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Benzyl alcohol differently affects fluid phase endocytosis and exocytosis in renal epithelial cells

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Abstract

The effects of benzyl alcohol, a local anaesthetic commonly used for modification of membrane fluidity, on fluid phase endocytosis and on exocytosis have been investigated in MDCK cells. Fluid phase endocytosis in confluent cells monolayer grown on solid support was determined, at 37° C, by the uptake of the fluorescent dye Lucifer Yellow (LY). Exocytosis was estimated from the release of LY by cells preloaded with the dye. Addition of benzyl alcohol resulted in a concentration dependent inhibition of fluid phase endocytosis. For 30 mM benzyl alcohol, the inhibition obtained (83%) compared with that produced by preincubating the cells in a solution made hypertonic with 0.25 M sucrose. The inhibitory effect of benzyl alcohol was reversed within 30 min by washing. Endocytosis inhibition by benzyl alcohol was also observed in LLC-PK1 cells and OK cells, two renal epithelial cell lines of proximal tubule origin. In contrast, benzyl alcohol had no effect on exocytosis in LLC-PK1 cells, a limited but significant (15% at 30 mM) stimulatory effect on exocytosis in MDCK cells and a marked stimulatory effect (75% at 30 mM) in OK cells. These data demonstrate that benzyl alcohol affects endocytosis and exocytosis processes in renal epithelial cells. They suggest that membrane fluidity may alter membrane trafficking in living renal epithelial cells.

Keywords: MDCK cell; LLC-PK1 cell; OK cell; Endocytosis; Exocytosis; Fluidity

1. Introduction

Lipid composition and/or physical state affect the activity of numerous membrane enzymes and transport systems [1–3]. In isolated renal brush border membranes, a decrease in lipid order inhibits Na-coupled glucose transport [4] via a decrease in the number of operative carriers accessible at the membrane surface [5], but stimulates the P_i uptake by the Na-P_i transport system [6]. In intact cells, the activity of transporters and enzymes can be modulated via pathways other than changes in their lipidic environment. For instance, in renal epithelial cells, changes in the endocytic rate have a marked effect on sodium-phosphate co-transport [7–10]. To what extent changes in membrane fluidity affect endocytic processes, and thus might indirectly alter transport properties in renal epithelial cells remains unknown.

To answer this question, we have investigated the effect of the fluidizing agent benzyl alcohol on endocytosis and exocytosis properties of three renal epithelial cell lines, MDCK cells of distal segments origin [11], and LLC-PK1 and OK cells of proximal origin [12,13]. Benzyl alcohol was recently reported to inhibit low density lipoprotein receptor-mediated endocytosis in leukemic guinea pig B lymphocytes [14].

Because the endocytic pathway followed by renal transporters is not clearly defined [7–10], fluid phase endocytosis which results from both the non-mediated and the mediated coated-pits pathways was determined using Lucifer Yellow (LY) as a marker. This fluorescent molecule was shown to be a good marker of fluid phase endocytosis in numerous cell types [15–18] including MDCK cells [19], LLC-PK1 cells and OK cells [7,8]. Efflux of fluid phase endocytosis markers from preloaded cells is of common use to determine exocytosis [16,20–22]. Accordingly, renal epithelial cells were incubated with LY and exocytosis was estimated from the release of the dye in the presence or absence of benzyl alcohol.

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The results demonstrate that benzyl alcohol has a marked inhibitory effect on endocytosis in the three cell lines. This effect is rapidly reversed by simple washing. On the other hand, benzyl alcohol stimulates exocytosis in MDCK cells and OK cells. These data suggest that alterations in membrane fluidity markedly affect intracellular membrane trafficking in renal epithelial cells.

2. Materials and methods

2.1. Materials

Lucifer Yellow CH (LY) was purchased from Molecular Probes (Eugene, OR). Culture media were from Gibco BRL (France). dDAVP was obtained from Ferring (Malmo, Sweden). Other hormones and growth factors were from Sigma. Plastic ware was from Costar (Dutscher, France). All other reagents were of analytical grade.

2.2. Cell culture

MDCK and LLC-PK1 cells were cultured as previously described [23] in serum-free fully defined medium (SFFD) which consists of a 50:50 mixture of DMEM and Ham's F12 containing 15 mM Hepes, 15 mM sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 nM sodium selenite. For MDCK cells, SFFD was supplemented with: 50 ng/ml PGE1, 5 μg/ml transferrin, 5 μg/ml insulin, $5 \cdot 10^{-9}$ M triiodothyronine and $5 \cdot 10^{-8}$ M hydrocortisone. For LLC-PK1 cells, SFFD was supplemented with: 10 μg/ml transferrin, 25 μg/ml insulin, $5 \cdot 10^{-9}$ triiodothyronine, 10^{-8} M cholesterol, 10^{-7} M dDAVP, and $2 \cdot 10^{-7}$ M hydrocortisone.

For OK cells, 20 mM Hepes, 10 mM sodium bicarbonate and 0.5 mM sodium pyruvate were added to SFFD which was supplemented with: 5 μg/ml transferrin, 10 μg/ml insulin, $5 \cdot 10^{-9}$ M triiodothyronine, $5 \cdot 10^{-8}$ M hydrocortisone and 2.5% fetal calf serum. The three cell lines were plated either directly on 6-well plastic plates or on glass coverslips (30 mm diameter) disposed in Petri dishes and maintained in a humidified 5% CO_2-95% air at 37° C. Cells were used 2 or 3 days after they reached confluence.

2.3. Uptake of Lucifer Yellow

Cells were washed with SFFD and preincubated in either SFFD alone at 37° C or 4° C, or in SFFD made hyperosmotic (550 mosmol/l) with 0.25 M sucrose. After 30 min, LY was added from a stock solution (5 mg/ml), to give a 250 μg/ml final concentration. Benzyl alcohol at various concentrations was added simultaneously. After 30 min incubation at the desired temperature, endocytosis was stopped by six rinses with ice-cold phosphate saline buffer (PBS) plus 0.1% bovine serum albumin (BSA), pH 7.4, and four rinses with PBS alone. For blanks, cells were treated in the same way, but without LY addition. Cells were extracted with 1 ml/well Triton X-100 0.1% in bidistilled water during 60 min, at 37° C. Each well was

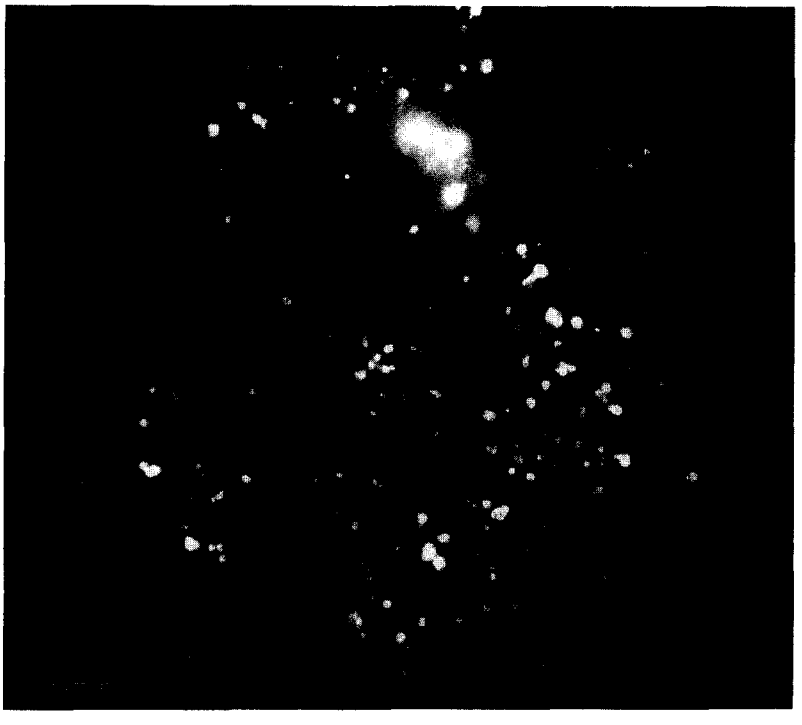

Fig. 1. Internalization of Lucifer Yellow by MDCK cells. MDCK cells incubated at 37° C with culture medium containing 0.25 mg/ml LY for 30 min were washed with ice-cold PBS plus BSA before examination under the microscope. Bar = 10 μm.

rinsed with 0.5 ml water and the pooled 1.5 ml extract was used for LY fluorescence quantitation and protein determination.

2.4. Exocytosis

Cells grown on glass coverslips were loaded with LY as previously. At the end of the loading period (usually 90 min), coverslips were rapidly transferred to beakers filled with 40 ml medium at the desired temperature (37° C or 4° C) containing or not benzyl alcohol. After 30 min, coverslips were rapidly rinsed with ice-cold PBS and cells were extracted as above for determination of remaining intracellular LY. Exocytosis was calculated as the difference between the intracellular LY content of cells incubated at 37° C and that of control cells maintained at 4° C during the same period. Benzyl alcohol had no significant effect on the intracellular LY content at 4° C.

2.5. Fluorescence measurements

LY content of cell extracts was determined by spectrofluorometry (SLM 4800S, Urbana, IL). Excitation wavelength was 428 nm. Emission was measured at 535 nm with a 495 nm cut-off filter placed in the emission light path. After blank subtraction, LY concentration of extracts was calculated from a standard curve generated with standard solution of LY in Triton X-100.

2.6. Fluorescence microscopy

After internalization of LY, cells on coverslips were examined under an inverted microscope (IMT2, Olympus), with a filter combination specially set for LY (Omega Optical, Brattleboro, VT). Micrographs were taken with 400 ASA Kodak Tri-X Pan films.

2.7. Protein determination

The protein content of cell extracts was assayed with the Coomassie Blue Reagent (Pierce, USA) according to Bradford [24], using bovine serum albumin as a standard.

2.8. Statistical analysis

Comparisons were done by one-way analysis of variance and significant differences were estimated with an interval of confidence of 95%.

3. Results

3.1. MDCK cells

Lucifer Yellow as a fluid phase endocytosis marker

In most circumstances, incubation of MDCK cells monolayer for 30 min at 37° C in LY resulted in a fluorescence pattern typical of endocytosis (Fig. 1). In particular, no significant labeling of the plasma membrane could be observed in accordance with the use of this probe as a fluid phase endocytosis marker. The fluid uptake by control cells was 428 ± 48 nl/mg protein per 30 min ($n = 18$) in this series of experiments. In accordance with the data of Tooze and Hollinshead [25], the presence of a tubular network filled with LY, which seemed in continuity with the typical endosomes, was observed in some of the cells monolayers (data not shown). From time to time, for unknown reasons, examination of the monolayers from a cell batch revealed an abundant labeling of the plasma membrane and of the intercellular spaces. These batches were not used for further experimentations.

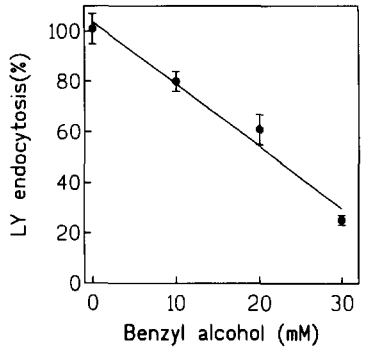

Fig. 2. Concentration-dependent inhibition of Lucifer Yellow uptake by benzyl alcohol in MDCK cells. Benzyl alcohol (10 to 30 mM) was added simultaneously with LY. Cells were incubated at 37° C for 30 min and the amount of LY internalized was determined as described in Materials and methods. The results are the means ± S.E. of three independent series of cell monolayers.

Effects of benzyl alcohol on fluid phase endocytosis

Addition of increasing amounts of benzyl alcohol, within a range of concentrations which do not impair cell viability [14,23,26] resulted in a linear decrease in fluid phase

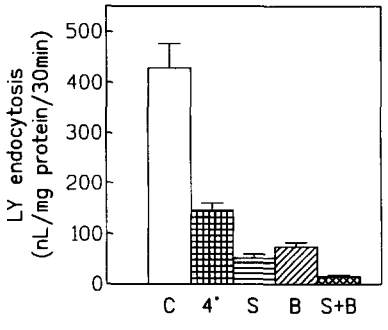

Fig. 3. Comparative effects of benzyl alcohol, hypertonicity and cold on Lucifer Yellow uptake by MDCK cells. Cells were preincubated in either SFFD alone or in SFFD made hypertonic (550 mosmol/l) with sucrose. LY uptake was determined after a 30 min incubation period with the dye. C: control at 37° C; 4: incubation at 4° C; S: incubation at 37° C in hypertonic SFFD; B: incubation at 37° C in SFFD plus 30 mM benzyl alcohol; S + B: incubation at 37° C in hyperosmotic SFFD plus 30 mM benzyl alcohol. Data are the means ± S.E. ($n \geq 9$).

endocytosis (Fig. 2). At 30 mM benzyl alcohol, endocytosis was inhibited by 83%. As shown by Fig. 3, addition of 0.25 M sucrose to the incubation medium, an experimental condition known to inhibit endocytosis in various cell types [16,27,28], reduced by 88% the uptake of LY by MDCK cells. Adding both sucrose and benzyl alcohol nearly abolished (97% inhibition) endocytosis. As expected, low temperature (4° C) inhibited the uptake, the inhibition was 66% after 30 min incubation.

The effect of benzyl alcohol on endocytosis was fully reversible. After a 30 min preincubation with 30 mM benzyl alcohol (37° C) followed by two washes in PBS, incubation for 30 min in absence of the fluidizing agent restored normal endocytic rates (treated/control = 0.99 ± 0.05, $n = 8$). A 15 min incubation in absence of benzyl alcohol prior the endocytosis assay resulted in a partial recovery (treated/control = 0.69 ± 0.05, $n = 7$).

Effects of benzyl alcohol on exocytosis

The effect of benzyl alcohol on exocytosis was determined on cells loaded with LY for 90 min at 37° C. Incubation of cell monolayers in an excess of medium devoid of LY resulted in a rapid exit of the marker (Fig. 4). During 30 min incubation at 37° C, control cells released 63 ± 3% ($n = 17$) of their intracellular LY, indicating the existence of an efficient recycling process. In contrast with its effect on endocytosis, benzyl alcohol did not affect exocytosis at 10 mM and had a limited but significant ($P < 0.05$) stimulatory effect (+15%) on the exocytosis at 30 mM (Fig. 5).

3.2. Effects of benzyl alcohol on proximal cell lines

The MDCK cell line originates from distal parts of the kidney tubule. Because the physical state properties of the plasma membrane of proximal cells likely differ from

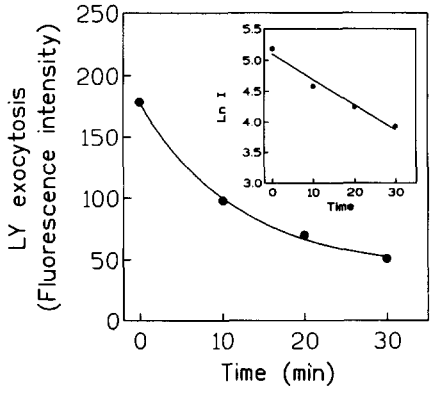

Fig. 4. Efflux of Lucifer Yellow from preloaded cells. MDCK cells were incubated for 90 min with LY as described in Materials and methods. At the end of the loading period cells on glass coverslips were rapidly transferred to beakers filled with 40 ml medium ($t = 0$). Remaining intracellular LY was determined as a function of time ($n = 3$). The curve was best fitted by a monoexponential plus a constant (R squared = 0.998). Inset: Log of LY fluorescence intensity remaining in cells as a function of time.

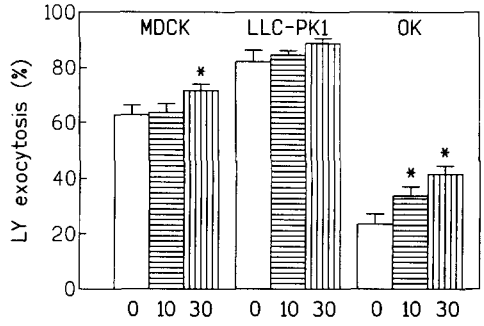

Fig. 5. Exocytosis of LY from preloaded renal epithelial cells. Cells were loaded with LY during 90 min at 37° C and transferred to beakers containing SFFD only (0) or SFFD + 10 mM benzyl alcohol (10) or SFFD + 30 mM benzyl alcohol (30). The intracellular LY remaining after 30 min incubation at 37° C was determined. For each cell line, the intracellular LY of cells maintained at 4° C during the same period was taken as a reference (intracellular LY content at $t = 0$). $^*P < 0.05$ as compared to controls.

those of distal cells [2], the effects of the fluidizing agent on fluid phase endocytosis were also investigated in LLC-PK1 and OK cells, two epithelial cell lines originating from the proximal tubule. In control conditions, fluid phase endocytosis was 180 ± 8 ($n = 10$) and 290 ± 30 ($n = 25$) nl/mg protein per 30 min for LLC-PK1 cells and OK cells, respectively. Although less efficient than in MDCK cells, benzyl alcohol at 30 mM still decreased fluid phase endocytosis by 55–60% in both proximal cell lines (78 ± 4 and 160 ± 10 nl/mg protein per 30 min for LLC-PK1 cells and OK cells, respectively). This inhibition was comparable to that obtained with hyperosmolar sucrose medium (sucrose/control = 47 ± 4% and 55 ± 4% for LLC-PK1 and OK, respectively). As for MDCK cells, adding benzyl alcohol to hyperosmolar treatment resulted in a further significant decrease in endocytosis in LLC-PK1 cells (78% inhibition). The effect of a similar treatment to OK cells was however not significant. The inhibitory effect of low temperature incubation (4° C) was less pronounced for LLC-PK1 (50% inhibition) than for OK cells (75% inhibition).

The efficiency of the process recycling LY in LLC-PK1 was even higher than in MDCK cells with 82 ± 4% of the marker endocytosed released in the medium within 30 min. On the other hand, the amount of LY that OK cells loaded for 90 min at 37° C released during the subsequent 30 min incubation period represented only 24 ± 6% ($n = 3$) of the intracellular load. Again, contrasting with its inhibitory effect on endocytosis, benzyl alcohol up to 30 mM had no significant effect on the exocytosis of LY by LLC-PK1 cells, and significantly enhanced (treated/control = 1.43 ± 0.10 and 1.76 ± 0.06, for 10 and 30 mM, respectively, $n = 3$) LY exocytosis in OK cells (Fig. 5).

4. Discussion

The present experiments demonstrate that benzyl alcohol is a potent inhibitor of fluid phase endocytosis in

MDCK, LLC-PK1 and OK renal epithelial cell lines. In contrast, benzyl alcohol has no effect on exocytosis in LLC-PK1 cells, exerts a limited but significant stimulatory effect on exocytosis in MDCK cells, and a marked stimulatory effect on exocytosis in OK cells. These data indicate that, for a range of concentrations used to probe the sensitivity of proteins to the physical state of their lipidic environment, benzyl alcohol markedly affects membrane trafficking in living renal epithelial cells. They suggest that, besides its effects on proteins activities in purified membranes, benzyl alcohol might also affect transport processes in living cells via alterations in endocytic/exocytic rates.

4.1. Effects of benzyl alcohol on endocytosis

Validity of LY as a marker of fluid phase endocytosis in various cell types including MDCK cells, as well as LLC-PK1 cells and OK cells was established earlier [7,8,15–18]. For OK cells, the endocytosis values obtained in the present experiments compared with those reported in the literature [7]. For MDCK cells, the values for endocytosis were much higher than those reported from cloned MDCK cells grown on filters [29,30], which likely resulted from the use of non-permeant support for the cultures in our study [19]. Although in the same range, the values determined for LLC-PK1 cells were significantly lower than those previously reported in the literature [7,8]. Again slight differences in the growth conditions might be involved in this observation.

Receptor-mediated endocytosis of transferrin and of low density lipoproteins in lymphocytes was shown to be markedly inhibited by benzyl alcohol [14]. Our results demonstrate that this inhibitory effect is also effective in renal epithelial cells: for the three cell lines benzyl alcohol had a marked inhibitory effect on fluid phase endocytosis. At 30 mM benzyl alcohol, a concentration that decreased by 80% transferrin endocytosis in lymphocytes, fluid phase endocytosis was inhibited by more than 80% in MDCK cells and by 55–60% in the two proximal cell lines. It was previously shown that for this range of concentration, benzyl alcohol does not impair cell viability [14,23,26], a conclusion supported here by the reversibility of the effect by simple washing. Fluid phase endocytosis results from the uptake of fluid during vesicles formation from both clathrin-coated and non-coated area of the plasma membrane. The relative importance of each pathway in the uptake of LY by renal cells in culture might depend on culture conditions and remains poorly known. Thus, whereas the fluid uptake into coated vesicles accounts for the fluid phase uptake in baby hamster kidney cells [31], its accounts for only 50% of the fluid phase uptake in CV-1 African green monkey kidney cells [15]. Endocytosis of horseradish peroxidase, a common used fluid phase endocytosis marker, by kidney collecting duct intercalated cells occurs via a non-clathrin coated vesicle pathway [32]. In several cell types, hyperosmolar sucrose interfers with receptor-mediated endocytosis by disrupting the coated pit cycle [16,28]. In the present experiments, addition of 0.25 M sucrose to the incubation medium resulted, for the three cell lines, in an inhibition of fluid phase endocytosis comparable with that induced by 30 mM benzyl alcohol. Although this might suggest that the inhibition of endocytosis by benzyl alcohol is entirely accounted for by an effect on coated pits, the absence of data on the effect of sucrose on the non-clathrin vesicle pathway in renal cells does not allow to reach such a conclusion. In fact, it has been proposed that in the coverslip-grown MDCK cells, fluid phase endocytosis primarily occurs via nonclathrin-coated vesicles [19]. Moreover, the observation that in MDCK cells and in LLC-PK1 cells, hyperosmolarity plus benzyl alcohol further reduced endocytosis suggests that the two maneuvers affected either slightly different populations of vesicles or different steps of the endocytosis processes.

4.2. Effects of benzyl alcohol on exocytosis

Efflux of fluid phase endocytosis markers, including LY, from cells loaded with such markers is a common method for estimating exocytosis [16,20–22]. Recycling of the endocytosed fluid back to the medium is associated with recycling of endosomes to the plasma membrane and occurs essentially prior to the delivery of internalized markers to lysosomes [30,33]. Following 90 min preincubation with LY, the amount of LY released in the medium by the two proximal cells lines during the subsequent 30 min incubation period varied from approximately 25% to 80% of the endocytosed marker for OK cells and LLC-PK1 cells, respectively. For MDCK cells, recycling accounted for 63% of the internalized LY. Likely due to the differences in culture conditions, this value was higher than that determined from MDCK cells grown on filter, where 45% of the content of apical early endosomes is recycled [30]. It is noteworthy, however, that in this last work the LY transcytosis rate was equal to the recycling, resulting in an intracellular LY accumulation of less than 10% of the total for 60 min incubations. The important variations in fluid recycling between the three renal epithelial cell lines we used might correspond to differences in their membrane traffic between early endosomes and lysosomes.

In contrast with its inhibitory effect on endocytosis, benzyl alcohol had either no effect (LLC-PK1 cells) or stimulated LY exocytosis in MDCK cells and OK cells. The effect of benzyl alcohol was in inverse correlation with the recycling efficiency of the cells, being nil for LLC-PK1 cells and maximal for OK cells. Because fluid recycling in MDCK cells essentially involves early endosomes [30], this suggests benzyl alcohol may alter membrane trafficking between endosomes and lysosomes. In accordance with this view, benzyl alcohol has been indicated to inhibit the fusion of endosomes with lysosomes [34].

Benzyl alcohol affects the activity of enzymes and of various transport systems in isolated membranes including the plasma membrane of renal epithelial cells [2,35] essentially via its effect on membrane fluidity. It also modifies the fluidity of the plasma membrane of living renal epithelial cells [36]. Alteration in membrane fluidity via modification of the fatty acyl composition of phospholipids in macrophages and fibroblasts, or via modification of the cholesterol content of lymphoblasts, was shown to influence the endocytic processes [37–39]. This strongly suggests that the membrane fluidizing effect of benzyl alcohol is involved in the inhibition of fluid phase endocytosis in renal cells. The observation that this local anaesthetic either unaffected or even stimulated the exit of LY from preloaded cells suggested a lower sensitivity of exocytic processes to the fluidizing properties of benzyl alcohol. Alteration of phospholipid transverse mobility [40,41], and a specific effect on trimeric G-proteins [42] might also participate to the effect of benzyl alcohol on intracellular membrane trafficking [43].

In renal epithelial cells, modifications of fluid phase endocytosis under various experimental conditions, like hyper- and hypotonicity [8,10], parathyroid hormone addition [44], gentamicin treatment [7], are associated with changes in the activity of sodium-coupled transport systems. It has been proposed that the number of carriers in the plasma membrane is regulated via membrane recycling [7]. Accordingly, our data suggest that changes in the transport activities of renal cells associated with modifications of their membrane lipid composition and physical state [2] might be partly mediated by an effect on endocytosis processes.

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