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Expression of K^+–Cl^- cotransporters in the α-cells of rat endocrine pancreas

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Abstract

The expression of K^+–Cl^- cotransporters (KCC) was examined in pancreatic islet cells. mRNA for KCC1, KCC3a, KCC3b and KCC4 were identified by RT-PCR in islets isolated from rat pancreas. In immunocytochemical studies, an antibody specific for KCC1 and KCC4 revealed the expression of KCC protein in α-cells, but not pancreatic β-cells nor δ-cells. A second antibody which does not discriminate among KCC isoforms identified KCC expression in both α-cell and β-cells. Exposure of isolated α-cells to hypotonic solutions caused cell swelling was followed by a regulatory volume decrease (RVD). The RVD was blocked by 10 μM [dihydroindenyl-oxy] alkanoic acid (DIOA; a KCC inhibitor). DIOA was without effect on the RVD in β-cells. NEM (0.2 mM), a KCC activator, caused a significant decrease of α-cell volume, which was completely inhibited by DIOA. By contrast, NEM had no effects on β-cell volume. In conclusion, KCCs are expressed in pancreatic α-cells and β-cells. However, they make a significant contribution to volume homeostasis only in α-cells.

Keywords: K^+–Cl^- cotransporter; Pancreas; Islet of Langerhans; α-cell; β-cell; Insulin; Glucagon; Regulatory volume decrease; Cell volume

1. Introduction

The cation chloride cotransporters are a family of seven membrane transport proteins [1]. Each member of this family mediates the electroneutral transport of Cl^- coupled to either Na^+ or K^+ (or both). In mammals, three Na^+-coupled transporters have been identified: the widely expressed Na^+–K^+–$2Cl^-$ (NKCC1); the kidney-specific NKCC2 and the thiazide-sensitive Na^+–Cl^- (NCCl [1]). All three transporters mediate Cl^- influx driven by the Na^+ gradient. By contrast the K^+–Cl^- cotransporters (KCC) mediate Cl^- efflux driven by the K^+ gradient. KCC are encoded by four separate genes transcribing: KCC1, KCC2, at least two KCC3 isoforms (KCC3a and KCC3b which differ only in their N-terminus) and KCC4 [1]. Furthermore, the presence of additional splice variants of KCC is predicted by sequences in the EST database.

The main functional properties of all five KCCs are similar. For example, they are all blocked by furosemide and [dihydroindenyl-oxy] alkanoic acid (DIOA; [2]), they are activated by cell swelling [1,2] and by *n*-ethyl maleimide (NEM; [2]). However, the different isoforms of KCC all exhibit distinct tissue specific patterns of expression. KCC2 for instance is expressed only in neurons, whereas KCC1 and KCC3a are widely expressed in many tissues [1].

Previous studies in this laboratory [3] determined the expression of NKCC1 in the endocrine pancreas. Functional and molecular experiments showed that this protein is expressed in the insulin-secreting β-cells, but not in the glucagon-secreting α-cells [3]. In the present study, the

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expression of KCC in cells of the endocrine pancreas has been investigated.

2. Materials and methods

2.1. Pancreatic cell isolation

Isolated islet cells were prepared from pancreata of Sprague–Dawley rats (250–300 g; Charles River, UK). Animals were killed by stunning and cervical dislocation. Islets were isolated by collagenase digestion and dispersed into single cells by a brief incubation in Ca^{2+}-free medium [4]. Single cells were harvested by centrifugation and resuspended in MEM medium (HEPES-buffered) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine (Gibco). The resuspended cells were plated at the centre of 35 mm sterile plastic culture dishes (Nunc). Cells were maintained in a humidified incubator at 37 °C, and used in cell volume measurement experiments between 24 and 48 h following isolation.

2.2. Reverse transcription and polymerase chain reaction

RNA was extracted from intact islets, and the positive control tissues (kidney and brain), using an RNeasy mini kit (Qiagen) according to the manufacturers' instructions. cDNA was prepared from extracted RNA using the *avain myeloblastosis* virus reverse transcriptase enzyme (Roche). RNAse-free DNAse (Invitrogen) treatment was performed to minimise contamination of RNA by genomic DNA. The quality of the islet mRNA was assessed by performing RT-PCR for pre-pro glucagon (PPG; a marker for α-cells) using the following primers: sense 5′TGCTGGTACAAGGCA GCTGG3′ and antisense 5′GTTTGGCAATGTTGTT CCGG3′. Potential contamination of islet mRNA with mRNA from exocrine pancreas was examined by performing RT-PCR for CFTR (a marker for pancreatic duct cells; [5,6]) using these primers; sense 5′CTGCTGACCACCTGT CTGAA3′ and antisense 5′GAAGAAGGCGGAGCTA GTGA3′. Primer pairs specific for five KCC isoforms were employed: KCC1 sense 5′GTTCGCCTCACTCTTCCTG GC3′ and antisense 5′TGGGCCACCACATACAGGGA3′ (rat sequence; U55815[1]); KCC2 sense 5′CATCACAGA TGAATCTCGGG3′ and antisense 5′TTCTCTGGGTCTG TCTCCC3′ (rat sequence; U55816); KCC3a sense 5′CAG AAGCCACCACCAAGATG3′ and antisense 5′ATGAAA GTACCCATTTGGGG3′ (based on mouse sequence; AF21 1854); KCC3b sense 5′AGTAAAAGCCCGGATTCAGG3′ (based on mouse sequence; AF211855) and antisense as for KCC3a; KCC4 sense 5′AGGAAGCTGCTGAGCGCAC3′ and antisense 5′CAGCATTGTACAGGTGCAGC3′ (based on mouse sequence; AF087436).

Reaction mixtures (25 μl final volume) contained: 10× PCR buffer (100 mM Tris–HCl; pH 9.0, 500 mM KCl, 1% Triton X-100), 2.5 mM of each dNTP, 50 μM of each primer, 1 mM $MgCl_2$ and 5 U *Taq* Polymerase. Cycle parameters for KCC and PPG were an initial denaturation step at 94 °C for 4 min, initial annealing step at 58 °C for 2 min and initial extension step at 72 °C for 3 min. Followed by 40 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min 15 s with final extension for 10 min. CFTR cycle parameters were: an initial denaturation step of 94 °C for 4 min followed by 40 cycles of 94 °C for 45 s, 54 °C for 45 s and 72 °C for 1 min. All reactions were performed in a PCR sprint thermal cycler (Hybaid). PCR products were separated on an agarose gel stained with ethidium bromide, and characterised by DNA sequencing.

2.3. Immunofluorescence

Immunofluorescence methods were used to determine KCC expression in pancreatic islet cells. The affinity-purified rabbit polyclonal antibodies employed, were raised against the (N-acetylated) NH_2-terminal amino acids 1–14 of mouse KCC1 (αNT-KCC1, 1:50 dilution), and the COOH-terminal amino acids 1074–1085 of mouse KCC1 (αCT-KCC, 1:50 dilution) as previously described [7,8]. A rabbit polyclonal antibody against glucagon (1:100) and a mouse monoclonal antibody against insulin (1:100) were purchased from BioGenex (USA). A mouse monoclonal antibody against somatostatin (1:200) was obtained from BioGenesis (UK). Donkey anti-rabbit or anti-mouse IgG coupled to indocarbocyanin (CY3), and goat anti-rabbit or anti-mouse IgG coupled to FITC (Jackson Immunoresearch) were used as secondary antibodies.

Male Wistar rats were anesthetized with pentobarbital (Nembutal; 65 mg/kg i.p.) and perfusion-fixed with 2% paraformaldehyde/75 mM lysine/10 mM sodium periodate (PLP) as described by McLean and Nakane [9]. Pancreata were removed, cut into blocks and further fixed in fresh fixative overnight at 4 °C. PLP-fixed tissue was kept in PBS containing 0.02% sodium azide at 4 °C until further use.

Immunofluorescence light microscopy was performed as described by Roussa et al. [8]. Sections were rehydrated in PBS, treated with 1% SDS for 5 min, and blocked with 1% BSA/PBS for 15 min. Slides were incubated overnight at 4 °C with primary antibody. Slides were washed three times with PBS for 5 min and incubated for 1 h at 20 °C with the appropriate secondary antibodies: CY3-coupled donkey anti-rabbit IgG (1:600 dilution) and FITC-coupled goat anti-rabbit or anti-mouse IgG (1:100 dilution). Sections were washed three times with PBS for 5 min, mounted with Vectashield, and viewed with a confocal laser scan microscope (Leica, Germany). For double immunofluorescence labeling with antibodies raised in the same species, i.e. the anti-KCC and anti-glucagon antibodies all raised in rabbits,

[1] The antisense primer differs at nucleotide 2 from the sequence in U55815 (G not C).

the protocol described by Lan et al. [10] was applied with minor modifications [11]. Briefly, fixed tissue cryosections were treated with 10% FCS and 10% normal goat serum (NGS) for 10 min, and incubated with the first primary antibody overnight at 4 °C. After washing with PBS, sections were incubated with the first secondary antibody for 1 h at 20 °C, placed in 0.01 M sodium citrate buffer (pH 6.0) and heated in a microwave oven twice for 5 min at 800 W. Sections were washed with PBS, followed by an additional treatment with FCS and NGS. Subsequently, slides were incubated with the second primary antibody overnight at 4 °C, washed with PBS and incubated with the second secondary antibody for 1 h at 20 °C.

3. Cell volume measurement

Cell volume was measured by video imaging using a Nikon Diaphot 200 microscope fitted with an ECD-1000 camera (Electrim Corporation, USA). Cells were viewed through a 40× objective and saved as TIF files for subsequent analysis. The area of each image was measured using Scion image software (Scion Corporation, USA), and cell volume was calculated assuming that cells maintained a spherical shape as previously described [4]. α-Cells and β-cells were selected for study on the basis of size as discussed by Majid et al. [3], i.e. cells with volumes less than 0.8 pl were deemed α-cells while cells with volumes greater than 1.4 pl were assumed to be β-cells.

KCC activity was assessed by studying cell volume regulation in response to anisotonic solutions. Cells were superfused at 5 ml/min in an isotonic bath solution which contained (mM): 140 NaCl, 5 KCl, 5 HEPES, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 20 mannitol, (pH adjusted to 7.4 with NaOH; osmolality 300 mosM/kg H_20). Following an equilibration period (10 min) and control period (2 min), the cells were exposed to a hypotonic solution for 15 min. The hypotonic solution had the following composition (mM): 90 NaCl, 5 KCl, 5 HEPES, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose (pH adjusted to 7.4 with NaOH; osmolality 190 mosM/kg H_20). Images of the cells were recorded at 1 or 2 min intervals during the experiment. DIOA (10 μM, Sigma; an inhibitor of KCC [2]) or 5 mM sodium tetraethylammonium (TEA^+; Sigma; a K^+ channel inhibitor) were added to the hypotonic solution in some experiments. KCC expression was also investigated by exposing α-cells to isotonic solution containing 0.2 mM NEM which is a KCC activator [2].

4. Data analysis

Data (mean±S.E.) are expressed as relative cell volume, i.e. volume divided by control volume measured in isotonic solution. For statistical analysis of the volume regulation data, volume recoveries were compared by unpaired Student's *t*-test (where volume recovery is the difference between the maximum and minimum volume observed in hypotonic solution). For analysis of data from the NEM experiments volume changes were compared by ANOVA followed by Dunnett's post hoc test. The volume change was estimated from the relative cell volume *v* time plot, and was the total area of the plot which differed from a volume of 1.0.

5. Results

5.1. Expression of KCC isoforms in pancreatic islets

Expression of mRNA for KCC isoforms was examined by RT-PCR, using mRNA isolated from rat pancreatic islets. Control experiments confirmed that this mRNA contained message for PPG (α-cell marker), but not CFTR (a pancreatic duct cell marker). RT-PCR with specific primers for KCC isoforms yielded products of the expected size for KCC1, KCC3a, KCC3b and KCC4 (Fig. 1). Products were obtained with the primers for KCC2 from brain mRNA (positive control), but not from islet mRNA (Fig. 1). Sequencing of the products from islet mRNA revealed 100%, 95%, 100% and 99% identity with the published sequences for rat KCC1 (NM_019229), mouse KCC3a (AF211854), rat KCC3b (XM_342489) and rat KCC4 (XM_217744), respectively.

Fig. 2A–C illustrates the labeling pattern with the αNT-KCC1 antibody and either insulin (A), glucagon (B) or somatostatin (C) in sections of rat pancreas. Intense KCC1 immunoreactivity (Fig. 2A, red and B, green) was localized to a subpopulation of cells located at the periphery of the islet (i). Staining of acinar cells (a) was also observed (Fig. 2A, red and B, green) as previously described [8]. The KCC immunoreactivity was abolished in competition experiments when sections were incubated with the antibody in the presence of the peptide antigen (data not shown). No co-localization of KCC1 (red) and insulin (green) could be observed (Fig. 2A). By contrast, Fig. 2B shows that the glucagon (red) and αNT-KCC1 (green) antibodies stained the same subpopulation of cells. There was no co-localization of somatostatin antibody (Fig. 2C, green) and the αNT-KCC1 antibody (red).

Fig. 2D–F shows the distribution of KCCs observed using the αCT-KCC antibody, together with antibodies for insulin (D), glucagon (E), or somatostatin (F). Acinar cells (a) exhibited specific KCC staining at the basolateral cell membranes (Fig. 2D, E, F). Diffuse KCC immunoreactivity was also detected in the islet tissue (i). This immunoreactivity was reduced when sections were incubated with αCT-KCC antibody which had been pre-absorbed with the peptide antigen (data not shown). There was some co-localization of the αCT-KCC (red) and the insulin antibody (Fig. 2D, green) or glucagon antibody (Fig. 2E, green). Co-localization of the αCT-KCC and somatostatin antibodies, however, was not observed (Fig. 2F).

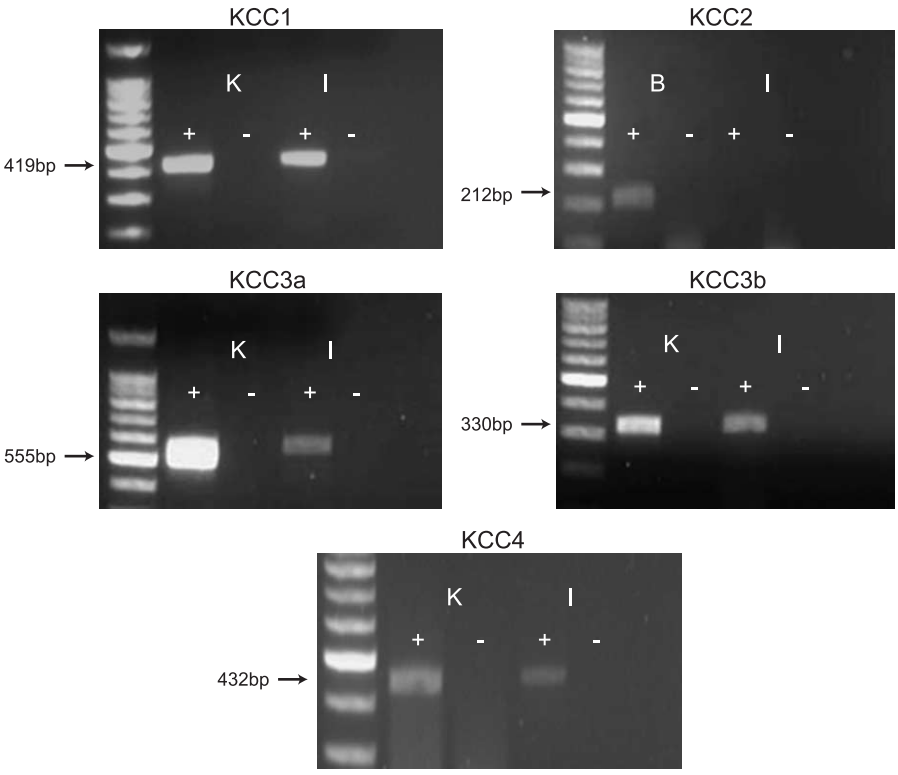

Fig. 1. RT-PCR for the KCC family on islet mRNA (I) and the appropriate positive controls (K; kidney or B; brain). (+) Indicates reverse transcriptase positive reaction products, and (−) indicates products from reverse transcriptase negative controls. Molecular mass markers are in the left-hand lane and the mass of the products indicated by the arrows.

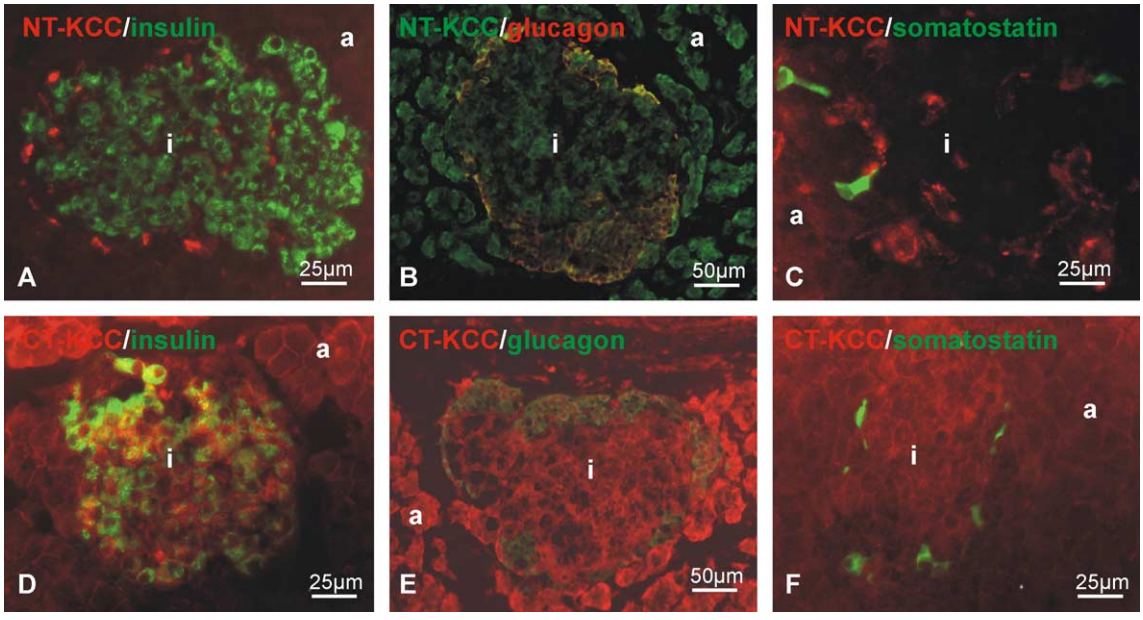

Fig. 2. Immunofluorescent localization of KCC expression in pancreatic islets. (A) αNT KCC antibody staining (red) is observed at the periphery of an islet (i) counterstained for insulin (green), and in exocrine acinar cells (a). (B) The αNT KCC antibody (green) and glucagon antibody (red) both stain the same population of cells at the periphery of the islet. (C) The αNT KCC antibody and a somatostatin antibody stain different populations of islet cells (i). (D, E) Co-staining of islet cells with the αCT KCC antibody (red) and insulin (D, green) or glucagon (E, green) antibodies. (F). The somatostatin antibody (green) and the αCT KCC antibody stain different populations of islet cell (i).

5.2. KCCs contribute to the regulatory volume decrease in pancreatic α-cells

Fig. 3A illustrates the changes in cell volume when eight α-cells were exposed to the hypotonic solution (190 mosM/kg H_2O). The response to the challenge was biphasic. There is an initial rapid increase in volume to 1.25±0.03, followed by a second phase of volume recovery to 1.05±0.02. This type of volume recovery is commonly referred to as a regulatory volume decrease (RVD). On return to the isotonic superfusate the cell volume quickly decreased below the original volume to 0.88±0.03. This response is typical of a cell which has undergone an RVD, and demonstrates that the cell has lost osmotically active contents during the RVD (e.g. K^+ and Cl^-).

In Fig. 3B six α-cells were exposed to a hypotonic solution which contained 10 μM DIOA, which is a specific KCC inhibitor at this concentration [12,13]. Cell swelling reached a maximum of 1.26±0.04 within 4 min of hypotonic exposure (not significantly different to control experiments Fig. 3A; $P>0.1$). In the presence of DIOA however, the subsequent RVD was greatly reduced so that the relative cell volume at the end of the hypotonic exposure remained elevated at 1.20±0.05. Fig. 3C shows that volume recovery is significantly reduced by DIOA compared to control ($P<0.01$ by unpaired t-test). By contrast, the RVD in α-cells was not inhibited by 5 mM TEA^+, which is a non-specific K^+ channel inhibitor. The maximum volume in the hypotonic solution was 1.31±0.04 ($n=7$; not different from control $P>0.1$) and the volume recovery in the presence of TEA^+ was 0.22±0.02 ($P>0.1$). These data suggest that RVD in α-cells is mediated by the activation of KCC.

Similar experiments examined the effects of DIOA on β-cells, in which RVD is thought to be dependent on activation of K^+ and Cl^- channels [14,15]. In the absence of DIOA, the volume recovery in β-cells was not significantly different to that in α-cells (Fig. 3C; $P>0.1$ by ANOVA). DIOA, however, did not significantly reduce the volume recovery in β-cells (Fig. 3C; $P>0.1$ by unpaired t-test).

5.3. Activation of KCCs by NEM causes a decrease in α-cell volume

NEM is a sulfhydryl alkylating agent which activates KCCs [2]. Fig. 4A illustrates the effects of exposing five α-cells to 0.2 mM NEM in the isotonic solution. Addition of NEM resulted in α-cell shrinkage, so that relative cell volume reached a minimum value of 0.85±0.03. This decrease in cell volume was partially reversed when NEM was removed from the cell (Fig. 4A). The effect of 0.2 mM NEM on cell volume was completely inhibited in the presence of 10 μM DIOA (Fig. 4B). Fig. 4C shows the effect of 10 μM DIOA on cell volume in isotonic solutions, and Fig. 4D is a time-matched control with isotonic solution.

The results from all of these experiments are summarized in Fig. 4E. This shows the volume change observed during the total period of exposure to the drugs, and the data are compared statistically to the changes in the isotonic control. NEM caused a significant decrease in cell volume compared to control ($P<0.01$), while NEM in the presence of DIOA caused a significant increase in volume ($P<0.05$). The effect of DIOA alone was significantly different to neither the isotonic control ($P>0.1$) nor the effect of NEM plus DIOA

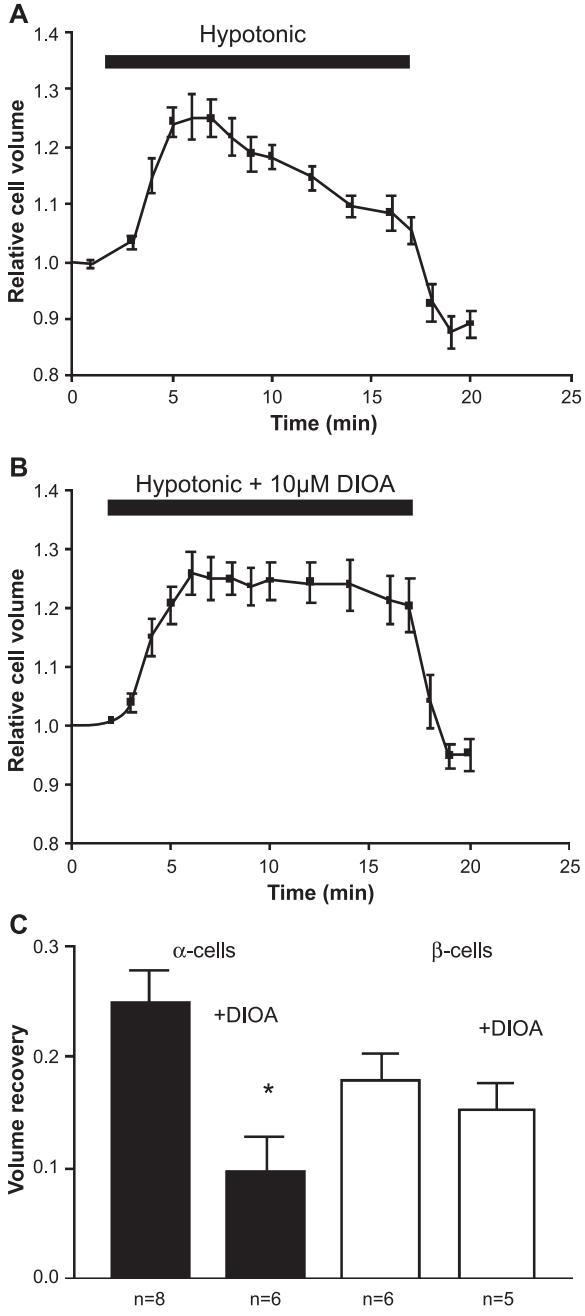

Fig. 3. The RVD in pancreatic α-cells is inhibited by the KCC inhibitor (DIOA). (A) RVD in eight α-cells in response to exposure to hypotonic solution (bar; 190 mosM/kg H_2O). (B) The RVD is inhibited when 10 μm DIOA is added to the hypotonic solution ($n=6$). (C) Volume recovery in response to hypotonic solutions in α-cells and β-cells in the presence or absence of 10 μM DIOA. *=significantly different ($P<0.05$) to control by unpaired t-test.

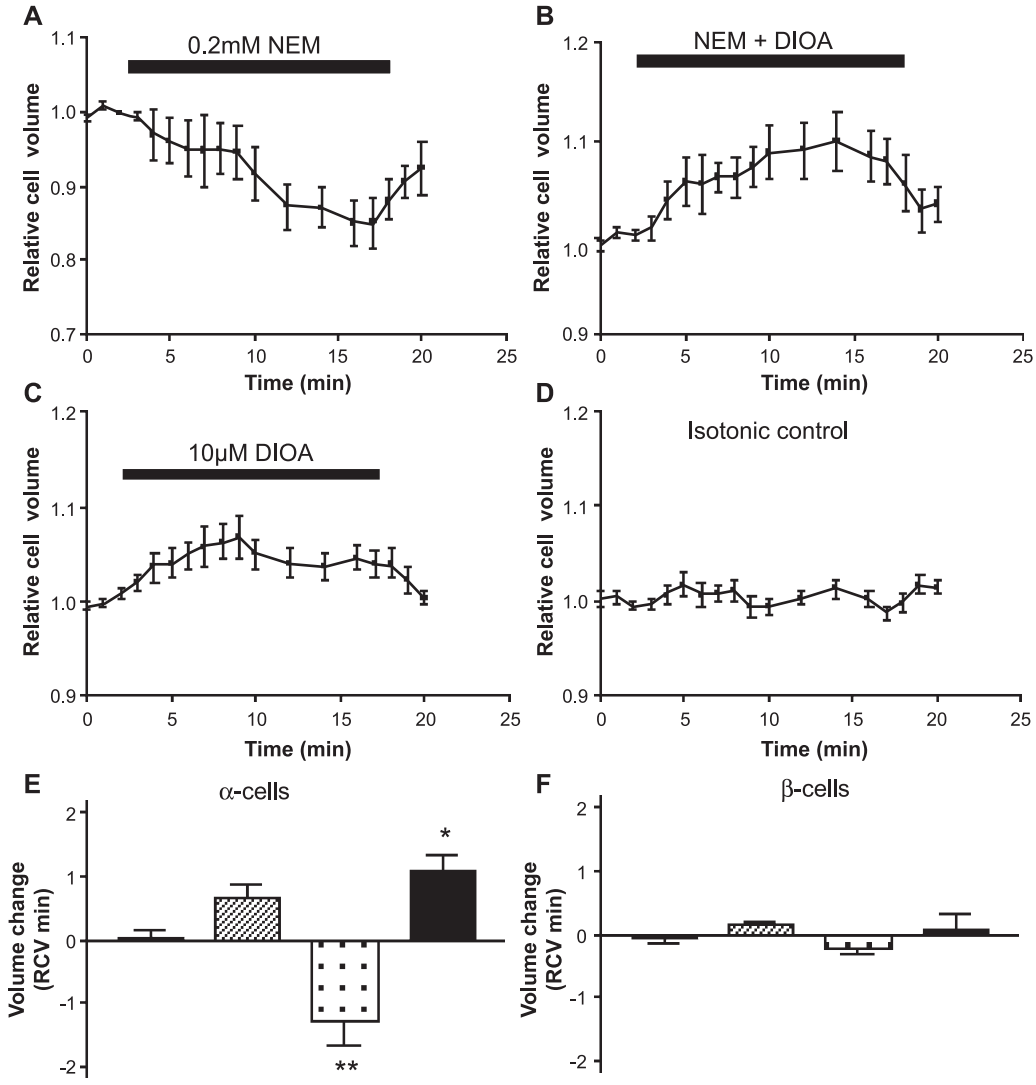

Fig. 4. NEM induced changes in α-cell volume. (A) Exposing five α-cells to 0.2 mM NEM (bar) caused cell volume to decrease. (B) α-Cells exposed to 0.2 mM NEM and 10 μM DIOA show an increase in cell volume (n=6). (C) The effect of 10 μM DIOA alone, and (D) time-matched control experiments in the isotonic solution with no drugs added. (E and F) Volume changes in relative cell volume.min (RCV.min) in α-cells (E) and β-cells (F). The bars represent: isotonic controls (open), 10 μM DIOA (shaded), 0.2 mM NEM (stippled) or DIOA and NEM (solid). ** and * are significantly different to control at P<0.01 or <0.05, respectively by ANOVA with Dunnett's post hoc test.

(P>0.1). Similar experiments were also performed on isolated β-cells. Fig. 4F shows that no significant changes in β-cell volume were observed in the presence of 0.2 mM NEM, DIOA, 0.2 mM NEM+10 μM DIOA or in isotonic control experiments (P>0.1).

6. Discussion

6.1. The expression of mRNA for KCCs and KCC protein in pancreatic islet cells

Majid et al. [3] demonstrated the expression of the cation-chloride cotransporter NKCC1 in pancreatic islets. Furthermore, both functional and immunocytochemical experiments in this study indicated that NKCC1 is expressed in the pancreatic β-cells, but not in α-cells [3]. The present study has investigated the expression of KCCs in pancreatic islet cells.

mRNAs encoding four isoforms of the KCC family: KCC1, KCC3a, KCC3b and KCC4 were identified in pancreatic islets by RT-PCR. However, there was no evidence for expression of KCC2 (the neuron-specific isoform; [16]). There are no previous reports of KCC expression in the endocrine pancreas. However, KCCs have been identified in the whole pancreas and in exocrine pancreas. KCC1, KCC3 and KCC4 have been found to be expressed in mRNA extracted from the whole pancreas [17–19]. Previous immunocytochemical studies performed have also demonstrated the expression of KCC1 in the basolateral membranes of the acinar cells in the exocrine pancreas [8]. It was also suggested that KCC3 and KCC4 may be

expressed in the exocrine pancreas [8]. The KCC expression observed in the present study is unlikely to be due to mRNA contamination from the exocrine tissue, however, because there was no evidence of mRNA for CFTR (a marker for the exocrine pancreas; [5,6]) in control RT-PCR experiments.

To investigate expression of KCC proteins in the major cell types of the endocrine pancreas (i.e. the α-, β- and δ-cells; [20]), immunocytochemical studies were performed using the antibodies previously employed by Roussa et al. [8]. Application of the αNT-KCC1 antibody revealed intense staining at the islet periphery. This staining co-localized with that for glucagon (α-cell marker), but not insulin nor somatostatin (markers of β- and δ-cells, respectively). The αNT-KCC1 antibody is known to recognize KCC1 and KCC4 in immunocytochemical experiments, but not KCC2 or KCC3a [8]. Reactivity with KCC3b has not been determined, however, there is significant homology between the first 14 amino acids in the N-terminus of mouse KCC1 (AF12118; against which the antibody was raised) and rat KCC3b (XM_342489; 50% identity), so that the antibody may also detect this protein. The data with the αNT-KCC antibody therefore suggest that KCC1 (and possibly KCC3b or KCC4) are expressed in the α-cells, but not the β-cells or δ-cells of the endocrine pancreas.

The αCT-KCC antibody showed diffuse staining throughout the islet. This antibody recognizes KCC1, KCC3a and KCC4 [8]. The data therefore suggest the possible expression of KCC3a or KCC4 throughout the islet, although not in the δ-cells. The lack of isoform specificity of the two antibodies used in the present study prevents conclusions about precisely which KCC polypeptides are expressed in the islet cells. The pattern of staining with the αNT-KCC antibody, however, suggests that there are differences between KCC expression in α-cells and β-cells. Furthermore, KCC expression in the pancreatic δ-cells was not detected.

6.2. Functional evidence for KCC expression in pancreatic α-cells

Functional studies were employed to further elucidate the cellular location of KCCs in the islet. In a first series of experiments, α-cells were shown to exhibit an RVD when exposed to hypotonic solutions. Most cells exhibit an RVD in these conditions [21], but this has not previously been reported in α-cells. RVD occurs by the loss of K^+ and Cl^- from cells either through individual K^+ and Cl^- channels or via KCCs [21]. DIOA blocked the RVD in α-cells suggesting the involvement of KCC in α-cell RVD. The RVD in α-cells was not affected, however, by the K^+ channel inhibitor TEA^+. This is in marked contrast to β-cells, where RVD is TEA^+-sensitive and occurs due to K^+ and Cl^- efflux via ion channels [14,15]. Indeed in control experiments DIOA had no significant effect on the RVD in β-cells. Taken together, these results suggest that KCCs are functional in α-cells, but not β-cells.

This conclusion is further supported by experiments performed with NEM. NEM is a thiol alkylating agent which activates KCCs by dephosphorylation of either the KCC proteins themselves or their postulated regulatory proteins which include phosphatases [2]. Addition of NEM caused α-cells to shrink, presumably by activating KCCs and thus causing the loss of K^+ and Cl^- from cells. Indeed, the effect of NEM on α-cell volume was blocked by DIOA the KCC inhibitor. Control experiments performed on β-cells revealed that NEM had no effect on cell volume. These data therefore suggest that KCCs make a significant contribution to ion transport in α-cells, but not the β-cells.

6.3. Physiological consequences of KCC activity in α-cells

The data presented in this paper together with the previous study of Majid et al. [3] indicate that there are major differences in the expression of cation-chloride cotransporters in α-cells and β-cells. KCC expression appears to be greatest in α-cells, whereas only β-cells express NKCC1. One physiological consequence of this differential distribution is that Cl^- is likely to be accumulated in β-cells, but extruded from α-cells. Indeed it is quite well established that Cl^- is accumulated in pancreatic β-cells, i.e. maintained above electrochemical equilibrium [22]. Less is known about Cl^- distribution across the α-cell membrane. Several studies however, have shown that GABA inhibits glucagon secretion [23], probably via $GABA_A$ receptor activation resulting in membrane hyperpolarization [24]. If activation of $GABA_A$ receptors is inhibitory, then Cl^- must be maintained below electrochemical equilibrium in α-cells. This would be consistent with the expression of KCCs, but not NKCC1 in α-cells. A similar pattern of cation-chloride expression has been observed in the central nervous system [25,26]. The ratio of KCC2 expression to NKCC1 expression is much greater in neurons where $GABA_A$ activation is inhibitory than in neurons where GABA is excitatory (e.g. in the immature CNS; [26]).

A study of Cl^- activities in pancreatic islet cells is now warranted. Such a study would help determine the role of the cation-chloride cotransporters in islet cells, by testing directly the hypothesis that Cl^- is distributed above and below electrochemical equilibrium in β-cells and α-cells, respectively. The timeliness of such a study is also highlighted in a recent paper which used patch clamp methods to study $GABA_A$ transmission in islets [27]. The authors were unable to conclude, because of a lack of data on Cl^- distribution, whether GABA hyperpolarizes or depolarizes Vm in α-cells [27].

In conclusion, this paper provides evidence that KCCs are expressed in the endocrine pancreas. Immunocytochemical data indicate that KCC expression differs between α-cells and β-cells, and that KCCs are not detectably expressed in δ-cells. Furthermore, functional studies indi-

cate that KCCs make a much greater contribution to ionic homeostasis in α-cells than in β-cells.

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