Evidence for Up-regulation of the Endogenous Na-K-2Cl Co-transporter by Molecular Interactions with the Anion Exchanger tAE1 Expressed in Xenopus Oocyte*

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Expression of trout anion exchanger 1 (tAE1) in X. oocyte led to the stimulation of a Na\(^+\)- and Cl\(^-\)-dependent Rb influx. Functional features and pharmacological data strongly suggest that this Rb influx is mediated by the endogenous Na-K-2Cl (NKCC) co-transporter. The functional relationship between expression of tAE1 and activation of the NKCC co-transporter was investigated. Indeed, it was shown previously that tAE1 expressed in X. oocyte induces a strong anion conductance, which is correlated with an increased taurine permeability. Measurements of intracellular ion contents ruled out the involvement of any modification of known electrochemical parameters in NKCC co-transporter activation by tAE1. Furthermore, using chimera of tAE1 made with AE1 from other species unable to exhibit anion conductance led to the conclusion that there was no correlation between tAE1 anion conductance and NKCC co-transporter stimulation. Therefore, a possible molecular interaction between tAE1 and the NKCC co-transporter was investigated. Our results clearly show that NKCC activation is dependent upon the C-terminal part of tAE1. Chimeric constructions where tAE1 C-terminal part was substituted by the corresponding part of mouse AE1 abolished co-transporter activation. Moreover, steric encumbrance on the C-terminal end of tAE1 with a specific antibody or with a protein fusion also prevented the co-transporter activation. These data suggest a new role for some anion exchangers in controlling other transporter activity by molecular interactions.

Membrane permeability is an essential property that governs cell physiology. Concentration of intracellular osmolites and, therefore, of intracellular enzyme activities, specific cell functions, and metabolism depends on cell membrane permeability. To ensure control of intracellular medium, the functioning of membrane transporters must be regulated and coordinated.

Regulation of membrane permeability could be achieved by direct modifications on transporters such as phosphorylation by means of kinase or phosphatase action that could change transport features (1). Another regulation possibility is obtained by interactions between transporters. These interactions could be functional, which means that the functioning of one transporter may create electrochemical conditions favorable to stimulate the functioning of other transporters. Many examples of such functional interactions between membrane transporters are known (for instance, electric coupling between channels, thermodynamic coupling between Na-K-2Cl (NKCC)\(^1\) co-transporter and KCl transporter (2) or autocrine mechanism; Refs. 3, 4). But transporter coupling could also be by means of direct protein-protein interaction. Interest in such interactions has been focused on the example of CFTR (cystic fibrosis transmembrane conductance regulator). Different studies report possible molecular interaction of the CFTR with other membrane transporters, such as the Na\(^+\) channel ENaC (epithelial Na\(^+\) Channel) (5–8) and possibly with the Cl\(^-/\)HCO\(_3\)\(^-\) exchanger (9) (in this last example, stimulation of members of the SLC26 family through molecular interactions with the CFTR is only documented by co-immunoprecipitation experiments). To our knowledge, CFTR is the only example of transporter susceptible to regulate other transporters by protein-protein interactions.

In the present paper, we provide another example of membrane transporter regulation by direct protein interactions that are not related to transport functions. It concerns interaction between the trout anion exchanger, tAE1, and the NKCC co-transporter. This protein catalyzes the simultaneous transport of one Na\(^+\) with one K\(^+\) and two Cl\(^-\) across cell plasma membrane. It can be detected by measuring a Na\(^+\)- and Cl\(^-\)-dependent K\(^+\) influx. Moreover, this co-transport is inhibited by micromolar concentrations of bumetanide. Two different isoforms of this co-transporter are known (NKCC1 and NKCC2), and it has been extensively studied in a great number of cell types (for review, see Ref. 10).

TAE1 is the anion exchanger of trout erythrocytes. Anion exchangers (AEs) are transmembrane proteins catalyzing the electroneutral exchange of 1 Cl\(^-\) for 1 bicarbonate. Up to now, three different genes, named slc4a4, slc4a2, and slc4a3, have been known, each of them coding for different polypeptide products, depending on the splicing and transcription initiation site (for reviews, see Refs. 11, 12). All of the AE polypeptides can be divided into two main domains of about the same size: an N-terminal cytoplasmic domain interacting with cytoskeleton and a membrane spanning domain, which is responsible for ion translocation, with a short C-terminal end in the cytoplasm. By expression studies of tAE1 in X. oocytes, we have demonstrated previously that this normally electroneutral anion exchanger is able to form an anion conductive path-

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\(^{‡}\) The abbreviations used are: NKCC, Na-K-2Cl; CFTR, cystic fibrosis transmembrane conductance regulator; AE, anion exchanger; tAE1, trout anion exchanger 1; hAE1, human AE1; mAE1, mouse AE1; skAE1, skate AE1.

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way permeable to different charged or neutral osmolytes such as urea, choline, sorbitol, Na\textsuperscript{+}, and K\textsuperscript{+} (13–15). In physiological conditions, these transport properties of tAE1 are activated by a decrease in intracellular ionic strength in swollen erythrocytes, and they are involved in the cell regulatory volume decrease response (16, 17). Here, we focused on another unexpected feature of this anion exchanger. Independently of its own transport ability, this exchanger is able to stimulate the endogenous NKCC co-transporter in Xenopus oocyte. We show that this stimulation is not linked to tAE1 activity but rather to the interaction between the C-terminal end of tAE1 and the NKCC co-transporter. This interaction could involve a direct contact between the two transporters, or it could involve intermediary regulatory proteins. Even though this interaction between tAE1 and the NKCC co-transporter was observed in Xenopus oocyte, it is likely that such interactions could take place in physiological conditions to coordinate transporter activity and regulate cell membrane permeability.

**Experimental Procedures**

**RNA Synthesis**—tAE1, tAE1gyrB, TZM, T-4M, T-5M, and tAE1AN cloned in pSP64polyA vector (Promega) were linearized by EcoRI. mRNA was synthesized in pSP64 linearized by HindIII. skAE1 and S2T, cloned in a modified pSP64polyA vector, were linearized by ApaI. Linearized DNA were transcribed by SP6 RNA polymerase following instructions from Ambion mMessage mMachine SP6 transcription kit. cRNA were then stored at 100 ng/μl in −20 °C before use.

**Taking of Oocytes**—Xenopus laevis were cooled on ice with MS222 until completely anesthetized and were kept covered with ice during the surgery, according to the procedure recommended by our ethics committee. The surgery consisted of removing about five ovarian lobes containing oocytes. After surgery, the animals were placed in cold water between 0 and 4 °C to recover from anesthesia, monitored for 3 hours, and then placed back in their aquaria.

**Oocyte Injection**—Collected oocytes were washed in a modified Barth’s saline (MBS) (85 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 0.82 mM MgSO\textsubscript{4}, 0.33 mM Ca(NO\textsubscript{3})\textsubscript{2}, 0.41 mM CaCl\textsubscript{2}, 10 mM HEPES, 4 mM NaOH, pH 7.4, supplemented with 10 units/ml penicillin and 10 μg/ml streptomycin). After washing with MBS, defolliculation was obtained by overnight incubation at 19 °C in MBS containing 0.8 and 1.3 mg/ml collagenase (Serva) corresponding to 1 unit/ml, followed by a 30-min incubation in Ca\textsuperscript{2+}−free MBS. Stage V-VI oocytes were then injected with 50 nl of 100 ng/μl cRNA and maintained at 19 °C in MBS. This cRNA concentration was found to induce maximal expression of the corresponding proteins in oocyte.

**Influx Measurements**—Cl− influx measurements were done as described previously (13, 15). Rb influx measurements were done in media containing 10−4 m ouabain to avoid looking at K\textsuperscript{+} movements through the Na-K-ATPase. Before incubation with radioactive medium, oocytes were quickly washed three times with 1.5 ml of radioactive-free medium containing ouabain 10−4 M to adapt the oocyte to the eventually different influx conditions. Eight oocytes were incubated at 19 °C in 80 μl of influx medium containing 40Rb used as a radioactive substitute for Na\textsuperscript{+} before use. The effect of 10−4 M ouabain. These data are the means of eight oocytes individually counted and then averaged. This experiment is one representative experiment of three. B, The Rb influx was measured in non-injected oocyte either in control isosmotic media (Ctrl) or in hyperosmotic media (Hyper). The oocyte incubation media were Ctrl-containing MBS (white bars), NO\textsubscript{3}−-containing MBS (dotted bars), or N-methyl-D-glucamine-containing MBS (striped bars). The effect of 10−6 M bumetanide was assessed in hyperosmotic MBS (containing Cl). These data are the means of eight oocytes individually counted and then averaged. This experiment is one representative experiment of three.

**Determination of Ion and Water Concentrations of Oocytes**—To determine the intracellular Cl concentrations, oocytes were incubated in Cl− containing MBS up to the equilibrium of specific radioactivity (36Cl dpm/nmol of Cl). By counting intracellular 36Cl dpm accumulated, it was possible to deduce the intracellular Cl concentration.

Na\textsuperscript{+} and K\textsuperscript{+} contents were measured as described previously (15). Intracellular ions were extracted from dried oocytes in 4 ml of milliQ water overnight at 4 °C. Measurements of sodium and potassium were done with a flame photometer (Eppendorf). Results are expressed in mM (see Table 1). The oocyte water content was measured by weighing a group of five oocytes after a quick wash in milliQ water (to remove extracellular ion contaminants) containing 3H-inulin (to be able to determine the extracellular water volume). The oocytes were weighed...
wet and then dry (they were dried overnight at 80 °C). Subtraction of the dry weight from the wet weight after correction for extracellular water trapped between the five oocytes, gives the water content of five oocytes. Assuming that the five oocytes are equivalent, we deduced the intracellular water content of an oocyte to be around 500 nl.

**Electrophysiology**—Electrophysiological parameters were measured as described previously (13) using the two-electrode voltage clamp technique with a TEV 200 amplifier (Dagan, M with a Li, MN) monitored by computer through Digidata 1200 A/D converter PC clamp software (Axon Instruments Inc., Foster City, CA). Measurements were done in MBS at room temperature.

**Chimera Construction**—Except for TB3-gyraseB, all other chimera constructions are described in previously published papers (13). The chimera tAE1-gyraseB was made by PCR with plasmids pSP64tAE1 and pcDNA3Raf-gyrB. This last plasmid was a kind gift from Dr. Michael Farrar (19). tAE1 was amplified with primers 5′-tAE1 (AAGCTT-GGCTTCGAGTCCGA) and 3′-tAE1 (GGCGGCCGCTGCAAGCG-GACTCGTA). The gyrase B was amplified with primers 5′-GyrB (GGCGGCCGAGCAATCTTTATGAC) and 3′-GyrB (GAGCTGTCAACTAGTGGTAC). The primers 3′-tAE1 and 5′-gyraseB have an overlapping part of nine nucleotides. 100 µl of PCR contained 100 ng of tAE1 or gyraseB plasmids, 200 µM 2′-deoxyinososine 5′-triphosphate (dNTP), 0.4 µM of each primer, 1 µl of VentDNA polymerase (NEBio-labs); denaturation was for 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min. The PCR products tAE1 (2.7 kb) and gyraseB (0.7 kb) were then diluted 10 times and mixed in a secondary PCR to amplify the fusion tAE1-gyraseB with primers 5′-tAE1 and 3′-gyrB in a final volume of 100 µl containing 1 µl of the two 10×-diluted first PCR products, 200 µM dNTP, 0.4 µM of each primer, 1 µl of Expand high fidelity DNA polymerase (Roche Applied Science). The amplification protocol was the same as for the first PCR. This yielded a 3.4-kb product corresponding to the fusion tAE1-gyraseB that was cloned in pGEMT-easy vector (Promega). Then tAE1-gyrB was subcloned in pSP64polya (Promega) using Fast-Link DNA ligase (Epicenter) and restriction enzymes from NEBio-labs. Top10F competent cells (Invitrogen) were transfected, and positive recombinant pSP64polya-tAE1GyrB containing plasmids were selected and analyzed.

**Western Blot of Oocyte Membrane Proteins**—Oocyte membranes were prepared by homogenization of 20 oocytes (control or injected) in cooled 20 mM Tris-HCl buffer, pH 7.4, with 0.5 mM dithiothreitol and 0.5 mM protease inhibitor (Pefabloc, Roche Applied Science). The mixture was centrifuged at 4 °C at 2000 rpm, 4000 rpm and finally at 6000 rpm in an Eppendorf tube; after each centrifugation, the pellet was discarded. Collected supernatant after the third centrifugation was ultracentrifuged at 65,000 rpm for 30 min at 4 °C, and the membrane pellet was solubilized in the homogenization buffer. Protein concentration was measured with a Bio-Rad kit, and 50 µg of proteins were then loaded per lane of SDS-PAGE electrophoresis gel. Western blot transfer was done with a semi-dry transfer system from Biometra on nylon membrane (Hybond C extra, Amersham Biosciences). The presence of tAE1 was detected by the antibodies Abi145 directed against a synthetic peptide corresponding to the last 15 amino acids of the C-terminal part of tAE1 (Neosystem, Strasbourg). Immunoglobulins Ab145 and Ab146 were purified from rabbit serum by precipitation with caprilic acid as described by Reik et al. (20). The secondary antibody was a goat IgG-peroxidase (Sigma) that was detected by chemiluminescence (Super Signal West Pico, Pierce).

**Chemicals and reagents** were from Sigma, unless otherwise stated. Plasmid DNA preparations were done with Qiagen Miniprep kit from Qiagen, and DNA purifications were done with Machery Nagel nucleospin extract kit.

**RESULTS**

The presence of the endogenous NKCC co-transport in Xenopus oocyte plasma membrane was characterized by measuring a K⁺ influx that is Cl⁻ and Na⁺ dependent, and sensitive to micromolar concentrations of bumetanide (21, 22). Fig. 1A illustrates the ouabain-insensitive K⁺ permeability in Xenopus oocytes between 1 and 5 days after oocyte collection in isos-
motic or hyperosmotic media. As shown previously by others, this K⁺ permeability is largely Na⁺- and Cl⁻-dependent because substitution of Na⁺ by N-methyl-D-glucamine or Cl⁻ by NO₃⁻ abolished the Rb influx (Fig. 1B). Hyperosmotic stimulation of the NKCC co-transporter increased about 9× the Rb influx of control oocytes. There is no significant difference in the activation of NKCC co-transporter by hyperosmolarity as a function of time. The co-transporter is present and can be activated by hyperosmolarity in oocyte plasma membrane for 5 days after oocyte collection. Moreover, the Rb influx stimulated by hyperosmolarity is highly sensitive to bumetanide as expected (Fig. 1B).

In the next experiments, it was observed that expression of the anion exchanger of trout erythrocyte, tAE1, stimulated a Cl⁻-dependent Rb uptake in *Xenopus* oocyte that was inhibited by bumetanide, by a NO₃⁻ substitution of Cl⁻, and by substitution of Na⁺ by N-methyl-D-glucamine (Fig. 2A). This Rb influx is not additive with the Cl⁻-dependent Rb uptake stimulated by hyperosmolarity (Fig. 2B); it has the features of the Rb influx mediated by the NKCC co-transporter. In previous work, it was shown that tAE1 is functionally expressed in oocyte plasma membrane where it increases by 6-fold Cl influx of control oocytes as early as 24 h after injection (12). Moreover, it was observed that this Cl influx increase is proportional to the amount of tAE1-cRNA injected in oocytes, and therefore, it was considered as a relevant marker of tAE1 expression level in oocyte plasma membrane. To correlate activation of NKCC co-transporter to the presence of tAE1 in oocyte plasma membrane, Cl⁻-dependent, bumetanide-sensitive Rb uptake was measured as a function of the amount of tAE1-cRNA injected in *Xenopus* oocyte (Fig. 3). This experiment shows a good correlation between NKCC activity and tAE1 expression level.

It should be noticed that the activation of NKCC co-transporter was observed as early as 1 day after tAE1 injection in *Xenopus* oocyte; then, it persisted over time (Rb influx in tAE1-expressing oocyte was 252 ± 33 pmol per oocyte per h; n = 7, 4 days after injection).

The anion exchanger of trout erythrocyte, tAE1, was shown to form an anion conductance permeable to ta urine as well as to different organic and inorganic compounds when expressed in *Xenopus* oocyte (9, 10). This large anion conductance imposes a large anion conductance equal between control oocytes and tAE1-expressing oocytes 1 day after injection. Thus, there is no variation of intracellular Cl⁻ concentration 1 day after injection, when activation of the co-transporter was measured. Table I shows that intracellular Na⁺, K⁺, and Cl⁻ concentrations are equivalent between control oocytes and tAE1-expressing oocytes 1 day after injection. Thus, there is no variation of intracellular ion concentrations that could explain the activation of the co-transporter in tAE1-expressing oocytes when we did our measurements.

In the absence of an obvious functional relationship between tAE1 expression and activation of the endogenous NKCC co-transporter in *Xenopus* oocytes, stimulation by protein-protein interaction was considered.

To assess whether tAE1 is able to stimulate the NKCC co-transporter by protein-protein interactions, different chimeras were done between trout, mouse or skate AE1. Fig. 6A represents a diagram of the different constructions. All of these constructions are functionally expressed in *Xenopus* oocyte. Except for tAE1ΔN, they have the same Cl⁻-transport features as does tAE1 (13); this finding suggests a similar amount of protein in oocyte plasma membrane. This possibility is con-
firmed by Western blot analyses of oocyte membrane proteins (Fig. 6B). In tAE1LN, 300 amino-acids in the putative cytoplasmic N-terminal domain (counting 400 amino-acids) were removed; the deletion of almost all of the cytoplasmic domain of the exchanger has an effect upon this protein expression at oocyte plasma membrane, as indicated by a 69% decrease of the Cl− exchange activity. However, despite a lower Cl− exchange activity, this deletion does not prevent stimulation of the NKCC co-transport, as illustrated in Fig. 7. Thus, the N-terminal cytoplasmic domain of tAE1 is not important for the NKCC co-transport, as illustrated in Fig. 7. The conductance was measured 2 days after injection in MBS containing 10−4 M ouabain and glybenclamide 2×10−4 M. Results are expressed as % of tAE1-induced Rb influx in the absence of inhibitors (control = 100%). Rb influx in non-injected oocyte with or without inhibitors was subtracted to corresponding tAE1-induced Rb influx. Means ± S.E., n = 3.

Fig. 5. Glybenclamide effect on tAE1 anion conductance and tAE1-stimulated NKCC co-transport. A, conductance (G μS) of tAE1-expressing oocyte was measured in the presence or absence of 2×10−4 M glybenclamide. Measurements were done at day 6 post-injection when tAE1 conductance was high (113). Glybenclamide was prepared at 10−3 M in Me2SO just before use. Results are expressed in % of the conductance of tAE1-expressing oocyte in the absence of inhibitor (control 100%). Means ± S.E., n = 3. Inset, dose-response curve of tAE1 anion conductance (G μS) as a function of glybenclamide concentration (Log M). Oocytes expressing tAE1 for 3 days were preincubated for 15 min in glybenclamide-containing MBS, and conductance was measured in the presence of the corresponding dose of glybenclamide. Data are the means obtained on six oocytes ± S.E.; B, Rb influx in tAE1-expressing oocytes was measured 2 days after injection in MBS containing 10−4 M ouabain and glybenclamide 2×10−4 M. Results are expressed as % of tAE1-induced Rb influx in the absence of inhibitors (control = 100%). Rb influx in non-injected oocyte with or without inhibitors was subtracted to corresponding tAE1-induced Rb influx. Means ± S.E., n = 3.

of the different chimeras was indicated under each construction in Fig. 7. The conductance was measured 2 days after oocyte injection. These data show a large discrepancy between chimera regarding their anion conductance. Furthermore, these differences are not correlated with differences in Rb influx activity.

All together, these data suggest the possible interaction between the last four putative helices in the C-terminal part of tAE1 and the NKCC co-transporter. To further investigate this possibility, we attempted to modify the C-terminal end of tAE1 by two means. First, we increased the size of tAE1 C-terminal domain by the fusion of a cytosolic protein, the B subunit of gyrase composed of about 200 amino acids. GyrB domain comes from the bacterial DNA gyrase. As illustrated in Fig. 8A, the fusion protein called tAE1-GyrB is expressed in Xenopus oocyte. Moreover, the addition of GyrB does not have any effect upon the anion conductance and Cl− transport characteristics (Fig. 8B). However, Fig. 8C shows that the presence of GyrB in the C-terminal part of tAE1 abolished 67% of NKCC co-transporter activation.

Second, we used an antibody raised against the 15 last amino-acids in the C-terminal end of tAE1 to prevent putative interactions between the C-terminal end of tAE1 and the NKCC co-transporter, thus, inhibiting the Cl−-dependent Rb influx stimulated by tAE1 expression. This antibody was shown to react with tAE1 in a Western blot (Figs. 6A and 8A). Injection of the antibody was done 1, 2, 3 or 5 hours before assessing the Cl−-dependent Rb influx in either tAE1-expressing oocytes or control oocytes. As control, oocytes expressing tAE1 were injected with water. It was observed that injection of the antibody at any time did not impair anion conductance or
Cl influx mediated by tAE1. In control condition, conductance and Cl influx of tAE1-expressing oocytes were 18.3 ± 2.6 μS and 192 ± 14 pmol/min per oocyte (n = 3), and they were 19.8 ± 4.1 μS and 202 ± 15 pmol/min per oocyte, n = 3, in these oocytes previously injected with 50 nl of Ab145). Moreover, this antibody was tested regarding its possible inhibitory effect on the endogenous NKCC co-transporter. The hyperosmotically stimulated Rb influx was measured in oocytes previously injected with 50 nl of water or antibody 145. The NKCC co-transporter activation was equivalent in both conditions (387 ± 85 pmol/h per oocyte in water-injected oocytes versus 355 ± 65 pmol/h per oocyte in Ab145-injected oocytes, n = 5). In contrast, Fig. 9 shows that injection of antibody 145 inhibits the Cl-dependent Rb influx induced by tAE1. The maximal inhibition was obtained 2 h after antibody injection. The presence of the antibody abolished 41 ± 9% of the tAE1-induced stimulation of NKCC (p < 0.05). It should be noticed that injection of the same amount of an irrelevant antibody (IgG against rabbit actin) did not impair the Cl-dependent Rb influx in tAE1-expressing oocytes (Fig. 9).

**DISCUSSION**

In the present report, we show that the Rb influx stimulated by tAE1 expression in *Xenopus* oocyte is sensitive to micromolar concentrations of bumetanide and to Na⁺ or Cl⁻ substitution. Moreover, it is not additive with the Rb influx activated by hyperosmotic medium, which strongly suggests that the same transporter is the target of the hyperosmotic shock and tAE1. This transporter has the features of the NKCC co-transporter that is present in oocyte plasma membrane, where it has been studied by different groups (21, 22). The stimulation of this co-transporter is observed as soon as tAE1 starts to be expressed in oocyte plasma membrane (1 day after injection; Ref. 13), and the activation is immediately maximal. The simplest interpretation of these results is that tAE1 expression in oocyte membrane activates the endogenous NKCC co-transporter.

Different kinds of interactions between membrane transporters could be envisioned; for example, they could be functional coupling or direct protein-protein interactions. Because tAE1 modifies oocyte membrane permeability, we first examined how these changes may be involved in NKCC co-transporter activation.

As previously shown, tAE1 is able to form an anion channel in oocyte plasma membrane. Up-regulation of NKCC co-transporter by Cl⁻ channels has been reported in different papers. For instance, in epithelial cells, opening of Cl⁻ channels in the apical membrane stimulates NKCC uptake in the basolateral membrane (27, 28). This stimulation seems to be related to intracellular Cl⁻ depletion by a molecular mechanism still not well understood. Co-expression of NKCC co-transporter and
the KCl co-transporter in human embryonic kidney cells showed that KCl depletion induced by KCl co-transporter activates NKCC co-transporter (2). Moreover, in immature neuronal cells, activation of GABA<sub>A</sub> receptors decreases intracellular Cl<sup>-</sup> concentration, and this consequently stimulates NKCC co-transporter.

tAE1-expressing oocytes exhibit a high Cl<sup>-</sup> conductance that allows the cell to reach equilibrium potential for Cl<sup>-</sup> (13). Our data clearly show that the depolarization in tAE1-expressing oocytes is not associated with a decrease in intracellular Cl<sup>-</sup> concentration at day 1 post-injection (Table I). Indeed, in control oocytes, equilibrium potential for Cl<sup>-</sup>, calculated from data Table I, is −35.6 mV. This value properly fits to membrane potential measured in tAE1-expressing oocytes at day 1 post-injection (Em = −36.2 ± 1.9 mV). Thus, tAE1 expressing oocytes reach the equilibrium potential for Cl<sup>-</sup> without any change in intracellular Cl<sup>-</sup> concentration. To conclude, the absence of intracellular Cl<sup>-</sup> depletion ruled out this possibility in the observed stimulation of the NKCC co-transporter.

Furthermore, tAE1 anion conductance also does not seem to be the triggering factor of NKCC co-transporter. Indeed, the conductive properties of tAE1 could be inhibited by glybenclamide (Fig. 5A) without modifying activation of the co-transporter (Fig. 5B). This result suggests the absence of functional links between tAE1 and the co-transporter.

In a previous work, different chimeras between tAE1 and other AE1s unable to form a Cl<sup>-</sup> channel were used to determine the domain of the protein involved in the channel formation. These chimeras strengthened the absence of correlation between conductance of tAE1 and co-transporter activation. As illustrated in Fig. 7, all of the chimeras ending with mAE1 sequences were unable to stimulate the NKCC co-transporter despite the fact that they have an anion conductance (T-4M and T-5M) or no anion conductance (TZM). In contrast, chimera SZT, which has a similar conductance to T-4M or T-5M, is able to maximally stimulate the co-transporter. Activation of NKCC co-transporter is maximal for different values of conductance: G = 15.2 μS (tAE1), 6.1 μS (SZT), or 2.8 μS (TAE1N); for equivalent conductances, activation is either maximal (SZT with G = 6.1 ± 1.0 μS) or null (T-5M with G = 4.53 ± 1.07 μS). This definitely rules out a role for the tAE1 chloride channel in NKCC co-transporter activation.

It seems obvious that activation of the co-transporter in tAE1-expressing oocytes is not related to known thermodynamic events because of tAE1 functioning but rather because of the presence of this protein in the plasma membrane. This stimulation is dependent upon at least the last four putative spanning domains in the C-terminal part of tAE1, because their substitution by corresponding helices of mAE1 abolished stimulation of the co-transporter. It is possible to interfere in the activation by increasing the size of the cytoplasmic tail of

FIG. 8. Fusion protein tAE1-gyrase B. A, Western blot of tAE1 or tAE1-GyrB fusion protein expressed in Xenopus oocyte membrane. Membranes were prepared 2 days after injection, as described under “Experimental Procedures.” The presence of tAE1 was detected by Ab146 (against C-terminal end, lane b) or Ab146 (against N-terminal end, lane d). TAE1-GyrB was detected by Ab146. The lower bands around 100 kDa correspond to unglycosylated or partially glycosylated tAE1, and the upper bands correspond to glycosylated tAE1 (controlled by deglycosylation experiments, not shown). The fusion tAE1-GyrB, unglycosylated and glycosylated, migrated with an apparent molecular mass of 120 kDa for the unglycosylated form and 140 kDa for the glycosylated form (lane e) instead of 100 kDa and 120 kDa for the wild tAE1. The B gyrase has a molecular mass of 24 kDa. Mass markers in kDa are drawn on the left of the picture. Lane a corresponds to control oocytes (non-injected) reacting with Ab145, and lane c corresponds to control oocytes reacting with Ab146. This Western blot is representative of at least five different experiments. B, Cl<sup>-</sup> influx was measured in oocyte-expressing tAE1 or the fusion protein tAE1-gyraseB, 2 days after injection of cRNA. Uptake was done in 15 min (during the linear phase of the uptake) in MBS with a specific radioactivity of 363 dpm/nmol of Cl<sup>-</sup>. Control is non-injected oocytes. Data presented are from one representative experiment of three. Means ± S.E., n = 8 oocytes. C, Rb influx was measured in MBS containing 10<sup>-5</sup> M ouabain with tAE1 or tAE1-gyraseB-expressing oocytes 2 days after injection. Data presented are from one representative experiment of three different experiments. Means ± S.E., n = 8 oocytes.
tAE1 (fusion tAE1gyraseB). The same result is observed when a specific antibody to the C-terminal extremity is injected in oocytes. These data strongly suggest that tAE1 is able to interact with the co-transporter. Further experiments should determine whether this is a direct interaction between the two transporters or if it involves an intermediary regulatory protein. It is well known that anion exchangers are able to interact by their large N-terminal cytoplasmic domain with different proteins in erythrocytes (29, 30). The only described interaction site localized in the short cytoplasmic C-terminal domain of the protein is a binding site for carbonic anhydrase (31). However, our results provide further evidence for the role of the C-terminal domain of tAE1 in interactions with other proteins.

The ability of tAE1 to stimulate a co-transporter in Xenopus oocyte could be extrapolated to the erythrocyte where tAE1 is the most abundant plasma membrane protein. It was shown that this anion exchanger has a key role in trout red cell regulatory volume decrease response, acting as a channel permeable to taurine, Na⁺, and K⁺ (32). Hyposmotic swelling of trout erythrocytes induces a K⁺ loss mediated by two pathways: a K-Cl co-transporter and a Cl⁻ independent pathway. The stoichiometry of solute loss during regulatory volume decrease is seven taurine with three cations (K⁺ and Na⁺) and three Cl⁻ (32). Such finely tuned osmolyte transport requires precise coordination between the two transporters KCl and tAE1. It is tempting to assume that, by interacting with the K-Cl co-transporter, tAE1 is able to regulate its activity. Moreover, it is well known that AE1 is able to interact with hemoglobin by means of its binding to erythrocyte cytoskeleton. This binding is sensitive to hemoglobin oxygenation (33). The data associated with our results, which suggest a role of tAE1 in regulating other transport functioning, might provide an explanation of oxygen control of the KCl cotransporter as well as the Na⁺/H⁺ exchanger in trout erythrocyte (34, 35). Indeed, the oxygen sensitivity of these two transporters could be mediated by tAE1 interacting with hemoglobin on one side and with these transporters on the other.

The CFTR, a Cl⁻ channel, was the first transporter studied for the possibility it might regulate other transporters (6, 9, 36, 37, 38). Our data provide an example other than the CFTR that supports the existence of a mechanism of membrane transport regulation by molecular interactions (direct or indirect) between transporters themselves. We have no explanation about the molecular mechanism underlying activation of NKCC co-transporter by tAE1, but regulation through interactions with a common regulatory protein could be hypothesized. Indeed, there is increasing evidence that membrane transporters may coordinate their functions through their common interactions with PDZ domains of regulatory proteins such as, for instance, EBP50 (39) or NHE3 kinase A regulatory protein (40).

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Evidence for Up-regulation of the Endogenous Na-K-2Cl Co-transporter by Molecular Interactions with the Anion Exchanger tAE1 Expressed in Xenopus Oocyte

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