Isoforms of endoplasmic reticulum Ca^{2+}-ATPase are differentially expressed in normal and diabetic islets of Langerhans

Anikó VÁRADI*§, Elek MOLNÁR†, Claes-Göran ÖSTENSON‡ and Stephen J. H. ASHCROFT*

*University of Oxford, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K., †Medical Research Council, Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, U.K., and ‡Department of Molecular Medicine, Endocrine and Diabetes Unit, Karolinska Hospital, S-171 76 Stockholm, Sweden

Glucose-dependent sequestration of Ca^{2+} into endoplasmic reticulum and its subsequent release play an important role in the control of intracellular Ca^{2+} concentration, which regulates insulin secretion in pancreatic β-cells. The active uptake of cytosolic Ca^{2+} into endoplasmic reticulum is mediated by sarco-(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs). We found, using RT-PCR with isoform-specific primers, that SERCA 2 and SERCA 3 mRNAs are co-expressed in human and rat islets of Langerhans and in the RINm5F β-cell line. Immunochemical analysis also revealed the existence of two SERCA proteins with molecular masses of 110 and 115 kDa in β-cell membranes. The 115 kDa protein was identified as SERCA 2b by its reaction with an isoform-specific antibody and the 110 kDa protein most probably corresponds to SERCA 3. The presence of two functionally different SERCA isoforms raises the possibility that they are located in distinct Ca^{2+} stores. There is evidence that altered Ca^{2+} handling in the β-cell may contribute to the decreased insulin secretion seen in non-insulin dependent diabetes mellitus (NIDDM). We therefore investigated SERCA 2 and SERCA 3 mRNA expression by quantitative RT-PCR in islets prepared from Goto–Kakizaki (GK) rats, a non-obese spontaneous model of NIDDM. We found a significant reduction (about 68 %) in SERCA 3 isoform expression. Since SERCA 2 expression was not significantly reduced, these genes are independently regulated and probably play distinct roles in islets of Langerhans. The marked decrease of SERCA 3 expression may constitute a defect in Ca^{2+} signalling in GK rat islets which could be a component of NIDDM.

INTRODUCTION

Glucose stimulation of insulin secretion from islet β-cells is initiated by membrane depolarization and an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due, in part, to the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels [1,2]. Glucose-dependent sequestration of cytosolic Ca^{2+} into endoplasmic reticulum Ca^{2+} stores via activation of sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs), and its subsequent release, have also been shown to play an important role in β-cell Ca^{2+} signalling [3,4]. There is evidence that the extent of filling of the endoplasmic reticulum Ca^{2+} store regulates the membrane potential of islet β-cells, thereby controlling Ca^{2+} influx and, consequently, insulin secretion [4]. Furthermore, abnormalities in glucose-stimulated Ca^{2+} signalling are connected with the development of non-insulin-dependent diabetes mellitus (NIDDM) [5–11]. In particular, it has been shown that the abnormal glucose-induced intracellular Ca^{2+} signalling in islets isolated from *db/db* mice is associated with reduction of SERCA activity [5].

Thus far, three different SERCA genes have been described (SERCA 1–3) [12–14]. SERCA 1 is exclusively expressed in fast-twitch skeletal muscle [15]. SERCA 2 gene transcripts give rise to two distinct protein isoforms, SERCA 2a and SERCA 2b, which differ only in the C-terminal part [16–19]. SERCA 2a, the muscle isoform, is expressed in slow-twitch skeletal muscle, in smooth muscle and in cardiac muscle. SERCA 2b, the non-muscle isoform, is widely expressed in non-muscle tissues and also in smooth muscle. SERCA 3 is in all cases found to be co-expressed with SERCA 2b at least and the overall picture of its tissue distribution is unclear. SERCA 3 is expressed in haematopoietic cells and in the embryologically related endothelial cells [20,21], in epithelial cells, in Purkinje neurones and in hippocampal and CA1 pyramidal cells [22]. In addition to their tissue- or cell-specific expression, there are functional differences between the non-muscle isoforms (SERCA 2b and 3) and their muscle counterparts (SERCA 1 and 2a) [23]. Moreover, SERCA 3 shows markedly different functional properties compared with the other members of the SERCA family [24].

Despite the clear importance of SERCA for β-cell stimulus–response coupling, little is known of its expression in the β-cell at the molecular level. In a previous study [25] the presence of SERCA 2b in rodent β-cells was demonstrated by phosphorylation and immunochemical methods. However, the presence of additional isoform(s) was not excluded. Moreover, there is no information available on SERCA expression in human β-cells. In this study we have investigated which SERCA isoform(s) are expressed and have determined their relative quantities in human (HIL) and rat (RIL) islets of Langerhans and an RINm5F pancreatic β-cell line (RIN). It is not known if the down-regulation of SERCA observed previously is a general feature in NIDDM models other than the *db/db* mouse or whether this down-regulation is the result of reduced SERCA gene expression. Therefore we have measured the changes in the expression level of SERCA isoform-specific mRNAs in islets from Goto–Kakizaki Wistar (GK) rat, a non-obese, spontaneous model of NIDDM [26,27].

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DEPC, diethyl pyrocarbonate; GK, Goto–Kakizaki Wistar rat strain; HIL, human islets of Langerhans; NIDDM, non-insulin dependent diabetes mellitus; RIL, rat islets of Langerhans; RIN, RINm5F β-cell line; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

§ To whom correspondence should be addressed.

EXPERIMENTAL

Materials

HIL were obtained from the Transplant Centre, Churchill Hospital, Oxford, U.K. The RIN cell line was generously provided by Professor C. B. Wollheim, University of Geneva. Rabbit anti-peptide antibody, raised against the 12 C-terminal amino acids of the SERCA 2b Ca^{2+}-ATPase [28], was kindly supplied by Dr R. L. Dormer, University of Wales College of Medicine, Cardiff, U.K. The production and characterization of rabbit anti-rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase antibody (EM-2) were described previously [25,29–33]. Collagenase (Type Ia), TRI-Reagent and all tissue culture materials were obtained from Sigma, Poole, Dorset, U.K. Protein molecular-mass markers were from Pharmacia Biosystems, Milton Keynes, U.K., and the DNA molecular-mass marker (*pBR*322/*Hae*III + *pBR*322/*Taq*I) was purchased from Appligene, Durham, U.K. DNA oligonucleotide primers were obtained from Cruachem Ltd. Glasgow, Scotland. Restriction endonucleases were from Boehringer, Mannheim, Germany (*Bam*HI, *Eco*RI, *Pst*I, *Rsa*I, *Xba*I), Promega Co., Madison, WI, U.S.A. (*Bsa*HI), and from Sigma, (*Sty*I). All other chemicals were of reagent grade.

Animals

GK rats of the Stockholm colony were bred at the Karolinska Hospital. The higher plasma glucose concentration and the lower body weight of GK rats from this colony (10.65 ± 0.64 mM and 304.5 ± 4.2 g respectively) compared with normal Wistar rats (6.97 ± 0.28 mM and 340.4 ± 6.3 g respectively) have been described previously [34,35].

Cell culture

RIN were cultured in RPMI 1640 tissue culture medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml) and foetal calf serum 10 % (v/v) at 37 °C in an atmosphere of humidified air/5 % CO_2 as described previously [36]. Cells were passaged weekly and harvested using 0.5 % (w/v) trypsin/0.2 % (w/v) EDTA. They were seeded in culture flasks at a density of 4×10^7 cells per flask and cultured for 5 days before RNA or membrane preparation.

Isolation of HIL and RIL

The pancreas was removed (with permission) from a heart-beating cadaver of a 62 year-old female at the same time as retrieval of other organs for organ donation. The pancreas was perfused with cold hypertonic citrate solution and processed for islet isolation using intraductal collagenase digestion [37]. The islets were purified using continuous Ficoll density-gradient centrifugation [38], and were then washed in University of Wisconsin solution at 4 °C [39] and left in this solution overnight.

RIL were obtained by collagenase digestion of the rat pancreas [40]. The isolated islets were washed three times in 1.28 mM $CaCl_2$/0.82 mM $MgSO_4$/5.5 mM KCl/0.35 mM K_2HPO_4/142 mM NaCl/0.4 mM NaH_2PO_4/5.5 mM D-glucose/20 mM Hepes/0.5 % (w/v) BSA, pH 7.4 (HBSS buffer) and selected under a dissecting microscope. For RNA preparation, all solutions were prepared using diethyl pyrocarbonate- (DEPC) treated water.

RNA preparation

Total RNA from RIN and HIL was prepared as described previously [41]. Total RNA was prepared from 100 normal or diabetic RIL by addition of 100 μl TRI-Reagent according to the manufacturer's protocol and the final RNA pellet was dissolved in 20 μl DEPC-treated water.

Reverse transcription and PCR

The total RNA (5 μl of RIL RNA or 5 μg of RIN or HIL RNA) was reverse-transcribed at 42 °C for 60 min in 50 μl of reaction mixture containing 1 × reverse transcriptase buffer (50 mM Tris/HCl, pH 8.3 containing 40 mM KCl, 1 mM dithiothreitol and 6 mM $MgCl_2$) containing 100 pmol of each 3′-end primer, 0.5 mM of each dNTP, 10 mM dithiothreitol, 40 units rRNasin (Promega) and 500 units M-MLV reverse transcriptase (Gibco BRL, Uxbridge, Middlesex, U.K.).

One-twentieth of the cDNA was subjected to a PCR in 50 μl reaction mixture containing 1 × PCR buffer (50 mM KCl/10 mM Tris/HCl, pH 9.0, containing 5 mM dithiothreitol), which contained 2 or 4 mM $MgCl_2$ (for SERCA 3- and SERCA 2-specific primers, respectively), 0.1 mM of each dNTP, 20 pmol of each primer and 0.5 unit of *Taq* DNA polymerase (Promega). PCR was carried out in a programmable thermal controller (PTC-100, MJ Research Inc., Essex, U.K) at 95 °C for 2 min, followed by 30 cycles (except in the experiment in which the exponential phase was determined) at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 10 min. After 30 cycles, the samples were concentrated by vacuum centrifugation and subjected to electrophoresis in a 1.9 % (w/v) agarose gel containing 0.4 μg ethidium bromide/ml in 1 × TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA). For DNA sequencing and restriction enzyme digestion, 500 μl PCR samples were precipitated with ethanol and then fractionated by electrophoresis in 1.3 % (w/v) low-melting-point agarose gels. The separated bands were isolated and the PCR products were extracted from gel slices by Wizard PCR preps DNA purification system (Promega).

Specific primer pairs (1/2, 1/3 and 1/4) for rat SERCA were used to detect the different isoform-specific mRNAs (Table 1). The sequences were derived from data published previously [13,14]. Primer pair 1/2 is specific for all SERCA isoforms in both rat and human. This primer pair was selected so that it completely matched the SERCA 2 and 3 sequences. Therefore the efficiency of the primers for these isoforms was equal; this enabled us to determine the ratio of SERCA 2 to SERCA 3 mRNA and also to detect simultaneous expression in HIL by restriction digestion at specific sites [20]. Primer pairs 1/3 and 1/4 are specific for rat SERCA 2 (including both SERCA 2a and SERCA 2b alternatively spliced forms) and SERCA 3 isoforms respectively.

All measurements were repeated at least three times. The following controls were used to check for possible amplification of contaminant DNA and RNA by PCR: RNA blanks taken through the cDNA synthesis step in the absence of reverse transcriptase were used in every PCR reaction and for each set of primers; samples without templates were run for every primer pair for each PCR experiment.

Sequencing of PCR products

Each non-cloned RT-PCR product was completely sequenced using the Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, OH, U.S.A.). Purified PCR products (1.5–3.0 pmol) were denatured at 95 °C for 3 min in reaction mixture containing a 10-fold molar excess of sequencing primers, and 1 × Sequenase buffer (40 mM Tris/HCl, pH 7.5, containing 20 mM $MgCl_2$ and 50 mM NaCl). The sequencing primers were the original primers used to produce the PCR products. The reaction mixture

Table 1 Position and sequences of PCR primers used

The primers were based on rat β-actin gene [54] and rat SERCA cDNA sequences (SERCA 2 [13]; SERCA 3 [14]). The numbers specify the 5′ position of primer sequence on the SERCA 2 and SERCA 3 (labelled with *) cDNA according to EMBL/GenBank database accession numbers X15635 and M30581 respectively.

Gene specificity	Direction	Sequence	Starting base	Length of PCR products	Name of primer
SERCA 1, 2 and 3	Forward	5′TGCCTGGTAGAGAAGATGAA3′	1545 (*1469)		1
	Reverse	5′CCCTTCACAAACATCTTGCT3′	1752 (*1679)	206 bp 209 bp	2
SERCA 2	Reverse	5′CTGTTTAACACCAGGCGTCA3′	1833	288 bp	3
SERCA 3	Reverse	5′CTCTCTGGAGGTGGCGCTTA3′	*1760	291 bp	4
β-Actin	Forward	5′ATCCGTAAAGACCTCTATGC3′	2746	255 bp	β-Actin
	Reverse	5′ATTTGCGGTGCACGATGGAGG3′	3126		

containing the purified PCR products was kept on ice for 10 min, at room temperature for a further 20 min, and then sequenced by the dideoxynucleotide chain termination method according to the manufacturer's protocol. All sequencing products were separated on 6% (w/v) polyacrylamide gels using SequaGel-6 sequencing gel solutions (National Diagnostic Inc., Hessle, Yorks., U.K.).

Determination of the exponential phase of RT-PCR

The kinetics of PCR were determined for 1/3 and 1/4 SERCA 2 and SERCA 3 specific primer pairs respectively. PCR of RIL RNA was performed in triplicate (50 μl volume) in the presence of 0.15 μCi of [α-^{32}P]dCTP with these primer pairs. After 15, 20, 25, 30, 35 and 40 cycles the reaction samples were concentrated by vacuum centrifugation and loaded on to a 1.9% (w/v) agarose gel. Bands were visualized under UV light, cut out, melted at 100 °C in 1 ml of double-distilled water and then transferred to scintillation vials and the radioactivity was measured after the addition of 5 ml Liquiscint (National Diagnostics, Manville, NJ, U.S.A.). The radioactivity recovered from the excised bands was plotted as a function of the number of cycles. Because of the low amount of SERCA template molecules in the tissue, the reaction was still in the exponential phase in the interval between 25 and 35 cycles with both primer pairs.

Determination of the optimal primer concentration for quantification of β-actin transcripts

For quantification of PCR products, co-amplification experiments were performed using primers for SERCA and a primer pair for β-actin (Table 1) as internal standard. To prevent the amplification reaction of β-actin from reaching the plateau phase under the standardized conditions (30 cycles), the optimal concentration of the primers for β-actin was determined. RT-PCR of RIL RNA was performed as described above in the presence of 0.15 μCi of [α-^{32}P]dCTP with the primer pair for β-actin at different concentrations (1, 2, 3, 4, 5, 7.5, 10 and 12.5 pmol/50 μl sample). The relative amount of amplified DNA was determined as described above and the radioactivity measured was plotted against the primer concentration. Saturation of the reaction was reached at primer concentrations greater than 4 pmol at 2 mM $MgCl_2$ and 5 pmol at 4 mM $MgCl_2$ in 50 μl of reaction medium. Therefore 3.5 and 2.5 pmol of β-actin primers were used for quantification of SERCA 3 and SERCA 2 respectively.

Quantification of PCR products of SERCA isoforms in normal and diabetic RIL

In PCR co-amplification experiments, 0.15 μCi of [α-^{32}P]dCTP and primers for either SERCA 2 (20 pmol) and β-actin (3.5 pmol) or SERCA 3 (20 pmol) and β-actin (2.5 pmol) were included in 50 μl of reaction medium. All initial PCR reactions were carried out at 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 10 min. The samples were concentrated and separated on 1.9 % (w/v) agarose gels and the radioactivity of the bands was counted. The amount of amplified products from either SERCA was divided by that from the actin internal standard. A single sample of normal Wistar RIL mRNA was used as a reference control for each quantitative PCR reaction. Values for the SERCA/β-actin mRNA ratio for each sample were expressed relative to the reference control values for each isoform in the same experiment. Three samples were obtained in each experiment which was repeated three times and the standard deviation was determined. Student's t test was used to evaluate the significance of differences in isoform mRNA abundance in normal and diabetic islets of Langerhans.

Immunoblot analysis of membrane samples

The membranes from RIN were prepared as described previously [25]. SDS/PAGE on 1 mm 9% (w/v) polyacrylamide gels was performed using the buffer system of Laemmli [42]. Proteins were transferred electrophoretically on to poly(vinylidene difluoride) microporous membrane (Immobilon, Millipore, Bedford, MA, U.S.A.) using an AE-6675 Horizoblot electrophoretic transfer unit with a discontinuous buffer system for 2 h at room temperature as recommended by the manufacturer (ATTO, Tokyo, Japan). The Immobilon sheets were blocked overnight at 4 °C with 5% (w/v) non-fat dry milk and 1:50 dilution of normal swine serum in 10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 (TBST). Immunostaining was performed for 12–16 h at 4 °C using either a SERCA-specific polyclonal rabbit antibody (EM-2, dilution 1:10000) or a SERCA 2b isoform-specific antipeptide polyclonal rabbit antibody (SERCA 2b, dilution 1:5000) diluted in blocking solution. After incubation the membranes were washed with 20 mM Tris/HCl, pH 7.5, containing 60 mM NaCl, 0.4% (w/v) Triton X 100, 0.4% (w/v) SDS, 0.4% (w/v) deoxycholate, 2 mM EDTA and TBST. The membranes were incubated with a

horseradish peroxidase-coupled anti-rabbit IgG (Dakopatts, Denmark) at a dilution of 1:1000 for 2 h at room temperature. Detection was by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, Bucks., U.K.) using the method recommended by the manufacturer. The membranes were stripped of all bound antibodies at 70 °C for 30 min in 62.5 mM Tris/HCl, pH 6.7, containing 100 mM 2-mercaptoethanol and 2% (w/v) SDS before re-probing.

RESULTS

Identification of SERCA isoform specific mRNAs

The expression of different SERCA mRNAs in HIL, RIL and RIN was determined by RT-PCR using 1/2, 1/3 or 1/4 primer pairs (Table 1). Primer pair 1/2 is specific for all known SERCA isoforms and its PCR amplification resulted in 206/209 bp fragment(s) in the samples analysed (Figure 1, lanes 1–6). For rat and human the predicted lengths of the amplified DNA fragments would be 206 bp for SERCA 2 and 209 bp for SERCA 3. Because of the broad specificity of this primer we tested the resulting PCR fragment to see if it was an amplified product of one, two or more SERCA isoforms. For this approach, restriction endonuclease digestion was used. Because of the sequence variation of SERCA isoforms between the different animal species, distinct restriction enzymes were applied for cutting human (*Sty*I, *Xba*I, *Pst*I and *Rsa*I) (Figure 2A, lanes 1–6) and rat (*Bam*HI, *Bsa*HI, *Sty*I and *Eco*RI) (Figure 2B, lanes 7–12) SERCA isoforms [20]. Digesting the PCR-amplified human SERCA fragments with *Xba*I converted the 206 bp SERCA 2 product into 114 bp and 92 bp fragments leaving the 209 bp fragment undigested (Figure 2A, lane 2). Digestion with *Pst*I reduced the 209 bp SERCA 3 product into a 169 bp fragment, leaving the 206 bp SERCA 2 fragment unaffected (Figure 2A, lane 3). When a combination of the above enzymes was applied, the 206/209 bp

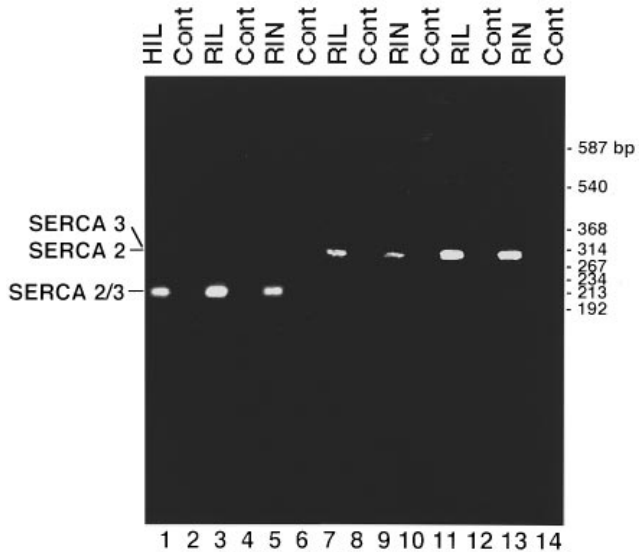

Figure 1 PCR amplification of SERCA isoforms in HIL, RIL and RIN

Detection in HIL, RIL and RIN of mRNAs for SERCA isoforms was performed by RT-PCR as described in the Experimental section. The products of the PCR reactions were separated on a 1.9 % (w/v) agarose gel and stained with ethidium bromide. Primer pair 1/2 was chosen to co-amplify SERCA 2 and 3 simultaneously (lanes 1–6); primers 1/3 and 1/4 are specific for SERCA 2 (lanes 7–10) and SERCA 3 (lanes 11–14), respectively. The 206/209 bp fragments correspond to SERCA 2/3 (lanes 1, 3 and 5), the 288 bp band represents SERCA 2 (lanes 7 and 9) and SERCA 3 yielded a product of 291 bp (lanes 11 and 13). Cont: control (PCR reaction without template) is shown in lanes 2, 4, 6, 8, 10, 12 and 14. Markers: 587, 540, 368, 314, 267, 234, 213, 192 bp.

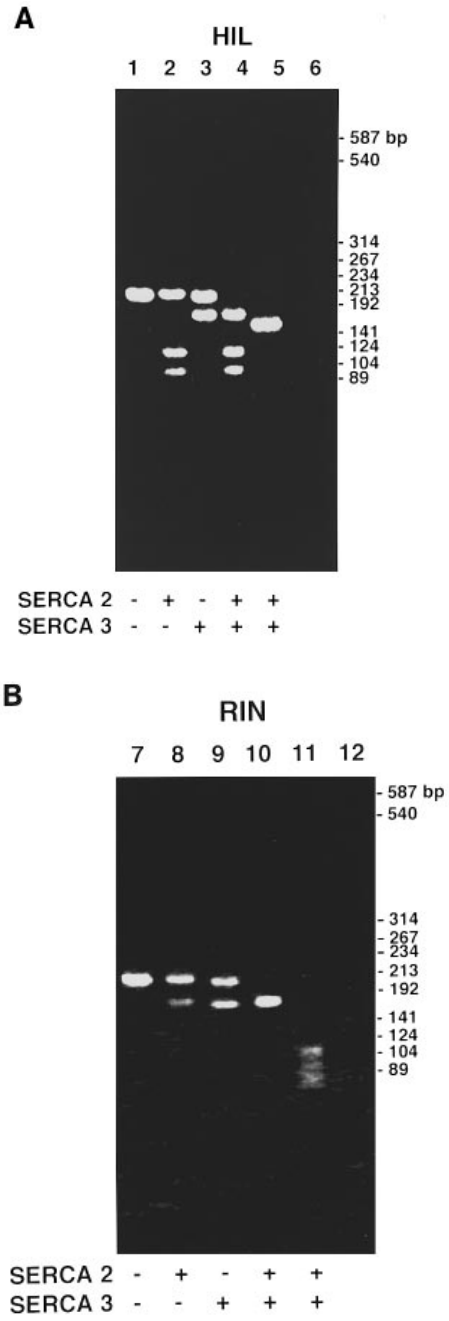

Figure 2 PCR amplification by primer pair 1/2 of SERCA 2 and SERCA 3 fragments from HIL (A) and RIN (B)

Primer 1/2 co-amplified SERCA 2 and SERCA 3 simultaneously from HIL (**A**, lanes 1–6) and RINm5F (RIN) β-cells (**B**, lanes 7–12). The products were separated by agarose gel electrophoresis and stained with ethidium bromide. The Figure shows the combined SERCA 2/3 products (lanes 1 and 7); the SERCA 2-specific fragments obtained after digestion with *Xba*I (lane 2) or *Bsa*HI (lane 8); the SERCA 3-specific fragments obtained after digestion with *Pst*I (lane 3) or *Sty*I (lane 9); the combination of digestion with *Xba*I + *Pst*I (lane 4) or with *Bsa*HI + *Sty*I (lane 10); simultaneous digestion of SERCA 2/3 products with *Rsa*I (lane 5) or *Eco*RI (lane 11). Controls (PCR without template) (lanes 6 and 12). Markers: 587, 540, 314, 267, 234, 213, 192, 141, 124, 104, 89 bp.

fragment was completely digested into 169, 114 and 92 bp fragments (Figure 2A, lane 4). *Rsa*I digested both SERCA isoforms resulting in 155/152 bp fragments (Figure 2A, lane 5). Digestion of the rat PCR fragments by *Bsa*HI converted SERCA 2 product into a 177 bp fragment leaving the 209 bp SERCA 3 undigested (Figure 2B, lane 8). *Sty*I reduced the SERCA 3 product into a 176 bp fragment, leaving SERCA 2 unaffected (Figure 2B, lane 9).

The expression of SERCA 2 and 3 was further confirmed using 1/3 or 1/4 primer pairs for RT-PCR. In rat samples the amplification resulted in products of 288 bp for SERCA 2 (Figure 1, lanes 7 and 9) and 291 bp for SERCA 3 (Figure 1, lanes 11 and 13). The non-cloned 288 bp and 291 bp fragments were completely sequenced using 1/2 and 1/3 or 1/2 and 1/4 primers respectively. The sequence obtained from each PCR product was identical to the published sequence of rat SERCA 2 and 3 cDNAs. The human SERCA 3 is only partially sequenced. Therefore it was not possible to detect SERCA 2 and 3 separately in human samples.

The efficiency of primer pair 1/2 is equal for SERCA 2 and SERCA 3 since the primer sequences are identical in both isoforms. This enabled the determination of the ratio of SERCA 2 to SERCA 3 messages, which was calculated from the intensity of enzyme-digested fragments. About 50 % of the amplified DNA was cut by the restriction enzyme *Xba*I (or *Bsa*HI in rat samples) and is thereby characterized as a SERCA 2 DNA fragment. The remaining 50 % is SERCA 3 since all the DNA product not digested by *Xba*I (or *Bsa*HI) was digested by *Pst*I (or *Sty*I in rat samples), yielding a fragment of the predicted length and in the predicted quantity (results not shown).

These data suggest that in HIL, RIL and RIN both SERCA 2 and 3 are expressed simultaneously in comparable amounts and there is no other known SERCA form present.

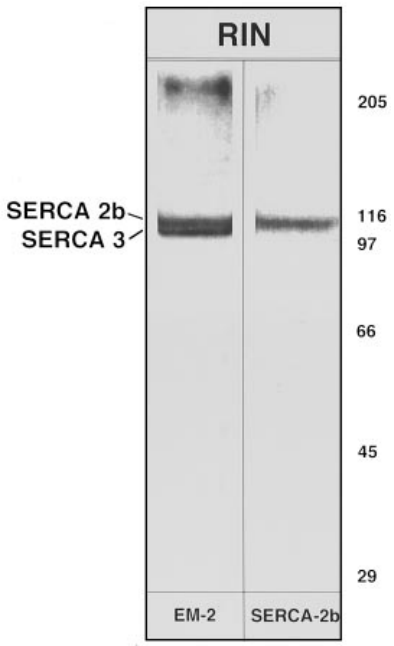

Figure 3 Immunochemical detection of SERCA proteins in RIN

Membrane proteins (100 μg) from RIN were separated by SDS/PAGE on 9 % Laemmli gels. The gels were electroblotted on to Immobilon membranes and incubated with a rabbit anti-rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase antibody at 1:10000 dilution (EM-2) and a rabbit anti-peptide antibody, raised against the 12 C-terminal amino acids of SERCA 2b Ca^{2+}-ATPase at 1:5000 dilution (SERCA 2b). The bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit IgG and visualized by ECL. The positions and sizes (kDa) of the molecular-mass standards are indicated on the right.

Detection of SERCA proteins

Translation of different SERCA proteins in RIN was studied by a SERCA-specific antibody, EM-2, which recognizes all known isoforms of SERCA [25,29–33]. Immunoblotting of RIN membranes with EM-2 labelled two distinct bands of 110 and 115 kDa molecular-mass (Figure 3, lane 1). When the same immunoblot was re-probed with SERCA 2b antibody only the upper 115 kDa band was labelled (Figure 3, lane 2). This antibody was raised against the 12 C-terminal amino acids of the SERCA 2b isoform and it does not cross-react with SERCA 1 or 3 [25–28]. The 115 kDa protein was previously demonstrated to correspond to the SERCA 2b isoform by autophosphorylation and immunoblotting [25]. We strongly suggest that the 110 kDa protein is SERCA 3 because the expression of the SERCA 1 and 2a isoforms is restricted to muscle tissues; β-cells express mRNAs for SERCA 2 and 3 forms exclusively; SERCA 3 protein migrates with lower molecular mass (110 kDa) than SERCA 2b [20], which coincides with our observation.

Quantification of SERCA 2 and 3 mRNA expression in normal Wistar and GK RIL

In order to investigate whether SERCA 2 or 3 mRNA levels alter in GK RIL, it was first necessary to assess the variability in their expression in the normal Wistar RIL population. Five separate batches of 100 islets of Langerhans were obtained, each from one normal Wistar rat. From each batch of islets, total mRNA was prepared and used for RT-PCR. To make sure that any differences observed were not due to the presence of different amounts of total mRNA we devised co-amplification experiments with SERCA 2 (or SERCA 3) and β-actin mRNAs, using the latter as an internal control. SERCA 2 and 3 were amplified by 1/3 and 1/4 primer pairs respectively. Each batch of islets was assayed in triplicate in three separate PCR experiments. The overall ratio of SERCA mRNA to β-actin mRNA was 0.29 ± 0.03 and 0.87 ± 0.12 (means $\pm$ S.D.) for SERCA 2 and SERCA 3 respectively. This difference in the SERCA/β-actin mRNA ratio for the two isoforms is attributable to the different primer efficiency and the greater (C + G) content of SERCA 3. The values obtained in this way for expression of both SERCA isoforms were highly reproducible both between batches of islets and between individual PCR amplifications. There was no significant difference in the expression of any of the SERCA forms between any of the islet batches obtained from normal Wistar rats. Therefore, for quantitative comparative PCR experiments a single sample of normal Wistar RIL mRNA was used as a reference control for each PCR reaction. Values of SERCA/β-actin mRNA for each sample were expressed relative to the reference control values for each isoform in the same experiment. Primer pair 1/3 was used for the amplification of SERCA 2 isoform in GK and control samples. For amplification of SERCA 3 isoform, primer pair 1/4 was used. Table 2 shows the results for SERCA isoform expression in normal Wistar and GK RILs.

The relative expression of SERCA 2 mRNA was 0.95 ± 0.09 ($n = 5$) in the control and 0.80 ± 0.15 ($n = 4$) in GK islets. This difference was not significant ($P > 0.05$). The relative expression of SERCA 3 mRNA was 1.10 ± 0.15 ($n = 5$) and 0.35 ± 0.11 ($n = 4$) in the control and GK samples respectively. SERCA 3 isoform-specific mRNA levels were significantly lower in GK

Table 2 Relative amount of SERCA 2 and 3 mRNAs in GK and normal Wistar RIL

Primer pairs 1/3 (SERCA 2), 1/4 (SERCA 3) and β-actin were used in the presence of [α-^{32}P]dCTP to co-amplify the two different SERCA isoforms and β-actin in normal Wistar (N 1–N 5) and GK (GK 1–GK 4) RIL. The radioactivity of the amplified products was measured and the amounts of SERCA were divided by the amounts of the β-actin internal standard. The mean value obtained in the control reference sample included in each PCR experiment was set to 1.0, and all other values obtained in diabetic (GK) and normal (N) samples were expressed relative to this value. Results are presented as means ± S.D. For the individual and the reference control values, n = the number of triplicate RT-PCR experiments. For the average values n = the number of experimental animals. *$P > 0.05$; †$P < 0.001$

	Relative amount of RIL mRNA		
Rats	SERCA 2	SERCA 3	n
Reference control	1.00 ± 0.07	1.00 ± 0.10	15
N 1	0.89 ± 0.01	1.17 ± 0.05	3
N 2	1.04 ± 0.05	1.22 ± 0.11	3
N 3	0.89 ± 0.07	0.98 ± 0.07	3
N 4	1.05 ± 0.06	0.90 ± 0.06	3
N 5	0.91 ± 0.03	1.18 ± 0.09	3
N (average)	0.95 ± 0.09	1.10 ± 0.15	5
GK 1	0.68 ± 0.15	0.41 ± 0.07	11
GK 2	0.68 ± 0.12	0.48 ± 0.11	11
GK 3	0.85 ± 0.08	0.24 ± 0.02	4
GK 4	1.00 ± 0.10	0.27 ± 0.00	4
GK (average)	0.80 ± 0.15*	0.35 ± 0.11†	4

than in control islets ($P < 0.001$).Thus there is a marked (about 68 %) and specific decrease in SERCA 3 expression in GK RIL.

DISCUSSION

We found that HIL, RIL and RIN co-express mRNAs for SERCA 2b and SERCA 3 isoforms. SERCA 2b isoform is expressed ubiquitously and probably represents the normal 'housekeeping' SERCA pump [14,43,44]. The tissue and cellular distribution of SERCA 3 in cerebellar Purkinje cells, secretory epithelial cells, lymphocytes and endothelial cells [24,44,45] reveals no simple or single correlation with a specific biological or cellular function. However, SERCA 3 seems to be expressed selectively in cells, including β-cells, in which Ca^{2+} signalling plays a critical and sensitive role in regulating physiological processes.

The expression of SERCA isoforms in COS cells has revealed clear functional differences between SERCA 2b and SERCA 3 [20]. The latter showed an altered pH optimum and an unusually low apparent Ca^{2+} affinity ($K_{0.5}$ of 1.1 μM versus 0.4 μM for SERCA 2b). It is not clear why these functionally different SERCA isoforms co-exist in a few cell types. One possibility is that the two isoforms may be located in functionally distinct Ca^{2+} storage pools of the cell. In support of this hypothesis, morphological and physiological studies in Purkinje cells, which co-express SERCA 2b and SERCA 3, have defined spatially distinct Ca^{2+} stores. It has also been shown that in platelets these SERCA isoforms are indeed located in distinct Ca^{2+} storage pools [46]. This could be the case in β-cells. It is possible also that SERCA 3 is specifically modulated by regulatory factors involved in Ca^{2+} signalling which could underlie its selective expression. SERCA 3 could play an important role in regulation of $[Ca^{2+}]_i$ in islets because they express SERCA 3 to at least the same level as the housekeeping SERCA 2b isoform. There are just a few cell types (platelets, B- and T-lymphocytes, mast cells, arterial endothelial cells) in which the amount of SERCA 3 mRNA is comparable with SERCA 2b [24]. In all these cells the SERCA 3 transcript resulted in a functional protein with a slightly higher electrophoretic mobility than the SERCA 2b isoform [20]. Our immunochemical-analysis data show that in β-cells, along with SERCA 2b protein there is another organellar Ca^{2+} pump present which most likely corresponds to SERCA 3. However, in different non-muscle cells the existence of a not-yet-characterized novel Ca^{2+}-transport ATPase was proposed [24,47,48], which probably co-migrates with SERCA 3 which could further complicate the quantification of SERCA 3 to SERCA 2b at the protein level.

We found a significant reduction of SERCA 3 mRNA levels in islets prepared from GK rats. Since SERCA 2 expression showed only a minor, non-significant decrease, SERCA genes are independently regulated and probably play distinct roles in the islets of Langerhans. In view of the central importance of intracellular Ca^{2+} for control of insulin secretion, altered expression of SERCA genes could be involved in the reduced insulin secretory response to glucose, which is a major pathological defect in NIDDM. Studies in animal models [5,11,34, 35,49–53] indicate that abnormal glucose-stimulated insulin release may result from defects at several sites, including glucose metabolism, ionic fluxes and Ca^{2+} action. In GK rats several metabolic abnormalities have been documented [34,35,52,53]. In this animal model it has not yet been clarified whether Ca^{2+} handling and events subsequent to elevation of $[Ca^{2+}]_i$ are also defective. However, significant reduction of L-type Ca^{2+} channel α1-subunit mRNA levels was observed in Zucker diabetic obese rats [11] and glucose-stimulated intracellular Ca^{2+} mobilization is decreased in diabetic Chinese hamsters [50] and in *db/db* mice [5,51]. Recently, abnormal glucose-induced intracellular Ca^{2+} signalling in islets isolated from *db/db* mice was found to be because of reduction of thapsigargin-sensitive SERCA activity [5]. Our finding that SERCA 3 gene-expression is down-regulated in GK islets supports the view that molecular defect(s) in Ca^{2+} signalling may be a component of NIDDM.

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