Biochemical localization of hepatic surface-membrane Na\(^+\),K\(^+\)-ATPase activity depends on membrane lipid fluidity
(Na\(^+\)/K\(^+\)-transporting ATPase/sinusoidal membrane/bile canalicular membrane)

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ABSTRACT Membrane proteins of transporting epithelia are often distributed between apical and basolateral surfaces to produce a functionally polarized cell. The distribution of Na\(^+\),K\(^+\)-ATPase [ATP phosphohydrolase (Na\(^+\)/K\(^+\)-transporting), EC 3.6.1.37] between apical and basolateral membranes of hepatocytes has been controversial. Because Na\(^+\),K\(^+\)-ATPase activity is fluidity dependent and the physiochemical properties of the apical membrane reduces its fluidity, we investigated whether altering membrane fluidity might uncover cryptic Na\(^+\),K\(^+\)-ATPase in bile canalicular (apical) surface fractions free of detectable Na\(^+\),K\(^+\)-ATPase and glucagon-stimulated adenylate cyclase activities. Apical membranes exhibited higher diphenylethriene-fluorescence polarization values when compared with sinusoidal (basolateral) membrane fractions. When 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate (A2C) was added to each fraction, Na\(^+\),K\(^+\)-ATPase, but not glucagon-stimulated adenylate cyclase activity, was activated in the apical fraction. In contrast, further activation of both enzymes was not seen in sinusoidal fractions. The A2C-induced increase in apical Na\(^+\),K\(^+\)-ATPase approached 75% of the sinusoidal level. Parallel increases in apical Na\(^+\),K\(^+\)-ATPase were produced by benzyl alcohol and Triton WR-1339. All three fluidizing agents decreased the order component of membrane fluidity. Na\(^+\),K\(^+\)-ATPase activity in each subfraction was identically inhibited by the monoclonal antibody 9-A5, a specific inhibitor of this enzyme. These findings suggest that hepatic Na\(^+\),K\(^+\)-ATPase is distributed in both surface membranes but functions more efficiently and, perhaps, specifically in the sinusoidal membranes because of their higher bulk lipid fluidity.

Membrane proteins that polarize function across cell surfaces of transporting epithelia are generally distributed asymmetrically at the cellular apical or basolateral pole (1–3). For example, leucine aminopeptidase is present on the apical membranes of enterocytes, proximal renal tubules, and hepatocytes (3–8), whereas IgA receptor (secretory component) is found in basolateral surfaces of most epithelial cells (9). On the other hand, Na\(^+\),K\(^+\)-transporting ATPase [ATP phosphohydrolase (Na\(^+\)/K\(^+\)-transporting); EC 3.6.1.37] is generally believed to be localized to the basolateral surface of renal (10), intestinal (11), and cultured MDCK (kidney) cells (12, 13) but on the apical surface of the choroid plexus (14) and possibly on both poles of the exobital and parotid gland cells (15–17).

The localization of Na\(^+\),K\(^+\)-ATPase in hepatocytes has been controversial (18). With histochemical, biochemical, and cell-fractionation techniques, Na\(^+\),K\(^+\)-ATPase activity has been seen in the basolateral membrane (19–22), whereas immunocytochemistry using a Na\(^+\),K\(^+\)-ATPase-specific antibody has localized Na\(^+\)/K\(^+\)-pump sites to the bile canalicular (or apical) surface, as well as the sinusoidal (or basolateral) surface (23, 24). Contradictory results also have been reported with these techniques (25–27).

Activities of many intrinsic membrane enzymes depend on the composition of lipids surrounding them and the general physical state of these membrane lipids (28–30). For example, Na\(^+\),K\(^+\)-ATPase activity has been directly related to membrane fluidity (31–33). Because differences in the lipid and biophysical properties of hepatic apical and basolateral surfaces result in a less-fluid apical surface, we wondered whether the biochemical localization of Na\(^+\),K\(^+\)-ATPase activity in sinusoidal surfaces might be due to the relatively decreased fluidity of bile canalicular membranes (BCMs). The results of our investigation indicate that Na\(^+\),K\(^+\)-pump \(\alpha\) subunits are present in BCM fractions in a cryptic form and that they become functionally active when canalicular membrane lipid fluidity is elevated in vitro to levels seen for the sinusoidal surface.

MATERIALS AND METHODS

Reagents. All inorganic chemicals or solvents were purchased from Fisher and were the highest grade available. 2-(2-Methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate (A2C) was obtained from Sigma and Triton WR-1339 (oxyethylated tertiary octylphenol-polyethylene polymer) was obtained from Ragu (Irvington, NJ).

Plasma Membrane Isolation. Male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 180–220 g were used after overnight fasting. Liver plasma membrane (LPM) subfractions were prepared as described (34). Sinusoidal membrane (SM) and BCM fractions were isolated concomitantly from rat-liver homogenates by sucrose density centrifugation and Mg\(^2+\) precipitation (15 mM), respectively.

Enzyme Studies. Na\(^+\),K\(^+\)-ATPase and Mg\(^2+\)-ATPase were measured after overnight freeze-thawing, by means of an enzyme-coupled kinetic assay with pyruvate kinase and lactate dehydrogenase, assuming ouabain (2.5 mM) inhibition measures the Na\(^+\)/K\(^+\) pump (35). Other enzymes were assayed according to standard procedures: alkaline phosphatase (36), leucine aminopeptidase (37), ouabain-sensitive K\(^+\)-dependent p-nitrophenyl phosphatase (38), and adenylic cyclase (39, 40). Protein was measured using bovine serum albumin (Sigma) as standard (41).

Fluorescence Polarization Studies. Fluorescence polarization (P), angle of hindrance (\(\phi\)), and time of fluorescence (\(\tau\)) measurements were done on a model 4800 polarization spectrophotometer (SLM Industries, Urbana, IL) with fixed angles of hindrance.

Abbreviations: A2C, A2C membrane mobility agent, 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate; BCM, bile canalicular membrane; SM, sinusoidal membrane; LPM, liver plasma membrane; DPH, 1,6-diphenyl-1,3,5-hexatriene; \(P\), polarization; \(\tau\), time of fluorescence; mAb, monoclonal antibody.

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emission and excitation filters as described (34). Fluorescence intensity was measured perpendicularly and parallel to the polarization phase of the excitation light [excitation wavelength, 360 nm; KV 389 emission filters (Schott, Durylea, PA)]. Measurements were made at 37°C. 1-6-Diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Junction City, OR) was dissolved in tetrahydrofuran (0.3 µg of DPH/ml) and added to LPM subfractions (72 µg of protein) directly before addition of either A2C (in dimethyl sulfoxide), benzyl alcohol, or Triton WR-1339 in a final total volume of 1.3 ml of 1 mM NaHCO3. Concentration of each test chemical is given in Results. Samples were mixed for no longer than 15 min, and measurements were done in triplicate.

Statistical Analysis. All results are expressed as mean ± SE. A two-tailed Student’s t test was used to compare differences between mean values. Linear regression was calculated by regression analysis.

RESULTS

Relative enrichment of putative plasma membrane marker enzymes for LPM subfractions is shown in Fig. 1. Na+,K+-ATPase and glucagon-stimulated adenylate cyclase activities were enriched (43 ± 2)-fold and (17 ± 3)-fold, respectively, in SM fractions. In contrast, these enzyme activities were not detected in canicular fractions. The BCM enzyme markers leucine aminopeptidase and Mg2+-ATPase were increased (34 ± 4) and (46 ± 5)-fold, respectively. Leucine aminopeptidase and Mg2+-ATPase were modestly increased (8- and 9-fold, respectively) in the SM fraction. Marker enzymes of intracellular organelles were enriched 2-fold or less in both fractions (data not shown). Total recovery of Na+,K+-ATPase was 85 ± 5% with 32 ± 5% accounted for in the SM fraction. Thus, BCM fractions prepared under these conditions were devoid of detectable Na+,K+-ATPase activity and another predominantly SM enzyme marker—adenylate cyclase (42-44).

Fluorescence-polarization values were significantly higher for BCM fractions than for SM preparations (Table 1). Fluidity measured with DPH is determined largely by a structural component (28). Although τ3 for BCM fractions was longer than τ3 for SM fractions, r∞ was significantly increased, indicating that the BCM fraction was significantly less fluid than the SM fraction.

Fluidizing agents are known to expand the packing order of membrane lipids and thus increase the motional freedom of the phospholipid fatty acid acyl chains (45). Fig. 2 shows the effect of additions of the synthetic ester A2C (46) on P values for BCM and SM fractions. A2C increased membrane fluidity in a linear fashion in both LPM subfractions. Addition of 7.7 µM A2C to BCM fractions reduced DPH P values to those measured in the basal state of the SM fraction (Table 1). Although time-of-fluorescence values in these studies were minimally but significantly reduced, r∞ measurements showed that the BCM is less ordered after treatment with A2C. Benzyl alcohol and A2C similarly decreased τ3 and r∞ in BCM fractions. Measurements of the effect of Triton WR-1339 on membrane fluidity could not be made because the detergent markedly quenched fluorescence. However, previous electron spin resonance studies with LPM fractions have shown that Triton WR-1339 also decreased order parameter (47).

Fig. 3 shows the effect of in vitro addition of A2C on Na+,K+-ATPase activity in SM and BCM fractions. As BCM fractions were made more fluid by A2C, Na+,K+-ATPase activity increased progressively, reaching a plateau at 3.3 µM, corresponding to a P value of 0.276 ± 0.003. In contrast, Na+,K+-ATPase activity in SM remained stable after the addition of A2C over the same concentration range. Similar effects of A2C on Na+,K+-ATPase activity were also seen when this same enzyme was measured with the phosphate-release assay (47) (data not shown).

To determine whether this increase in Na+,K+-ATPase activity in the BCM fraction was related to the ability of A2C

<table>
<thead>
<tr>
<th>Membrane fractions (n)</th>
<th>τ3, nsec</th>
<th>P</th>
<th>r∞, °</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM (6)</td>
<td>8.5 ± 0.2</td>
<td>0.254 ± 0.006</td>
<td>0.162 ± 0.003</td>
</tr>
<tr>
<td>BCM (7)</td>
<td>9.8 ± 0.1*</td>
<td>0.303 ± 0.0002*</td>
<td>0.210 ± 0.002*</td>
</tr>
<tr>
<td>+ 7.7 µM A2C (3)</td>
<td>9.1 ± 0.1†</td>
<td>0.258 ± 0.001†</td>
<td>0.172 ± 0.001†</td>
</tr>
<tr>
<td>+ 95 mM benzyl alcohol (4)</td>
<td>9.0 ± 0.1†</td>
<td>0.231 ± 0.003†</td>
<td>0.143 ± 0.002†</td>
</tr>
</tbody>
</table>

Parentheses indicate number of separate determinations. Fluidity parameters were determined at 37°C using DPH as the probe. The data are means ± SE.

*Probability < 0.001 compared with SM.
†Probability < 0.001 compared with BCM.
to increase lipid fluidity or rather to nonspecific activation, other fluidizing agents were tested. At their optimal concentrations, benzyl alcohol (95 mM) activated Na⁺,K⁺-ATPase to 69 ± 7% and Triton WR-1339 (0.015%) to 77 ± 6% of SM enzyme activity. Neither one of these agents increased Na⁺,K⁺-ATPase activity in the SM fraction. Taken together, these results indicated that activation of cryptic Na⁺,K⁺-ATPase activity in BCM fractions was independent of the structure of fluidizing agents and acted somewhat specifically on the BCM fraction.

To investigate whether these increases in BCM Na⁺,K⁺-ATPase activity induced by fluidizing agents might have been due partly to detergent-like increases in the accessibility of substrate, the effects of A2C were compared to the addition of 0.1% Triton X-100 on the activities of Na⁺,K⁺-ATPase, Mg²⁺-ATPase, ouabain-sensitive K⁺-stimulated p-nitrophenyl phosphatase, leucine aminopeptidase, and alkaline phosphatase (Table 2). The addition of A2C (6 μM) to BCM fractions selectively increased Na⁺,K⁺-ATPase and ouabain-sensitive K⁺-p-nitrophenyl phosphatase, whereas other enzyme activities were unaltered. In contrast, Triton X-100 (0.1%) did not change either Na⁺,K⁺-ATPase or ouabain-sensitive K⁺-p-nitrophenyl phosphatase, but Mg²⁺-ATPase was decreased.

Hormone-stimulatable adenylate cyclase activity is also influenced by the physical state of membrane lipids (48). Hepatic glucagon receptors are linked functionally to the catalytic unit through membrane-associated GTP-binding proteins (49); therefore, the effects of GTP and glucagon were also studied. The effects of A2C on adenylate cyclase activity are presented in Table 3. In SM fractions, basal adenylate cyclase activity was increased 2.5-fold by glucagon alone and 15-fold by glucagon plus GTP. This response was unaltered by A2C (6 μM) in the SM fraction. In contrast, in both untreated and A2C-fluidized BCM fractions, neither glucagon nor GTP-stimulated adenylate cyclase activities were measurable to significant levels. In additional experiments, forskolin (10⁻⁴ M) failed to activate the trace levels of catalytic subunit in BCM subfractions, even after addition of 6 μM A2C (data not shown).

A monoclonal antibody (mAb) (9-A5), shown previously to inhibit the α subunit of rat hepatic and renal Na⁺,K⁺-ATPase, was used to examine whether BCM Na⁺,K⁺-ATPase activity was immunochemically identical to the Na⁺,K⁺-ATPase activity of α subunits seen in SM fractions (23, 50). In both SM and BCM fractions treated with A2C, mAb 9-A5 inhibited Na⁺,K⁺-ATPase activity similarly (Fig. 4). mAb 9-A5 had no effects on Mg²⁺-ATPase activity (data not shown). The ID₅₀ values for SM and BCM Na⁺,K⁺-ATPase inhibition were 90 ± 7 and 93 ± 7 ng of mAb 9-A5 per μg of membrane protein, respectively.

**DISCUSSION**

The purpose of this study was twofold. First, to find whether differences in membrane lipid fluidity regulate, in part, Na⁺,K⁺-ATPase activity. Because the apical domain of epithelial cells is well known to be less fluid than the basolateral surfaces (28), we proposed that the decreased BCM lipid fluidity masks a cryptic Na⁺,K⁺-ATPase catalytic activity. Our second aim was to resolve the controversy regarding the polarized distribution of the Na⁺,K⁺ pumps in hepatocytes. This report presents biochemical and immunochemical evidence that the catalytic subunits of Na⁺,K⁺-ATPase are present in bile canaliculus as well as in sinusoidal liver plasma membrane subfractions. These observations indicate that previous controversial findings in situ showing immunologically reactive Na⁺,K⁺-ATPase α subunits as well as enzyme cytochemistry showing no Na⁺,K⁺-ATPase catalytic activity in BCM are both correct.

![Figure 2](image2.png)

**Fig. 2.** Effect of A2C on SM and BCM fluidity. DPH-fluorescence polarization was measured at 37°C as described. Linear regression curves were as follows: BCM (●) (n = 5), y = −0.005x + 0.296 and r = 0.994°, SM (○) (n = 5), y = −0.005x + 0.236 and r = 0.969°.

![Figure 3](image3.png)

**Fig. 3.** Effect of A2C on Na⁺,K⁺-ATPase activity in LPM subfractions. SM (○) and BCM (●) fractions were isolated as described. Na⁺,K⁺-ATPase activity was measured at 37°C 5 min after addition of A2C to LPM subfractions (67 μg of protein/ml) with vigorous mixing.

**Table 2. Effect of agents on enzyme-specific activities in bile canaliculus membrane fractions**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>BCM enzyme-specific activitya</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>109.2</td>
</tr>
<tr>
<td>Ouabain-sensitive K⁺-p-nitrophenyl phosphatase</td>
<td>0</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>95.5</td>
</tr>
<tr>
<td>Alkaline phosphatase†</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Values are reported as the means (n = 3–6); measurement errors differed by <10%.

aEnzyme activities reported as μmol of substrate hydrolyzed/hr per mg of protein.

†Measured routinely with 0.1% Triton.
Table 3. Adenylate cyclase-specific activity in LPM subfractions

<table>
<thead>
<tr>
<th>Hormone/nucleotide</th>
<th>SM</th>
<th>BCM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-A_2C)</td>
<td>(+A_2C)</td>
</tr>
<tr>
<td>None</td>
<td>9.2 ± 0.4</td>
<td>8.6 ± 0.9</td>
</tr>
<tr>
<td>Glucagon</td>
<td>23.4 ± 3.6</td>
<td>19.8 ± 1.8</td>
</tr>
<tr>
<td>Glucagon + GTP</td>
<td>126.6 ± 7.4</td>
<td>119.4 ± 7.2</td>
</tr>
</tbody>
</table>

n, Three separate determinations presented as mean ± SE. Glucagon (10^-6 M) and GTP (10^-5 M) were added to separate assays.

Three independent lines of evidence suggest Na\(^{+}\),K\(^{+}\)-ATPase is located on the bile canalicular domain: (i) increasing BCM fluidity by different agents activates Na\(^{+}\),K\(^{+}\)-ATPase; (ii) increases in fluidity in these membranes selectively activates Na\(^{+}\),K\(^{+}\)-ATPase and in parallel ouabain-sensitive K\(^{-}\)-p-nitrophosphatase activity; and (iii) monospecific anti-\(\alpha\) subunit mAb 9-A5 inhibits BCM and SM Na\(^{+}\),K\(^{+}\)-ATPase activity with similar kinetics.

Our preparations of BCM were apparently devoid of contaminating SM vesicles, which might be attributed to the isolation of these BCM fractions by Mg\(^{2+}\)-precipitation after vigorous homogenization (34). Such preparations yield a BCM fraction devoid of detectable Na\(^{+}\),K\(^{+}\)-ATPase and adenylate cyclase (either glucagon- or GTP-stimulated) activity but are still highly enriched in enzyme markers located at the biliary pole (21). In addition, fluorescence \(p\) measurements on BCM fractions indicate that their lipid structure behaves rigidly, unlike the more fluid SM fraction (28).

Addition of A\(_2\)C, benzyl alcohol, and Triton WR-1339 each selectively increased Na\(^{+}\),K\(^{+}\)-ATPase activity in the BCM. All these agents are structurally different; yet, their effects upon the BCM fractions were similar. It would appear, therefore, that the decreased membrane fluidity of untreated BCM fractions normally does mask a cryptic Na\(^{+}\),K\(^{+}\)-ATPase activity. A cryptic Na\(^{+}\),K\(^{+}\)-ATPase, by this criterion, seems absent from SM fractions, because when SM fluidity increases beyond its apparent physiological level, no stimulation of Na\(^{+}\),K\(^{+}\)-ATPase occurred. Previous work also demonstrated that benzyl alcohol increased Na\(^{+}\),K\(^{+}\)-ATPase activity and decreased order parameter (51), but interpretation of these results was complicated because of the use of mixed-LPM fractions.

Previous studies from our laboratory have shown that detergents increase Na\(^{+}\),K\(^{+}\)-ATPase activity in freshly analyzed preparations, but do not increase this activity after freeze-thawing (52). In experiments reported here, addition of 0.1% Triton X-100 to both membrane fractions after freeze-thawing did not further increase Na\(^{+}\),K\(^{+}\)-ATPase activity. Moreover, ouabain-sensitive K\(^{-}\)-p-nitrophosphatase, which is closely associated with Na\(^{+}\),K\(^{+}\)-ATPase and reflects an externally oriented enzymatic activity (53), showed similarly increased ouabain-sensitive K\(^{-}\)-p-nitrophosphatase activity with A\(_2\)C but not Triton X-100 in BCM fractions. Such lipid fluidity regulation may be somewhat specific for Na\(^{+}\),K\(^{+}\)-ATPase, as leucine aminopeptidase and Mg\(^{2+}\)-ATPase, BCM ectoenzymes, were not altered by A\(_2\)C (54).

An alternative interpretation of these findings is that Na\(^{+}\),K\(^{+}\)-ATPase undergoes lateral movement from its SM location to the BCM during membrane isolation (5, 55). To investigate this possibility, basal and stimulated adenylate cyclase activities were measured in LPM subfractions. No functional components (glucagon receptors, catalytic subunits, or G proteins) of this complex were identified in the BCM fraction. Thus, glucagon-stimulated adenylate cyclase was localized to the SM fraction, a finding indirectly supporting the conclusion that SM proteins do not redistribute during the isolation procedure.

A recent study, however, failed to identify Na\(^{+}\),K\(^{+}\)-ATPase in BCM by either immunocytochemistry or immunoblotting (27); the reasons for the negative results are unclear. With regard to the immunocytochemical findings (27), it is likely that the positive epitopes were limited by the stability, conformational state, or accessibility of their anti-bodies to Na\(^{+}\),K\(^{+}\)-ATPase (18, 23, 50). Failure to demonstrate Na\(^{+}\),K\(^{+}\)-ATPase catalytic subunits in the BCM fraction by immunoblotting may be due to isolation of BCM fractions in low yield (21). Because apical enzymes may be localized in microdomains (56, 57), these workers could have selectively discarded the BCM fraction containing Na\(^{+}\),K\(^{+}\)-ATPase. In addition to Na\(^{+}\),K\(^{+}\)-ATPase their study also failed to identify secretory component, a receptor that has been shown to reside, at least transiently, in the BCM before specific proteolysis (58).

Additional experimental findings in the present work also demonstrated similar inhibition curves for Na\(^{+}\),K\(^{+}\)-ATPase activities in A\(_2\)C-activated BCM and SM fractions using mAbs against the Na\(^{+}\)/K\(^{+}\) pump. Thus, immunologically identical protein epitopes are present on both surfaces of LPM subfractions, strongly suggesting that the Na\(^{+}\),K\(^{+}\)-ATPase activities in each fraction are probably due, at least in part, to similar \(\alpha\)-subunit isoforms.

These studies demonstrate the functional importance of membrane lipid fluidity in governing hepatic Na\(^{+}\),K\(^{+}\)-ATPase activity. Cryptic Na\(^{+}\),K\(^{+}\)-ATPase activity in the canalicular membrane domain raises the possibility that hepatic Na\(^{+}\)/K\(^{+}\) pump activity is activated without any net increase in number of pump sites—i.e., by increasing plasma membrane fluidity. Indeed, we recently demonstrated this change with bile duct obstruction (59) and, in addition, hepatic regeneration is associated with increased Na\(^{+}\),K\(^{+}\)-ATPase activity, bile flow, and plasma membrane fluidity without any increase in the number of enzyme units (60–62).

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