

NCX1 Na/Ca exchanger splice variants in pancreatic islet cells

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Abstract

In the rat pancreatic β -cell, Na/Ca exchange displays a quite high capacity. The cell is equipped with two alternatively spliced Na/Ca exchanger-1 (NCX1) isoforms, namely NCX1.3 and NCX1.7. To examine the existence of a possible functional difference between these splice variants, they were cloned, together with the heart variant NCX1.1, and expressed in human embryonic kidney-293 (HEK293) and Chinese hamster ovary (CHO) cells. In these two systems, the three splice variants showed a comparable level of intracellular Na^+ ($[\text{Na}^+]_i$)-dependent extracellular Ca^{2+} (Ca^{2+}_o) uptake. Different

levels of NCX1.3 and NCX1.7 transcripts were found in four rodent species, together with a marked inter-species difference in Na/Ca exchange activity. Three additional splice variants were found (NCX1.2, NCX1.9 and NCX1.13) in guinea-pigs, hamsters and mice, again in different proportions. Our data provide evidence for the activity of the three NCX1 splice variants. They also show the existence of a differential and species-specific transcription pattern of NCX1 gene products in pancreatic islet cells.

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Introduction

The Na/Ca exchanger (NCX) is an electrogenic transporter, located at the plasma membrane, that couples the exchange of Na^+ and Ca^{2+} with a stoichiometry of 3 Na^+ for 1 Ca^{2+} (Eisner & Lederer 1985). In the heart, Na/Ca exchange is the predominant mechanism for ^{45}Ca extrusion, being able to restore and control the basal Ca^{2+} level on a beat-to-beat basis (Bers 1991). The exchanger may also reverse during the heart cycle and hence allow Ca^{2+} entry during systole (Levesque *et al.* 1994). Na/Ca exchange is also present in a variety of other tissues, including neurons and kidney. In neurons, Na/Ca exchange may play a role in Ca^{2+} extrusion and mediate net Ca^{2+} influx (Blaustein *et al.* 1996). In the kidney, Na/Ca exchange participates in the active reabsorption of Ca^{2+} in the distal nephron (Lytton *et al.* 1996).

Three mammalian isoforms of the NCX have been cloned: NCX1 (Nicoll *et al.* 1990), NCX2 (Li *et al.* 1994) and NCX3 (Nicoll *et al.* 1996), representing the products of three distinct genes. Two non-mammalian NCX isoforms have also been cloned from *Drosophila* (Schwarz & Benzer 1997) and from squid (He *et al.* 1996). Whilst NCX1 is widely distributed in various tissues, NCX2 and NCX3 seem to be restricted to the brain and skeletal muscle (Li *et al.* 1994, Nicoll *et al.* 1996). NCX1, initially cloned in dog heart, has an open reading frame of 970 amino acids, of which a 32-residue NH_2 -terminal seg-

ment corresponds to a cleaved signal peptide (Nicoll *et al.* 1990). NCX1 contains 11 putative transmembrane segments, five upstream and six downstream of the large cytoplasmic loop (Nicoll *et al.* 1990). Alternative splicing in this cytoplasmic loop generates tissue-specific variants of NCX1 displaying high homology ($\geq 90\%$). Sequence analysis of the intron–exon boundaries in this region revealed the presence of two ‘mutually exclusive’ exons (A and B) and four ‘cassette’ exons (C, D, E and F) (Kofuji *et al.* 1994, Lee *et al.* 1994). In the heart, only one NCX1 splice variant appears to be present (NCX1.1), whereas in other tissues (e.g. kidney, brain, aorta, β -cells) two or even three (eye tissue) variants are expressed (Kofuji *et al.* 1994, Lee *et al.* 1994, Van Eylen *et al.* 1997). To date, the reason for the presence of two or more NCX1 exchanger splice variants in one single tissue is unknown. Likewise, study of the expression pattern of the splice variants among different tissues has started only recently (Van Eylen *et al.* 1997).

The existence of a process of Na/Ca exchange in the pancreatic β -cell was suggested about 20 years ago (Hellman *et al.* 1980, Herchuelz *et al.* 1980), but the process was characterized only recently (Plasman *et al.* 1990, Plasman & Herchuelz 1992). In the rat pancreatic β -cell, Na/Ca exchange displays quite a high capacity (Plasman *et al.* 1990) and participates in the control of the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and in the control of insulin release (Plasman *et al.* 1990, Van Eylen *et al.* 1994). The rat pancreatic β -cell is equipped with two

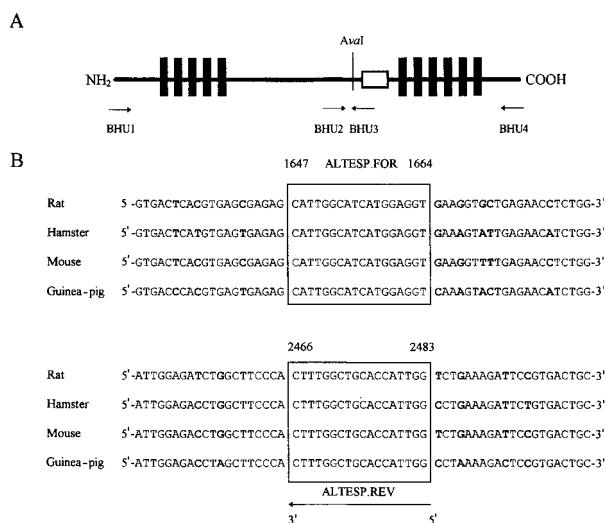


Figure 1 (A) Cloning strategy of human heart NCX1.1 and pancreatic β -cell NCX1.3 and NCX1.7. Putative transmembrane segments 1–11 are indicated by black bars and the putative splicing area by a white box. The primer pairs BHU1/BHU3 and BHU2/BHU4 (arrows) were designed to amplify NCX1.1, NCX1.3 and NCX1.7 cDNAs into two fragments overlapping the unique *AvaI* restriction site of NCX1. (B) Nucleotide sequences of parts of pancreatic islet NCX1 cDNAs from the rat, the hamster, the mouse and the guinea-pig. Numbering is according to the rat heart cDNA sequence (accession number X68191). PCR primers used in the experiments are framed. The two primer regions ALTESP.FOR and ALTESP.REV are 100% conserved in the four species. Non-conserved bases are shown in bold.

NCX1 splice variants (NCX1.3 and NCX1.7), but does not express NCX2 (Van Eylen *et al.* 1997). Recently, we observed that differences in the expression pattern of Na/Ca exchange splice variants (NCX1.3 and NCX1.7) in pancreatic β -cells and an insulinoma cell line could be correlated with differences in Na/Ca exchange activity in these cells (Van Eylen *et al.* 1997). The aim of the present study was to characterize the possible functional differences between NCX1.3 and NCX1.7 compared with the cardiac splice variant NCX1.1. The results provide evidence of the activity of the three NCX1 splice variants. They also show the existence of a differential and species-specific transcription pattern of NCX1 splice variants in islet cells, which may explain, in part, the difference in Na/Ca exchange activity seen in these species.

Materials and Methods

Design of polymerase chain reaction (PCR) primers

Cloning of human pancreatic islet NCX1.3 and NCX1.7 and right-ventricle NCX1.1 Primers were designed to amplify NCX1 cDNAs into two fragments overlapping the unique restriction site *AvaI* (Fig. 1A). On the basis of the human cardiac cDNA sequence (accession

number M91368), the sense primers BHU1 5'-GTCATGTACAACATGCGGCG-3' and BHU2 5'-CATCATGGAGGTGAAAGTATTG-3' correspond to nucleotides -3-17 and 1662-1683 respectively, and the antisense primers BHU3 5'-CCCCTCCACCTCTG GCAG-3' and BHU4 5'-CCTTTAGAAGCCTTTTA TGTGG-3' correspond to nucleotides 1739-1756 and 2905-2926 respectively. The nucleotides were synthesized using the phosphoramidite method performed on an Applied Biosystems 394 synthesizer (Perkin Elmer, Zaventem, Belgium).

Quantitative analysis of NCX1 splice variants The sense primer 5'-ACCCAGAAGGAAATCAGAG-3' and the antisense primer 5'-GGAGAAGAAAATGTACA AGAGCC-3' corresponding to nucleotides 1402-1420 and 2858-2880 respectively and largely encompassing the putative splicing area of NCX1 cDNA were designed on the basis of the rat cardiac cDNA sequence (accession number X68191). PCR amplification and sequencing of the obtained PCR products allowed us to synthesize two nested primers common to the rat, hamster, mouse and guinea-pig islet cell NCX1 cDNAs, around the splicing zone (Fig. 1B). The following primers were used for the identification and quantitative analysis of NCX1 splice variants in the islet cells of four mammals: the sense primer ALTESP.FOR (5'-CATTGGCATCATGGAGGT-3') and the antisense primer ALTESP.REV (5'-CCAATGG TGCAGCCAAAG-3') corresponding to nucleotides 1647-1664 and 2466-2483 respectively. To make the distinction between the guinea-pig islet splice variants NCX1.3, NCX1.9 and NCX1.13, the following additional primers were used for the quantitative analysis: the sense primer GUIALT.FOR (5'-GCTCGAATTCCAG AACGATG-3') corresponding to nucleotides 1772-1791, and the antisense primers GUIALT.REV (5'-CTGGT TAACGGCTGTTTGTGTC-3') and HAMALT.REV (5'-GTCAGTGGCTGCTTGTGTCATC-3') corresponding to nucleotides 2044-2063 and 2041-2060 respectively.

Polymerase chain reaction

Total RNA was isolated from guanidine-isothiocyanate-solubilized human insulinoma (kindly given by Dr J Rahier, Cliniques Universitaires St Luc, Brussels, Belgium), human right ventricle, and rat, mouse, hamster and guinea-pig islet cells by centrifugation on a cesium chloride gradient, as described by Sambrook *et al.* (1989). RNA (1 μ g) was reverse-transcribed for 20 min at 42 °C and for 40 min at 37 °C with 200 U Superscript II (Gibco BRL, Merelbeke, Belgium), using random primers (Promega, Leiden, The Netherlands) and triphosphate nucleosides (Boehringer Mannheim, Brussels, Belgium) in the buffer supplied by the manufacturer, in a total volume of 20 μ l. The medium was then diluted with 30 μ l 16 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0) and the

reaction was terminated by heating it up to 99 °C for 5 min. For the quantitative analysis of NCX1 splice variants, 1 μ l single-strand cDNA was amplified by the PCR in a 20 μ l volume, using the GoldStar DNA polymerase kit (Eurogentec SA, Seraing, Belgium) with dATP, dCTP, dGTP and dTTP (200 μ M each) (Boehringer, Mannheim), 10 pmol each primer and 0.5 U GoldStar DNA polymerase. The amplification was conducted in a thermal cycler (GeneAmp PCR system 2400; Perkin Elmer) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 36 (or 31, 30, 29) cycles at 94 °C, 54 °C (or 60 °C with the primers GUIALT.FOR/REV and 62 °C or 64 °C with GUIALT.FOR/HAMALT.REV) and 72 °C (1 min each), and a final extension at 72 °C for 5 min. Products were size-fractionated by electrophoresis in a 1% or 3% agarose gel and visualized by ethidium bromide staining.

For the cloning of human NCX1.3, NCX1.7 and NCX1.1, 1.5 μ l single-strand cDNA was amplified by PCR in a 20 μ l volume, using the Expand Long Template PCR system (Boehringer Mannheim) with dATP, dCTP, dGTP and dTTP (1 mM each; Boehringer Mannheim), 20 pmol each primer and 2 U Expand polymerase. The amplification was conducted in a thermal cycler (GeneAmp PCR system 2400; Perkin Elmer) under the following conditions: 94 °C 2 min (initial melt); 10 cycles of 94 °C for 15 s, 58 °C for 30 s, 68 °C for 4 min; 25 cycles of 94 °C for 15 s, 58 °C for 30 s, 68 °C for 4 min increased by 20 s at each cycle; and then 68 °C for 7 min (final extension). Products were size-fractionated by electrophoresis in a 0.7% agarose gel and visualized by ethidium bromide staining.

Cloning and sequencing of PCR products

PCR products were subcloned into the TA-cloning plasmid vector pCRII (Invitrogen Corp., San Diego, CA, USA) according to the manufacturer's protocol. DNA sequencing of selected clones was determined using cycle sequencing with AmpliTaq polymerase (Perkin Elmer).

Engineering of full-length clones

PCR amplification with the pair of primers BHU1/BHU3 yielded a fragment of 1759 bp common to the NCX1.3, NCX1.7 and NCX1.1 splice variants; the pair of primers BHU2/BHU4 yielded two fragments of 1224 bp and 1155 bp in pancreatic islets and one fragment of 1264 bp in heart, containing the NCX1 splice region of NCX1.7, NCX1.3 and NCX1.1 respectively. Human full-length cDNA clones NCX1.7, NCX1.3 and NCX1.1 were constructed by digestion and ligation of two partially overlapping clones. The 5' 1759 bp NotI-AvaI- (Gibco BRL) digested fragment, common to the NCX1.7, NCX1.3 and NCX1.1 splice variants, was ligated to 3' 1224 bp, 1155 bp or 1264 bp AvaI-BamHI-

(Gibco BRL) digested fragments containing the splice region of NCX1.7, NCX1.3 or NCX1.1. The three final full-length NCX1 fragments were then subcloned into the multicloning site of the pcDNA3(-) expression vector (Invitrogen) digested by NotI and BamHI. The positive clones were verified by restriction-enzyme mapping and sequencing.

Cell cultures and transient and stable transfection

Human embryonic kidney (HEK) 293 cells (ATCC CRL-1573) were cultured in Dulbecco's modified Eagle's medium (43%) and Ham-F12 (43%) media supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml fungizone, 10 μ g/ml gentamicin and 1 mM sodium pyruvate (Gibco BRL) in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were plated at 1.2×10^6 on a 100-mm-diameter plastic dish and incubated for 24 h prior to transfection. The HEK293 cells were then transfected transiently by the DNA/calcium phosphate coprecipitation procedure (Chen & Okayama 1988) with the mammalian expression vector pcDNA3(-) (Invitrogen Corp.) containing a 2.9 kb insert expressing human pancreatic β -cell NCX1.3 or NCX1.7 or human heart NCX1.1. Ten μ g DNA/dish and 5 μ g Green-Lantern fluorescent marker (Gibco BRL) were introduced simultaneously into the HEK293 cells. The Green-Lantern was used to determine the efficiency of transfection (as a percentage); the average was 60–80% as assessed by FACScan (Becton Dickinson, San Jose, CA, USA). For the fluorescence experiments, the controls were untransfected cells and cells transfected with 10 μ g pcDNA3(-) cat vector and 5 μ g Green-Lantern. Forty-eight hours later, cells were harvested by trypsinization and used for functional studies.

The electroporation method was used for stable transfection of Chinese hamster ovary (CHO) dhfr⁻ cells (4×10^6 cells, 330 V, 1000 μ F) with 20 μ g vector pcDNA3(-) containing a 2.9 kb insert expressing human NCX1.1, NCX1.3 or NCX1.7 (Electroporator II; Invitrogen Corp.). Transfected cells were selected and maintained in Ham-F12 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 500 μ g/ml geneticin (Gibco BRL). NCX1.1, NCX1.3 and NCX1.7 clones were each obtained from a single colony of transfected cells. Parental (control) CHO cells were maintained in Dulbecco's modified Eagle's medium without geneticin and were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and non-essential amino acids (Gibco BRL).

Preparation of HEK293 and CHO plasma membranes

Plasma membranes were isolated from control HEK293 and CHO cells as well as from cells transfected with

the expression vector pcDNA3(-) containing human NCX1.3, NCX1.7 or NCX1.1 cDNA, as previously described (Juhászová *et al.* 1994). In brief, cells were scraped and washed with chilled phosphate-buffered saline. After centrifugation, the pellet was resuspended in a 20 mM Tris, 1 mM EDTA and 250 mM sucrose solution containing additional protease inhibitors (phenylmethylsulfonyl fluoride, 500 μ M; *o*-phenanthroline, 1 mM; bacitracin, 200 μ g/ml; pepstatin, 1 μ g/ml; benzamide, 17 μ g/ml (Sigma-Aldrich SA, Bornem, Belgium); and leupeptin, 1 μ g/ml (Boehringer Mannheim)). Inhibitors were included in all solutions used to isolate membranes, and all preparative steps were carried out at 4 °C. The suspension was homogenized and centrifuged for 10 min at 1000 *g*. The supernatant containing the membranes was collected, centrifuged at 17 000 *g* for 20 min, aliquoted and then frozen.

Immunoblotting

Proteins from SDS-PAGE (7.5% gel) were transferred onto nitrocellulose for 120 min at 10 V. Immunoblotting was performed using monoclonal antibody (dilution 1:30 000) raised against the canine cardiac sarcolemmal NCX (R3F1; SWant, Bellinzona, Switzerland) and anti IgG (goat)–horseradish peroxidase-labeled antibodies (1:10 000; NEN Life Science Products). The blots were developed with Chemiluminescence Reagent Plus (NEN Life Science Products) and visualized by autoradiography on Kodak BioMax Light Film (NEN Life Science Products).

Quantitative comparison of PCR products

To determine the relative amounts of the different NCX1 gene products, the quantitative reverse-transcribed (RT)–PCR method was used (Van Eylen *et al.* 1997). Total RNA was serially diluted and amplified by RT-PCR. After amplification, the samples were analyzed on a 1% agarose gel (or 3% with the primers GUIALT.FOR/REV and GUIALT.FOR/HAMALT.REV) stained with ethidium bromide; the cDNA bands were quantified by scanning densitometry. The quantitative analysis of the NCX1 splice variants was realized using 1 μ g total RNA.

Cell preparations

Pancreatic islets were isolated by the collagenase technique (Lacy & Kostianovsky 1967) from the pancreases of Wistar rats, Gold hamsters, NMRI mice and Dunkin–Hartley albino guinea-pigs. The method used to isolate pancreatic islet cells has been described elsewhere (Gobbe & Herchuelz 1989).

⁴⁵Ca uptake

The medium used to incubate the islet, HEK293 and CHO cells consisted of a Krebs–Ringer Hepes-buffered solution (pH 7.4, 37 °C) of the following composition (mM): NaCl (135), CaCl₂ (1), MgCl₂ (1), Hepes/NaOH (10). The medium was gassed with ambient air. In some experiments, NaCl was iso-osmotically replaced by sucrose (241 mM; Merck, Darmstadt, Germany) or KCl, and Hepes/NaOH by Hepes/KOH. The different media also contained glucose (2.8 or 10 mM; Merck) and nifedipine (5 μ M; Calbiochem, La Jolla, USA). The method used for the measurement of ⁴⁵Ca uptake in islet or HEK293 cells has been described previously (Plasman *et al.* 1990). In brief, the cells were pre-incubated in 1 ml non-radioactive solution for 30 min and then incubated for various periods of time in 1 ml of the same medium containing, in addition, ⁴⁵Ca (10 μ Ci/ml). The (pre) incubation solutions contained, or did not contain, 1 mM ouabain to inhibit the Na⁺–K⁺ pump and raise the intracellular Na⁺ concentration. At the end of the incubation, the cells were separated from the incubation medium by using a combined lanthanum and oil technique (Plasman *et al.* 1990). Na/Ca exchange was evaluated by measuring intracellular Na⁺ (Na_i⁺)-dependent extracellular ⁴⁵Ca (⁴⁵Ca_o) uptake. After pre-incubation, the islet cells were exposed to Na⁺-depleted media containing ⁴⁵Ca. No measurable Na_i⁺-dependent ⁴⁵Ca_o uptake could be observed in non-transfected HEK293 or CHO cells.

Statistics

The results are expressed as means \pm s.e.m. The statistical significance of differences between data was assessed by using analysis of variance followed by the Tukey post test.

Results

Cloning and functional analysis of human Na/Ca splice variants

NCX1.3 and NCX1.7 cDNAs were isolated from a human insulinoma, whilst NCX1.1 was isolated from the right ventricle of a human heart, using the RT-PCR technique (see the Materials and Methods). The nucleotide and amino acid sequences of human NCX1.7 are shown in Fig. 2 (GenBank accession number AF108388). The sequences of NCX1.3 are identical to that of NCX1.7, except for the absence of a 23-amino-acid and

Figure 2 Nucleotide and amino acid sequences of the cloned human pancreatic β -cell Na/Ca exchanger splice variants. The nucleotide and amino acid sequences presented are those of NCX1.7. The sequences of NCX1.3 are identical to those of NCX1.7 except that a stretch of 23 amino acids and 69 nucleotides corresponding to exon F (in bold) is missing. The putative transmembrane regions predicted by hydropathy analysis are underlined.

1 ATGTACAACATGCGCGGATTAAGTCTTTCACCCACCTTTTCAATGGGATTTTCATCTGTTAGTTACTGTGAGTCTCTTATTTCCCATGTGGACCATGTAA
 1 M Y N M R R L S L S P T F S M G F H L L V T V S L L F S H V D H V I
 101 TTGCTGAGACAGAAATGGAAGGAGAAGAAATGAACTGGTGAATGACTGGATCATATTACTGTAAGAAAGGGGTGATTTGCCCATTTGGGAACCCCA
 35 A E T E M E G E G N E T G E C T G S Y Y C K K G V I L P I W E P Q
 201 AGACCCCTTCTTTGGGGACAAAATGCTAGAGCTACTGTGTATTTTGGGCCATGGTCTACATGTTTCTGGAGTCTCTATCATAGCTGATCGGTTTCATG
 68 D P S F G D K I A R A T V Y F V A M V Y M F L G V S I I A D R F M
 301 TCCTCTATAGAAGTCATCACATCTCAAGAAAAAATAACATAAAGAAACCAATGGAGAGACCACCAAGACAACCTGTGAGGATCTGGAATGAAACAG
 101 S S I E V I T S Q E K E I T I K K P N G E T T K T T V R I W N E T V
 401 TTTCTAACCTGACCTTGATGGCCCTGGGATCTTCTGCTCCTGAGATTTCTCCTTTTCAGTAATTGAAGTGTGTGGCCAACTTCTAGCGAGGACCTCGG
 135 S N L T L M A L G S S A P E I L L S V I E V C G H N F T A G D L G
 501 TCCTAGCACCATCGTGGGAAGTGTGCATTCAATATGTTTCATCATTTATGCACCTCTGTGTTTATGTGGTGCCTGACGGAGAGACAAGGAAGATTAAGCAT
 168 P S T I V G S A A F N M F I I I A L C V Y V V P D G E T R K I K H
 601 TTGCGTGTCTTCTTTGTGACAGCAGCCTGGAGCATCTTTCCTACACCTGGCTTTACATTATTTGTCTGTATATCTCCTGGTGTGTGGAGGCTCGG
 201 L R V F F V T A A W S I F A Y T W L Y I I L S V I S P G V V E V W E
 701 AAGGTTGCTTACTTCTTCTTCTTCCCATCTGTGTGTGTGCTTGGGTAGCGGATAGGAGACTTCTGTTTTACAAGTATGTTCTACAAGAGGTATCG
 235 G L L T L T F F F F P I C V V F A W V A D R R L L P Y K Y V Y K R Y R
 801 AGCTGGCAAGCAGAGGGGATGATTGAACATGAAGGAGACAGGCCATCTTCAAGACTGAAATGAAATGGACGGGAAAGTGGTCAATTTCTCATGTT
 268 A G K Q R E M I I E H E G D R P S S K T E I E M D G K V N T S H V
 901 GAAAATTTCTAGAGTGTCTGCTGCTGGTCTGGAGGTGGATGAGAGGGACCAAGATGATGAAGAAGCTAGGCGAGAAATGGCTAGGATTTCTGAGGAACCTTA
 301 E N F L D G A L V L E V D E R D Q D D E E A R R E M A R I L K E L K
 1001 AGCAGAAGCATCCAGATAAAGAAATAGAGCAATTAATAGAATTAGCTAACTACCAAGTCTTAAGTCAAGCAGCAAAAAGTAGAGCATTTTATCGCATTC
 335 Q K H P D K E I E Q L I E L A N Y Q V L S Q Q Q K S R A F Y R I Q
 1101 AGCTACTCGCCTCATGACTGGAGCTGGCAACATTTAAAGAGGCATCGACTGACCAAGCAAGGAAGGCTGTGACGATGCACGAGGTCAACACTGAAGTG
 368 A T R L M T G A N I L K R H A A D Q A R K A V S M H E V N T E V
 1201 ACTGAAAATGACCTGTAGTAAAGCTTCTTTGAACAAGGCATCATGCTGTGGAGAAGTGTGACTGTGGCCCTTACCATTATCCCGCAGAGGTG
 401 T E N D P V S K I F F E Q G T Y Q C L E N C G T V A L T I I R R G G
 1301 GTGATTTGACTAACACTGTGTTGTTGACTTCAAGACAGAGGATGGCACAGCAAAATGCTGGGTCTGATTATGAATTTACTGAAGAACTGTGGTGTAA
 435 D L T N T V F V D F R T E D G T A N A G S D Y E F T E G T V V F K
 1401 GCCTGGTATACCCAGAAGAAATCAGAGTGGGTATCATAGATGATGATATCTTTGAGGAGATGAAAATTTCTTGTGCATCTCAGCAATGTCAAAGTA
 468 P G D T Q K E I R V G I I D D D I F E E D E N F L V H L S N V K V
 1501 TCTTCTGAAGCTTCAAGATGACACTGGAAGCAATCATGTTTCTACACTTGCTTGCCTCGGATCTCCCTCCACTGCCACTGTAACATTTTTGTAGT
 501 S S E A S E M I L E A G D R P S S T A C L E G S P S T A T V T I F D D
 1601 ATGACCACGCAGGCATTTTACTTTTGGGAACCTGTGACTCATGTGAGTGAGAGCATTGGCATCATGGAGGTGAAAGTATGAGAACATCTGGAGCTCG
 535 D H A G I F T P E E P V T H V S E S I G I M E V K V L R T S G A R
 1701 AGGAAATGTTATCGTTCCATATAAAAACCATCGAAGGGACTGCCAGAGGTGGAGGGGAGGATTTTGGAGACACTGTGAGAGCTCGAATTCAGAATGAT
 568 G N V I V P Y K T I E G T A R G G G E D F E D T C G E L E F Q N D
 1801 GAAATGTGAAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAGAGTGCAGTTTCTCCCTTGTGCTTGGAGAACAAAATGGATAAGAA
 601 E I V K I I T I R I F D R E E Y E K E C S F S L V L E E P K W I R R
 1901 GAGGAATGAAAGGTGGCTTCAACAATAACAGGCCAACCTGTCTCAGGAAGVTCATGCTAGAGAACATCCGATTTCTTACTGTAAATCACCATTGCGA
 635 G M K G P T I T G Q P T F R K G V H A R E H P I L S T V I T I A D
 2001 CGAATATGATGACAAGCAGCCACTGACCAGCAAGAGGAGAGGAGCGCATTTGCAGAAATGGGGCGCCCATCTGGGAGAGCACACCAAGTTGGAA
 668 E Y D D K Q P L T S K E E E E R R I A E M G R P I L G E H T K L E
 2101 GTGATCATGAAGAATCCTATGAATCAAGAGTACTGTGGACAAACTCATTAGAAGACAAACCTGGCCCTTGTGGTGGGACTAACAGCTGGAGAGA
 701 V I I E E S Y E F K S T V D K L I K K T N L A L V V G T N S W R E Q
 2201 AGTTCATTGAAGCTATCACTGTGCTGCTGGGAAGATGATGACGACGATGAATGTGGGAAGAGAAGCTGCCCTCTGTTTCGATTACGTGATGCACTT
 735 F I E A I T V S A G E D D D D D E C G E E K L P S C F D Y V M H F
 2301 TCTGACTGTGTTCTGGAAGTCTGTTTTCCTTCCCTTACTGAAATGCTGGAATGGCTGGGCGTGTTCATTGTCTCCATCTCATGATTGGCCTA
 768 L T V F W L F A F V P T E Y W N G W A C F I V S I L M I G L
 2401 CTGACAGCTTTCATTGGAGACCTGGCTTCCCACCTTGGCTGACCATTGGCCCTGAAAGATTTCTGTACTGCAGTCTGCTGCGCATTTGGAATCAG
 801 L T A F I G D L A S H F G C T I G L K D S V T A V V F V A L G T S V
 2501 TGCCAGACACATTTGCCAGCAAAGTGGCAGCCACCCAGGACCAGTATGACAGCGCTCCATAGGTAACGTCACGGGCAGCAACCGGCTGAATGCTTCCCT
 835 P D T F A S K V A A T Q D Q Y A D A S I G N V T G S N A V N V F L
 2601 GGAATCGGTGGCCCTGGTCCATCGCTGCCATCTACCACGACCAATGGGGAACAGTTCAAAGTGTCCCTGGCACACTAGCTTTCTGTGCTACTCTC
 868 G I G V A W S I A A I Y H A A N G E Q F K V S P G T L A F S V T L
 2701 TTCACCATTTTTGTCTTTCATCAATGTGGGGTGTGCTGTATCGGCGGAGGCCAGAAATCGGAGGTGAGCTGGTGGGCCCGGACTGCCAAGCTCCTCA
 901 F T I F A F I N V G V L L Y R R R P E I G G E L G G P R T A K L L T
 2801 CATCTGCCTCTTGTGCTCCTATGGCTCTGTACATTTCTTCTCCTCCCTGGAGGCTACTGCCACATAAAAGGCTTCTAA
 935 S C L F V L L W L L Y I F P S S L E A Y C H I K G F *

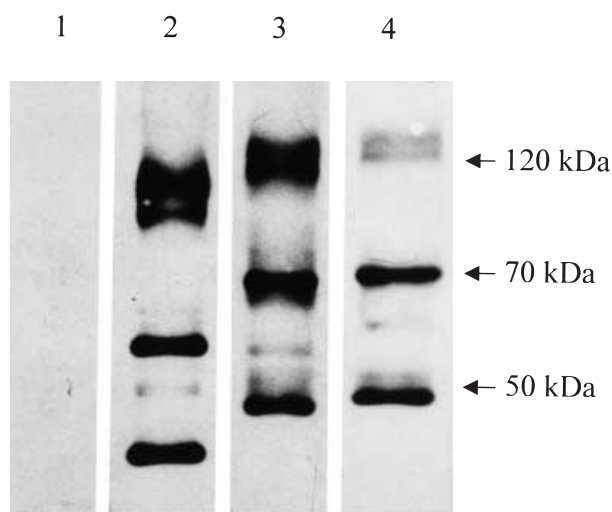


Figure 3 Immunoblot analysis of human NCX1 splice variants expressed in HEK293 cells. Lanes: 1, non-transfected HEK293 cells; 2, HEK293 cells transfected with human NCX1.3; 3, HEK293 cells transfected with human NCX1.7; 4, HEK293 cells transfected with human NCX1.1. The lanes were loaded with 5, 2, 5 and 6 μ g protein respectively. Antibodies to the Na/Ca exchanger labeled three major bands at 120, 70 and 50 kDa.

69-nucleotide segment (shown in bold) corresponding to exon F (accession number AF108389). The sequence of human NCX1.1 was similar to that previously published by Komuro *et al.* (1992). The three splice variants were expressed separately in both a transient and a stable expression system, in HEK293 cells and in CHO cells, respectively. Na/Ca exchange activity was measured as Na^+ -dependent $^{45}\text{Ca}_o$ uptake (reverse Na/Ca exchange). In HEK cells, the data were normalized for the percentage of transfection, using the reporter gene Green-Lantern and fluorescence examination by FACScan.

Figure 3 illustrates immunoblots performed under reducing conditions to assess the expression of NCX protein in HEK-transfected cells. Purification of NCX protein has revealed three major protein bands at 160, 120 and 70 kDa (Philipson *et al.* 1988). The 120 kDa band is widely accepted as representing the mature glycosylated protein, the 70 kDa protein being a proteolytic fragment of it (Philipson *et al.* 1988). The 160 kDa protein is seen only under non-reducing conditions (Philipson *et al.* 1988). Other protein bands at 50, 36 and 33 kDa, immunologically related to the 120 and 70 kDa bands, have been observed occasionally (Barzilai *et al.* 1987, Michaelis *et al.* 1992, Nicoll *et al.* 1990). Figure 3, lane 1 shows a control experiment loaded with protein from HEK cells transfected with the vector only (without the insert). Under our working conditions, no bands were observed. In HEK cells transfected with NCX1.3, NCX1.7 or NCX1.1, three major bands were observed at 120, 70 and 50 kDa. There were slight differences between the three mi-

HEK293 transfected cells

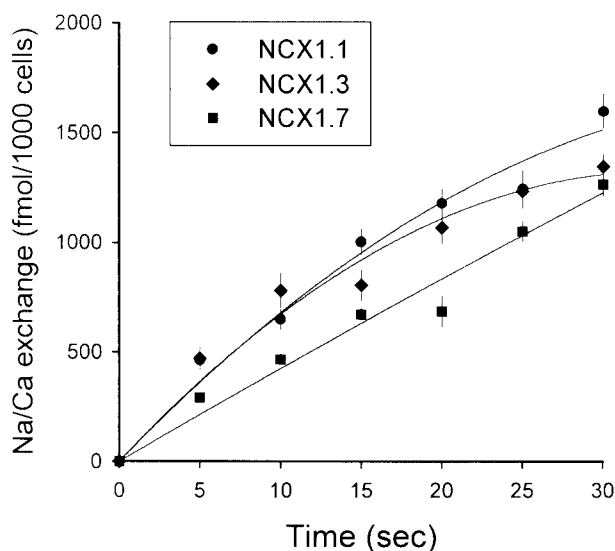


Figure 4 ^{45}Ca uptake by reverse Na/Ca exchange in NCX1.1-, NCX1.3- and NCX1.7-transfected HEK cells. Na/Ca exchange was measured as intracellular Na-dependent $^{45}\text{Ca}_o$ uptake. The time-courses of ^{45}Ca uptake were measured in NCX1.1-, NCX1.3- and NCX1.7-transfected cells (●, ◆ and ■ respectively) after reversal of Na/Ca exchange (by replacing Na (139 mM) with K (139 mM) in the presence of 1 mM ouabain). Means \pm S.E.M. relate to three to four individual experiments, comprising six replicates in each case.

grations, because of the size differences of the three splice variants. Figure 4 shows the time-courses of ^{45}Ca uptake by reverse Na/Ca exchange in NCX1.3-, NCX1.7- and NCX1.1-transfected cells. Na^+ -dependent $^{45}\text{Ca}_o$ uptake was measured over short periods of time in order to estimate the initial rate of Ca^{2+} inflow. Irrespective of whether the cell expressed NCX1.1, NCX1.3 or NCX1.7, a rapid increase in ^{45}Ca uptake was observed which was of comparable magnitude in the three preparations.

Figure 5 shows the time-course of ^{45}Ca uptake by reverse Na/Ca exchange in CHO transfected cells. Na^+ -dependent $^{45}\text{Ca}_o$ uptake was also measured over short periods of time. As in HEK cells, the uptake was rapid and of comparable magnitude in the three preparations, although the magnitude of the uptake was clearly lower in CHO cells than in HEK cells.

Identification of NCX1 gene products present in rat, hamster, mouse and guinea-pig islets

With the common primers ALTESP.FOR and ALTESP.REV, PCR amplification yielded three products in hamster, mouse and guinea-pig islets (data not shown).

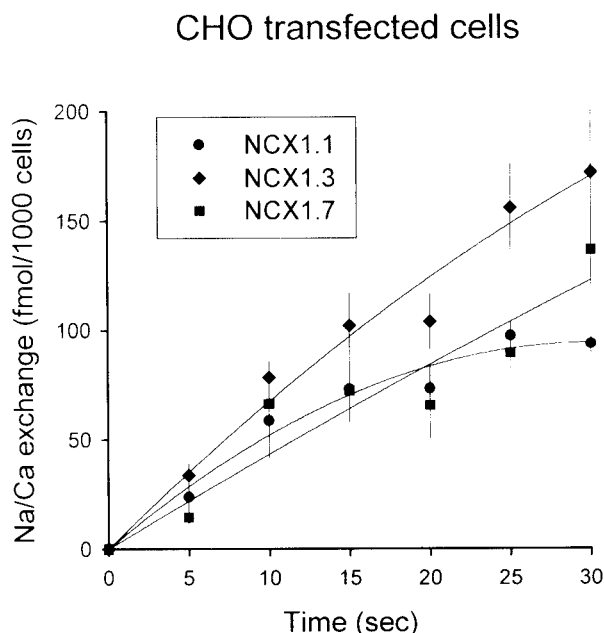


Figure 5 ^{45}Ca uptake by reverse Na/Ca exchange in NCX1.1-, NCX1.3- and NCX1.7-transfected CHO cells. Na/Ca exchange was measured as intracellular Na-dependent $^{45}\text{Ca}_o$ uptake. The time-courses of ^{45}Ca uptake were measured in NCX1.1-, NCX1.3- and NCX1.7-transfected cells (●, ◆ and ■ respectively) after reversal of Na/Ca exchange (by replacing Na (139 mM) with K (139 mM)) in the presence of 1 mM ouabain. Means \pm S.E.M. relate to two individual experiments, comprising six replicates in each case.

The identities of the PCR products were determined by subcloning and sequencing at least two independent clones in each case. Hamster and mouse pancreatic islets expressed two splice variants (NCX1.3 and NCX1.7) previously found in rat islets (Van Eylen *et al.* 1997), plus a third splice variant, NCX1.2 (Kofuji *et al.* 1994) (accession numbers AF108390, AF108392, AF108396, AF108398, AF108391 and AF108397 respectively). NCX1.3, NCX1.7 and NCX1.2 are characterized by the presence of exons BD, BDF and BCD, respectively, in the splice region of the NCX1 cDNA (Table 1). The guinea-pig islets expressed NCX1.3 and two additional NCX1

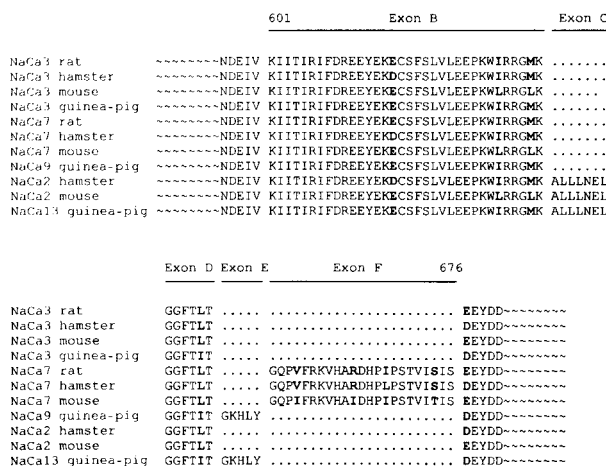


Figure 6 Comparison of the amino acid sequences of the various splice variants in the splice region of NCX1 cDNA in rat, hamster, mouse and guinea-pig pancreatic islet cells. Deduced amino acid sequences of reverse-transcribed PCR amplification products were aligned. Points denote gaps (due to missing exons) introduced to align the sequences. Mismatched amino acids are shown in bold. The amino acid numbering scheme assumes the deduced initiator methionine as the first amino acid.

splice variants; one of these was identified as NCX1.9 and characterized by the presence of exons BDE (Quednau *et al.* 1997), and the other was a newly identified splice variant named NCX1.13 and characterized by the presence of exons BCDE (accession number AF108393, AF108394 and AF108395 respectively). The comparison of the amino acid sequences of the various splice variants in the splice region is shown in Fig. 6.

Quantitative analysis of the NCX1 splice variants present in rat, hamster, mouse and guinea-pig islets

To determine the relative amounts of NCX1 gene products in the four mammalian species, the quantitative RT-PCR method was used. Several precautions must be taken to ensure that the amount of the amplified fragment is quantitatively related to the amount of the template. Indeed, after a certain number of cycles, PCR reaches a

Table 1 Tissue distribution of alternative splicing-region of splice variants of NCX1 identified in rat, hamster, mouse and guinea-pig pancreatic islet cells

	NCX1 splice variants (and exons used)				
	NCX1.3 (BD)	NCX1.7 (BDF)	NCX1.9 (BDE)	NCX1.2 (BCD)	NCX1.13 (BCDE)
Islet cells from:					
Rat	+	+			
Hamster	+	+		+	
Mouse	+	+		+	
Guinea-pig	+		+		+

Table 2 Quantitative RT-PCR analysis of the NCX1 splice variants present in rat, hamster, mouse and guinea-pig pancreatic islet cells

	No. of cycles	NCX1.7 (%)	NCX1.3 (%)	NCX1.9 (%)	NCX1.2 (%)	NCX1.13 (%)
Islet cells from:						
Rat	31	51.5±0.9	48.5±0.9	0	0	0
Hamster	29	31.2±1.8	59.8±1.8	0	9.0±1.8	0
Mouse	29	6.7±0.2	77.5±0.5	0	15.8±0.7	0
Guinea-pig	30	0	39.1±0.3	59.0±0.4	0	1.9±0.7

The proportions of NCX1.7, NCX1.3, NCX1.9, NCX1.2 and NCX1.13 are expressed in percentages and are given as means±S.E.M. of 2–4 determinations.

plateau according to different individual factors. Therefore, the number of cycles corresponding to the exponential phase of the PCR amplification was first determined. Three pairs of primers to the NCX1 splice variants of the different types of islet-cell preparations were chosen (ALTESP.FOR and ALTESP.REV, GUIALT.FOR and GUIALT.REV, GUIALT.FOR and HAMALT.REV), flanking the putative splicing area of NCX1 cDNA. PCR amplification was carried out, focusing on cycles 26–36 (data not shown); the numbers of cycles chosen for further work were 31, 30 and 29 for the rat, guinea-pig and hamster plus mouse respectively. To verify the accuracy and reliability of the RT-PCR method, total RNA was serially diluted, as previously proposed by Golde *et al.* (1990), and each RNA dilution (0.5–2 μ g) was reverse-transcribed and amplified for the specified number of cycles. There was an excellent and linear correlation between the amount of RNA used and the amount of corresponding cDNA obtained after 31, 30 and 29 cycles ($r \geq 0.99$) (data not shown). Table 2 summarizes the relative proportions of the different NCX1 transcripts found in each of the four mammalian species. In rat pancreatic islets, NCX1.3 and NCX1.7 transcripts were present in equal amounts, as previously observed (Van Eylen *et al.* 1997). In the other mammalian islets, NCX1.7 was detected in lower amounts than NCX1.3, or even was absent. Indeed, the level of NCX1.7 transcription averaged 31, 7 and 0% of the total NCX1 transcription in hamster, mouse and guinea-pig islets respectively ($P < 0.001$). In the hamster and the mouse, the level of NCX1.2 transcription averaged 9 and 16% respectively, whereas in the guinea-pig, the levels of NCX1.9 and NCX1.13 transcripts averaged 59 and 2% of the total.

Finally, to compare the amounts of each NCX1 transcript of the splice variants in the four islet preparations, the amounts of cDNA obtained after a 31 cycles of PCR amplification were quantified (Fig. 7). Whilst similar amounts of NCX1.3 products were observed in rat, hamster and mouse islets ($P > 0.05$), the amount of NCX1.3 product found in guinea-pig islets was only half of that found in the other species ($P < 0.005$). Compared with rat-islet NCX1.7 product, the amounts of NCX1.7 products found in hamster, mouse and guinea-pig islets averaged only 59% ($P < 0.01$), 15% ($P < 0.001$) and 0%

respectively. In the hamster and the mouse, the level of NCX1.2 transcript averaged 15 and 14% respectively relative to the levels of NCX1.3 or NCX1.7 in the rat. In the guinea-pig, the level of NCX1.9 and NCX1.13 transcripts averaged about 70 and 11% respectively relative to that of NCX1.3 or NCX1.7 in the rat.

Na/Ca exchange activity in rat, hamster, mouse and guinea-pig islets cells

Na/Ca exchange activity was measured as Na^+_{i-} -dependent $^{45}\text{Ca}_o$ uptake, but was analyzed over a longer period of time than that in HEK293 cells and CHO cells (5 min). Indeed, in β -cells, Na/Ca exchange activity was much lower than in the latter cells, precluding accurate measurements over short periods of time. Therefore, the value of Na/Ca exchange activity could be underestimated. Extracellular Na^+ removal induced a marked (3.7-fold) increase in ^{45}Ca uptake in rat pancreatic islet cells, as previously described (Van Eylen *et al.* 1997). In the other species, the uptake was less marked, 2.4-fold, 1.9-fold and 2.1-fold increases in ^{45}Ca uptake being

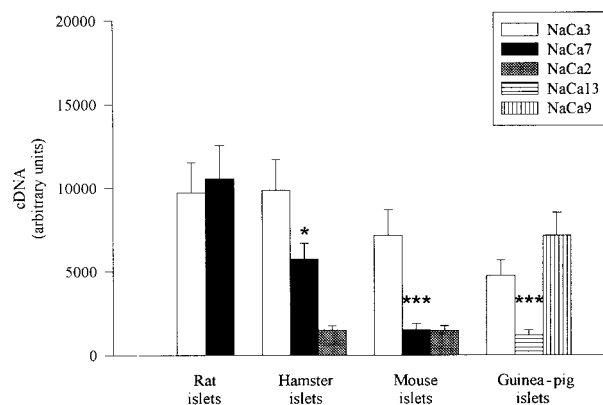


Figure 7 Expression and transcription pattern of the five NCX1 splice variants present in rat, hamster, mouse and guinea-pig islet cells (NCX1.3, NCX1.7, NCX1.2, NCX1.13 and NCX1.9). The cDNA values were determined from the fluorescence of PCR fragment by densitometry (arbitrary units). Means ± S.E.M. refer to five determinations. (* $P < 0.05$, *** $P < 0.001$).

observed in response to Na/Ca exchange reversal in hamster, mouse and guinea-pig islet cells respectively.

Discussion

Tissue-specific expression of NCX1 splice variants has been evidenced but it is unknown whether structural differences between NCX1 splice variants are translated into functional differences. In a previous work, we showed that the pancreatic β -cell was equipped with two NCX1 splice variants (NCX1.3 and NCX1.7) and suggested, on the basis of indirect evidence, that there could be difference in activity between Na/Ca exchange splice variants.

The present study provides direct evidence of the activity of the three NCX1 splice variants. This does not preclude the possibility that the three splice variants may nevertheless display differences in intrinsic activity. Indeed, the present data do not provide any figure for the level of expression of each splice variant, nor do they show that the splice variants were equally targeted to the plasma membrane. Supporting the existence of a difference in intrinsic activity between NCX1 splice variants, Dyck *et al.* (1999) recently showed that NCX1.3 and NCX1.4 differed in terms of ionic regulatory properties.

NCX1.7 differs from NCX1.3 by the presence of an additional stretch of 23 amino acids, coded by exon F. NCX1.1 differs from NCX1.3 and NCX1.7 by the presence of exon A, which is mutually exclusive with respect to exon B, and by the presence of additional stretches of 35 and 12 amino acids coded by the cassette exons CEF and CE respectively. The region of alternative splicing, (amino acid residues 601–676) that is located towards the C-terminal end of the intracellular loop, was shown to be implicated in the secondary regulation of the exchanger by Ca^{2+} . In fact, the high-affinity Ca^{2+} -binding domain implied in secondary Ca^{2+} regulation is located upstream of the region of alternative splicing (Levitsky *et al.* 1994). Although the latter region does not bind Ca^{2+} , it appears that structural integrity of the entire intracellular loop is necessary for Ca^{2+} regulation. Thus, in the absence of regulatory Ca^{2+} , the activity of the exchanger is inhibited by an unfavorable conformation of the intracellular loop. When Ca^{2+} binds to the loop, a conformational change occurs and inhibition is relieved (Matsuoka *et al.* 1993). Matsuoka *et al.* (1993) showed that a mutant of the cardiac NCX (NCX1.1) with amino acid residues 594–717 deleted is still capable of Na/Ca exchange activity but is no longer regulated by Ca^{2+} . Thus, the exchanger current was not inhibited in the absence of Ca^{2+} . It was concluded that the exchanger loop could not adopt the conformation of the inhibited state when residues 594–717 were deleted.

In a previous study, we examined the transcription pattern of NCX1.3 and NCX1.7 in rat pancreatic β -cells and in a clonal insulin-producing cell line (RINm5F cells;

Van Eylen *et al.* 1997). NCX1.3 was transcribed equally in the two cell preparations, but in RINm5F cells, NCX1.7 was three times less transcribed. Because this lower transcription was accompanied by a threefold lower Na/Ca activity, it was suggested that functional differences between NCX1 splice variants may exist. In order to examine this possibility further, we compared Na/Ca exchange activity and the transcription pattern of NCX1 splice variants in pancreatic islet cells from different species, i.e. the rat, the mouse, the hamster and the guinea-pig. The present study shows the existence of a differential and species-specific expression pattern of Na/Ca exchange splice variants in pancreatic islet cells. Indeed, compared with the rat, which expresses NCX1.3 and NCX1.7, the hamster and the mouse express an additional splice variant, NCX1.2, and the guinea-pig expresses only NCX1.3 plus two other splice variants, NCX1.9 and NCX1.13. This difference in the expression and transcription pattern could explain, at least in part, the differences in Na/Ca exchange activity observed in these species.

In response to glucose, the mouse pancreatic β -cell displays a characteristic pattern of electrical activity consisting of slow oscillations between (1) a depolarized plateau potential on which action potentials are superimposed and (2) repolarized, electrically silent intervals (Henquin & Meissner 1984). In a recent work, we showed that the β -cell Na/Ca exchange was electrogenic and that the inward current generated by the exchanger, although being of small amplitude (15% of the voltage-gated Ca^{2+} conductance), could modulate (prolong) the duration of the burst of electrical activity (Gall & Susa 1999, Gall *et al.* 1999). Although the present study was carried out in islet cells instead of a pure β -cell population, which represents up to 70% of the islet cell population, it is interesting that the rat pancreatic β -cell, instead of presenting slow oscillations of membrane potential (as in the mouse) displays continuous electrical activity (no repolarized, electrically silent intervals) (Antunes *et al.* 1998). Therefore, it is conceivable that the species difference in burst pattern could result, in part, from a higher Na/Ca exchange activity in the rat compared with the mouse, as evidenced in the present study. Thus, the exchanger current, by being of larger magnitude in the rat than in the mouse, would prolong the duration of the burst, leading to continuous electrical activity in the rat. Further work is required to evaluate this hypothesis.

Taken as a whole, our data provide evidence for the activity of the three NCX1 splice variants. In addition, pancreatic islet cells display a differential and species-specific expression and transcription pattern of NCX splice variants that may explain, in part, the difference in Na/Ca exchange activity (and perhaps electrical activity) seen in the β -cells of these species. It would be interesting to evaluate the expression pattern and activity of NCX1.1 splice variants in other tissues and examine to what extent

the differences seen in islet cells can be generalized or are tissue specific.

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