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Erika Moreno
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

Consuelo Plata
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

Alejandro Rodríguez-Gama
Universidad Nacional Autónoma de México

Eduardo R. Argaiz
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

Norma Vázquez
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

See next page for additional authors

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Erika Moreno[‡], Consuelo Plata[‡], Alejandro Rodríguez-Gama[§], Eduardo R. Argaiz[‡§], Norma Vázquez[‡§], Karla Leyva-Ríos[§], León Islas[¶], Christopher Cutler[‖], Diana Pacheco-Alvarez[**], Adriana Mercado[‡‡], Raquel Cariño-Cortés[§§], María Castañeda-Bueno[‡], and Gerardo Gamba[‡§1]

*From the [‡]Department of Nephrology and Mineral Metabolism, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Tlalpan, 14080 Mexico City, Mexico, the [§]Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Tlalpan, 14080 Mexico City, Mexico, the [¶]Department of Physiology, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 Coyoacan, Mexico City, Mexico, the [‖]Biology Department, Georgia Southern University, Statesboro, Georgia 30460, the **Escuela de Medicina, Universidad Panamericana, 03920 Mexico City, Mexico, the [‡‡]Department of Nephrology, Instituto Nacional de Cardiología Ignacio Chávez, Tlalpan, 14080 Mexico City, Mexico, and the [§§]School of Medicine, Universidad Autónoma del Estado de Hidalgo, 42034 Pachuca, Hidalgo, México*

The thiazide-sensitive Na-Cl cotransporter (NCC) is the major pathway for salt reabsorption in the mammalian distal convoluted tubule. NCC plays a key role in the regulation of blood pressure. Its inhibition with thiazides constitutes the primary baseline therapy for arterial hypertension. However, the thiazide-binding site in NCC is unknown. Mammals have only one gene encoding for NCC. The eel, however, contains a duplicate gene. NCCα is an ortholog of mammalian NCC and is expressed in the kidney. NCCβ is present in the apical membrane of the rectum. Here we cloned and functionally characterized NCCβ from the European eel. The cRNA encodes a 1043-amino acid membrane protein that, when expressed in *Xenopus* oocytes, functions as an Na-Cl cotransporter with two major characteristics, making it different from other known NCCs. First, eel NCCβ is resistant to thiazides. Single-point mutagenesis supports that the absence of thiazide inhibition is, at least in part, due to the substitution of a conserved serine for a cysteine at position 379. Second, NCCβ is not activated by low-chloride hypotonic stress, although the unique Ste20-related proline alanine-rich kinase (SPAK) binding site in the amino-terminal domain is conserved. Thus, NCCβ exhibits significant functional differences from NCCs that could be helpful in defining several aspects of the structure-function relationship of this important cotransporter.

The thiazide-sensitive Na-Cl cotransporter (NCC)[2] is the major salt reabsorption pathway in the distal convoluted tubule of the mammalian nephron. The activity of NCC is critical to define blood pressure levels as well as renal salt, potassium, calcium, and acid-base metabolism (1). The decreased activity of NCC in inherited diseases such as Gitelman syndrome (2) and seizures, sensorineural deafness, ataxia, mental retardation and electrolyte imbalance (SeSAME) syndrome (3) results in arterial hypotension accompanied by hypokalemic metabolic alkalosis and hypocalciuria; however, the increased activity of NCC is involved in the development of a salt-dependent form of human hypertension known as familial hyperkalemic hypertension or pseudohypoaldosteronism type II because of mutations in the with-no-lysine kinases WNK1 and WNK4 (4) or the ubiquitin ligase complex proteins KLHL3 and Cul3 (5, 6), whose molecular targets are the WNKs. NCC is the receptor for thiazide-type diuretics, which are used extensively for the treatment of arterial hypertension (7, 8). Thus, thiazides are among the most commonly prescribed drugs worldwide. However, the binding site of thiazides to NCC is unknown.

Another member of the same family (SLC12), the Na-K-2Cl cotransporter NKCC2, is the receptor for loop diuretics (furosemide, bumetanide, or ethacrynic acid), which are also used extensively for the treatment of edematous conditions associated with chronic failure of the heart, liver, or kidney (9). Mammalian NCC and NKCC2 exhibit an overall 50% identity degree at the amino acid level. The amino- and carboxyl-terminal domains, which are intracellularly located, are believed to be mainly regulatory and exhibit identities between 5% and 30%. The central hydrophobic domain, containing the 12 membrane-spanning regions, exhibit up to 75% identity. It is known that residues defining the specificity for the diuretics (thiazide *versus* bumetanide) and translocated ions (Na-Cl *versus* Na-K-2Cl) reside within the central domain (10).

We have been interested in the analysis of the structure-function relationship in these cotransporters that can eventually unmask the diuretic binding site, which could lead to the design of more potent diuretic drugs. With this in mind, we first demonstrated that flounder NCC exhibits affinity differences for the transported ions and thiazide diuretics compared with those observed in mammalian orthologs (11–13). Next, using chimeric proteins between the flounder and rat NCC, we demonstrated that differences in the affinity for chloride or thiazide diuretics are located within the transmembrane regions 1–7 or 8–12, respectively (14). We could define one specific amino

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[1] To whom correspondence should be addressed: Vasco de Quiroga No. 15, Tlalpan 14080, Mexico City, México. Tel.: 5255-55133868; E-mail: gamba@biomedicas.unam.mx.

[2] The abbreviations used are: NCC, Na-Cl cotransporter; WNK, with-no-lysine kinase; SPAK, Ste20-related proline alanine-rich kinase; TEA, triethanolamine.

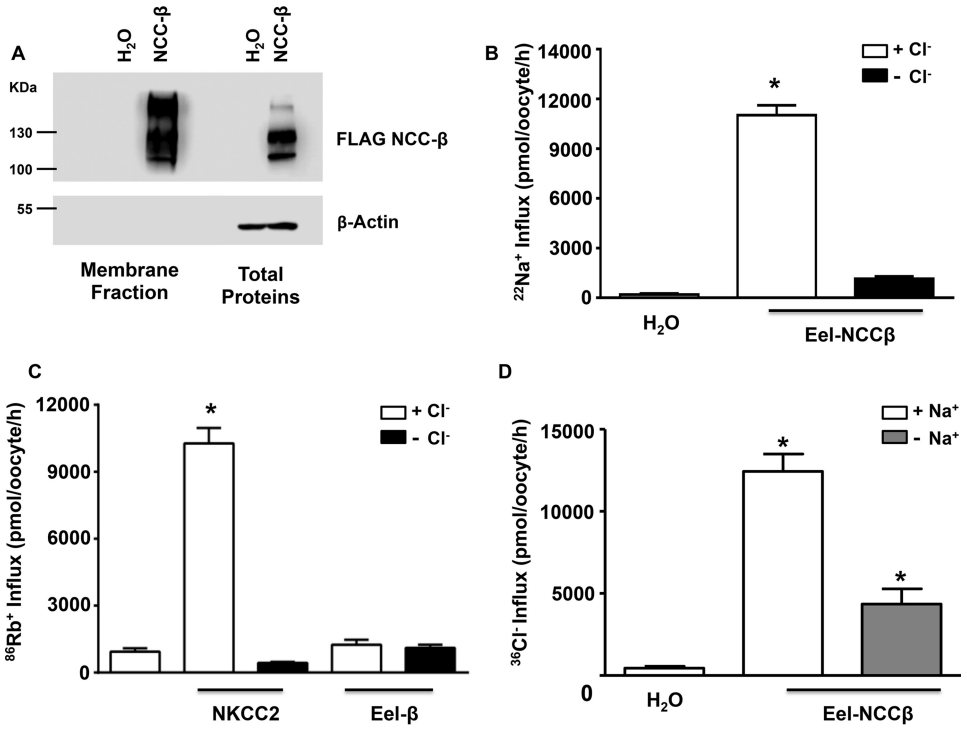

FIGURE 1. **Eel NCCβ encodes an Na-Cl cotransporter.** *A*, Western blotting analysis of total and membrane proteins (as stated) extracted from *Xenopus* oocytes injected with water or FLAG-NCCβ cRNA. The membrane was blotted with anti-FLAG antibody or anti-actin antibody. *B*, $^{22}Na^+$ influx in oocytes injected with water or with NCCβ cRNA (as stated) in the presence (*white columns*) or absence (*black columns*) of extracellular chloride. *C*, $^{86}Rb^+$ influx in oocytes injected with water, NKCC2, or eel NCCβ cRNA (as stated) in the presence (*white columns*) or absence (*black columns*) or extracellular chloride. *D*, $^{36}Cl^-$ influx in oocytes injected with water or with NCCβ cRNA (as stated) in the presence (*white columns*) or absence (*gray columns*) of extracellular sodium. *, $p < 0.0001$ *versus* water-injected control or *versus* absence of chloride or sodium of the same group.

acid residue, cysteine 575 of rat NCC (serine 575 in the flounder) located in transmembrane region 11, as responsible for the difference in the affinity for thiazides between fish and mammalian NCC (15). However, the amino acid residues defining the specificity for thiazide inhibition of NCC remain unknown.

In contrast to all mammalian species studied to date that contain only one NCC gene (16, 17), the European eel possesses two genes encoding NCC proteins (18). NCCα, expressed in the kidney, is 65–70% identical to the mammalian and flounder NCC. An additional paralogous gene, not expressed in mammals and referred to as NCCβ, is expressed in the eel intestine and exhibits ~55% identity with either the mammalian NCC or eel NCCα genes. This is higher than the 45% identity between NCCβ and mammalian NKCC2, suggesting that, indeed, NCCβ is likely to encode a Na-Cl cotransporter. Additionally, NCC-like genes have been reported in zebrafish (19). In this work, we studied the functional properties of the eel NCCβ, revealing a cotransporter with significant differences to that shown by all other NCC proteins studied to date.

Results

Cloning of NCCβ cDNA—The full-length cDNA encoding eel NCCβ was amplified using RT-PCR and cDNA using primers made based on the sequence of the eel NCCβ cDNA that was reported previously (18). Custom primers were used to add a FLAG epitope in-frame after the first methionine residue. The complete sequence of both strands of the resultant cDNA clone revealed that it contains 3437 base pairs exhibiting an open reading frame of 3132 bp and encoding a 1043-amino acid residue protein.

Eel NCCβ Is an NaCl Cotransporter—Xenopus laevis oocytes microinjected with 5 ng of FLAG-NCCβ cRNA produced by *in vitro* transcription from the NCCβ cDNA expressed a protein of the expected molecular size (~110 kDa) that was detected in the cRNA but not in the water-injected oocytes via anti-FLAG antibody (Fig. 1*A*). In addition, the plasma membrane expression of FLAG-NCCβ protein was corroborated by its finding in the biotinylated membrane fraction (Fig. 1*A*).

To study the functional properties of NCCβ, the tracer $^{22}Na^+$ influx was assessed in the presence or absence of extracellular Cl^- in *Xenopus*. As shown in Fig. 1*B*, NCCβ cRNA injection in oocytes induced a significant increase in Na^+ influx that decreased in the absence of extracellular Cl^-. These experiments strongly suggested that eel NCCβ operates as a Na-Cl transporter because the influx solution used for these experiments had no K^+, and the Na^+ influx was dependent on extracellular Cl^-. Nevertheless, it was necessary to analyze whether eel NCCβ could transport Rb^+ (as a surrogate of K^+) and Cl^- by assessing the influx of tracer $^{86}Rb^+$ or $^{36}Cl^-$ in oocytes injected with NCCβ cRNA. As shown in Fig. 1*C*, although rat NKCC2 cRNA injection induced significant furosemide-sensitive $^{86}Rb^+$ influx, no increased influx was observed in eel NCCβ-injected oocytes, supporting the idea that NCCβ is a K^+-independent cotransporter. In addition, as shown in Fig. 1*D*, robust $^{36}Cl^-$ influx in NCCβ cRNA-injected oocytes was significantly reduced in the absence of extracellular Na^+. Thus,

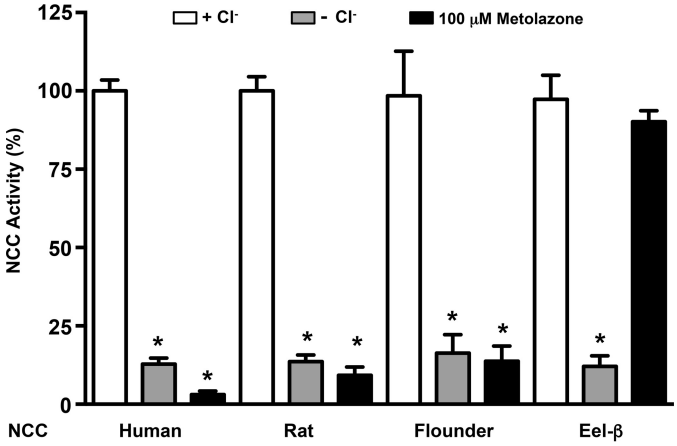

FIGURE 2. **Effect of the thiazide-type diuretic metolazone on the activity of human, rat, and flounder NCC or eel NCCβ.** *Xenopus* oocytes were injected with the corresponding cRNAs as stated. Three days later, the tracer Na^+ influx was assessed in the presence of NaCl (*white columns*), in the absence of extracellular Cl^- (*gray columns*), or in the presence of NaCl and 100 μM metolazone (*black columns*). The data observed for each control group were taken as 100%, and the *gray* and *black* groups were normalized accordingly. *, $p < 0.01$ *versus* the same group under control conditions.

eel NCCβ cRNA induced the appearance of a Na^+ transport mechanism that was dependent on extracellular chloride. However, it is worth noting that, although the Na^+ and Cl^- influxes were interdependent, we observed that the reduction of the influx in the absence of the counterion was different between $^{22}Na^+$ and $^{36}Cl^-$. In the absence of extracellular Cl^-, the Na^+ influx was decreased by more than 90%, as usually observed in the overexpression of other NCC orthologs (12, 20) (Fig. 1*B*). In contrast, in the absence of extracellular Na^+, the Cl^- influx was decreased by ~65% (Fig. 1*D*). Although the Cl^- influx under control conditions in NCCβ-injected oocytes was 12,432 ± 1060 pmol oocyte^{-1} h^{-1}, it was 4353 ± 927 pmol oocyte^{-1} h^{-1} in the absence of extracellular Na^+ ($p < 0.05$, $n = 3$). Thus, a significant amount of remaining $^{36}Cl^-$ influx was observed in the absence of extracellular Na^+.

Eel NCCβ Is an NaCl Cotransporter That Is Not Sensitive to Thiazides—We compared the effect of thiazides in oocytes microinjected with human, rat, or flounder NCC or eel NCCβ cRNA. Three days after cRNA injection, we assessed $^{22}Na^+$ influx in the presence or absence of extracellular Cl^- or in the presence of the thiazide-type diuretic metolazone at a concentration of 100 μM. For these experiments, the data observed in the control group were set as 100%, and those of the other groups of cRNA-injected oocytes were normalized according to the control group. As has been shown previously (14), human, rat, and flounder NCC cRNA injection induced an increase in the $^{22}Na^+$ influx that was completely dependent on extracellular Cl^- and that was highly sensitive to metolazone (Fig. 2). Interestingly, the behavior exhibited by oocytes injected with eel NCCβ cRNA was different regarding the sensitivity to metolazone. Although increased $^{22}Na^+$ influx was similarly prevented by the absence of extracellular Cl^-, it was not sensitive to metolazone at a concentration that usually inhibits more than 90% of activity of other NCC orthologs from humans, rats, or other teleost fish (Fig. 2).

Because it has been shown previously that rat and flounder NCC exhibit differences in the profile of inhibition between thiazides and in the affinity for thiazides (12–14), the absence of a metolazone effect could be due to a difference in the sensitivity or profile between known NCCs and eel NCCβ. Thus, oocytes were injected with flounder NCC cRNA and eel NCCβ cRNA, and the dose response to metolazone and effect of several different thiazides were assessed at a concentration of 100 μM. As shown in Fig. 3*A*, although rat NCC was inhibited by metolazone in a dose-dependent fashion, no effects on eel NCCβ cRNA-injected oocytes were observed at any concentration used. Similarly, as depicted in Fig. 3*B*, at the 100 μM concentration used, all of the thiazides tested inhibited flounder NCC cRNA-injected oocytes by more than 60% and by more than 90% in some cases, whereas none of them had any effect on eel NCCβ cRNA-injected oocytes. Note that, in the same experiments, the tracer influx was completely inhibited in both flounder NCC and eel NCCβ cRNA-injected oocytes in the absence of extracellular Cl^-, demonstrating the specificity of the Na^+ influx depending on the corresponding NCC cRNA injected. These data together show that eel NCCβ encodes a K^+-independent, NaCl cotransporter that is not sensitive to thiazide diuretics.

A C379S Substitution in Eel NCCβ Confers a Partial Sensitivity to Thiazides—In addition to the European eel (18), Watanabe *et al.* (21) cloned and studied the NCCβ from the Japanese eel (*Anguilla japonica*). They found that NCCβ is expressed in the apical membrane of rectal epithelia and that expression of this cotransporter is higher in freshwater eels than in seawater eels, suggesting a role in absorption when the ion concentrations are very low. In that study, they observed in rectum sac preparations that hydrochlorothiazide at a 1 mM concentration significantly reduced Na^+, Cl^-, and water absorption, suggesting thiazide sensitivity in the Japanese eel NCCβ. Cluster alignment analysis (Fig. 4) of the sequences of NCCβ reported by Cutler and Cramb (18) and Watanabe *et al.* (21) shows that the degree of identity between these orthologs from *Anguilla* is 98%. The 2% difference is due to six amino acid residues located in the NH_2-terminal domain, three residues located in the transmembrane (TM) regions, and four residues located at the COOH-terminal domain. As shown in Fig. 4, in the transmembrane regions, the differences are a serine at position 194 in the middle of TM2 in the European eel corresponding to a phenylalanine in the Japanese eel, a cysteine at position 379 in the European eel at the end of TM6 that corresponds to a serine in the Japanese eel, and an alanine at position 482 instead of a glycine in the middle of TM8 in the Japanese eel.

These single-amino acid changes could be responsible for abolishing the effect of thiazides on NCCβ. Accordingly, we assessed the effect of single point mutations in European eel cDNA to substitute these residues for those present in the NCC from the Japanese eel. The A482G substitutions had no effect on conferring thiazide sensitivity to eel NCCβ (data not shown). By contrast, C379S and S194F had a positive effect on thiazide sensitivity. The C379S substitution consistently conferred a certain degree of sensitivity. As shown in Fig. 5, the compilation of ten experiments showed that wild-type eel NCCβ had no sensitivity to thiazides, whereas the mutant C379S NCCβ was inhibited by ~45% using two different thiazides. This effect could be due to the elimination of cysteine or

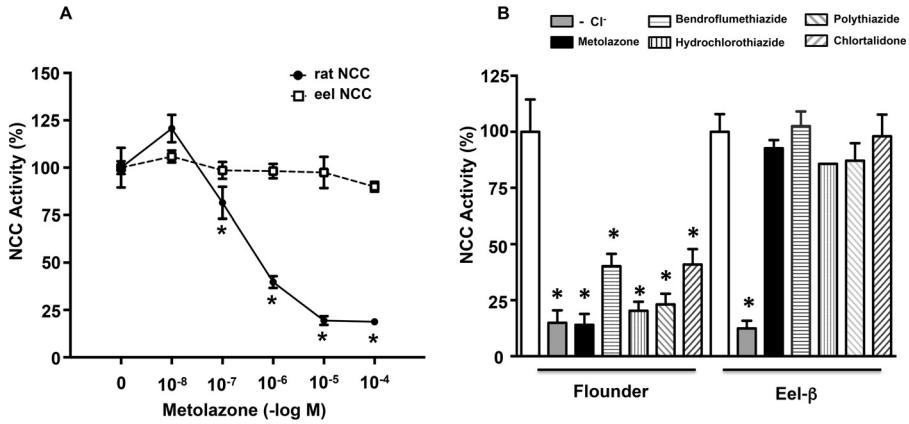

FIGURE 3. **Thiazide inhibitory kinetics and profile in NCC and NCCβ.** *A*, *Xenopus* oocytes were injected with rat NCC cRNA (*circles*, *continuous line*) or eel NCCβ cRNA (*boxes*, *discontinuous line*), and 3 days later a dose-response curve for metolazone was obtained by assessing the Na^+ influx in the absence of the diuretic or in its presence from 10^{-8} to 10^{-4} M concentration. No effect was observed in any of the NCCβ cRNA groups, whereas an inhibitory effect of metolazone was observed for rat NCC, as described previously (12, 14). *, $p < 0.05$ *versus* control group. *B*, oocytes injected with flounder NCC or eel NCCβ cRNA were exposed to tracer $^{22}Na^+$, and the influx was measured under control conditions (*white columns*), in the absence of extracellular Cl^- (*gray columns*), or in the presence of 100 μM metolazone (*black columns*), bendroflumethanizde (*horizontally hatched columns*), hydrochlorothizide (*vertically hatched columns*), polythiazide (*right hatched columns*), or chlortalidone (*left hatched columns*). The data observed for each control group were taken as 100%, and the thiazide groups were normalized accordingly. *, $p < 0.01$ *versus* control conditions.

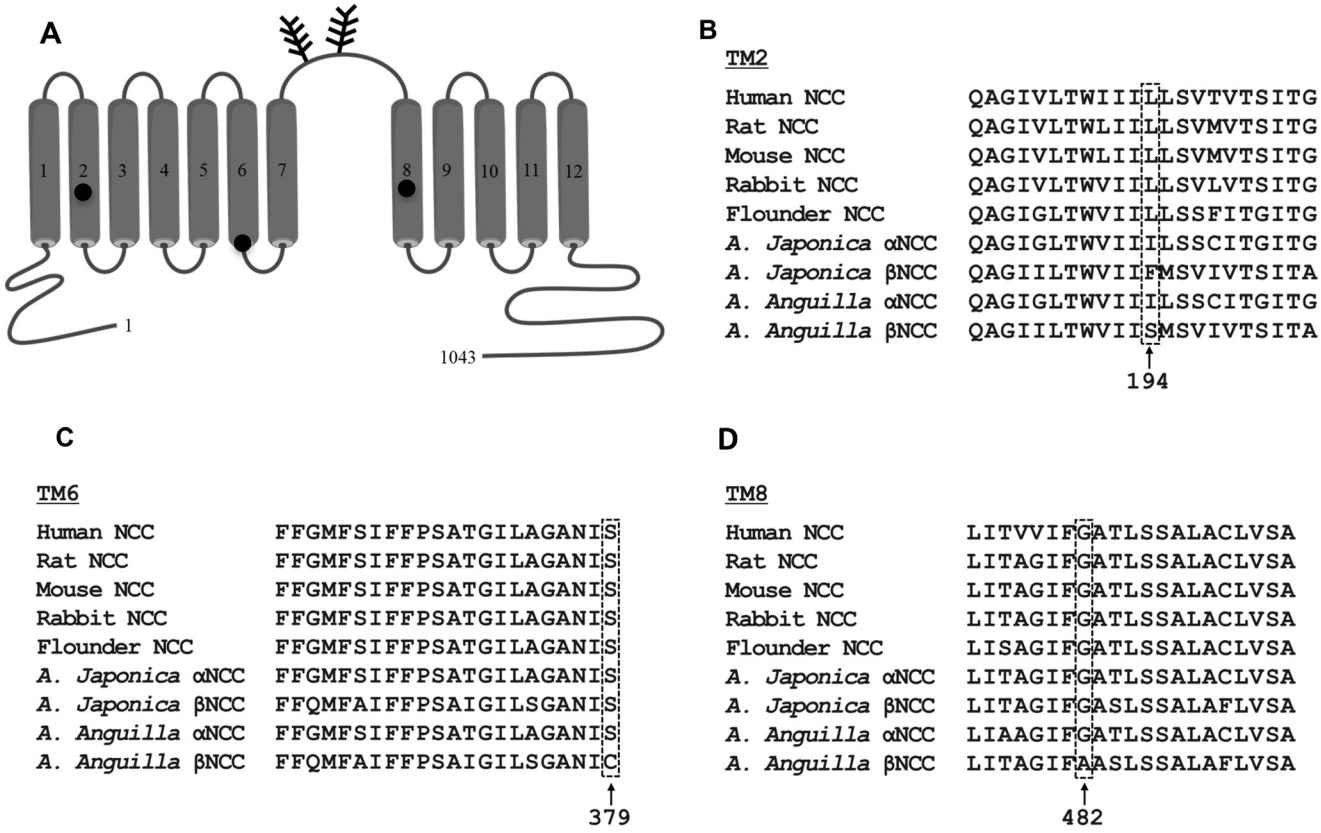

FIGURE 4. **Current model of NCC secondary structure and an alignment of NCC orthologs.** *A*, NCC secondary structure is depicted, with a central hydrophobic domain containing 12 putative transmembrane segments and an extracellular glycosylated loop facing the extracellular side of the cell located between transmembrane segments 7 and 8. The *black dots* depict the localization of the non-conserved amino acid residues between European and *A. japonica* eel NCCβ. *B–D*, alignment of TM segment 2 (*B*), TM segment 6 (*C*), and TM segment 8 (*D*) between human, rat, mouse, rabbit, and flounder NCC and *A. japonica* and *Anguilla anguilla* NCCα and NCCβ transporters. Amino acid residues shown in *boxes* are conserved in all NCCs, except for *A. anguilla* NCCβ.

the substitution of cysteine for a serine residue. To define these possibilities, a C379A substitution was also analyzed in which cysteine was substituted with alanine instead of serine. As shown in Fig. 5, the substitution of cysteine 379 for alanine did not confer sensitivity to thiazides, suggesting that it is the presence of the serine that makes the difference between the wild-type and C379S mutant version of NCCβ. In a set of three experiments, it was observed that the single mutation S194F in the European eel NCCβ cDNA also conferred ~30% sensitivity to the same thiazides. Neither the combination of the double substitution mutant NCCβ-C379S,S194F nor the triple substitution mutant (NCCβ-C379S,S194F,A482G) added further

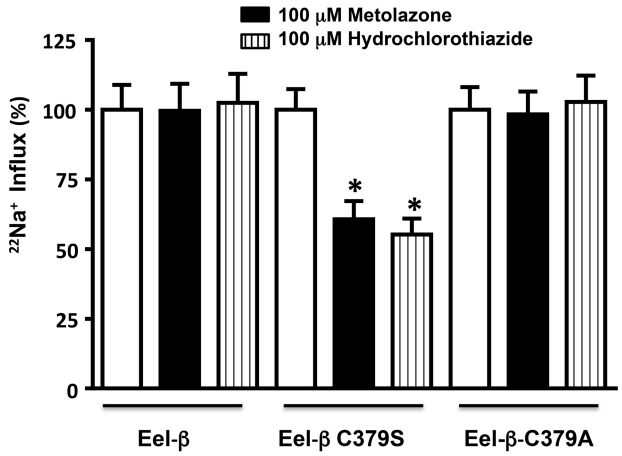

FIGURE 5. **The C379S substitution confers thiazide sensitivity to eel NCCβ.** Single point-directed mutagenesis was used for C379S or C379A substitution in eel NCCβ cDNA. The mutations were confirmed by DNA sequencing. Oocytes were injected with wild-type or mutant C379S or C379A NCCβ cRNA. Three days later, Na^+ influx was assessed under control conditions (*white columns*) or in the presence of 100 μM metolazone (*black columns*) or hydrochlorothiazide (*vertically hatched columns*). The data observed for each control group were taken as 100%, and the thiazide groups were normalized accordingly. The influx in the absence of thiazides for each group was similar. $n = 10$. *, $p < 0.01$ *versus* influx under control conditions in the same group.

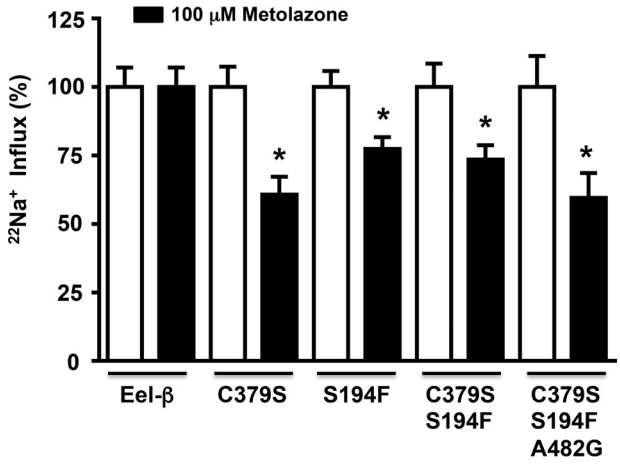

FIGURE 6. **Effect of C379S, S194F, double C379S,S194F or triple C379,S194F,A482G mutations on the thiazide sensitivity of eel NCCβ.** Oocytes injected with cRNA from the wild type or mutant NCCβ (as stated) were exposed to Na^+ influx medium in the absence (*open columns*) or presence (*black columns*) of 100 μM metolazone. The data observed for each control group were taken as 100%, and the metolazone groups were normalized accordingly. $n = 2$. *, $p < 0.05$ *versus* control group for each clone.

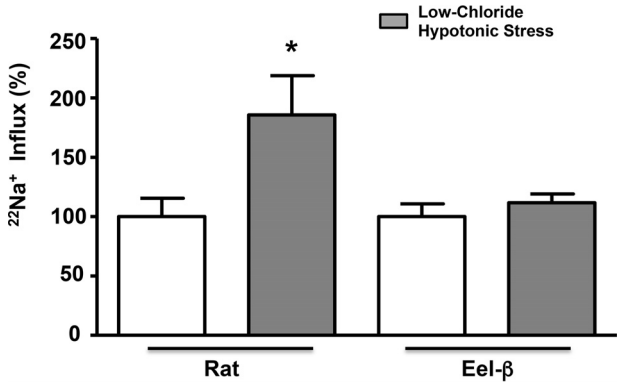

FIGURE 7. **Effect of low-chloride hypotonic stress on the activity of rat NCC and eel NCCβ.** Two days after microinjection with corresponding cRNAs (as stated), oocytes were incubated overnight in regular ND96 (*white columns*) or a slightly hypotonic medium (170 mosmol/kg) without chloride (substituted with isothionate) (*gray columns*). The next day, the Na^+ influx was assessed in influx media containing NaCl with a similar osmolarity as that used overnight. The data observed for each control group were taken as 100%, and the hypotonic medium groups were normalized accordingly. The influx in the absence of thiazides for each group was similar. $n = 3$. *, $p < 0.05$ *versus* control group.

sensitivity to thiazides (Fig. 6), suggesting that the single mutants C379S and S194F might have similar consequences on the structure that allow thiazides to partially inhibit the cotransporter.

The Eel NCCβ Is Not Activated by Low-chloride Hypotonic Stress—Members of the SLC12 family of transporters are modulated by the intracellular chloride concentration ($[Cl^-]_i$). When $[Cl^-]_i$ is reduced, NCC is activated by the phosphorylation of certain amino-terminal domain threonine residues through the WNK-SPAK kinase pathway (22, 23). The effect of low-chloride hypotonic stress (which is known to reduce the $[Cl^-]_i$ (23)) on the activity of rat and eel NCCβ was assessed. Interestingly, as shown in Fig. 7, although the activity of rat NCC was significantly increased by lowering the $[Cl^-]_i$, no change in eel NCCβ activity was observed under similar conditions. Thus, in contrast to what has been observed for mammalian NCC and other members of the SLC12 family (24, 25), eel NCCβ is not activated by the reduction of the $[Cl^-]_i$.

Na^+ and Cl^- Transport Kinetics in Eel NCCβ—We have shown previously that mammalian and teleost NCC exhibit significant differences in their ion transport kinetics (11–14). The affinity for both ions is higher in mammalian orthologs than in flounder NCC. In addition, the K_m values for Na^+ and Cl^- are similar in mammalian NCCs; however, in flounder NCC, the K_m values for Cl^- are lower than those observed for Na^+ (12–14). Thus, the ion transport kinetics in oocytes injected with NCCβ cRNA were assessed. As depicted in Fig. 8*A*, Cl^- transport kinetics are similar between eel NCCβ (K_m of 15.6 ± 4.5 mM) and flounder NCC (K_m of 15.0 ± 2.0 mM; see Ref. 14). As Fig. 8*B* depicts, the Hill coefficient of the transport kinetics was 0.99, suggesting that one Cl^- ion was translocated.

In sharp contrast to what has been observed previously for rat, mouse, and flounder NCC (11–14), we could not define the Na^+ transport kinetics of eel NCCβ in oocytes by assessing $^{22}Na^+$ influx because the Na^+ influx increased progressively in influx media containing 0–80 mM Na^+ (Fig. 9) without reaching saturation. No more than 100 mM was tested because this is the maximum amount within normal oocyte tonicity. An attempt was made to express NCCβ in HEK293 cells, but this was unsuccessful. As occurs with mammalian NCC, no functional expression was observed in mammalian transfected cells (data not shown).

We were able to assess the Na^+ transport kinetics by measuring $^{36}Cl^-$ influx with increased concentrations of extracellular Na^+ from 0–96 mM. Values in the absence of Na^+ were subtracted from each of the studied influx points. As shown in Fig. 10*A*, a saturation curve was observed with a K_m of 31.2 ± 4.0 mM, a value similar to the previously observed of 30 ± 6.0 mM for flounder NCC (14). The slope of the Hill plot was 0.98 ± 0.11(Fig. 10*B*). Thus, eel NCCβ cRNA exhibits ion transport kinetics and stoichiometry that are similar to those present in flounder NCC.

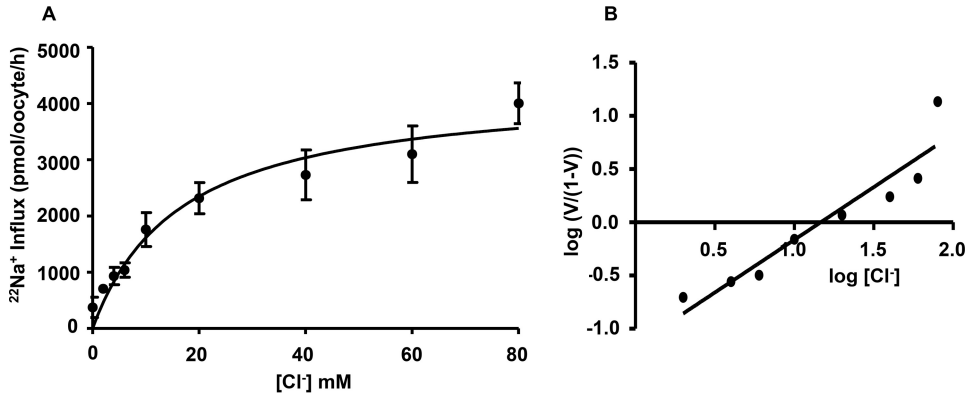

FIGURE 8. **Chloride transport kinetic analysis in oocytes injected with NCCβ cRNA.** *A*, Na^+ influx in oocytes exposed to influx medium with increased concentration of Cl^- from 0–96 mM. *B*, Hill plot analysis based on kinetic data obtained from *A*. The slope of the Hill plot is 0.99.

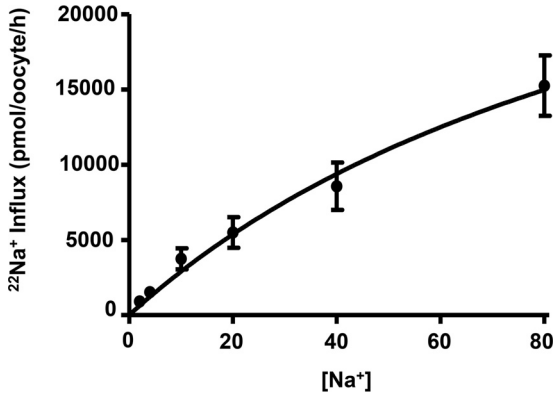

FIGURE 9. **Sodium transport kinetic analysis in oocytes injected with NCCβ cRNA assessed by tracer $^{22}Na^+$ influx.** Shown is Na^+ influx in oocytes exposed to influx medium with increased concentration of Na^+ from 2–80 mM in solutions with pH 7.4. A non-saturation curve was observed.

Discussion

Our data show that European eel NCCβ cDNA encodes an Na-Cl cotransporter that is K^+-independent but nevertheless resistant to thiazide-type diuretics. It has been generally considered that, in the SLC12 family, the Na-Cl cotransporter is sensitive to thiazides and resistant to furosemide, whereas the Na-K-2Cl cotransporter is sensitive to furosemide but resistant to thiazides. In the mouse SLC12A1 gene encoding the renal Na-K-2Cl cotransporter (NKCC2), there is one alternatively spliced isoform that, by losing most of the carboxyl-terminal domain, encodes an Na-Cl cotransporter that remains sensitive to furosemide (26). That is, the variant lost the K^+ transport ability without changing its sensitivity to loop diuretics. The latter finding is in agreement with the idea that diuretic affinity is determined by sequences within the central transmembrane domain (10). Here we observed that NCCβ behaves as an Na-Cl cotransporter that is nevertheless not sensitive to thiazides. Thus, eel NCCβ can be helpful in elucidating the amino acid residues required for NCC cotransporters to be inhibited by thiazides.

An initial approach revealed that residues located in the second and sixth TM regions could be important for thiazide sensitivity. According to Watanabe *et al.* (21), the Japanese eel expresses NCCβ in the rectum, and functional analysis of this region of the intestine using rectal sac preparations showed that NaCl transport is sensitive to hydrochlorothiazide. Thus, presumably, the Japanese eel NCCβ is sensitive to thiazides. By comparing the protein sequences of NCCβ from Japanese and European eels, we observed that, in the central transmembrane domain, there are only three amino acid residues that are not identical in NCCβ from both species. We observed in several experiments that wild-type NCCβ was not inhibited at all by thiazides; however, the NCCβ-C379S was significantly inhibited every time by thiazides of 45% to 55%. Thus, the presence of the serine in this position turns NCCβ into a thiazide-sensitive cotransporter. Mutant NCCβ with double or triple substitutions also showed thiazide sensitivity but not higher than that shown for the single mutant C379S, suggesting that it is this residue that is critical for thiazide affinity. Substituting cysteine 379 for alanine did not change the thiazide resistance of eel NCCβ. Thus, it is apparently not the presence of the cysteine at 379 but the absence of serine that makes NCCβ resistant to thiazides. According to the alignment shown in Fig. 4, serine 379 is conserved in all NCCs, from teleosts to humans. Thus, the absence of serine at this position is unique for European eel NCCβ.

The structural requirements for thiazide sensitivity or affinity in NCC are largely unknown but seem to be very complex. It is known that the diuretic binding site is located within the central transmembrane domain because swapping the hydrophilic amino- and/or carboxyl-terminal domains between the Na-Cl and the Na-K-2Cl cotransporter did not change the affinity for the thiazides in NCC or furosemide on NKCC2. That is, a chimeric protein containing the central hydrophobic domain of NCC with the amino- and/or carboxyl-terminal domains of NKCC2 remains sensitive to thiazides (10). Elimination of the glycosylation sites in rat NCC decreases the cotransporter activity by 95%, but the affinity for thiazides in the remaining active transporter increased by 2 orders of magnitude, suggesting that perhaps the presence of glycosylation prevents the thiazides from reaching the binding site within the transmembrane domain (27). However, this was not observed in flounder NCC. Elimination of glycosylation sites in the teleost NCC decreased the cotransporter by 50% but had no effect in the affinity for thiazides (14). Finally, the difference in the affinity for thiazides between the mammalian and teleost NCC (12, 13) is due to a single-residue difference located in the 11 transmembrane segment (15). Here we observed that substi-

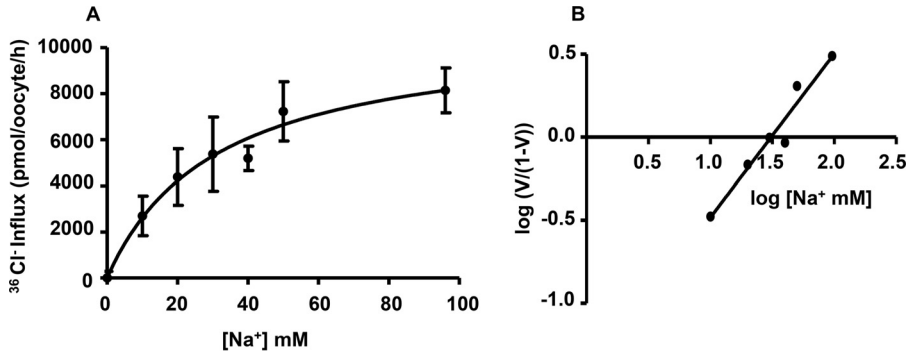

FIGURE 10. **Sodium transport kinetic analysis in oocytes injected with NCCβ cRNA assessed by $^{36}Cl^-$ influx.** *A*, Cl^- influx in oocytes exposed to influx medium with increased concentrations of Na^+ from 0–96 mM. *B*, Hill plot analysis based on kinetic data obtained from *A*. The slope of the Hill plot is 0.98.

tuting a cysteine for a serine at the position 379 confers sensitivity to thiazides in the eel NCCβ. The serine 379 is predicted to be located at the very end of the sixth transmembrane domain, which, according to the current model of NCC, should be facing the inner part of the membrane. However, on one hand it is known that NCC forms dimers to be functional (28), and on the other hand, the tridimensional structure of NCC is not known. Thus, further studies are required to understand what serine 379 could be doing to the structure of the transporter to confer sensitivity to thiazides.

Another interesting difference between NCCβ and the other characterized NCCs is the insensitivity to $[Cl^-]_i$. It is known that NCC is modulated by $[Cl^-]_i$ (25) through the WNK-SPAK pathway. WNKs are chloride-sensitive kinases (29). When $[Cl^-]_i$ is decreased, WNK1 and WNK4 are activated by autophosphorylation, resulting in the activation of SPAK/OSR1, which, in turn, stimulates NCC by phosphorylating key residues in the amino-terminal domain (23). Mammalian NCC and eel NCCβ share the SPAK/OSR1 binding site and the main phosphorylation sites in the amino-terminal domain (Thr-58 and Ser-71 in rat NCC and Thr-60 and Ser-73 in human NCC). However, the third known site, Thr-53 in rat NCC, is not conserved in NCCβ. Our previous work showed that, in rat NCC, the elimination of Thr-53 did not preclude the activation of NCC by the reduction of $[Cl^-]_i$ (25), whereas the elimination of Thr-58 or Ser-71 completely prevented activation of the cotransporter. Thus, NCCβ contains the SPAK/OSR1 binding site and the phosphorylation sites that are required but, interestingly, is not activated by depletion of $[Cl^-]_i$. This observation suggests that an additional unknown motif could be required for the cotransporter to be modulated by $[Cl^-]_i$. Thus, NCCβ could be helpful in future studies to find out the potential motif.

European eel NCCβ belongs to the SLC12 family of solute carriers in which all studied members today are electroneutral cation-coupled chloride cotransporters with cation-chloride 1:1 stoichiometry, precluding changes in the transmembrane potential. The ion transport kinetics of NCCβ revealed a stoichiometry of 1:1 for Na^+ and Cl^- because the slope of the Hill plot in both cases was almost one. In fact, the K_m for both ions was similar to that observed previously for flounder NCC (13, 14). Thus, it is likely that the NaCl transport mode by eel NCCβ is similar to that observed for NCC in other teleosts.

However, our observation suggest that, in addition to the 1:1 Na-Cl translocation, NCCβ could have an electrogenic mode of transport. This is based on the remaining Na^+-independent Cl^- influx. As shown in Fig. 1*D*, the Na^+-dependent fraction accounts for 80% of the Cl^- influx, leaving about 20% of Cl^- transport as a Na^+-independent fraction. Because NCCβ is not sensitive to thiazide-type diuretics, it is not possible to define whether the remaining Cl^- influx in the absence of extracellular Na^+ observed in NCCβ-injected oocytes is indeed due to Cl^- transport through the cotransporter protein. One possibility to consider is that the presence of NCCβ induces the opening of another endogenous transport pathway involving Cl^- and, thus, that the remaining Cl^- influx is not due to NCCβ. The other possibility is that indeed NCCβ could have a small portion of activity as an electrogenic transporter. This type of behavior has been documented previously for other transport proteins. For instance, the Na^+/monocarboxylate cotransporter SLC5A8 has been shown to exhibit Na^+ leaking behavior (30). Another example is SLC26A9, which is an anion exchanger but also has anion channel properties (31, 32). Thus, further experiments, including two-electrode voltage clamp analysis and assessment of intracellular chloride concentration, will be required to define the nature of the Cl^- influx observed in the absence of extracellular Na^+ in the NCCβ-injected oocytes.

With the functional properties observed for NCCβ in this work, it is possible that this transporter, located at the very end of the intestine, helps freshwater eels to absorb Cl^- ions either with or without Na^+, maximizing the absorption capacity in an environment where ion concentrations are very low. This is supported by the observation of Watanabe *et al.* (21) that NCCβ expression in the rectum is up-regulated in freshwater-acclimated eels.

In summary, we have identified a series of functional characteristics of eel NCCβ that are unique to this NCC ortholog that could potentially help elucidate structure-function relationships. As an example, we have shown that serine 379 at the end of the six transmembrane segments seems to be a key residue conferring affinity for thiazide diuretics in NCC.

Experimental Procedures

NCCβ Cloning—Eel *NCCβ* isoform full-length cDNA was amplified by RT-PCR from pre-existing seawater-acclimated eel rectal cDNA using Phusion DNA polymerase (New England Biolabs) using the 5′ and 3′ UTR-located primer sequences CTGAATTCATGGGGCAGCGCATATCTGGTC and ACT-

CTAGATCACTGGCAGTAGAAGGTGAGCACATTCT, respectively. The eel *NCCβ* cDNA was inserted into the *Xenopus* expression vector *PGHEM* flanked with the restriction sites SmaI and XbaI. The complete cDNA insert was sequenced and contained 3437 bp, with an open reading frame of 3132, encoding a 1043-amino acid residue protein. During the cloning, a FLAG epitope was inserted by PCR into the open reading frame after the first methionine.

Site-directed Mutagenesis—Site-directed mutagenesis was achieved using the QuikChange site-directed mutagenesis system (Stratagene) as described previously (33) and according to the recommendations of the manufacturer. All of the mutations were confirmed by automatic DNA sequencing. All of the primers used for mutagenesis were custom-made (Sigma).

In Vitro cRNA Translation—To prepare cRNA for microinjection, human and rat NCC cDNA, rat NKCC2 cDNA, and eel NCC*β* cDNA were digested at the 3′ end using NheI from Invitrogen, and cRNA was transcribed *in vitro* using the T7 RNA polymerase mMESSAGE mMACHINE (Ambion) transcription system. cRNA product integrity was confirmed on agarose gels, and the concentration was determined using an absorbance of 260 nm (DU 640, Beckman, Fullerton, CA). The cRNA was stored frozen in aliquots at −80 °C until ready for use.

X. laevis Oocyte Preparation—Oocytes were harvested surgically from adult female *X. laevis* frogs (Nasco) under 0.17% tricaine anesthesia and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES-Tris (pH 7.4)) in the presence of collagenase B (2 mg/ml) for 1 h. After four washes in ND96, the oocytes were manually defolliculated and incubated overnight at 16 °C in ND96 supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml gentamicin. The next day, stage V-VI oocytes (34) were injected with 50 nl of water or 10–20 ng of NCC*β* cRNA/oocytes. Next, the oocytes were incubated for 2 or 3 days in ND96 with sodium pyruvate and gentamicin, which were changed every 24 h (35).

Assessment of NCCβ Cotransporter Function—The function of NCC*β* was determined by assessing tracer $^{22}Na^+$, 86 Rb^+ and $^{36}Cl^-$ influx in groups of 10–15 oocytes following our protocol. The normal extracellular osmolarity for *Xenopus* oocytes is around 200 mosM/kg, whereas the extracellular osmolarity of rectal epithelial cells of the eel is more variable, depending on the presence of eels in fresh or salt water. However, we have seen that the behavior of SLC12 family members is maintained when expressed in *Xenopus* oocytes even with their inherent extracellular osmolarity (35–37). For instance, the mammalian Na-K-2Cl cotransporter expressed in oocytes is activated by hypertonicity and inhibited by hypotonicity. Also, the K-Cl cotransporters are inhibited by hypertonicity and activated by hypotonicity. Thus, oocytes are a good expression system to study SLC12 family members, although the osmolarity at which oocytes live is different from the cells from which the cotransporters were identified.

Assessing $^{22}Na^+$ Tracer Influx—A 30-min incubation in Cl^--free ND96 medium containing 1 mM ouabain, 0.1 mM amiloride, and 0.1 mM bumetanide was followed by a 60-min influx period in K^+-free, NaCl-containing medium with ouabain, amiloride, bumetanide, and 2 μCi of $^{22}Na^+$/ml. The affinity for thiazide diuretics was assessed by exposing the groups of cRNA-injected oocytes to concentrations of drugs from 10^{-9} to 10^{-4} M. Human, rat, and or flounder NCC was used as a control signal.

To determine the ion transport kinetics of NCC*β*, we performed experiments varying the concentrations of Na^+ and Cl^-. To maintain osmolarity and ionic strength, gluconate was used as a Cl^- substitute, and *N*-methyl-D-glucamine was used as an Na^+ substitute. NCC*β* was subjected to at least four different ion transport kinetic experiments with each set of solutions (12, 14).

Assessing $^{86}Rb^+$ Tracer Influx—A 30-min incubation in K^+- and Cl^--free medium with 1 mM ouabain was followed by a 60-min influx period in the presence of Na^+, K^+, and Cl^- medium containing 1 mM ouabain and in the absence or presence of furosemide (10^{-4} M). NKCC2 was used as a control signal.

All of the influx experiments were performed at 32 °C. At the end of the influx period, oocytes were washed five times in ice-cold influx solution without isotope to remove extracellular fluid tracer. After the oocytes were dissolved in 10% sodium dodecyl sulfate, tracer activity was determined for each oocyte by *β*-scintillation counting (38).

Assessing $^{36}Cl^-$ Tracer Influx—30-min incubation in Cl^--free ND96 medium containing 1 mM ouabain, and 0.1 mM bumetanide was followed by a 60-min influx period in K^+-free, NaCl-containing medium with ouabain and bumetanide and with 2 μCi of $^{36}Cl^-$/ml. To determine the Na^+ dependence of the $^{36}Cl^-$ transport of NCC*β*, $^{36}Cl^-$ influx was assessed in parallel groups using K^+-free and Na^+-free (replaced with *N*-methyl-D-glucamine or choline) Cl^--containing medium.

Western Blotting—Western blotting analysis was used to assess the protein expression of FLAG-NCC*β*-injected oocytes. Proteins extracted from 50 oocytes were quantified by Bradford's technique, and 50 μg of each protein per lane was run using sample buffer containing 6% SDS, 15% glycerol, 0.3% bromophenol blue, 150 mM Tris (pH 7.6), and *β*-mercaptoethanol, resolved by Laemmli SDS-polyacrylamide (7.5%) gel electrophoresis, and transferred to a PVDF membrane. Immunoblotting was performed using an anti-FLAG monoclonal antibody (Sigma). The membranes were exposed to anti-FLAG antibody overnight at 4 °C and washed again. The protein bands were detected using Immun-Star chemiluminescent protein detection systems (Bio-Rad).

Cell Surface Biotinylation of X. laevis Oocytes—The oocytes were injected with cRNA encoding FLAG-NCC*β*, washed five times in ND-96 TEA buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 8.8), and 10 mM TEA), and incubated for 30 min with 1.5 mg/ml Sulfo-NHS-LC-Biotin (Thermo, Pierce) in ice-cold ND-96-TEA. The oocytes were then washed five times in ND-96-TEA buffer and homogenized using a 25-gauge needle in a sucrose-based buffer (5 μl/oocyte) comprised of 250 mM sucrose, 0.5 mM EDTA, 5 mM Tris·HCl (pH 6.9), 1 mM PMSF, and 10 μl/ml protease inhibitor mixture (P8340, Sigma). The samples were centrifuged for 7 min at 8000 rpm, the supernatant was collected, and protein concentration was assessed utilizing the Bradford assay (Bio-Rad). Streptavidin precipitation was carried out by adding 75 μl of streptavidin-agarose beads in a 50% slurry (Cell Signaling Solutions,

Upstate) to 400 μg of biotinylated total protein diluted in 1 ml of Tris-buffered saline (100 mM NaCl, 50 mM Tris·HCl (pH 7.4)). The samples were continuously rolled overnight at 4 °C. The beads were then washed once with buffer A (5 mM EDTA, 50 mM NaCl, 50 mM Tris·HCl (pH 7.4)), twice with buffer B (500 mM NaCl, 20 mM Tris·HCl (pH 7.4)), and once with buffer C (10 mM Tris·HCl (pH 7.4)) with a 2-min, 5000 × *g* spin between each wash. After the last wash, buffer C was replaced with 30 μl of Laemmli sample buffer with 5% 2-mercaptoethanol (Sigma, Bio-Rad). The protein samples were heated to 65 °C for 15 min before separation on a 7.5% polyacrylamide gel (39).

Statistical Analysis—The results are presented as mean ± S.E., and significance was defined as two-tailed $p < 0.05$. The significance of the differences between the groups was tested using Student's *t* test. For three or more groups, one-way analysis of variance with multiple comparisons using Bonferroni correction was applied.

Author Contributions—E. M., C. P., A. R. G., E. R. A., N. V., K. L. R., D. P. A., A. M., M. C. B., and R. C. carried out the experiments, analyzed the data, and approved the final manuscript. E. M., C. P., L. I., C. C., and G. G. analyzed the data, wrote the manuscript, and approved the final version. E. M. and G. G. conceived the study.

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The European Eel NCCβ Gene Encodes a Thiazide-resistant Na-Cl Cotransporter

Erika Moreno, Consuelo Plata, Alejandro Rodríguez-Gama, Eduardo R. Argaiz, Norma Vázquez, Karla Leyva-Ríos, León Islas, Christopher Cutler, Diana Pacheco-Alvarez, Adriana Mercado, Raquel Cariño-Cortés, María Castañeda-Bueno and Gerardo Gamba

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