Role of Bumetanide on Insulin Secretion

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By

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ABSTRACT

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Insulin secretion depends on the electrical properties of the β-cells in pancreatic islets. The consensus mechanism of insulin secretion involves glucose-stimulated metabolic increase in the ATP/ADP ratio. This increase closes ATP-sensitive K+ (K_{ATP}) channels resulting in β-cell plasma membrane depolarization, which in turn activates voltage-dependent Ca^{2+} channels further resulting in Ca^{2+} influx and release of insulin stored in granules. In addition to this well-known consensus mechanism, insulin secretion also depends on electrogenic efflux of Cl^{-} ions. The Na^{+}K^{+}-2Cl^{-} cotransporters (NKCCs) maintain the intracellular concentration of Cl^{-} ([Cl^{-}]_{i}) at values higher than those predicted at thermodynamic equilibrium. This distribution of Cl^{-} makes possible the electrogenic exit of the anion from β-cells upon opening of volume-regulated anion channels (VRAC). Therefore, the prevailing notion states that disruption of the β-cell Cl^{-} gradient by inhibition of NKCCs with loop diuretics such as furosemide or bumetanide (BTD) impairs insulin secretion. Our results challenge that long-standing concept by demonstrating that long-term BTD does not impair insulin secretion in mouse β-cells in vitro and that this effect is related to changes in the NKCC expression pattern and Cl^{-} uptake in response to the diuretic.
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INTRODUCTION

Glucose Metabolism

In the post-absorptive (fasting) state, normal plasma glucose levels \textit{i.e.}, euglycemia is kept within the range of 60-115 mg/dl (3.6 mM to 6.3 mM) mainly as a result of the homeostatic balance between glucose use or disposal and its endogenous production. Under fasting conditions, \(\sim75\%\) of total glucose is normally used by tissues such as the brain (50\%) liver, gastrointestinal tissues and kidneys (25\%). The muscle and adipose tissues utilize the 25\% left of glucose in an insulin-dependent manner. Overall, whilst glucose under basal fasting conditions is used by tissues at a rate of \(\sim2\) mg/kg/min, endogenous glucose production matches that rate, thus keeping basal blood glucose levels relatively constant (DeFronzo 2004).

Two main mechanisms are involved in glucose production in the post-absorptive state. These are: glycogenolysis (glucose production from stored glycogen) and gluconeogenesis (\textit{de novo} glucose production from non-glucose precursors) (Bisschop, et al. 2000). Most of total endogenous glucose comes from the liver whereas a relatively small proportion comes from the kidneys and intestines (Figure 1) (Gerich 2010). The liver, kidney and intestines are the only organs expressing enough glucose-6-phosphatase activity; the key enzyme involved in the delivery of endogenously produced glucose into the circulation (Mithieux, et al. 2004). Although in the liver glycogen stores and \textit{de novo} biosynthesis contribute equally to the basal rate of glucose production, the kidneys provide glucose via gluconeogenesis only (Stumvoll, et al. 1997). In fact, the kidney may represent \(\sim40\%\) of total gluconeogenesis in the post-absorptive state (Meyer, et al. 2002b). In
Figure 1. Glucose metabolism: Biosynthesis and utilization.  

A. Proportional view of total endogenous glucose production through glycogenolysis (glycogen breakdown) or gluconeogenesis from *de novo* biosynthesis or from gluconeogenic substrates such as glycerol, pyruvate, lactate or amino acids. 

B. Post-absorptive *i.e.*, fasting (<12-16 hs) sources of endogenous glucose. 

C. Post-prandial (~2 hs after a meal) sources of endogenous glucose. 

D. Proportional view of total glucose uptake from tissues and its use. 

E-F. Proportional approximate tissue distribution of glucose uptake during fasting (E) or after a meal (F). Data plotted from results published in: DeFronzo (2004) and Gerich (2010).
addition, because hepatic glycogenolysis lasts until glycogen stores are depleted, a task that may require up to 48 hours (Gerich 2010), after that period of time virtually all hepatic glucose delivery comes from de novo synthesis of glucose from a variety of substrates (Consoli, et al. 1987; Landau, et al. 1996). Therefore, the balance between glycogenolysis and gluconeogenesis depends on the duration of fasting and usually, after a prolonged fasting (overnight or longer), all of endogenous glucose may be provided by hepatic, intestinal and renal gluconeogenesis (Gerich 2010). Although different substrate sources including lactate, glycerol, alanine and other amino acids can be used for gluconeogenesis, the kidney and liver may share some but not all gluconeogenic precursors. For instance, lactate is the main source of hepatic and renal glucose. In fact, the kidney prefers glutamine (Meyer et al. 2002b) whilst almost all of alanine is converted to glucose in the liver (Stumvoll, et al. 1998).

While gluconeogenesis is activated, in parallel, tissues completely oxidize endogenously produced glucose to provide energy or to produce lactate by anaerobic glycolysis, which in turn may be used mostly by the liver or in less extent by the kidneys to produce glucose via gluconeogenesis. The hepatic glucose produced from lactate returns to the muscles to produce lactate again i.e., the Cori’s cycle (Lehninger, et al. 2013). It is important to mention here that the kidneys and intestines by themselves are able to sustain euglycemia in the absence of hepatic gluconeogenesis (Mutel, et al. 2011).

In the post-prandial state or after a meal, plasma levels of glucose are heavily dependent on insulin and glucagon release from the endocrine pancreas (see bellow) as well as other hormones. It is the combination of transient high levels of plasma glucose (hyperglycemia) and glucose-stimulated plasma insulin (hyperinsulinemia) the responsible for increased glucose uptake by tissues, principally muscles, liver, kidneys and brain
and in a less extent by the gut and fat tissues (~5%). Glucose uptake facilitated or promoted by increased insulin in some but not all tissues is accompanied by inhibited glycogenolysis and a virtual cease of gluconeogenesis in the liver (Meyer, et al. 2002a). In the kidneys, insulin initially inhibits glucose release (Cersosimo, et al. 1994). However, renal gluconeogenesis is not blocked. In fact, it doubles after a meal as a consequence of a gradual increase in glucagon release (Figure 1) (Gerich 2010).

The endocrine pancreas

The endocrine unit of the pancreas, i.e., the islet of Langerhans consists of several endocrine cells, some of them with the remarkable ability to respond to changes in blood glucose levels with appropriate amounts of insulin, glucagon or other hormones. The β-cells, which account for most of all endocrine cells of the islet (Chen, et al. 2012), are the ones that sense hyperglycemia, releasing insulin, which restores euglycemia. As mentioned earlier, insulin increases glucose uptake from many peripheral tissues, promotes excess glucose storage as glycogen and prevents glycogenolysis i.e., glycogen breakdown in the liver (MacDonald, et al. 2005). However, insulin is not the only hormone produced by the islets. In fact, the islet of Langerhans regulates glucose and carbohydrate homeostasis by producing and releasing several hormones from unique sets of cells. So far the mammalian islet has five different cell types with the ability to produce different hormones. These cells are: α-cells (glucagon), δ-cells (somatostatin), PP-cells (pancreatic polypeptide), enterochromaffin-cells (serotonin), G-cells (gastrin) and a recently discovered cell type which produces a hormone able to stimulate growth hormone release and originally named GH-relin (ghrelin) (Wierup, et al. 2014). Of all the islet hormones, insulin is the only one with hypoglycemic function and therefore, impairment of the ability of β-cells to respond adequately to hyperglycemia represents a key component in the
pathogenesis of diabetes mellitus (DeFronzo 2004).

**Most common types of Diabetes Mellitus**

Diabetes mellitus (DM) can be defined as a poly-endocrine and multi-metabolic syndrome, which results from chronic, uncontrolled high levels of blood glucose due to impaired insulin secretion, action or both (DeFronzo 2004). DM is multi-metabolic, because all aspects of metabolism are impacted by the reduced or absent capacity of tissues to properly utilize glucose as the main source of energy as a consequence of insulin deficiency. Poly-endocrine, because the relative or absolute deficiency of insulin unbalances the ability of other hormones to properly exert their function aggravating the diabetic picture (DeFronzo 2004). Therefore, it is important to keep in mind that diabetes mellitus is not a single entity but comprises a group of metabolic diseases with the most notable characteristic of chronic hyperglycemia as a result of impaired insulin secretion, insulin action, or both. It is the chronically elevated glycemia the one associated with the long-term neural, micro- or macro-vascular and many other co-morbid complications observed in patients with diabetes, which in turn make the diabetic picture very difficult to control. This is highlighted in some of the most recent reviews (Ackermann and Hart 2013; Aslam, et al. 2014; Bordier, et al. 2014; Charnogursky, et al. 2014; Cunha-Vaz, et al. 2014; Mora-Fernandez, et al. 2014; Paneni, et al. 2014; Prieto, et al. 2014; Siddiqui 2014; Szuszkiewicz-Garcia and Davidson 2014; Wang, et al. 2014).

Of the many forms of DM classified by the American Diabetes Association *(Diabetes Care 27:Suppl1, S5-S10, 2004)*, the vast majority of cases fall within two main groups named Type 1 and Type 2 DM (T1DM and T2DM, respectively). T1DM is characterized by virtually absent insulin secretion due to autoimmune destruction of insulin-secreting ß-cells of the pancreas. The diabetic syndrome in T1DM appears suddenly and early in life and its treatment depends exclusively on daily external insulin injections in
order to normalize blood glucose and to allow tissues to use it. This type of diabetes is not commonly associated with obesity and usually is diagnosed in childhood. T2DM, is a significantly more prevalent entity considered non-insulin dependent and characterized by relative deficiency of insulin manifested either by impaired insulin secretion, tissue resistance to the action of insulin or a variable combination of both. The impaired insulin secretory response characteristic of T2DM correlates with variable hyperglycemia often times sufficient to impact the normal function of various tissues, but initially without clinical symptoms. Typically, impaired insulin secretion is first reflected in abnormal carbohydrate metabolism, usually diagnosed when plasma glucose levels are higher than 7.8 mM either under fasting conditions or 2 hours after a standardized oral glucose load i.e., glucose tolerance test. The detailed full diagnostic criterion adopted worldwide can be obtained from the World Health Organization Diabetes Program (www.who.int/diabetes/publications/en/).

T2DM is a common syndrome with almost endemic proportions. According to the International Diabetes Federation (www.idf.org), T2DM impacts the quality of life of ~380 million individuals in the world and its prevalence is expected to almost double by the year 2035. There are several factors involved in pathogenesis of T2DM such as genetic and environmental factors. In fact, the multifactorial nature of T2DM has made virtually impossible to elucidate the pathogenic mechanisms of the disease. As mentioned above, the main characteristic of patients with T2DM is chronic hyperglycemia whereas the myriad of complications brought about hyperglycemia is highly dependent on the population studied, environmental factors, lifestyle, obesity, stress, aging, and other factors. (Leahy 2005).

**The Biphasic Nature of Insulin Secretion**

Glucose has a direct, concentration- and time-dependent impact on β-cells promoting insulin release in a characteristically biphasic time course
The biphasic nature of insulin secretion is chiefly observed in rat and human islets but is usually weak in mouse islets and, with few exceptions, mostly absent in several rodent β-cell lines (Nunemaker, et al. 2006). The first phase of insulin secretion occurs rapidly after insulinotropic blood glucose levels, usually higher than 5.5-6.0 mM. The raise in insulin secretion stimulated by insulinotropic glucose is sharp and accounted for the insulin contained in primed secretory granules of the β-cell (Henquin, et al. 2002). This first phase of insulin secretion is the responsible for adequate postprandial glucose uptake by tissues and therefore, its impairment is clinically manifested as glucose intolerance. In other words, insufficient insulin secreted in response to glucose is not enough to clear plasma glucose at a normal rate.

The first phase of insulin secretion does not last long. It is overcome by the second phase characterized by a slow but steady increase in plasma insulin levels that last a couple of hours. The insulin secreted in this second phase represents de novo biosynthesis of the hormone by β-cells as well as insulin stored in granules not released during the first phase (Henquin et al. 2002). It is this phase the one responsible of restoring euglycemia. In T2DM patients this second phase is not usually as compromised as the first one, explaining the fact that these patients may reach values close to euglycemia, although after longer periods of time relative to normal individuals (Figure 2) (DeFronzo 2004).

**The First Phase of Insulin Secretion: The ATP-dependent (K<sub>ATP</sub>)-channel-dependent mechanism**

The best-known mechanism involved in the first phase of insulin secretion is the one dependent on ATP-sensitive K⁺ (K<sub>ATP</sub>) channels (Komatsu, et al. 2013). This mechanism has its foundations on the seminal observations of Dean & Matthews in 1968, the characteristic, albeit not unique electrophysiology of β-cells and the cloning followed by molecular,
Figure 2. Impairment or absence of the first phase of insulin secretion causes Diabetes Mellitus. In normal individuals, insulin is secreted in response to glucose in a typical biphasic manner where the first phase is prominently high and fast. In individuals with T2DM, however, the first phase of insulin secretion is lost. However, the second phase is usually seen. Contrary to the normal population or T2DM, patients with T1DM do not secrete insulin in response to glucose due to destruction of β-cells. Plots made from data published in Pfeifer et al. Am. J. Med 1981 70:579–588.

The $K_{\text{ATP}}$-dependent mechanism of insulin secretion can be summarized as follows: glucose transporters present in the surface of $\beta$-cells facilitate glucose entry into the cell where it is immediately phosphorylated by the action of a glucokinase and further metabolized via glycolysis. Glucose metabolism increases the ATP/ADP ratio resulting in the closure of $K_{\text{ATP}}$ channels and membrane depolarization due to decreased $K^+$ conductance. Plasma membrane depolarization activates voltage-dependent $Ca^{2+}$ channels promoting massive $Ca^{2+}$ influx, the ultimate trigger of insulin release from storage granules (Komatsu et al. 2013). At non-insulinotropic glucose levels (<5.5-6.0 mM) the probability of $K_{\text{ATP}}$ channels to be in the open state is high. This permits the normal "leakage" of $K^+$ ions out of the cell, which with the action of the Na/K-ATPase help maintain a resting hyperpolarized membrane potential of $-70$ mV (Ashcroft et al. 1984; Cook and Hales 1984; Dean and Matthews 1968, 1970; MacDonald et al. 2005). This mechanism, considered the consensus model of glucose-induced insulin secretion is schematized in Figure 3.

Notably, the consensus mechanism of insulin secretion has been dogmatically considered the exclusive and only one, until a series of experiments in the nineties demonstrated that $K_{\text{ATP}}$ channels are dispensable for insulin secretion [reviewed in (Best, et al. 2010)]. Further, the cloning and reconstitution of $\beta$-cell $K_{\text{ATP}}$ channels in 1995 by Aguilar-Bryan, Seino and collaborators (Aguilar-Bryan et al. 1995; Inagaki et al. 1995; Nichols et al. 1996) opened up several new avenues to study the consensus mechanism of insulin secretion. The elucidation of the minimum requirements for the $K_{\text{ATP}}$ channel to form and transport $K^+$ i.e., 4 subunits of the sulphonylurea receptor (SUR1) and 4 subunits of the inwardly rectifier $K^+$ channel 6.2
Figure 3. Simplified version of the consensus model of insulin secretion: the K\textsubscript{ATP}-dependent pathway. **A (left).** Under basal conditions, the K\textsubscript{ATP} channels of the \( \beta \)-cells are open allowing the exit of K\(^+\) ions and, with the functional activity of the Na/K-ATPase (not represented in the diagram) maintain the cell hyperpolarized at \(-70\) mV. Under this conditions, glucose metabolism within the \( \beta \)-cells is minimum and therefore the ATP/ADP ratio is low. **A (right).** Upon hyperglycemia, glucose enters the \( \beta \)-cell by facilitated diffusion via glucose transporters and is metabolized via glucolysis. The resulting increase in the ATP/ADP ratio inhibits K\textsubscript{ATP} channels depolarizing the plasma membrane to values enough to activate voltage-dependent Ca\(^{2+}\)-channels (VDCC). This allow the entrance of Ca\(^{2+}\) and the increase in its intracellular concentration; the trigger of insulin secretion. **B.** Represented are the changes in membrane potential (red), intracellular concentration of Ca\(^{2+}\) (blue) and insulin secretion in fold changes (green) under basal low or high glucose conditions.
(Kir$_{6.2}$) permitted not only to determine the mechanisms of action of sulfonylureas, the most commonly prescribed drugs to manage hyperglycemia, but also the creation of mice lacking either of the two subunits of the K$_{ATP}$ channel \textit{i.e.}, Kir-KO (Miki, et al. 1998) and SUR-KO (Seghers, et al. 2000). Surprisingly, these mice presented normal glucose homeostasis (Dufer, et al. 2004; Miki et al. 1998; Rosario, et al. 2008; Seghers et al. 2000; Szollosi, et al. 2007). These results confirmed earlier and almost forgotten experiments demonstrating that pancreatic $\beta$-cells depolarize, increase [Ca$^{2+}$],i and release insulin in response to physiological glucose concentrations in the absence of functional K$_{ATP}$ activity (Best and Brown 2009; Best, et al. 1992; Gembal, et al. 1993; Gembal, et al. 1992; Sato, et al. 1992). Altogether, these results were considered proof for the presence of additional K$_{ATP}$ channel independent mechanisms involved in insulin secretion. One of these mechanisms is directly related to the ability of $\beta$-cells to depolarize via volume-regulated anion channels (VRAC).

**The first phase of insulin secretion: Mechanisms independent of the K$_{ATP}$ channel**

In addition to ATP, glucose metabolism produces osmotically active metabolites with the natural ability to promote water influx (Beauwens, et al. 2006; Best 1999, 2002; Best and Brown 2009). Therefore, glucose metabolism swells $\beta$-cells, which in turn results in the activation of VRAC and the consequent exit of anionic osmolytes thus bringing cell volume back to normal (Hoffmann, et al. 2009). The physiology of VRAC has been extensively studied in $\beta$-cells after the pioneering work of Len Best (Best 1999), Les Satin (Kinard, et al. 2001), collaborators and others [\textit{extensively reviewed in} (Di Fulvio, et al. 2014)]. Notably, the molecular identity of VRAC remained unknown until early 2014 when two papers published almost simultaneously identified the product of the leucine-rich repeat-containing protein 8A (LRRC8A) gene as an essential component of VRAC, a multimeric
complex composed of several subunits of homologous LRRC8 genes with the ability to reconstitute in vitro a VRAC with the typical functional properties which have been known for many years, in particular hypotonicity (cell swelling)-stimulated anion fluxes and high permeability to Cl\(^-\) ions (Qiu, et al. 2014; Voss, et al. 2014).

Indeed, VRAC transports several anions but prefers Cl\(^-\) (Best 2002; Best and Brown 2009; Best, et al. 1997; Best and Yates 2010), which together with bicarbonate constitutes the most abundant anion in living cells. This implies that VRAC transports Cl\(^-\) inward or outward according to the electrochemical gradient of the anion in a given cell (see Figure 4). Now we know that in β-cells the net flux of Cl\(^-\) is outward and therefore, electrogenic and depolarizing. To explain the electrogenic, depolarizing and VRAC-mediated Cl\(^-\) efflux induced by glucose in β-cells, we have to understand how Cl\(^-\) ions are transported into the cell in order to attain an intracellular concentration ([Cl\(^-\)]\(_i\)) high enough to support its depolarizing efflux from β-cells holding a membrane potential (Em) of −70 mV (Dean and Matthews 1968). Indeed, at such Em and a physiological extracellular concentration of Cl\(^-\) around 130 mM, the Nernst equation predicts [Cl\(^-\)]\(_i\) of ~10 mM [reviewed in (Di Fulvio et al. 2014)]. Therefore, if β-cells had such [Cl\(^-\)]\(_i\), the net movement of Cl\(^-\) ions \emph{i.e.}, the algebraic sum of ions going in or going out of the β-cell would be zero and therefore, no electrogenic Cl\(^-\) fluxes could be induced by glucose. However, β-cells maintain [Cl\(^-\)]\(_i\) above thermodynamic equilibrium, \emph{i.e.}, higher than the [Cl\(^-\)]\(_i\) predicted by the Nernst equation.

Indeed, in 1978 Sehlin first suggested that \(^{36}\text{Cl}^-\) accumulates against its electrochemical gradient in isolated rat β-cells (Sehlin 1978). Twenty years later, Kozak & Logothetis demonstrated for the first time that [Cl\(^-\)]\(_i\) is maintained above thermodynamic equilibrium in rodent β-cells as calculated from its reversal potential (Kozak and Logothetis 1997; Kozak, et al. 1998) and Eberhardson and collaborators directly measured [Cl\(^-\)]\(_i\) by using
microfluorometric methods and demonstrated its levels to be above equilibrium (Eberhardson, et al. 2000). Now we known that \([\text{Cl}^-]\), in rodent \(\beta\)-cells is \(~40\ \text{mM}\) \(\textit{i.e.}\), \(3-4\) times the one predicted by thermodynamic equilibrium. Therefore, this \([\text{Cl}^-]\) does explain the electrogenic efflux of \(\text{Cl}^-\) originally demonstrated in response to insulinotropic glucose (Sehlin 1978; Sehlin and Meissner 1988).

If \([\text{Cl}^-]\) in \(\beta\)-cells is kept above thermodynamic equilibrium, it must have mechanisms for its active accumulation. These mechanisms were suspected in early experiments using \(\beta\)-cells of several species treated with loop-diuretics such as furosemide and bumetanide (Engstrom, et al. 1991; Sandstrom 1988, 1990; Sandstrom and Sehlin 1987, 1988a, b, c, d), nowadays well-known inhibitors of some \(\text{Cl}^-\) transporters of the \(SLC12A\) family of genes (Arroyo, et al. 2013) \(\textit{see bellow}\). Almost thirty years ago, Sandstrom & Sehlin (1987) established for the first time that \(\beta\)-cells contain a bumetanide-sensitive mechanism for \(\text{^36Cl}^-\) uptake (Sandstrom and Sehlin 1987). This seminal finding prompted the immediate test of the hypothesis that loop-diuretics inhibit insulin secretion. In a series of three papers, the same authors reported that a single-dose of furosemide provokes transient hyperglycemia in animal models, that this diuretic may induce glucose intolerance and that the negative impact of furosemide on insulin secretion was directly related to inhibition of \(\text{Cl}^-\) and \(\text{Ca}^{2+}\) fluxes in the \(\beta\)-cell (Sandstrom 1990; Sandstrom and Sehlin 1987, 1988a, c). The parallel discovery that \(\beta\)-cells express functional cotransport systems for \(\text{Na}^+\), \(\text{K}^+\) and \(\text{Cl}^-\) ions and that this mechanism is sensitive to furosemide (Sandstrom and Sehlin 1987, 1988a, b, c, d) gave immediate support to the hypothesis that accumulation of \(\text{Cl}^-\) in \(\beta\)-cells is active, sensitive to loop-diuretics and involved in insulin secretion. The definitive link between electrogenic \(\text{Cl}^-\) efflux and insulin secretion was established in an elegant series of experiments in mouse pancreatic \(\beta\)-cells originally performed by Sehlin & Meissner (Sehlin and Meissner 1988). Even though the pharmacological and
functional impact of bumetanide or furosemide on insulin secretion appeared well established at that time, the molecular identity of the diuretic’s targets in β-cells had to wait another ten or so years. Nevertheless, the simplified model presented in Figure 4 represents the concept that Cl⁻ exits β-cells depolarizing the plasma membrane

Cation-coupled chloride co-transporters: The SLC12A family

Of the numerous ion transporters involved in the regulation of \([\text{Cl}^-]\), several of them, nucleated in the solute carrier 12 group A (Slc12a in rodents or SLC12A in non-rodents or primates) family of cation-coupled Cl⁻ cotransporters (CCCs) are considered the prototypical Cl⁻ accumulators (Slc12a1-3 isoforms and splice variants v1, v2, v3, and so on.) or extruders (Slc12a4-7 isoforms and splice variants v1, v2, v3, and so forth) (see Figure 5). NKCC1 (Slc12a2), NKCC2 (Slc12a1) and the thiazide-sensitive Na⁺Cl⁻ (NCC) cotransporters (Slc12a3) accumulate Cl⁻ ions against its electrochemical gradient increasing \([\text{Cl}^-]_i\) to values typically above thermodynamic equilibrium whereas K⁺Cl⁻ cotransporters collectively known as KCCs and individually named as KCC1 (Slc12a4), KCC2 (Slc12a5), KCC3 (Slc12a6) and KCC4 (Slc12a7) are the ones with Cl⁻ extrusion capabilities and responsible for keeping \([\text{Cl}^-]_i\) bellow thermodynamic equilibrium. Since NKCCs are the targets of bumetanide, one of the most commonly prescribed diuretics and β-cells keep a high \([\text{Cl}^-]_i\) in a bumetanide-sensitive manner, the next sections concentrate on NKCC1 and NKCC2. For a detailed molecular analysis of KCCs, the reader is directed to other sources (Arroyo et al. 2013; Gagnon and Di Fulvio 2013).

Na⁺K⁺2Cl⁻ cotransporters

As mentioned above, two isoforms of NKCC produced by different genes are known. These are: NKCC1 and NKCC2, encoded by Slc12a2 and Slc12a1 genes, respectively (Arroyo et al. 2013; Markadieu and Delpire
Figure 4. The high intracellular concentration of Cl\(^-\) in \(\beta\)-cells. Insulin-secreting \(\beta\)-cells exhibit [Cl\(^-\)]\(_i\) above thermodynamic equilibrium due to the predominant activity of Cl\(^-\) uptake mechanisms relative to those which extrude the anion. It is this high [Cl\(^-\)]\(_i\) the one that supports the exiting of the anion upon opening of any Cl\(^-\) channel expressed in \(\beta\)-cells, including volume-regulated anion channels (VRAC). Adapted from Di Fulvio & collaborators (2014).
2014). NKCC1 is expressed ubiquitously *i.e.*, abundantly in all cells studied so far (Hoffmann et al. 2009). However, there are some exceptions to this rule. These include glucagon-secreting rat α-cells (Majid, et al. 2001) or tubular epithelial cells of the thick ascending loop of Henle (TALH) (Kaplan, et al. 1996). It is important to note that additional cell types might be found in the future lacking NKCC1 and therefore, the use of the epithet "ubiquitous" might not be appropriate to designate NKCC1. At any rate, contrary to NKCC1, the most abundant and almost exclusive source of NKCC2 is the kidney. Indeed, NKCC2 is expressed in the mammalian kidney in such a high level that it is considered "kidney-specific" (Arroyo et al. 2013; Markadieu and Delpire 2014). However, NKCC2 has been found in several cell types where it was searched, including insulin secreting rodent β-cells (Alshahrani, et al. 2012). Further, NKCC2 has also been found in rat neurons of mesenteric ganglia, secretomotor enteric neurons (Xue, et al. 2009; Zhu, et al. 2011), cells of the gastric, colonic, intestinal, endolymphatic sac and olfactory epithelia (Akiyama, et al. 2010; Akiyama, et al. 2007; Kakigi, et al. 2009; Nickell, et al. 2007; Nishimura, et al. 2009; Xue et al. 2009; Xue, et al. 2014; Zhu et al. 2011) and in starburst amacrine cells of the retina (Gavrikov, et al. 2006). In fact, large-scale analysis of the human transcriptome demonstrated that *Slc12a1* transcripts are widely distributed among tissues, although at much lower levels when compared to kidney (Su, et al. 2004). Notably, the main functional difference between NKCC1 and NKCC2 is that the former has the ability to transport water (Hamann, et al. 2010) and therefore, it is directly involved in cell volume regulation (Hoffmann et al. 2009). NKCC2, however, is a "dry" cotransporter (Zeuthen and Macaulay 2012) mainly involved in NaCl reabsorption by the TALH (Ares, et al. 2011), where NKCC2 is heavily expressed and from where most if not all of our knowledge comes from (Arroyo et al. 2013; Markadieu and Delpire 2014). Outside the kidney, the functional impact of NKCC2 is poorly understood.

At the molecular level, the mouse *Slc12a2* gene produces at least three
Figure 5. Cladogram of Slc12a isoforms and splice variants. In silico phylogenetic neighbor-joining analysis using the genetic distance model of Jukes & Cantor (Evolution of Protein Molecules. New York: Academic Press. pp. 21–132, 1969). Shown are the two main branches of the Slc12a family (Cl loaders and Cl extruders) as well as their respective sensitivities to diuretics. Graph generated by using Geneious R7 (Biomatters Ltd. NZ).
alternative splice variants. These are annotated in the RefSeq database with the following accession numbers: NM_009194 (Slc12a2, NKCC1a), XM_006525732 (Slc12a2 x1, NKCC1b) and XR_385981 (Slc12a2 x2). Except for the latter, which was recently predicted by automated computational analysis, mouse NKCC1a and NKCC1b mRNAs differ among themselves by the inclusion of exon 21. Indeed, NKCC1b lacks the 16 C-terminal residues encoded by that exon (Randall, et al. 1997) (Figure 6A). Both transcripts are predicted to encode proteins of ~130 kDa in its core/high-mannose N-glycosylated form and of ~170 kDa when hybrid/complex N-glycosylated (Singh, et al. 2014). Hydropathy analysis of NKCC1 sequences predicts proteins with 12-transmembrane domains and two large intracellular N- and C-termini (Di Fulvio and Alvarez-Leefmans 2009). Both NKCC1a and NKCC1b exhibit similar functional properties but differ in their expression pattern (Vibat, et al. 2001). The mouse Slc12a1 gene appears to produce at least three variants as the result of the alternative election of one of three mutually exclusive exons (Arroyo et al. 2013; Markadieu and Delpire 2014). Two of these variants are annotated in RefSeq with the following accession numbers: NM_183354 [Slc12a1 v1, NKCC2A (exon 5)], NM_001079690 [Slc12a1 v3, NKCC2F (exon 6)]. The third mouse NKCC2 variant originally named NKCC2B (exon 4) (Igarashi, et al. 1995) has yet to be referenced in the database (Figure 6B).

**Chloride uptake mechanisms and insulin secretion**

After the initial functional demonstration of Na⁺K⁺2Cl⁻ cotransport in β-cells by Sandström & Sehlin (Sandstrom and Sehlin 1987), molecular studies determined the identity of this cotransport system in β-cells as NKCC1 (Majid et al. 2001). At that time, NKCC2 was assumed exclusive of the kidney and its presence was not evaluated (Majid et al. 2001). It was not until 2012 when NKCC2 splice variant A was cloned from two rat β-cell lines (INS-1E and RIN-5mF) (Alshahrani et al. 2012) and subsequently found in rodent
Figure 6. The *Slc12a2* (NKCC1) and *Slc12a1* (NKCC2) isoforms and splice variants.  

**A.** Represented are NKCC1a and NKCC1b splice variants differing in the sequence encoded by the 21st exon depicted in red.

**B.** Three of the several known NKCC2 splice variants are represented. They differ by the inclusion of mutually exclusive exons depicted in red (exon 5), green (exon 6) and blue (exon 4).
islets, that the participation of this cotransporter in insulin secretion was suggested. Indeed, bumetanide impaired insulin responses from WT islets or mice lacking NKCC1 (NKCC1-KO) suggesting that bumetanide-sensitive mechanisms different from NKCC1 are operative in NKCC1-KO islets (Alshahrani and Di Fulvio 2012). Notably, NKCC1-KO mice exhibited exaggerated insulin responses to glucose associated with flat glucose tolerance curves, further suggesting that NKCC1 is not necessary for insulin secretion and that NKCC2, present in the islet, may not adequately replace NKCC1 function. This is intriguing inasmuch as NKCC2 constitutively increases net Cl⁻ influx in cells without changing cell volume (Zeuthen and Macaulay 2012) and β-cells lacking NKCC1 are significantly shrunken (Alshahrani and Di Fulvio 2012). This suggests the possibility that [Cl⁻]ᵢ in NKCC1-KO β-cells might be kept within normal ranges \( \text{i.e., } \sim 40 \text{ mM or even higher as a consequence of constitutive NKCC2 function, but overall decreased as per unit of } \beta \text{-cell mass, which in turn is increased in NKCC1-KO mice relative to WT (Alshahrani and Di Fulvio 2012). In addition, absence of NKCC1 might impair the ability of } \beta \text{-cells to respond appropriately to changes in cell volume induced by glucose metabolism.}

Although the mechanisms involved in insulin secretion in response to glucose in NKCC1-KO mice are not fully understood, it is important to keep in mind that plasma membrane depolarization is not exclusively achieved by closure of \( K_{\text{ATP}} \) channels and that glucose homeostasis in turn is finely regulated not only by insulin, but also by glucagon, which might be deregulated in NKCC1-KO mice. Further, absolute global absence of NKCC1 in β-cells, for instance, may set, up-regulate, down-regulate, organize, establish or generate alternative regulatory routes which may eventually have an impact in overall islet physiology. Clearly, additional experiments are needed to elucidate the mechanisms of insulin secretion in islets lacking NKCC1.

**Diuretics and insulin secretion**
"Loop diuretics also can cause (...) hyperglycemia (infrequently precipitating diabetes mellitus)...", [quoted from Edwin Jackson, Chapter 28, Diuretics, p753 (Goodman, et al. 2006)]

As mentioned earlier, NKCCs are sensitive to furosemide and bumetanide (BTD) (Haas and McManus 1983), and probably other loop diuretics including azosemide, torasemide, etacrinic acid, etozoline, muzolimine, piretanide or tienilic acid. Thiazides, however, are a different group of diuretics which main targets are NCCs (Hilal-Dandan, et al. 2011). Many studies tested the effect of thiazides on carbohydrate metabolism in humans and animal models. The notion that all diuretics may cause diabetes sparked after a single manuscript published soon after the introduction of this loop diuretic in the clinics in late 50’s (Wilkins 1959). Subsequently, textbooks dedicated to the study of hypertension discussed the impact of thiazides in diabetes and cautioned about their use in hypertensive patients (see Finnerty F.A.: Discussion of Special Problems in Therapy of Hypertension. Hahnemann Symposium on Hypertensive Diseases 1958: Hypertension, edited by Moyer J.F., Philadelphia: W.B. Saunders Company, 1959, p653; and Freis E.: Discussion of Special Problems in Therapy of Hypertension, Hahnemann Symposium on Hypertensive Diseases, 1958: Hypertension 1959, p652).

The earliest reports relating thiazides and carbohydrate metabolism showed impaired glucose tolerance and even glycosuria in some but not all of a variety of patient groups treated in different ways with the diuretic [briefly summarized in (Giugliano, et al. 1980)]. For instance, Goldner and collaborators treated 20 patients with no clinical manifestations of diabetic and 20 diabetic patients with oral thiazide derivatives. Contrary to initial expectations, thiazides did not precipitate glucose intolerance or hyperglycemia in non-diabetic patients, whereas 30% of the diabetic group showed hyperglycemia and glycosuria (Goldner, et al. 1960). In 1961,
Zatuchni & Kordasz demonstrated that thiazides elevated glycemia in 56% of 25 patients suffering various cardiac conditions (Zatuchni and Kordasz 1961). The diabetogenic long-term effects of benzothiadiazines were suggested later on by Wolf and collaborators in non-obese and obese hypertensive patients with family history of diabetes (Wolf, et al. 1963). Thirteen years later, however, Lewis and coworkers demonstrated that two years of thiazide administration to normal subjects did not result in hyperglycemia, which could only be observed after six years of treatment (Lewis, et al. 1976).

The impairment of glucose tolerance observed after thiazide treatment of some but not all subjects studied is still controversial. In particular within the context of the observation that K⁺ replenishment prevented glucose intolerance in patients chronically treated with chlorothiazide. Indeed, hypokalemia, a common finding in thiazide-treated patients was originally considered as the cause of hyperglycemia in patients chronically treated with thiazides (Rapoport and Hurd 1964; Seltzer and Allen 1969). These observations, together with those of Cornish and collaborators in 1961, who demonstrated morphological and pathological changes induced by chronic thiazide treatment in the pancreas of 21 out of 300 mice (Cornish, et al. 1961), precipitated the conclusion that thiazides, a strong inhibitor of NCCs and other Cl⁻ transporters (Leviel, et al. 2010) may cause diabetes. Interestingly, that concept was also extended to furosemide and later to BTD developed in 1964 and 1971, respectively (Feit 1971; Ingram 1964), in spite of the fact that few studies dealt with this. The first one, for instance, performed in 1966 showed that furosemide impairs glucose tolerance after three-month administration of the diuretic to normal and hypertensive diabetic patients in response to systemic injection of the sulphonylurea tolbutamide, a K_ATP channel inhibitor (Jackson and Nellen 1966). The second study done 6 years later, demonstrated no impact of furosemide or the newly developed BTD on glycemia in 4 normal subjects
treated with different concentrations of the diuretics at 4 days intervals (Asbury, et al. 1972). The third study came in 1980 demonstrating that BTD administered acutely and systemically did not change basal or stimulated insulin secretion, and did not change glucose tolerance after ten days of BTD administration in 14 normal subjects (Giugliano et al. 1980). The next year, Robinson and collaborators wrote in the abstract of their paper: "Glucose tolerance was significantly improved with bumetanide but not with furosemide", at least in eleven healthy individuals treated with the diuretics for 8 days or T2DM patients for 6 weeks (Robinson, et al. 1981). Soon after these publications, Hermansen and collaborators demonstrate that thiazides actually increased basal insulin, glucagon and somatostatin release from perfused isolated dog pancreas whereas BTD did not have a significant effect on the same parameters (Hermansen, et al. 1985). When taken together, these results indicate that the impact of diuretics on glucose homeostasis depends on the population studied and the diuretic used. In fact, BTD may actually have beneficial potential rather than being diabetogenic!

The "inconsistency" of the effects of diuretics on carbohydrate metabolism, as referred to in Flamenbaum & Friedman (Flamenbaum and Friedman 1982), became more problematic when Sandström demonstrated that a high single intraperitoneal (ip) dose of BTD (100 mg/kg) transiently increases glycemia and impairs glucose tolerance in mice (Sandstrom 1988) and that hydrochlorothiazide inhibits insulin secretion directly from the mouse islet by a mechanism not related to Cl− but to Ca2+ fluxes (Sandstrom 1993), thus indicating that NCC, the main target of thiazides cannot be involved in the effects of the thiazide. Notably, the response to 50-100 mg BTD/kg in mice was not different from that of 200 mg furosemide/kg observed in earlier experiments (Asbury et al. 1972; Cohen, et al. 1976) consistent with the idea that BTD is ~40 times more potent than furosemide (Hilal-Dandan et al. 2011). Therefore, when taken together these results suggest that NKCCs might regulate insulin secretion, a concept that was
supported in subsequent experiments \textit{in vitro}, as discussed earlier in this Introduction, but mostly when the effects of BTD were tested acutely (Best 2005; Sandstrom 1990; Sandstrom and Sehlin 1988c).

\textbf{Acute versus long-term effects of BTD and furosemide on NKCCs}

Interestingly enough, most of our knowledge regarding the pharmacological or functional properties of NKCCs comes from experiments performed \textit{in vitro} wherein acute actions of diuretics were studied (Gamba 2005). In a more realistic clinical scenario, diuretics, in particular BTD and furosemide are administered chronically and as expected, the pharmacologic outcome of such administration is increased diuresis (Roush, et al. 2014). This clinically relevant and easy to observe effect of BTD is the consequence of its concentration in the tubules of the kidney and inhibition of NKCC2 in the TALH (Schlatter, et al. 1983). However, chronic treatment with BTD may impact gene expression (Rizzo, et al. 2004). In addition, NKCC1 is not only the most sensitive target of BTD but also a widely expressed one (Gamba 2005). Therefore, several questions arise: What is the long-term effect of BTD on the ubiquitously expressed NKCC1? What effect would long-term BTD have if administered at low doses? Would long-term administration of BTD impair insulin secretion?

The answer to the first question is unknown, but \textit{assumed} to be negligible (Loscher, et al. 2013) as BTD is highly ionized at physiological pH and \textasciitilde{}90\% of it circulates bound to plasma proteins (Cohen et al. 1976). The answer to the second question is partial but encouraging inasmuch as low doses of BTD are used to treat neonatal seizures and other GABAergic disorders where central neuronal NKCC1 is thought to play a major role (Ben-Ari 2012; Cotton, et al. 2012; Hochman 2012; Kahle and Staley 2008; Pressler and Mangum 2013; Walcott, et al. 2012). As for the third question, the previous section provided the notion that the impact of BTD and other diuretics on carbohydrate metabolism is clearly inconsistent and that more
research needs to be done in order to improve our knowledge. This Thesis aims to that.
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Long-term BTD does not impair insulin secretion due to BTD-induced changes in NKCC expression in β-cells.

Specific aims

To determine the long-term effect of BTD on insulin secretion in β-cells

To correlate NKCC expression and total chloride uptake in β-cells

To determine the long-term effects of BTD on NKCC expression in β-cells
MATERIALS AND METHODS

Antibodies

The primary antibodies used in our experiments are indicated in Table 1. Conjugated secondary antibodies for Western blotting and immunofluorescence applications were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Stocks of these antibodies were immediately prepared upon arrival by adding an equal volume of glycerol (Affimetrix/USB, Cleveland, OH) to avoid freezing and thawing cycles while storing. Aliquots of the stocks were stored at -80°C whereas working aliquots were kept at -20°C. Secondary antibodies for Western blotting applications were dissolved in appropriate volume of water upon arrival, then fractionated and stored at -80°C. Working aliquots of these antibodies were kept at -20°C after diluting with glycerol 1:1 (v/v).

Cell culture

The mouse insulinoma β-cell line MIN6 was kindly provided by Dr. Jun-Ichi Miyazaki (University of Kumamoto, Medical School, Kumamoto, Japan) (Miyazaki, et al. 1990). We selected this pancreatic β-cell line for our studies because their insulin response to physiologic glucose closely matches that of primary β-cells (Ishihara, et al. 1993). In addition, this cell line has been thoroughly characterized at the functional and molecular levels by numerous researchers throughout the years and is the most commonly used β-cell line to study mechanisms of insulin secretion (Skelin, et al. 2010). Adherent MIN6 β-cells were cultured essentially as described (Ishihara et al. 1993; Miyazaki et al. 1990). In brief, MIN6 β-cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 11 mM glucose, 4 mM L-
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glutamine, 1 mM sodium pyruvate, 10 % (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin or 100X antimycotic-antibiotic mix. Cells were grown in 5 % CO₂ at 37°C and media changed every 3 days until desired confluence was reached. Cells were cultured in 75 cm² flasks (T75) treated for cell culture and used until passage 30. Although MIN6 β-cells have been used at higher passages with satisfactory results (Cheng, et al. 2012), the passage limit of 30 was chosen to avoid potential genotypic or phenotypic changes. Cells were propagated by enzymatic digestion of the extracellular matrix by using 3 ml 0.05 % (w/v) trypsin supplemented with 0.53 mM EDTA for 5 min at room temperature. Cell detachment was followed under an inverted microscope until all cells were detached. Trypsin-mediated digestion was inhibited by adding culture media fully supplemented. The total number of detached cells was then used to seed either four T75 flasks or the appropriate number of 6/12-well plates to perform experiments.

Kidney fibroblast COS7 cells (ATCC, Manassas, VA) were grown as control cells for the standardization of Cl⁻ fluxes and gene expression analyses considering that this cell line has been extensively characterized and are relatively simple to maintain. COS7 cells were maintained in 6-well plates (BioLite, Thermo Scientific) in fully supplemented DMEM in a 5% CO₂ incubator at 37°C. COS7 cells depleted of endogenous NKCC1 or NKCC2 expression (COS7shNKCC1 and COS7shNKCC2, respectively) and control cells (COS7shControl) were generated as we have recently described in detail elsewhere (Singh et al. 2014). These cells were cultured in fully supplemented DMEM plus the continuous presence of 2.5 µg/ml puromycin.

Mice

The use of mice for our experiments was approved by the Institutional Animal Care and Use Committee of WSU-BSoM. Mice had ad libitum access to a standard chow diet and water and were housed under 12-12 hour light:
darkness cycles. Wild-type mice (WT, C57BL/6J genetic background) of both sexes aging 4-6 weeks were used in most of our experiments. These mice were routinely generated in our Animal Facility by crossings genetically engineered mice harboring a single functional allele of NKCC2A (heterozygous, NKCC2-HE) (Figure 7A). These breeders were kindly provided by Dr. Hayo Castrop (University of Regensburg) (Oppermann, et al. 2007). To identify WT, HE or KO mice, we took advantage of the genetic strategy followed to create those breeders. Briefly, premature stop codons were introduced in the 96 bp exon 5 (exon A) of the Slc12a1 gene in the form of a 97 bp FLAG-tagged sequence. The new targeted exon A of 192 bp and flanking sequences was used to design primers for the polymerase chain reaction (PCR) to determine the genotypes of mice in our colony (Figure 7B). The genotyping primers recognizing flanking intronic sequences 5' and 3' exon A are indicated in Table 2. The primer set is predicted to amplify a single band of 206 bp or 302 bp from genomic DNA obtained from WT or KO mice, respectively, or the two bands in the case of heterozygous mice i.e., those harboring both alleles: WT and KO (Figure 7B). The procedure for genotyping mice was developed and set up in our laboratory by Victor Otaño-Rivera (Undergraduate STREAMS Student) and Amma Boayke (Undergraduate HONORS Student) and was based on the original protocol written by Glenn Travis in Laboratory of Molecular Systematics, Smithsonian Institution. Washington, DC 20560, and the protocols of Walsh and collaborators (Walsh, et al. 1991) and the one described by Morin & Woodruff in Paternity exclusion using multiple hypervariable microsatellite loci amplified from nuclear DNA of hair cells. Pages 63-81 in RD Martin, AF Dixon & EJ Wickings (eds). Paternity in primates: genetic tests and theories. Basel, Karger 1992.

Mice tissue samples were placed in tubes containing 100 µl 10% chelex resin (Sigma C7901) and centrifuged 30 secs at 12,000 rpm in a centrifuge (Sorvall Legend Micro 21R centrifuge, Thermo Scientific) to collect
Figure 7. The NKCC2A mice colony and genotyping. A. Breeding strategy followed to produce mice lacking one (heterozygous, HE) or two alleles (knock-out, KO) of the NKCC2A transgene or wild-type (WT) mice. B. Genotyping strategy to determine HE, KO or WT genotypes in NKCC2A mice. On top, represented is exon A WT (red) of the NKCC2 gene, the two flanking primers used for PCR and the targeted exon A including additional 97 bp inserted (Flag-Stop). PCR performed with the indicated primers (red arrowheads) are predicted to produce a single fragment of 205 bp (WT), 302 bp (KO) or both (HE).
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the tissue sample at the bottom of the tubes. Then, tubes were incubated at 100°C for 20 minutes with casual swirl. The PCR amplification mixture consisted in (for each sample): 0.25 µl Taq DNA polymerase (GenScript E00007), 2.5 µl 10X Taq buffer (Gen Script B0005), 0.5 µl dNTPs 10 mM (USB/Affimetrix #77119), 0.25 µl each primer, 21 µl of nuclease-free water (Hyclone sh30529.02) and 2 µl DNA samples (or 2 µl water as negative control) set in a thin-walled PCR tube (Fisher 14230205). Then, PCR was performed in a thermal cycler (Bio Rad, BioRad Hercules, CA) for 40 cycles with the usual thermal settings. The products of PCR were then resolved in 2% agarose (Axygen AX-LE120060) gels prepared in Tris-Acetate-EDTA buffer (Teknova, T1346) and visualized by using ethidium bromide (USB/Affimetrix #75809). The molecular weights of the PCR products were compared against a 100 bp DNA ladder (Fermentas SM0243) (see Figure 7B).

**Islet preparations**

WT islets were isolated following the method of Carter and collaborators (Carter, et al. 2009) and under the supervision of Dr. Lydia Aguilar-Bryan’s team (Pacific Northwest Diabetes Research Institute, Seattle, WA, USA). Briefly, mice were euthanized with compressed CO2. Then, abdomen was opened and pancreas, liver and duodenum were located and then bile duct was exposed. Bile duct was closed tightly using a piece of thread at the conjunction with duodenum. A hole in the bile duct was poked using 27G*1/2 surflo-winged infusion set (Terumo SV*27EL) that connected with a 10 ml syringe containing 3 ml of collagenase solution (Vorthington #4210 at 4.3 mg/ml in DMEM medium). Then, the pancreas was inflated with collagenase solution gently and slowly. After inflation process was performed, the pancreas was cut starting from the side close to the duodenum toward the small intestines and then close to the stomach and colon. Then, the pancreas was grabbed and placed in a new 15 ml conical
tube containing ~2 ml collagenase solution to perform the digestion process as follows: the tube was placed in the water bath (37_°C) for 10 min and then shaken firmly and vigorously ~12 times. After the digestion process was completed i.e., no tissue chunks were visible, 5 ml of cold DMEM medium was added to stop digestion and then the tube centrifuged 3 min at 1000 rpm. The supernatant was discarded and the pellet re-suspended in 3 ml DMEM medium. Islets were then purified by filtration through 0.22 µm pore filter (Millex SLGP033RS) by centrifugation for 3 min at 1000 rpm. The pellet obtained and containing islets was used for protein/RNA extraction or culture in RPMI/10 % FBS medium. Islets usually attached in 6-well plates for 3 days at 37_°C/5 % CO₂ before functional experiments.

In some occasions were islet yield was predicted low, islets were purified by Ficoll gradient centrifugation. Briefly, removed pancreases were washed twice with ice-cold DMEM and then finely chopped by using surgical blades. Chopped tissues were transferred to tubes containing 2 ml of collagenase solution prepared as indicated above, incubated in water bath at 37_°C for 10 min and shaken firmly and vigorously ~12 times. After the digestion process was performed, DMEM medium was added to stop the action of collagenase and centrifuged at 1000 rpm for 3 min. The supernatant was discarded, the pellet re-suspended in 3 ml RPMI (Gibco 1640) and filtered through 0.22 µm pore filters. The filtrate was centrifuged at 1000rpm for 3 min and then the supernatant discarded (RPMI was removed as much as possible). The pellet was re-suspended in 4 ml Histopaque (Sigma 1077) using pipette 4-5 times. Then, 5 ml RPMI was added slowly (drop by drop) to the tube’s side above Histopaque. To separate islets from Histopaque, tubes were centrifuged at 2000 rpm at 4_°C without rotor brake and for 5 min. The islets were then collected at the interface of Histopaque and RPMI and placed in a new tube containing 5 ml of RPMI. The final islet pellet was obtained by centrifugation 3 min at 1000 rpm. The supernatant was removed and the pellet used for protein/RNA analysis or re-suspended with RPMI/10 % FBS.
medium for islets culture.

**Protein Determination**

Total protein content was estimated in control and treated samples by using the Bradford method (BioRad, Hercules, CA) and relating the results to standard curves built by using increasing concentrations of bovine serum albumin (BSA, 0-5 µg/µl).

**Western Blotting**

Western blot analysis of protein extracts obtained from MIN6 β-cells was performed essentially as described in detail elsewhere (Singh et al. 2014) and adapted to our experiments. Briefly, ~90% confluent MIN6 cells growing in 6 well plates were placed on ice and washed three times with cold phosphate buffer saline (PBS). Then, 50 µl/well of lysis buffer (25 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.1 % v/v β-mercaptoethanol, 1% Triton 100X, 50 mM NaF, 150 mM NaCl, 1.5 mM MgCl₂) were added. To extract proteins from purified islets or tissues RIPA buffer (Teknova R3792) was used at a proportion of 3 ml per 1 g of tissue). All lysis buffers were supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease/phosphatase inhibitors cocktails (Thermo Scientific 78443) composed of aprotinin, bestatin, E-64, leupeptin, soldium fluoride, sodium orthovanadate, sodium pyrophosphate and β-glycerophosphate were added in each well. Cell lysates were collected and placed in labeled micro-tubes on ice for ~1 h with vortex every 5 min. 50-100 µg of total protein in a final volume of 10-20 µl was mixed with equal amount of loading buffer (8 % SDS, 125 mM Tris-HCl, pH- 6.8, 20 % glycerol, 0.02% bromophenol blue, 100 mM dithiothreitol) and boiled 5 min before loading onto pre-casted Tris-HEPES 4-20 % SDS-PAGE protein gels (Thermo Scientific-Pierce, Rockford, IL). Gel wells were washed before loading samples by using Tris-HEPES buffer to eliminate traces of polyacrylamide and improve the electrophoretic mobility.
of proteins. Proteins were separated for ~1 h at 100V. The separated proteins were electro-transferred onto PVDF membranes (Millipore, MA) at 4 °C for ~2.5 hours at 72V. Membranes were air-dried and cut according to the desired proteins size to allow immunoblotting with multiple antibodies. For instance, the upper part of the membrane containing proteins of molecular weight (MW) ranging from 70-260 kDa was immunblotted against NKCC1 (130-170 kDa) or NKCC2 (120-160 kDa) whereas the bottom one was probed against β-actin (~45 kDa). Membranes were blocked with 5 % BSA or 3 % dry milk in 50 mM Tris, 150 mM NaCl supplemented with 0.05 % Tween 20 (TBST) for ~1 hour at room temperature (RT). After that, membranes were incubated with primary antibodies and diluted in TBST as indicated in Table 1. After incubation overnight at 4 °C, membranes were washed twice in TBST for 5 minutes at RT and gentle rocking. Then, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies directed against each primary antibody diluted in TBST (Table 1) at RT for ~1 hour. Membranes were washed twice with TBST for 5 min. The antigen-antibody complexes were then developed by chemiluminiscence (West Pico 34080) following the instructions of the manufacturer, visualized by using the BioRad image analyzer (Chemi-Doc MP Imaging system, Hercules, CA) and digitized for densitometric analysis.

Densitometry

The bands developed by using anti-NKCC1, -NKCC2 and β-actin antibodies in Western blotting were digitally acquired by using Image Lab Quantitative software (BioRad, Hercules, California) and adjusted to 8-bit images in ImageJ (NIH) before densitometry analysis. The detailed protocol for acquiring densitometry data by using ImageJ can be found at lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. The optical density of the bands corresponding to NKCC1 and NKCC2 were normalized to that of β-actin by dividing the intensity volume of
each of them by the respective intensity volume of ß-actin band. Once the relative intensity (NKCC1 or NKCC2/ß-actin) of each lane was calculated, it was further related to Control values *i.e.*, those obtained in the absence of treatment or treated with vehicle alone. These values were used to plot a curve of relative expression levels of NKCC1 or NKCC2 (y-axis) vs. bumetanide concentrations (x-axis) (*see* Fig 13B).

**Total RNA extraction and reverse transcription (RT) coupled to PCR**

Total RNA from MIN6 ß-cells, mouse islets or tissues in general was obtained by using the GenJet RNA purification kit (Thermo Scientific K0731) following the manufacturers’ instructions. First-strand cDNA synthesis and PCR was performed as described in detail elsewhere (Alshahrani et al. 2012). Simultaneous screening of NKCC1a and NKCC1b transcripts in MIN6 and islets was done following the original strategy developed by Mao and collaborators (Mao, et al. 2012) and successfully applied to ß-cells (Alshahrani et al. 2012) and COS7 cells (Singh et al. 2014). NKCC1, NKCC2 and control transcripts were amplified by using the primers sets indicated in Table 2. All primer sets were designed by using Geneious R7 (Biomatters, Auckland, NZ). The nucleotide sequence of RT-PCR products was determined to confirm molecular identity (Beckman Coulter Genomics, Beverly, MA).

**Immunofluorescence Microscopy**

Pancreas tissues were obtained from transcardially perfused mice of the WT or NKCC2A-KO genotypes and placed in 4% p-formaldehyde (PFA). Perfusion was performed as follows: The mouse was anesthetized by using Euthasol (Virbac 302085-020, 390 mg/ml) diluted to reach final concentration of 78 mg/ml and then the necessary volume in µl injected to reach a dose of 150 mg/kg. After the complete absence of mouse reflex responses, abdominal and thoracic cavities were opened to expose the heart. First, tissues were washed with PBS and a needle inserted into the apex of
left ventricle and then the right ventricle was cut to allow the blood and perfusion solutions to exit the circulation. Blood was drained by continuous infusion of PBS by using a peristaltic pump (Masterflex 77200-62). When the exiting fluid became clear, fixation process of tissues was performed by perfusing 4 % PFA solution into the heart to allow tissue distribution. After tissues color became white and muscle contraction stopped, perfusion was considered completed. Then, perfused tissues were collected and placed in labeled tubes containing 4 % PFA and kept overnight at 4_°C. Next day, tissues were placed in fresh 20 % sucrose/4 % PFA for paraffin embedding. Paraffinized tissues were sent to AML laboratories, Inc (Baltimore, MD) to generate 5 µm slides sections. After slide sections were received, the following steps were performed.

Tissues sections on slides were fixed by using 4 % PFA overnight at 4_°C and deparaffinized with xylene for 5 min twice. Then, slides were washed twice with 95% ethanol and successively with 80 %, 70 %, and 50 % ethanol for 5 minutes each. After that, sections were rinsed with distilled water before rinsed again with PBS for 10 min. Slides then were transferred to water bath in a staining dish containing sodium citrate buffer (10 mM) at 95-100_°C for 30 min. Slides were incubated at room temperature 30 min before washing with PBS-0.1 % triton. Sections then were blocked with goat serum diluted in PBS (1:10) 30 min at room temperature and then incubated with primary antibodies as indicated in Table 1 diluted in PBS-0.1 % Triton overnight at 4_°C. Next day, sections were washed three times with PBS/0.1 % Triton for 10 minutes each and then incubated with appropriate secondary antibodies (Table 1) for 2 hours at room temperature in the dark. When appropriate, 4’,6-diamidino-2-phenylindole (DAPI)-containing mounting media was used in the sections to counterstain cell nuclei. Digital images were taken by using the BX51 system fluorescence microscope (Olympus Corp, Tokio, Japan) connected to a Spot 5.1 digital camera (SPOT Imaging Solutions, Diagnostic Instruments, Inc. Sterling Heights, MI) coupled
to MetaVue software (Molecular Devices, Sunnyvale, CA). Slides of tissues from mice lacking NKCC1 were also used for control purposes. NKCC1-KO mice were generated by Dr. Gary Shull, University of Cincinnati (Flagella, et al. 1999) and generously provided by Dr. Alvarez-Leefmans (WSU-BSoM).

**Insulin Secretion**

The supernatant of sub-confluent MIN6 β-cells growing under normal or glucose/drug-stimulated conditions was used to determine the amount of insulin released to the media, a simple criterion of insulin secretion. The assay was designed as follows: MIN6 β-cells were plated in 12-well plates (Falcon 353043) in complete DMEM media (Hyclone, Cat# sh30243.01) supplemented with sodium pyruvate and 450 mg/dl glucose, 10 % FBS, glutamine and 2 µl of 2-mercaptoethanol. On day one, cells were washed twice with sterile glucose-free Krebs-Ringer Bicarbonate/HEPES buffer (KRBH) containing per 100 ml of deionized water the following: 2.7 ml of NaCl 5 M (Sigma S5150), 4.8 ml KCl 75 mM (Sigma P9327), 562 µl NaHCO3 0.89 M (Sigma S8761), 100 µl NaH2PO4 0.5 M (Sigma 94046), 50 µl MgCl2 1 M (Affimetrix/USB #78641), 150 µl CaCl2 1 M (Sigma 21114), 1 ml HEPES 1 M (Sigma H0887) and 100 mg BSA (Sigma A7906). After washing, cells were incubated for 30 min with KRBH (1 ml per well) freshly supplemented with 5.5 mM glucose (22 µl of glucose stock 250 mM). Cells were then washed twice with glucose-free KRBH. After this washing, cells were incubated for 2h in fresh KRBH supplemented with several concentrations of glucose (non-insulinotropic: 1-5.5 mM and insulinotropic: 5.5-20 mM) to generate a curve of insulin secretion as that shown in Figure 16A in the Results section. To test the effects of bumetanide (BTD, Sigma B3023) on insulin secretion, MIN6 β-cells were incubated with KRBH plus the diuretic at the concentrations indicated in relevant each sections. Then, glucose was added as explained above. After 2 hs, 800 µl of supernatants were collected, fractionated and stored at −20°C overnight. The next day, supernatants were centrifuged at
12,000 rpm for 10 min (Sorvall Legend Micro 21R centrifuge, Thermo Scientific) to remove any cell debris. The supernatants were diluted 1:20 by adding appropriate amounts of deionized water and used for insulin assays (see below). To estimate the proportion of insulin secreted relative to the total amount of insulin in cells, the total insulin content of MIN6 β-cells was estimated after extraction of the hormone in acidified ethanol (75 % ethanol, 1.5 % concentrated HCl). Briefly, after supernatants were collected, cells were extracted by adding 1 ml of acidified ethanol per well overnight at −20 °C. Next day, 800µl of acidified ethanol extracts were collected and centrifuged at 12,000 rpm for 10 min as above. The cellular extracts obtained were diluted 1:250 with de-ionized water and assayed for insulin content (see below) i.e., the total amount of insulin contained in cultured MIN6 β-cells.

To test the long-term effect of BTD on insulin secretion, MIN6 β-cells were pre-incubated with several concentrations of the diuretic for 16 hours before insulin secretion assays were performed. Insulin secretion is expressed either as percentage change relative to basal values i.e., those obtained with non-insulinotropic glucose concentrations (<5.5 mM) or relative to total insulin content calculated as follows: insulin in supernatant / insulin in supernatants + insulin content = fraction of insulin secreted.

Insulin concentration in samples (plasma, supernatants or extracts) was determined by using a mouse ultrasensitive "sandwich-type" enzyme-linked immunoabsorbent assay (ELISA, APLCO 80-INSMSU-E01) in the 96 microplate format and following the instructions of the manufacturer. Briefly, the assay consisted in the following: 5 µl of insulin Standards having 0, 0.188, 0.5, 1.25, 3.75, and 6.9 ng/ml of mouse insulin, two plasma controls containing low or high insulin concentration (see inset Figure 8), plasma obtained from mice or samples from MIN6 β-cells added in duplicate to wells containing a monoclonal antibody against mouse insulin attached to the
bottom of the wells. Then, 75 µl of HRP-conjugated antibodies directed against insulin (a different epitope relative to the first antibody) were added to the wells and incubated for 2 hours at room temperature on a rocker at 700-900 rpm. Wells were then carefully washed six times by using 300 µl of Wash Buffer supplied in the kit. Then, 100 µl per well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate to detect HRP activity were added and allowed additional 30 min of incubation at room temperature on the rocker (700-900 rpm). To develop the color of the reaction, 100 µl per well of a Stop solution were added and the optical density or absorbance determined at 450 nm in a multi-well plate reader (Packard Bioscience Company 425228). The relationship between absorbance and insulin content in this assay follows a linear function over the range of insulin concentrations shown in Figure 8.

**Determination of cellular Cl⁻ uptake**

The total intracellular content of Cl⁻ ions in cultured cells or islets was determined by potentiometry by using calibrated Cl⁻-selective electrodes (Thermo Sci-Orion) following essentially the methods described by Northrop, Sanderson and Weinstein & Jennings (Northrop 1948; Sanderson 1952; Weinstein and Jennings 1959) and the initial supervision of Dr. Kenneth Gagnon (Anatomy and Cell Biology, University of Saskatchewan, Canada). The relationship between the concentration of Cl⁻ ions in moles/L and the response of the Cl⁻ electrode in mV at 25 °C is described by the Nernst equation:

\[
E (\text{mV}) = k - 59.2 \times \log[\text{Cl}^-]
\]

Therefore, the electrode response to calibrated and accurately prepared NaCl solutions of concentrations ranging from 0.0001 mM (pCl = -4) to 500 mM (pCl = 2.7) were converted to [Cl⁻] by using standard curves plotting E vs. pCl (Figure 9). To determine the accuracy of the Cl⁻ electrode,
Figure 8. Insulin standard curve. Non-linear regression analysis performed on the relationship between optical density or absorbance measured at 450 nm and increasing concentrations of mouse insulin (0 - 6.9 ng/ml) as determined by ELISA. The standard curve produced is used to determine the concentration of insulin in cell culture supernatants, cell extracts or plasma samples. Inset: Internal controls for ELISA using 10 different plasma samples with low or high levels of insulin (red and blue dots, respectively).
preliminary experiments were performed at high and constant ionic strength by supplementing samples and standards with NaNO₃ 1 M. There were no differences in the electrode responses in the presence or absence of NaNO₃ indicating that the ionic strength of the samples were low enough to impact the electrode responses and therefore, this step was omitted.

To determine Cl⁻ content in cells, COS7 or MIN6 were seeded onto 6-well plastic plates (Thermo Scientific #130184) and grown until ~80 % confluence was reached. In the case of islets, they were plated onto 6-well plates for two days to allow attachment. Cells or islets were then carefully washed and depleted of endogenous Cl⁻ by pre-incubating them in an isotonic solution lacking Cl⁻ ions and the composition indicated in Table 3. The osmolarity of the solutions, as determined by using an osmometer (Advanced Instruments, Inc. 3300), averaged 310 mOsm/kg. After the pre-incubation period, Cl⁻-free ISO was aspirated and replaced by an isotonic solution containing physiological concentrations of Cl⁻ (ISO, Table 3). Cells or islets were incubated at room temperature in ISO solution for variable periods of time in order to obtain either a mono-exponential, first-order kinetic curve of Cl⁻ uptake or end-point uptake of the anion. For kinetic analysis, Cl⁻ uptake into cells was terminated at 0, 5, 10, 30 or 60 min after incubation in ISO media by placing cells on ice, aspirating the media and immediately applying three washes of ice-cold Cl⁻-free ISO solution at those time points. The ionic content of washed cells was then released by incubating cells in 0.25 N NaOH at room temperature for 30 min. After neutralization with glacial acetic acid, aliquots were accurately taken for protein determination and for the potentiometric determination of Cl⁻ ions present in the samples. Net Cl⁻ uptake in cells was calculated and expressed as nmol of Cl⁻ per µg of total protein content. BTD-sensitive Cl⁻ uptake i.e., NKCC-mediated was defined as the difference between Cl⁻ accumulated in 5 minutes and that obtained in the presence of BTD. To test the long-term effects of BTD on Cl⁻ accumulation, cells were incubated with the diuretic for
Figure 9. Calibration curve of the ion-selective electrode. The response of the ion-selective electrode in mV to increasing concentrations of Cl ions ranging from 0.00001 to 500 mM was plotted following the relationship determined by the Nernst equation. The electrode responses for each $pCl$ represents the mean ± range ($n=12$). The grey zone represents the minimum values of Cl concentrations detected by the electrode. The effective linear range of Cl concentration in which the electrode gives a response in mV with a slope of $\sim 56$ mV occurs from 0.1 µM to 500 mM.
<table>
<thead>
<tr>
<th>Stock Solutions &amp; salts</th>
<th>ISO</th>
<th>Cl-free</th>
<th>Final Concentration</th>
</tr>
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<tbody>
<tr>
<td>NaCl 5M</td>
<td>13 ml</td>
<td>0</td>
<td>130 mM</td>
</tr>
<tr>
<td>KCl 75mM</td>
<td>33.3 ml</td>
<td>0</td>
<td>5 mM</td>
</tr>
<tr>
<td>CaCl2 1M</td>
<td>1 ml</td>
<td>0</td>
<td>2 mM</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ 0.5M</td>
<td>0.83 ml</td>
<td>0.83 ml</td>
<td>0.83 mM</td>
</tr>
<tr>
<td>MgSO$_4$ 1M</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>1 mM</td>
</tr>
<tr>
<td>HEPES 1M</td>
<td>10 ml</td>
<td>10 ml</td>
<td>20 mM</td>
</tr>
<tr>
<td>Na-gluconate</td>
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<td>130 mM</td>
</tr>
<tr>
<td>K-gluconate</td>
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<td>5 mM</td>
</tr>
<tr>
<td>Ca-gluconate</td>
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<td>0.4304 g</td>
<td>2 mM</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.5g</td>
<td>2.5g</td>
<td>10 mM</td>
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pH 7.4, Water to 500 ml Osmolarity ~300 mOsm/k

<table>
<thead>
<tr>
<th>500 ml</th>
<th>500 ml</th>
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<tbody>
<tr>
<td>~300 mOsm/k</td>
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16 hours and then depleted of endogenous Cl⁻ by incubating them 1 hour in Cl⁻-free ISO containing BTD followed by 5 min ISO incubation, also in the presence of BTD. For end-point determination of Cl⁻ uptake, islets depleted of the anion were incubated 30 minutes in ISO with or without BTD.

**Statistical analysis**

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare between multiple groups. Student's two-tailed t-test was used for paired comparisons. To calculate kinetic parameters of chloride data, one-phase decay best fitting analysis was used. A $p<0.05$ was used as criterion of statistical significance.
RESULTS

NKCC1 and NKCC2 are expressed in MIN6 β-cells and mice islets

To determine and identify expression of NKCC1 and NKCC2 in our mouse β-cell model MIN6, we used RT-PCR and the sets of primers shown in Table 2. A graphic representation of NKCC1 and NKCC2 primers is depicted in Figure 10A. Note that some of these primer sets are designed to co-amplify more than one splice variant of NKCC1 or NKCC2 in a single reaction. For instance, primer set NKCC1-599a/551b encompasses a region of NKCC1 transcripts involving exon 21, a 48 bp sequence cassette eliminated in NKCC1b (see Fig 6A). Therefore, co-amplification of NKCC1 transcript fragments of 599 bp and 551 bp indicates expression of NKCC1a and NKCC1b transcripts, respectively. In the case of the primer set NKCC2-700, no difference in RT-PCR product length is expected because alternative exons A, B or F are all of 96 bp (see Fig 6B) (Di Fulvio and Alvarez-Leefmans 2009). Therefore, NKCC2-700 is predicted to co-amplify NKCC2A, NKCC2B and NKCC2F as a single amplicon of 700 bp, which may be identified by direct sequencing.

As shown in Figure 10B, NKCC1a transcripts of expected sizes are found in MIN6 β-cells. In addition, and contrary to rat β-cell lines (Alshahrani et al. 2012), faint bands of 551 bp and 453 bp corresponding to NKCC1b were also co-amplified when using NKCC1-599a/551b or NKCC1-501a/453b primer sets (see red arrows in Fig 10B) suggesting that both, NKCC1a and NKCC1b mRNAs are co-expressed in MIN6 β-cells. Figure 10C also shows that NKCC2 is expressed at the mRNA level in MIN6 β-cells, extending previous results (Alshahrani et al. 2012). To determine the molecular
Figure 10. Molecular and cellular expression of NKCC1 and NKCC2 in MIN6 β-cells. A. Schematic representation of NKCC1 (top) and NKCC2 (bottom) transcripts, their exons (grey arrowed boxes), coding regions (yellow arrowed boxes) and the relative positions of the primer sets used. They are named according to the transcript amplified and followed by three numbers indicating the predicted size in base pairs (bp) of the RT-PCR products. B-C. Representative RT-PCR experiments performed showing bands of predicted sizes for NKCC1 (B) and NKCC2 (C). D. Sequence identity of the NKCC2-700 amplicon (red asterisk in C). Shown is the partial nucleotide sequence aligned against the sixth exon of NKCC2 gene (exon F). E-F. Immunofluorescence analysis of MIN6 β-cells growing in glass coverslips and probed against NKCC1 (E) or NKCC2 (F) by using the primary and secondary antibodies shown in Table 1.
identity, the NKCC2 amplicon of 700 bp obtained in MIN6 ß-cells (*indicated with a red asterisk in Fig 10C*) was purified and sequenced. As shown in **Figure 10D**, the partial nucleotide sequence of NKCC2 transcripts detected in MIN6 ß-cells correspond to the F (exon 6) variant. At the cellular level, both immunoreactive NKCC1 and NKCC2 proteins are detected in cultured MIN6 cells. Representative images of these results are shown in **Figures 10E-F**.

To further extend these results to the mouse islet, NKCC1 and NKCC2 transcripts were amplified by RT-PCR using some of the same primer sets used to detect NKCC1 and NKCC2 in MIN6 ß-cells (Figs 10A-B). As shown in **Figure 11A**, NKCC1a and faint NKCC1b (*red arrows*) mRNAs are detected in total RNA extracts from purified mice WT islets. In addition, **Figure 11B** shows that NKCC2 is also detected at the transcript level in mice islets confirming previous results (Alshahrani et al. 2012; Alshahrani and Di Fulvio 2012). To determine the molecular identity of NKCC2 expressed in those islets, the RT-PCR fragment obtained with NKCC2-700 (*red asterisk in Fig 11B*) was purified and sequenced. As shown in **Figure 11C**, the partial nucleotide sequence of NKCC2 transcripts detected in mouse islets corresponds to the F (exon 6) variant.

To correlate NKCC transcripts with their products, protein expression and localization of NKCC1 were determined at the cellular level in mice islets by co-immunolabeling NKCC1 or NKCC2 with insulin, the latter to identify ß-cells of the islets. To this end, mice pancreas slides were probed against NKCC1 by using validated primary chicken antibodies directed against human NKCC1 (Singh et al. 2014) and one of the most commonly used and thoroughly characterized antibodies against insulin (see Table 1). As shown in **Figure 11D**, NKCC1 is expressed in the islet of WT but not in NKCC1-KO mice, as expected (Flagella et al. 1999). Notably, NKCC1 locates in insulin-positive ß cells in mice islets. Following a similar strategy, **Figure 11E** shows
Figure 11. Molecular and cellular expression of NKCC1 and NKCC2 in mouse islets. A. NKCC1a and NKCC1b transcript expression in WT islets was analyzed by RT-PCR using the primer sets NKCC1-515 (first lane), 501a/453b (second lane) and 599a/551b (third lane). B. NKCC2 mRNA expression in WT islets as determined by using RT-PCR and the NKCC2-700 primer set. As positive control of RT-PCRs transcripts of GAPDH were amplified (555 bp). As negative control, water was used instead of total RNA. C. Partial sequence chromatogram obtained from a representative DNA sequencing reaction using purified NKCC2-700 amplicons (red asterisk). Note that the DNA sequence obtained is 100% identical to that of exon 6 (exon F) of NKCC2 (Slc12a1 v2, NM_001079690). Experiments performed by Shams Kursan. D-E. Representative immunolocalization experiments for NKCC1 (D) and NKCC2 (E) and insulin using pancreas slides from mice of the indicated genotypes. The antibodies used in these experiments are indicated in Table 1. Immunoanalysis performed by Eduardo Dias Junior.
immunoreactive NKCC2 locating in insulin-positive β-cells in WT islets, as we have previously demonstrated (Alshahrani and Di Fulvio 2012). Further, immunoreactive NKCC2 is also detected in β-cells of islets of pancreas from NKCC2A-KO mice supporting the notion that, contrary to rat β-cells, NKCC2 splice variant F is expressed in mice islets.

**MIN6 β-cells and islets accumulate Cl⁻ ions**

The presence of NKCC1 and NKCC2 in MIN6 predicts active BTD-sensitive accumulation of Cl⁻ ions by these cells. Therefore, to determine functional NKCC expression, MIN6 β-cells were depleted of endogenous Cl⁻ and subsequently allowed to accumulate this anion as a function of time. As shown in **Figure 12A**, Cl⁻-depleted MIN6 β-cells accumulates Cl⁻ in isotonic media containing physiological concentrations of Cl⁻ (ISO) following a time curve typical of first-order kinetics with a rate constant $k = 0.124$ min⁻¹, a maximal half-life $T_{1/2} = 5.6$ min and a plateau $P = 3.96$ nmol/µg protein. However, such accumulation kinetics is reduced in the continuous presence of 10 µM BTD ($k = 0.111$ min⁻¹, $T_{1/2} = 6.2$ min and $P = 2.58$) suggesting that MIN6 β-cells accumulate Cl⁻ in a BTD-sensitive manner a finding consistent with the notion that these cells express functional NKCCs.

To add support to this conclusion, dose-response inhibition curves of BTD-sensitive Cl⁻ uptake (i.e., the fraction of total Cl⁻ accumulation that is sensitive to BTD. In other words, NKCC-mediated) as a function of increasing concentrations of BTD were performed to determine the half-maximal inhibitory concentration (IC₅₀) of the diuretic on NKCC function. As shown in **Figure 12B**, the BTD-sensitive component of Cl⁻ accumulation in MIN6 β-cells follows a typical inhibitory dose-response curve with an estimated BTD IC₅₀ of ~7-9 µM.

To extend these results to mice islets, Cl⁻ uptake experiments were
Figure 12. Kinetics of Cl− uptake. A. Cl− uptake into MIN6 ß-cells depleted of intracellular Cl− under control (red dots) or in the presence of the indicated concentration of BTD (blue dots). Cl− uptake reaches equilibrium after ~10 minutes at room temperature and a final physiological Cl− concentration of ~140 mM. Results are expressed as mean ± SEM (n>10). The initial uptake rate is approximately linear during the first 5-10 minutes of the reaction. Thereafter, a mono-exponential decay in the uptake rate can be observed. B. Dose-response curve of Cl− uptake in Cl-depleted MIN6 ß-cells determined 5 minutes after readmission of physiological Cl− in the presence of the indicated concentrations of BTD. Results are expressed as the mean ± SEM (n>5). C. Maximal Cl− uptake into Cl-depleted NKCC2A islets. Results are expressed as the mean ± SEM (n=3).
performed in cultured islets obtained from WT and NKCC2A-KO mice expressing NKCC2F and NKCC1 (Figs 12D-E). To that end, islets were initially depleted of endogenous Cl– and subsequently exposed to physiological Cl– concentrations for an hour in order to determine the maximal content of Cl– accumulated in the presence or absence of 10 µM BTD. As shown in Figure 12C, the maximal uptake of Cl– ions in mice islets of both genotypes exhibited a BTD-sensitive component corresponding fairly to 40% of the total uptake of the anion. These results, when taken together indicate that MIN6 β-cells and mice islets of both genotypes express functional NKCCs.

**Down-regulation of NKCC1 increases NKCC2 expression**

The findings that NKCC1-KO islets respond to glucose (Alshahrani and Di Fulvio 2012), and that mice islets express NKCC2 prompted the question as to what extent NKCC2 might participate in long-term regulation of Cl– in β-cells co-expressing both transporters. Therefore, to gain insights into the potential long-term effects of NKCC inhibition on either NKCC1 or NKCC2 expression, MIN6 β-cells were treated for 16 hours with 1, 5, 10 and 50 µM BTD and NKCC protein expression levels determined by immunoblot analysis. As shown in Figure 13A, BTD progressively reduces NKCC1 expression in MIN6 β-cells until reaching a maximum inhibitory effect at 10-50 µM, a phenomenon that closely paralleled increased NKCC2 protein expression levels. Indeed, densitometric analysis of at least three independent Western blotting experiments shows the relationship between the expression levels of NKCC1 and NKCC2 in MIN6 β-cells (Figure 13B).

To ascertain if increased levels of NKCC2 expression occurs in response to decreased NKCC1 in a general rather than specific setting, we first determined whether BTD impacted NKCC1 expression in COS7, a fibroblast-like cell line wherein NKCC1 expression has been thoroughly characterized and validated (Singh et al. 2014) and in which low levels of NKCC2 can be detected. As shown in Figure 13C, incremental BTD
Figure 13. Impact of BTD or NKCC1 depletion on NKCC expression. A. Representative immunoblot analysis of protein extracts obtained from MIN6 β-cells (Control and treated with the indicated concentrations of BTD 16 hours) and probed against NKCC1 or NKCC2 by using NKCC1 or NKCC2 antibodies respectively. B. Densitometric analysis of at least three different immunoblots as the one shown in A. C. Representative immunoblot analysis of protein extracts obtained from COS7 cells treated with the indicated concentrations BTD 16 hours. D-E. Representative Western blots of COS7 cells and COS7 cells lacking NKCC1 (shNKCC1) (D) or COS7 Control and COS7 depleted of endogenous NKCC2 (shNKCC2) (E) probed against NKCC1 and NKCC2 (experiments performed by Mohamed Almiahuob). F. Representative immunofluorescence experiments performed on COS7 cells treated with the indicated concentrations of BTD or Control conditions. Immunoreactive NKCC1 is shown in green. The cell nuclei were counterstained using DAPI (experiments performed by Richa Singh).
concentration correlates with decreased NKCC1 protein expression. Notably, the NKCC1 expression response to BTD in COS7 cells follows a pattern resembling that of MIN6 β-cells (Fig 13A). Further, to add support to the notion that NKCC1 down-regulation may result in increased NKCC2 expression levels, we determine NKCC2 protein expression in COS7\textsuperscript{shNKCC1}, a genetically engineered cell line stably expressing minimum if any endogenous NKCC1 through stable expression of shRNAs against the transporter’s RNA (Singh et al. 2014) or in COS7\textsuperscript{shNKCC2} recently developed in our laboratory. As shown in Figures 13D-E, elimination of endogenous NKCC1 expression in COS7\textsuperscript{shNKCC1} cells results in increased NKCC2 expression levels whereas elimination of endogenous NKCC2 does not impact NKCC1 expression in COS7\textsuperscript{shNKCC2} cells. Taken together, these results demonstrate that NKCC2 expression responds to decreased NKCC1 levels, but not the other way around.

To visualize the ability of BTD to deplete endogenous NKCC1 expression, COS7 cells, which are big, flat and exhibit negligible auto-fluorescence (Singh et al. 2014) were treated for 16 hours with BTD (10-100 µM) and immunoreactive NKCC1 assessed by using the primary antibodies indicated in Table 1. As shown in Figure 13F, endogenous NKCC1 expression not only decreases with increasing concentrations of BTD, but also redistributes toward intracellular compartments, a phenomenon that correlates with visible cell shrinkage.

**Long-term BTD increases the rate of Cl\textsuperscript{-} uptake**

Since pharmacological or molecular depletion of NKCC1 correlates with increased NKCC2 expression in MIN6 β-cells (Figs 13A-B) and COS7 (Figs 13C-E), we determined the initial rate at which MIN6 β-cells upload Cl\textsuperscript{-} ions after 16 hours of BTD treatment. As shown in Figure 14, increasing concentrations of BTD within the range observed to progressively inhibit NKCC1 expression and increase NKCC2 levels (see Fig 13) result in higher
Figure 14. Long-term effect of BTD on the initial rate of Cl uptake in MIN6 β-cells. MIN6 β-cells were depleted of Cl in Cl-free medium for an hour (black dots) and then incubated five minutes in ISO media containing vehicle (red dots) or the indicated concentrations of BTD (colored dots). Results are expressed as nmol/µg protein/min. Each dot represents a single independent determination (* p<0.05).
initial rates of Cl\(^-\) accumulation (*i.e.*, nmol of Cl\(^-\) loaded per µg of protein per unit of time computed during the first 5 minutes of the linear phase of the reaction) relative to vehicle-treated cells. These results indicate that additional transport mechanisms are activated by long-term treatment with BTD. We have hypothesized that this mechanism is NKCC2.

**Elimination of NKCC1 decreases steady-state total Cl\(^-\) content**

The following observations and reasoning are key to interpret the long-term impact of BTD on NKCC expression: *i*) long-term treatment of MIN6 β-cells or COS7 cells with BTD decreases expression of NKCC1 (Fig 13), *ii*) NKCC1 is a cotransporter directly involved in water uptake and cell volume regulation (Hamann et al. 2010), *iii*) β-cells lacking NKCC1 are shrunken (Alshahrani and Di Fulvio 2012), *iv*) BTD-induced down-regulation of NKCC1 increases the levels of NKCC2 (Fig 13), and *v*) NKCC2 is a "dry" cotransporter (Zeuthen and Macaulay 2012). It follows that NKCC2 may not restore total Cl\(^-\) content in cells chronically depleted of NKCC1 in spite of increased initial rates of Cl\(^-\) uptake (Fig 14). To test this hypothesis, Cl\(^-\) uptake experiments were performed in COS7 WT or COS7\(^{shNKCC1}\). As shown in **Figure 15A**, elimination of NKCC1 in COS7 cells results in decreased total Cl\(^-\) content incorporated in 30 minutes per µg of protein when compared to WT cells (filled and emptied red dots, respectively, in Fig 15A) indicating that NKCC1 is a major player in Cl\(^-\) uptake. To determine the relative contribution of NKCC2 to the total pool of Cl\(^-\) uploaded by COS7\(^{shNKCC1}\), these cells were treated with 10 µM BTD during the length of the uptake experiments. As shown in **Figure 15A**, BTD decreases even further the total Cl\(^-\) content of COS7\(^{shNKCC1}\) confirming that NKCC2 is operative in the absence of NKCC1. However, since NKCC2 function cannot replace that of NKCC1 in terms of water transport and therefore cell volume regulation (Hamann et al. 2010; Zeuthen and Macaulay 2012), it follows that [Cl\(^-\)]\(_i\) may be restored to normal values by NKCC2 function when NKCC1 is absent, but not the total content of
Figure 15. Kinetics of Cl⁻ uptake in COS7 cells. A. Cl⁻ uptake into COS7 cells WT (red dots) or depleted of endogenous NKCC1 (shNKCC1, blue squares), treated with vehicle (filled dots or squares) or 10 µM BTD (empty dots or squares) during the uptake reaction. Cl⁻ uptake reaches equilibrium after ~5 minutes at room temperature and a final physiological Cl⁻ concentration of ~140 mM. Results are expressed as mean ± SEM [n= 66 and 34 (WT and shNKCC1, respectively)]. The initial uptake rate is approximately linear during the first 5-10 minutes of the reaction. Thereafter, a mono-exponential decay in the uptake rate can be observed. B. Kinetic parameters calculated from one-phase decay best fitting analysis.

<table>
<thead>
<tr>
<th>COS7</th>
<th>rate k (min⁻¹)</th>
<th>half-life T₁/₂ (min)</th>
<th>Plateau (nmol/µg protein)</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.25 ± 0.06</td>
<td>2.73</td>
<td>3.3 ± 0.2</td>
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<tr>
<td>WT + BTD</td>
<td>0.234 ± 0.07</td>
<td>2.98</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>shNKCC1</td>
<td>0.378 ± 0.40</td>
<td>1.84</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>shNKCC1 + BTD</td>
<td>0.279 ± 0.24</td>
<td>2.48</td>
<td>1.5 ± 0.1</td>
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Long-term BTD treatment does not impact insulin secretion \textit{in vitro}

When applied acutely during minutes to islets or β-cell lines \textit{in vitro} or to mice, BTD transiently inhibits insulin release (Sandstrom 1990) or impairs glucose tolerance (Sandstrom 1988), even in mice lacking NKCC1 (Alshahrani and Di Fulvio 2012). Further, if NKCC2 function restores \([\text{Cl}^–]\) in β-cells lacking NKCC1, then long-term treatment of these cells with BTD may not negatively impact insulin secretion. To test the long-term effect of BTD on insulin secretion \textit{in vitro}, we first confirmed the ability of MIN6 β-cells to secrete insulin after challenging them with physiological concentrations of glucose, as shown in Figure 16A. Then, we either pre-incubated MIN6 β-cells with 10 µM BTD for 16 hours or incubated them for the length of time required to assay insulin secretion in response to glucose \textit{in vitro} \textit{i.e.}, 2 hours. As shown in Figure 16B, end-point basal or glucose-stimulated insulin secretion in the presence of BTD was not different from that of Control. These results suggest that long-term action of BTD does not impact the total amount of insulin secreted by MIN6 β-cells during the assayed period of time.
Figure 16. Glucose-induced insulin secretion. A. MIN6 β-cells were incubated with the indicated concentrations of glucose (GLU) for 2 hours. Then, insulin secreted into the media and total cellular insulin content were measured using ELISA. Results are expressed as % increase of insulin secreted into the media over basal levels i.e., those obtained at GLU <5.5 mM (n=13). B. Short and long term BTD (10 µM) effects on insulin secretion from MIN6 cells: Insulin secretion and content from MIN6 control (red), treated with BTD 2h (green) or 16 h (blue) incubated with low (5.5 mM) and high (20 mM) glucose concentration were measured using ELISA. Insulin secretion was related to total insulin content (insulin secretion/total insulin content) and then correlated to basal levels.
Discussion

Previous experiments have demonstrated that NKCC1 and NKCC2 are co-expressed in the rodent islet, precisely in insulin-secreting β-cells but not in other endocrine cells of the islet (Alshahrani et al. 2012; Alshahrani and Di Fulvio 2012). These results coupled to the fact that NKCC1a and NKCC2A were the splice variants detected at the molecular levels in two different rat β-cell lines i.e., INS-1E and RIN-m5F (Alshahrani et al. 2012), gave origin to the suggestion that rodent islets express these NKCC variants and not others. However, our results indicate that NKCC1a, NKCC1b and NKCC2F are co-expressed in the mouse β-cell line MIN6 and islets (Fig 10B and 11A, respectively). The finding of very low transcript levels of NKCC1b in mouse but not in rat β-cells cell lines suggests either i) species differences, or ii) higher expression levels of NKCC1 mRNAs in the mouse islet or β-cell lines relative to the rat counterpart. Examples of the former argument abound in the literature. It is estimated that >11% of conserved exon cassettes are spliced out in human but constitutively included in mouse transcripts (Barbosa-Morais, et al. 2012; Pan, et al. 2005). Relative to β-cells, the most prominent example of species-specific gene expression is the vesicular monoamine transporter 2 (VMAT2), which is abundantly expressed in porcine and human β-cells but undetectable in rat or mouse laboratory strains (Schafer, et al. 2013). In addition, NCC is detected in MIN6 β-cells (Di Fulvio et al. 2014) but not in mouse islets (results not shown). Moreover, we have to keep in mind that undetectable levels of NKCC1b in rat β-cells or any transcript in any cell type or tissue is always subjected to our ability to detect it, in particular when expressed at low levels and when the source of transcripts is a pure cell line growing under specific conditions or purified
from tissues, as we have recently observed in COS7 cells and brain tissues (Singh et al. 2014). In addition, NKCC1b appears to contribute minimally to the total pool of NKCC1 transcripts in β-cells, although its relative mRNA expression varies widely in different tissues (Vibat et al. 2001). Indeed, we estimated that NKCC1b corresponds roughly to ~1% of the total NKCC1 transcripts in MIN6 β-cells, explaining the undetectable levels of NKCC1b in previous reports (Alshahrani et al. 2012). Although additional experiments are required to determine potential inter-species differences in the expression pattern of Slc12a genes in β-cells, the fact that some genes are clearly detected in cell lines relative to primary cells or tissues suggests that caution should be paid before extending results from cell lines to tissues or from one species to another.

Relative to NKCC2 expression in rodent β-cells and islets, molecular cloning and sequence analysis demonstrated that NKCC2A is expressed in INS-1E and RIN-m5F rat β-cells lines. However, the molecular identity of NKCC2 expressed in rat or mouse islets, was not determined at that time (Alshahrani et al. 2012). The finding that NKCC2F is expressed in mouse β-cells and mice islets, including those of the NKCC2A-KO genotype, but not in rat β-cell lines minimizes the possibility that NKCC2F is the compensatory result of NKCC2A absence in the islet. However, this possibility may remain open due to the fact that MIN6 β-cells are derived from in vivo immortalized β-cells, which may not represent the normal population of β-cells. In addition, mice β-cells may express NKCC2A mRNAs at levels low enough to be under-represented by our RT-PCR assays. Indeed, nested-PCR of NKCC2-700 amplicon obtained from WT islets was necessary to yield sufficient product for DNA sequencing and the nucleotide sequence chromatogram corresponded to exon F of NKCC2 (see Figs 10C-D and 11B-C). Because, no other DNA sequences were detected, we concluded that NKCC2A or NKCC2B are either expressed at levels bellow the detection limit or not expressed at all. Irrespective of the splice variants of NKCC2 or NKCC1 expressed in rat
and mouse β-cells, our results clearly showed that both transporters colocalize in insulin-positive cells of the islet (Figs 11D-E). These results not only confirm and extend previous findings (Alshahrani et al. 2012; Alshahrani and Di Fulvio 2012), but also support the notion that both cotransport systems may participate in the regulation of $[\text{Cl}^-]$ in β-cells, regardless of the rodent species.

Indeed, our experiments demonstrated that MIN6 β-cells depleted of endogenous $\text{Cl}^-$ accumulate the anion as a function of time in a way resembling first-order kinetics (Fig 12A) with a constant rate of 0.124/min and a half-life of 5.6 min. However, short-term BTD treatment at concentrations known to inhibit NKCC function (Gamba 2005) significantly reduced total $\text{Cl}^-$ accumulation in these cells (Fig 12A) but it did not completely block it, indicating that β-cells uptake $\text{Cl}^-$ ions by using at least two different mechanisms: one of them, which accounts for ~40% of total $\text{Cl}^-$ uptake (Fig 12A), is BTD-dependent and has an estimated $IC_{50}$ for the diuretic of ~8 µM (Fig 12B). In analogy, approximately 40% of the total $\text{Cl}^-$ accumulated in mice islets was sensitive to 10 µM BTD as well (Fig 12C). Since BTD inhibits NKCC1 and NKCC2 with an $IC_{50}$ reported within the range of 0.5-20 µM (Gamba 2005; Haas and McManus 1983; Homma, et al. 1990; Markadieu and Delpire 2014; Plata, et al. 2002; Russell 2000; Vigne, et al. 1994) and both, NKCC1 and NKCC2 are expressed in MIN6 β-cells and mice islets, our results support the notion that these cells express functional NKCCs and that they accumulate $\text{Cl}^-$ actively to concentrations above thermodynamic equilibrium. This concept is in agreement with $[\text{Cl}^-]_i$ calculated and directly measured in other rodent β-cells and islets (Eberhardson et al. 2000; Kozak and Logothetis 1997). However, our $\text{Cl}^-$ uptake experiments performed in MIN6 β-cells or islets do not distinguish whether NKCC1, NKCC2 or both transport systems are actively involved in that process. The very low levels of NKCC2 detected in β-cells or islets (Figs 10C, 11B and 13A) (Alshahrani et al. 2012) relative to NKCC1 may argue
against the idea that NKCC2 participates in [Cl\(^-\)], physiology, at least under normal conditions. Our novel findings that COS7\textsuperscript{shNKCC1} cells lacking NKCC1, but expressing NKCC2 (Figs 13D-E) accumulated Cl\(^-\) ions in a lesser extent relative to WT cells and that this accumulation decreased even further after BTD treatment (see Fig 15A) strongly suggest that NKCC2 is active, at least when NKCC1 is absent. Clearly, these experiments cannot answer the question as to what is the relative proportion of each cotransporter involved in Cl\(^-\) uptake when both are co-expressed. Based on the very high NKCC1/NKCC2 expression ratio, it is tempting to speculate that NKCC1 plays a major role in Cl\(^-\) uptake in MIN6 and COS7 cells and that NKCC2 may have a secondary, less important, if any role under normal conditions.

The previous conclusion begs the question: What is the function of NKCC2 in β-cells? Our results demonstrate that long-term treatment of MIN6 β-cells or COS7 cells with BTD results in decreased endogenous NKCC1 expression (Figs 13A-C). To the best of our knowledge, this surprising effect of BTD was not observed before, probably due to the fact that the effects of BTD on NKCCs have been studied in short-term experiments looking almost exclusively at the functional properties of the transporter (Gamba 2005; Russell 2000). Notably, BTD-induced NKCC1 down-regulation was dose-dependent and reached a maximum inhibitory effect at concentrations around 5 µM (Figs 13A-B), a value very close to that required to inhibit half of functional endogenous NKCC1 in many cell systems (Gamba 2005; Russell 2000) and NKCC-mediated Cl\(^-\) uptake in rodent β-cells (Sandstrom 1990; Sandstrom and Sehlin 1987), including MIN6 (Fig 12B). These results suggest that functional inhibition of NKCC with BTD and the observed BTD-induced down-regulation of NKCC1 expression may be related. Interestingly, the latter phenomenon was observed in MIN6 and COS7 cells (see Fig13), further suggesting that the inhibitory effect of BTD on NKCC1 expression is independent of the cell type and that similar mechanisms are operative in both cell types. Although the mechanisms whereby BTD decreases NKCC1
expression in these cells remain unknown, NKCC1 immuno-localization analysis of COS7 cells treated long-term with BTD revealed an interesting feature: vacuolization of endogenous immunoreactive NKCC1 (Fig 13F) which resulted in apparent retention of the transporter in the cytoplasm of the cells and down-regulation. This result resembles that of receptor-mediated endocytosis, whereby ligands e.g., BTD may be internalized and their receptors e.g., NKCC1 degraded not only ceasing trans-membrane signaling but also transducing signals from the cell surface to the nucleus in the form of long-term responses i.e., gene expression (Alberts, et al. 2008). Our results demonstrate that long-term pharmacological down-regulation of NKCC1 in MIN6 and COS7 cells increases NKCC2 expression (Figs 13A-C) and that this phenomenon appears related to a chronic down-regulation of NKCC1 expression rather than an independent increase in NKCC2 in response to BTD in vitro. Indeed, molecular elimination of endogenous NKCC2 did not result in increased NKCC1 expression (Fig 13E) thus suggesting that up-regulation of NKCC2 in MIN6 or COS7 cells treated long-term with BTD occurs in response to NKCC1 depletion and that NKCC1 expression is refractory to absent NKCC2.

The most parsimonious functional consequence of long-term BTD treatment is a chronic drop in \([Cl^-]_i\), cell volume or both, providing no fully compensatory mechanisms are triggered in a given cell and the effects of BTD are specific of NKCCs. Clearly, NKCC2 cannot compensate for NKCC1 function as the former is incapable of moving water across plasma membranes (Zeuthen and Macaulay 2012) whereas the latter upload ~500 molecules of water per ion transport cycle (Hamann et al. 2010). These concepts are consistent with the finding that: i) β-cells lacking NKCC1 COS7\textsuperscript{shNKCC1} are shrunken [(Alshahrani and Di Fulvio 2012) and data not shown] and ii) MIN6 β-cells or COS7 cells treated long-term with BTD accumulate Cl\textsuperscript{−} at higher initial rates relative to non-treated cells (Figs 14 and 15, respectively) whereas total Cl\textsuperscript{−} accumulation in COS7\textsuperscript{shNKCC1} cells is
decreased relative to WT (Fig 15A). Therefore, these results are compatible with the suggestion that up-regulation of NKCC2 in response to pharmacological inhibition of molecular depletion of NKCC1 may restore \([\text{Cl}^−]\), but not cell volume. However, we have to keep in mind that BTD may have off-target effects that might potentially impact the overall physiology of \([\text{Cl}^−]\).

For instance, the *BioActivity* data for BTD posted in *PubChem* database indicates that this diuretic at half-maximal concentrations of 6-25 µM is active in 28 out of 1209 bioassays performed ([pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?cid=2471](http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?cid=2471)). These include inhibition of the nuclear factor erythroid 2-related factor 2 isoform 2 (NRF2), a basic leucine zipper transcription factor that maintains cellular redox homeostasis protecting cells from toxic xenobiotics (Ma 2013), 17-β-hydroxysteroid dehydrogenase-4, an enzyme involved in the conversion of estrogens and androgens from 17-hydrox steroids (de Launoit and Adamski 1999), carbonic anhydrases (CA5B, CA13 and CA14) involved in the bidirectional inter-conversion between bicarbonate and protons from carbon dioxide and water (Lindskog 1997), or MEK5, a dual specificity protein kinase of the STE7 family of kinases (Kato, et al. 2000). Additional complexity to the interpretation of functional data obtained by using BTD comes from the facts that BTD is also a direct inhibitor of the cystic-fibrosis conductance regulator (CFTR), an anion/Cl channel (Ju, et al. 2014) and the human G-protein coupled receptor-35 (GPR35) (Yang, et al. 2012).

Since acute insulin secretion depends on \([\text{Cl}^−]\); above thermodynamic equilibrium (Best 2005) and the depolarizing efflux of \(\text{Cl}^−\) via VRAC requires glycolysis-induced β-cell swelling (Best et al. 2010), it follows that the up-regulation of NKCC2 in MIN6 β-cells observed in response to long-term down-regulation of NKCC1 (Figs 13A-B) may protect the insulin-secretory capacity of the β-cell to the action of BTD. This hypothesis is consistent with our results showing that long-term treatment of MIN6 β-cells with BTD did not result in impaired insulin secretion in response to glucose (Fig 16B). This
result cannot be seen as challenging those previously published studies wherein BTD or furosemide inhibited insulin secretion in isolated rodent β-cells, islets or glucose homeostasis, simply because the action of the diuretics were tested for minutes (Sandstrom 1988, 1990; Sandstrom and Sehlin 1988a, b, c). In fact, even an hour of BTD treatment resulted in decreased insulin concentrations in the media of INS-1E β-cells (Alshahrani and Di Fulvio 2012). Notably, the amount of insulin released into the cell culture media as a proportion of the total cellular content of the hormone did not change when MIN6 β-cells were co-incubated with BTD for the time period involved in the insulin assay (2 hours, Fig 16C). However, our results cannot discard the possibility of impaired kinetics of insulin release in particular the rate of insulin release during the first minutes of glucose challenge. When these results are placed together with those related to the impact of BTD on NKCC expression and Cl⁻ regulation (Figs 12-16), it is reasonable to support the hypothesis that long-term BTD does not impair insulin secretion from β-cells in vitro due to up-regulation of NKCC2, a transporter expressed in these cells at very low levels under normal conditions. However, these results must be taken with caution because NKCC2 is also a target of BTD in vitro and in vivo (Gamba 2005) although at concentrations higher than those required to fully inhibit NKCC1 (Plata et al. 2002).

**Conclusion**

Taken together, the results presented in this Thesis suggest that the functional long-term balance between NKCC1 and NKCC2 co-expressed in β-cells keeps insulin secretion within normal ranges and that long-term pharmacological inhibition or molecular depletion of NKCC1 activates mechanisms aimed at restoring [Cl⁻]ᵢ in β-cells and therefore it may protect β-cells against inhibition of insulin secretion.
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