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Water permeation in Madin-Darby canine kidney cells is modulated by membrane fluidity

Marie-Cécile Giocondi and Christian Le Grimellec

INSERM U 251, Département de Physiologie, Faculté de Médecine Xavier Bichat, Paris (France)

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Simultaneous determinations of water and antipyrine permeations in monolayers of Madin-Darby canine kidney (MDCK) cells grown on a permeant support were done to study the relationships between water transport and membrane fluidity in these epithelial cells. The changes in permeation of the lipophilic non-electrolyte antipyrine were used to probe the modifications in membrane fluidity. In controls, the apparent diffusional permeability coefficient for water (P_{Dw}) was three times higher than the antipyrine's one, P_{DAp} ($4.2 \cdot 10^{-5}$ vs. $1.4 \cdot 10^{-5}$ cm s^{-1}). Addition of vasopressin or dibutyryl cyclic AMP to the monolayers induced a biphasic increase in antipyrine permeation with peak values at $t = 2$ min, 3–4-fold that of controls. Variations in water permeation were of similar amplitude and obeyed the same time course, leaving the water to antipyrine permeation ratios unchanged. Compound H7, an inhibitor of protein kinases, blunted the increase in permeation for both antipyrine and water. Finally, addition of the fluidizing agent benzyl alcohol to the monolayers resulted in a parallel increase in P_{DAp} and P_{Dw}. These results suggest that the physical state of membrane lipids may control water permeation in MDCK cells.

Introduction

Monolayers of Madin-Darby canine kidney (MDCK) cells, a renal epithelial cell line which retains differentiated properties of distal nephron segments [1,2], grown on a permeable support transport water at a low rate [3,4]. Addition of antidiuretic hormone to the cell monolayers induces a modest but significant biphasic increase in water permeation [4]. Using direct determination of membrane lipid order by fluorescence polarization, we recently reported that MDCK cells in suspension or grown on glass coverslips respond to vasopressin by a transient increase in membrane fluidity [5] whose time course resembled that of variations in water transport. From these observations, we hypothesized that, in this cell line, water transport might be regulated via the lipidic phase of the membrane.

Validity of this hypothesis was tested in the present study by the simultaneous measurements of water and antipyrine transport through MDCK cell monolayers grown on a porous support and treated by either vasopressin, dibutyryl cyclic AMP or the fluidizing agent benzyl alcohol [6]. Because fluorescence spectroscopy can hardly, for technical reasons, be applied to such preparation, antipyrine, a moderately lipophilic solute which enters cells via the membrane lipids, was used as a 'physiological probe' of membrane fluidity [7–9]. The results indicate that, under the various experimental conditions, the changes in the physical state of the membrane lipids can account for the changes in water transport in MDCK cells.

Correspondence: C. Le Grimellec, INSERM U 251, Faculté X. Bichat, 16 rue H. Huchard, 75018 Paris, France.

Materials and Methods

Materials

Tritiated water, [^{14}C]sucrose and [^{14}C]antipyrine were purchased from CEA (Saclay, France), and New England Nuclear (Boston, MA, U.S.A.). Culture media and reagents were from Eurobio (Paris, France). Plasticware and tissue culture treated, 3.0-μm pore, polycarbonate membranes (24 mm Transwell), were from Costar (Cambridge, MA, U.S.A.). Insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin E_1, sodium selenite, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H_7), and N^6,$O^{2'}$-Dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (DBcAMP) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). 1-Deamino[D-Arg^8]vasopressin (DDAVP) was obtained from Ferring

AB (Malmö, Sweden). MDCK cells (passage 60), were from Eurobio (Paris, France).

Methods

Cell culture. MDCK cells (passages 68–78) in a 50 : 50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 10% fetal calf serum were seeded on Costar Transwell and grown at 37 °C, in a 5% CO_2/95% air atmosphere, 48 hours later, the medium was changed for fresh medium without (apical side) or with (basal side) fetal calf serum, and the media were changed subsequently every four days. 24 hours prior to the experiment, both apical and basal media were changed, the basal medium, without serum, being supplemented as described by Taub et al. [10].

Transport experiments

The upper and lower medium of cells plated for 8–12 days on Transwell cell culture chambers was replaced by 1.8 and 3.2 ml, respectively, of phosphate-buffered saline (PBS) containing 0.5 mM $CaCl_2$, and 2 mM glutamine (pH 7.4). Transwell chambers disposed in 6-well cluster plates, were placed in a Lab Line (Lab Line Inc., IL. U.S.A.) incubator, equipped with an orbital shaker, set at 37 °C and 50 rpm. After a 10 min equilibration period, apical medium was replaced by 1.8 ml of the same buffer, pre-equilibrated at the same temperature and containing 0.5 μCi/ml of tritiated water (THO) and 0.2 μCi/ml of either [^{14}C]antipyrine or [^{14}C]sucrose ($t = 0$). At $t = 2$, 4, 6, 9, 12, 17, 22 and 27 min, 50 μl of the medium bathing the basal side of the cells was collected, transferred to scintillation vials, and counted by liquid scintillation. When added, DDAVP was injected in the lower chamber at $t = 7$ min. The same time was chosen for the addition in both the upper and lower chamber of DBcAMP or benzyl alcohol. The protein kinases inhibitor H7 [11], when used, was added in both the upper and lower media, 15 min prior to the addition of DBcAMP.

The permeability coefficients (cm s^{-1}) for diffusion of tritiated water (P_{Dw}) and antipyrine (P_{DAp}) was computed directly from a modified form of Fick's law [12]:

$$P_D = (C_{Bl} \cdot V)/(C_{Ap} \cdot A \cdot \Delta t)$$

where C_{Bl} and C_{Ap} are, respectively, the concentration of the isotope (cpm ml^{-1}) on the basolateral and apical side, V is the volume of basolateral medium, A is the membrane area (4.7 cm^2) and Δt is the duration of the flux period in seconds.

Results

Using the fluorescent probe TMA-DPH, we previously reported biphasic changes in membrane fluidity of MDCK cells, grown as a monolayer on glass support or suspended in phosphate-buffered saline, upon addition of DDAVP. The peak response occurred 2–5 min after the agonist addition, was dependent on intracellular cAMP, and partially inhibited by compound H7, an inhibitor of protein kinases [5]. Water and antipyrine fluxes through MDCK cells monolayers grown on transwell filter were therefore examined under comparable experimental conditions.

DDAVP induces a biphasic increase in both water and antipyrine permeation

As shown by Fig. 1, addition of 100 nM DDAVP to the medium bathing the basal side of MDCK cells grown on Transwell filters resulted in a marked increase in the transport of both [^{14}C]antipyrine and THO from the apical to the basal compartment defined by the monolayer. This effect was not observed when adding the V_2 agonist to the apical medium (data not shown). Calculation of the corresponding diffusional permeability coefficients (Fig. 2A), indicated that water permeability of MDCK monolayers, P_{Dw}, was modified in a complex manner: a maximal increase in permeation (from $(4.2 \pm 2.2) \cdot 10^{-5}$ to $(14.6 \pm 2.8) \cdot 10^{-5}$ cm s^{-1}, $n = 5$) was obtained during the first 2 min following the agonist addition. Permeability then rapidly dropped to a new equilibrium value approximately twice (183%) that of controls run in parallel (Fig. 2B). Antipyrine permeation obeyed the same pattern with a maximal increase in its diffusional permeability coefficient P_{DAp}, from $(1.4 \pm 0.6) \cdot 10^{-5}$ to $(5.8 \pm 1.8) \cdot 10^{-5}$ cm s^{-1} occurring within 2 min after the DDAVP addition, followed by a second phase in which the mean permeability ratio of treated to control monolayers was 177%. Thus, as illustrated by Fig. 2B, both the extent and the time course of the permeability changes brought by DDAVP were comparable for water and antipyrine.

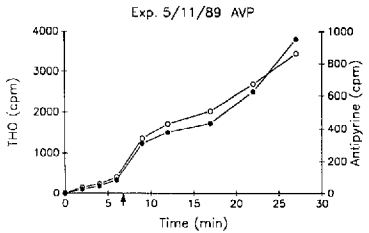

Fig. 1. Typical experiment. 50 μl samples of the medium bathing the basal side of the monolayer were collected at various times and counted by liquid scintillation. At $t = 7$ min (arrow) 100 nM DDAVP was added to the lower chamber. ○, [^{14}C]Antipyrine; ●, tritiated water (THO).

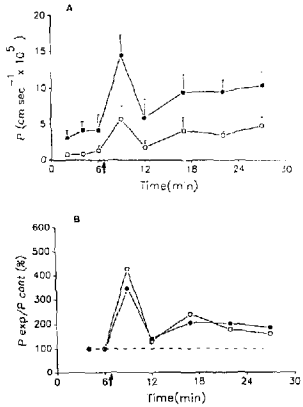

Fig. 2. Effect of DDAVP on antipyrine and water diffusional permeabilities. Diffusional permeability coefficients P, were calculated as described in Materials and Method. At $t = 7$ min 100 nM DDAVP was added to the lower chamber. ○ [^{14}C]Antipyrine; ●, tritiated water. (A) Results are means ± S.E. of five experiments. (B) Permeability ratio of treated cells to control cells run in parallel.

Determination of [^{14}C]sucrose permeation across control and DDAVP-treated monolayers, indicated that the increase in P_{Dw} was not linked to an increase in sucrose flux (Fig. 3). This made unlikely that the regulation of the water and antipyrine fluxes occurred via a transient opening of the tight-junctions.

Dibutyryl cAMP mimicks the effects of DDAVP on water and antipyrine permeation

The cyclic AMP dependence of the phenomenon was examined by replacing DDAVP by DBcAMP. Addition

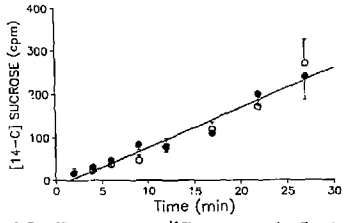

Fig. 3. DDAVP has no effect on [^{14}C]sucrose permeation. Experimental conditions are similar to those of Fig. 1. Addition of 100 nM DDAVP (arrow) had no effect on the apical to basolateral transfer of sucrose. Results are means ± S.E. of six experiments. ○, Treated cells; ●, control cells.

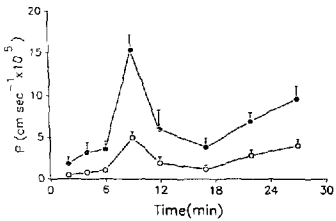

Fig. 4. Effect of DBcAMP on antipyrine and water diffusional permeabilities. DBcAMP (5 mM) was added to both apical and basal medium at $t = 7$ min. Results are means ± S.E. of six experiments. Symbols as in Fig. 2.

of 5 mM DBcAMP to the medium bathing MDCK cell monolayers resulted in an increase in antipyrine and THO permeation whose time course, biphasic character and amplitude were close to those induced by the V_2 agonist. Thus, P_{DAp} and P_{Dw} increased to $(5.0 \pm 0.7) \cdot 10^{-5}$ and $(15.3 \pm 1.9) \cdot 10^{-5}$ cm s^{-1} ($n = 6$), respectively, within the 2 min following DBcAMP addition (Fig. 4). The transient return towards controls although more marked than that obtained with DDAVP, was followed by a second increase of P_{Dw} and P_{DAp} towards the 'plateau' values reached using the V_2 agonist. As previously, the relative changes in antipyrine and water permeability brought by the cAMP analog were similar. Accordingly, the P_{Dw}/P_{DAp} ratio of treated monolayers at the peak (3.1) was not significantly different from that determined at $t = 6$ min (3.5), just before DBcAMP addition.

As previously observed for the changes in membrane fluidity detected by the fluorescent probe TMA-DPH, pretreatment of the monolayers by compound H7 (100 μM) inhibited by more than 60% (Fig. 5) the increase in antipyrine and THO permeation promoted by

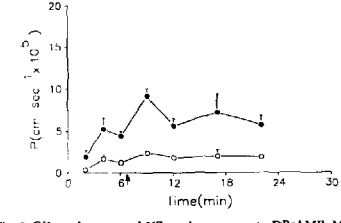

Fig. 5. Effect of compound H7 on the response to DBcAMP. Monolayers were incubated with 100 μM H7 during 15 min before starting the flux experiments. The same concentration of inhibitor was maintained throughout the flux experiment. DBcAMP (5 mM) was added at $t = 7$ min. Results are means ± S.E. of five experiments.

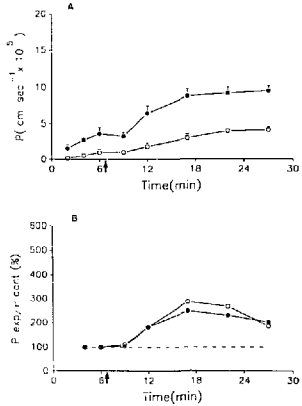

Fig. 6. Effect of benzyl alcohol on antipyrine and water permeations. Benzyl alcohol (30 mM) was added on the apical side of the monolayer at $t = 7$ min. Symbols as in Fig. 2(A) Means ± S.E. of five experiments. (B) Permeability ratio of treated cells to control cells run in parallel.

DBcAMP: peak values were $(2.4 \pm 0.3) \cdot 10^{-5}$ and $(9.2 \pm 0.9) \cdot 10^{-5}$ cm s^{-1} ($n = 5$) for P_{DAp} and P_{Dw}, respectively. This pretreatment alone had no significant effects on the basal values of P_{Dw} ($4.4 \cdot 10^{-5}$ cm s^{-1}) or of P_{DAp} ($1.2 \cdot 10^{-5}$ cm s^{-1}).

Benzyl alcohol increases identically water and antipyrine fluxes

If, as suggested by fluorescence experiments and by determinations of THO and antipyrine permeability coefficients changes in water fluxes upon DDAVP addition are, in MDCK cell monolayers dependent on changes in membrane fluidity, then conversely, modification of membrane fluidity must affect THO and antipyrine transport similarly. As shown by Fig. 6, addition of 30 mM of the fluidizing agent benzyl alcohol, a concentration that, in this cell line, decreases by approx. 5% the anisotropy of TMA-DPH, and has no effect on the basal level of intracellular cAMP [6], more than doubled the permeability for both solutes (benzyl alcohol to control ratios of 2.9 for P_{DAp} and 2.5 for P_{Dw}, at $t = 10$ min).

Discussion

The present experiments demonstrate that, in MDCK cell monolayers, variations in the permeation of the lipophilic non electrolyte antipyrine were accompanied by parallel modifications in water permeation. These data suggest that, in MDCK cells, water movement essentially occurs through the lipidic phase of the plasma membrane. They also suggest that the increase in water permeation that occurs in MDCK cells treated by AVP is mediated by changes in membrane fluidity.

Antipyrine and water permeation in control cells

Permeation of lipophilic non electrolytes generally occurs through the lipidic phase of membranes and, for a given membrane, is a function of its fluidity or physical state [13–15]. Changes in the permeation of these lipophilic non electrolytes have therefore been used as a physiological probe of modifications in the physical state of membrane lipids [7,8,16]. Diffusional permeability coefficients of antipyrine in the various experimental series were in the range of that reported for toad urinary bladder [7] and about two times less than those reported for rabbit cortical collecting tubules [8]. Comparison with the data obtained by Al-Zahid et al. [8] and by Finkelstein [15] shows that such low values are found in lipid bilayer membranes made of sphingomyelin and cholesterol.

This would agree with the highly ordered character of the apical membrane of MDCK cells [17], likely related to the high content of sphingolipids in renal plasma membranes [18,19].

In accordance with previous data [3,4], water permeability of MDCK cell monolayers was in the lower range of the values reported for biological membranes: the P_{Dw} values obtained were 2–3-times lower than those reported for amphibian skin or toad urinary bladder [9]. However, it is likely that in these epithelia even when unstimulated, water crosses the luminal membrane primarily through aqueous pores [20]. In absence of literature data available on the free diffusion coefficient of antipyrine in water at 37°C, the diffusional permeability coefficients determined from the present experiments were not corrected for the presence of unstirred layers or post-apical membrane resistances [9,12] and have therefore to be considered as apparent diffusional permeability coefficients. The relative influence of unstirred layers on the water diffusional permeability coefficient measured in MDCK cells can be evaluated [9,12] using a free diffusion coefficient for water of $3 \cdot 10^{-5}$ cm^2 s^{-1} at 37°C [21]. Assuming a 1000 μm thickness for the unstirred layer, i.e., a value exceeding those determined from gallbladder and, at zero stirring rate, for isolated cornea and jejunum [22,23], the P_{Dw} value would be increased by less than 20% ($0.53 \cdot 10^{-4}$ cm s^{-1}). In sphingomyelin/cholesterol lipid bilayer membranes the ratio of the water permeability coefficient to the permeability coefficient of *n*-butyramide, a lipophilic solute of diffusional permeability coefficient close to that of antipyrine, is between 2.8 and 4.1 [15]. The P_{Dw}/P_{DAp} ratios determined from the

control periods of the different experimental series fall within this range, thus suggesting that, like in tracheal [24] and intestinal [25] epithelial cells, the movement of water across the plasma membrane of unstimulated MDCK cells occurs essentially via a solubility-diffusion mechanism [26,27]. Comparisons with osmotic water permeability coefficients, determinations of the activation energy for water permeation and determination of the sensitivity to mercurial sulfhydryl reagents would be necessary to firmly establish water permeation mechanisms in MDCK cells [32]. On the other hand, a strong support to the view that water permeates through the membrane lipids was provided by benzyl alcohol experiments: should the water permeate through 'water channels', benzyl alcohol would have promoted a much larger increase in antipyrine than in water fluxes, which clearly was not the case, both antipyrine and water permeation being more than doubled upon addition of 30 mM of the fluidizing agent. The observation that P_{Dw} and P_{DAp} increased by the same factor also rendered unlikely that the change in membrane fluidity might have acted indirectly by increasing the flux of water through putative water channels.

Antipyrine and water permeation in treated cells

Qualitatively, the present measurements of antipyrine fluxes in DDAVP or DBcAMP treated monolayers are in good agreement with the fluorescence spectroscopy data obtained on MDCK cell suspensions or on monolayers grown on glass coverslips [5]. Thus, cAMP-dependent changes in membrane fluidity also occur for MDCK cell monolayers grown on a permeable support. In fluorescence experiments on monolayers, however, the peak value was recorded 5 min after the agonist addition and was equivalent to the fluidization induced by 30 mM benzyl alcohol. Considering that the changes in antipyrine permeation reflect the changes in the membrane fluidity of the cell monolayer, the difference in the time course and the amplitude of the response between the two series of experiments might be related to the cell heterogeneity of cultures: MDCK cells have multiple cellular origin and the growth and differentiation of a cell type, and its responsiveness to antidiuretic hormone are affected by the culture conditions [6,28,29]. In the present experiments the maximal effect, reached within 2 min after the stimulation by DDAVP or by DBcAMP, corresponded to a 4-fold increase in P_{DAp} and was accompanied by an equivalent increase in water permeation. Although of lesser amplitude, a similar biphasic increase in water permeation of MDCK cell monolayers in response to [Arg^8]vasopressin has been reported previously [4]. The difference in amplitude of the responses might be related to cell heterogeneity as discussed above. In the absence of any significant changes in sucrose permeation, an index of the intactness of tight-junctions, the observation that P_{Dw}/P_{DAp} ratios were not significantly modified by DDAVP or DBcAMP additions, suggests that, as in controls, water permeates essentially through the lipidic phase of treated cells [20]. In accordance with such a view, an inhibition of water transport, leading to an unmodified P_{Dw}/P_{DAp} ratio, was also obtained when inhibiting the DBcAMP-stimulated antipyrine permeation by compound H7. Moreover, a highly significant correlation ($r = 0.975$, slope 0.38) was obtained when antipyrine permeability was plotted against water permeability in control and at the peak response evoked by DDAVP, DBcAMP, DBcAMP + H7, and benzyl alcohol.

The present experiments do not allow to know if the DDAVP-dependent changes in membrane fluidity were associated or not to the fusion of intracellular vesicles having different permeability properties [30–32]. However, (a) no evidences for fusion events were found when investigating by fluorescence the changes in fluidity of the cells grown on glass supports [5]; (b) if fusion had occurred it was in a way such as to maintain the water/antipyrine permeability ratio unchanged making unlikely that the vesicles contained significant amounts of water transporters.

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