Increase in Membrane Fluidity and Opening of Tight Junctions Have Similar Effects on Sodium-coupled Uptakes in Renal Epithelial Cells*

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Apical membranes of renal epithelial cells were shown to be more rigid than other plasma membranes, due in part to the abundance of sphingomyelin among their constituent phospholipids. Tight junctions play a key role in maintaining differences between the apical and the basolateral domains of the plasma membrane with respect to their lipid composition and fluidity. To evaluate the influence of alterations of membrane fluidity on the activity of two apically located transport systems, we compared the effect of opening of tight junctions, by a preincubation period in calcium-deprived medium and of increasing fluidity, with benzyl alcohol, on Na-dependent uptakes of Pi and α-methyl-D-glucopyranoside (MGP) in intact, confluent LLC-PK1 cells and MDCK cells. Benzyl alcohol, at 10 mM, increased the $V_{\text{max}}$ of Pi uptake by 55 and 42% in LLC-PK1 cells and MDCK cells, respectively, but decreased the $V_{\text{max}}$ of MGP uptake in LLC-PK1 cells by 23%. Similarly to 10 mM benzyl alcohol, opening of tight junctions also increased the $V_{\text{max}}$ of Pi uptake by 45 and 46% in LLC-PK1 cells and MDCK cells, respectively, and depressed MGP uptake in LLC-PK1 cells by inducing a 15% decrease of the $V_{\text{max}}$. None of the two maneuvers (i.e. addition of benzyl alcohol or opening of tight junctions) affected the $K_m$ values of the transport systems. From these results it is concluded that (i) the increase in membrane fluidity, achieved either by benzyl alcohol or by opening of tight junctions, affects Na-Pi and Na-glucose cotransports differently, reflecting differences in the lipid environments of the two transport systems, and (ii) membrane fluidity might play a physiological role in the modulation of the activity of transport systems.

Modifications of membrane lipid order have been shown to affect the activity of numerous membrane-bound proteins, among which are enzymes and transport systems (1). Increasing the fluidity of renal brush-border membrane vesicles with the local anesthetic drug benzyl alcohol elicited a dramatic impairment of Na-dependent D-glucose uptake, demonstrating that this transport system was exquisitely sensitive to variations of membrane physical state (2). Indirect evidence suggested, as well, that renal phosphate (Pi) reabsorption could be modulated by membrane physical state. (i) In rats fed a low phosphate diet, brush-border membrane fluidity was increased as a consequence of the decrease of cholesterol content, while Na-dependent Pi uptake was increased (3). (ii) Administration of 1,25-dihydroxycholecalciferol to vitamin D-depleted rats induced simultaneously an increase in fluidity of brush-border membranes and a stimulation of Pi uptake (4-6). It appears therefore that the activity of both Na-coupled Pi and D-glucose transport systems may depend on the lipid composition and/or physical state of the apical membrane in which the involved proteins are embedded. The apical domain of the plasma membrane of renal epithelial cells was shown to be uncommonly rigid, likely as a consequence of the abundance of sphingomyelin in its lipid composition (7-9). Maintenance of the lipid composition of the apical membrane is partly ensured by tight junctions, which prevent lateral diffusion of lipids toward the basolateral domain (10, 11). We hypothesized that opening of tight junctions should mimick the effects of a fluidizing agent such as benzyl alcohol on the activity of Na-Pi, and Na-glucose cotransports.

Renal epithelial cell lines such as LLC-PK1 cells and MDCK cells, which retained differentiated properties of nephron segments (12-16), proved to be useful tools for studying thoroughly the mechanisms of Na-coupled solute transports (17). In intact MDCK cells we have previously demonstrated that increasing membrane fluidity with the fluidizing agent benzyl alcohol affected in a complex manner the activity of adenylate cyclase, a basolaterally located protein (18). In the present study we sought (i) to examine the influence of membrane physical state on the activity of two apically located transport proteins by studying the effects of benzyl alcohol on Na-dependent Pi, and hexose uptakes in LLC-PK1 cells and MDCK cells, and (ii) to compare the effects elicited by benzyl alcohol with those induced by the opening of tight junctions in confluent cellular monolayers. We show that increasing membrane fluidity with benzyl alcohol or opening tight junctions stimulated Pi uptake whereas it depressed hexose uptake.

**EXPERIMENTAL PROCEDURES**

**Materials**

Insulin, transferrin, hydrocortisone, triiodothyronine, cholesterol, prostaglandin E$_2$, α-methyl-D-glucopyranoside (MGP),† and sodium selenite were purchased from Sigma, and 1-deamino-(8-D-arginine) vasopressin from Ferring AB (Malmö, Sweden). Tracers were from the following sources: K$_4$H$_2$PO$_4$, from Du Pont-New England Nuclear

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† The abbreviations used are: MGP, α-methyl-D-glucopyranoside; Heparin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
and methyl-(a-D-(U-"C)gluco)pyranoside (["C]MGP) from Amersham Corp. (Amersham, United Kingdom (U.K.)) LLC-PK1 cells (passage 190) and MDCK cells (passage 65), as well as culture media and reagents were from Flow Labs (Irvine, U.K.). Plasticware was from Falcon (Oxnard, CA).

**Methods**

**Cell Culture**—LLC-PK1 cells (passages 220-220) and MDCK cells (passages 75-96) were seeded in 24-well plastic trays and grown at 37 °C, in a 5% CO2, 95% air atmosphere, in serum-free medium. For both cell lines, medium consisted of 1:1 (%/v) Ham’s F-12/Dulbecco’s modified Eagle medium mixture supplemented as described by Taub et al. (19) and Chuman et al. (16) for MDCK cells and LLC-PK1 cells, respectively. Subcultures were performed once weekly, using a trypan blue solution, with a splitting ratio of 1:4. After 1 month cultured cells were discarded, and a new culture was initiated from a new batch of cells kept frozen in liquid nitrogen.

**Uptake Studies**—Procedures were adapted from Caverzasio et al. (20) and Biber et al. (14). Uptakes were performed at 37 °C in a buffered solution with the following composition (mmol/liter): 137 NaCl, 5.4 KCl, 2.8 CaCl2, 1.2 MgSO4, 15 Hepes. A sodium-free solution was made by replacing sodium chloride with choline chloride. The solution pH was adjusted to 7.4 with Tris base. Confluent cells of both cell lines were used 6-7 days after seeding. The culture medium was removed and cells were washed with 1 ml/well of the uptake solution. Cells were then incubated for various periods of time in 400 μl/well of the uptake solution in the presence of K2H3PO4 (1 μCi/ml) or ["C]MGP (0.5 μCi/ml), and appropriate concentrations of KH2PO4, or MGP. All these steps were performed at 37 °C. At the end of incubation, the uptake was stopped by washing the cells three times with 1 ml/well of ice-cold solution (137 mM NaCl, 15 mM Hepes, pH = 7.4). Cells were then solubilized in 0.5% Triton X-100 (500 μl/well), and aliquots were counted by liquid scintillation.

In experiments with benzyl alcohol, this drug was added to the uptake solution. Opening of tight junctions was achieved by the benzyl alcohol, 10 or 40 mM, was added to the incubation balanced salt solution, and then preincubated in this solution for 30 min of incubation (Fig. 1). Linearity was maintained when increasing concentrations of benzyl alcohol were added over a period of 30 min. Increasing concentrations of benzyl alcohol affected Pi uptake in a bimodal manner (Fig. 2). When present at low concentration (less than 10 mM), benzyl alcohol affected the Na-coupled uptake by membrane fluidity.

**Presentation of Data**—Pi or MGP uptake was expressed as nanomol/mg protein (21). Na-dependent uptake was calculated by subtracting the uptake value measured in the presence of choline from that measured in the presence of sodium. Results were presented as mean ± S.E. of three to four experiments in which duplicates or triplicates were obtained. One-way or two-way analyses of variance were performed and, when allowed by the F value, results were compared by the modified t test (22).

**RESULTS**

**Effect of Benzyl Alcohol on Pi Uptake**—In LLC-PK1 cells, Na-dependent Pi uptake increased linearly with time, up to 30 min of incubation (Fig. 1). Linearity was maintained when benzyl alcohol, 10 or 40 mM, was added to the incubation medium. In subsequent experiments Pi uptake was measured after 10 min of incubation. Increasing concentrations of benzyl alcohol affected Pi uptake in a bimodal manner (Fig. 2). When present at low concentration (less than 20 mM), benzyl alcohol enhanced Pi uptake, while higher concentrations of benzyl alcohol depressed the uptake. In contrast the Na-independent component of Pi uptake was not influenced by benzyl alcohol up to 40 mM. The bimodal effect of benzyl alcohol on Pi uptake was confirmed by the kinetic analysis of Na-dependent Pi uptake in the absence and in the presence of 10 or 40 mM benzyl alcohol (Fig. 3, left panel). Analysis of the Pi uptake by the Eadie-Hofstee plot (Fig. 3, right panel) showed that benzyl alcohol affected the Vmax but not the Km value of the transport system. Because MDCK cells were recently shown to possess a Na-Pi cotransport (23, 24), we also studied the influence of benzyl alcohol on Na-dependent Pi uptake in this cell line. As shown in Fig. 4, the effects of benzyl alcohol were similar to those elicited by this drug in LLC-PK1 cells. Changes in the kinetic parameters of Na-dependent Pi uptake under the influence of benzyl alcohol in both cell lines were summarized in Table I.

**Effect of Benzyl Alcohol on MGP Uptake**—Hexose transport was evaluated by studying Na-dependent uptake of MGP, a nonmetabolized analog of D-glucose, which competes with the latter on the Na-D-glucose cotransport system (13, 25). In preliminary experiments we verified that no Na-dependent MGP uptake could be evidenced in MDCK cells under our culture conditions, a result in agreement with previously reported data (17). The effect of benzyl alcohol on Na-MGP cotransport was therefore studied in the LLC-PK1 cell line.
Na-dependent MGP uptake was linear with time of incubation up to 60 min (not shown). Benzyl alcohol decreased MGP uptake in a concentration-dependent manner (Fig. 5). MGP uptake was decreased by 50% in the presence of 30 mM benzyl alcohol. The Na-independent component of MGP uptake was not affected by benzyl alcohol, up to 80 mM. As shown in Fig. 6, the decrease of Na-dependent MGP uptake in the presence of 10 or 40 mM benzyl alcohol resulted from a selective decrease of the $V_{\text{max}}$ of the transport system, while the $K_m$ value was not significantly changed.

**Effect of Opening of Tight Junctions on $P_i$ and MGP Uptakes**—In order to open tight junctions, confluent monolayers of LLC-PK1 cells and MDCK cells were preincubated in a calcium-deprived medium [26, 27], and the uptake of $P_i$ was subsequently measured in the presence of calcium, as described under "Materials and Methods." This treatment resulted, in LLC-PK1 cells, in an increase in Na-dependent $P_i$ uptake (Fig. 7, left panel). Analysis of the kinetic parameters of $P_i$ uptake by the Eadie-Hofstee plot revealed that the $V_{\text{max}}$ of the transport system was increased whereas the $K_m$ value was unchanged. The magnitude of the increase in $V_{\text{max}}$ was similar to that induced by 10 mM benzyl alcohol. Treatment of MDCK cells by calcium-deprived medium also increased the $V_{\text{max}}$ of Na-dependent $P_i$ uptake. Although the $K_m$ value tended to decrease, the difference did not reach significance (Fig. 8). In contrast with the enhancement of Na-$P_i$ cotransport, preincubating LLC-PK1 cells without calcium resulted in a decrease of Na-dependent MGP uptake by LLC-PK1 cells, which resembled that elicited by 10 mM benzyl alcohol. Again, only the $V_{\text{max}}$ of the transport system but not the $K_m$ value was affected (Fig. 9).

**DISCUSSION**

The results of this study showed that, in renal epithelial cells, the local anesthetic drug benzyl alcohol, an agent known to increase membrane fluidity, induced changes in the activity of the transport system but  not the $K_m$ value, preincubating LLC-PK1 cells without calcium resulted in a decrease of Na-dependent MGP uptake by LLC-PK1 cells, which resembled that elicited by 10 mM benzyl alcohol. Again, only the $V_{\text{max}}$ of the transport system but not the $K_m$ value was affected (Fig. 9).

![Fig. 3. Effect of benzyl alcohol (BA) on the kinetics of Na-dependent $P_i$ uptake by LLC-PK1 cells.](image)

Cells were incubated during 10 min with the indicated concentrations of BA, in the absence (closed circles) or in the presence of benzyl alcohol, at the concentration of 10 mM (closed triangles) or 40 mM (closed squares). Na-dependent $P_i$ uptake (left panel) was calculated as the difference between $P_i$ uptakes measured in the presence of sodium or of choline. $K_m$ and $V_{\text{max}}$ values determined from Eadie-Hofstee plot (right panel) are summarized in Table I. The data represent the mean ± S.E. of three different experiments ($n = 3$) in which duplicates were obtained.

![Fig. 4. Effect of benzyl alcohol (BA) on the kinetics of Na-dependent $P_i$ uptake by MDCK cells.](image)

Cells were incubated during 10 min with the indicated concentrations of BA, in the absence (closed circles) or in the presence of benzyl alcohol, at the concentration of 10 mM (closed triangles) or 40 mM (closed squares). Na-dependent $P_i$ uptake (left panel) was calculated as the difference between $P_i$ uptakes measured in the presence of sodium or of choline. $K_m$ and $V_{\text{max}}$ values determined from Eadie-Hofstee plot (right panel) are summarized in Table I. The data represent the mean ± S.E. of three different experiments ($n = 3$) in which duplicates were obtained.

![Fig. 5. Concentration-dependent effect of benzyl alcohol on MGP uptake by LLC-PK1 cells.](image)

The uptake of MGP (1 mM) was measured after 60 min of incubation in the presence of sodium (closed circles) or of choline (open circles). The data represent the mean ± S.E. of four different experiments ($n = 4$) in which duplicates were obtained. *Significantly different from the value without benzyl alcohol, p < 0.05.

**TABLE I**

| Effect of benzyl alcohol on the kinetic parameters of Na-dependent $P_i$ uptake in LLC-PK1 cells and MDCK cells |
|---|---|---|---|---|
| Cells were incubated with increasing concentrations of BA, during 10 min at 37°C. $K_m$ and $V_{\text{max}}$ values were calculated using the Eadie-Hofstee plot. The results represent the mean ± S.E. of three different experiments ($n = 3$) in which duplicates were obtained. |
| $K_m$ (μM) | $V_{\text{max}}$ (nmol/10 min/mg protein) |
| LLC-PK1 | MDCK | LLC-PK1 | MDCK |
| Benzyal alcohol 0 | 162 ± 21.5 | 340 ± 22.2 | 12.9 ± 0.80 | 8.8 ± 0.51 |
| Benzyal alcohol 10 mM | 151 ± 20.0 | 371 ± 36.3 | 20.0 ± 1.65* | 12.5 ± 1.23* |
| Benzyal alcohol 40 mM | 165 ± 18.4 | 292 ± 31.5 | 8.4 ± 0.12* | 6.8 ± 0.26* |

*Significantly different from the value without benzyl alcohol, $n = 3$, $p < 0.05$. 
of two apically located transport proteins, namely those involved in the Na-dependent uptakes of P, and glucose. Low concentrations of benzyl alcohol had opposite effects on the two transport systems since P uptake was increased while MGP uptake was decreased. Furthermore, opening of tight junctions mimicked the effects elicited by benzyl alcohol at low concentration: increase in Na-P cotransport in LLC-PK1 cells and in MDCK cells and decrease of Na-MGP cotransport in LLC-PK1 cells.

FIG. 6. Effect of benzyl alcohol (BA) on the kinetics of Na-dependent MGP uptake by LLC-PK1 cells. Cells were incubated during 60 min with the indicated concentrations of MGP, in the absence (closed circles) or in the presence of benzyl alcohol, at the concentration of 10 mM (closed triangles) or 40 mM (closed squares). Na-dependent MGP uptake (left panel) was calculated as the difference between MGP uptakes measured in the presence of sodium or of choline. The data represent the mean ± S.E. of three different experiments (n = 3) in which duplicates were obtained. KmA and Vmax values were determined from Eadie-Hofstee plots (right panel). KmA values (μM) were as follows: 679 ± 35.7, 578 ± 46.7, 635 ± 135.0 in the presence of 0, 10, and 40 mM benzyl alcohol, respectively. Vmax values (nmol/60 min/mg protein) were as follows: 38.5 ± 1.5, 29.6 ± 0.3, 10.9 ± 0.8 in the presence of 0, 10, and 40 mM benzyl alcohol, respectively. Vmax values in the presence of benzyl alcohol were significantly different from that in the absence of benzyl alcohol (p < 0.05).

FIG. 7. Effect of a preincubation period in calcium-deprived medium on the kinetics of Na-dependent P uptake by LLC-PK1 cells. Cells were preincubated during 15 min at 37 °C in Hank's balanced salt solution containing (closed circles) or not (open circles) 2 mM CaCl2. Cells were then washed with the uptake solution containing 2.8 mM CaCl2, prior to P uptake. Na-dependent P uptake (left panel) was calculated as the difference between P uptakes measured in the presence of calcium or of choline. The data represent the mean ± S.E. of three different experiments (n = 3) in which duplicates were obtained. KmA and Vmax values were determined from Eadie- Hofstee plot (right panel). KmA values (μM) were 167 ± 25.0 and 185 ± 12.0 for cells preincubated with and without calcium, respectively. Vmax values (nmol/10 min/mg protein) were 12.2 ± 0.8 and 17.7 ± 0.7 for cells preincubated with and without calcium, respectively (p < 0.05).

Opposite Effects of Low Concentrations of Benzyl Alcohol on Na-P, and Na-MGP Cotransports—The benzyl alcohol-induced inhibition of MGP in intact LLC-PK1 cells is in line with the reported inhibition of Na-glucose cotransport induced by benzyl alcohol in renal brush-border membrane vesicles (2) and by aliphatic alcohols in enterocyte brush-border membranes (28). In these studies the decrease of the order of membrane lipids was correlated with the inhibition of transport. It is noteworthy that benzyl alcohol, at 10 mM, inhibited to a similar extent Na-MGP cotransport in intact LLC-PK1 cells (this study) and Na-D-glucose cotransport in renal brush-border membrane vesicles (2). In the latter study
benzyl alcohol-induced modifications of transport occurred without any change in membrane permeability to D-glucose or Na. Moreover, benzyl alcohol, at 20 mM, was shown to decrease by 75% the number of phlorizin-binding sites in renal brush-border membranes (29). From these results it appears very likely that, in intact LLC-PK1 cells, the inhibition exerted by low concentrations of benzyl alcohol is related to a direct effect of the drug on the lipidic environment of the transport system. Similarly, an inhibitory effect of vitamin D3 on Na-coupled D-glucose uptake was also reported in renal brush-border membranes, together with a vitamin D3-induced decrease of membrane lipid order (4).

As regards the uptake of Pi, by brush-border membranes, it was shown to be enhanced by maneuvers which decreased the order of membrane lipids, i.e. increase in temperature (30), low phosphate diet (3), or administration of 1,25-dihydroxycholecalciferol in vitamin D-depleted rats (5, 6, 31). With low concentrations (up to 10 mM) of benzyl alcohol, we provide evidence for a direct, stimulatory effect of membrane fluidization on Pi uptake. Because Pi uptake is dependent on acidic-base status, the possibility that benzyl alcohol might exert an indirect effect on Pi uptake, related to a modification of the membrane proton gradient, must be considered. Indeed, increase in the fluidity of renal brush-border membranes was reported to lead to an increased proton permeability (32) or to an inhibition of sodium-proton exchange (33). Both events would result in intracellular (intravesicular) acidification, which stimulates Pi uptake (34, 35), while a decrease of extravesicular (extravesicular) pH has opposite effects (14, 34, 35). The effect of changes in membrane physical state on proton gradient is not univocal, however, since other studies reported a stimulatory effect of membrane fluidization on Na/H antiport (36).

That higher concentrations of benzyl alcohol inhibited both cotransports might be due either to a direct effect of the drug on the lipidic environment of the transport proteins, or to a dissipation of the sodium gradient as a consequence of an increased permeability to sodium, or of an inhibition of Na,K-ATPase activity (37).

Similar Effects of Opening of Tight Junctions and of Benzyl Alcohol on Na-coupled Uptakes—Preincubating cultured epithelial cells in calcium-deprived medium was demonstrated to open tight junctions (26, 27), which separate the apical domain of the plasma membrane, should induce changes in the activity of transport proteins located in the apical membrane similar to those induced by the fluidizing agent benzyl alcohol. As expected we observed that changes in Na-coupled P, and MgP uptake induced by opening of tight junctions resembled those elicited by benzyl alcohol at low concentration. These results are in line with those reported by Rabito (27) who observed that opening of tight junctions in confluent LLC-PK1, induced a decrease of apical MgP uptake. Phorbol esters, through stimulating protein kinase C (Ca++/phospholipid-dependent enzyme), induced both an opening of tight junctions, as judged from increased paracellular permeability (39), and an increased Na-dependent Pi uptake (40). Finally, experimental renal ischemia induced a loss of cellular polarity (7) and decreased sphingomyelin over phospholipids ratio, fluorescence anisotropy, and sodium-dependent D-glucose uptake in brush-border membranes (29). The consistency of these studies, in which opening of tight junctions was achieved by various means, argues against a direct role of calcium on transports, exerted through an effect of this cation on membrane fluidity (9), but rather for a role of tight junctions per se in preserving the functional characteristics of transport systems.

In conclusion the increase in fluidity of apical membranes of renal epithelial cells, resulting either from the effect of benzyl alcohol or from the opening of tight junctions, induced an increase in Pi uptake and a decrease of hexose uptake. These results might reflect differences in the lipid environments of the transport systems or differences in the sensitivity of these proteins to variations of their lipid environments (2, 37). Moreover, these results suggest that membrane fluidity might play a physiological role in the modulation of renal tubular, carrier-mediated transport.

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REFERENCES

Modulation of Na-coupled Uptakes by Membrane Fluidity