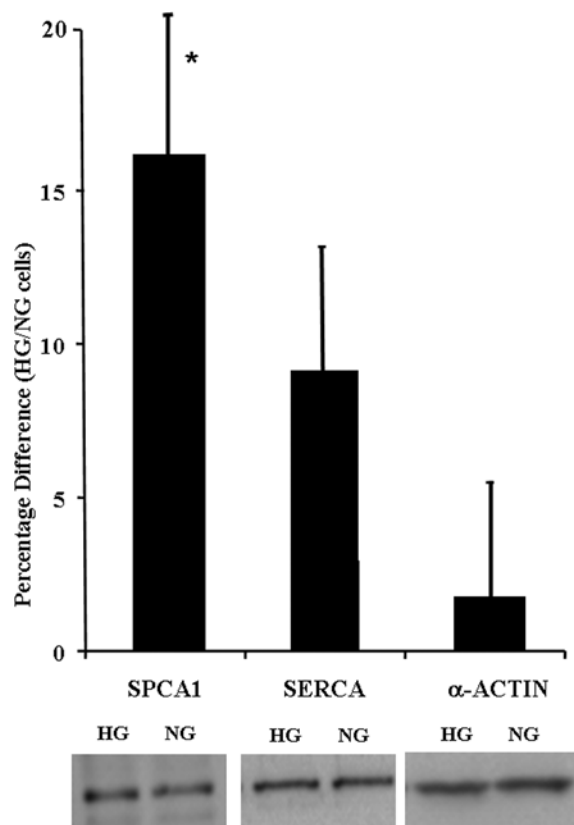

Figure 2 Differences in protein content for SPCA1, SERCA and α-actin in hyperglycaemic compared with normal glucose-cultured A7r5 cells

Western blots were repeated twice for each of three replicate cell cultures for each primary antibody and glucose culture condition. These were then used to calculate a final mean relative band intensity value for each protein of interest, and percentage changes in these proteins for HG cells relative to NG cells were calculated. Error bars represent the S.E.M. of the differences. Statistically significant P values (where $P < 0.05$) are indicated by *. The images under each bar show the associated typical protein band intensities observed for each of the target proteins, whereby HG and NG represent the proteins derived from cells grown under hyperglycaemic and NG culture conditions respectively. The molecular mass for each protein was, from left to right, ∼100 kDa for both SPCA1 and SERCA and 42 kDa for α-actin.

HG-cultured cells had an increased abundance of SPCA1 (by $15 \pm 5\%$), as well as a small or negligible change in SERCA (by $9 \pm 4\%$) and α-actin (by $2 \pm 4\%$) protein when compared with NG-cultured cells. The increase in SPCA1 protein levels between HG- and NG-cultured cells was found to be statistically significant ($P < 0.05$). Figure 2 also shows representative Western blots for SPCA1, SERCA and α-actin protein levels detected in samples from NG- and HG-cultured A7r5 cells.

To determine whether increased SPCA1 expression resulted in increased SPCA-specific Ca^{2+}-ATPase activity, microsomal membranes were isolated from HG- and NG-cultured cells, and measured using the phosphate liberation assay (Table 1). Thapsigargin (1 μM) was used to distinguish the contributions made by SERCA and SPCA. The assay buffer also con-

tained both 2 mM azide and 2 μM vanadate to inhibit the mitochondrial Ca^{2+} uptake/activity and plasma membrane Ca^{2+}-ATPase activity respectively. Under these experimental conditions, the Ca^{2+}-dependent ATPase activities were deemed to be predominantly due to either a combination of SERCA and SPCA (in the absence of thapsigargin) or SPCA alone (in the presence of thapsigargin) [20]. Due to the inherent variability in Ca^{2+}-ATPase activities from different membrane preparations, experiments were done using samples of microsomal membranes isolated from three replicate pairs of HG and NG cell cultures. Table 1 shows that the Ca^{2+}-ATPase activities from the three membrane preparations, isolated from NG- and HG-cultured cells, were similar in the absence of thapsigargin.

Table 1 Ca²⁺-dependent ATPase activities of microsomal membranes from normal glucose and hyperglycaemic cultured A7r5 cells

| | Ca²⁺-dependent ATPase activity (nmol/min per mg of protein)(mean $\pm$ S.E.M.) | | | |
| | Absence of thapsigargin | | 1 μM Thapsigargin | |
Membrane preparation	NG	HG	NG	HG
1	3.2 ± 0.5	3.1 ± 0.7	0.62 ± 0.09	1.05 ± 0.17
2	4.1 ± 1.6	2.7 ± 0.4	0.38 ± 0.07	2.89 ± 1.21
3	4.4 ± 0.5	3.7 ± 0.2	1.26 ± 1.17	1.63 ± 0.48

(The mean activities were 3.9 ± 0.5 and 3.2 ± 0.4 nmol/min per mg of protein for NG- and HG-cultured cells respectively). On the other hand, in the presence of 1 μM thapsigargin, the Ca²⁺-ATPase activities did show significant differences between the membranes isolated from NG- and HG-cultured cells, with higher activities consistently obtained from HG-cultured cell membranes. Mean activities for the three membrane preparations were 0.75 ± 0.27 nmol/min per mg of protein for NG cells and 1.86 ± 0.55 nmol/min per mg of protein for HG cells. Statistical analysis showed that the activity differences between the two culture conditions were significant ($P < 0.05$).

A comparison of total Ca²⁺-ATPase activity (from SERCA and SPCA) in the membranes, with the activity for SPCA alone, showed that SPCA accounted for between 20 and 60% of the Ca²⁺-ATPase activity in A7r5 cultured cells. This is not dissimilar from our previous findings in aortic VSMC membranes, where we showed that approx. 50% of total ATP-dependent Ca²⁺ uptake into these membranes was due to SPCA [11]. Therefore these findings again strongly suggest that SPCA plays a significant role in Ca²⁺ uptake into Ca²⁺ stores within VSMCs.

To assess whether increased expression of SPCA1 can alter the profile of agonist-induced cytosolic [Ca²⁺] transients, NG- and HG-cultured cells, loaded with Fluo-3, were treated with 10 nM AVP. These cells were first pre-treated with 1 μM thapsigargin in order to minimize the effects of SERCA on their response to AVP. Figure 3 shows the averaged traces (with each trace comprising results from ten individual cells) of Fluo-3 fluorescence change in both NG (Figure 3A) and HG (Figure 3B) cells. Although the peak values in AVP-induced Ca²⁺ transients were similar in cells from the two glucose culture conditions, their kinetic profiles were notably different. The Ca²⁺ transients observed for NG-cultured cells were much longer than those for HG-cultured cells, for both the rise and the decay phases of the transients. However, if the cells were not initially pre-treated with thapsigargin, the kinetics of the Ca²⁺ transients for both NG- and HG-cultured cells were very similar to each other and shorter (insets), consistent with observations from previous studies [24]. The mean peak heights between cells not treated and those pre-treated with thapsigargin, for both NG and HG culture conditions, were each reduced by approx. 60%. This shows that the contribution made by SERCA-containing Ca²⁺ stores to the AVP-mediated Ca²⁺ responses in these cells is not overwhelmingly dominant and SPCA-containing Ca²⁺ stores are also likely to contribute significantly.
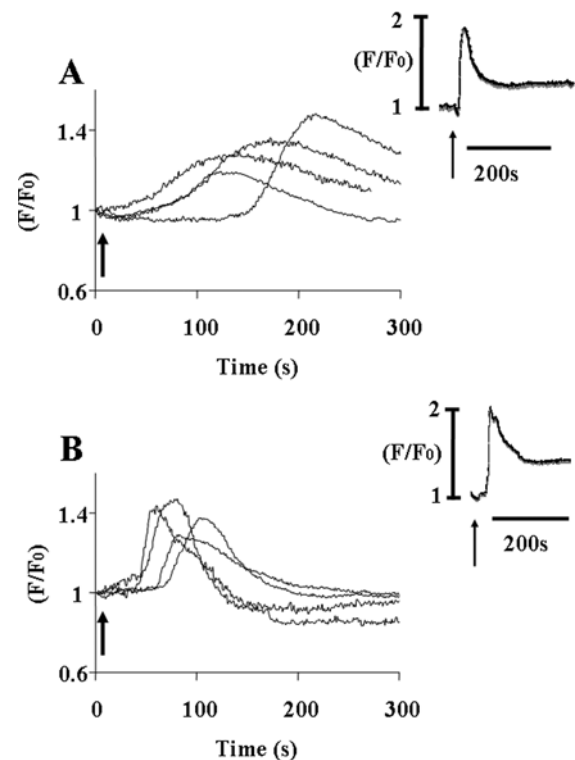
Figure 3 Ca²⁺ transients induced by AVP after pre-treatment with thapsigargin in hyperglycaemic and normal glucose-cultured A7r5 cells
For both (A) NG and (B) HG culture conditions, the main Figures show results from four coverslips, whereby each trace represents averaged fluorescence values from ten cells, analysed for Fluo-3 fluorescence changes (F/F_0) in response to 10 nM AVP, after pre-treatment of cells with thapsigargin. The insets show the typical response profile (averaged from ten cells) for 10 nM AVP after pre-treatment with DMSO (vehicle control for thapsigargin) in cells from the same glucose culture conditions. Arrows indicate when AVP was added.

The kinetic measurements taken from the Ca²⁺ transient traces representing post-thapsigargin AVP responses showed a quantitative difference between cells cultured in NG and HG media (Table 2). The mean time taken to increase from basal to peak [Ca²⁺]$_i$ levels of the AVP response was approx. 3-fold longer in NG-cultured cells compared with HG-cultured cells. The half-life for the decay in [Ca²⁺]$_i$ from the peak back towards basal levels

Table 2 Kinetics measurements from AVP-mediated Ca²⁺ responses in normal glucose and hyperglycaemic cultured A7r5 cells, after pre-treatment with thapsigargin

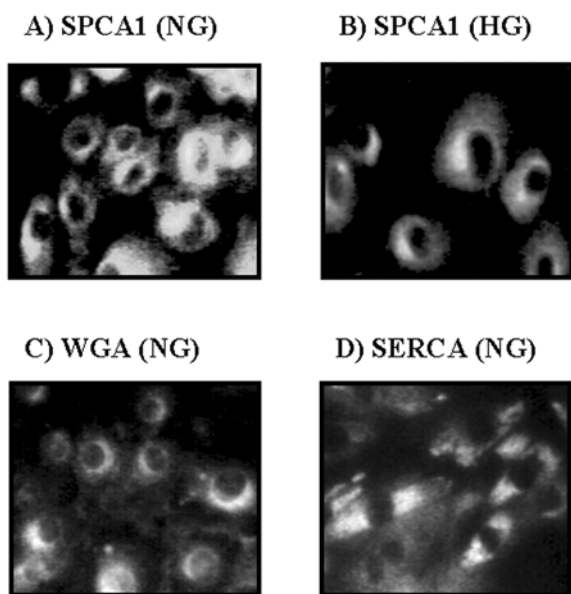|	Value	
Measurement	NG	HG
Time taken to reach half-maximal response (s)	48.3 ± 5.1	18.2 ± 3.2
Half-life of decay in response (s)	76.9 ± 7.4	39.7 ± 5.7

Figure 4 Immunolocalization of SPCA1, Golgi apparatus and SERCA in A7r5 cells

Immunostaining for SPCA1 using the anti-SPCA1 antibody (1:30 dilution) in (A) NG- and (B) HG-cultured cells. NG cultured cells were used to identify the location of the Golgi apparatus with TMR–WGA (10 µg/ml) (C) and the Y1F4 antibody (1:100 dilution) for SERCA (D). Cells were viewed at ×200 magnification under epifluorescence microscope.

SERCA showed a more uniform distribution throughout the cell that was distinct from those observed for both SPCA1 and WGA and which we have previously reported elsewhere for primary VSMCs [11].

DISCUSSION

Results obtained by Wootton et al. [11] showed rat aortic tissue and primary human aortic VSMCs express relatively high levels of SPCA1 protein compared with many other tissues and cell types. From Ca²⁺-dependent ATPase measurements we show that between 20 and 60 % of the Ca²⁺-ATPase activity associated with intracellular membranes of A7r5 cells is derived from SPCA. Again this is similar to our previous findings, which showed that in aortic VSMC membranes approx. 50 % of the ATP-dependent Ca²⁺ uptake is via SPCA [11]. This is also corroborated by the fact that substantial increases in AVP-induced intracellular [Ca²⁺] were observed in thapsigargin-pre-treated cells, which suggests that these VSMCs must contain a sizeable SERCA-independent mobilizable Ca²⁺ store. Therefore, taken together, these findings highlight the substantial role SPCA plays in Ca²⁺ store refilling within VSMCs.

It has also been well established that vascular tissues prone to developing atherosclerotic plaques (as observed in diabetes mellitus [26]) have a tendency to change their VSMCs from a contractile to a secretory phenotype [27]. This is characterized by their increased ability to produce secreted proteins, such as collagen and proteoglycans found in the extracellular matrix [28]. Since SPCA1 plays an important role in both post-translational modification of proteins in the Golgi apparatus and transit of proteins through the secretory pathway, the aim of the present study was to investigate whether SPCA1 expression and/or activity is different between NG and HG-cultured A7r5 cells, a commonly used cell line for VSMCs.

Measurements of mRNA and protein levels in this study showed A7r5 cells grown in HG culture medium expressed measurably more SPCA1 than cells grown in NG culture medium, which suggests that glucose availability can influence the expression of this protein. There was a notable difference, however, in the extent of change between SPCA1 mRNA and protein expression levels, whereby a 60 % increase in mRNA resulted in only approx. 15 % increase in protein. A similar discrepancy was observed by Reinhardt et al. [29] when they compared SPCA expression levels between rat mammary and brain tissue, and attributed their finding to a difference in protein turnover. A similar explanation may, at least in part, explain our observation in A7r5 cells. Transcriptional control of SPCA1 may also play a role, since Sp1 and YY1, which are the transcription factors of the SPCA1 gene [30], possess increased activity in cells grown in high, compared with normal, glucose media [31,32]. One possibility could be that a combination of enhanced transcription and near-maximal translation of the SPCA1 gene in hyperglycaemic A7r5 cells may result in an increase in its mRNA,

was also approx. 2-fold lower in NG cells. Statistical analysis of these kinetic measurements for both HG- and NG-cultured cells showed that these differences were significant (i.e. $P < 0.05$, for both the rise and decay phases comparing NG- and HG-cultured cells).

Immunolocalization of SPCA1, together with WGA-based lectin affinity staining, in NG- and HG-cultured cells showed that SPCA1 and the Golgi apparatus share a very similar distribution pattern, based on its characteristic perinuclear staining (Figure 4). WGA binding is commonly used as a marker for the Golgi apparatu because it binds to glycoproteins highly abundant in this organelle [25]. Comparison of the staining patterns for SPCA1 showed no notable differences between NG- and HG-cultured cells. Thus localization of SPCA1 in A7r5 cells was largely unaffected by the glucose culturing conditions. Immunostaining for

but not necessarily increase in its protein levels to the same extent.

Consistent with results from PCR-based mRNA measurement and immunochemical detection of SPCA1 protein, thapsigargin-insensitive Ca^{2+}-dependent ATPase activity in microsomal membranes (representative of SPCA activity) was also found to be higher from HG, compared with NG, cultured cells. However, the mean activity for the HG-cultured cells was approx. 2-fold higher than that for the NG-cultured cells, which would also appear to be disproportionate to the increase in SPCA1 protein levels observed. Such differences may be explained by the differences in Ca^{2+} transport activities between different SPCA1 splice variants [33]. In our present study, we measured all SPCA activity and the expression of all SPCA1 slice variants. However, one could envisage that an increased expression of SPCA1 splice variants that have higher levels of activity could contribute to the higher than normal SPCA activity observed in HG-cultured cells. However, this possibility needs to be further explored.

In order to assess whether higher than normal expression of SPCA1 within HG-cultured A7r5 cells can alter agonist-induced cytosolic $[Ca^{2+}]$ transients, the effects of AVP on cytosolic $[Ca^{2+}]$ profiles were investigated. AVP (working through the phospho-inositide pathway) initiates Ca^{2+} mobilization from internal Ca^{2+} stores and stimulates Ca^{2+} entry from the extracellular medium. Cytosolic Ca^{2+} removal (by uptake into intracellular stores and extrusion out of the cell) then follows, resulting in a typical Ca^{2+} transient profile [24]. In our present study, using thapsigargin-pre-treated A7r5 cells, we showed that, although the peak heights of SERCA-independent AVP-induced Ca^{2+} transients were not significantly different between NG- and HG-cultured cells, these transients were different with regard to both their rise and decay phase kinetics. The differences in the rise and decay phase kinetics could be attributed to differences in the abundance of thapsigargin-insensitive IP_3-releasable Ca^{2+} stores (i.e. Golgi Ca^{2+} stores). Thus, if HG-cultured cells had more of these Ca^{2+} stores than NG-cultured cells, this could result in higher rates of Ca^{2+} release, as well as higher rates of Ca^{2+} re-uptake (due to their higher levels of SPCA). This becomes even more apparent once SERCA-containing Ca^{2+} stores have been emptied by thapsigargin treatment.

Our immunostaining studies confirm that SPCA1 remains located at the distinct regions within the cell consistent with being the Golgi apparatus, rather than more widely distributed throughout the endoplasmic reticulum like SERCA. Furthermore, increased SPCA expression, as observed in HG-cultured cells, does not appear to affect its subcellular location (Figure 4).

In summary, the present study has shown that culturing A7r5 cells (a commonly used VSMC model) in HG medium (which mimics the hyperglycaemic state associated with severe diabetes mellitus) can lead to: (i) higher SPCA1 expression levels (both at the mRNA and protein levels); (ii) increased thapsigargin-insensitive Ca^{2+}-ATPase activity, which is comparable with the activity observed for SERCA; and (iii) higher rates of both the rise and decay phases of AVP-induced Ca^{2+} transients in thapsigargin-pre-treated cells. These findings suggest that gluc-

ose availability can substantially affect the Ca^{2+}-handling properties within the Golgi apparatus and therefore affect the secretory pathway and post-translational protein modification processes, as well as Ca^{2+}-mediated hormonal responses, in A7r5 VSMCs. If these changes in SPCA1 expression and activity also occur in primary VSMCs, this might potentially contribute towards cardiovascular diseases often associated with diabetes. Therefore further studies are required, using primary VSMCs cultured in different glucose media, as well as studying cells isolated from normal and severely diabetic animals, in order to corroborate this hypothesis.

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