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Identification and subcellular localization of the Na⁺/H⁺ exchanger and a novel related protein in the endocrine pancreas and adrenal medulla

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Abstract

Na⁺/H⁺ exchangers (NHE) constitute a family of membrane antiporters that contribute to the regulation of cellular pH and volume in many tissues, including pancreatic islets. We investigated the molecular identity of NHE in rodent and human endocrine pancreas, and determined its cellular and subcellular localization. NHE1 was the most abundantly expressed isoform in rat islets, and was also expressed in mouse and human islets. By western blot, an antiserum raised against the C-terminus end of NHE1 confirmed the presence of a ~100 kDa protein corresponding to NHE1 in islets and unexpectedly unveiled the existence of a ~65 kDa cross-reactive NHE1-related protein. By immunohistochemistry, the antiserum labelled the membranes of pancreatic acini and ducts, but also diffusely stained the cytoplasm of insulin, glucagon and somatostatin cells as well as endocrine cells of the adrenal medulla. Electron microscopy localized the NHE1 immunoreactivity in the membrane of secretory granules, an unexpected finding supported by a decrease in immunohistochemical signal in degranulated β-cells. Islets of SIc9A1^{swe/swe} mice, which lack full NHE1 protein, were found to express an mRNA corresponding to the 3' end of NHE1 as well as the ~65 kDa protein. They still showed the cytoplasmic labelling but no plasma membrane was stained. We conclude that both the full-length and the shorter-splice variant of NHE1 are expressed in all cell types of the endocrine pancreas and in the adrenal medulla of rodents and humans. The complete protein is addressed to the plasma membrane and the shorter one to the membrane of secretory granules where its function remains to be established.

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Introduction

Pancreatic β cells adjust insulin secretion to the ambient concentration of glucose and other nutrients through changes in their metabolism (Newgard 2002, MacDonald et al. 2005a, Matschinsky et al. 2006). Oxidative glycolysis increases the ATP:ADP ratio, which closes ATP-sensitive K⁺ channels in the plasma membrane, thereby causing Ca²⁺ influx through voltage-dependent Ca²⁺ channels and rise in the concentration of cytosolic Ca^{2+} (Seino *et al.* 2000, Gilon et al. 2002, MacDonald et al. 2005b). This rise triggers exocytosis of insulin-containing granules. Simultaneously, but independently of its action on ATP-sensitive K⁺ channels, the metabolism of glucose produces amplifying signals that augment secretion without further increasing Ca^{2+} (Henquin 2000, Aizawa et al. 2002, Straub & Sharp 2002). During stimulation by nutrients, the β cell cytosolic pH (pH_i) Lindstrom & Sehlin 1984, Best et al. 1988, Juntti-Berggren et al. 1991, Shepherd & Henquin 1995, Salgado et al. 1996, Shepherd et al. 1996) and volume

(Milev et al. 1997) increase. The functional significance of these changes is still debated (Pace et al. 1983, Lindstrom & Sehlin 1986, Bertrand et al. 2002, Gunawardana & Sharp 2002) and the underlying mechanisms are incompletely elucidated. However, experiments using ionic substitutions in the extracellular medium or pharmacological tools (e.g. dimethyl-amiloride to block Na⁺/H⁺ countertransport) have established that, besides $HCO3^{-}/Cl^{-}$ exchangers, a Na^{+}/H^{+} exchanger is implicated in the regulation of β cell pH_i (Juntti-Berggren et al. 1991, Shepherd & Henquin 1995, Shepherd et al. 1996) and possibly volume (Miley et al. 1998). Similarly, Na^+/H^+ exchange has been implicated in the control of pH_i and volume of adrenal chromaffin cells (Delpire et al. 1988, Kao et al. 1991).

Sodium-proton exchangers (NHE) are widely distributed integral membrane proteins that regulate cellular volume and pH (Orlowski & Grinstein 1997, Ritter et al. 2001). The SLC9 family comprises many pseudogenes and genes that encode at least nine isoforms of the NHE proteins (Orlowski & Grinstein 2004, Nakamura et al. 2005). Several SLC9 genes are

Journal of Molecular Endocrinology (2007) 38, 409-422

also known to give rise to multiple transcripts or partial mRNA (Orlowski & Grinstein 2004). The first five isoforms (NHE1 to NHE5) are well characterized and display distinct physiological and pharmacological properties. NHE1 is ubiquitous. In polarized cells, it is usually inserted in the basolateral domain of the plasma membrane where it fulfils housekeeping regulation of cell volume and pH (Orlowski & Grinstein 2004). NHE2 and NHE3 are mainly found at the apical pole of epithelial cells in kidney (Chambrey et al. 1998), intestine (Chu et al. 2002) and duct cells of salivary glands and pancreas (Lee et al. 1998, 2000), where they play a role in Na⁺ and fluid absorption, and secretion of protons (Orlowski & Grinstein 2004). NHE4 has been identified in the macula densa of the kidney (Peti-Peterdi et al. 2000) and in the stomach (Rossmann et al. 2001). NHE5 has mainly been found in the brain where it seems to behave like NHE3 (Baird et al. 1999). Although NHE1 to NHE5 are usually localized in the plasma membrane, NHE3 and NHE5 have also been observed in recycling vesicles (Kurashima et al. 1998, Szaszi et al. 2002). In contrast, the ubiquitously distributed NHE6 to NHE9 have not been localized to the plasma membrane but to intracellular organelles (endosomes, trans-Golgi network or mitochondria; Nakamura et al. 2005).

The primary aims of the present study were to determine the molecular identity of NHE in human and rodent islets and to establish its subcellular localization. The initial results unexpectedly led to the discovery of a novel protein related to NHE1 and heavily concentrated in the membrane of secretory granules in all cell types of the endocrine pancreas, and also in the adrenal medulla.

Materials and methods

Samples

All studies were approved by and conducted in accordance with the regulations of our institutional ethics committees. Human pancreases were obtained either at autopsy of normoglycaemic patients (n=4) performed within 6 h after death, or from organ donors (n=6). None of these patients had a disease affecting the pancreas. Autopsy samples from human kidney and heart were used as control tissue for RT-PCR. Human islets isolated (Dufrane *et al.* 2005) from the pancreas of one organ donor were provided by Dr D Dufrane (Experimental Surgery, Université Catholique de Louvain, Brussels, Belgium).

Normal male Wistar rats and National marine research institute (NMRI) mice were from a local colony. To evaluate NHE1 distribution after massive insulin secretion, Wistar rats received two i.p. injections

of glibenclamide (5.0 mg/kg body weight) or saline at 12 h interval. Animals were fed ad libitum and decapitated 4 h after the second injection. C57BL/6.SJL, +/swe (slow wave epilepsy) mice (Cox et al. 1997) were purchased from the Jackson Laboratory (Bar Harbor, MI, USA). These heterozygous mice were mated and the resulting homozygous NHE1 null mutant (*Slc9A1^{swe/swe}*) and wild-type (*Slc9A1^{+/+}*) were studied at 5-7 weeks of age. Homozygous mutant mice exhibited a neurological phenotype including ataxia in the hind limbs, and seizures. Genotyping was performed as described (Cox et al. 1997) to confirm the phenotype. Rat islets were obtained by collagenase digestion of the pancreas (Jonas et al. 1998), and were used freshly or after 7 days of culture in RPMI 1640 medium containing 10 mM glucose and 0.5 g/100 ml BSA. A similar procedure was used to obtain mouse islets except that they were cultured for 18-42 h in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 mm glucose.

Antibodies

Most experiments were performed with a rabbit serum directed against the C-terminus end of NHE1 (Goss *et al.* 1994), which was raised, characterized and kindly given by Dr M Donowitz (Johns Hopkins University School of Medicine). A mouse monoclonal antibody also directed against the C-terminus end of NHE (clone 4E9, MAB3140, Chemicon, Temecula, CA, USA) was used in a few, clearly identified, control experiments. Mouse monoclonal antibodies against insulin, glucagon and somatostatin were obtained from NovoBiolabs (HUI-018, GLU-001 and SOM-018 respectively; Bagsvaerd, Denmark).

Other reagents and supplies

Biotinylated secondary antibodies against mouse and rabbit Fab' Ig fragments were obtained from Vector Laboratories (Burlingame, CA, USA) and Chemicon respectively. Streptavidin-peroxidase (SP) and Streptavidin-Texas-Red (STR) complexes were obtained from Roche-Diagnostics and Zymed (San Francisco, CA, USA). En-Vision detection system (EV) and Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Dako (Glostrup, Denmark). Immunogold reagents including nanogold-labelled anti-rabbit Ig (NG-Ig) and silver enhancement solutions were obtained from Nanoprobes (Stony Brook, NY, USA). Electrophoresis reagents were from Bio-Rad and Amersham. Other reagents were obtained from Amresco (Solon, OH, USA), Sigma and J T Baker (Philipsburg, NJ, USA).

 Na^+/H^+ exchanger in the endocrine pancreas · P MOULIN and others 411

Tissue processing

Human or rodent samples were frozen in liquid nitrogen or fixed in 4% paraformaldehyde for 6 or 24 h.

Tissue proteins were extracted from snap-frozen samples (~500 mg) homogenized in (~10 ml/g tissue) ice-cold buffer (20 mM Imidazole, pH 7·2, 1 mM EDTA, 250 mM sucrose) containing protease inhibitors (Complete, Roche) by ten passes of P6 Ultra-Turrax (Labortechnik, Staufen, Germany) and sonicated (Branson Sonifier B12, Danbury, CT, USA). The homogenate was centrifuged at 1000 g for 15 min at 4 °C. The postnuclear supernatant was centrifuged at 80 000 g for 1 h at 4 °C to separate membrane and cytosolic fractions (Combet *et al.* 1999). The membrane pellet was suspended in the homogenization buffer and protein concentrations were determined using the Bradford method (Bio-Rad) with BSA as standard.

Total RNA was extracted from snap-frozen samples in Trizol according to manufacturer's instructions (Life Biotechnologies, Gibco-BRL). RNA was reverse-transcribed in cDNA using random hexamers and the Superscript RNA reverse transcriptase (Gibco-BRL).

Immunoblot analysis

SDS-PAGE and immunoblotting were performed as described (Combet *et al.* 1999). The extracts were solubilized by heating at 95 °C for 3 min in sample buffer. Proteins (10–40 μ g/lane) were separated by electrophoresis through 7.5% acrylamide slabs and transferred to nitrocellulose. Membranes were blocked for 30 min at room temperature in blotting buffer, followed by incubation with the primary antibody (anti-NHE1 at 1/2000). The membranes were then washed and incubated for 1 h at room temperature with anti-rabbit Fab' peroxidase-labelled antibody (Dako). After washing, immunoblots were visualized with ECL-Plus reagent (Amersham).

Immunohistochemistry

Paraffin-embedded specimens were cut into 3 µm thick sections and processed as described elsewhere (Sempoux et al. 1998) including, when necessary, an antigen retrieval treatment. Primary antibodies were diluted in Tris (pH 7.4) supplemented with 1% BSA and applied overnight at 4 °C. All subsequent incubations lasted 1 h at room temperature. For double immunofluorescence experiments, anti-hormone and NHE1 antisera incubations were carried out sequentially. When necessary, a tyramine amplification step was added (Sempoux et al. 2003). The peroxidase activity was revealed by 3,3'-diaminobenzidine hypochloride (DAB: 50 mg/100 ml, pH 7·4; Fluka Chemie, Buchs, Switzerland) for 10 min. Antibodies and detailed conditions are described in Table 1. Specific optical density of the immunohistochemical signal was measured as described previously (Rahier et al. 1989).

Electron microscopy and immunogold labelling

After 24-h fixation in paraformaldehyde, small pancreas blocks were cryoprotected in PBS containing 15% sucrose for 48 h, before being frozen into liquid nitrogen. Forty micrometre thick cryosections were cut and incubated with NHE1 antiserum (1/50) for 45 min at 4 °C. After rinsing in PBS, NG-Ig (1/40) was applied for 1 h. After short fixation in 2.5% glutaraldehyde, the signal was amplified by a silver enhancer solution according to the manufacturer's instructions. The sections were rinsed and embedded into Epon 812 and processed for electron microscopy (Rahier *et al.* 1989).

Radioactive RT-PCR analysis of rat NHE isoforms mRNA

The primers are indicated in Table 2. Radioactive PCR was performed as described previously (Jonas *et al.* 1999), with a thermal cycle profile consisting of a

	Antibody; dilution	Amplification system	Detection system
Antigen retrieval			
Yes	Polyclonal rabbit anti-NHE1; 1/2000	EV	DAB
Yes	Polyclonal rabbit anti-NHE1; 1/1000	EV	DAB
No	Monoclonal mouse anti-insulin; 1/1000	2B-SP	DAB
No	Polyclonal rabbit anti-NHE1; 1/50	2B-SP-BT-STR	Fluorescence
No	Monoclonal mouse anti-insulin; 1/80	2F	Fluorescence
No	Monoclonal mouse anti-glucagon; 1/80	2F	Fluorescence
No	Monoclonal mouse anti-somatostatin; 1/80	2F	Fluorescence

EV, En-Vision; 2B, biotinylated secondary antibody (1/500); 2F, secondary antibody FITC conjugate (1/20); BT, biotinylated tyramine; SP, streptavidin-peroxydase complex (1/500); STR, streptavidin-Texas Red conjugate (1/50).

Table 1 Immunohistochemical staining

	Size (bp)	5'-Sense primer-3'	5'-Antisense primer-3'	Reference or GenBank Accession no.
Gene				
Rat NHE1	298	GAA CAT CCA CCC CAA GTC TG	CAG TGG GTC TGA GCC TAT GC	NM_012652
Rat NHE2	333	AAA CCA ACC CAA GTC TAG CAT TGT	CGG TTT AAG CTG TTG TCC TTC CTA	NM_012653
Rat NHE3	282	AGA GCT TCA CAT CCG TCT TAT GG	GAA AGT CGC TTG ATT CCC TGT ATC	M85300
Rat NHE4	396	CAG CGT GTT TAC CCT CTT CTA TGT	TTC CGC AAA TAT CTG TGG TCA AAC	M85301
Rat NHE5	235	GCG GTC AGC CTA TCG TAT CC	GCC ACT GCG TAC TGT GTC AAT C	NM138858
Human NHE1	492	ACC ACC ACT GGA AGG ACA AG	ATA GGC CAG TGG GTC TGA GC	M81768
Mouse NHE1: 5/ex2-P4	1834	ACG TCT TCT TCC TCT TCC TGC TG	GGC TCC TTG CTC CGA ATC ATG	Bell et al. 1999; NM_012652
Mouse NHE1: P1-P4	706	AGG ACA TCT GTG GTC ATT ATG GC	GGC TCC TTG CTC CGA ATC ATG	NM_012652
<i>Slc9A1</i> ^{+/+} : MR0975–MR0977	200	CCT GAC CTG GTT CAT CAA CA	TCA TGC CCT GCA CAA AGA CG	Cox et al. 1997
SIc9A1 ^{swe/swe} : MR0976–MR0977	200	CCT GAC CTG GTT CAT CAA CT	TCA TGC CCT GCA CAA AGA CG	Cox et al. 1997

 Table 2
 Sequences of oligonucleotide primers used for PCR analysis of NHE isoforms

10 min denaturing step at 95 °C followed by 30 cycles of amplification (1 min steps at 94, 60 and 72 °C each) and a final extension step of 10 min at 72 °C. TATAboxbinding protein (TBP) was used as control gene and amplified by a 24 cycles PCR (Jonas *et al.* 1999). The amplimers were then separated on a 6% polyacrylamide gel in Tris borate EDTA buffer, in parallel with a 100-bp DNA ladder. The gel was dried, and the amount of $[\alpha$ -³²P]dCTP incorporated in each amplicon was quantified with a Cyclone Storage Phosphor System (Packard, Meriden, CT, USA). The ratio of specific

Amplification of NHE1 species-specific cDNA and human NHE1 probe production for *in situ* hybridization

product/control gene was then calculated for each

sample.

The primers used for human and rodent NHE isoforms are described in Table 2. PCRs were performed in a thermocycler 2400 (Applied Biosystems, Foster City, CA, USA) with a total volume of 25 μ l mixture containing GeneAmp PCR buffer, 1.5 mM MgCl₂, 200 μ M deoxyNTP, 0.5 μ M primers and 1 U Taq Gold polymerase (Applied Biosystems). The thermal cycle profile was 10 min denaturation at 94 °C followed by 35 cycles (30 s at 94 °C, 45 s at 62 °C and 1 min at 72 °C) and a final extension of 10 min at 72 °C. Abelson protooncogene or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were taken as controls.

The amplified DNA samples were electrophoresed on ethidium bromide agarose gel and quantified by GelDoc 2000 scanning device (Bio-Rad). The identity of NHE1 PCR product was confirmed by DNA sequence analysis using the dye terminator sequencing system on a Genetic analyser 3600 (Applied Biosystems). The PCR procedure was repeated with a no-reverse transcription control to exclude genomic DNA contamination and carry-over.

The probe production procedure for *in situ* hybridization was similar to that described previously for insulin-like growth factor-II including negative and sequence controls (Sempoux *et al.* 2003) except that a human NHE1 hybridization probe was produced from human heart using specific primers (Table 2).

In silico analyses

Electronic database searches for mouse *Slc9A1* gene or mRNA matching sequences, structure and theoretical alternative splicing or start sites were available at http://www.ensembl.org/Mus_musculus/geneview? gene = ENSMUSG00000028854&db = core (Stalker *et al.* 2004). RT-PCR primers were tested using *blastn* against all

nucleotide databases available from the National Center for Biotechnology Information (NCBI) at http://www. ncbi.nlm.nih.gov/BLAST/ (Altschul *et al.* 1990). Alternate splicing sites were searched with online tool available at http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl.

Results

Identification of NHE isoforms expressed in the endocrine pancreas

Different isoforms of NHE (NHE1–NHE5) were searched by semi-quantitative radioactive RT-PCR analysis of isolated rat islets. We found a major expression of NHE1 (Fig. 1A), whereas NHE2 was expressed in lower amounts. No signal was seen for NHE3 and NHE4, and only a weak signal was obtained for NHE5. Similar results were obtained with several preparations of both fresh and cultured islets. Control experiments carried out with a no-reverse transcription control did not yield any signal. Since NHE1 was the predominant isoform in rat islets, we studied its expression in the human pancreas. RT-PCR showed expression of NHE1 mRNA in heart and kidney (Fig. 1B, lanes 1 and 2) and in extracts of total pancreas from three subjects (lanes 3–5). The PCR products obtained in pancreatic samples were sequenced and shown to correspond to human NHE1.

By western blot, a polyclonal antiserum raised against the C-terminus part of NHE1 (CtNHE1) detected a major protein band at ~100 kDa in pancreas membrane extracts (Fig. 1C) from three different subjects, with a similar pattern as in kidney extracts (positive control). The band pattern was also similar in rat and mouse total pancreas. However, in isolated human, rat or mouse islets, the ~100 kDa band was weaker and the predominant signal corresponded to a lower molecular weight (low MW, ~65 kDa). This low MW band was also visible in total pancreas extracts at least from rat and mouse, but with a low intensity (Fig. 1C). This inverse pattern is compatible with a greater abundance of the ~100 kDa protein in the exocrine pancreas (98% of



Figure 1 Expression of NHE isoforms in the pancreas and control tissues. (A) Radioactive RT-PCR analysis of NHE isoforms and TBP after 30-cycles amplification. RT-, non-reverse transcript negative control; Islets, cDNA library from isolated rat islets (representative of three experiments). Kidney and brain, cDNA libraries taken as positive controls. (B) RT-PCR for NHE1 on human tissues including heart, kidney and three different pancreas extracts. (C) Western blot analysis (20 µg/lane) of human and mouse pancreas extracts. Preimmune, membrane fraction of human pancreas incubated with preimmune serum. Cytosol, cytoplasmic fraction of the same human pancreas incubated with preimmune serum. Cytosol, cytoplasmic fraction of the CtNHE1 antiserum. Human kidney, membrane extracts from human and rouse pancreas and isolated islets extracts from human pancreas incubated with the CtNHE1 antiserum (representative of three different subjects). Human islets, membrane extracts from one human islet preparation. Rat, rat pancreas and isolated islets extracts respectively. Low MW band (approximately 65 kDa, arrow) is present with a low intensity in total pancreas extract and high intensity in islet extract.

414 P MOULIN and others $\cdot Na^+/H^+$ exchanger in the endocrine pancreas

the tissue) and of the ~65 kDa protein in the islets (2% of the tissue). No similar signal was found when cytosolic extracts of pancreas were incubated with the antiserum. Several minor bands were variably observed depending on the preparations, and a weak signal was detected above 75 kDa in human kidney and in human and rat pancreas and islets. They were considered non-specific either because of the lack of reproducibility or because a similar band was observed when membrane extracts were incubated with preimmune serum (Fig. 1C).

Localization of NHE1 in pancreas and adrenal gland

In histological sections of human pancreas, in situ hybridization identified a specific signal corresponding to NHE1 mRNA in islets and ducts (Fig. 2A). No signal was seen with an irrelevant probe (not shown). By immunohistochemistry, the CtNHE1 antiserum labelled islets in human pancreas. Islets were stained irrespective of the duration of tissue fixation (6 or 24 h), whereas labelling of the plasma membrane of acinar cells was only observed when a higher antibody concentration was used on shortly fixed tissues (Fig. 2B). In islets, the immunohistochemical signal was diffusely distributed over the cytoplasm with a stronger labelling in pericapillary regions (Fig. 2C). A linear staining suggestive of a membrane pattern was seen in selected areas of packed endocrine cells (Fig. 2D). The staining distinctly delineated the cell membrane in pancreatic ducts (Fig. 2E), but was fainter in exocrine acini (Fig. 2B). The specificity of immunolabelling was attested by the distinct basolateral labelling in proximal convoluted tubules in the kidney (Fig. 2F) and by the absence of staining when the preimmune rabbit serum was used as the primary antibody (not shown).

In sections of rat pancreas, the islets were considerably more immunolabelled with CtNHE1 antibody than the exocrine tissue. Double immunofluorescence for CtNHE1 (red) and insulin (green) resulted in a yellow signal in the β -cell core of the islets, while peripheral cells remained red (Fig. 3A). However, when the CtNHE1 antibody was combined with an antiglucagon (Fig. 3B: green) or anti-somatostatin (Fig. 3C: green) antibody, peripheral cells of the islets were stained in yellow. These results indicate that NHE1 is present in β , α and δ cells. In all cell types, the staining was diffuse over the cytoplasm.

In human adrenals, the staining pattern by CtNHE1 differed strikingly between medulla and cortex (Fig. 4A). The serum diffusely stained the cytoplasm in the medulla (Fig. 4B), but only stained the plasma membranes in the cortex (Fig. 4C). This staining pattern was also observed in rat and mouse adrenals, irrespective of the duration of fixation (not shown).



Figure 2 (A) In situ hybridization of human pancreas for NHE1. The digestion procedure has degraded the tissue but the signal can unambiguously be localized in islets (arrow heads) and ducts (>-). (B-E) Immunohistochemical staining with CtNHE1 antiserum in human pancreas. After short tissue fixation (6 h) and use of the antiserum at 1/1000, acinar plasma membranes are labelled (arrow heads) but the staining still predominates in islets (B). Longer fixation (24 h) and lower serum concentration (1/2000) unmask variations in the staining intensity of islet cells, with a major labelling of the cytoplasmic area surrounding capillaries (C: asterisk). Similar results were obtained using the antiserum at 1/200 without antigen retrieval. A faint membrane-staining pattern can be observed in selected islet areas (D: arrow heads), whereas the membrane labelling is more clear in ducts (E). (F) Immunohistochemical staining with CtNHE1 antiserum in kidney cortex. A distinct basolateral membrane pattern is present in proximal convoluted tubules whereas glomeruli (\times -) are negative. Bars: A, B, $F = 50 \mu m$; C, D, $E = 20 \mu m$.

Ultrastructural localization and prominence of the membrane localization after exocytosis

Since the signal obtained with CtNHE1 antiserum was predominantly cytoplasmic, the subcellular localization of the epitope was investigated by electron microscopy on rat pancreas. The immunogold signal was localized in areas rich in endocrine granules (Fig. 5A). The background was very low outside the granule area (nucleus, mitochondria and endoplasmic reticulum) so that the labelling density was at least tenfold higher over insulin granules and fivefold higher over glucagon granules than other intracellular structures (Fig. 5B). The membranes are not visible in our preparations because preservation of immunoreactivity imposed suboptimal fixation procedures and precluded the use of membranecontrasting agents. Therefore, to assess the visual impression that gold particles are located at the periphery of endocrine granules, the distance between each gold particle and the centre of the closest granule was measured. The frequency distribution of these distances showed that most gold particles are separated from the centre of the granule by a distance corresponding to the average radius of the insulin (Fig. 5C) or glucagon (Fig. 5D) granules, as illustrated by the corresponding micrographs. Because of poor preservation of cell membranes and the relatively low abundance of CtNHE1 signal in acinar when compared with the



Figure 3 Double immunofluorescence for CtNHE1 (red) and islet hormones (green) in rat pancreas. The superimposition of red and green fluorescence yields a yellow signal which indicates the co-localization of CtNHE1 and the hormone. (A) Insulin: β cells in the central part of the islet display an orange to yellow signal indicating the colocalization of CtNHE1 with insulin. Peripheral cells stained in red (CtNHE1) with no yellow signal are probably non-ß cells. (B) Glucagon: the central core of the islet composed of ß cells is only stained in red (CtNHE1). Peripheral cells display a green to yellow fluorescence indicating the presence of CtNHE1 in glucagon cells. (C) Somatostatin: the same pattern can be observed as for glucagon. The superimposition of red and green signals is less easily found because of the stellate shape of δ cells. However, in several areas, there is a distinct colocalization of CtNHE1 and somatostatin. Cross-reactions between antibodies were excluded by comparison with fluorescent staining obtained after simple and double immunodetection. Bars: $A = 50 \mu m$; B, $C_{1} = 20 \ \mu m.$



Figure 4 Immunohistochemical staining with CtNHE1 in human adrenal. (A) Low magnification view of the adrenal medulla (left) and cortex (right). (B) High magnification view of the medulla. The neuroendocrine cells are diffusely stained by the antiserum and some cell membranes can be seen. (C) High magnification view of the cortex. The membranes of endocrine cells are stained by the antiserum, but no cytoplasmic labelling is observed. Bars: A: 100 μ m; B, C: 50 μ m.

endocrine cells, no specific labelling could be observed in exocrine cells (not shown).

Since insulin granules showed a major immunoreactivity against CtNHE1, we investigated the impact of a strong stimulation of insulin secretion on the signal distribution in β cells. We compared the pancreas of control rats with that of rats treated with high doses of glibenclamide. The immunoreactivity of insulin in islets from test animals was markedly decreased (by $56\pm 8\%$, s.D., Fig. 6A and B), reflecting β -cell degranulation (Rahier *et al.* 1989). As compared with controls, the cytoplasmic CtNHE1 staining was fainter in degranulated islets but the plasma membrane labelling was stronger so that the overall signal intensity was only slightly reduced (by $20\pm 9\%$, s.D.; Fig. 6C and D).

Identification of a novel NHE1-like protein

In control $Slc9A1^{+/+}$ mice, as in humans and control rats, the islets were diffusely stained by CtNHE1 antiserum, whereas only membrane staining was observed in acinar cells (Fig. 7A). In $Slc9A1^{swe/swe}$ mice that lack functional NHE1 protein, a diffuse labelling unexpectedly persisted in islets, while exocrine cells were negative (Fig. 7B).



Distance between gold particles and center of the nearest granule



Figure 5 Ultrastructural localization of CtNHE1 immunohistochemical signal. (A) Portion of β cell cytoplasm (80 000×). The signal is restricted to the peripheral zone of the insulin granules. (B) Immunogold particles distribution between endocrine granules and other sub-cellular structures. Values represent the mean number (±s.E.) of gold particles by μ m², measured on 27 micrographs of islets from 3 animals. The signal is five- to tenfold higher over endocrine granules than in the rest of the cytoplasm. Gold particles are twice more abundant around insulin than glucagon granules. (C and D) Distribution of distances between gold particles and centre of insulin (C: n=347) and glucagon (D: n=200) granules. Values correspond to the proportion of gold particles found at a given distance interval from the centre of the closest insulin or glucagon granule. The electron micrograph below each histogram shows two secretory granules at a magnification matching the geometric scale of the *X*-axis in the histogram. The origin of this axis (zero value) is projected on the centre of the granule on the left.

By contrast, hepatocytes showed no cytoplasmic staining and the selective labelling of their plasma membranes in $Slc9A1^{+/+}$ mice (Fig. 7C) was completely absent in $Slc9A1^{swe/swe}$ mice (Fig. 7D). Adrenal medulla from $Slc9A1^{+/+}$ and $Slc9A1^{swe/swe}$ mice showed a diffuse cytoplasmic staining pattern by CtNHE1 antiserum (Fig. 7E and F). In adrenal cortex from $Slc9A1^{+/+}$ mice, the antiserum distinctly stained the cell membranes but not the cytoplasm (Fig. 7G). CtNHE1 did not stain the membranes from *Slc9A1^{swe/swe}* mice adrenal cortex (Fig. 7H).

The persistence of a labelling in islets or adrenal medulla from *Slc9A1*^{swe/swe} mice was surprising, particularly since the exorrine pancreas, the liver and the adrenal cortex were negative, as expected. Therefore, to determine whether this reflected a non-specific binding or revealed the presence of a fragment of NHE1 in *Slc9A1*^{swe/swe} mice, RT-PCR was used to amplify



Figure 6 Influence of β -cell degranulation on CtNHE1 detection in rat pancreas. (A and B) The insulin content was markedly decreased in islets from glibenclamide-treated rats as compared with control animals. (C and D) CtNHE1 staining of the same islets (step sections). Islets from control rats (C) showed a dense, diffuse labelling whereas those of glibenclamide-treated rats (D) displayed a fainter cytoplasmic labelling with a strong membrane pattern. These experiments were performed on 24 h-fixed tissue, which explains why little signal was observed in acini. Bar: 50 μ m.

defined fragments of NHE1 mRNA from liver, isolated islets and adrenals. The strategy was to seek out expression variations between full-length mRNA and a 3' short-length mRNA downstream of the mutation point (Fig. 8A). A common 3' primer was designed at the end of the epitope-coding region (P4). A short-end 5' primer was chosen between the *swe* mutation point and the epitope-coding region (P1), and a full-length forward primer was chosen at the 5'-end exon 2 as described previously (Bell et al. 1999). The largest amplified segment of 1834 bp $(5'ex2 \rightarrow P4)$ encompasses a large portion of the full-length NHE1 mRNA (Fig. 8B). It was only observed in tissues from $Slc9A1^{+/+}$ animals. A smaller segment of 706 bp $(P1 \rightarrow P4)$ corresponding mostly to the 3'-end of the mRNA includes the region coding for the C-terminus epitope. It was observed in liver, islets and adrenals from $Slc9A1^{+/+}$ as well as $Slc9A1^{swe/swe}$ mice (Fig. 8B). A RT-PCR using primers designed for genotyping confirmed the mutated or wild-type sequence of mRNA extracted from these tissues (Fig. 8B: 200 bp). These results indicate that the mRNA sequence which encodes the epitope recognized by CtNHE1 antiserum is present in both $Slc9A1^{+/+}$ and $Slc9A1^{swe/swe}$ mice even if the latter lack full-length mRNA and NHE1 protein (Cox et al. 1997).

By western blot, isolated islets and adrenals from both $Slc9A1^{+/+}$ and $Slc9A1^{swe/swe}$ mice showed a low

MW band at ~ 65 kDa, which was absent in the liver. In contrast, the ~ 100 kDa protein observed in $Slc9A1^{+/+}$ tissues was absent in $Slc9A1^{swe/swe}$ mice (Fig. 8C). These observations were confirmed by reprobing western blot membranes with the 4E9 monoclonal antibody directed against the same region of NHE1. In islets, the ~100 kDa band was fainter than that of ~65 kDa. In adrenals, the ~100 kDa band appeared much more abundant than that of \sim 65 kDa. We attribute this inverse pattern to the high proportion of cortical cells in the total adrenal protein extract. These findings show that although NHE1 is effectively absent from Slc9A1^{swe/swe} islets and adrenals, the low MW protein is still present. This is consistent with the persistence of an immunohistochemical signal (Fig. 7B) and the presence of a 3'-end of NHE1 mRNA (Fig. 8B) in Slc9A1^{swe/swe} islets and adrenal medulla. In contrast, no similar protein (Fig. 8C) and cytoplasmic labelling (Fig. 7D) were observed in liver cells.

Discussion

This study identified NHE1 as the major isoform of the Na^+/H^+ exchanger in rat pancreatic islets. Expression of NHE1 was also established by RT-PCR in isolated



Figure 7 CtNHE1 immunostaining in control and *Slc9A1*^{swe/swe}mutant mice. (A and B) Pancreas. Both diffuse insular and membrane acinar immunohistochemical stainings were observed in the pancreas from *Slc9A1*^{+/+} mice (A). Only the diffuse islet labelling was observed in *Slc9A1*^{swe/swe} mice (B). (C and D) Liver. The membrane labelling observed in *Slc9A1*^{+/+} mice (C) was absent in *Slc9A1*^{swe/swe} mice (D). (E–H) Adrenals. A diffuse cytoplasmic staining pattern was observed in medulla from both control (E) and *Slc9A1*^{swe/swe} (F) mice. A distinct membrane labelling was observed in adrenal cortex of control (G), but not of *Slc9A1*^{swe/swe} (H) mice. Bars: A–D: 50 µm; E–H: 25 µm.

mouse islets and by *in situ* hybridization on histological sections of human islets.

At the protein level, the presence of NHE1 was documented by western blot and immunohistochemistry using a polyclonal antiserum directed against a 147 aa epitope at the C-terminus (CtNHE1; Goss *et al.* 1994). In the three examined species, the immunohistochemical labelling of CtNHE1 was stronger in islets than the exocrine pancreas. Whereas the abundance of CtNHE1 in the plasma membrane of duct cells (Roussa *et al.* 2001) was readily confirmed, detection of CtNHE1 in the membrane of exocrine acini (Roussa *et al.* 2001) was more difficult and required shorter fixation of the tissue and higher concentration of the antiserum.



Figure 8 (A) Graphical representation of NHE1 mRNA. Exons are represented with alternate pattern. Introns are not represented. The mutation point in *Slc9A1^{swe/swe}* (AAG-TAG: STOP) and the CtNHE1 epitope-coding sequence (Ct 147aa) are indicated. Arrow heads below represent the primers used in (B). (B) RT-PCR products using the selected primers on cDNA of liver, isolated islets and adrenals from *Slc9A1^{+/+}* and *Slc9A1^{swe/swe}* mice. 5'ex2-P4 and P1-P4 correspond to the full-length and the 3'-end of NHE1 mRNA respectively. MR0975–MR0977 and MR0976–MR0977 detect wild-type and mutated sequences respectively. (C) Western blot using polyclonal or monoclonal antibodies with liver, isolated islets and adrenal extracts (40 µg/lane) from *Slc9A1^{+/+}* and *Slc9A1^{swe/swe}* mice. The ~ 100 kDa band corresponds to NHE1. The low MW band is similar to that shown by the arrow in Fig. 1C (mouse islets).

We acknowledge that our experimental conditions were selected for islet studies and were not optimal for the exocrine pancreas.

In islets, double immunofluorescence demonstrated the presence of CtNHE1 in α , β and δ cells of the islets. First, CtNHE1 labelled more cells than any single anti-hormone antibody, indicating the presence of CtNHE1 in additional islet cells. Second, true co-localization of CtNHE1 and glucagon, insulin or somatostatin was demonstrated by the superimposition of the fluorescent signals. Plasma membranes were clearly labelled, but the major staining was diffuse, though not homogeneous, over the cytoplasm. Often, the signal was most abundant at the *advascular* pole of islet cells, which is known to contain the highest density of secretory granules (Bonner-Weir 1988).

Using electron microscopy, we found CtNHE1 labelling at the periphery of secretory granules but not in other structures. Although the imposed fixation technique was not optimal to preserve intact membrane structures, the spatial distribution of the immunogold signal localized the epitope in the granule membrane. Further evidence for this localization of CtNHE1 was obtained by strong degranulation of β cells with glibenclamide. The efficacy of the treatment was attested by a major decrease in insulin immunolabelling, which corresponds to a decrease in the number of secretory granules (Channaoui & Rahier 1992). Decreasing the cytoplasmic CtNHE1 labelling also unveiled a strong labelling of the plasma membrane, compatible with the presence of NHE1 at this level. It is also possible that the protein was translocated from the granule to the plasma membrane during stimulation of exocytosis.

Western blot analysis of membrane extracts from the whole pancreas with CtNHE1 antiserum showed a major band at ~100 kDa (for a predicted MW of 91 kDa of NHE1) as previously observed in PS127A cultured cells (Goss et al. 1994) and rat exocrine pancreas (Anderie et al. 1998, Roussa et al. 2001). Several of the minor bands present in the gels (Fig. 1C) have also been observed by others (Anderie et al. 1998, Roussa et al. 2001). However, in extracts of isolated islets from humans, mice or rats, the NHE1 band at ~ 100 kDa was weak, whereas a low MW band at ~65 kDa was prominent. The same ~65 kDa band was present but faint in whole pancreas extracts. This inverse pattern reflects the dilution of the prominent low MW protein in the islets by the exocrine tissue, in which the ~ 100 kDa protein is more abundant. Our failure to detect the low MW band in extracts of human total pancreas, in spite of a cytoplasmic staining of islets and of the presence of a low MW band in isolated islets, may also be linked to a poorer preservation of autopsy material in comparison with animal organs.

Staining of adrenal glands from the three species with the CtNHE1 antiserum consistently yielded strikingly different results in the two parts of the organ. In the cortex, plasma membranes were distinctly labelled, whereas the medulla was diffusely stained. Moreover, the ~65 kDa protein was identified in extracts of adrenal gland as in total pancreatic extracts. The cytoplasmic diffuse labelling was thus present in islets and adrenal medulla, two tissues rich in densecore neuroendocrine secretory granules, but was absent from the exocrine pancreas, the adrenal cortex and the liver, tissues devoid of neuroendocrine secretory granules.

The unique results obtained with CtNHE1 antiserum in islets and adrenal medulla, viz the detection of a low MW protein and a predominant cytoplasmic topography, led us to study *Slc9A1*-mutant mice bearing a point-mutation, which introduces an aberrant stop codon within the coding sequence. Homozygous mutant mice (*Slc9A1*^{swe/swe}) do not have the NHE1 protein in membranes from kidney, brain and stomach (Cox *et al.* 1997). We further show here that NHE1 is also absent from the membrane of pancreatic acini and ducts, hepatocytes and adrenal cortex in *Slc9A1*^{swe/swe} animals. Totally unexpected, therefore, was the similar staining of the islets and adrenal medulla by the CtNHE1 antiserum in both *Slc9A1*^{swe/swe} and *Slc9A1*^{+/+}

The existence of splice variants of NHE1 (Dewey et al. 2001, Zerbini et al. 2003) or other NHE isoforms (Miyazaki et al. 2001), prompted us to search for variants in islets. We examined the NHE1 mRNA expression profile and confirmed that the full-length mRNA was present in liver and islets from $Slc9A1^{+/+}$ but not Slc9A1^{swe/swe} mice. In contrast, a downstream short-length mRNA was found in tissues from both $Slc9A1^{+/9+}$ and $Slc9A1^{swe/swe}$ mice. To exclude the hypothetical expression of a non-mutated mRNA in *Slc9A1^{swe/swe}* mice, the mutation site was studied with primers designed for genotyping (Cox et al. 1997). No aberrant wild-type sequence was found in mRNA from mutated animals. Again, cross-matching of the primers with non-relevant gene products was ruled out by testing their sequence against electronic mouse genome databases. At the protein level, NHE1 in its complete form of ~ 100 kDa was only found in $Slc9A1^{+/+}$ tissues. The low MW band was found in both *Slc9A1*^{+/+}and *Slc9A1*^{swe/swe} islets, which is consistent with the mRNA studies, but was not detected in the liver despite the presence of the corresponding mRNA. It is possible that the protein, which is concentrated in large secretory granules of endocrine cells, is not synthesized or is rapidly degraded in hepatocytes that do not contain similar secretory granules.

A cross-reaction between the anti-serum and a known intracellular isoform of NHE (NHE6–NHE9) is unlikely. NHE6 (Numata *et al.* 1998) and NHE7 (Numata & Orlowski 2001) have higher predicted MW (~80 and ~76 kDa respectively) than the ~65 kDa band. Moreover, the intracellular NHE6, NHE7, NHE8 and NHE9 are present in the liver (Nakamura *et al.* 2005), but CtNHE1 did not stain liver cell cytoplasm. Finally, alignments of the cDNA sequence of these four isoforms did not identify regions corresponding to the C-terminus end of NHE1 (Nakamura *et al.* 2005).

The presence of a limited Slc9A1 mRNA sequence corresponding to the CtNHE1 epitope in both $Slc9A1^{swe/swe}$ and $Slc9A1^{+/+}$ mice suggests that a truncated gene product comprising the C-terminus end of the original protein is constitutively produced in neuroendocrine cells. One possible explanation could be the presence of alternate transcripts of Slc9A1. Indeed, analysis of the whole Slc9A1 sequence identifies 119 donor and 195 acceptor sites for possible splicing using a probability threshold of 0.70. In addition to these *in silico* analyses, support for alternate splicing can be found in previous work. First, early after the cloning of NHE1, short-length transcripts have been described in rabbit myocardium (Dyck et al. 1992). Second, amongst two alternate transcripts detected in brain and lung from engineered Slc9A1 knockout mice, one was shown to have an in-phase downstream sequence that can produce an intact C-terminus peptide (Bell et al. 1999). However, this transcript should produce a protein of predicted MW at ~ 80 kDa, which does not correspond to the present ~65 kDa protein. A splice variant of NHE1 with an intact C-terminus sequence exists in erythrocytes and kidney, not in liver (pancreas was not examined), and could mediate Na:Li countertransport (Zerbini et al. 2003). Again, the predicted MW of the product ($\sim 80 \text{ kDa}$) differs from the present ~ 65 kDa protein. Finally, there are reports of splice variants of a unique gene that are either differentially expressed in tissues (Zhang et al. 2004) or addressed to different subcellular locations (Ozaita et al. 2002). All these observations are in keeping with our proposal that a splice variant of SLC9A1 is specifically expressed in the neuroendocrine cells of the pancreas and the adrenal, where it produces a ~ 65 kDa protein that is addressed to the membrane of the secretory granules.

In conclusion, the present study shows that both the full-length and a shorter-splice variant of NHE1 are expressed in pancreatic islets and adrenal medulla of rodents and humans. As in other cell types (Cavet et al. 1999), NHE1 is addressed to the plasma membrane where it serves its functions of cytosolic pH (Juntti-Berggren et al. 1991, Shepherd & Henquin 1995, Shepherd et al. 1996) and volume (Miley et al. 1998) regulation. The shorter protein is associated with neuroendocrine secretory vesicles. In β cells, insulin granule acidification by a proton-pump is important for proinsulin conversion by the pH-sensitive prohormone convertases (Orci et al. 1994). Recent studies also indicate that an acidic granular pH is important for Ca²⁺-induced insulin secretion (Barg *et al.* 2001, Stiernet et al. 2006). Whether the NHE1-like protein is involved in the regulation of granular pH is an interesting possibility to be investigated in future work.

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