**Thyroid Na\(^+\)/I\(^-\) Symporter**

**MECHANISM, STOICHIOMETRY, AND SPECIFICITY**

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Sepehr Eskandari‡, Donald D. F. Loo‡, Ge Dai‡, Orlic Levy‡, Ernest M. Wright‡, and Nancy Carrasco‡

From the ‡Department of Physiology, UCLA School of Medicine, Los Angeles, California 90095-1751 and the ¶Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

The rat thyroid Na\(^+\)/I\(^-\) symporter (NIS) was expressed in *Xenopus laevis* oocytes and characterized using electrophysiological, tracer uptake, and electron microscopic methods. NIS activity was found to be electrogenic and Na\(^+\)-dependent (Na\(^+\) \(\gg\) Li\(^+\) \(\gg\) H\(^+\)). The apparent affinity constants for Na\(^+\) and I\(^-\) were 28 ± 3 mM and 33 ± 9 \(\mu\)M, respectively. Stoichiometry of Na\(^+\) anion cotransport was 2:1. NIS was capable of transporting a wide variety of anions (I\(^-\), ClO\(_4\)^\(-\), SCN\(^-\), SeCN\(^-\), NO\(_3\)^\(-\), Br\(^-\), BF\(_4\)^\(-\), IO\(_4\)^\(-\), BrO\(_3\)^\(-\)), but perchlorate (ClO\(_4\)^\(-\)) was not transported. In the absence of anion substrate, NIS exhibited a Na\(^+\)-dependent leak current (−35% of maximum substrate-induced current) with an apparent Na\(^+\) affinity of 74 ± 14 mM and a Hill coefficient of 1. In response to step voltage changes, NIS exhibited current transients that relaxed with a time constant of 8–14 ms. Presteady-state charge movements (integral of the current transients) versus voltage relations obey a Boltzmann relation. The voltage for half-maximal charge translocation \((V_{0.5})\) was −15 ± 3 mV, and the apparent valence of the movable charge was 1. Total charge was insensitive to [Na\(^+\)]\(_o\), but \(V_{0.5}\) shifted to more negative potentials as [Na\(^+\)]\(_o\) was reduced. NIS charge movements are attributed to the conformational changes of the empty transporter within the membrane electric field. The turnover rate of NIS was 22 s\(^{-1}\) in the Na\(^+\) uniport mode and 36 s\(^{-1}\) in the Na\(^+\)/I\(^-\) cotransport mode. Transporter density in the plasma membrane was determined using freeze-fracture electron microscopy. Expression of NIS in oocytes led to a 2.5-fold increase in the density of plasma membrane protoplasmic face intramembrane particles. On the basis of the kinetic results, we propose an ordered simultaneous transport mechanism in which the binding of Na\(^+\) to NIS occurs first.

It is now firmly established that active accumulation of iodide (I\(^-\)) by the thyroid gland epithelium, previously referred to as the "iodide pump" or "iodide trap," is a Na\(^+\)-dependent secondary active transport process mediated by the Na\(^+\)/I\(^-\) symporter (NIS),\(^1\) an integral plasma membrane protein of the basolateral membrane of the thyroid gland follicular cells. Iodide transport into the thyroid gland has attracted substantial scientific and clinical interest due to the importance of I\(^-\) in the biosynthesis of thyroid hormones triiodothyronine and tetraiodothyronine, and to the significance of NIS in the diagnosis and treatment of thyroid disorders (1). A cDNA clone encoding NIS has recently been isolated, sequenced, and expressed in *Xenopus laevis* oocytes (2). Oocytes injected with NIS cRNA exhibit a 700-fold increase in perchlorate-sensitive I\(^-\) uptake.

This study reports a comprehensive characterization of rat NIS function expressed in *X. laevis* oocytes. NIS activity is Na\(^+\)-dependent and electrogenic, and the stoichiometry of cotransport is 2 Na\(^+\)/1 anion. Kinetics of transport as a function of external Na\(^+\) and substrate concentration suggest an ordered binding of Na\(^+\) and substrate to the transporter in which binding of Na\(^+\) occurs first. Substrate selectivity experiments show that a variety of anions are transported into the cell via NIS. However, perchlorate, the most potent known inhibitor of NIS, is not transported. Measurements of charge movements associated with NIS conformational changes, and substrate-uncoupled Na\(^+\)-dependent leak currents of NIS have provided insight into the nature of Na\(^+\)/I\(^-\) cotransport. Combined data from electrophysiological measurements and freeze fracture electron microscopy suggest that NIS may be multimeric in its functional form.

**EXPERIMENTAL PROCEDURES**

NIS cRNA (50 ng) was microinjected into stage V-VI *X. laevis* oocytes (2, 3), and the oocytes were maintained in Barth's solution at 18 °C until used for experiments. Oocytes were superfused with buffers containing (in mM): 100–0 NaCl, 0–100 choline chloride, 2 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, pH 7.5. Chloride was replaced with glutamate in Cl\(^-\)-free solutions. For cation selectivity experiments, NaCl was replaced with choline chloride or LiCl at pH 7.5 or choline chloride at pH 5.0 (adjusted with MES).

**Electrophysiological Measurements**—Electrophysiological recordings were done using the two-microelectrode voltage clamp technique at 22 ± 1 °C (4). To obtain current/voltage (I/V) or charge/voltage (Q/V) relations, the pulse protocol (pCLAMP, Axon Instruments) consisted of 100-ms voltage steps from a holding potential \((V_{0.5})\) of −50 mV to a series of test voltages \((V_{m})\) from +50 to −150 mV in 20 mV decrements. Currents from three sweeps were averaged, low-pass filtered at 500 Hz, and sampled at 10 μs.

**Tracer Uptake under Voltage Clamp**—Uptake of \(^{125}\)I\(^-\) (15 μCi/ml; Amersham Corp.) was determined in NIS cRNA-injected oocytes in the presence of 100 mM Na\(^+\) and 50 μM I\(^-\) \(\cdot\) V\(_{0.5}\) was −90 mV, and substrate-evoked inward currents were recorded for 10 min. Total inward charge movement was determined by integration of the current with time. At the end of the recording period, oocytes were washed with ice-cold choline buffer, solubilized with 10% sodium dodecyl sulfate, and assayed for \(^{125}\)I\(^-\) content. \(^{23}\)Na\(^+\) (2.5 μCi/ml; DuPont) uptake was deter-

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\(^{1}\) The abbreviations used are: NIS, Na\(^+\)/I\(^-\) symporter; MES, 2(N-morpholino)ethanesulfonic acid; FRTL, Fisher rat thyroid line; hPEPT1, human intestinal oligopeptide transporter; IMP, intramembrane particle; \(n\), Hill coefficient; \(N\), sample size; SGLT, Na\(^+\)/glucose cotransporter; SMIT, Na\(^+\)/myo-inositol cotransporter.
Fig. 1. Electrogenicity of the Na⁺/I⁻ symporter. Current was recorded from a NIS cRNA-injected oocyte at V₅ = -50 mV, and the oocyte was superfused with the solutions indicated in the top panel. Base line was established in the Na⁺ buffer (100 mM NaCl). Addition of I⁻ (500 μM) to the bath caused a large inward current. ClO₄⁻ (500 μM) completely inhibited the I⁻-evoked inward current. Perchlorate, by itself, does not generate an inward current.

Anion Selectivity—Addition of 500 μM anionic substrate. Itself, does not generate an inward current.

Data Analysis—Substrate-evoked currents were obtained as the difference in steady-state current measured in the presence and absence of substrate and were fitted to:

\[ I = \frac{I_{\text{max}}^S}{[S]^n} + [S] \]  

where \( I \) is the evoked current, \( I_{\text{max}}^S \) is the maximum current, \( S \) is the substrate (anion or Na⁺), \( K_{S,5} \) is the substrate concentration at half-maximal current, and \( n \) is the Hill coefficient. To obtain the presteady-state currents, total currents were fitted to Equation 2, and transport-domain-mediated transients were determined by subtracting the capacitive and steady-state components,

\[ I_{\text{total}}(t) = I_{\text{cap}} \exp(-t/\tau_c) + I_{\text{tp}} \exp(-t/\tau_p) + I_{\text{SS}} \]  

where \( I_{\text{total}} \) is the total current, \( I_{\text{cap}} \) is the initial membrane capacity current, \( \tau_c \) is the time constant of \( I_{\text{cap}} \), \( I_{\text{tp}} \) is the initial presteady-state current, \( \tau_p \) is the time constant of \( I_{\text{tp}} \), and \( I_{\text{SS}} \) is the steady-state current. Q/V relations were obtained by integration of the presteady-state current with time for various voltages and were fitted to the Boltzmann relation,

\[ \frac{Q - Q_{\text{hyp}}}{Q_{\text{max}}} = \frac{1}{1 + \exp[(V_m - V_{\text{hyp}})/RT]} \]  

where the total charge \( Q_{\text{max}} = Q_{\text{hyp}} - Q_{\text{loq}} \) (where \( Q_{\text{hyp}} \) and \( Q_{\text{loq}} \) represent \( Q \) at depolarizing and hyperpolarizing limits), \( z \) is the apparent valence of the moveable charge, \( V_m \) is the membrane voltage during the pulse, \( V_{\text{loq}} \) is the membrane voltage at which half of the total charge has moved, \( F \) is Faraday's constant, \( R \) is the gas constant, and \( T \) is the absolute temperature. Unless otherwise indicated, results obtained from experiments on individual oocytes are presented, but all experiments were repeated on at least three oocytes from different donor frogs. Data fits were performed using Clampfit (Axon Instruments) or Sigma Plot (Jandel Scientific). Errors are reported as S.E. of the estimate obtained from the fit or as S.E. of the mean obtained from data from several oocytes.

RESULTS

Steady-state Currents—Electrogenicity of NIS is shown in Fig. 1. Addition of 500 μM I⁻ to the bathing medium caused an inward current of ~400 nA in an oocyte expressing NIS. ClO₄⁻ (500 μM), a specific blocker of I⁻ transport by the Na⁺/I⁻ symporter, abolished the I⁻-evoked inward current. Fig. 2 shows typical I/V relationships in a NIS-expressing oocyte before (A) and after (B) addition of I⁻ (500 μM) to the perfusion solution. The pulse protocol is shown. In A the presteady-state currents associated with NIS are evident (see also Fig. 8). B, addition of I⁻ (500 μM) apparently eliminated the presteady-state currents and induced an inward current. Dotted traces show current at the holding potential and emphasize the difference caused by the addition of I⁻. C, net I⁻-evoked inward current was taken as the difference between the steady-state current in the presence (1–100 μM) and absence of I⁻ and plotted as a function of \( V_m \).

Anion Selectivity—Fig. 4 shows the relative substrate selectivity of NIS. In this experiment, current was monitored as anions were added (500 μM) to the perfusion solution. The best transported substrates were I⁻, ClO₄⁻, SCN⁻, SeCN⁻, and...
NO\textsubscript{3} was transported to a significant extent, but ClO\textsubscript{4} was not transported at all. The transported anions did not induce an appreciable inward current in H\textsubscript{2}O-injected oocytes from the same batch (see below). NO\textsubscript{3}, HCO\textsubscript{3}, acetate, succinate, SO\textsubscript{4}\textsuperscript{2-}, CO\textsubscript{3}\textsuperscript{2-}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-}, and P\textsubscript{2}O\textsubscript{4}\textsuperscript{2-} were not transported (not shown).

The relative apparent affinity of NIS for anions (Table I) were: I\textsuperscript{-} (1.00) > SeCN\textsuperscript{-} (0.87) > SCN\textsuperscript{-} (0.34) > ClO\textsubscript{4}\textsuperscript{-} (0.12) > NO\textsubscript{3} (0.04). The relative \(I_{\text{max}}\) values (\(I_{\text{max}} = 1\)) ranged from 0.8 to 1.5 (Table I). The voltage dependence of \(K_{\text{0.5}}\) and \(I_{\text{max}}\) was the same for all of the above anions (not shown).

**Inhibition of Iodide-induced Inward Current—**ClO\textsubscript{4} completely blocked the current generated by 50 \(\mu\text{M}\) I\textsuperscript{-}, with an apparent half-inhibition constant (\(K_{\text{IClO4}}\)) of 1.8 \(\pm\) 0.4 \(\mu\text{M}\) (\(N = 4\)). \(K_{\text{IClO4}}\) was also very low (3.2 \(\pm\) 0.4 \(\mu\text{M}\); \(N = 5\)), but this inhibitor could only block the I\textsuperscript{-}-induced current by 86 \(\pm\) 3%.

This could be due to the fact that at high concentrations (500 \(\mu\text{M}\)), ReO\textsubscript{4}\textsuperscript{3-} itself can induce a very small inward current (Fig. 4). BF\textsubscript{4} and IO\textsubscript{3} (500 \(\mu\text{M}\)) reduced the I\textsuperscript{-}-evoked (50 \(\mu\text{M}\)) inward currents by 74 \(\pm\) 10% and 21 \(\pm\) 6%, respectively (\(N = 3\)).

In some control (non-injected) oocytes, I\textsuperscript{-} induced a small but detectable inward current, which was prominent at high iodide concentrations (>500 \(\mu\text{M}\)) and at depolarizing membrane potentials (−10 to +50 mV). These iodide currents were insensitive to perchlorate. Of the anions that are readily transported by NIS (I\textsuperscript{-}, ClO\textsubscript{4}\textsuperscript{-}, SCN\textsuperscript{-}, SeCN\textsuperscript{-}, and NO\textsubscript{3}), only ClO\textsubscript{4} did not exhibit this behavior. Therefore, we chose to use ClO\textsubscript{4} as a model anion for further kinetic studies.

**Cation Selectivity—**When Na\textsuperscript{+} in the perfusion solution was isotonically replaced with choline, no I\textsuperscript{-}-induced (500 \(\mu\text{M}\)) inward current was observed at either pH 7.5 or 5.0. Li\textsuperscript{+}, however, was able to drive transport at a reduced level. At −150 mV, the Li\textsuperscript{+}/I\textsuperscript{-} current was 10–20% of the Na\textsuperscript{+}/I\textsuperscript{-} current (not shown).

**Na\textsuperscript{+} and ClO\textsubscript{4}\textsuperscript{-} Activation of Inward Currents—**In Fig. 5A, inward currents induced by 0.25, 1, and 5 mM ClO\textsubscript{4} are plotted as a function of external Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]\textsubscript{o}). At each substrate concentration, inward currents saturated with increasing [Na\textsuperscript{+}]\textsubscript{o}. The Hill coefficient for Na\textsuperscript{+} was −2 regardless of the substrate concentration and \(V_{\text{m}}\); at [ClO\textsubscript{4}]= 1 mM and \(V_{\text{m}} = -50\) mV, \(n = 2.2 \pm 0.1\). \(I_{\text{max}}\) increased with increasing substrate concentration (Fig. 5B) and the apparent affinity of NIS for Na\textsuperscript{+} increased as [ClO\textsubscript{4}] was increased (Fig. 5C). At −50 mV, at [ClO\textsubscript{4}]= 0.25, 1, and 5 mM, \(K_{0.5}\) was 57 \pm 7, 39 \pm 3, and 28 \pm 3 mM, respectively.

Examination of substrate kinetics at different [Na\textsuperscript{+}]. (Fig. 6A) showed that although \(K_{\text{IClO4}}\) remained constant as [Na\textsuperscript{+}]\textsubscript{o} was lowered (Fig. 6B), the apparent affinity of NIS for substrate decreased dramatically (Fig. 6C); \(K_{0.5}\) was 271 \pm 5 \(\mu\text{M}\) at 100 mM [Na\textsuperscript{+}]\textsubscript{o} and 1671 \pm 263 \(\mu\text{M}\) at 20 mM [Na\textsuperscript{+}]\textsubscript{o}.

**Voltage Dependence of Kinetic Parameters—**Fig. 7A shows the voltage-dependence of \(K_{0.5}\) at various [Na\textsuperscript{+}]\textsubscript{o}. Regardless
of [Na\(^+\)]_o, \(K_{0.5}^{\text{ClO}_3}\) approached 150 \(\mu M\) at hyperpolarizing limits. At less negative membrane potentials \(K_{0.5}^{\text{ClO}_3}\) varied greatly depending on [Na\(^+\)]_o. As with \(K_{0.5}^{\text{Na}}, K_{0.5}^{s}\) varied with voltage and with the concentration of cosubstrate (Fig. 7B).

**Presteady-state Charge Movement**—In the absence of substrate, NIS cRNA-injected oocytes exhibited presteady-state current transients in response to step changes in \(V_m\) (Figs. 2A and 8A). These current transients were not observed in control H\(_2\)O-injected oocytes (see Ref. 3). Fitting of the current traces (both ON and OFF; Fig. 8A) to Equation 2 resolved three components: (i) a fast component (\(\tau \sim 0.5\) ms) due to oocyte membrane capacitive currents (also seen in control oocytes); (ii) a second slower component (\(\tau \sim 8–14\) ms), which was the NIS-mediated current; and (iii) a steady-state current due to “leak” pathways in NIS and the membrane. To obtain the carrier-mediated transients, the membrane capacitive and steady-state components were subtracted from the total current (Fig. 8B). At each clamped voltage, integration of the carrier-mediated currents (Fig. 8B) with time yielded the charge \(Q\) moved by NIS within the membrane electric field. Fig. 8C shows a \(Q/V\) relationship for NIS. \(Q_{\text{ON}}\) and \(Q_{\text{OFF}}\) were equal and opposite in sign and reversed at \(V_m (\sim 50\) mV). The \(Q/V\) curve fitted a single Boltzmann relation (Equation 3) with a \(V_{0.5}\) of -15 ± 3 mV and a \(z\) of 0.9 ± 0.1 (\(N = 8\)). The time constant of the slow current transient was voltage-dependent for the ON response. \(\tau_{\text{ON}}/V\) was bell-shaped and ranged from 8 to 14 ms with its maximum value at \(-55\) mV (\(V_{\text{max}}^{\text{ON}}\)) (Fig. 8D). \(\tau_{\text{OFF}}\) was voltage-independent at \(-10\) ms (C; Fig. 8D).

Fig. 9A shows \(Q/V\) curves at 0–100 mm [Na\(^+\)]_o. There was no loss in \(Q_{\text{max}}\) as [Na\(^+\)]_o was reduced from 100–20 mm (Fig. 9B), but \(V_{0.5}\) shifted from -17 mV at 100 mm [Na\(^+\)]_o to -90 mV at 20 mm [Na\(^+\)]_o (Fig. 9C). \(z\) was \(-1\) at all Na\(^+\) concentrations. The maximum value of the time constant of the relaxation currents \(\tau_{\text{max}} \sim 14\) ms did not change as [Na\(^+\)]_o was reduced (not shown), but \(V_{\text{max}}^{\text{ON}}\) shifted from \(-55\) mV at 100 mm [Na\(^+\)]_o to \(-74\) mV at 20 mm [Na\(^+\)]_o (not shown).

Addition of either substrate or inhibitor led to a reduction in \(Q_{\text{max}}\) (Fig. 10A). As the concentration of substrate or inhibitor was increased, the decrease in \(Q_{\text{max}}\) followed a hyperbolic function (not shown). With Cl\(_3\)O\(_2\)\(^-\), 50 percent reduction in \(Q_{\text{max}}\) was reached at 586 ± 80 \(\mu M\) (\(N = 3\)). The Cl\(_3\)O\(_2\)\(^-\)-induced reduction in \(Q_{\text{max}}\) was directly proportional to the steady-state Cl\(_3\)O\(_2\)\(^-\)induced inward current (Fig. 10B). At -50 and -150 mV, the slope of the plot \(I\) versus \(Q\) was 36 ± 2 s\(^{-1}\) and 61 ± 4 s\(^{-1}\), respectively.

**Na\(^+\)-dependent Leak**—In NIS-expressing oocytes, replacement of 100 mM choline chloride with NaCl caused an inward current that was much larger (>100 nA; Fig. 11A) than that seen in H\(_2\)O-injected oocytes (<20 nA at -50 mV). Addition of 500 \(\mu M\) I\(^-\) caused a further increase in the inward current. The Na\(^+\)-dependent inward current, in the absence of substrate, is referred to as the NIS Na\(^+\) leak current. The Na\(^+\) leak current was saturable with increasing [Na\(^+\)]_o. At -50 mV, the [Na\(^+\)]_o at which the leak current was half-maximal \((K_{0.5}^{\text{leak}})\) was 74 ± 14 mM (\(N = 3\); Fig. 11B), and the Hill coefficient was 0.9 ± 0.1 (\(N = 3\)). Control H\(_2\)O-injected oocytes exhibited inward Na\(^+\) currents that had a half-saturation constant of 9 ± 2 mM (\(N = 3\)). The magnitude of the leak current increased linearly with the level of expression, such that there was a direct linear correlation between the leak current and the substrate-induced current. At -150 mV, the plot of maximum leak current as a
function of maximum ClO₃⁻-induced current (I_max versus ClO₃⁻_max) yielded a slope of 0.34 ± 0.04 (N = 7; not shown).

Stoichiometry—Fig. 12A shows a current record from a NIS-expressing oocyte held at −90 mV and perfused with a solution containing I⁻ (100 mM Na⁺, 50 µM I⁻, and 15 µCl/mol ¹²⁵I⁻) for 10 min. Integration of the I⁻-evoked inward current yielded the net positive charge that entered the oocyte during the recording period (shaded region). In the same oocyte, I⁻ uptake was measured by determination of ¹²⁵I⁻ content. A plot of the net inward charge versus I⁻ uptake from 10 oocytes revealed a linear relation with a slope of 0.76 ± 0.03 inward charge per iodide uptake (Fig. 12B). In Fig. 12C, inward charge is plotted as a function of Na⁺ uptake. Inward current was induced by 5 mM ClO₃⁻ for 10 min in the presence of 30 mM Na⁺ and 2.5 µCl/mol ²²Na⁺. The slope of the line was 0.42 ± 0.04 inward charge per Na⁺ uptake (N = 6).

NIS Intramembrane Particles—Freeze-fracture electron micrographs of the P fracture face from the plasma membrane of a control H₂O-injected oocyte and an oocyte expressing NIS are shown in Fig. 13. In the control oocyte, the density of IMPs in the P face was 556 ± 69/µm² (mean ± S.D.; Fig. 13A). The endogenous intramembrane particles showed a relatively homogenous distribution with a mean diameter of 7.6 ± 1.2 nm (N = 875). Oocytes expressing NIS showed a ~2.5-fold increase in the density of P face particles to 887 ± 146/µm² (Fig. 13B). In contrast, the density of IMPs in the E face was not altered by expression of NIS (not shown; see Ref. 5). In addition, P face intramembrane particles of NIS-expressing oocytes showed a greater heterogeneity in size. Analysis of the diameter of P face IMPs (N = 856) in oocytes expressing NIS showed two prominent populations: one at 7.2 ± 0.5 nm corresponding to the endogenous particles and another at 9.0 ± 0.6 nm due to NIS particles. In the oocyte in Fig. 13B, Q_max was 18 nanocoulombs and the total number of transporters (N_{NIS}) in the plasma membrane was 3.5 × 10¹⁰. Q_max = N_{NIS}Z e, where Z is the valence of the moveable charge per NIS particle, and e is the electronic charge. Therefore, Z was estimated to be ~3 electronic charges.

DISCUSSION

General Properties of NIS—Cloning of the Na⁺/I⁻ symporter and its expression in X. laevis oocytes has made it possible to carry out a thorough functional characterization of this transporter. Iodide transport via NIS generates a net influx of positive charge (an inward current) that depolarizes the membrane. The inward current is Na⁺-dependent, stimulated by I⁻, and coupled to Na⁺ and I⁻ influx. Uptake studies indicate that 2 Na⁺ ions are transported with one anion, resulting in inward movement of one positive charge. Previously, the electrogenic nature of the Na⁺/I⁻ symporter had been suggested in experiments using plasma membrane vesicles from hog thyroid (6).

The apparent affinity constant of NIS for I⁻ (33 ± 9 µM at −50 mV) is in general agreement with those obtained in uptake
ions are transported by NIS: I
\(^{-2}\) spherical while SeCN
\(^{-2}\) transported (10). No conclusion, however, can be drawn regarding the geometry of the anion, the qualitative similarity of transport kinetics of I
\(^{-}\) and SCN
\(^{-}\) is also comparable with that found in other studies (6, 9).

Anion Selectivity—In addition to I
\(^{-}\), a number of other anions are transported by NIS: I
\(^{-}\) \text{SeCN}^{-} > SCN^\(-) > ClO_4^\(-) > NO_3^\(-). The only apparent common denominator for the well transported substrates is anionic monovalency. The closer the size of the monovalent anion to that of I
\(^{-}\), the better it is transported (10). No conclusion, however, can be drawn regarding the molecular geometry of a good substrate. Iodide is nearly spherical while SeCN
\(^{-}\) and SCN
\(^{-}\) are near-linear; ClO_4
\(^{-}\) has a trigonal pyramidal geometry; and NO_3
\(^{-}\) is planar. Regardless of the geometry of the anion, the qualitative similarity of transport kinetics of I
\(^{-}\), SeCN
\(^{-}\), SCN
\(^{-}\), ClO_4
\(^{-}\), and NO_3
\(^{-}\) suggests that their mechanism of transport may be the same.

Inhibition of Iodide Transport—A number of anions can significantly inhibit I
\(^{-}\) transport. Most notable is ClO_4
\(^{-}\), the most potent known inhibitor of NIS (K_i ClO_4 = 1.8 \pm 0.4 \mu M). Previous studies in NIS-expressing X. laevis oocytes (36 \mu M) (2), FRTL-5 cells (30 \mu M) (7), and membrane vesicles derived from porcine thyroid (5 \mu M) (6, 8) suggest that their mechanism of transport may be the same.

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The choroid plexus, salivary glands, lactating mammary glands, gastric mucosa, placenta, ciliary body of the eye, and kidney tubules have also been shown to possess a Na⁺-dependent I⁻ transport system (see Refs. 1 and 14). The anion selectivity of NIS found in this study (Kᵢᵢ or Kᵢ) was: ClO₄⁻ > ReO₄³⁻ > I⁻ > SeCN⁻ > SCN⁻ > Cl⁻ > NO₃⁻. This is very similar to that in thyroid (10, 13) and choroid plexus (15), with the exception that in those tissues, SCN⁻ was found to interact with a higher apparent affinity than I⁻.

**Cation Selectivity**—The specificity of Na⁺-dependent cotransporters for Na⁺ as the driving cation is not absolute. Proton can substitute for Na⁺ in the Na⁺/glucose cotransporter (SGLT1) (16) and the serotonin transporter (17). Sugar transport by SGLT1 is also driven by Li⁺ (18). Iodide transport through NIS was not driven by H⁺, but Li⁺ was able to drive transport at a reduced level (10–20% of Na⁺-driven transport). This is consistent with results on porcine thyroid plasma membrane vesicles (6).

**Stoichiometry**—Na⁺ activation of I⁻ influx in porcine thyroid plasma membrane vesicles revealed Na⁺ Hill coefficients of 1.6–1.8 (6, 8). Hill analysis of Na⁺ activation curves (e.g. Fig. 5A) provides an index of the number of Na⁺ ions necessary to activate the transport process (n = 2.2 ± 0.1), but does not determine the number of Na⁺ ions that actually enter the cell as a result of transporter activity. The actual stoichiometry can be inferred by simultaneous monitoring of inward charge and Na⁺ and substrate influx under voltage-clamp conditions. Measurement of Na⁺- and anion-evoked inward currents under voltage-clamp with determination of Na⁺ and anion uptake revealed that for every anion taken up, 0.76 positive charge entered the cell (Fig. 12). The point represents Na⁺/I⁻ ± 0.03 charge/I⁻ uptake for NIS-expressing oocytes (○) and control oocytes (□) represents Na⁺ uptake through the leak pathway.

(Fig. 4). ClO₄⁻ and SCN⁻ were traditionally used as competitive inhibitors of I⁻ uptake in the thyroid gland, and both were believed to be transported via the Na⁺/I⁻ cotransport system (1, 11). In our system, SCN⁻ is transported, but perchlorate is not. That perchlorate is not transported by NIS is not unique to the Xenopus oocyte expression system as similar results have been obtained with rat NIS expressed in Chinese hamster ovary cells (12). Our data, however, cannot exclude electroneutral Na⁺/ClO₄⁻ transport (1:1 coupling ratio).

Thus, anions that effectively interact with NIS can be subdivided into three groups: (i) anions that are readily transported; e.g. I⁻, SeCN⁻, SCN⁻, ClO₄⁻, and NO₃⁻; (ii) anions that partially inhibit I⁻ transport, but are themselves transported to some extent; e.g. IO₃⁻, BF₆⁻, and ReO₄³⁻; and (iii) anions that completely inhibit transport; ClO₄⁻. Although no conclusion can be drawn about the molecular commonality of the first group, anions belonging to the second and third groups all have a tetrahedral molecular geometry with an anionic volume very similar to that of I⁻ (13, 14).

The choroid plexus, salivary glands, lactating mammary glands, gastric mucosa, placenta, ciliary body of the eye, and kidney tubules have also been shown to possess a Na⁺-dependent I⁻ transport system (see Refs. 1 and 14). The anion selectivity of NIS found in this study (Kᵢᵢ or Kᵢ) was: ClO₄⁻ > ReO₄³⁻ > I⁻ > SeCN⁻ > SCN⁻ > Cl⁻ > NO₃⁻. This is very similar to that in thyroid (10, 13) and choroid plexus (15), with the exception that in those tissues, SCN⁻ was found to interact with a higher apparent affinity than I⁻.

**Cation Selectivity**—The specificity of Na⁺-dependent cotransporters for Na⁺ as the driving cation is not absolute. Proton can substitute for Na⁺ in the Na⁺/glucose cotransporter (SGLT1) (16) and the serotonin transporter (17). Sugar transport by SGLT1 is also driven by Li⁺ (18). Iodide transport through NIS was not driven by H⁺, but Li⁺ was able to drive transport at a reduced level (10–20% of Na⁺-driven transport). This is consistent with results on porcine thyroid plasma membrane vesicles (6).

**Stoichiometry**—Na⁺ activation of I⁻ influx in porcine thyroid plasma membrane vesicles revealed Na⁺ Hill coefficients of 1.6–1.8 (6, 8). Hill analysis of Na⁺ activation curves (e.g. Fig. 5A) provides an index of the number of Na⁺ ions necessary to activate the transport process (n = 2.2 ± 0.1), but does not determine the number of Na⁺ ions that actually enter the cell as a result of transporter activity. The actual stoichiometry can be inferred by simultaneous monitoring of inward charge and Na⁺ and substrate influx under voltage-clamp conditions. Measurement of Na⁺- and anion-evoked inward currents under voltage-clamp with determination of Na⁺ and anion uptake revealed that for every anion taken up, 0.76 ± 0.03 positive charge entered the cell and, conversely, for every Na⁺ taken up, 0.42 ± 0.04 positive charge entered the cell (Fig. 12). The stoichiometry obtained from the ratio is 1.8 ± 0.2 (0.76/0.42). This suggests a 2 Na⁺:1 anion stoichiometry.

**Steady-state I/V Relationship**—The I/V curves (Figs. 2C) and both Iₛₐₓ max (Fig. 3C) and Iₛₐₓ max (not shown) increase in a linear or superlinear fashion with hyperpolarizing voltages with no evidence of saturation. Thus, in the voltage range tested (~150 to ~10 mV), there is at least one rate-limiting voltage-dependent step in the transport cycle (19). This behavior is unlike that of SGLT1 (3), which shows a saturation of I/V curves with voltage, but is similar to that of the Na⁺/myo-inositol cotransporter (SMIT) (20) and the taurine transporter (21).

**Voltage Dependence of Kinetic Parameters**—That negative membrane potentials increased the apparent affinity of NIS for Na⁺ is consistent with the presence of a Na⁺ well (19). Increased cation affinity with membrane hyperpolarization has
been observed in other mammalian Na$^+$-driven cotransporters, e.g. SGLT1 (3), SGLT2 (22), and SMIT (20), and in a mammalian proton-driven oligopeptide transporter (hPEPT1) (23). Further evidence for the existence of a Na$^+$ well was provided by the fact that at hyperpolarizing potentials, $K_{Q,5}^{\text{Na}}$ was independent of [Na$^+$], (Fig. 7A), indicating that a negative $V_m$ could offset the effect of reduced [Na$^+$].

The apparent affinity for I$^-$ was relatively insensitive to voltage at hyperpolarizing potentials, but exhibited a sharp voltage dependence at potentials more positive than $-50$ mV. It may seem counterintuitive that at negative membrane potentials, the apparent affinity for an anion is voltage-independent. One possibility is that the putative conformational change associated with Na$^+$ binding to the transporter (see below) would position the I$^-$ binding site at or beyond the membrane electric field/water interface such that, at hyperpolarized potentials, it no longer senses the membrane electric field. Alternatively, Na$^+$ binding to the transporter induces a conformational change that creates a low access resistance path for I$^-$ entry, and the voltage drop across such path may be very small to allow for detection of a voltage dependence of $K_{Q,5}^{\text{Na}}$.

**Na$^+$-dependent Leak**—In the absence of substrate, there was a Na$^+$-dependent inward current via NIS, which was $-35\%$ of the current induced at saturating substrate concentration. Up-take studies also showed that, in the absence of substrate, there was increased Na$^+$ influx in NIS-expressing oocytes (Fig. 12C). The apparent affinity constant for the Na$^+$ leak ($K_{Q,\text{leak}}^{\text{Na}}$) was greater than twice the apparent affinity constant of the Na$^+$/I$^-$ transport pathway ($K_{Q,5}^{\text{Na}}$); at $-50$ mV, $K_{Q,\text{leak}}^{\text{Na}} \approx 75$ mM, whereas $K_{Q,5}^{\text{Na}} \approx 30$ mM. The Hill coefficient for Na$^+$ activation of the leak current was 1. This implies that in the absence of substrate, NIS behaves as a Na$^+$ uniporter, and may confer a resting Na$^+$ conductance to the cell.

**Presteady-state Charge Movements**—In the absence of substrate and in response to step-changes in $V_m$, presteady-state currents are observed for NIS, as for other cloned cotransporters expressed in *X. laevis* oocytes; e.g. SGLT1 (4, 24), SGLT2 (22), hPEPT1 (23), SMIT (20), GABA transporter (GAT1) (25), and plant H+/hexose cotransporter (STP1) (26). Presteady-state currents represent charge translocations and provide clues about partial reactions in the transport cycle. Also similar to other cotransporters, NIS total charge translocation ($Q_{\text{max}}$) appears to decrease in the presence of substrate and/or inhibitor.

We observed no reduction in $Q_{\text{max}}$ with decreasing [Na$^+$], from 100–20 mM, and only a small decrease was seen in $Q_{\text{max}}$ at [Na$^+$], = 0 mM (Fig. 9B). This behavior is similar to that of SGLT2 (22), but unlike that of SGLT1 (24) and hPEPT1 (23), which show an apparent reduction in $Q_{\text{max}}$ ($-20\%$) as the external concentration of the driving cation (Na$^+$ or H+) is reduced. At nominal zero external sodium, NIS $Q_{\text{max}}$ appeared to be smaller, but this is most likely due to the large left-shift in $V_{Q,5}$, which precludes us from obtaining a reliable Boltzmann fit in the voltage range tested. Therefore, charge movements in NIS are primarily due to the conformational changes of the empty transporter within the membrane electric field (shaded region in Fig. 14), but there may also be a minor contribution to the total charge due to Na$^+$ binding/dissociation.

For a 10-fold reduction in [Na$^+$], $V_{Q,5}$ shifted by $-100$ mV to negative potentials, similar to that seen for other transporters (e.g. SGLT1, SGLT2, hPEPT1). This indicates that in the absence of Na$^+$, Na$^+$ can bind to NIS, and it is possible that the shift in $V_{Q,5}$ is due to Na$^+$ binding-induced conformational changes of NIS. Similar to other cotransporters, the apparent valence of the moveable charge for NIS is 1. Therefore, the basic features of charge translocation by NIS are similar to what has been reported for other cotransporters (see Table II in Ref. 24).

**Substrate-coupled and Substrate-uncoupled Turnover Rate**—Both $I_{\text{max}}$ and $Q_{\text{max}}$ are dependent on the number of transporters present in the plasma membrane. $Q_{\text{max}}$ is an index of transporter density in the plasma membrane (see “NIS Intramembrane Particles”). NIS turnover rate was estimated using two different approaches. First, in several NIS-expressing oocytes, both $Q_{\text{max}}$ (in the absence of substrate) and $I_{\text{max}}$ (in the presence of saturating substrate) were measured. NIS turnover rate was then estimated from the slope of $I_{\text{max}}$ versus $Q_{\text{max}}$ plot; $37 \pm 2$ s$^{-1}$ at $-50$ mV and $66 \pm 4$ s$^{-1}$ at $-150$ mV ($N = 18$). These values are comparable with those found for other cotransporters (see Table I in Ref. 23). Second, in individual oocytes, $Q_{\text{max}}$ was measured in the absence and presence of various concentrations of substrate, with simultaneous recording of the substrate-induced inward current. When substrate-induced inward current is plotted as a function of $Q_{\text{max}}$ in the same oocyte, nearly identical turnover numbers result from the slope of the line; $36 \pm 2$ s$^{-1}$ at $-50$ mV and $61 \pm 4$ s$^{-1}$ at $-150$ mV (Fig. 10B). Nonetheless, both approaches underestimate maximum NIS turnover rate, since the I/V curve does not saturate in the voltage range tested (see Fig. 2C). The existence of a large leak pathway leads to a large turnover rate in the substrate-uncoupled (Na$^+$ uniport) mode relative to that found for other cotransporters. In the absence of substrate and at 100 mM [Na$^+$], the substrate-uncoupled turnover rate for NIS is $22 \pm 2$ s$^{-1}$ at $-50$ mV and $27 \pm 2$ s$^{-1}$ at $-150$ mV ($N = 7$), whereas that for SGLT1 is less than $5$ s$^{-1}$ (27).

**Mechanism of Na$^+$/I$^-$ Cotransport**—The steady-state kinetic data point to an ordered, simultaneous transport mechanism in which Na$^+$ binds first to the transporter followed by iodide (Fig. 14). This ordered mechanism was inferred from the observation that $P_{\text{Na}}^{\text{out}}$ was dependent upon the substrate concentration (Fig. 5B), whereas $P_{\text{Na}}^{\text{in}}$ was independent of [Na$^+$],
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(Fig. 6B) indicating that binding of Na\(^+\) to the transporter occurs first (28). Thus, regardless of [Na\(^+\)]\(_o\), once Na\(^+\) is bound to the transporter, greater concentrations of substrate can drive transport to the same maximum velocity. Transport is simultaneous because decreases in the concentration of Na\(^+\) or substrate lead to decreases in the apparent affinity of the other (Figs. 5C and 6C) (29).

According to the scheme in Fig. 14, at physiological Na\(^+\) concentrations and membrane voltages, the Na\(^+\) binding site of NIS faces the extracellular solution with one bound Na\(^+\) ion (CaNa\(^+\)). Reorientiation of the transporter within the membrane exposes the bound Na\(^+\) to the intracellular compartment (CaNa\(^+\) \(\rightarrow\) CaNa\(^+\)) followed by its release into the cytoplasm. Return of the empty Na\(^+\) binding site to the external solution completes the cycle (C \(\rightarrow\) C). This pathway constitutes the Na\(^+\) leak or Na\(^+\) uniport pathway and has a turnover rate of \(\approx 22\) s\(^{-1}\). In the presence of I\(^-\), CaNa\(^+\)I\(^-\) is formed which undergoes a transition resulting in the bound ions facing the cytoplasmic space (CaNa\(^+\) \(\rightarrow\) CaNa\(^+\)I\(^-\)). Release of the bound ions is followed by the return of the empty carrier. This second pathway constitutes the Na\(^+\)-dependent I\(^-\) transport pathway and has a turnover rate of \(\approx 36\) s\(^{-1}\).

**NIS Intramembrane Particles**—Expression of NIS in oocytes led to a \(\approx 2.5\)-fold increase in the density of intramembrane particles in the P face of the oolemma. This increase in the density of IMPs reflects the insertion of NIS particles into the membrane. Using the total number of transporters in the membrane obtained by freeze-fracture electron microscopy, and \(Q_{\text{max}}\) obtained electrophysiologically from the same oocyte, the valence of NIS moveable charges was estimated to be \(-3\) electronic charges per particle (\(Q_{\text{max}} = N_{\text{NISZe}}\)). This is in contrast with electrophysiological measurements, where a single Boltzmann fit of the \(Q/V\) curves predicts the apparent (effective) valence of the moveable charge of NIS to be 1. However, effective valence as measured electrophysiologically would only be equal to the actual moveable charge if, in response to a voltage jump, all of the moveable charge of NIS moved in one step (30). This is highly unlikely and the discrepancy between the two values is not surprising. A similar result has been reported for SGLT1 and the Shaker K\(^+\) channel (5).

The cloned cDNA for NIS codes for a 618 amino acid protein with a molecular weight of 65 kDa, and expression of NIS in X. laevis oocytes led to the appearance of 9-nm (diameter) particles. In comparison, the particles associated with the Shaker K\(^+\) channel (70 kDa) and the water channel, CHIP28 (28 kDa), in oocytes were 10.7 and 9.3 nm, respectively (5). There is strong evidence that Shaker K\(^+\) channel (31, 32) and CHIP28 (33) form functional homotetramers. Thus, based on these observations and the possible existence of a leucine-zipper motif in NIS (2), it is tempting to suggest that NIS may function as a multimeric protein.

**Conclusion**—From a mechanistic viewpoint, NIS steady-state and presteady-state kinetics are very similar to those of other cotransporters. This points to the possibility that, although the ionic nature of the substrate may vary (neutral, anionic, or cationic), the mechanism by which Na\(^+\)-coupled transporters perform their function remains similar. This is substantiated by the fact that NIS belongs to the SGLT1 gene family and exhibits 25% amino acid identity with SGLT1. Subtle differences do exist and are related to the specific function performed by the transporter. Therefore, it is possible that a common ancestor gene existed which then upon divergence coded for different proteins able to couple various substrates to Na\(^+\) transport while preserving the general mechanistic aspects of the cotransport process (34).

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