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Rapid report

Temperature dependence of endocytosis in renal epithelial cells in culture

Zahra Mamdouh [a], Marie-Cécile Giocondi [c], Raynald Laprade [b], Christian Le Grimellec [c,*]

[a] *INSERM U 426, Faculté de Médecine Xavier-Bichat, Paris, France*
[b] *G.R.T.M., Université de Montréal, Montréal, Québec, Canada*
[c] *C.B.S., INSERM U 414, Faculté de Pharmacie, 15 av. Ch. Flahault, 34060 Montpellier Cedex, France*

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Abstract

Temperature dependence of fluid-phase endocytosis was determined in two renal epithelial cell lines, MDCK cells and LLC-PK1 cells, using Lucifer Yellow or horseradish peroxidase as markers. For both cell lines, grown on solid support as a confluent monolayer, biphasic curves of marker uptake vs. temperature were obtained. The changes in slope occurred around 27°C, a critical temperature at which the lipids of the plasma membrane of MDCK cells enter in the gel state. Activation energies were significantly higher above 27°C (15–22 kcal/mol) than below that critical temperature (9–12 kcal/mol). These data indicate that changes in membrane physical state have marked effects on endocytic processes. They suggest that two mechanisms, with different activation energies are involved in the fluid phase endocytosis by renal epithelial cells in culture.

Keywords: Fluid-phase endocytosis; Endocytosis; Membrane physical state; Epithelium; MDCK cell; LLC-PK1 cell

Plasma membranes isolated from either the proximal tubule of the mammalian kidney or the intestine undergo lipid phase separations in a temperature range of physiological interest (for a review, see [1]). For the kidney, the phase separation, as detected by EPR and fluorescence polarization experiments, extends from ≈ 20°C to 42–48°C [2,3]. Below 20°C, membrane lipids are essentially in a highly ordered, gel-like, phase of low fluidity whereas temperatures higher than 42–48°C are required to obtain a liquid crystal ('fluid') behaviour. For renal epithelial cells in culture (MDCK cells), the lower end of the phase separation occurs around 27°C [4]. Numerous kidney membrane functions, including the activities of several transport systems, are sensitive to the physical state (membrane thickness?)/lipid composition of the plasma membrane [1]. Endocytosis, besides its essential role in proteins reabsorption by the proximal tubule [5], appears as an other way to regulate the activity of transport systems in renal epithelial cells [6]. We recently reported that the fluidizing agent benzyl alcohol alters the uptake of Lucifer Yellow (LY), a marker for fluid phase endocytosis, in cultured renal epithelial cells [7]. This suggests that, in renal cells, the endocytic process itself may also depends on the plasma membrane physical state. To further characterize the relationships between membrane physical state and endocytosis in renal cells, we have investigated in the present experiments the temperature dependence of fluid-phase endocytosis in cultured MDCK cells and LLC-PK1 cells, two epithelial cell lines that originate from the distal and proximal parts of the kidney tubule, respectively [8,9].

LLC-PK1 and MDCK cells were cultured in serum-free fully defined medium (SFFD) which consists of a 50:50 mixture of DMEM and Ham's F12 (Gibco BRL, France) containing 15 mM Hepes, 15 mM sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 nM sodium selenite [7]. The two cell lines were plated directly on 6-well plastic plates (Costar, Dutscher, France) and maintained in a humidified 5% CO_2-95% air at 37°C. Cells were used two or three days after they reached confluence.

Uptakes of LY (Lucifer Yellow CH, Molecular Probes, Eugene, OR) at various temperatures were performed as previously described [7]. Briefly, cells were washed two times with SFFD and preincubated for 30 min at the chosen temperature in the same medium. Care was taken

* Corresponding author. Fax: +33 67529623.

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to adjust the pH of the medium at 7.4 for each temperature assay. LY was then added from a stock solution (5 mg/ml) to give a 250 μg/ml final concentration. After incubation at the desired temperature, LY uptake 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. LY content of cell extracts was determined by spectrofluorometry (SLM 4800S, Urbana, IL), using 428 nm excitation and 535 nm emission wavelengths. The protein content of cell extracts was assayed with the Coomassie blue reagent (Pierce, USA), using bovine serum albumin as a standard. Endocytosis rates were calculated by correcting the total uptake of the markers for non-specific adsorption, determined at 4°C. For horseradish peroxidase (HRP, Sigma) experiments, at the end of the 30 min preincubation period at a given temperature, 1 ml of SFFD containing 0.1% BSA and 2 mg/ml HRP (final HRP concentration 1 mg/ml), pre-equilibrated at the same temperature was added. The uptakes were stopped by six washes with PBS containing 10^{-3} M mannose followed by four rinses with PBS. Cells were then extracted by Triton X-100 as described above. The amount of HRP taken up by each cell monolayer was determined according to Steinman and Cohn [10].

Temperature dependence of fluid-phase endocytosis in the epithelial cells LLC-PK1 grown as confluent monolayers was investigated in two series of experiments using either LY or HRP as markers. In these series, uptakes of the markers at 37°C (and at lower temperatures) were linear over 15 min period of incubation. At 37°C, endocytosis rates were 162 ± 18 (6 cells cultures) and 110 ± 22 (5 cultures) nl/mg of cell protein/10 min for HRP and LY, respectively. As shown by Fig. 1, both markers gave a similar temperature dependence for the fluid phase endocytosis. The fluid phase endocytosis was much more sensitive to temperature variations between 37°C and 25°C (≈ 75% inhibition) than between 25°C and 4°C. This biphasic character was also clearly observed (Fig. 1 inset) from the Arrhenius plot of total marker total uptake, i.e., before substraction of the non-specific adsorption. Fitting the curvilinear Arrhenius plot of Fig. 1 led to two main slopes intersecting at an inflexion point situated around 28°C (28 ± 2°C). LLC-PK1 cells originate from the proximal part of the kidney tubule. The apical membrane of kidney proximal tubular cells undergoes phase separation phenomena around room temperature [1]. It seemed reasonable to assume that the changes in slope recorded for LLC-PK1 cells corresponded to a change in their plasma membrane physical state. To strengthen the hypothesis of an effect of membrane physical state on fluid phase endocytosis in renal epithelial cells, we next investigated the effect of temperature on fluid phase endocytosis in MDCK cells, a cell line of distal origin in which we previously

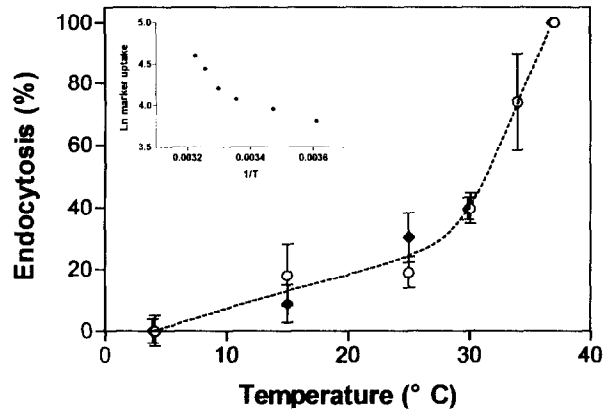

Fig. 1. Effect of temperature on fluid-phase endocytosis in LLC-PK1 cells. Monolayers of confluent LLC-PK1 cells were incubated with either HRP (filled symbols) or LY (open symbols) at the selected temperature. Data were standardized by giving a 100% value for the endocytosis, at 37°C, determined in each experiment. Data are the means ± S.E. of three independent experiments carried out in duplicate, for each marker. Inset: Arrhenius plot of the markers total uptake: values of HRP and LY uptake at each temperature were averaged.

established that plasma membrane in situ undergoes a marked change in physical state around 27°C [4]. For MDCK cells, uptake of fluid phase markers was linear for incubation times longer than 30 min. We previously demonstrated that the fluorescent membrane probe TMA-DPH added from the apical side labels only the apical membrane of MDCK cells grown on solid support [11]. This indicates that the cell monolayers are tight and, accordingly, that the fluid phase markers enter the cells via the apical plasma membrane. Endocytosis rate at 37°C was 421 ± 34 nl/mg of cell protein/ 30 min (8 cells cultures), a value comparable to those previously reported [7]. Fig. 2 illustrates the temperature dependence of LY endocytosis in MDCK cells. The curvilinear curve was close to that obtained for LLC-PK1 cells, most of the temperature-de-

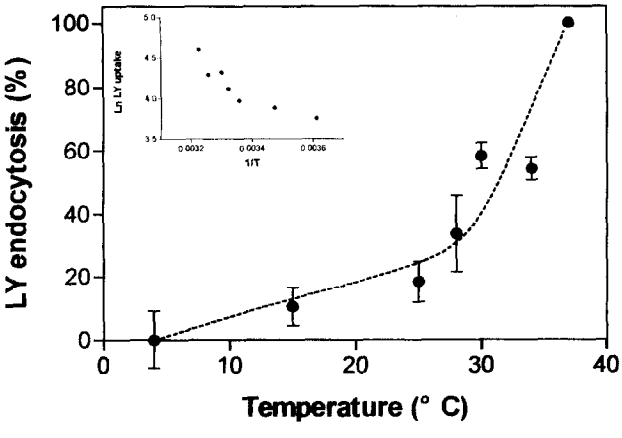

Fig. 2. Effect of temperature on fluid-phase endocytosis in MDCK cells. Monolayers of confluent MDCK cells were incubated with LY as described in Fig. 1. Data are the means ± S.E. of five independent experiments carried out in duplicate. Inset: Arrhenius plot of the total LY uptake.

pendent inhibition of endocytosis (70%) occurring between 37 and 25°C. Again, the best fit in the corresponding Arrhenius plot was obtained using two straight lines intersecting at 27 ± 2°C (see inset).

It is noteworthy that in an other epithelial cell type, i.e., the isolated rat hepatocyte, the endocytosis mediated by the asioaloglycoprotein receptor is also characterized by a biphasic Arrhenius plot, with a 'break-point temperature' of 20°C [12]. As in the kidney, a broad lipid phase transition occurs in isolated rat hepatocyte plasma membranes [13] and its lower end (15–18°C) is close to the 'break-point temperature' determined for receptor-mediated endocytosis. On the other hand, Arrhenius plots of fluid phase endocytosis in cells where phase separations have not been observed, like fibroblasts [14] and mouse peritoneal macrophages [15] give a single slope with activation energies between 17 and 25 kcal/mol. Taken together these data strongly support the view that changes in membrane physical state markedly affect fluid-phase endocytosis in renal cells.

A further insight into the relationships between membrane physical state and endocytosis was provided by the endocytosis activation energies. For LLC-PK1 cells, estimated activation energies for endocytosis were 22.1 ± 1.5 and 11.6 ± 1.5 kcal/mol for temperatures above and below the inflexion point temperature, respectively. The corresponding values for MDCK cells were 15.6 ± 3.2 and 8.7 ± 0.6 kcal/mol for temperatures above and below 27°C, respectively.

For temperatures above the inflexion point temperature, the energies determined in the present experiments were within the range of those previously reported for various cell types [14–16]. On the other hand, for temperatures below 27°C, fluid phase endocytosis activation energies decreased by 2–2.5-fold. This represented a very unusual situation: liquid crystal to gel phase lipid transitions and phase separations generally result in a large increase in the activation energy of transport processes and enzymes activities (for a review see [17]). Our observations therefore suggest that two different pathways, with different energy requirements were involved in fluid phase endocytosis by renal epithelial cells in culture. Multiple pathways for ligand internalization with different energy requirements were proposed to account for the effects of anoxia, phenylarsine oxide and monensin in rat hepatocytes [18]. In the same cells it was also shown that fluid phase endocytosis is less sensitive to ATP depletion than receptor-mediated endocytosis [19]. Finally, in MDCK cells, endocytosis occurs by clathrin-dependent as well as clathrin-independent mechanisms that can be regulated selectively [20]. Taken together these data suggest that, in renal epithelial cells in culture, fluid phase endocytosis might occurs by a non-coated pit pathway with low energy requirements for temperatures at which the membrane lipids are highly ordered, i.e., below 27°C. For this temperature range, fluid phase markers would not enter the cells via the receptor-mediated, clathrin-dependent, pathway which is blocked. Above this threshold, fluidization of the membrane might allow to turn on this second pathway, allowing the endocytosis of fluid phase markers by both pathways.

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