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Sodium-dependent taurocholate uptake by isolated rat hepatocytes occurs through an electrogenic mechanism

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The uptake mechanism for the bile salt, taurocholate, by the liver cell is coupled to sodium but the stoichiometry is controversial. A one-to-one coupling ratio would result in electroneutral transport, whereas cotransport of more than one sodium ion with each taurocholate molecule cause an electrogenic response. To better define the uptake of this bile salt, we measured the effect of taurocholate on the membrane potential and resistance of isolated rat hepatocytes using conventional microelectrode electrophysiology. The addition of 20 μM taurocholate caused transient but significant depolarization accompanied by a significant decrease in membrane resistance. The electrical effect induced by taurocholate mimicked that induced by L-alanine (10 mM), the uptake of which is known to occur through an electrogenic, sodium-coupled mechanism. The sodium dependence of taurocholate-induced depolarization was further confirmed by: (1) replacing Na^+ with choline$^+$, and (2) preincubating cells with ouabain (2 mM) or with the Na^+-ionophore, gramicidin (25 μg/ml); both suppressed the electrogenic response. Further, cholic acid, which inhibits sodium-coupled taurocholate uptake in hepatocytes, inhibited taurocholate evoked depolarization. These results support the hypothesis that sodium-coupled taurocholate uptake by isolated hepatocytes occurs through an electrogenic process which transports more than one Na^+ with each taurocholate molecule.

Introduction

The osmotic gradients generated by the uptake and excretion of bile salts by the liver cell provide the primary driving force for bile formation. The uptake of taurocholate, a conjugated bile salt, occurs predominantly through a sodium-dependent mechanism which is energy dependent and exhibits saturation kinetics [1–7]. Another, sodium-independent carrier mechanism accounts for approximately 20% of the total uptake of the bile salt [1,5,6]. The stoichiometry of the sodium cotransport process has not yet been resolved and remains an issue of considerable controversy. On the basis of some radioisotope flux studies [3,7] an electroneutral uptake process involving the transport of one sodium ion with one taurocholate molecule has been proposed. Conflicting evidence has also been reported which indicates that sodium, taurocholate cotransport may be electrogenic and more than one sodium ion is transported with each taurocholate anion [1,8,9].

In order to resolve the existing controversy and to elucidate the mechanism for taurocholate uptake we measured intracellular potential and electrical resistance in isolated rat hepatocytes. Our

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results show that taurocholate uptake is an electrogenic process causing cellular depolarization with increased membrane conductance. The Na^+ dependence of this electrical event was determined in ion substitution studies and in experiments using the ($Na^+ + K^+$)-ATPase inhibitor, ouabain, and gramicidin, a Na^+ ionophore. The specificity of the taurocholate-stimulated response was also assessed using an inhibitor of Na^+-dependent taurocholate uptake, cholic acid [1]. Furthermore, we compared the electrogenic effect evoked by taurocholate with that induced by L-alanine, the uptake of which is known to occur primarily through an electrogenic, sodium-coupled uptake mechanism [10–12].

Methods and Materials

Isolated hepatocyte suspensions were prepared from the livers of male Wistar rats (150–200 g) using the collagenase perfusion method [13]. Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (30 mg/kg) and the liver perfused with a physiological electrolyte solution at 37 °C containing 150 units/ml collagenase (type IV). The liver was then finely minced in the same collagenase solution for 5 min. At the end of the digestion period, single hepatocytes or hepatocyte couplets were harvested for experimentation. Small aliquots of cells were transferred to a 3 ml chamber on the stage of a Zeiss inverted microscope and viewed at 400 × magnification. The experiments were conducted at 37 °C. The physiological electrolyte solution contained: 140 mM NaCl/4.7 mM KCl/1.13 mM $MgCl_2$/10 mM glucose, 2.5 mM $CaCl_2$/10 mM Hepes. In Na^+ substitution experiments, 140 mM NaCl were replaced with 140 mM choline chloride. The pH of the solution was adjusted to 7.2. Membrane potentials of isolated cells were measured using conventional microelectrodes. Microelectrodes were made by pulling borosilicate glass capillary tubes (1.2 mm outer diameter, WPI, New Haven, CT, U.S.A.) using a Haer ultrafine microelectrode puller. The microelectrodes were filled with 1 M KCl and had tip resistances of approximately 50 MΩ. The electrodes were inserted into appropriate Ag-AgCl-pellet microelectrode holders (WPI) and mounted on a Narishige hydrostatic micromanipulator. The potentials were measured using a Dagan amplifier (Model 8700) and recorded on a Gould chart recorder. A grounded, chloride-coated silver wire was used as a reference electrode. Input resistance was continuously monitored by injecting current pulses (0.2 nA) applied through the recording electrode using a Grass stimulator (Model S48).

Taurocholic acid, cholic acid, alanine and ouabain-containing solutions were prepared using the physiological electrolyte solution previously described. Gramicidin was first dissolved in dimethyl sulphoxide and then subsequently dissolved in the electrolyte solution. These solutions were administered by continuous superfusion of the cell from one of a series of line tubes permitting flow at a rate of approximately 100 μl/min [14]. The mouth of a tube containing a particular solution was manipulated to close proximity to the impaled cell. The addition of the vehicle, dimethyl sulphoxide, did not result in a significant change in membrane potential in five studies.

Materials

Taurocholic and cholic acid were supplied by Calbiochem; CA; gramicidin, collagenase (type IV), ouabain and alanine were purchased from Sigma. St. Louis, MO, U.S.A.

Statistical methods

The results were expressed as mean ± S.E. Data were analyzed using the *t*-test for the significance of the difference between two sample means. Mean values were considered significantly different at a P value of less than 0.05.

Results

The resting membrane potential of isolated rat hepatocytes

A continuous recording of the electrical potential measured in the extracellular solution and following cell penetration is shown in Fig. 1. Hyperpolarizing current pulses were applied through the recording electrode in order to measure input resistance. Penetration of the cell resulted in a sudden negative deflection of the potential measured, indicative of the resting membrane potential. Stable intracellular recordings were obtained within 10 s and the electrotonic potential changes

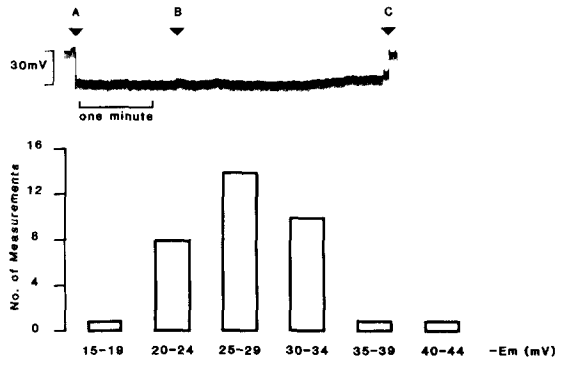

Fig. 1. The resting membrane potential in isolated rat hepatocytes. The upper panel shows a typical intracellular recording from an isolated rat hepatocyte. Hyperpolarizing current pulses have been applied through the recording electrode (0.2 nA) to provide a measure of input resistance. A indicates the point in the recording at which the electrode punctures the cell. The downward deflection in the recording of 25 mV is indicative of the resting membrane potential of the cell. The superfusion of the normal salt solution, identical to that bathing the cells was started at B. No change occurred. The electrode was withdrawn from the cell at C. The lower panel shows the frequency histogram for potentials measured in individual hepatocytes obtained from 35 different liver cell preparations.

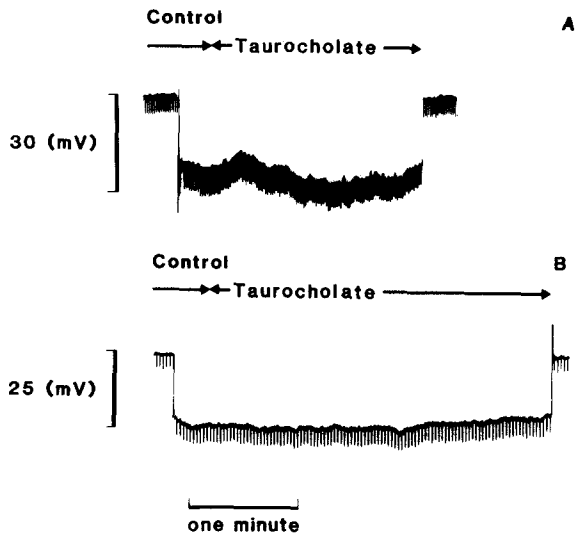

Fig. 2. The electrogenic effect of sodium-dependent taurocholate uptake. A. Superfusion of the normal salt solution containing taurocholate (20 μM) was started 20 s after the impalement of an isolated hepatocyte. Depolarization ensued, then repolarization to below the resting membrane potential. B. Taurocholate (20 μM) was added to cells bathed in Na^+-free solution, containing (140 mM) choline chloride. No change occurred.

assumed a constant amplitude reflecting the membrane resistance. In 35 cell impalements where stable potentials were observed for at least 4 min, the mean membrane potential was 25.9 ± 0.1 mV. The mean input resistance was 83.6 ± 4.5 (MΩ). The frequency histogram of the potentials measured is shown in the lower panel in Fig. 1.

The electrogenic effect of sodium-coupled taurocholate uptake by isolated rat hepatocytes

The addition of sodium taurocholate evoked an electrogenic response in isolated rat hepatocytes; a representative experiment is shown in Fig. 2A. In nine such experiments, a transient, statistically significant ($P < 0.05$), depolarization was observed within 20–30 s with a mean value of 4.4 ± 0.6 mV. Cellular depolarization was accompanied by a slight but significant ($P < 0.05$) decrease in membrane resistance, a mean change of 16.8 ± 1.6 MΩ. In the presence of continuous taurocholate superfusion, the cells repolarized after the initial depolarization event and in five of the nine experiments became hyperpolarized with respect to the resting membrane potential within 1–2 min. The hyperpolarization response had a magnitude of 6.5 ± 1.7 mV and the membrane resistance remained significantly decreased from resting values.

To assess the sodium dependence of the taurocholate-induced electrogenic response, we determined the effect of taurocholate addition to cells bathed in a Na^+-free solution, with choline as the substituting cation. The resting membrane potential and membrane resistance measured in hepatocytes incubated in the Na^+-free solution for approximately five min, i.e., 26.7 ± 2.9 mV and 90.0 ± 4.8 MΩ, respectively, were not significantly different from those measured in normal solutions, containing 140 mM NaCl (140 mM).

The addition of taurocholate (also dissolved in choline chloride) produced no change in membrane potential when the cells were bathed in a Na^+-free solution (Fig. 2B, lower panel). This result was observed in five separate experiments, indicating that the electrogenic response was Na^+ dependent.

L-Alanine uptake by hepatocytes provides a classic example of electrogenic, sodium-coupled solute transport [10,11]. We therefore examined the electrical effect elicited by L-alanine in isolated

hepatocytes and compared this response to that induced by taurocholate. Addition of L-alanine evoked a biphasic electrical response: an immediate depolarization followed by cellular repolarization and hyperpolarization (Fig. 3A). In a total of five such experiments, the initial depolarization had a mean value of 10.0 ± 1.6 mV accompanied by a decrease in membrane resistance of 28.0 ± 3.3 MΩ. The final hyperpolarization of 15.0 ± 4.4 mV below resting values was associated with only a partial recovery in membrane resistance, an increase of 14.8 ± 3.2 MΩ above those values associated with the depolarization event. The changes in membrane potential caused by L-alanine are significantly different from resting values, $P < 0.005$ and $P < 0.01$, respectively. Similarly, membrane resistance measurements associated with membrane depolarization were significantly ($P < 0.002$) less than resting values and significantly greater ($P < 0.01$) during membrane hyperpolarization than during the preceding depolarization ($P < 0.01$). The electrogenic effects produced by L-alanine were clearly due to its' sodium-coupled entry as alanine failed to induce potential changes when hepatocytes were in a Na^+-free solution (Fig. 3B).

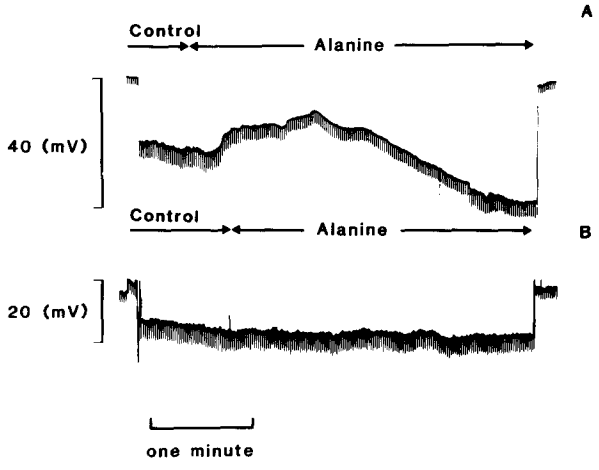

Fig. 3. Electrogenic effect of sodium coupled L-alanine uptake by isolated rat hepatocytes. A. Alanine (10 mM) when added to hepatocytes bathed in a normal electrolyte solution caused prompt depolarization followed about 2 min later by hyperpolarization. With the initial depolarization, membrane resistance decreased. B. Alanine (10 mM) superfusion was started as marked. In this case the cells were bathed in a Na^+-free electrolyte solution and no change occurred.

The effect of ouabain and gramicidin on taurocholate-induced depolarization

Treatment of hepatocytes with ouabain (2 mM) or gramicidin (25 μg/ml) should dissipate the inward transmembrane sodium gradient, ouabain by inhibiting the $(Na^+ + K^+)$-ATPase [2,6,15] and gramicidin through its' action as a Na^+ ionophore [16]. In six experiments, the addition of ouabain alone caused transient cellular depolarization (5.3 ± 1.3 mV) followed by complete repolarization with 1–3 min. The addition of taurocholate, in nine experiments, to cells pretreated with ouabain for 5 min resulted in a 66% reduction in the depolarization produced by taurocholate in control experiments, as shown in Fig. 4. Addition of gramicidin alone resulted in an immediate, transient depolarization of 8.3 ± 2.7 mV, with complete repolarization to resting values within 3 min in six experiments. As in the case of ouabain pretreatment, the addition of taurocholate to cells pretreated with gramicidin for at least 3 min resulted in a 46% reduction in the depolarization produced by taurocholate in control experiments (Fig. 4).

Effect of cholate on taurocholate-induced depolarization

Isotope flux studies have previously shown that

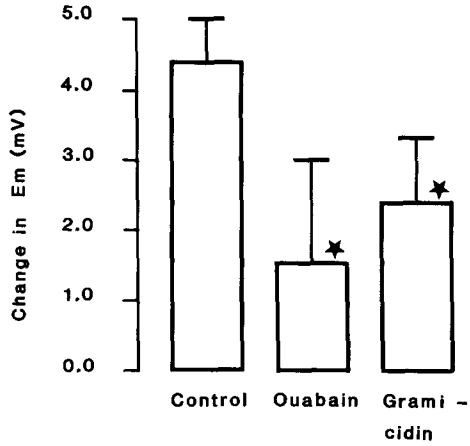

Fig. 4. Effect of ouabain and gramicidin on taurocholate-induced depolarization. This bar graph shows the effect of taurocholate (20 μM) on membrane potential in control cells, cells pretreated with ouabain (2 mM) for 5 min or with gramicidin (25 μg/ml) for at least 3 min. Both inhibitors significantly suppress the electrogenic effect induced by taurocholate (*) ($P < 0.05$).

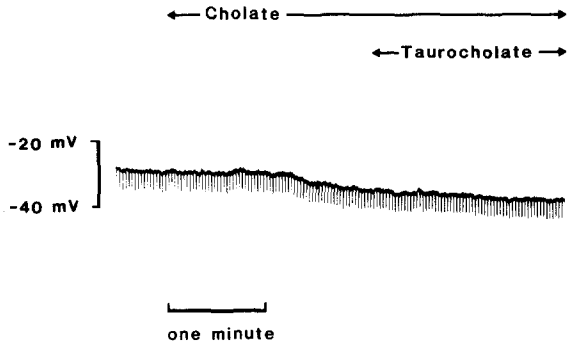

Fig. 5. Effect of cholate on taurocholate induced depolarization. The superfusion of cholate (100 μM) caused cellular hyperpolarization after 1 min. No electrogenic effect was evoked by the subsequent addition of taurocholate (20 μM).

the treatment of hepatocytes with cholic acid inhibits Na^+-dependent taurocholate uptake [1]. Our electrophysiological studies showed that the addition of cholate (100 μM) caused cellular hyperpolarization of 6.7 ± 2.2 mV within 1 min with a slight but significant decrease in membrane resistance of 9.2 ± 4.5 MΩ. Pretreatment of hepatocytes with cholate for 1–1.5 min prevented taurocholate-induced depolarization in the experiment shown in Fig. 5 as well as in five similar experiments.

Discussion

In the present study we determined that the uptake of the bile salt, taurocholate, causes cellular depolarization with a concomitant decrease in membrane resistance. This electrogenic event is due to sodium-dependent taurocholate uptake as it is inhibited by drugs such as ouabain and gramicidin, which act to dissipate the transmembrane sodium gradient and is inhibited by replacement of Na^+ ion with $choline^+$. Furthermore, taurocholate-induced depolarization is inhibited by cholic acid, a known inhibitor of sodium-dependent taurocholate uptake [1]. The electrogenic effect evoked by taurocholate suggests that the stoichiometry for the Na^+-coupled taurocholate uptake mechanism is not one-to-one, but rather more than one sodium ion is transported with each taurocholate molecule.

Measurements of resting membrane potential and membrane resistance obtained in this study, i.e. 26 mV and 84 MΩ, respectively, are close to other reports for isolated liver cell preparations [17]. The membrane potentials measured in the in vivo preparations are greater, close to -35 mV, possibly reflecting the hyperpolarizing effect of neurotransmitters and hormones which are present in the intact animal [18,19].

The electrogenic response produced by taurocholate in isolated hepatocytes is biphasic. The addition of taurocholate causes an immediate and transient depolarization, followed by repolarization and, finally, the cell become slightly hyperpolarized with respect to the resting membrane potential. Taurocholate-stimulated depolarization was also observed by Fitz and Scharschmidt [9] using microelectrodes in an in vivo preparation. In contrast to the present investigation, no secondary hyperpolarization phase was observed. Furthermore, Fitz and Scharschmidt's study did not characterize the electrogenic response with respect to membrane conductance changes or ionic requirements. In our study, the conductance changes which accompany taurocholate-induced depolarization were quantitated and the sodium dependence of the response established.

Sodium dependency of the taurocholate-evoked electrogenic response was determined by replacing Na^+ with choline in the bathing solution. Na^+ replacement with choline has previously been used to inhibit Na^+-dependent taurocholate uptake in isolated rat hepatocytes [1,5,6]. The membrane potential and input resistance measured in cells incubated in Na^+-free solution for 5 min were not significantly different from those measured in cells bathed with the normal salt solution. This observation is consistent with the reports by Graf and Petersen [18] that replacement of Na^+ by choline resulted in a transient hyperpolarization of 3 mV followed by a gradual depolarization which did not significantly change the resting cell membrane potential even 15 min after ion replacement. Gramicidin and ouabain were chosen to further investigate the sodium dependency of the taurocholate-evoked electrogenic response. Gramicidin pretreatment suppressed the taurocholate-activated depolarization suggesting, therefore, that taurocholate-stimulated depolarization is dependent upon the normal inwardly directed sodium

gradient. However, this interpretation should be considered with caution, since gramicidin produces significant changes in membrane resistance, an effect which may account for inhibition of the taurocholate response. Ouabain produced cellular depolarization within 2–3 min in our studies, a result consistent with it's role as an inhibitor of the $(Na^+ + K^+)$-ATPase. The magnitude of the depolarization was small, 5 mV, but comparable to values reported by Williams et al. [21] in in vivo experiments. It appears that in liver cells as well as in other epithelial cells, the ouabain-sensitive $(Na^+ + K^+)$-ATPase contributes relatively little to the resting membrane potential. As in the case of gramicidin, pretreatment of hepatocytes with ouabain significantly inhibited taurocholate-induced depolarization. This result is consistent with the observations reported by Van Dyke et al. [6] in which ouabain (1.0 mM) inhibited taurocholate uptake by 60%.

The specificity of the electrogenic response for sodium-coupled taurocholate uptake was further substantiated when cholate pretreatment inhibited taurocholate-evoked depolarization. Cholic acid, as shown by Inoue et al. [1] inhibits Na^+-dependent taurocholate uptake, possibly through a competitive mechanism. Recently, Blitzer et al. [22] examined cholate uptake in basolateral liver plasma membranes. Although cholate uptake by vesicles was most profoundly enhanced by an outwardly directly hydroxyl gradient, uptake was stimulated to 140% of control values in the presence of an inside-positive electrical potential. Our present study supports the role for an electrogenic component of cholate uptake, as the addition caused cellular hyperpolarization with a decrease in membrane resistance.

The electrogenic response evoked by taurocholate is similar to that stimulated by L-alanine uptake. The mechanisms contributing to the electrogenic response induced by L-alanine have been well investigated in the liver [10,11] and in other tissues such as the pancreas [12] and intestine [20]. The initial depolarization which occurs following alanine addition is due to sodium-driven alanine uptake. As alanine is a neutral molecule, depolarization can be explained with a one-to-one coupling ratio for the sodium cotransport mechanism. This symmetric coupling ratio was confirmed recently in the pancreas using the method of patch-clamp electrophysiology [12]. On the other hand, a one-to-one coupling ratio cannot be used to describe the Na^+ cotransporter for taurocholate. The depolarization which occurs following taurocholate addition must be due to the net transport of more than one sodium ion with each taurocholate anion. The exact stoichiometry of the cotransporter may await the application of novel techniques such as patch-clamp electrophysiology which permit the exact quantitation of the electrical currents associated with the operation of the cotransporter.

Alanine-induced depolarization is followed by repolarization and eventual hyperpolarization to values more negative than the resting membrane potential. Cellular hyperpolarization is due to increased activity of the $(Na^+ + K^+)$-ATPase and increased K^+ conductance [11]. Alanine-activated K^+ channel opening has been recently observed using patch-clamp electrophysiology in the rat liver cell (Bear and Petersen [23]). Such mechanisms may be involved in the repolarization phase observed with taurocholate.

In conclusion, the results of this study indicate that sodium-dependent taurocholate uptake occurs through a rheogenic process. The role for such an electrical effect in the regulation of biliary water and electrolyte secretion remains uncertain. Perhaps the sodium ions cotransported with taurocholate provide a further osmotic gradient for the transport of water, thereby potentiating bile formation.

Acknowledgements

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