Electrogenicity of Na\(^+\)-Coupled Bile Acid Transporters

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The Na\(^+\)-bile acid cotransporters NTCP and ASBT are largely responsible for the Na\(^+\)-dependent bile acid uptake in hepatocytes and intestinal epithelial cells, respectively. This review discusses the experimental methods available for demonstrating electrogenicity and examines the accumulating evidence that coupled transport by each of these bile acid transporters is electrogenic. The evidence includes measurements of transport-associated currents by patch clamp electrophysiological techniques, as well as direct measurement of fluorescent bile acid transport rates in whole cell patch clamped, voltage clamped cells. The results support a Na\(^+\):bile acid coupling stoichiometry of 2:1.

INTRODUCTION

The transport of solute molecules by membrane transport proteins is electrogenic if electric charge is translocated as part of the transport cycle. Electrogenic transport processes generate a transport current and may themselves be influenced by the transmembrane electrical potential. The charge movement provides an additional driving force for transport, may greatly increase the maximal transmembrane gradients that can be achieved, makes transport sensitive to membrane potential and provides an important mechanism for regulation of transport. Kinetic studies such as those performed for glucose and amino acid transporters [1-6] have the potential to determine which steps in the transport mechanism are electrogenic and, in conjunction with structural studies, have the potential to provide direct insight into the conformational changes associated with the transport cycle. This review will discuss the current state of knowledge of the electrogenicity of the Na\(^+\)-coupled bile acid transporters and specifically summarize recent work from our laboratory on bile acid transport in hepatocytes and intestine.

CHARACTERISTICS OF EXPERIMENTAL PREPARATIONS

There are two general categories of systems that can be used as experimental preparations for the study of the function of biological transport proteins. First are systems containing all or part of the transport protein in their native milieu. These can be whole organs or tissues, primary isolated cells, such as hepatocytes and ileal epithelial cells, or they can be membrane fragments from these cells, such as isolated membrane vesicles. The advantage of these systems are that the transporter is in its native membrane. Any

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\(^{b}\) Abbreviations: NTCP, Na\(^+\)-taurocholate cotransport polypeptide; ASBT, apical bile salt transporter; DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid; TMA, tetramethylammonium; FBA, fluorescent bile acid analogue; NBD, nitrobenzoxadiazolyl; CGamF, cholyl glycy l amido fluorescein; C-NBD-L, cholyl-N-\(\varepsilon\)-nitrobenzoxadiazol-lysine.

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accessory proteins that might be necessary for transport are present, and the transporter is inserted correctly. In addition, physiological regulation of the transporter may be present in some of these systems. However, a major disadvantage of native membrane systems is that other transport proteins may be present. If these have overlapping substrate specificity, they may contribute to the parameters being measured and could obscure functional measurements of the protein of interest. In addition, the transporter abundance in native membrane preparations is not easily changed, and transport rates in some cases may be below the threshold required for measurement.

Alternatively, it may be advantageous to study transport in preparations in which transport proteins themselves are heterologously expressed. To date, Xenopus oocytes, insect cells and stably and transiently transfected mammalian cells have proven most useful in this regard. The use of purified transport proteins inserted in artificial lipid bilayers has been important in the study of ion channels, but because of the much lower turnover numbers and the less sensitive transport measurement methods for organic ion transporters, this latter approach has not yet been successful for organic ion cotransport.

The advantage of the heterologous expression system is that it increases the probability that the measured transport results from the protein of interest and provides an opportunity to enhance expression and increase transport rate. The disadvantage is that an unphysiological milieu may be present, and the function of the transport proteins may be altered. Therefore, although heterologous expression of cloned transporters has provided an important opportunity to study transport, it has not obviated the need to study the transport proteins in their native cells as well.

METHODS OF DETERMINING ELECTROGENICITY

Transport-associated currents

Methods to measure electrogenicity of transport can be divided into three general categories. First is the direct measurement of transport-associated currents. This approach involves initiating transport and measuring transmembrane current flow. This classic electrophysiological approach has been successful for determining the electrogenicity of Na+-K+ ATPase [7] and other ion transporting ATPases and has also proven very successful for electrogenic Na+-glucose [8] and Na+-amino acid transporters [9]. This really is what is meant by electrogenicity of transport. When the transporter cycles, it creates an electric current. There are advantages of this approach, particularly the time resolution that current measurement allows. Transport can be measured on a millisecond time scale. The disadvantage of this system is that what is being measured is electrical current before and after the initiation of transport. It is not certain that the current is actually generated by the transporter. This is particularly a problem for the study of the bile acid transporters since transport is generally initiated by the rapid addition of bile acid to the solution. If this stimulus alters the activity of ion channels or other electrical currents that flow across that membrane, the measured currents may not be resulting from the transporter.

When currents through the transporter are relatively large, much larger than other changes in membrane currents induced by the substrate, this method works well. This is the case for Na+-glucose cotransport in intestine, Na+-amino acid cotransport in pancreatic acinar cells and many other systems. However, it is potentially a problem for bile acids since the flux of bile acids into hepatocytes is relatively small compared to these other substrates, and because bile acids are relatively good detergents, may alter membrane fluidity, and have even been postulated to act as ion channels [10]. High bile acid concentrations are quite likely to alter some other ion channels presently in the membrane.
Direct current measurement is, therefore, not the ideal method for studying the electrogenicity of bile acid cotransporters.

**Voltage-dependence of transport**

The second general method for determining whether organic substrate transport is electrogenic is to directly measure the flux of the organic substrate as a function of voltage. Strictly speaking this method determines whether transport is voltage-dependent, not electrogenic (which implies current flow); but in practice, in order for a net transport process to remain sensitive to voltage, it must involve charge translocation in the steady-state. This has been the underlying principle behind many classic studies with membrane vesicles in which ionophore-induced voltage changes have been used. The advantage of this method is that it directly measures substrate transport rate and not current. Therefore interference from other conductances is not important. However, the need to be able to measure and control the imposed voltage gradient is crucial and has been lacking in some studies. Another disadvantage of this general approach is that substrate flux must be measured directly. This can be done either with radioactive fluxes or with fluorescence measurements (see below). The time resolution of these measurements is much slower than that for electric current. One cannot generally examine as many conditions as rapidly as with direct current measurement. For this reason, detailed kinetic modeling has generally involved current measurements. However, this method has advantages if the currents are very low or if substrate activates other ion channels such as may be the case for bile acids.

**Kinetic measurements of stoichiometry**

Finally, there is a third method for inferring electrogenicity of cotransport processes. This is the kinetic method. In this approach, the activation kinetics of each of the cotransported substrates is examined and fitted to models of n independent binding sites. For a cotransport process that involves both anions and cations, activation kinetics that are best described by an unequal n for binding of the two substrates could be explained by a transport cycle that translocates a greater number of one molecule that the other. A 2:1 coupling stoichiometry, for instance, of an anion and a cation would result in electrogenic transport. The problem with this approach is that it does not demonstrate that there is 2:1 transport, only 2:1 binding and thus does not prove that all bound substrates are transported. In addition, the possibility of cooperativity of binding can make the estimates of binding site number from kinetic models uncertain. Although determining kinetic coefficients is helpful and provides important information about the protein, this method by itself cannot prove electrogenicity.

**ELECTROGENICITY OF Na⁺-BILE ACID COTRANSPORT IN HEPATOCYTES**

**Current measurements**

Studies in hepatocytes and hepatocyte-derived membranes have used each of the approaches described. Several studies have looked at membrane depolarization or inward currents associated with taurocholate addition to hepatocytes. These studies have generally shown that depolarization was induced by taurocholate addition. Bear et al. [11, 12] observed depolarizations of 4.4 to 8.4 mV when 20 mM taurocholate was added to hepatocytes. Although this suggests electrogenicity, these depolarizations are much greater than expected for a 2:1 or 3:1 cotransport and either imply a coupling stoichiometry 5-10 or more Na⁺ per taurocholate or the possibility that changes in membrane conductances could be partly responsible for the depolarization. Fitz and Scharschmidt [13] measured rat hepatocyte membrane potential in vivo while taurocholate was injected intravenously.
Figure 1. Taurocholate-induced membrane depolarization in hepatocytes. Each of the three traces is a voltage record from a single hepatocyte impaled with microelectrodes. During the periods indicated by the bars each cell was exposed to taurocholate (100 mM). The upper trace demonstrates the result in control, Na+-Ringers solution. The middle trace was obtained in solution in which all Na+ was replaced with tetramethylammonium. In the lower trace DIDS (0.5 mM) was added to the taurocholate containing solution.

The observed 2 mV depolarization could have resulted from electrogenic transport but limitations of the in vivo model made it impossible to rule out other membrane conductance changes.

In order to optimize the ability to detect taurocholate-induced depolarizations and exclude the possibility that the bile acid was altering membrane conductance, we conducted microelectrode impalements and membrane voltage measurements in hepatocytes after first blocking K+-conductance with quinine [14]. This served both to prevent changes in K+-conductance from influencing the results, and it decreased the background conductance making transport currents easier to measure. Under these conditions (Figure 1), addition of 100 μM taurocholate resulted in a rapid and reversible depolarization of approximately 5 mV. Omitting Na+ from the medium completely blocked that depolarization and the anion transport and Na+-taurocholate cotransport inhibitor, DIDS, also blocked the depolarization. When we examined depolarization as a function of membrane potential we found a potential dependence of the calculated transport current for taurocholate, consistent with electrogenic transport (Figure 2). In the absence of Na+, there was no bile acid associated current. Ion substitution experiments and blockage of K+ conductance with quinine could not provide any evidence that taurocholate was changing other ion conductances. It is unlikely that the depolarization resulted from a non-specific detergent effect of the bile acid since maximum depolarization occurred at 50-100 μM. This is predicted for the known $K_m$ of transport of approximately 15 μM, but would be hard to explain for detergent effects, which become more significant at higher concentrations. Finally, the ability of DIDS to block the Na+-dependent taurocholate-induced depolarization strongly suggests that it results from a transport current and not a bile-acid induced increase in Na+-conductance.

Lidofsky et al. [15] measured taurocholate associated whole cell currents in hepatocytes and were able to observe a Na+-dependent inward current present at holding potentials greater than −50 mV. These currents, approximately 5 pA/cell induced by 100 μM taurocholate were similar in magnitude to those observed by ourselves. Together, these
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30
0
140 mM Na\(^+\)

180°
20
0
0
140 mN Na\(^+\)

20
0
0
0
0
-60 -40 -20
Membrane Voltage (mV)

Transport current (pA/cell)

Figure 2. Voltage-dependence of taurocholate-induced currents. Transport currents associated with taurocholate uptake into hepatocytes were calculated from the measured depolarization and input resistance and plotted as a function of voltage. Open circles were obtained in the presence of 140 mM Na\(^+\). Closed circles were obtained in the absence of Na\(^+\).

Studies demonstrate that taurocholate induces Na\(^+\)-dependent inward current flow and provide strong direct evidence of electrogenicity of the overall process of Na\(^+\)-taurocholate cotransport in primary isolated hepatocytes. The currents measured demonstrate that positive current flows into the cell in association with bile acid uptake. These data are consistent with either a 2:1 or 3:1 Na\(^+\)-bile acid coupling stoichiometry [14].

Voltage-dependence of bile acid uptake

The second approach, imposing a voltage gradient and measuring effects on bile acid transport, has also been applied to hepatocytes. These were among the first studies to examine electrogenicity of Na\(^+\)-bile acid cotransport and were performed once the techniques for isolation of liver sinusoidal membrane vesicles were developed. The protocol is conceptually simple. It involves using valinomycin, a K\(^+\)-selective ionophore, to increase the K\(^+\) permeability of the vesicles. The vesicles are preloaded with a high internal K\(^+\) solution and incubated in a relatively low K\(^+\) external solution. Uptake rates are measured before and after addition of valinomycin. If the vesicle permeability was not already highly K\(^+\) selective, the sudden increase in K\(^+\) permeability should shift intravesicular membrane voltage close to the K\(^+\) equilibrium potential, thus hyperpolarizing the vesicles.

Studies in membrane vesicles preparations, however, have produced inconclusive results. Duffy, Meier, Boyer and colleagues showed that valinomycin did not stimulate Na\(^+\)-dependent uptake of taurocholate into sinusoidal membrane vesicles [16, 17] supporting electroneutrality of transport. However, other studies using similar techniques showed that valinomycin-enhanced K\(^+\)-gradients did increase Na\(^+\)-taurocholate transport [18] and permeant anions stimulate the uptake of taurocholate [19].

The reason for these discrepant results is not entirely clear. One possible explanation is that since the vesicle techniques are not able to directly measure or precisely control the intravesicular voltage, it is not absolutely certain that the imposed maneuvers produced the expected hyperpolarization. For instance, if the vesicles already had a high potassium
selectivity, increasing the potassium conductance with valinomycin may not have made a significant change in vesicle membrane voltage.

To resolve some of these problems, we used whole-cell patch clamp to directly clamp cell voltage and determine if bile acid transport rate is voltage-dependent. This approach took advantage of several of the fluorescent bile acid analogs (FBA) developed by Hofmann, Schteingart and colleagues. These molecules are substrates for both the Na\textsuperscript{+}-coupled uptake transporters such as Ntcp and the canalicular bile acid transporter [20]. They allow transport rate to be measured as the rate of change of cell fluorescence, while voltage is clamped with a patch clamp pipet. We used two different FBA, both derivatives of cholate with either an NBD group or a fluorescein moiety attached to the side chain (Figure 3). Both of these derivatives, C-NBD-L and CGamF are taken up specifically by hepatocytes by a high affinity process with \( K_m \) approximately 4 and 10 \( \mu \)M, respectively. For each molecule, uptake is only partially Na\textsuperscript{+}-dependent, however, suggesting that these FBA may be substrates for transporters other than Ntcp [20].

In order to test whether fluorescent bile acid uptake in hepatocytes is electrogenic, whole-cell patch clamp was performed, and cells were voltage clamped at constant intracellular potential for periods of one to two minutes. At the same time, a photometer-based system was used to measure cell fluorescence [21]. The rate of change of cell fluorescence during the period of each voltage clamp is a measure of FBA transport rate. Figure 4 shows the effects of voltage-clamp on C-NBD-L uptake. When the cell is patched and voltage was held at \(-30\) mV, there was a linear uptake process which persisted for approximately eight minutes. This results from the relatively high degree of intracellular binding of the FBA [22]. However, the uptake rate changed when voltage was clamped at either higher or lower values. Figure 5 shows the voltage dependence of uptake of C-NBD-L in the presence of Na\textsuperscript{+}, and when Na\textsuperscript{+} was replaced by the impermeant cation, tetramethylammonium (TMA\textsuperscript{+}). Only the Na\textsuperscript{+}-dependent fraction of uptake was influenced by voltage. Uptake increased at more negative voltages and Na\textsuperscript{+}-dependent uptake was abolished at more positive voltages. This voltage dependence is similar to that seen in the transport
Figure 4. Effects of voltage changes on C-NBD-L uptake. Records show the uptake of fluorescent bile acid during whole-cell patch clamp of single hepatocytes. In the upper panel (A) cell voltage was clamped constant at -30 mV. In the lower panel (B) voltage was clamped as indicated.

Figure 5. Voltage dependence of C-NBD-L uptake. Uptake rates were measured in the presence and absence of Na+. For each cell, initial uptake was measured at -30 mV and then voltage was changed. Subsequent uptake rates were normalized to the initial uptake rate in Na+ at -30 mV.
current measurements (Figure 2), and suggests that Na⁺-dependent, and electrogenic transport are mediated by the same transport protein, presumably Ntcp.

We repeated this experiment with a second bile acid derivative, CGamF (Figure 3). Although uptake of this fluorescein derivative was Na⁺-dependent, it was not electrogenic [21]. The different results for the two different FBA can be explained by the different net charge at neutral pH of these two compounds. The electrogenic bile acid, C-NBD-L has a net charge of −1. The neutral bile acid, CGamF, has a second titratable hydrogen. Titration studies confirmed that at neutral pH it has a net charge of −2. This fortuitous example of adding a single extra charge converting the Na⁺-dependent uptake from electrogenic to electroneutral is consistent with a 2:1 Na⁺:bile acid stoichiometry.

Kinetics of Na⁺-bile acid cotransport

Finally, kinetic evidence also suggests the involvement of multiple Na⁺ ions in the transport cycle. Studies of the Na⁺-dependence of taurocholate uptake in hepatocytes observed Hill coefficients for Na⁺ of approximately 2-3 [23, 24] and recent studies of taurocholate uptake in oocytes expressing Ntcp show a Hill coefficient for Na⁺ of approximately 2.

Taken together these three lines of evidence provide a compelling case that the coupled Na⁺-bile acid cotransport present in hepatocytes and mediated by Ntcp is electrogenic. Electrogenicity provides several advantages for the function of the transporter. First, membrane potential serves as a driving force for bile acid entry, in addition to the chemical Na⁺-gradient. Since back-leakage of bile acids across the hepatocyte basolateral membrane occurs, the ability to achieve the previously measured steady state 10-fold concentration gradient [22] requires a transporter capable of generating an even larger gradient [25]. The added driving force contributed by electrogenicity makes this possible. In addition, electrogenic transport provides another opportunity for transport regulation by the cell. Under conditions in which the cell membrane is depolarized, bile acid entry will be reduced. This phenomenon has been measured for bile acid transport in hepatocytes [21].

Electrogenicity of Na⁺-bile acid cotransport in intestine

Bile acid uptake from the gut lumen into the ileal intestinal epithelial cell is mediated by a second Na⁺-bile acid cotransport protein located on the apical membrane of the epithelial cell [26]. This same transporter has been identified in kidney and in bile duct epithelium and has been designated the apical bile salt transporter (ASBT) [27]. ASBT and NTCP are closely related proteins. While there is only approximately 35 percent amino acid identity between rat ASBP and Ntcp, there is 65 percent similarity, and the two proteins share an almost identical hydrophathy plot. Both are predicted to have 7 membrane spanning domains, and there are 4 highly conserved regions, all within the membrane spanning domains [26-28]. Although both proteins appear to function similarly, there are important substrate specificity differences with ASBT preferring dihydroxy bile acids and being less tolerant of bulky side-chain substitutions [29].

There have been no direct bile acid-dependent current measurements in intestinal cells or in systems expressing ASBT. Evidence for electrogenicity, therefore, depends on the latter two approaches as described above. There have been several studies in intestine-derived membrane vesicles, which looked at the effects of valinomycin and permeable anions on bile acid uptake rate. Similar to the situation in liver, these studies have been inconclusive, with both electroneutrality [30-32] or electrogenicity [33, 34] demonstrated. Some of the same issues of adequacy of the voltage clamp procedure are raised by these studies as were the case for hepatocytes.
We have recently used the fluorescent bile acid patch clamp approach to measure electrogenericity of transport in a CHO cell line stably expressing the human apical sodium-bile acid transporter. These cells have been shown to specifically express the transport protein and they transport taurocholate and cholate by a high affinity and entirely Na⁺-dependent manner [35]. Fluorescent bile acid transport was then examined by pre-equilibrating cells with CDC-NBD-L and determining voltage-dependent shifts in cell fluorescence. As has been preliminary reported [36], this protocol produced voltage-dependent shifts in cell fluorescence consistent with an electrogenic transport process. Transport behaves as if net bile acid uptake moves positive charge into the cell. Further studies to define the stoichiometry of this process are underway. However, kinetic studies in this cell line by Dawson and colleagues demonstrate a 2:1 Hill coefficient for Na⁺ [35].

CONCLUSION

In conclusion, substantial evidence exists that the Na⁺-bile acid cotransporters present in both liver and intestine perform an electrogenic transport cycle and move 2 Na⁺ for each bile acid. For the liver transporter, the data derive primarily from studies in hepatocytes but recent work in oocytes expressing Ntcp is consistent with this. In the intestine, the primary evidence derives from recent studies of stably transfected mammalian cells expressing the hASBT protein. In this case, transport is also electrogenic, as would be expected from the strong structural similarity of NTCP and ASBT. Electrogenericity of transport increases the maximal gradients of bile acid that can be achieved across the cell membrane and allows regulation of transport to result from changes in membrane voltage.

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