BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 94–101 (1996)
ARTICLE NO. 1473

Establishment and Characterization of a Chinese Hamster Ovary Cell Line, CHO-4J, Stably Expressing a Number of Na^+/I^- Symporters

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Received August 21, 1996

The cDNA of the Na^+/I^- symporter playing a key role in thyroid iodide transport was cloned very recently. To characterize its function, we transfected the Na^+/I^- symporter gene into Chinese hamster ovary (CHO) cells and established a cell line stably expressing a number of rat Na^+/I^- symporters, named CHO-4J. Iodide uptake was dependent on $[Na^+]$ and reached a plateau within 30 min. Kinetic studies revealed that the K_m for iodide was 35 μM, similar to that of FRTL-5 thyroid cells. The maximal velocity (V_{max}) at cell protein level was 6–10-fold higher than in FRTL-5 cells and that at single cell level was ~1000-fold higher. ClO_4^- and SCN^- dose-dependently inhibited iodide uptake in a competitive manner. The K_i was 1.5 and 16 μM, respectively. Iodide efflux from CHO-4J cells was apparently slower ($t_{1/2}$ = 15 min) than FRTL-5 cells ($t_{1/2}$ = 2 min). Electrophysiological characteristics were examined using the whole cell patch clamp technique. Rapid inward current was observed when CHO-4J cells were perfused with 50–1000 μM NaI, suggesting a transport stoichiometry of at least 2 Na^+ per I^-. The current-voltage relation revealed that this current was membrane potential-dependent. The reversal potential was very close to that of Na^+ in agreement with dependency on the Na^+ electrochemical gradient. CHO-4J cells with a slow iodide efflux, expressing a number of Na^+/I^- symporters whose characteristics are identical to those of FRTL-5 cells will function as a new tool for sensitive analysis of iodide uptake.

The specific functions of the thyroid gland, the formation and secretion of iodinated thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), are initiated by the active transport of iodide at the basolateral plasma membrane against an electrochemical gradient. This initial iodide uptake is the rate-limiting step and it is achieved by the Na^+/I^- symporter, of which the cDNA was cloned very recently by Dai et al. (1). Analysis of the structure and function of the Na^+/I^- symporter is one of the most important subjects for understanding the overall metabolism of iodide in the thyroid gland; subsequent translocation into colloid through the apical membrane, organification, catalization by thyroid peroxidase (TPO) and incorporation into thyroglobulin (Tg) molecule, the phagolysosomal hydrolysis of iodinated Tg, and the release of T_3 and T_4 into the blood stream (2). In this study, we established a Chinese hamster ovary (CHO) cell line stably expressing a number of recombinant rat Na^+/I^- symporter and characterized its basic biochemical and electrophysiological features using a pure and specific system.

Electrogenecity of the Na^+/I^- symporter has been suggested in several reports. O'Neill et al. (3) reported that the presence of a negative membrane potential resulted in a slight stimulation of I^- transport. They (3) and Nakamura et al. (4) revealed that the Na^+-dependence of I^- accumulation was sigmoidal, with a Hill coefficient of 1.6 to 1.8 and suggested that at least

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0006-291X/96 $18.00

two Na^+ are transported with I^-. Dai et al., reported that the rat Na^+/I^- symporter shares the highest degree of homology with the human Na^+/glucose cotransporter among all Na^+-dependent transporters sequenced to date (1). At least two sodium ions are involved in Na^+/glucose cotransport and an inward current was identified by whole cell recording (5,6). If the Na^+/I^- transport ratio is more than one, like that of the Na^+/glucose cotransporter, an inward current should be observed electrophysiologically when NaI is added to the bathing solution. However, an electrophysiological study of Na^+/I^- symporter has not been performed because the low density of the symporter in thyroid cells has made it difficult to measure the inward current induced by Na^+/I^- symport. In this report, we performed an electrophysiological study using CHO-4J cells expressing a number of Na^+/I^- symporter.

MATERIALS AND METHODS

Cloning of the rat Na^+/I^- symporter gene. Poly(A)$^+$ RNA was extracted from FRTL-5 functional rat thyroid cells cultured in 6H medium containing thyrotropin (TSH) (7) using a Micro-Fast Track kit (Invitrogen). Reverse transcription and polymerase chain reaction (PCR) were performed using an oligo-(dT) primer and Avian myeloblastosis virus reverse transcriptase, and Taq polymerase and the following primers according to the reported sequence (1); forward primer, 5′-PO_4-TGCGACTCTCCCACTGACCGAGA-3′; reverse primer, 5′-CAACCATCCAGAGAGGAGGCTGC-3′. The PCR product that corresponded to nucleotide numbers 40-2028 of the rat Na^+/I^- symporter (1) was directly subjected to TA-cloning using the pCR3-Uni vector (Invitrogen) which contains cytomegalovirus-promoter and neomycin-resistance gene. Obtained cDNA clone was confirmed by digestion with restriction enzymes (Hind III/Bam HI/Eco RI/Apa I/Nar I/Pst I), and transfection into Cos-7 cells and examination of $^{125}I^-$ uptake.

Establishment of Chinese hamster ovary cell lines stably expressing Na^+/I^- symporter. Purified plasmid DNA was transfected into CHO-K1 cells by electroporation (Bio-Rad). Selection was performed with 4 mg/ml geneticin (true concentration; GIBCO-BRL) in Ham's F-12 medium containing 10% fetal calf serum for 3 weeks starting from the day after transfection. Surviving well-isolated colonies were picked up by cylinder technique and were subjected to screening for $^{125}I^-$-uptake. A cell line termed CHO-4J that accumulated the highest level of $^{125}I^-$ among 24 colonies screened, was selected for further investigation. Another cell line termed CHO-2E, which was selected with 2 mg/ml geneticin and whose gross iodide uptake was about 90-95% of that of CHO-4J cells, was also obtained.

Iodide uptake. CHO-4J cells were plated in 24-well plates and cultured with Ham's F-12 medium containing 10% fetal calf serum. When the cells reached confluence, I^- uptake was examined as described below. Unless otherwise noted, iodide uptake was determined by incubating cells with 500 μl HBSS incubation buffer (Hanks' balanced salt solution containing 0.5% bovine serum albumin (BSA) and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-NaOH pH 7.4) with $\sim$0.1 μCi carrier-free $Na^{125}I$ and 10 μM NaI to give a specific activity of $\sim$20 mCi/mmol at 37°C for 2 or 60 min. After finishing the incubation, cells were washed twice on wet ice with ice-cold 2 ml HBSS incubation buffer as quickly as possible ($<$15 sec). Cells were solubilized with 1 ml 0.1 M NaOH/0.1% (w/v) SDS/2% Na_2CO_3 and subjected to protein concentration determination by Bradford method (8) using BSA as standard, and to counting of radioactivity by a gammer counter. Some wells of cells were trypsinized for cell number counting. Data of iodide uptake are expressed as pmol/μg cell protein unless otherwise noted. Identical procedures were applied to FRTL-5 cells in 24-well plates cultured in 6H medium (including TSH) with 5% calf serum.

Iodide efflux. Iodide efflux studies were performed at 37°C as described by Weiss et al. (9) using CHO-4J and FTRL-5 cells cultured in 6H medium. After incubating the cells with 10 μM NaI and $\sim$0.1 μCi $Na^{125}I$ in 500 μl HBSS incubation buffer at 37°C for 60 min and washing them rapidly once with 2 ml HBSS incubation buffer, the medium was removed gently so as not to dislodge the cells and replaced with 500 μl fresh non-radioactive medium every 3 (0-15 min) or 6 min (15-27 min). The medium was HBSS incubation buffer with 10 μM NaI which was kept at 37°C. After the last medium removal (27 min), the cells were solubilized for counting along with the previously collected medium samples. The total radioactivity present at the initiation of the efflux study (100%) was calculated by adding the counts in the final cells and the summation of the medium counts.

Voltage-clamp and recording. Monolayer cultures of CHO-4J cells were perfused with Tyrode solution consisting of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES at pH7.4 with or without 50-1000 μM NaI. A single CHO-4J cell was voltage-clamped using the whole-cell configuration of the patch-clamp technique (10). The glass suction pipette had a tip diameter of $\sim$2 μm and a resistance of 3 to 5 M Ω when filled with the internal solution. The series resistance was $<$8 M Ω, as examined from the time course of the capacitive current recorded at the start of the whole-cell voltage clamp. The current and voltage signals were stored on a digital magnetic tape (RD101, TEAC) for later computer analysis (PC98, NEC). The liquid junction ($-$10 mV) was not corrected for all membrane potential recordings. The pipette was filled with an internal solution consisting 90 mM CsOH, 100 mM aspartate, 1.0 mM NaH_2PO_4, 5.0 mM $MgCl_2$, 20 mM tetraethylammonium chloride, 10 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM adenosine 5′-triphosphate

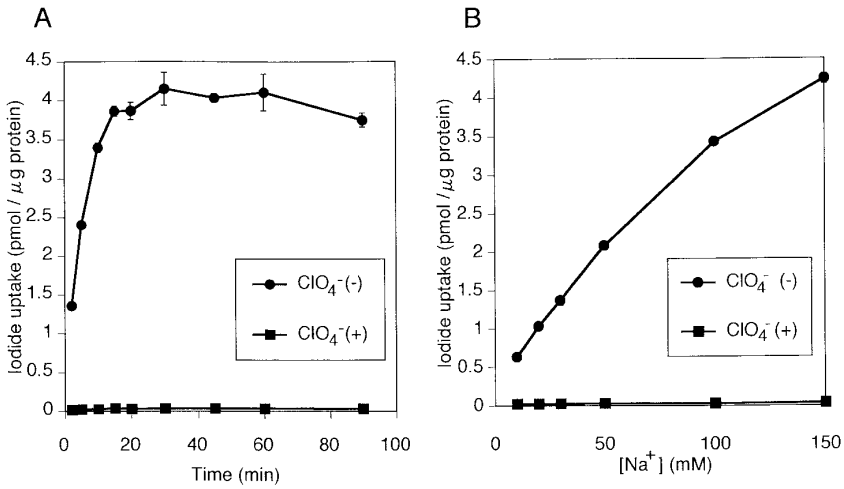

FIG. 1. Time course (A) and Na^+-dependency (B) of iodide uptake by CHO-4J cells. All data are expressed as means ± S.D. (pmol/μg protein) of triplicate wells. Some error bars are hidden within symbols.

magnesium salt, 5.0 mM phosphocreatine disodium salt and 10 mM HEPES at pH 7.4. The currents were investigated by determining the current-voltage (I-V) relation using ramp pulses. The holding potential was −40 mV and the ramp pulse was of a sawtooth configuration, and its dV/dt was 0.84 V/s.

RESULTS

Time Course of Iodide Uptake

We examined the time course of iodide uptake by incubating CHO-4J cells for 2-90 min at 37 °C with 10 μM NaI and ~0.1 μCi $Na^{125}I$. As shown in Fig. 1A, the iodide uptake reached the half maximal level within 5 min and became saturated at 20-30 min. These results are quite consistent with those observed for FRTL-5 thyroid cells (9). The saturated, steady state seems to reflect a balance of influx and efflux of iodide as reported for FRTL-5 cells (9). Iodide uptake was completely inhibited by 1 mM ClO_4^-. Thus, iodide uptake with ClO_4^- was less than 1/100 of that without ClO_4^- at every time point tested (Fig. 1A). In addition, the iodide uptake in CHO-K1 cells without transfection was at the baseline level (data not shown) and it was indistinguishable from that in CHO-4J cells with 1 mM ClO_4^-.

Na^+-Dependency of Iodide Uptake

By substituting choline chloride for NaCl, the effect of the Na^+ concentration on iodide uptake in CHO-4J cells was examined by incubating the cells for 60 min at 37°C. As shown in Fig. 1B, the increase of iodide uptake was almost linear, depending on the extracellular Na^+ concentration within the range of 10-150 mM, as seen in FRTL-5 cells (9). These findings suggest that the iodide uptake in CHO-4J is a symport of Na^+ and I^-.

Kinetics

The initial velocity of the iodide uptake was estimated by incubation for 2 min with 5-500 μM iodide. As shown in Fig.2A, the velocity of iodide uptake increased depending on extracellular iodide concentration and reached saturation at ~500 μM. A double reciprocal plot (inset of Fig. 2A) revealed that it followed the Michaelis-Menten equation. The apparent K_m for iodide was 34.7 ± 6.6 μM (mean ± S.E.M. of 4 experiments) which is similar to that reported

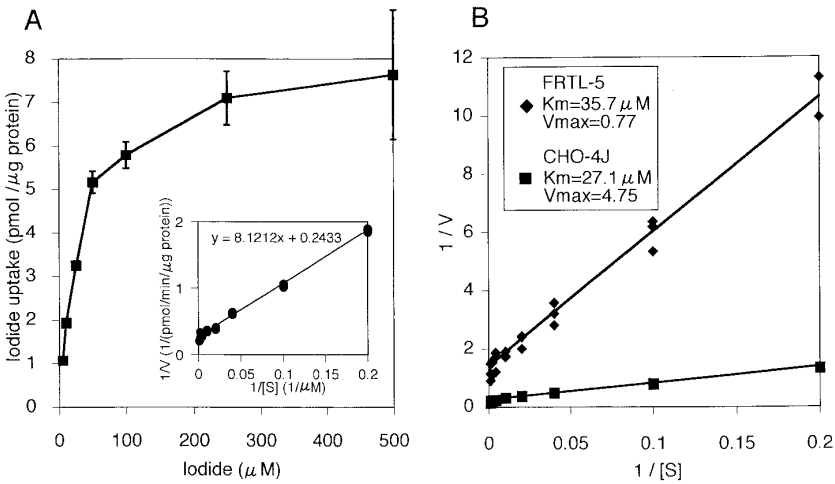

FIG. 2. Dependency of initial velocity of iodide uptake (2 min) on the extracellular iodide concentration in CHO-4J cells (A), and comparison of the kinetics of iodide uptake between CHO-4J and FRTL-5 cells (B). A, all data are expressed as means ± S.D. (pmol/μg protein) of triplicate wells. Some error bars are hidden within symbols. Inset of A, double reciprocal plot of the data shown in A. Every point of triplicate wells are plotted. B, scales of both axes are identical to those shown in the inset of A; 1/V (ordinate) expressed as 1/(pmol/min/μg protein) and 1/[S] (abscissa) as 1/μM. All points of triplicate wells are plotted.

for FRTL-5 cells (31 μM; Ref. 9), that obtained for FRTL-5 cells simultaneously examined (Fig. 2B), and that reported for oocytes expressing the symporter (36 μM; Ref. 1).

Comparison with FRTL-5 cells

The kinetics of iodide uptake was simultaneously studied in CHO-4J and FRTL-5 cells. As shown in Fig. 2B, the maximal velocity of iodide uptake (V_{max}) in CHO-4J cells expressed as pmol/min/μg cell protein was 6-10-fold of that of FRTL-5 cells (from three experiments). When expressed per single cell, the V_{max} was about 1000-fold of that of FRTL-5 cells.

Effect of ClO_4^- and SCN^-

Over 30 years ago, Wolff and Maurey reported that perchlorate (ClO_4^-) and thiocyanate (SCN^-) competitively inhibited iodide uptake of the thyroid gland (11). However, the exact mechanism of action of ClO_4^- and SCN^- on Na^+/I^- symporter yet remains to be elucidated using a specific system. CHO-4J cells seem to be an ideal tool for examining the agents influencing iodide transport. Kinetic studies should be simplified with less assay variability using CHO-4J cells expressing a large number of Na^+/I^- symporter.

We examined the effects of ClO_4^- or SCN^- with iodide on the kinetics of iodide uptake in CHO-4J cells. As shown in the lower panel of Fig. 3A, ClO_4^- dose-dependently increased the K_m for iodide uptake in CHO-4J cells without changing the V_{max}, indicating that ClO_4^- competitively inhibited of iodide uptake. When the slopes of each line on the double reciprocal plot were plotted against the ClO_4^- concentration (Fig. 3A, upper panel), they formed a straight line and the calculated K_i value was $\sim$1.5 μM. Similar experiments using SCN^- were also performed (Fig. 3B). SCN^- also competitively inhibited iodide uptake in CHO-4J cells with the K_i of $\sim$15.9 μM.

Iodide Efflux

Iodide efflux was simultaneously studied in CHO-4J and FRTL-5 cells by replacing HBSS incubation solution with 10 μM NaI without $^{125}I^-$ every 3-6 min, after incubating the cells

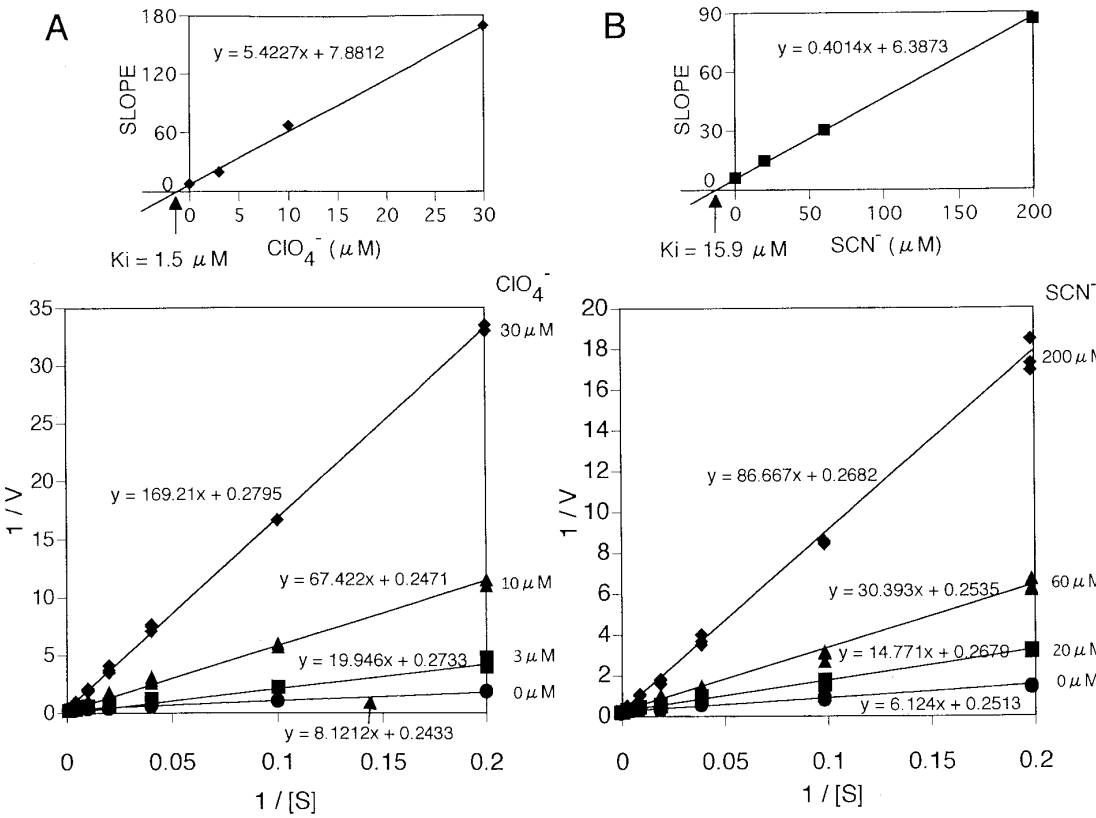

FIG. 3. Effect of ClO_4^- (A) and SCN^- (B) on the kinetics of iodide uptake in CHO-4J cells. All points of triplicate wells are plotted. Scales of both axes are identical to those in Fig. 2. Upper panels, slopes of each line are plotted against concentration of ClO_4^- (A) and SCN^- (B), resulting in determination of the K_i values for ClO_4^- and SCN^-.

with 10 μM NaI and ~0.1 μCi $Na^{125}I$ for 60 min (Fig. 4). As previously reported (9), I^- accumulated in FRTL-5 cells was rapidly exchangeable. Thus, FRTL-5 cells released 77% of the $^{125}I^-$ within 3 min ($t_{1/2}$ = ~2 min). In contrast, CHO-4J cells very slowly released $^{125}I^-$.

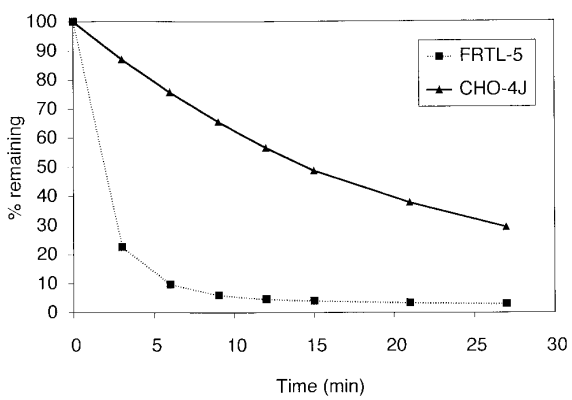

FIG. 4. Iodide efflux of CHO-4J and FRTL-5 cells. All data are expressed as means ± S.D. (% remaining of $^{125}I^-$) of quadriplicate wells, but all error bars are hidden within symbols.

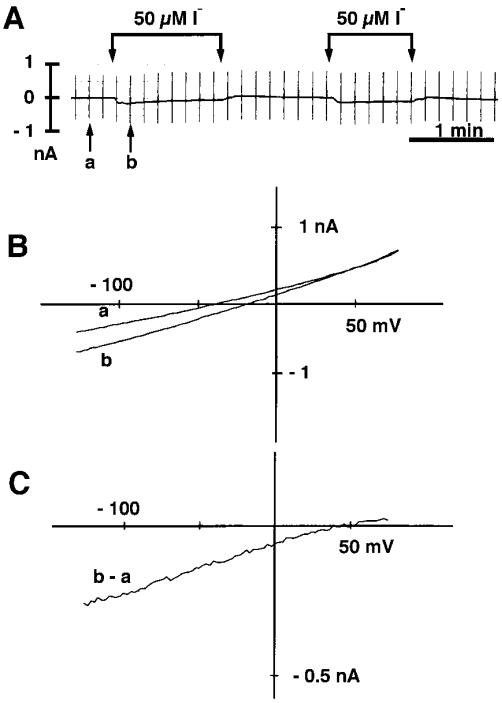

FIG. 5. Activation of the transporter current with NaI in CHO-4J cells. A, chart recording of the membrane current changes induced by switching the bathing solution as indicated. Vertical deflections were due to ramp pulses. B, current–voltage relationships determined in Tyrode solution without (a) and with (b) 50 μM NaI. C, current–voltage relationship for the current induced by NaI determined by subtracting the recording in (a) from (b) in panel B.

The initial 3 min release was only 13% and half of the $^{125}I^-$ was released at ~15 min. When the incubation solution was replaced every minute and when replacement solution without NaI was used, no difference was observed (data not shown). These results indicate that the contribution of re-uptake of released iodide by the Na^+/I^- symporter is very small.

Electrophysiological Study

We performed whole cell voltage-clamp studies of CHO-4J cells. As shown in Fig. 5A, the holding current at −40 mV rapidly shifted inwardly after switching the bathing solution to Tyrode solution with 50 μM NaI. On washing out the NaI, this response disappeared. The second application of NaI induced a similar inward shift of the holding current. Figure 5B shows the current-voltage (I-V) relationships using ramp pulses determined in Tyrode solution representing NaI-unrelated current (a) and in Tyrode solution with 50 μM NaI representing NaI-induced and -unrelated currents (b). The specific current induced by Na^+/I^- symporter was determined by subtracting the recording in (a) from (b) in Fig. 5B (Fig. 5C).

The I-V relationship in Fig. 5C revealed that the observed inward current was membrane potential-dependent and the mean conductance calculated from the slope of current-voltage relationship was 23.5 ± 3.2 pS/pF (mean ± S.D. of 4 experiments). The membrane current became zero at a membrane potential ~+50mV which was very close to the Na^+ reversal potential. This result is in agreement with the fact that iodide transport depends on electrochemical gradient of Na^+.

An electrophysiological study of the current induced by 500 and 1000 μM NaI revealed

similar features (data not shown) indicating that these concentrations were electrophysiologically saturable.

DISCUSSION

We confirmed the basic characteristics of the Na^+/I^- symporter by expressing it in CHO-cells. These features included the time course, Na^+-dependency, kinetics, and competitive inhibition by ClO_4^- and SCN^-, and were essentially identical to those reported for FRTL-5 thyroid cells (9), providing the validity of using CHO-4J cells to investigate the Na^+/I^- symporter instead of thyrocytes. The major differences of CHO-4J cells from FRTL-5 cells are the number of Na^+/I^- symporter and very slow iodide efflux.

From the iodide efflux study comparing CHO-4J and FRTL-5 cells, it is noteworthy that iodide efflux is markedly slower in CHO-4J cells than in FRTL-5 cells. This might be related to highly active uptake of iodide in CHO-4J cells. However, because the amount of remaining iodide did not differ between 3-min and 1-min medium replacements, and in the presence or absence of 10 μM NaI, the contribution of re-uptake of released iodide seems very small. It is strongly suggested that the iodide efflux of FRTL-5 thyroid cells is accelerated by some thyroid specific mechanism such as facilitated diffusion carrier or iodide channel (12,13), while that of CHO-4J cells seems to be through non-selective ion channels. By comparison with FRTL-5 cells, CHO-4J cells may provide new insight into the mechanism of iodide efflux in thyroid cells.

CHO-4J cells are very useful tools with which to examine iodide transport in terms of the following. (i) CHO-4J cells express high levels of Na^+/I^- symporter, making assay of iodide uptake more sensitive. (ii) Because iodide is not highly exchangable in CHO-4J cells due to the slow efflux unlike FRTL-5 cells, iodide uptake can be more simply and accurately assessed than in FRTL-5 cells. In FRTL-5 cells, significant amounts of accumulated iodide seem to be released during the washing process even when performed quickly and at low temperature. This property of CHO-4J cells may also contribute to higher sensitivity for the iodide uptake assay and is ideal for investigating pure Na^+/I^- symport activity. (iii) FRTL-5 cells lose iodide uptake activity when cultured without TSH (14). In contrast, CHO-4J cells show very high iodide uptake without agents that increase intracellular cAMP levels. To activate the Na^+/I^- symporter in FRTL-5 cells, it is postulated that cAMP-dependent transcription of unknown protein is necessary (14). It remains to be elucidated whether or not activation of the cAMP-cascade in CHO-4J cells further strengthens iodide uptake activity. (iv) Electrophysiological studies using whole cell clamp are more easily performed using CHO-4J cells because electrode injection is easier and recording current is much easier than in FRTL-5 cells. This is because CHO-4J cells are larger than FRTL-5 cells and express abundant levels of the symporter.

CHO-4J cells that express high levels of Na^+/I^- symporter may be also useful for detecting antibodies against the Na^+/I^- symporter that might be present in sera of patients with autoimmune thyroid disorders (15). CHO-4J cells provide a source of a large amount of symporter protein that can be used in Western blotting to detect autoantibodies. Furthermore, iodide uptake using patient samples may be sensitively assayed using CHO-4J cells. Not only antibodies as a result of thyroid inflammation like anti-TPO and anti-Tg antibodies, but also antibodies which have stimulatory or inhibitory activity on Na^+/I^- symporter and are causative of diseases like thyroid stimulating antibodies in Graves' disease and blocking type TSH receptor antibodies in idiopathic myxedema, may be detected using CHO-4J cells which can be used for highly sensitive assay of iodide uptake.

We showed that an inward current is induced by NaI for the first time. The present results confirmed that at least two sodium ions are transported with one iodide ion. The I-V relationship of the current also revealed that the Na^+/I^- symporter is electrogenic and potential-dependent. An electrogenic gradient acts as the driving force for I^- accumulation against its electrochemical

gradient. We described that a K^+ channel in FRTL-5 cells, which is activated by the TSH-cAMP-A-kinase system (16), hyperpolarizes the membrane, and causes an increase in the electrochemical gradient across the cell membrane. The coexistence of the K^+ channel and Na^+/I^- symporter in the cell membrane would be consistent with their physiological function.

ACKNOWLEDGMENTS

This study was in part supported by grants-in-aid from the Japanese Ministry of Education and Culture (07557353, 07671129, and 08671152), Kyoto University Research Foundation, Kurozumi Medical Research Foundation, Clinical Pathology Research Foundation of Japan, and Inamori Foundation (all to S.K.), and SRF for Biomedical Research (to T.M.). We are grateful to Miss Aiko Tamada for her excellent technical assistance.

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