Lack of Function of an N-Ethylmaleimide-Sensitive Thiol Protein in Erythrocyte Membrane of Autosomal Dominant Polycystic Kidney Disease

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Abstract. The polycystic kidney disease 1 (PKD1) gene product polycystin has been predicted to be an integral membrane protein involved in cell–cell and cell–matrix interactions. The erythrocyte membrane fluidity in autosomal dominant polycystic kidney disease (ADPKD) patients is increased, and this may be due to a membrane cytoskeletal abnormality. The abnormal erythrocyte sodium–lithium countertransport kinetics in ADPKD are related to an altered thiol protein in the cytoskeleton. The possibility that a similar thiol protein abnormality causes the increased erythrocyte membrane fluidity in ADPKD was investigated. The membrane fluidity of intact erythrocytes from 12 ADPKD patients and 12 healthy control subjects was assessed from the fluorescence anisotropies of 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenyl-hexatriene (TMA-DPH). The effect on membrane fluidity of N-ethylmaleimide (NEM), cytochalasin D, heating at 48°C for 20 min, or more specifically, liposomes containing antibodies to actin or ankyrin, was determined. In erythrocytes from healthy control subjects, the fluorescence anisotropy of DPH (mean ± SEM: 0.223 ± 0.001) was decreased after treatment with NEM (0.200 ± 0.003, P < 0.001), cytochalasin D (0.206 ± 0.006, P < 0.001), heating (0.199 ± 0.002, P < 0.001), and antibodies to actin (0.194 ± 0.002, P < 0.001) or ankyrin (0.196 ± 0.002, P < 0.001). The TMA-DPH anisotropy (0.279 ± 0.001) was also decreased after treatment with NEM (0.264 ± 0.001, P < 0.001), cytochalasin D (0.264 ± 0.001, P < 0.001), heating (0.265 ± 0.001, P < 0.001), and antibodies to actin (0.262 ± 0.002, P < 0.001) or ankyrin (0.262 ± 0.002, P < 0.001). NEM had no additional effect on the other treatments, suggesting that its target thiol protein was associated with the cytoskeleton. In untreated erythrocytes from ADPKD patients, fluorescence anisotropies of both DPH and TMA-DPH were reduced, and none of the treatments altered the anisotropy of either DPH or TMA-DPH. In ADPKD, a cytoskeletal thiol protein is abnormal and possibly explains abnormal lipid bilayer properties and transport protein function in erythrocytes in this disease. (J Am Soc Nephrol 9: 1–8, 1998)

Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary disease affecting approximately 1 in 1000 and accounts for approximately 10% of end-stage renal failure patients who are on renal replacement therapy (1). It is now clear that ADPKD is a systemic disease, and the phenotypic expression of ADPKD is characterized by enormous cystic enlargement of renal tubules with involvement of other extrarenal organs (2). Genetic heterogeneity has now been proposed for ADPKD. The first gene, PKD1, is located on chromosome 16p13.3 (3) and accounts for the majority of ADPKD patients worldwide. The second gene, PKD2, is on chromosome 4q13 through q23 (4), and recently there is evidence of a third gene, PKD3 (5), for which the gene locus still has to be mapped.

Although the pathogenesis of ADPKD is still unclear, there is evidence suggesting that abnormalities of the functions and properties of cell membranes and extracellular matrix are involved (6–9). From structure predictions it has been suggested that the PKD1 gene product polycystin is an integral membrane protein involved in cell–cell or cell–matrix interactions, which are important for normal basement membrane production (10–14). Polycystin has been shown to be expressed in several tissues and might play a major role in the maintenance of renal epithelial differentiation and organization of epithelial cells from fetal life (15). Recently, it has been postulated that one function of polycystin is to suppress renal epithelial cell growth (16), and loss of function of polycystin might result in hyperplasia of epithelial cells. The PKD2 gene protein has also been predicted to encode for an integral membrane protein that resembles polycystin, calcium, and sodium channels (17). It has been postulated that the PKD1 gene might act as the regulator of the PKD2 channel activity, and this raises the
possibility that the phenotype of ADPKD may be due partly to a defect of a transport system, which is at present unknown.

Erythrocyte sodium–lithium countertransport (Na/Li CT) is a sensitive membrane marker, and its kinetics are strongly associated with hypertensive-related disease, such as familial essential hypertension, diabetic nephropathy, and IgA nephropathy (18). We have previously found that the kinetics of Na/Li CT are abnormal in both normotensive and hypertensive ADPKD patients (19). Na/Li CT is sensitive to several aspects of membrane organization, and its kinetics are modified by two types of thiold groups in the membrane (20). We have found that in healthy control subjects the Michaelis constant (K_m) of Na/Li CT for external sodium (K_m(So)) after treatment with N-ethylmaleimide (NEM) or heating at 48°C for 20 min was significantly lower than before these treatments (19). This effect of lowering K_m(So) after treatment of erythrocytes with NEM or after heating was not found in ADPKD patients. In untreated intact erythrocytes from ADPKD patients, the K_m(So) of Na/Li CT was lower than that observed in healthy subjects. These observations indicate that the key thiol protein is part of a membrane protein that normally modulates the erythrocyte Na/Li CT but is nonfunctional in ADPKD. In addition, we have also shown that membrane fluidity of intact erythrocytes of ADPKD patients is higher than in healthy control subjects (21). This abnormality was not found in erythrocyte ghost membranes, where membrane cytoskeleton was lost during ghost membrane preparation. This suggests that the abnormality of erythrocyte membrane fluidity in ADPKD might be due to abnormal membrane lipid-cytoskeleton interactions.

The aim of this study was to explore further whether an NEM-sensitive thiol protein similar to that modifying the kinetics of erythrocyte Na/Li CT also plays a role in determining membrane lipid fluidity in healthy control subjects and whether this characteristic thiol protein can be used to probe the abnormality of cytoskeleton–membrane interactions in ADPKD patients.

Materials and Methods

Patients and Healthy Control Subjects

Twelve Caucasian patients with ADPKD, between 18 and 70 yr old, were studied. The diagnosis of ADPKD was based on the demonstration of multiple bilateral renal cysts by ultrasound and a family history of the disease. All had serum creatinine less than 120 μmol/L. Six ADPKD patients had hypertension, and six were normotensive. Twelve healthy subjects with no family history of hypertension or diabetes mellitus from laboratory and hospital staff were studied for comparison. The clinical details of ADPKD patients and healthy control subjects are given in Table 1. This study was approved by the Joint Ethics Committee of the Newcastle Health Authority and University of Newcastle-upon-Tyne. All subjects gave informed consent to participate in the study.

Preparation of Intact Erythrocyte Suspension

The method used was similar to that we have described previously (21). Venous blood was collected in lithium heparin tubes, centrifuged, and the erythrocytes were washed twice with isotonic phosphate-buffered saline (PBS; 290 ± 2 mosmol/kg, pH 7.4) and separated by centrifugation (3000 rpm, 5 min). The erythrocytes were resuspended in isotonic PBS to prepare a 0.1% packed cell volume (PCV) suspension to minimize the effect of scattering of emitted light by intact erythrocytes.

Table 1. Clinical details of healthy control subjects and ADPKD patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>ADPKD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>6/6</td>
<td>7/5</td>
</tr>
<tr>
<td>Age</td>
<td>40 ± 4</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>123 ± 2</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>78 ± 1</td>
<td>83 ± 1</td>
</tr>
<tr>
<td>Plasma creatinine (μmol/L)</td>
<td>99 ± 3</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>1.15 ± 0.14</td>
<td>1.83 ± 0.37</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>5.4 ± 0.4</td>
<td>5.8 ± 0.4</td>
</tr>
</tbody>
</table>

*Values are mean ± SEM. ADPKD, autosomal dominant polycystic kidney disease.

Measurement of Fluorescence Anisotropy

The membrane fluidities of core and superficial regions were determined by measuring fluorescence anisotropy of 1,6-diphenyl-
1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexatriene (TMA-DPH), respectively. DPH was obtained from Sigma (Poole, United Kingdom), and TMA-DPH was purchased from Molecular Probes (Eugene, OR).

For measurement of DPH anisotropy, 3 μl of 2 × 10⁻³ M DPH (in acetone) was added into 3 ml of intact erythrocyte suspension (0.1% PCV). The mixture was incubated at 37°C for 45 min. The mixture was centrifuged, and the erythrocytes were washed twice with isotonic PBS. After the final washing, the supernatant was removed and the cells were resuspended in 3 ml of isotonic PBS. This mixture was used to measure DPH anisotropy of intact erythrocytes.

For measurement of TMA-DPH anisotropy, 3 ml of intact erythrocyte suspension (0.1% PCV) was equilibrated at 37°C in a silica cuvette in the fluorometer. TMA-DPH (3 μl of 2 × 10⁻³ M, in dimethylformamide) was added directly into the cuvette. The fluorescence intensity and anisotropy of TMA-DPH in intact erythrocytes were dependent on the incubation time. Therefore, all measurements were taken after a 5-min incubation.

Fluorescence anisotropy values of both DPH and TMA-DPH were measured with a Perkin-Elmer LS 50B luminescence spectrometer at 37°C with excitation and emission wavelengths of 360 and 450 nm, respectively, and slit widths 10 nm. Each measurement was integrated over 3 s, and the mean of four measurements was taken. Anisotropy (A) was calculated according to the equations:

\[ A = \frac{I_{vv} - G \times I_{hh}}{I_{vv} + 2G \times I_{hh}} \]

\[ G = \frac{I_{vv}}{I_{hh}} \]

where \(I_{vv}\) and \(I_{hh}\) are the intensities with the excitation in the vertical (v) position and the analyzer in the vertical (v) or horizontal (h) position, respectively. \(G\) is the correction factor for the optical system and was determined before each measurement of anisotropy.
NEM Treatment of Erythrocytes

After each measurement of the fluorescence anisotropy of DPH or TMA-DPH, 10 μl of NEM solution (30 mM NEM in choline medium [139 mMol/L choline chloride, 1 mMol/L MgCl₂, 10 mMol/L glucose, and 10 mMol/L Tris-4-morpholinepropanesulfonic acid, pH 7.4; 290 ± 2 mosmol/kg]) was added directly into the cuvette. The mean of seven measurements was taken 3 min after adding NEM.

Cytochalasin D Treatment of Erythrocytes

To examine whether disruption of cytoskeletal actin affects the erythrocyte membrane fluidity, erythrocytes were exposed to cytochalasin D to depolymerize actin filaments. Three milliliters of 0.1% PCV erythrocyte suspension was incubated at 37°C with 10 μM/L cytochalasin D (Sigma) for 60 min. The suspension after incubation was used to determine the fluorescence anisotropy of DPH and TMA-DPH.

NEM Treatment of Erythrocytes Treated with Cytochalasin D

After reading the fluorescence anisotropy of the erythrocyte suspension treated with cytochalasin D, NEM was added to determine whether there was any additional change in the anisotropy of either dye.

Heat Treatment of Erythrocytes

Three milliliters of 0.1% PCV erythrocyte suspension was placed in a waterbath at 48°C for 20 min and then cooled to 37°C and used to determine the fluorescence anisotropies of DPH and TMA-DPH.

Liposome Preparation

Liposomes were prepared by dissolving 2 mg of cholesterol with 2 mg of L-α-phosphatidylcholine in 0.5 ml of chloroform in a glass tube and evaporating the chloroform with vigorous mixing. The thin film of lipid was then dried out and used to prepare liposomes containing antibody to actin, ankyrin, or albumin by vigorous vortex mixing with a solution of the appropriate antibody.

Anti-Actin, Anti-Ankyrin, and Anti-Albumin Treatments of Erythrocytes

Five microliters of actin-antibody (anti-actin [C terminus] polyclonal from rabbit, affinity-isolated; Sigma Chemical Co., Poole, United Kingdom), 4 μl of ankyrin-antibody (anti-ankyrin polyclonal from mouse, 25 μg in 150 μl of PBS; Zymed Laboratories, South San Francisco, CA), or 5 μl of albumin-antibody (anti-albumin polyclonal from rabbit, IgG fraction; Sigma Chemical Co.) was added to 2 ml of isotonic PBS and then used to prepare liposomes as above. The resulting mixture was centrifuged for 5 min (10,000 × g), and the supernatant was taken to be used as liposomes containing anti-actin, anti-ankyrin, or anti-albumin. Liposomes containing each antibody were added to each intact erythrocyte suspension in cell culture medium, TC 199, to make a final cell concentration of 0.1% PCV, and these mixtures were then incubated in a CO₂ incubator overnight. Afterward, these suspensions were washed three times with isotonic PBS (2000 g, 3 min) and then used to measure fluorescence anisotropies of TMA-DPH and DPH.

Statistical Analyses

When the variables measured were normally distributed, the results are given as mean ± SEM. The significance of differences between groups was assessed by using unpaired t test. When the variables were not normally distributed, the Mann–Whitney U test was used to test for the significance of differences.

Results

The fluorescence anisotropies of both DPH and TMA-DPH in intact erythrocytes of ADPKD patients were significantly lower than in erythrocytes of healthy control subjects (Table 2). Normotensive and hypertensive ADPKD patients showed no significant difference in the values of fluorescence anisotropy of either DPH or TMA-DPH in untreated erythrocytes or in erythrocytes after the various treatments. There was also no obvious effect of age, sex, or plasma levels of cholesterol and triglycerides on the fluorescence anisotropy measurements of either DPH or TMA-DPH.

Effect of NEM on the Membrane Fluidity

After treatment with NEM, the fluorescence anisotropies of both dyes in erythrocytes from healthy control subjects were decreased, but there were no significant effects of NEM on the fluorescence anisotropy of either DPH or TMA-DPH in erythrocytes from ADPKD patients (Table 2).

After addition of NEM to the erythrocytes from healthy control subjects, there was an immediate decrease in fluorescence anisotropy that recovered partially during the next minute before becoming stable (Figure 1). In the recovery period, the fluorescence anisotropy of DPH increased significantly with time (regression coefficient: [median, range]) (5.917, -5 to 31%/min⁻¹) and was stable for the next 2 min

Table 2. Fluorescence anisotropies of DPH and TMA-DPH in untreated intact erythrocytes and in erythrocytes after treatment with NEM, cytochalasin D, and heating at 48°C for 20 min in healthy control subjects and ADPKD patients

<table>
<thead>
<tr>
<th>Anisotropy</th>
<th>Control (n = 12)</th>
<th>ADPKD (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UT</td>
<td>0.223 ± 0.001</td>
<td>0.190 ± 0.002⁹b</td>
</tr>
<tr>
<td>NEM</td>
<td>0.200 ± 0.003c</td>
<td>0.193 ± 0.003</td>
</tr>
<tr>
<td>Cy D</td>
<td>0.206 ± 0.006c</td>
<td>0.194 ± 0.002</td>
</tr>
<tr>
<td>heating</td>
<td>0.199 ± 0.002c</td>
<td>0.195 ± 0.003</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UT</td>
<td>0.279 ± 0.001</td>
<td>0.266 ± 0.001⁹b</td>
</tr>
<tr>
<td>NEM</td>
<td>0.264 ± 0.001c</td>
<td>0.270 ± 0.001</td>
</tr>
<tr>
<td>Cy D</td>
<td>0.264 ± 0.001c</td>
<td>0.268 ± 0.001</td>
</tr>
<tr>
<td>heating</td>
<td>0.265 ± 0.001c</td>
<td>0.268 ± 0.001</td>
</tr>
</tbody>
</table>

⁹b P < 0.001 for healthy control subjects and ADPKD patients.

Values are mean ± SEM. DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, trimethylammonium-diphenylhexatriene; NEM, N-ethylmaleimide; UT, untreated erythrocytes; Cy D, cytochalasin D.
(regression coefficient: \(-0.7, -5.6\) to \(6.4\%\min^{-1}\), \(P = 0.02\) versus the first minute, Mann–Whitney U test). The TMA-DPH anisotropy also decreased immediately after addition of NEM (Figure 1) but was then stable over the next minute (regression coefficient: \(0.12, 0.05\) to \(0.15\%\min^{-1}\)) and subsequently (regression coefficient: \(0.117, -2.8\) to \(2.8\%\min^{-1}\), \(P = 0.25\) versus the first minute, Mann–Whitney U test). After addition of NEM to the erythrocytes from ADPKD patients, there was no significant decrease in fluorescence anisotropy of either DPH or TMA-DPH (Figure 2).

Effect of Cytoskeleton Modification on Membrane Fluidity

In erythrocytes from healthy control subjects, cytochalasin D treatment caused a decrease in fluorescence anisotropies of both DPH and TMA-DPH (Table 2), but there was no such effect on the erythrocytes from ADPKD patients (Table 2). After cytochalasin D treatment, NEM caused no further changes in fluorescence anisotropy of either dye in erythrocytes from healthy control subjects (Table 3).

Heating normal erythrocytes at \(48^\circ\text{C}\) for 20 min caused a significant decrease in the fluorescence anisotropies of both DPH and TMA-DPH (Table 2). This was very similar to the
Table 3. Lack of effect of NEM on fluorescence anisotropies of DPH and TMA-DPH in intact erythrocytes of five healthy control subjects previously treated with cytochalasin D

<table>
<thead>
<tr>
<th>Anisotropy</th>
<th>UT</th>
<th>Cy D</th>
<th>Cy D/NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>0.226 ± 0.001</td>
<td>0.202 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.202 ± 0.002</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>0.277 ± 0.002</td>
<td>0.265 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.266 ± 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± SEM. Abbreviations as in Table 2.

<sup>b</sup> P < 0.001 for UT and after treatment with Cy D.

effect of NEM and cytochalasin D. Heating erythrocytes from ADPKD patients had no effect on the fluorescence anisotropy of either DPH or TMA-DPH (Table 2), and this was similar to the lack of effect of NEM and cytochalasin D on the erythrocytes from ADPKD patients.

Effect of Liposomes Containing Antibodies to Actin, Ankyrin, and Albumin on Erythrocyte Membrane Fluidity

In healthy control subjects, after overnight incubation with liposomes alone there was no significant change of fluorescence anisotropy of either DPH or TMA-DPH. Incubation with liposomes containing antibodies to either actin or ankyrin caused significantly decreased fluorescence anisotropies of both DPH and TMA-DPH, but liposomes containing antibody to albumin had no significant effect on the fluorescence anisotropies of either DPH or TMA-DPH (Table 4). NEM caused no further changes in fluorescence anisotropy of either dye in erythrocytes previously treated with liposomes containing antibody to either actin or ankyrin. In marked contrast, NEM caused significant decreases in fluorescence anisotropies of both DPH and TMA-DPH in erythrocytes previously treated with liposomes containing antibody to albumin (Table 4).

In ADPKD patients, neither liposomes alone nor liposomes containing antibody to either actin or ankyrin caused a significant change in the fluorescence anisotropy of either dye (Table 4). Additionally, NEM did not cause any alterations of the fluorescence anisotropy of either dye in erythrocytes previously treated with liposomes containing antibody to either actin or ankyrin.

Discussion

The membrane fluidity measured by both DPH and TMA-DPH fluorescence probes in intact erythrocytes of ADPKD patients was higher (reciprocal-to-fluorescence anisotropy) than that in erythrocytes of healthy control subjects. This was similar to our previous study (21), in which the abnormality found in ADPKD was detected in intact erythrocytes, but not in ghost membranes. This abnormality could be due to an abnormal arrangement of the proteins that form the cytoskeleton-membrane complex in ADPKD.

In the healthy control group, the fluorescence anisotropies of both DPH and TMA-DPH were significantly decreased after treatment of erythrocytes with either NEM or cytochalasin D, or heating at 48°C. This indicates that these treatments, which alter the protein components of the cytoskeleton, cause an increase in fluidity in the membrane lipid bilayer. The effect of NEM on the fluorescence anisotropy of DPH was biphasic, with a very rapid decrease in fluorescence anisotropy that was maximal after 90 s, followed by a recovery to a new lower, but stable, fluorescence anisotropy. This may be due to disruption followed by reorganization. In marked contrast, in the ADPKD

Table 4. Effects of actin antibody, ankyrin antibody, and albumin antibody on fluorescence anisotropies of DPH and TMA-DPH in intact erythrocytes of healthy control subjects and ADPKD patients<sup>a</sup>

<table>
<thead>
<tr>
<th>Anisotropy</th>
<th>Control (n = 5)</th>
<th>ADPKD (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>NEM</td>
</tr>
<tr>
<td>DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UT</td>
<td>0.222 ± 0.001</td>
<td>0.198 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lp</td>
<td>0.222 ± 0.001</td>
<td>0.197 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ac-Ab Lp</td>
<td>0.194 ± 0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.196 ± 0.001</td>
</tr>
<tr>
<td>Ak-Ab Lp</td>
<td>0.196 ± 0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.196 ± 0.002</td>
</tr>
<tr>
<td>Al-Ab Lp</td>
<td>0.223 ± 0.001</td>
<td>0.200 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UT</td>
<td>0.277 ± 0.002</td>
<td>0.263 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lp</td>
<td>0.277 ± 0.002</td>
<td>0.263 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ac-Ab Lp</td>
<td>0.262 ± 0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.264 ± 0.001</td>
</tr>
<tr>
<td>Ak-Ab Lp</td>
<td>0.262 ± 0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.263 ± 0.002</td>
</tr>
<tr>
<td>Al-Ab Lp</td>
<td>0.280 ± 0.001</td>
<td>0.262 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± SEM. Lp, liposomes alone; Ac-Ab Lp, liposomes containing actin antibody; Ak-Ab Lp, liposomes containing ankyrin antibody; Al-Ab Lp, liposomes containing albumin antibody.

<sup>b</sup> P < 0.001 for before and after adding NEM.

<sup>c</sup> P < 0.001 for healthy control subjects and ADPKD patients.

<sup>d</sup> P < 0.001 for UT and after treatment with either actin antibody or ankyrin antibody.
patients, there was no significant effect of any of these treatments on the fluorescence anisotropy of either DPH or TMA-DPH.

These effects on membrane fluidity are similar to those we have reported on the erythrocyte membrane transporter Na/Li CT. We have shown that in healthy control subjects, the kinetics of this transporter are modified by two types of thiol groups (20). One of these thiol groups affects $K_m$ (So), and alklyation with NEM or heating at 48°C decreases $K_m$(So). The $K_m$(So) of Na/Li CT is low in ADPKD and is not affected by NEM or heating (19). In the same way, these treatments did not affect membrane fluidity in ADPKD patients, but caused a change in healthy control subjects to values seen in ADPKD. This is consistent with the abnormal behavior in ADPKD of a thiol protein(s) that modifies both Na/Li CT and membrane fluidity.

NEM alkylates thiol groups, and one reported effect is to cause dissociation of spectrin tetramers to dimers either by alklyation of thiol groups on spectrin or on one of the several accessory proteins that stabilize its organization (22–24). For example, ankyrin has a key thiol group necessary for its association with spectrin (25,26). Ankyrin also associates with the band 3 anion transporter and Na$^+$/K$^+$-ATPase (27). Additionally, it has been suggested that NEM increases osmotic fragility of human erythrocytes (28). These thiol group-dependent interactions give the cytoskeleton an effect on the lipid bilayer. Cytochalasin D specifically binds to F-actin, does not bind to the glucose transporter, and has a high affinity for barbed ends of actin (29). It therefore specifically binds to the association caps of actin and then causes dissociation of actin–spectrin membrane organization. There is evidence suggesting that cytochalasin D inhibits polymerization of actin by blocking the net polymerizing ends of actin nuclei and filaments in human erythrocytes (30–32). Cytochalasin D, therefore, disrupts the spectrin–actin band 4.1 complex and then leads to a rapid decrease in the viscosity (increase in the fluidity) of erythrocyte membranes (33). Heating normal erythrocytes at 48°C for 20 min causes partial dissociation of spectrin tetramers in the cytoskeleton to dimers (34). Thus, all of these treatments cause alterations of the erythrocyte membrane cytoskeleton. Integration of the proteins that constitute the cytoskeleton makes it difficult to assign a key role to any one of them, because disruption of any individual protein is likely to lead to malfunction of the complex as a whole.

In healthy subjects, cytochalasin D enhanced the membrane fluidity of erythrocytes up to the same level observed at the basal state in ADPKD patients. NEM, which can enhance the membrane fluidity of the untreated normal erythrocytes, caused no significant further change on the fluorescence anisotropies of either TMA- or DPH of the erythrocytes treated with cytochalasin D. This therefore suggests that NEM-sensitive thiol proteins are associated with the cytoskeletal proteins, because an abnormal cytoskeletal organization induced by cytochalasin D can nullify the effect of NEM on the membrane fluidity.

The ability of phospholipid/cholesterol liposomes to fuse with biological membranes has been widely used in biotechnolgy (35). Liposomes were used in this study to introduce antibody to actin or ankyrin to bind with their specific antigens inside cell membranes, using antibody to albumin as a control. The result clearly showed that in healthy control subjects either antibody to actin or ankyrin caused a significant increase of erythrocyte membrane fluidity up to the level that was observed at the basal state in untreated erythrocytes from ADPKD patients, whereas liposomes alone or antibody to albumin, which clearly have no effect on the membrane cytoskeleton, caused no significant alterations in erythrocyte membrane fluidity. This suggests that there are close interactions between the cytoskeleton and the lipid bilayer. Erythrocyte spectrin is known to play an important role in the regulation of membrane fluidity (36,37). However, this may be mediated by intermediate bridging proteins such as ankyrin. In addition, the organization of the mainly spectrin cytoskeleton is affected by the range of proteins that bind to it, particularly at junction points, such as actin. Hence, disruption or disorganization of either actin or ankyrin could lead to a similar change in lipid organization and lateral mobility of membrane proteins, causing alteration in the membrane fluidity. NEM, which caused a significant decrease of fluorescence anisotropies of both DPH and TMA-DPH in erythrocytes treated with liposomes alone or liposomes containing antibody to albumin, caused no significant further change in erythrocytes, in which cytoskeletal actin or ankyrin was blocked by its specific antibody. In ADPKD, in which the membrane fluidity in untreated erythrocytes was abnormally high, none of these three antibodies or liposomes alone caused significant further alterations in fluorescence anisotropies of either probe. This supports our hypothesis that abnormal membrane fluidity found in ADPKD is due to abnormal lipid–cytoskeleton associations and that the effect of NEM is due to a cytoskeletal protein.

It is now becoming clear that causative products of the PKD genes are integral membrane proteins with a wide tissue distribution (2). Unfortunately, the basic nature and function of PKD proteins are still unclear. However, several lines of investigation suggest that the phenotype of ADPKD might be caused by an abnormal membrane transporter that is unknown at present (17). Mutations or deletions of the PKD genes cause abnormal expression of these proteins, which account for the abnormalities of cell membrane and extracellular matrix and subsequently lead to cystic formation in many organs, particularly the kidneys (2,14). The lack of function of an NEM-sensitive thiol protein(s) that modifies the kinetics of erythrocyte Na/Li CT and also has an effect on the fluidity of membranes clearly indicates that the abnormality of cell membranes is also found in erythrocytes of ADPKD patients. It is possible that abnormal PKD proteins could cause abnormal imprinting of organization on the erythroblast membranes in the bone marrow during erythropoiesis, and this abnormality of the cell membrane persists in the erythrocytes.

Therefore, it appears that there is a key thiol protein(s) on the cytoskeleton of erythrocytes that is sensitive to NEM and that is important for the organization of the cytoskeleton. This
key thiol protein(s) has an effect on the membrane lipid fluidity and the function of integral transport proteins. This mechanism is nonfunctional in ADPKD and probably contributes to abnormal lipid bilayer properties and transport protein function in this disease.

Acknowledgments

We acknowledge the Northern Counties Kidney Research Fund for supporting this study. Dr. Vareesangthip is supported by the Siriraj Medical School, Mahidol University, Thailand.

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