Original Paper

Expression of the *Slc12a1* Gene in Pancreatic β -cells: Molecular Characterization and *in silico* Analysis

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Key Words*Slc12a1* • Gene • NKCC2A • β -cells • Insulin secretion**Abstract**

The solute carrier protein family 12 group A, member one (*Slc12a1*) and two (*Slc12a2*) encode several splice variants of the kidney-specific and the ubiquitous isoforms, respectively, of the bumetanide (BTD)-sensitive Na-dependent K2Cl co-transporter. The *Slc12a2* co-transporter is involved in the maintenance of a high intracellular chloride concentration $[Cl^-]_i$ in β -cells and its inhibition with BTD blocks glucose-induced insulin secretion. In β -cells, $[Cl^-]_i$ plays an important role in glucose-induced depolarization and insulin secretion. Glucose promotes electrogenic efflux of Cl^- contributing to β -cell's electrical and secretory activity. To identify the expression pattern of *Slc12a1* and *Slc12a2* genes in β -cells we have used RT-PCR, Western blotting and immunolocalization studies in mouse pancreatic islets, β -cell lines and rat tissues. Our results demonstrate expression of specific splice variants of *Slc12a1* and *Slc12a2* transcripts in β -cells *i.e.*, variants 1 of *Slc12a1* (NKCC2A) and *Slc12a2* (NKCC1a). Molecular cloning and characterization of *Slc12a1* variant 1 transcripts from β -cells revealed an alternative splicing event involving the 5'-UTR region. NKCC2A expression at the protein level in islets and β -cells was confirmed by immunoblotting and immunolocalization. Further, NKCC2A, NKCC1a and pro-insulin co-localized in β -cells but not in the exocrine pancreas. Therefore, our results provide for the first time evidence of NKCC2A expression in pancreatic β -cells where it may play a role in insulin secretion.

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Introduction

Pancreatic β -cells secrete insulin in response to a rise in plasma glucose levels thus preventing hyperglycemia. In contrast, when glycemia falls below a particular physiological threshold, β -cells stop secreting insulin, thus avoiding hypoglycemia [1]. The primary

mechanism involved in glucose-induced insulin secretion involves depolarization of β -cells. The best-characterized pathway whereby glucose stimulates secretion of insulin is the ATP-sensitive K^+ (K_{ATP})-channel-dependent pathway. In this mechanism, glucose metabolism in β -cells increases the ATP/ADP ratio resulting in closure of K_{ATP} -channels. The consequent gradual decrease in K^+ conductance depolarizes the β -cell. This depolarization opens voltage-dependent Ca^{2+} channels promoting Ca^{2+} influx and triggering exocytosis of stored insulin granules [2].

However, in addition to the well-known K_{ATP} -channel-dependent pathway, physiological concentrations of glucose can still promote β -cell's electrical activity and insulin secretion [3]. This mechanism is operative even in the absence of functional K_{ATP} channel activity [4-6] and involves glucose-induced electrogenic Cl^- efflux through activation of volume-sensitive anion channels [2, 3]. Such efflux of Cl^- is possible because the intracellular chloride concentration ($[Cl^-]_i$) in β -cells is maintained at a higher concentration than expected from the Nernst's equation [7]. The bumetanide (BTD)-sensitive NaK2Cl co-transporter 1 (NKCC1) is involved in the maintenance of a high $[Cl^-]_i$ in β -cells [8, 9]. Moreover, inhibition of this co-transporter mechanism with BTD or furosemide transiently blocks glucose-induced insulin secretion [8-11]. These findings support the suggestion that loop-diuretics may have diabetogenic consequences [12].

The BTD-sensitive NKCCs belong to the family of solute carriers *Slc12a* comprising NaK2Cl (*Slc12a1* and *Slc12a2*), NaCl (*Slc12a3*) and KCl (*Slc12a4-7*) co-transporters, commonly known as NKCCs, NCCs and KCCs, respectively [13]. NKCCs and NCCs are active transport mechanisms that accumulate Cl^- in the cell by using the energy stored in the Na^+ chemical gradient, kept in all cells by the Na/K-ATPase, whereas KCCs mediate active Cl^- extrusion using the K^+ gradient as the source of energy [14]. Each of the *Slc12a1*, *Slc12a2* and *Slc12a3* genes encode several splice variants that may be different among species (reviewed in [13]). For instance, the mouse *Slc12a2* gene produce at least two splice variants: NKCC1a (*RefSeq* NM_009194) and a variant where the 21st exon is spliced out (named here NKCC1b) [15]. Similarly, the rat *Slc12a2* gene may produce two splice variants. However, as in the mouse, only one rat full-length variant of NKCC1 has been cloned (*GenBank*: AF051561) [16]. *In silico* analysis of this rat NKCC1 variant has revealed high degree of identity to mouse NKCC1a [13]. The human *Slc12a2* gene produces at least three NKCC1 splice variants: NKCC1a (*GenBank*: U30246, AY280459) [17, 18], NKCC1b (*GenBank*: BC144221 and BC146839) and NKCC1c (*GenBank*: BC033003) [19]. The latter is predicted to express a protein of 1151 amino acids with the unique C-terminal sequence FYEPC. *In silico* analysis of the hNKCC1c variant demonstrates the presence of a splicing event involving the first 66 bp of the 26th exon resulting in the inclusion of a premature stop codon [13].

With some exceptions, including α -cells of Langerhan's islet [9] and most adult neurons [20], the pharmacological, functional or molecular presence of NKCC1 (assumed to be NKCC1a) has been established in all mammalian cells examined so far [21]. Pancreatic β -cells express NKCC1 at the molecular and functional levels [9-11, 22]. However, its variant identity remains unknown. Contrary to NKCC1, the products of the *Slc12a1* gene (NKCC2s) have long been considered exclusive of the kidney; precisely, of the apical membrane of the tubular cells of the thick ascending limb of Henle's loop [23, 24]. In this particular location, NKCC2s play a key role in the kidney's capacity to retain salt and concentrate urine [24]. Mutations affecting the functional properties of this co-transporter result in neonatal Bartter's syndrome type I, a disorder characterized by severe dehydration, electrolyte imbalance and polyuria [25]. The mouse *Slc12a1* gene produces at least three splice variants: NKCC2A (variant 1, U20973), NKCC2B (variant 2, U20974) and NKCC2F (variant 3, U20975) [26]. These splice variants differ among themselves by the inclusion of one of three mutually exclusive and adjacent exons *i.e.*, exon 4 (B), exon 5 (A) and exon 6 (F). Although three *Slc12a1* variants have been also cloned from the rabbit kidney [27], two of them have been cloned from rat kidney and only one from the human one *i.e.*, rNKCC2F (U10096), rNKCC2A (BC087017) and hNKCC2F (U5813), respectively [19, 23, 25].

The objective of the present study was to determine and characterize expression patterns involving *Slc12a* gene products in insulin-secreting β -cells where $[\text{Cl}^-]_i$ plays an important role in insulin secretion [8-11]. In this report we demonstrate for the first time that β -cells co-express NKCC2A and NKCC1a. Specific co-expression of these co-transporters known to regulate $[\text{Cl}^-]_i$ in β -cells of the pancreas provides novel insights into the processes and pathways involved in insulin secretion.

Materials and Methods

Materials and immunochemicals

Platinum *Pfx* polymerase, RNase OUT, SuperScript III reverse transcriptase, random hexamers, cloning vectors, competent bacteria and tissue culture media were from Invitrogen (Carlsbad, CA); dNTPs, alkaline phosphatase/exonuclease I, enzymes and molecular biology grade chemicals were from USB/Affimetrix (Cleveland, OH); custom primers were from Integrated DNA Technologies (Coralville, IA); the RNeasy minikit for RNA purification was from Qiagen (Valencia, CA). Restriction enzymes, T4 DNA ligase, DNA ladders were from New England Biolabs (Ipswich, MA). Culture supplements, general chemicals and collagenase were from Sigma (St. Louis, MO). Culture media low in glucose was from Lonza Inc. (Basel, Switzerland). Monoclonal antibodies against NKCC1 (T4c) and pro-insulin (GS-9A8) were from Developmental Studies Hybridoma Bank (University of Iowa). β -actin and secondary FITC/TRITC/Cy3/HRP-conjugated antibodies were from Cell Signaling Technology Inc. (Danvers, MA) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively. Rabbit polyclonal antibodies directed against the C-terminus of rat NKCC2 were kindly donated by Dr. Pablo Ortiz (Hypertension and Vascular Research Division, Henry Ford Hospital, Detroit, MI). Microscopy materials were from Electron Microscopy Sciences (Hatfield, PA).

Cell culture

β -cell lines derived from rat insulinoma INS-1E (a kind gift from Dr. Pierre Maechler [28]) and RIN-m5F (ATCC, Manassas, VA) were cultured and grown in RPMI-1640 (11.1 mM glucose), supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 10 % heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were grown in 5 % CO_2 at 37°C and media changed every 2-3 days until confluence. The mouse insulinoma β -cell line MIN6 (a kind gift of Dr. Jun-Ichi Miyazaki) was cultured and grown in DMEM supplemented with 10 % FBS and antibiotics as described in the original publication [29].

Islet isolation

Normal mice (WT), lacking one (HE) or two (KO) functional alleles of the *Slc12a2* gene were generated by crossing mice of the C57BL/6J genetic background to HE breeders kindly donated by Dr. Gary Shull (University of Cincinnati [30]). Islets were obtained immediately after sacrifice and cultured following the protocol of Li et al. [31]. Briefly, liver/intestines were exposed through an incision around the upper abdomen. After clamping the duodenum wall to block the bile path, the pancreas was slowly perfused and distended with 10 % FBS-RPMI/antibiotics through the common bile duct. Then, the pancreas was removed and digested in a plastic tube in HBSS-collagenase XI (1 U/ μl) for 15 min at 37.5°C. Digestion was stopped by placing the digestion reaction on ice and by adding cold 1 mM CaCl_2 -HBSS. Islets were purified/washed by centrifugation (290 $\times g$, 30 sec. at 4°C) and resuspension in cold 1 mM CaCl_2 -HBSS. Pure islets were immediately processed for protein/RNA purification.

Total RNA extraction and RT-PCR

Total RNA was obtained from freshly isolated mouse islets or β -cell lines using the RNeasy Protect Mini kit, following the instructions of the manufacturers. Total RNAs/cDNAs from tissues were purchased from BioChain (Hayward, CA) or Zyagen (San Diego, CA). First-strand cDNA synthesis was initiated with up to 2 μg of total RNA, 200 ng of random hexamers, 500 μM dNTPs, 10 mM DTT, 40 units RNase OUT and 200 units of SuperScript III reverse transcriptase. Reverse transcriptions (RT) reactions were incubated at 50°C for 50 min. After RT, reverse transcriptase was inactivated at 85°C for 5 min. Aliquots of cDNA reaction were amplified using the polymerase chain reaction (PCR) in a final volume of 50 μl and the following conditions: 0.5 mM of each dNTP, 1-2 mM MgSO_4 and 50 pmol of gene-specific primers. The thermal conditions for PCR (*i*Cycler, BioRad Hercules CA) were as follows: denaturation at 98°C for 2 min, followed by 40 cycles of 30 sec at 95°C; annealing, 30 sec at 58°C and extension, 0.5-1 min at 68°C, according to primers and/or

predicted amplicon length in bps. The sets of primers used to amplify NKCC1, NKCC2 and control transcripts are detailed in Table 1. These primers were also used to determine the nucleotide sequence of each RT-PCR fragment produced. The nucleotide sequence of RT-PCR fragments was obtained in at least three amplicons from separate RT-PCRs using different RNA samples. Sequences of overlapping RT-PCR fragments were aligned and assembled *in silico* using *Geneious software suite* v5.5 (Biomatters Ltd. NZ www.geneious.com) and rat/mouse sequences of reference (*RefSeq*) as template models.

Quantitative real-time PCR

NKCC1 and NKCC2A transcript abundance relative to β -actin mRNAs were determined using *iQ SYBR Green Supermix* (BioRad, Hercules CA). Total RNA from INS-1E β -cells growing under basal conditions was used as template. NKCC1, NKCC2A and β -actin mRNAs were amplified using the following primer sets: NKCC2-150 sense: 5'-TGC TAA TGG AGA TGG GAT GC-3', NKCC2-150 antisense: 5'-CAG GAG AGG CGA ATG AAG AG-3'; NKCC1-149 sense: 5'-CAC GAT GAG CTG GAA AAG GA-3', NKCC1-149 antisense: 5'-ATG CAG CGG ACT AAT ACA CC-3'; and β -actin-220 sense: 5'-ACG AGG CCC AGA GCA AGA GA-3', β -actin-220 antisense: 5'-GTG TTG AAG GTC TCA AAC ATG ATC TGG GT-3'; and the following thermal conditions: initial denaturation (hot-start) at 94 °C 8 min plus 40 cycles at 94 °C, 65°C and 72 °C 30 sec each. Control reactions in the absence of template were included for each primer set. Samples were amplified in triplicates. Transcript quantification was based on the ΔC_t method where C_t is the cycle number at which the amount of amplified target mRNA reaches the arbitrary threshold of 0.2 Relative Fluorescent Units above background. NKCC1 and NKCC2 mRNA levels relative to β -actin were estimated using the relation: $\text{target/actin} = 2^{-\Delta C_t}$, where ΔC_t is the difference between the mean C_t values for the target transcript (NKCC1 or NKCC2) and β -actin *i.e.*, $\Delta C_t = C_{t_{\text{Actin}}} - C_{t_{\text{NKCCs}}}$.

Western blotting

Total protein from islets and cell lines was obtained following standard methods [32]. Briefly, 50 μ g of total protein were loaded onto pre-casted Tris-HEPES protein gels (Thermo Scientific-Pierce) and run under denaturing conditions and electrotransferred onto PDVF membranes at 4°C. After blocking, membranes were blotted using antibodies directed against NKCC1, NKCC2 or β -actin. To characterize NKCC2 antibodies, PVDF membranes containing protein extracts (20 μ g) from anatomically and functionally defined regions of the rat kidney (Alpha Diagnostic Int., San Antonio TX) and rat tissues (Imgenex, San Diego CA) were used.

Immunofluorescence

Cell lines cultured on glass coverslips or tissue sections were fixed for 30 minutes in fresh 4 % *p*-formaldehyde and permeabilized using 0.5% Triton X-100 at room temperature following published methods [33] with modifications. Briefly, fixed and permeabilized cells/sections were incubated 2 h at 4°C in phosphate buffer containing 3 % serum and 0.1% Triton X-100 (PBS-T). Fixed and permeabilized cells were incubated with gentle rocking overnight at 4°C in the presence of primary antibodies (NKCC1, 1:500; NKCC2, 1:250; pro-insulin, 1:250). After washing off unbound primary antibodies, labeled cells/sections were incubated 1 h at 4°C with secondary antibodies [Cyanine3- (Cy3)-, fluorescein isothiocyanate- (FITC) or rhodamine- (TRITC)-conjugated (1:500)]. Cells/sections were finally washed with PBS and each coverslip mounted onto a glass slide with a 20 μ l of Vectashield mounting media per slide (Vector Labs) containing 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) to counterstain cell's nuclei. Slides were sealed and immediately viewed using an Olympus Epi Fluorescence Spot microscope equipped with RT color camera, a 40x, 60/100 x oil objective and FITC, TRITC or DAPI fluorescence filters. Photomicrographs were obtained using a Diagnostics Instrument Spot 6 digital camera (Spot Imaging Solutions, Sterling Heights, MI).

Results

β -cells express NKCC1a

The expression pattern of known splice variants of the *Slc12a2* gene was determined in mouse islets, rodent β -cell lines and tissues. To this end, we first used the Basic Local Alignment Search Tool (BLAST) to screen rat/mouse expressed sequence tags (ESTs) posted in the NCBI's EST database (dbEST). As a query, we used rat/mouse mRNA sequences corresponding to the joint of exons 20 and 22 *i.e.*, lacking the exon 21' sequence of the *Slc12a2* gene (NKCC1b, Fig. 1A, *top*). Only three rat ESTs clones matched our criteria: BG666200, BG663902 and BG672434 (Fig. 1A, *center*) suggesting that rat tissues are able to express

Fig. 1. β -cells express NKCC1a. A) Expression of NKCC1a and NKCC1b mRNA variants was screened in dbEST using BLAST and rat/mouse NKCC1b sequences encompassing exons 20/22 as queries. Shown are the three EST clones that matched the queries: BG663902, BG666200 and BG672434. To visualize the splicing event, these rat EST clones were aligned against rat NKCC1a mRNA sequence of reference (NM_031798) using the MAFFT algorithm. B) Partial schematic representation of the mouse *Slc12a2* gene (Gene ID: 20496, NC_000084.6 position 57878678 to 57946821) showing the predicted alternative spliced exon 21 that encode for mNKCC1a (NM_009194) or mNKCC1b (predicted). Shown are the last ten exons of the gene (exons 18-27, filled boxes). Exon numbering is relative to the first coding exon of the gene (i.e., exon 1, harboring the first in-frame ATG). The exon involved in alternative splicing (exon 21, 48 bp) is indicated as a grey box. Introns are represented with V-shaped lines connecting exons. White boxes represent the 3'-UTR of the mouse *Slc12a2* gene. The transcript region predicted to be amplified with primer set NKCC1-525a/477b is also shown. C) RT-PCR using total RNA from the β -cell line INS-1E, mouse islets [wild-type (WT)] and the indicated rat tissues. The NKCC1-525a/477b primer-set, represented in B, was used to detect and discriminate expression of NKCC1a and NKCC1b mRNAs in a single PCR reaction using rat or mouse total RNA as template. A band of predicted size (525 bp) corresponding to NKCC1a is detected in islets, INS-1E β -cells and in rat tissues tested, whereas two bands corresponding to NKCC1a and NKCC1b (577 bp) were detected in total RNA obtained from rat dorsal root ganglia (DRG). As negative control for RT-PCR reactions, water instead of RNA was used. The primer set used in the control reaction was GAPDH-555. D) DNA sequence chromatogram of the band of 525 bp obtained by RT-PCR from INS-1E β -cells. Indicated is the region encompassing exons 20, 21 and 22. E) Immunoblot analysis of protein extracts obtained from INS-1E β -cells, mouse pancreas or islets of the three genotypes i.e., WT, heterozygous (HE) and knock-out (KO) probed against NKCC1 using a monoclonal antibody directed against the C-terminus of human NKCC1.

NKCC1b and that this variant may not be exclusive of the mouse and/or any particular tissue. To confirm this, we used RT-PCR to detect NKCC1a and NKCC1b transcript expression in mouse islets, INS-1E β -cells and different rat tissues such as kidney, pancreas, stomach, colon and dorsal root ganglia. The latter tissue was used as positive control for the co-expression

Fig. 2. β -cells express NKCC2A. A) Schematic representation of the rat NKCC2A (member 1) mRNA sequence of reference (NKCC2A, NM_019134). This mRNA is arranged in ~4.6 kb of nucleotide sequence transcribed from the rat *Slc12a1* gene (Gene ID: 25065, NC_005102.2 positions 112455897 to 112534752). The gene contains 32 exons (filled grey arrows), 26 of which encode for a protein (NKCC2A NP_062007) including the unique exon A (exon 5, white arrow). Exon numbering is relative to the first coding exon *i.e.*, the one that harbors the first ATG (exon 1). The predicted encoded protein of 1095 amino acids is indicated as a black arrow atop the exons of the transcript. Underneath, the predicted RT-PCR fragments to be obtained by using NKCC2 primer sets indicated in Table 1. These fragments are represented as black arrows labeled NKCC2-533, NKCC2-1010, NKCC2-700 and NKCC2-878 to indicate that these primer sets are predicted to generate bands of 533, 1010, 700 and 878 bp, respectively. B-E) Expression of NKCC2 mRNAs in islets of WT mice (B), RIN-m5F (C), INS-1E (D) and MIN6 (E) β -cell lines. Reverse transcription coupled to PCR of islets or confluent cells' total RNA was performed using the primer sets indicated in A. Shown are representative 2 % agarose gels where bands of predicted sizes for NKCC2 are detected *i.e.*, 533 bp (WT islets), 1010 bp (RIN-m5F), 700 bp (INS-1E) and 878 bp (MIN6). As negative control, RT-PCR reactions were performed using H₂O instead of total RNA and a primer set designed to amplify transcripts for GAPDH. F) Real-time quantitative PCR analysis of NKCC1 and NKCC2A transcripts expressed in INS-1E β -cells. Bars represent NKCC1 and NKCC2A mRNA levels (*mean* $\pm$ SEM) relative to β -actin. Values obtained from three different experiments performed in triplicate. G) Partial nucleotide sequence of the RT-PCR amplicon obtained with NKCC2-700 primer set. The 700 bp RT-PCR product amplified from INS-1E cells was purified and sequenced in both directions by PCR using the same primers (sense and antisense) used in the original amplification. Shown is a representative chromatogram from 6 different amplification reactions. The sequence chromatogram corresponds to the region encompassing exons A/B/F of the rNKCC2 transcripts. H) Alignment of chromatogram's nucleotide sequence against rat NKCC2A sequence of reference. Showing is an exact match with exon A (exon 5) of NKCC2A (NM_019134).

of NKCC1a and NKCC1b [34]. To this end, we took advantage of the fact that rat and mouse ESTs potentially encoding for NKCC1b mRNA lack 48 bp due to alternative exclusion of the 21st exon (Fig. 1A) and this difference can be easily detected in PCR reactions designed to co-amplify both transcripts. We used an NKCC1-specific set of PCR primers named here NKCC1-525a/477b (Fig. 1B, Table 1) designed to co-amplify in a single RT-PCR reaction either rat or mouse NKCC1a and NKCC1b transcripts and to produce two amplicons differing in length by

Primer set	sense	antisense	Position	GeneBank	Species
NKCC1-525a/477b	CCTGGGATTTAAGAAAGATT	CCACCAATGAATACTCGGAT	2636-3161	NM_019194	mouse
NKCC2-533	TGCTCTTCATTGCGCTCTCCTGGATTGTAG	TGGCCCAAGTTTCCGCAATATAGATGC	572-1105	NM_019134	rat
NKCC2-1010	CACACAAACACATATTACCTACAAACCTTTG	CCTATGTAGGCGACGGTGGTGATGAAATG	262-1272	NM_019134	rat
NKCC2-700	TGCTCTTCATTGCGCTCTCCTGGATTGTAG	CCTATGTAGGCGACGGTGGTGATGAAATG	572-1272	NM_019134	rat
NKCC2-878	CACGGTGGTGATTCTCTAGGGATCTCAGT	CCGGCTCCATCCTGGCGATTGG	909-1786	NM_019134	rat
NKCC2-686	CAGAAGGCGGCTGAATGGGAGG	ATCTCCAGGCATCCCATCTCCATTAG	exon -2-471	NC_005102	rat
NKCC2-920A	ACACCAGGGGCTATGAGATGTTCTAA	TGACAACCCAGTGATAGAGGTTACCA	exon -1-exon A	BC087017	rat
NKCC2-929A	ACACCAGGGGCTATGAGATGTTCTAA	TGCAGAAGTTGACAACCCAGTGATAG	exon -1-exon A	BC087017	rat
NKCC2-943A	ACACCAGGGGCTATGAGATGTTCTAA	CCGTTTGTGCTATTGCAGAAGTTGA	exon -1-exon A	BC087017	rat
GAPDH-555	GTGAAGGTCGGAGTCAACGGATT	CACAGTCTTCTGGGTGGCAGTGAT	9-564	NM_002046	human

Table 1. Primer-set sequences and identification. The sense and antisense sequences of PCR primers used in this study are shown. PCR primers were designed according to mouse, rat or human cDNAs and/or *RefSeq* nucleotide sequences. PCR primers were designed to amplify transcript isoforms (*i.e.*, NKCC1 and NKCC2) and to co-amplify splice variants (*i.e.*, NKCC1a, NKCC1b, NKCC2A, NKCC2B and NKCC2F). Primer sets composed by sense (*forward*) and antisense (*reverse*) primers are named indicating the amplified transcript *i.e.*, NKCC1, NKCC2, GAPDH, followed by numbers reflecting the predicted length of the RT-PCR amplicon in base pairs (bp). *GenBank* accessions numbers for cDNA clones used as templates to generate primer sets are indicated as numbers preceded by the prefix BC, whereas *RefSeqs* accession numbers are shown as NM_ numbers. Positions are relative to the first in-frame coding ATG.

48 bp. As shown in Figure 1C, RT-PCR products of predicted sizes for NKCC1a transcripts (525 bp) are detected in islets, β -cell lines and rat tissues. As expected, transcripts corresponding to NKCC1a and NKCC1b were co-amplified in dorsal root ganglia. Expression of NKCC1a as the sole *Slc12a2* variant in islets and β -cell lines was further confirmed by direct sequencing of the purified cDNA fragment obtained with the NKCC1-525a/477b primer set in INS-1E β -cells (Fig. 1D).

At the protein level, NKCC1 expression was confirmed in INS-1E β -cells, pancreas and mouse islets by immunoblotting using a monoclonal antibody directed against the C-terminus of human NKCC1. As shown in Figure 1E, NKCC1a is expressed in these cells and tissues as a major band of ~170 kDa, consistent with its N-glycosylated molecular weight (MW) [18]. As expected, NKCC1 expression was not found in islets purified from mice deficient in *Slc12a2* expression (KO, Fig. 1E left panel). Taken together, these results suggest that only one splice variant of NKCC1 *i.e.*, NKCC1a is expressed in β -cells.

β -cells express NKCC2A

Next, we determined the expression pattern of *Slc12a1* gene products in β -cells. Expression of NKCC2 transcript variants was analyzed by RT-PCR in mice islets and in three β -cell lines: INS-1E, RIN-m5F and MIN6. Reverse transcribed total RNA from these tissues/cells was PCR-amplified using several species-specific sets of PCR primers designed to co-amplify splice variants of NKCC2 *i.e.*, NKCC2A (*Slc12a1* variant 1), NKCC2B (*Slc12a1* variant 2) and NKCC2F (*Slc12a1* variant 3) (Fig. 2A, Table 1). To amplify *Slc12a1* transcripts we used four different primer sets: NKCC2-533, -1010, -700 and -878 (Fig. 2A, Table 1). As shown in Figures 2B-E, mouse islets (B) and rat/mouse β -cell lines (C-E) express NKCC2 at the transcript level. Since NKCC2 transcripts appear to be present in extra-renal tissues at very low levels [13], we determined NKCC1 and NKCC2 mRNA levels in INS-1E β -cells by using reverse transcription coupled to quantitative real-time PCR. As shown in Figure 2F, NKCC2 transcripts are expressed at lower levels when compared to NKCC1 or to β -actin.

To establish molecular identity of RT-PCR amplicons, each PCR product obtained was sequenced in both directions. The partial nucleotide sequence of the RT-PCR fragment obtained from INS-1E β -cells (700 bp) is shown in Figure 2G. *In silico* analysis of this sequence demonstrated ~99 % identity to the corresponding region of rat NKCC2A sequence of reference (*RefSeq* NM_019134, Fig. 2H). Neither exon B nor F at the nucleotide sequence level was found in this fragment. The nucleotide sequence of PCR fragments of 533 and 1010 bp also confirmed the presence of exon A sequences (*not shown*).

Fig. 3. NKCC2A mRNAs contain alternatively spliced 5'-UTRs. A) *In silico* alignment of the nucleotide sequence of rat NKCC2As corresponding to the 5'-untranslated and coding regions. Shown are: the sequence or reference (NM_019134), the kidney-derived clone (BC087017) and the two sequences obtained from β -cells (JN579708 and JN579707). B) Representation of the rat NKCC2A 5'-UTR sequence of reference (NM_019134) aligned against the kidney-derived rat NKCC2A (BC087017). Shown is the non-coding exon of 64 bp (*exon -1*) located immediately up-stream exon 1. Also shown is the most distal non-coding exon of the rat *Slc12a1* gene (22 bp, *exon -2*) responsible for coding the first 22 nucleotides of the 5'-UTR. This exon is located 10 bp down-stream a putative transcriptional initiation site (TTS, red arrowhead). The sense and antisense PCR primers used to amplify the genomic region of the rat *Slc12a1* gene are indicated as opposite blue arrowheads. The alternative splicing of the 5'-UTR region of NKCC2A expressed in INS-1E and RIN-m5F is also indicated.

To provide convincing evidence of NKCC2A expression as the sole transcript of the *Slc12a1* gene expressed in β -cells, the complete open-reading frame (ORF) containing 3'/5'-UTR sequences of NKCC2A mRNAs were PCR-amplified from INS-1E and RIN-m5F cells in overlapping fragments and fully sequenced in both directions. The full 5'-UTR of NKCC2A mRNAs were obtained using exon-specific RT-PCR primers with sequences designed based upon the nucleotide sequence of the rat *Slc12a1* gene (NC_005102, position 112455897 to 112534752), *in silico* predicted exon/intron boundaries and deduced transcriptional start sites (TTS) [13]. The full-length nucleotide sequences of NKCC2A expressed in INS-1E and RIN-m5F β -cell lines have been submitted to *GenBank* under accession numbers JN579707 and JN579708, respectively.

Molecular characterization, *in silico* analysis and expression pattern of NKCC2A

In silico comparison of the predicted translated products of NKCC2A cDNAs cloned from rat β -cell lines (JN579707 and JN579708) and rat kidney (BC087017) reveals ~100 % amino acid identity (*not shown*). However, the full-length nucleotide sequence of rat β -cell NKCC2A

Fig. 4. Expression pattern and characterization of NKCC2A mRNAs with different 5'-UTRs. A) Non-scale, partial representation of rat NKCC2A transcripts encoding *exon -2* (white arrow, top) or *exon -1* (black arrow, bottom) in their 5'-UTRs. Exons are represented as grey arrows and are numbered according to the first coding one. Exon A (exon 5) is indicated as a red arrow whereas the coding region of this partial representation is indicated as a solid green bar on top of exons. Primer sets used for RT-PCR amplification of NKCC2A transcripts differing in 5'-UTRs are indicated. Primer set rNKCC2-686 (*exon -2*)-specific is designed to amplify rat NKCC2 transcripts expressing *exon -2* as a single band of 686 bp. Primer sets rNKCC2-900As (*exon -1* and A)-specific comprise three pairs of primers: rNKCC2-920A, -939A and -943A. These primer sets share identical sense primers but three different ones specific for rat exon A. B-E) Expression pattern of NKCC2A transcripts in INS-1E (B), rat colon (C), rat pancreas (D) and rat kidney (E). Bands of predicted sizes for NKCC2A transcript expressing *exon -2* (686 bp) are detected in these rodent tissues whereas mRNAs encoding for *exon -1* were only found in kidney. Asterisks denote the fragments sequences shown below. F) Partial nucleotide sequence of the RT-PCR amplicons obtained with NKCC2-689 and NKCC2-943A primer sets. The 689 and 943 bp RT-PCR products amplified from pancreas and kidney were purified and sequenced by PCR using the antisense primers. Shown is a representative chromatogram from 3 different amplification reactions. The sequence chromatogram corresponds to the region encompassing exons *exon -2*, *exon -1* and exon 1 of the rNKCC2A transcripts.

cDNAs and NKCC2A cDNAs cloned from rat kidney was different in many instances, including the third nucleotide of several codons and, in particular, their 5'-UTRs. As shown in Figure 3A, alignment of NKCC2A 5'-UTRs demonstrates that the rat *Slc12a1* gene generates two differentially spliced NKCC2A mRNAs. To provide experimental support to this new finding

Fig. 5. NKCC2A localize in β -cells of the islets. A) Immunoblot of INS-1E, pancreas from mice lacking NKCC1 (KO) and islets of the three genotypes (WT, HE and KO). Detected are bands corresponding to the core molecular weight of rat NKCC2A (~120 kDa) and to its fully N-glycosylated version (~165 kDa). B) Immunoblot of protein extracts obtained from whole rat kidney and different anatomical regions of the kidney: cortex (outer), medulla (outer), papilla and basolateral membranes purified from renal cortexes (BLM). Shown is the immunological presence in kidney extracts of NKCC2 as a single band of ~165 kDa corresponding to the fully N-glycosylated protein and as several bands of MWs ranging from ~120 kDa to ~165 kDa in outer medulla, corresponding to NKCC2 with various degrees of N-glycosylation. C-D) Immunoblot of protein extracts from the indicated rat tissues (C) and its amido-black stain (D) as loading control. For clarity, kidney samples were omitted from the original membrane due to the overwhelming signal with NKCC2 antibodies.

Note immunoreactive bands corresponding to NKCC2 proteins of 165 kDa, 132 kDa and 122 kDa in small intestine and stomach, but not in other tissues where NKCC1 is abundantly expressed. E) Representative indirect immunofluorescence of a pancreas slide obtained from a mouse lacking NKCC1 (NKCC1^{KO}) showing immunoreactivity in most but not all cells of the islet. F) Positive control for NKCC2 expression using kidney slides from NKCC1^{KO} mice. Shown is the typical immunoreactive distribution of NKCC2 in this tissue *i.e.*, the apical region of the tubular cells (white arrowheads) as well as intracellular compartments. G-J) Co-immunolocalization of NKCC2A and pro-insulin in rat pancreas. Pancreas slides were co-immunostained using a monoclonal antibody directed against pro-insulin (GS-9A8) and anti-NKCC2 antibodies. FITC- and Cy3-conjugates secondary antibodies were used to label pro-insulin and NKCC2A, respectively, whereas nuclei were counterstained using DAPI.

and to rule out the possibility of β -cell line-related re-arrangements of the *Slc12a1* gene, the genomic region encompassing the predicted TSS and the first A¹TG of the rat *Slc12a1* gene (~1.6 kb) was PCR-amplified from INS-1E β -cells, sequenced and compared to the relevant

Fig. 6. NKCC1a and NKCC2A co-localize in β -cell lines. INS-1E β -cells were grown under basal conditions on glass coverslips. Cells were fixed and co-immunostained using a monoclonal antibody directed against human NKCC1 and anti-NKCC2 antibodies. FITC- and Cy3-conjugated secondary antibodies were used to label NKCC1 and NKCC2A, respectively whereas nuclei were counterstained using DAPI.

region of the rat *Slc12a1* gene posted in NCBI map viewer (NC_005102). As illustrated in Figures 3B-D, sequence analysis of the amplified genomic region demonstrates the presence of two non-coding exons: named here *exon -2* (22 bp) and *exon -1* (64 bp), located 10 and 75 bp downstream TSS, respectively. *Exon -2* and *exon -1* are separated by a classic intron of 52 bp (*intron -2*), whereas an intron of 966 bp (*intron -1*) separates *exon -1* from exon 1, the one harboring the first in-frame A¹TG codon. Together, our results suggest that the *Slc12a1* variant 1 (NKCC2A) expressed in β -cells is the result of an alternative splicing involving *exon -2* and exon 1.

To determine the expression pattern of NKCC2A transcripts containing *exon -2/-1* we performed RT-PCR experiments using *exon -2/-1*-specific primers and total RNA from pancreas, β -cells and different rat tissues where NKCC2 mRNAs have been found *e.g.*, kidney [19] and colon [35, 36]. To this end we used different sets of primers designed to co-amplify NKCC2A mRNAs containing *exon -1* (NKCC2-920A, -929A and -943A bp, *i.e.*, ~900As bps) or *exon -2* (NKCC2-686) (shown in Fig. 4A, Table 1). As shown in Figure 4, *exon -1* in NKCC2A mRNAs is not found in INS-1E β -cells (Fig. 4B), colon (Fig. 4C) or pancreas (Fig. 4D), but in kidney (Fig. 4E), whereas NKCC2 mRNAs containing *exon -2* (686 bp) were found in these cells/tissues. To confirm molecular identity, the antisense strand sequence of RT-PCR fragments from kidney and pancreas was obtained using several nested reversed primers. Figure 4F shows that NKCC2A expressed in pancreas lacks *exon -1*. Hence, our results suggest that the *Slc12a1* gene produces at least two NKCC2A transcripts in the kidney, which are different in their 5' ends, but only one outside the kidney, the one that lacks *exon -1*.

NKCC2A and insulin are co-expressed in β -cells

NKCC1 is expressed in β -cells but not in glucagon secreting α -cells [9] where $[Cl^-]_i$ seems to be below equilibrium [37]. To correlate the presence of full-length NKCC2A mRNAs in insulin-secreting β -cells lines with expression of its predicted product, NKCC2A protein expression was determined in β -cell lines and control tissues. To this end, protein extracts from INS-1E cells, mouse pancreas and islets obtained from normal mice (WT) or lacking one or two alleles of NKCC1 (HE and KO, respectively) were immunoblotted against a polyclonal antibody directed against the C-terminus of rat NKCC2. As shown in Figure 5A, NKCC2A is detected in β -cells, mouse pancreas and islets as two bands, one of ~120 kDa and another of ~165 kDa. The presence of NKCC2A in β -cells, pancreas and islets as bands of 120 kDa (predicted core MW) and ~165 kDa (N-glycosylated) is consistent with the known MWs of NKCC2 when expressed in the kidney [38, 39]. To corroborate this, the immunological presence of NKCC2 was determined in blots of proteins extracts obtained from whole kidney, outer cortex, outer medulla, papilla and basolateral membranes (BLM) purified from kidney

cortexes by Percoll density gradient centrifugation. As expected, NKCC2 is expressed as a major band of ~165 kDa in kidney and as several bands of MWs ranging from ~120 kDa to 165 kDa in kidney medulla (Fig. 5B) [40-43]. Notably, no immunological reaction was observed with anti-NKCC2 antibodies in BLM where NKCC1 is known to be expressed [44], thus suggesting that NKCC2 antibodies are unable to detect NKCC1. To support this notion, the immunological presence of NKCC2 was determined in a panel of rat tissues. As shown in Figure 5C, NKCC2 is undetectable in protein samples from heart, liver, lung, muscle spleen and ovary, most tissues known to express abundant NKCC1 [18]. However, immunoreactive bands corresponding to NKCC2 proteins were found in intestine and stomach, in accordance with recent findings [35, 45].

To corroborate that NKCC2A is expressed in islets, particularly in insulin-containing β -cells and not in any cell of the islet, pancreas from NKCC1^{KO} mice were immunostained against NKCC2. Immunostaining of pancreas sections obtained from these mice revealed the presence of immunoreactive NKCC2A as a fine punctate distributed throughout the cytoplasm of most but not all cells of the islet (Fig. 5E). As control for NKCC2 immunostaining, kidney from a NKCC1^{KO} mouse was used (*inset*, Fig. 5F). To provide further evidence of NKCC2A protein expression in β -cells, we performed double labeling immunofluorescence experiments in mouse WT pancreas using a monoclonal antibody against pro-insulin, a marker of β -cells, and NKCC2. As shown in Figs. 5G-J, NKCC2A is detected in cells of the islet that express pro-insulin (β -cells), thus demonstrating that NKCC2A is expressed in β -cells. It is important to note that there was little if any NKCC2 staining in the exocrine secretory pancreas (Fig. 5H), where NKCC1 is abundantly expressed [46] thus minimizing the possibility that NKCC2 antibodies cross-react with endogenous NKCC1.

To extend and further confirm co-localization of NKCC1 α and NKCC2A in β -cells, co-immunofluorescence experiments were performed in the rat β -cell line INS-1E. As expected, NKCC1 α and NKCC2A are co-expressed in these β -cells (Figs. 6A-D). Similar results were obtained with mouse MIN6 β -cells (*not shown*). Taken together, these results suggest that at least two different mechanisms involved in $[\text{Cl}^-]_i$ regulation *i.e.*, NKCC2A and NKCC1 α are co-expressed in insulin secreting β -cells providing evidence in support of the notion that both co-transporters may be involved in the regulation of insulin secretion.

Discussion

The concept that $[\text{Cl}^-]_i$ in β -cells plays an important role in the first phase of glucose-induced insulin secretion has been recognized long ago [47] and confirmed thereafter by many investigators [7-9, 48-51]. The fact that β -cells maintain $[\text{Cl}^-]_i$ above equilibrium [7] formed the basis for the “volume regulated anion channel” hypothesis for glucose-induced insulin secretion [3]. Indeed, ample evidence supports the notion that electrogenic Cl^- efflux from β -cells modulates the well-known effect that glucose has on membrane depolarization and insulin release. This is supported by the finding that $[\text{Cl}^-]_i$ in β -cells is kept above equilibrium by a mechanism that is sensitive to loop-diuretics (*e.g.*, BTD, furosemide). Therefore, NKCC inhibition impairs glucose-induced insulin secretion, a concept that has found experimental support in several pharmacological experiments [7, 8, 11].

Using RT-PCR, NKCC1 mRNA expression was confirmed in β -cells and islets and its molecular identity established as NKCC1 α (Fig. 1). Interestingly, transcripts corresponding to NKCC1 β *i.e.*, lacking exon 21, were undetectable in other rat tissues such as pancreas, kidney, colon and stomach (Fig. 1C). These were expected findings based on the current notion that NKCC1 α , not NKCC1 β is abundantly expressed in polarized epithelia [52], which is abundant in our tested tissues (colon, stomach, pancreas and kidney). Further, in agreement with recent work [34], we found NKCC1 α and NKCC1 β transcripts in neurons of dorsal root ganglia, therefore minimizing the possibility that undetectable levels of NKCC1 β in β -cells are the result of inappropriate primer design. Based on chimeric constructs between

human NKCC1 α and rabbit NKCC2A and site-directed mutagenesis, it has been proposed that residues encoded in exon 21 are involved in basolateral sorting of NKCC1 α in polarized cells [52]. Since β -cells are non-polarized, the residues of exon 21 of NKCC1 α in these cells may be involved in different properties of the co-transporter. Interestingly, exon 21 in NKCC1 α encodes a putative phosphorylation site for protein kinase A [15], a potential modulator of NKCC1 activity [14]. However, absence of exon 21 in genetically engineered human NKCC1 constructs does not affect NKCC1 function when over-expressed in polarized cells [53]. Therefore, the role of exon 21 of NKCC1 α expressed in β -cells remains to be established.

Our results demonstrate that NKCC2 is expressed in insulin-secreting β -cells (Fig. 2). This unexpected finding argues against the paradigm that NKCC2 mRNAs are exclusive of the kidney [24]. Nevertheless, it has become clear that the *Slc12a1* gene can also express its transcripts outside tubular cells. Molecular, immunological and/or functional evidence of NKCC2 was found in neurons of mesenteric ganglia, secretomotor enteric neurons [35, 36], cells of the gastric, intestinal, endolymphatic sac, olfactory epithelia [35, 36, 54-57], and starburst amacrine cells of the retina [58]. In fact, *Slc12a1* gene's transcripts appear to be present widely among tissues, although at lower levels when compared to kidney [13]. In line, real time quantitative PCR experiments demonstrate that NKCC2A transcripts are expressed at lower levels when compared to NKCC1 (Fig. 2F) raising the possibility that NKCC2A may play a secondary/less prominent role in total cellular NKCC transport.

Our experiments also demonstrate that it is the *Slc12a1* variant 1 (NKCC2A) the NKCC2 spliced version expressed in β -cells and outside the kidney (Figs. 2-4). Expression of NKCC2A as the sole splice variant of the *Slc12a1* gene expressed in β -cells is supported by the following facts: *i*) the nucleotide sequences corresponding to NKCC2B and/or NKCC2F mRNAs were absent in sequence chromatograms of RT-PCR amplicons encompassing exons A, B and F (Fig. 2), *ii*) tile-cloning of whole *Slc12a1* mRNAs by PCR from two rat β -cell lines (INS-1E and RIN-m5F) shows only NKCC2A sequences, and *iii*) specific polyclonal antibodies directed against NKCC2 detect its immunological presence in INS-1E β -cells, islets and pancreas of WT mice (Fig. 5), but not in exocrine pancreas (Fig. 5C) where NKCC1 is abundantly expressed [46]. The latter finding argues against the notion that NKCC2 antibodies may cross-react with endogenous NKCC1. Indeed, no immunological reaction was observed with anti-NKCC2 antibodies in BLM purified from the renal cortex where NKCC1 is known to be expressed [44]. Furthermore, NKCC2 was undetectable in protein samples from heart, liver, lung and muscle, tissues known to express abundant NKCC1 [18]. Interestingly, NKCC2 proteins were found in intestine and stomach, in accordance with recent findings [35, 45]. Together, these results indicate that NKCC2 antibodies are unable to detect ubiquitous NKCC1 in protein samples and in tissues.

In silico analysis of full-length rNKCC2 cDNAs cloned from two different β -cell lines demonstrated the presence of an open reading frame (ORF) predicted to encode a protein of 1096 amino acids. However, the nucleotide sequence corresponding to the 5'-UTR region of β -cell NKCC2A was different from BC087017 and from the rat NKCC2A mRNA sequence of reference posted in *RefSeq* database (NM_019134). Indeed, an alternative splicing event was evident in NKCC2A 5'-UTR (Fig. 3). We found two short non-coding exons (*i.e.*, exon -2 and exon -1) located downstream from the putative TATA box and up-stream from the first coding exon of the rat *Slc12a1* gene. Hence, we provide for the first time evidence that NKCC2A mRNAs expressed in β -cells or outside the kidney are the result of alternative splicing involving exon -2 and the first coding exon 1 of the *slc12a1* gene. Although the differences in NKCC2A 5'-UTRs are due to a variation between NKCC2A mRNAs expressed in the kidney and non renal tissues, we did not find evidence of exon -2 expression in the form of ESTs from many species (*data not shown*). However, the nucleotide sequence of the first cloned full-length rat *Slc12a1* (variant 3, NKCC2F, U10096) does contain exon -2/exon 1 splicing, thus demonstrating that the rat *Slc12a1* gene can express exon -2 in the rat kidney, at least as part of variants 1 and 3. Exon -1, however, was found in a single EST from rat kidney (CK483746) and in a full-length NKCC2A cDNA (BC087017).

Therefore, two NKCC2A transcripts can be found in rat tissues which are different in their 5' ends: one encoding *exon -2* in the kidney and extra-renal tissues, and another encoding *exon -1* in the rat kidney only. The existence of two NKCC2A mRNAs encoding for one protein in rat kidney tissues suggests that either different TTSs and/or core promoter elements are functionally present in the rat *Slc12a1* gene, as bioinformatic analysis of *Slc12a1* genes have already predicted [13]. Notably, election of alternative promoters impacts most commonly the 5' non-coding region [59]. Further, the use of alternative promoters in the 5'-UTR region of a gene is often coupled to alternative exon splicing thus producing mRNAs with different 5' ends [60]. This difference may impact NKCC2A expression levels outside the kidney, either in a developmental stage-specific or temporal manner, or affect the stability of the mRNAs or the efficiency of its translation. Further, different 5'-UTRs may affect responsiveness to specific signals. In line with these concepts, several human *SLC12A6* mRNAs with different 5'-UTRs *e.g.*, AF105366, AK292550, AF531258, DQ138323, BC070107, have been described to be the result of alternative splicing of 5' non-coding exons/introns. In turn, *Slc12a6* mRNAs are differentially expressed in rodent tissues [61] and may be regulated at the post-transcriptional level [62, 63]. Additional experiments are needed to determine if different 5'-UTR NKCC2A variants are the result of activation of different promoters acting and/or regulated in a tissue-restricted manner.

Apart from NKCC2A and NKCC1a, other genes can potentially regulate $[Cl^-]_i$ in β -cells. Some members of the anion exchanger family *Slc4a* and *Slc26a* (Cl^-/HCO_3^- exchangers, AEs) and *Slc9a* (Na^+/H^+ exchangers, NHEs) are functionally expressed in β -cells [64]. However, it is known that expression of *Slc9a1* (NHE1), the most abundant member of the *Slc9a* gene family expressed in islets, has little or no impact on glucose-induced insulin secretion [65]. *Slc4a2* (AE2), a widely expressed disulfonic stilbene (DIDS)-sensitive Cl^-/HCO_3^- exchanger [66] may be involved in $[Cl^-]_i$ regulation in β -cells. Interestingly, a DIDS-sensitive Cl^-/HCO_3^- exchanger of unknown molecular identity was proposed to be involved in transient insulin secretion under hypotonic conditions [67]. Additional candidates potentially implicated in $[Cl^-]_i$ regulation in β -cells may be the products of the *Slc12a1* and *Slc12a3* genes [14]. It has been reported that hydrochlorothiazide, an inhibitor of the *Slc12a3* gene product NCC, was able to inhibit insulin secretion from cultured islets, although by mechanisms independent of NCC's ability to regulate $[Cl^-]_i$ [68]. Interestingly, we did not find clear evidence of NCC mRNA expression in β -cells (*not shown*). Hence, it remains to be determined if the observed effect of hydrochlorothiazide on insulin secretion is due to mechanisms independent of NCC.

Based on what is well-known about NKCC1 and NKCC2 function in polarized epithelia, expression of both co-transporters in β -cells may regulate $[Cl^-]_i$ under different physiological, pathophysiological or pharmacological conditions. Indeed, NKCC2 transport K^+/Cl^- under hypertonic conditions [69] and NKCC2 is less sensitive to bumetanide than NKCC1 [70]. Further, NKCC1 co-transport water, whereas NKCC2 does not [71], and both co-transporters have different ion affinities [14]. Therefore, NKCC1a and NKCC2A may have neither overlapping functions nor be subjected to similar regulatory mechanisms in β -cells. Interestingly, NKCC1a and NKCC2A appear not only to be expressed in the plasma membrane but also in intracellular granular compartments (Fig. 6), raising the possibility that NKCCs may have a functional impact in β -cell granule biology.

In conclusion, the identification of a specific splice variant of *Slc12a1* (NKCC2A) co-expressed with NKCC1a in β -cells suggests that these two co-transporters may contribute to the control of the ion environment within these cells, particularly $[Cl^-]_i$. Although the exact mechanisms whereby $[Cl^-]_i$ regulates insulin secretion in response to glucose remain to be detailed, the electrogenic efflux of Cl^- from β -cells enabled by the action of NKCCs contributes to glucose-induced membrane depolarization and therefore insulin secretion.

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Erratum

In the article by Alshahrani S. and Di Fulvio M., entitled “Expression of the *Slc12a1* gene in pancreatic β -cells: molecular characterization and *in silico* analysis” [Cell Physiol Biochem 2012;30(1):95-112 (DOI: 10.1159/000339050)], the name of Dr. Francisco J. Alvarez-Leefmans was omitted in the submitted manuscript. His name should be included as second author of the paper.

The changes made are as follows:

Authors: Saeed Alshahrani, Francisco J. Alvarez-Leefmans and Mauricio Di Fulvio

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