Anoctamin-1/TMEM16A is the major apical iodide channel of the thyrocyte

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Anoctamin-1/TMEM16A is the major apical iodide channel of the thyrocyte. *Am J Physiol Cell Physiol* 307: C1102–C1112, 2014.—Iodide is captured by thyrocytes through the Na+/I− symporter (NIS) before being released into the follicular lumen, where it is oxidized and incorporated into thyroglobulin for the production of thyroid hormones. Several reports point to pendrin as a candidate protein for iodide export from thyroid cells into the follicular lumen. Here, we show that a recently discovered Ca2+-activated anion channel, TMEM16A or anoctamin-1 (ANO1), also exports iodide from rat thyroid cell lines and from HEK 293T cells expressing human NIS and ANO1. The Ano1 mRNA is expressed in PCCl3 and FRTL-5 rat thyroid cell lines, and this expression is stimulated by thyrotropin (TSH) in rat in vivo, leading to the accumulation of the ANO1 protein at the apical membrane of thyroid follicles. Moreover, ANO1 properties, i.e., activation by intracellular calcium (i.e., by ionomycin or by ATP), low but positive affinity for pertechnetate, and nonrequirement for chloride, better fit with the iodide release characteristics of PCCl3 and FRTL-5 rat thyroid cell lines than the dissimilar properties of pendrin. Most importantly, iodide release by PCCl3 and FRTL-5 cells is efficiently blocked by T16Ainh-A01, an ANO1-specific inhibitor, and upon ANO1 knockdown by RNA interference. Finally, we show that the T16Ainh-A01 inhibitor efficiently blocks ATP-induced iodide efflux from in vitro-cultured human thyrocytes. In conclusion, our data strongly suggest that ANO1 is responsible for most of the iodide efflux across the apical membrane of thyroid cells.

Anoctamin-1; iodide release; pendrin; thyrocyte; TMEM16A

The function of the thyrocyte is to take up iodide, oxidize it, and bind it to tyrosine residues of thyroglobulin to finally couple these iodothyrosines into iodothyronines, the thyroid hormones (T3 and T4). The steps involved in this biosynthesis are the trapping of iodide in thyrocytes from the extracellular medium by the Na+/I− symporter (NIS), its transport from the thyrocyte to the follicular lumen, its oxidation there into iodothyrosine, and the coupling of iodothyrosines in thyroglobulin by thyroperoxidase. Pendrin has been proposed as the protein mediating the step of iodide export from the cell into the follicular lumen, i.e., of iodide crossing the thyrocyte’s apical membrane (3). However, several arguments indicate that pendrin is not critical for this transport (38, 44) as mice deficient for pendrin are euthyroid (4, 6, 42) and patients affected by the Pendred syndrome, i.e., homozygotic pendrin inactivation, may develop goiters in iodine-deficient conditions but usually not before the second decade of life (3). Another pathway for iodide transport across the apical membrane must therefore be considered, and the definition of its identity and hormonal responsiveness constitutes the aim of the present study. Most cells are equipped with ion channels permeable to anions. Among those, Ca2+-activated chloride channels have been identified in the 1980s on the basis of their Ca2+ sensitivity and biophysical properties as defined by patch-clamp experiments. These channels play a large variety of roles depending on the cell type in which they are expressed, from membrane excitability, olfactory transduction, photoreception, and transepithelial secretion, to preventing polyspermy (see Ref. 11 for review). However, their molecular identity remained elusive until 2008, when three groups independently cloned the gene encoding the transmembrane protein called TMEM16A or anoctamin-1 (ANO1) (5, 33, 46). This protein was observed to function as a Ca2+-activated chloride channel (26, 37) and to be widely expressed at the plasma membrane of many cells in liver, skeletal muscle, heart, pancreas, placenta, sensory neurons, kidney, airways epithelial cells, lung alveoli, and mammary and salivary glands where they concentrate at the apical membrane (8). The functional properties of ANO1 were found to be regulated by alternative splicing and some variants were observed to be expressed in the thyroid (9). As most variants transport iodide better than chloride (i.e., with a higher affinity; Ref. 8), we decided to investigate whether ANO1 might play a role in iodide export in thyroid. The present observations demonstrate that in the rat and human iodide release into the colloid space is carried out mainly by ANO1.

Materials and Methods

Materials

Enzymes were purchased from Invitrogen (Carlsbad, CA) and Roche (Penzberg, Germany). Oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media were purchased from Invitrogen. The ANO1 inhibitor T16Ainh-A01 was from Calbiochem (cat 613551; San Diego, CA) (21).125I as NaI (37 GBq) was purchased from Perkin Elmer (Zaventem, Belgium).

Cell Line Culture and Transfection

Human embryonic kidney 293T (HEK 293T) cells were grown in Dulbecco’s modified Eagle’s medium Glutamax (Invitrogen) supple-

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mented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Rat PCC13 (10) and FRTL-5 (1) cells were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% decomplemented fetal bovine serum (Invitrogen), bovine thyrotropin (TSH; 1 mIU/ml), transferrin (5 μg/ml), insulin (1 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml).

For iodide uptake/release experiments and Western blot analyses, cells were plated at a density of 10⁵ cells/well (HEK 293T cells) or 5 × 10⁵ cells/well (PCC13 and FRTL-5 cells) in six-well plates. HEK 293T cells were transfected the next day using 2 μg of plasmid DNA mixed with 6 μl of Fugene 6 (Roche) according to the manufacturer’s instructions. Typically, cells were transfected with 1 μg of NIS-pCDNA3 and 1 μg of a plasmid encoding either GFP (pEGFP-C2; Clontech, Mountain View, CA) or ANO1. For pendrin expression, the plasmid DNA amount was lowered to 100 ng and supplemented with 900 ng of the pEGFP-C2 plasmid.

Human Primary Thyrocyte Culture

Human thyroid tissue was obtained from patients undergoing thyroidectomy for solitary cold nodules or multinodular pathology. The protocol was approved by the ethics committee of the Erasme hospital. Only the healthy normal looking, nonnodular tissue was used within 30–45 min of surgical removal. The tissue was digested into follicles and grown in primary culture as described previously (29) except that forskolin (5 μM) was added to the culture medium the last 3 days to support differentiation.

RT-PCR Analysis of Ano1 Expression in Tissues

RNA extraction from tissue and subsequent classical PCR analysis were performed as described in Best et al. (2) except that reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). For the comparison of Ano1 expression in control and methimazole-treated rats, cDNA was prepared from thyroid tissue as described above, and a quantitative real-time PCR was performed on a CFX 96 Real-Time System C1000 Thermal Cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). The reference gene used for the comparison of transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). For the comparison of ANO1 expression in control and methimazole-treated rats, cDNA was prepared from thyroid tissue as described above, and a quantitative real-time PCR was performed on a CFX 96 Real-Time System C1000 Thermal Cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). The reference gene used for the comparison of transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

Analysis of Rat Ano1 Isoforms

Alternatively spliced Ano1 transcripts expressed in PCC13 cells and rat thyroid were detected by RT-PCR. Conditions for PCR were the following: 95°C for 5 min for the initial denaturation; 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s for 38 cycles; and 72°C for 10 min for the final extension. We used the following primers: for exon 6b, forward: 5’GAAAAACGTCAGCAGCAAGGAGCC3’ and reverse: 5’GCTCCAGACCGACAAATGC3’; for exon 13, forward: 5’GAAACGGAAGCAGATGAGAC3’ and reverse: 5’GGCTCTCATATCTGCTCTTG3’; and for exon 15, forward: 5’GATGAAAGCGCGCTCTTG3’ and reverse: 5’ATAAACGTAGTCACAGCCGCG3’. The amplified products were resolved on 2% agarose gels. Expected sizes of PCR products amplified from 6b+ and 6b– transcripts: 310 and 244 bp, respectively; 13+ and 13– PCR products: 100 and 88 bp, respectively; and 15+ and 15– PCR products: 553 and 475 bp, respectively.

Protein Extraction

HEK 293T cells were collected, washed twice in ice-cold PBS, and lysed in EBC buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2% NP40, and Complete EDTA-free protease inhibitor cocktail; Roche) for 30 min at 4°C. Thyroid tissues were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, and 0.25% sodium deoxycholate) containing phosphatase and proteases inhibitors. The lysates were centrifuged at 10,000 rpm for 10 min, and the supernatants were saved at −80°C.

Western Blotting

Ten micrograms of total protein extract from HEK 293T cells or 40 μg from thyroid tissue were electrophoresed on a 7.5% polyacrylamide gel and then transferred onto a nitrocellulose membrane for Western blot analysis. The membrane was blocked with PBST containing 2–5% nonfat dry milk, incubated overnight at 4°C with the anti-ANO1 antibody sc-69343 (1/500–1/1000 dilution), rinsed extensively with PBST, and incubated for 1 h at room temperature with the donkey anti-goat IgG-HRP sc-2020 (1/2500–1/2,000 dilution; Santa Cruz Biotechnology). The signals were detected by chemiluminescence using either the SuperSignal West Pico detection kit (Thermo Fisher Scientific, Waltham, MA) or the Western Lightning Plus-ECL detection kit (Perkin Elmer, Waltham, MA).

Detection of ANO1 by Immunofluorescence Staining of Rat Thyroid Sections

Ethics statement. Adult male Wistar rats were housed and used following the rules of the Belgian Regulations for Animal Care, with approval of the Ethics Committee of the School of Medicine of the Université Libre de Bruxelles.

Sample preparation. Fourteen-week-old male Wistar rats received or not methimazole for 2 wk (200 mg/l in drinking water). They were then anesthetized with ketamine hydrochloride and xylazine, and they were perfused intracardially with a fresh solution of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Thyroids were quickly dissected and further fixed by overnight immersion in 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The tissue was then transferred to successive graded sucrose solutions (10, 20, and 30%, overnight each) and finally embedded in Tissue-Tek OCT compound, snap-frozen in cold 2-methylbutane, and stored at a temperature of −80°C. Cryosections (10 μm) were cut on a cryostat (Leitz), mounted on slides coated with 0.1% poly-l-lysine (Sigma), and stored at −20°C until use. Hematoxylin and eosin staining was routinely used to evaluate the sections before immunostaining.

Immunofluorescence. Immunofluorescence was carried out at room temperature. The slides were dropped 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. They were then washed in water, preincubated 10 min in normal rabbit serum, and incubated overnight at room temperature with anti-ANO1 sc-69343 antibody in Tris-buffered saline (TBS) containing 1% of normal rabbit serum. Thereafter, the slides were washed in TBS and incubated for 30 min with a secondary goat anti-rabbit biotinylated antibody (1/300 dilution; Vector), rinsed in TBS, and incubated for 1 h with an avidin-biotin complex conjugated with peroxidase. The slides were washed again in TBS and incubated for 10 min with Tyramide Alexa350 (1/100 dilution; Molecular Probes). After being rinsed twice in TBS, the slides were mounted in Gelvatol/DABCO aqueous medium. Sections were examined with a Zeiss Axioplan microscope, and images were acquired using an AxioCam HRc camera. The ability of the sc-69343 antibody to recognize ANO1 in immunofluorescence experiments was validated by showing a colocalization of anti-Flag and anti-ANO1 signals at the membrane of HEK 293T cells transfected to express Flag-ANO1 (data not shown).

DNA Constructs

The NIS-pcDNA3 was previously described (40a). The pcDNA3 DNA construct encoding mouse pendrin was kindly provided by D. Eladari (Centre de Recherche Cardio-vasculaire, Inserm U970, Équipe 123, Paris, France). pcDNA3.1 constructs encoding human ANO1 ac and abc isoforms were previously described (9). We expressed the abc isoform unless stated otherwise. Flag-tagged ANO1 (abc) construct was generated by PCR amplification of ANO1 (abc) open reading frame (ORF) using a reverse primer encoding Flag epitope to generate the ORF with an in-frame Flag sequence at its N-terminus.
3’-end. The resulting PCR product was cloned in the XhoI site of the pcDNA3 plasmid. The DNA construct was subsequently sequenced.

Iodide Uptake and Release from Cells

Seventy-two hours after transfection, HEK 293T cells were rinsed with Krebs-Ringer HEPES buffer (KRH) and incubated for 45 min in 1 ml of KRH supplemented with $^{125}$I (Perkin Elmer; 1 μCi/ml, 10$^{-7}$ M KI) at 37°C. The wells were then rinsed twice with KRH at 22°C and further incubated at 22°C in 1 ml of KRH supplemented with $^{125}$I, 10$^{-7}$ M KI, 1 mM ClO$_4^-$, for the duration of cell loading, i.e., 45 min. Only preparations with a cell-to-medium ratio over 6 were used. The cells were then rinsed twice with 1 ml of KRH at 0°C and lysed in 1 ml of 1 M NaOH. The release at each time was expressed as percentage of total uptake, i.e., $^{125}$I released in the loading, i.e., 45 min. Only preparations with a cell-to-medium ratio over 6 were used. The cells were then rinsed twice with 1 ml of KRH at 0°C and lysed in 1 ml of 1 M NaOH. The medium aliquots and the cells were counted in a open air incubator with mild shaking. Fifty microliters of medium were withdrawn after 1, 2, 3, 5, 10, and 30 min. The cells were then rinsed twice with 1 ml of KRH at 0°C and lysed in 1 ml of 1 M NaOH. The medium aliquots and the cells were counted in a γ-counter (Perkin Elmer-Wallac Wizard 1470 auto γ counter). The release at each time was expressed as percentage of total uptake, i.e., $^{125}$I released in the medium plus the amount remaining in the cells. The uptake was always checked in parallel wells in which cells were incubated with $^{125}$I, $^{125}$I, 10$^{-7}$ M KI, plus or minus 1 mM ClO$_4^-$, for the duration of cell loading, i.e., 45 min. Only preparations with a cell-to-medium ratio over 6 were used. The cells were then rinsed twice with 1 ml of KRH at 0°C and counted.

In chloride deprived medium, KCl, NaCl, and CaCl$_2$ were replaced by gluconate salts of K, Na, and Ca.

The test agent (e.g., ionomycin) or test medium (i.e., chloride deprived) was present during the release incubation only, except for T16Ainh-A01, the ANO-1 inhibitor, which was added during the $^{125}$I loading and release incubations. Ionomycin and T16Ainh-A01 were dissolved in DMSO (final DMSO concentration in cell culture medium: 0.1%). Control wells analyzed in parallel with ionomycin- or the condition (e.g., chloride-free medium) under study. The six-well plates were incubated in an open air incubator with mild shaking. Fifty microliters of medium were withdrawn after 1, 2, 3, 5, 10, and 30 min. The cells were then rinsed twice with 1 ml of KRH at 0°C and lysed in 1 ml of 1 M NaOH. The release at each time was expressed as percentage of total uptake, i.e., $^{125}$I released in the medium plus the amount remaining in the cells. The uptake was always checked in parallel wells in which cells were incubated with $^{125}$I, 10$^{-7}$ M KI, plus or minus 1 mM ClO$_4^-$, for the duration of cell loading, i.e., 45 min. Only preparations with a cell-to-medium ratio over 6 were used. The cells were then rinsed twice with 1 ml of KRH at 0°C and counted.

Generation of PCCl3 Stably Expressing shRNA

The DNA oligos encoding shRNA sequences were designed and cloned into the expression vector pLV-HI-EF1a-puro using the single oligonucleotide RNAi technology developed by Biosettia (San Diego, CA). A shRNA targeting an irrelevant gene (β-galactosidase) was used as a negative control (control shRNA). The reference gene used was GAPDH. Primer sequences are available upon request.

RESULTS

Expression of ANO1 in Rat Tissues and in PCCl3 Cells

Expression studies in murine tissues indicated that Ano1 is predominantly expressed in epithelia like trachea, pancreas, colon, salivary gland, prostate tissue, and the thyroid (32). Ano1 primary transcript undergoes alternative splicing, thus resulting in the generation of multiple isoforms. Three alternative exons, 6b, 13, and 15, coding for segments of 22 (segment b), 4 (segment c), and 26 (segment c) amino acids, respectively, are differently spliced in human organs (9, 40). Functional studies on ANO1 isoforms indicated that the inclusion/exclusion of specific exons influenced ANO1 channel properties (9). In human thyroid, the predominantly expressed ANO1 transcript contains exons 6b and 13 but lacks exon 15 (9, 40). Here, we analyzed the expression of Ano1 in rat tissues as well as in the rat thyroid PCCl3 cell line by RT-PCR. As shown in Fig. 1A, Ano1 is expressed in several tissues including the thyroid, the pancreas, and the parotid and submaxillary glands. These results are in accordance with a previous expression profiling of Ano1 in mouse (32). In PCCl3 cells and rat thyroid tissue, the Ano1 gene is expressed as alternatively spliced mRNAs containing or not exon 6b in various ratios ($6b^- > 6b^+$ in PCCl3, $6b^- \sim 6b^+$ in rat thyroid). All
transcripts contained exon 13 but lacked exon 15 (Fig. 1B). The cloning and sequencing of Ano1 cDNA ORF obtained from PCCI3 and rat thyroid confirmed the alternative inclusion of exon 6b and the presence and absence of exons 13 and 15, respectively (data not shown). Of note, the peptide sequence encoded by rat Ano1 6b exon was identical to the one encoded by human ANO1 6b exon, contrarily to the one deduced from the current National Center for Biotechnology Information reference sequence for rat Ano1 mRNA (accession no.: NM_001107564).

Expression and Localization of ANO1 in Rat Thyroid Follicles

To evaluate the interaction between thyroid metabolism and ANO1 localization and expression, we treated rats with the antithyroid drug methimazole, which inhibits thyroid hormone synthesis and consequently increases serum TSH. We observed that this classical stimulating treatment increased Ano1 expression at the mRNA (Fig. 2A) and protein (Fig. 2B) levels.

We then immunostained thyroid tissues from rats treated or not with methimazole for ANO1. In the thyroids from control rats, the signal was generally low (Fig. 3A). However, a discrete but clear-cut labeling was observed in some follicles; in a few of them, ANO1 was detected at the apical membrane of rat thyrocytes (Fig. 3B). As expected from our previous results, the ANO1 signal was considerably stronger in the thyroid from methimazole-treated rats. Moreover, it was located at the apical side of the thyrocytes in almost all follicles (Fig. 3, C and D). This correlates with increased serum TSH level (20). ANO1 localization in rat thyroid is therefore compatible with a role in iodide export from thyrocytes to the follicle lumen.

Release of Iodide Is Induced by Ectopic Expression of ANO1 and Pendrin in HEK 293T Cells

In polarized thyroid cells, iodide release takes place at the apical but not at the basal membrane, as shown in porcine thyroid cells in primary culture (23). In unpolarized cells, influx and release take place at the same membrane sites, thus establishing a steady state. We tested the ability of ANO1 to mediate iodide release and compared it to that of pendrin in a heterologous expression setup. HEK 293T cells were transfected with plasmid DNA constructs encoding the sodium/iodide symporter NIS in combination with ANO1 (abc) isoform or pendrin or, as a control, with GFP. We then loaded these cells with radioiodide and measured the subsequent iodide release from these cells. As shown in Fig. 4, HEK 293T cells expressing GFP displayed a low ability to release iodide.

Fig. 2. The classical stimulating drug methimazole increases Ano1 expression in rat thyroid. A: quantitative real-time PCR showing the effect of a methimazole treatment on the expression of the Ano1 mRNA in rat thyroid tissue. Bars represent the average value ± SE over 3 independent experiments. B: Western blot showing the effect of a methimazole treatment on the expression of the ANO1 protein in rat thyroid tissue.

Fig. 3. Expression and localization of ANO1 in rat thyroid follicles. A–D: rat thyroid sections were immunostained for ANO1 (blue) and cell nuclei were counterstained with propidium iodide (red). A merged view of the 2 signals is presented except for B. A and B: representative pictures of ANO1 immunodetection in thyroid follicles of untreated rat. A discrete labeling is observed in some follicles (A); only a few follicles express ANO1 at the level of the apical side of thyrocytes (B, arrows). C and D: representative pictures of ANO1 immunodetection in thyroid of rats treated with methimazole (200 mg/l) in drinking water during 2 wk. Strong fluorescence intensity is detected at the apical side of almost all thyroid follicles. No significant signal was observed when the anti-ANO1 antibody was omitted during sample preparation (data not shown) suggesting that the weak signal observed in the interstitium is not artifactual, probably reflecting vascular ANO1.
Iodide Release Is Stimulated by Increased Intracellular Ca\(^{2+}\) Concentration in PCCl3 Cells and in ANO1-Expressing HEK 293T Cells but not in Pendrin-Expressing HEK 293T Cells

Ca\(^{2+}\) ionophore enhances the release of iodide from dog thyroid cells (28) as well as in kidney proximal tubular cells under the control of purinergic stimulation (7). We therefore studied iodide release by pendrin- and ANO1-expressing HEK 293T cells in the presence or the absence of the Ca\(^{2+}\) ionophore ionomycin. While ionomycin increased iodide release mediated by ANO1 isoforms, no change was observed in pendrin-expressing HEK 293T cells (Figs. 5 and 6). The iodide channel expressed in PCCl3 cells shared ANO1 activation properties, i.e., the Ca\(^{2+}\) ionophore stimulated iodide release from these cells, as previously reported for dog thyroid cells (28). Moreover, this enhanced iodide efflux is reproduced upon purinergic stimulation (Fig. 6). Thus, while increase of intracellular calcium concentration does not affect pendrin-mediated iodide efflux, it greatly stimulates iodide release from ANO1-expressing HEK 293T cells, PCCl3 cells, and dog thyrocytes. Of note, Pesce et al. (27) showed weak activation of iodide loss by TSH but not by forskolin in PCCl3 cells, which suggests a TSH effect through the weakly responding phospholipase C-PIP2-Ca\(^{2+}\) cascade in this model.

Ionomycin Stimulates \(^{99m}\)TcO\(_4^-\) Release from ANO1-Expressing HEK 293T Cells and from PCCl3 Cells

\(^{99m}\)TcO\(_4^-\) is often used as a substitute for radioiodide in uptake experiments, and \(^{99m}\)TcO\(_4^-\) is transported into thyroid cells by the iodide transporter NIS (22, 40a). We tested whether this absence of specificity also applied to the iodide channels. In NIS/GFP-expressing HEK 293T cells as well as in PCCl3 cells, \(^{99m}\)TcO\(_4^-\) was better concentrated and released than radioiodide. On the other hand, there was no increase in basal release from HEK 293T cells also expressing ANO1 or pendrin. In NIS/GFP and in NIS/pendrin-expressing HEK 293T cells, this release was not enhanced by ionomycin. However, ionomycin induced an increased release of \(^{99m}\)TcO\(_4^-\) from NIS/ANO1 HEK 293T and from PCCl3 cells (Fig. 7). Thus \(^{99m}\)TcO\(_4^-\) is not transported by ANO1, except upon stimulation of the cells with Ca\(^{2+}\) ionophore. Moreover, the similar capacity of PCCl3 cells to transport \(^{99m}\)TcO\(_4^-\) upon increased Ca\(^{2+}\) intracellular concentration supports the presence of active ANO1 channels in these cells.

The Release of Iodide Occurs in the Absence of Chloride in ANO1-Expressing HEK 293T Cells and in FRTL-5 and PCCl3 Cells but not in Pendrin-Expressing HEK 293T Cells

As pendrin exchanges iodide (or bicarbonate) for chloride, pendrin-mediated release of iodide is expected to be drastically decreased in the absence of chloride in the medium. To assess ANO1 and pendrin iodide transport activity in the presence or the absence of Cl\(^-\) ions, transfected HEK 293T cells were incubated in normal or chloride-free medium and iodide release was measured after prior loading incubation in normal medium. We observed that chloride deprivation markedly decreased iodide release from pendrin-expressing HEK 293T cells but did not affect iodide release from ANO1-expressing HEK 293T cells (Fig. 8). The same conditions were then applied to PCCl3 and FRTL-5 cells. As shown in Fig. 8,
chloride deprivation did not significantly modify iodide release from either cell line. These results indicate that ANO1, in contrast to pendrin, does not require chloride anions to promote iodide release and that the channel mediating basal iodide release from rat thyroid cells shares this characteristic with ANO1.

The T16Ainh-A01 Inhibitor Reduces Iodide Release from PCCl3 and FRTL-5 Cells

Novel ANO1 inhibitors have been recently developed, the most potent one being the aminophenylthiazole T16Ainh-A01 (21). We first tested the specificity of T16Ainh-A01 to block iodide release by 10.220.32.247 on June 22, 2017 http://ajpcell.physiology.org/ Downloaded from
from ANO1-expressing HEK 293T cells compared with GFP- and pendrin- expressing HEK 293T cells. As shown in Fig. 9, T16Ainh-A01 strongly inhibited iodide release from ANO1-expressing HEK 293T cells but not from pendrin-expressing HEK 293T cells, thereby confirming T16Ainh-A01 specificity towards ANO1 transport activity. We then tested T16Ainh-A01 inhibitory activity on iodide release by PCCl3 and FRTL-5 cells and showed an inhibition of basal iodide release and a complete inhibition of Ca²⁺ ionophore-stimulated release. The IC50 of the inhibitor is of the order of 30 μM.

Fig. 9. Effect of the ANO1 inhibitor T16Ainh-A01 on ¹²⁵I release from HEK 293T cells transfected with NIS in combination with pendrin or Ano1 or from PCCl3 cells or from FRTL-5 cells. ●, ■: Ionomycin-stimulated cells (1 μM for HEK 293T cells; 0.2 μM for PCCl3 and FRTL-5 cells); ●, ○: cells incubated with T16Ainh-A01 (100 μM). The data are representative of at least 3 independent experiments.
Knockdown of ANO1 Expression Downmodulates Iodide Efflux in Ionomycin- and ATP-Exposed PCCl3 Cells

To confirm that ANO1 is the Ca\(^{2+}\)-dependent iodide channel in thyroid cells, we knocked down ANO1 synthesis in PCCl3 cells by stable expression of interfering shRNA. In parallel, we established PCCl3 cell lines expressing a control shRNA or a shRNA targeting pendrin. As shown in Fig. 10A, shRNAs targeting ANO1 and pendrin led to a three- and sixfold decrease in ANO1 and pendrin mRNA, respectively, compared with their levels in PCCl3 expressing control shRNA. While ANO1 knockdown did not modify iodide efflux in basal conditions (Fig. 10B, left), it markedly reduced iodide efflux in ionomycin- and ATP-exposed cells (Fig. 10B, middle and right). In contrast, downmodulation of pendrin expression did not affect iodide efflux in the three conditions. All together, these results confirm the primary role of ANO1 in iodide efflux from stimulated thyroid cells.

The T16Ainh-A01 Inhibitor Reduces Iodide Release from Human Thyrocytes

We then examined the role of ANO1 in iodide efflux by human thyrocytes. First, we validated that Ano1 transcript and protein are expressed in human thyroid (Fig. 11, A and B). Second, we measured iodide efflux from primary human thyrocytes exposed or not to ATP in the presence or the absence of the T16Ainh-A01 inhibitor. As shown in Fig. 11C, ATP strongly increased iodide efflux as in PCCl3 cells and this increase was suppressed by T16Ainh-A01, thereby confirming the conserved role of ANO1 in human thyroid.

DISCUSSION

To be oxidized and bound to the tyrosines of thyroglobulin and for the following synthesis of thyroid hormones, iodide must be first transported into the lumen of the thyroid follicle (34, 44). This uptake requires a first active transport driven by a favorable electrochemical gradient. The latter has been ascribed to the apical Cl\(^{-}/\text{HCO}_3\) \((\text{I}^-)\) exchanger pendrin. However, there are several arguments against an exclusive role of pendrin: 1) in human congenital homozygotic defects of pendrin (the Pendred syndrome), hypothyroidism only appears in iodine-deficient areas (18, 25); and 2) the inactivation of pendrin gene in mouse does not induce a thyroid-deficient phenotype (6). The question therefore arises whether other membrane proteins fulfill this role. ANO1 is expressed within the apical membrane of several chloride secreting epithelia often in parallel with another chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR): they both constitute major rate-limiting steps for secretions controlled by increased cytosolic Ca\(^{2+}\) for ANO1 and by the cAMP cascade for the CFTR (10, 34). Both channels can also mediate HCO\(_3^-\) transport; yet, only ANO1 exhibits a greater selectivity for iodide than chloride and it has recently been suggested as a potential candidate mediating iodide exit into the follicular space (41). Expression of ANO1 gene is decreased in some papillary thyroid carcinoma and in 11 tested anaplastic carcinomas (15). It is slightly increased in five thyroids of nonimmune hyperthyroidism and is markedly upregulated in autonomous adenomas (16). Expression of pendrin/Slc26a4 gene is decreased in most papillary and in 11 anaplastic carcinomas investigated (15). It is slightly increased in autonomous adenomas (16). These expression patterns are consistent with a role of both proteins in functional differentiation.

In this work, we show that Ano1, which has been recently cloned and characterized, is expressed in human and rat thyroid primary cells and in PCCl3 and FRTL-5 rat thyroid cell lines. Among the alternatively spliced forms, both the abc and the ac forms are expressed. The ac protein form, which is more sensitive to calcium than the abc form (8, 9), is equally if not more expressed than the abc form in FRTL-5 and PCCl3 cells. In the thyroid of methimazole-treated rats in which serum TSH is elevated, Ano1 expression is increased and the ANO1 protein is detected at the apical membrane of most follicles. The higher permeability of ANO1 for iodide vs. chloride is consistent with the release observed here even though intracellular chloride is present at much higher concentration than...
iodide (9). Both pendrin and ANO1 confer to NIS-expressing HEK 293T cells the property of iodide release. They both differ in specificity from the NIS transporter, which takes almost exclusively $^{125}$I$^{-}$ and $^{99m}$TcO$_4^-$. While they transport many other anions including Cl$^{-}$ and HCO$_3^-$, the two mainly expressed isoforms of ANO1 (ac and abc isoforms) transport iodide. Moreover, our work suggests that iodide release by the thyroid PCCl3 and FRTL-5 cells reproduces the characteristics of ANO1 but not those of pendrin. Whereas release of iodide by pendrin-expressing cells is inhibited in a chloride-free medium, as expected for a chloride-anion exchanger, it is not downmodulated in ANO1-expressing cells nor is it in thyroid FRTL-5 and PCCl3 cells. On the other hand, iodide release is activated by increased intracellular Ca$^{2+}$ concentration in ANO1-expressing HEK 293T and in PCCl3 cells but not in pendrin-expressing HEK 293T cells. Moreover, the more physiological purinergic stimulation of PCCl3 cells reproduces the marked increase in iodide efflux observed upon ionomycin exposure. It is worth noting that this property is one of the major characteristics of dog thyroid cells in primary culture (28).

Fig. 11. Human Ano1: expression and functionality in iodide efflux from thyrocytes. A: detection of Ano1 transcripts in human tissues including thyroid by RT-PCR. B: detection of ANO1 protein in human thyroid and in thyroid from methimazole (MMI)-treated rats by Western blot. C: effect of the ANO1 inhibitor T16Ainh-A01 on $^{125}$I efflux from resting and ATP-stimulated human thyrocytes in primary culture. Resting cells: ⊗, ⊗; ATP (1 mM)-stimulated cells: ◊, ◊. Left: control values (without T16Ainh-A01). Right: cells incubated with T16Ainh-A01 (100 μM).

The pathway of iodide exit into the follicular space of human thyroid has not been solved so far. Here, we show that ANO1 is also expressed in human thyrocytes and is involved in iodide efflux from these cells under conditions of increased intracellular Ca$^{2+}$ (Fig. 11), thereby highlighting the conserved role of ANO1 in human. We confirm that pendrin is also able to export iodide from the cells as previously reported (12, 18, 36). Moreover, pendrin is present at the apex of thyroid cells and iodide release from a thyroid cell line derived from a Pendred patient is decreased (25). However, neither knockdown of pendrin expression nor chloride deprivation in the cell culture medium modified iodide efflux both under basal and activated conditions in PCCl3 cells. Yet, these experiments do not completely exclude a possible role of pendrin or of other channels in iodide efflux under basal conditions in these and...
other species. As a matter of fact, the relative role of these membrane proteins may vary between species as does the second messenger at play during TSH stimulation: cAMP increases iodide release from pig thyroid cells in primary cultures (24, 28) while increase in intracellular Ca2+ plays the prominent role in PCC13 and FRTL-5 rat thyroid cells as shown here and in dog and human thyroid. Many such biochemical interspecies differences have been observed among mammalian thyroids (19, 35).

Taken together, the present data support the conclusion that Tmem16A/Ano1 is expressed at the apex of the thyrocyte and that ANO1 constitutes the thyrocyte apical iodide channel accounting for stimulated iodide release under conditions of increased intracellular Ca2+.

NOTE ADDED IN PROOF

While this work was under revision, an article suggesting a role of ANO1 in iodide release from rat thyroid cells, based on other evidence, was published (17).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Akhatov A, L.J.V.G., and V.K. edited and revised manuscript; L.T., A.S., M.V., and V.K. designed research; L.T., A.S., M.V., C.M., J.V.S., C.W., A.W.B., and V.K. contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

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