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Chloride-activated Water Permeability in the Frog Corneal Epithelium

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Abstract. We have previously reported that the isolated frog corneal epithelium (a Cl^--secreting epithelium) has a large diffusional water permeability ($P_{dw} \approx 1.8 \times 10^{-4}$ cm/s). We now report that the presence of Cl^- in the apical-side bathing solution increases the diffusional water flux, J_{dw} (in both directions) by 63% from 11.3 to 18.4 $\mu l\ min^{-1} \cdot cm^{-2}$ with 60 mM [Cl] exerting the maximum effect. The presence of Cl^- in the basolateral-side bathing solution had no effect on the water flux. In Cl^--free solutions amphotericin B increased J_{dw} by 29% but only by 3% in Cl^--rich apical-side bathing solution, suggesting that in Cl^--rich apical side bathing solution, the apical barrier is no longer rate limiting. Apical Br^- (75 mM) also increased J_{dw} by 68%. The effect of Cl^- on J_{dw} was observed within 1 min after its addition to the apical-side bathing solution. $HgCl_2$ (0.5 mM) reduced the Cl^--increased P_{dw} by 31%. The osmotic permeability (P_f) was also measured under an osmotic gradient yielding values of 0.34 and 2.88 ($\times 10^{-3}$ cm/s) in Cl^--free and Cl^--rich apical-side bathing solutions respectively. It seems that apical Cl^-, or Cl^- secretion into the apical bath could activate normally present but inactive water channels. In the absence of Cl^-, water permeability of the apical membrane seems to be limited to the permeability of the lipid bilayer.

Key words: Water permeability — Frog cornea — Chloride activation — Unidirectional water fluxes — Net water fluxes — Chloride-secreting epithelium

Introduction

The external surface of the cornea is covered by a multilayered, tight epithelium, whereas a monolayered endothelium overlays the internal corneal surface in contact with the aqueous humor. Between these two cell layers is a collagenous stroma that has a marked tendency to swell, thereby becoming disorganized and rendering the cornea opaque. It is mainly due to the continuous functioning of the endothelium and its "fluid pump," which moves fluid from the stroma into the anterior chamber of the eye, that the stroma stays dehydrated and the cornea transparent (Maurice, 1972; Fischbarg & Lim, 1974; Hodson & Miller, 1976). Classically, the function of the epithelium has been relegated to that of a protective barrier with no defined role in fluid movement. A few studies have shown that the corneal epithelium may be capable of fluid secretion (Klyce, 1975; Candia, 1976), but the driving force and the pathways for water movement across the corneal epithelium remain poorly defined. During the past 25 years, the frog corneal epithelium has been characterized as a Cl^--secreting epithelium (Candia, 1984). In fact, after the initial finding of Cl^- transport by this epithelium (Zadunaisky, 1966) descriptions of the responses of the corneal epithelium to epinephrine (Chalfie, Neufeld & Zadunaisky, 1972), Ca^{2+} ionophore (Candia, Montoreano & Podos, 1977), and loop diuretics (Candia, 1973) were among the initial reports on what came to be regarded as the typical physiological and pharmacological responses of Cl^--secreting epithelia. Challenging the view that the corneal epithelium is a relatively impermeable barrier to water was a report indicating values in the toad cornea for diffusional permeability, P_{dw}, in the order of 10^{-4} cm/s, a level that was reduced by 30% by cell acidification (Parisi, Candia & Alvarez, 1980). More recently Fischbarg (1992) has pointed out that the rabbit corneal epithelium exhibits a comparatively high osmotic water permeability of about $80–140 \times 10^{-4}$ cm/s. Thus, it is possible that the corneal epithelium contains specific water pathways, and it could contribute to the dehydration of the corneal stroma.

With the recent discovery of CHIP28 (Neilsen et al., 1992; Preston et al., 1992; Van Hoek & Verkman, 1992) and other water channels, we decided to reinvestigate,

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from a physiological perspective, water movement across the isolated frog corneal epithelium. Our results suggest that a fraction of the transepithelial water movement is mediated by an apical element that is activated by Cl^- and blocked by $HgCl_2$.

Materials and Methods

All experiments were performed on the isolated corneal epithelium of the frog *Rana catesbeiana.* Frogs were obtained from a local supplier and kept in fresh-water tanks. They were double pithed just prior to the dissection of the cornea. The corneal endothelium was scraped off and the corneas, consisting of the epithelium with the stroma (about 150 μ thick), were mounted as a partition between hemichambers. Two types of chambers were used. Chamber "a," a two-compartment chamber for measurements of diffusional water permeability using tritiated water (^3H_2O), and Chamber "b," a three-compartment chamber accommodating two corneas for measurements of osmotic water permeability by volumetric detection. Chamber "a" was a modified Ussing-type chamber similar to that used for electrophysiological studies (Candia, 1972). The volume of the "hot" side compartment was increased to about 30 ml. This was necessary to maintain the specific activity of the "hot" side within 10% of its initial value. Typically, at a maximum flux rate of 0.8 ml/hr the specific activity will decrease by 8% (2.4/30) in 3 hr. Nevertheless, periodic samples were taken from the "hot" side and fluxes were calculated based on the activity corresponding to the time of the flux being calculated. Sampling and replacement kept the "cold" side activity below 2% of that in the "hot" side. Vigorous stirring was provided by continuous recirculation with a jet of fluid directed to the surface of the cornea (Candia, Mia, & Yorio, 1993). About 1.3 μCi/ml of ^3H_2O (ICN Biomedicals, Costa Mesa, CA) was added to one side, and samples were taken from the "cold" side every 15 min. At least four flux periods were determined for each experimental condition. A full experiment usually consisted of two or three conditions of the "before–after" type so that paired comparisons could be made. The chambers also provided the necessary salt bridges for electrical determinations. The corneas were continuously short circuited except for a few seconds every 10 min for measurements of the electrical resistance. In the tables, the diffusional water flux, J_{dw}, is expressed in $\mu l \cdot min^{-1} \cdot cm^{-2}$. The diffusion permeability coefficient, P_{dw}, is expressed in cm/s and is given by:

$$P_{dw} = J_{dw}/(A \cdot V_w \cdot C_w)$$

where A is the area of the membrane (cm^2), V_w is the partial volume of water (cm^3/mol), C_w is the concentration of water (mol/cm^3), and J_{dw} is the measured flux in cm^3/s.

Chamber "b" was a regular Ussing-type chamber with a center compartment separating the two halves. With this arrangement, two corneas facing each other with their stromal sides towards the center compartment were mounted. The center compartment was totally closed except for two openings. One, controlled by a valve, was used to fill and regulate the volume of the center compartment. The second was connected to the graduated cylinder of a 20 μl syringe, which allowed for visual detection of 1 μl changes (Fig. 1). The level of the cylinder could be reset by opening the valve controlling the flow from/to the filling syringe. Because of the rigidity of the stroma, setting the cylinder level 2–3 cm above the external compartment levels maintained the corneas in a fixed position. Swelling of the stroma does not affect the cylinder level because imbibed stromal water is retained in the center compartment. Changes in the cylinder level represent actual movement of fluid between the external compartments and the center compartment across the corneas. Since the exposed area of each cornea is 0.5 cm^2, the changes in transcorneal volume flow are recorded in μl/cm^2. Changes of solutions in the external compartments can be easily accomplished without opening or disturbing the center compartment. In the results, the volume flow, J_v, is expressed in $\mu l \cdot min^{-1} \cdot cm^{-2}$. The osmotic permeability coefficient, P_f, is expressed in cm/s and can be calculated from the following expression:

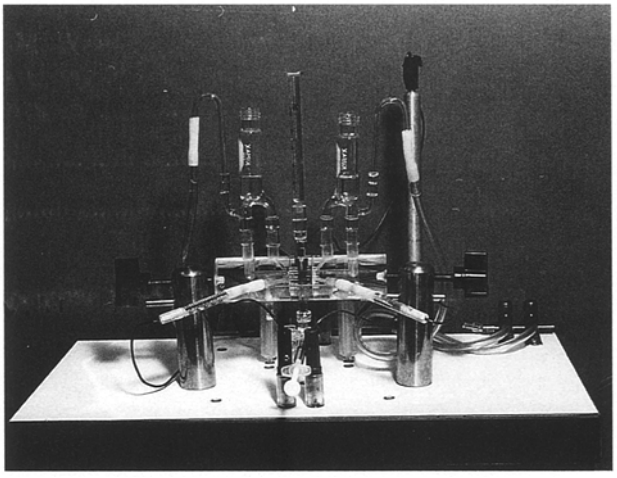

Fig. 1. Modified Ussing-type chamber for the measurement of net water flow (chamber "b" described in Materials and Methods). A center compartment connects to the vertically placed syringe cylinder and to another syringe (located below the chamber) used to calibrate the volume of the center compartment. Two corneas (one on each side) were placed between the center compartment and each outer hemichamber. Salt bridge-Ag/AgCl electrodes were connected to the outer compartments to monitor the transcorneal electrical resistance.

$$P_f = J_v/(A \cdot V_w \cdot \Delta C_s)$$

where J_v is in cm^3/s and ΔC_s is the difference in solute concentration (mol/cm^3). In this type of experiment, the corneas were at open circuit and the resistance of the two corneas in series was periodically monitored. The temperature of the system was carefully maintained constant at 22°C.

The basic Ringer's solution used during the dissection and some of the experiments had the following composition (in mM): 103.4 Na^+, 2.5 K^+, 1.0 Ca^{2+}, 1.2 Mg^{2+}, 75.0 Cl^-, 25.0 HCO_3^-, 1.8 SO_4^{2-}, 2.0 HPO_4^{2-}, 0.7 $H_2PO_4^-$, 2.0 gluconate, and 26.0 glucose. When a Cl^--free solution was necessary, SO_4^{2-} or gluconate replaced Cl^-, and the osmolarity was compensated for with sucrose when necessary. The pH of the solutions when bubbled with air was 8.6. Amphotericin B was from Squibb, Princeton, NJ and $HgCl_2$ was reagent grade from Sigma, St. Louis, MO.

Results

Since the cystic fibrosis transmembrane conductance regulator (CFTR) is a glycoprotein that can function not only as a cAMP-stimulated channel for Cl^- (Clarke et al., 1992), but also as an aqueous pathway (Hasegawa et al., 1992), we thought that water may also move through the Cl^- channel in the apical membrane of the corneal epithelium. Although our preliminary hypothesis turned out

Table 1. Unidirectional water fluxes across the isolated frog corneal epithelium ($\mu l \cdot min^{-1} \cdot cm^{-2}$)

	Cl-free Ringer		Cl-rich Ringer	
	T-to-S	S-to-T	T-to-S	S-to-T
	12.0	12.5	17.8	18.0
	12.2	9.3	18.3	17.2
	12.3	10.0	18.3	17.7
	12.0	10.5	19.3	17.0
	12.2	9.5	20.5	16.8
	12.5	11.2	18.5	17.0
	11.8	10.7	18.7	18.7
	12.2	9.7	19.7	18.8
	11.5	9.3	19.3	18.7
	11.5	11.2	20.5	18.8
	11.7	10.7	19.5	17.8
	11.7	9.3	18.8	18.2
	12.3	10.2	18.8	17.7
	10.7	9.7	18.0	17.3
	12.0	9.3	18.2	17.7
	12.0	13.0	18.3	17.3
	12.5	13.3	18.7	16.8
	11.8	11.0	20.3	17.3
	12.0	13.2	19.7	17.2
	12.0	12.5	20.3	18.3
Mean	11.9	10.8	19.1*	17.7*
± SE	0.09	0.31	0.19	0.15
P_{dw} (cm/s)	2×10^{-4}	1.8×10^{-4}	3.2×10^{-4}	3.0×10^{-4}

* Mean fluxes in Cl-rich Ringer were significantly larger than mean fluxes in Cl-free Ringer. $P < 0.01$ compared as groups. T-to-S: Tear-to-Stroma. S-to-T: Stroma-to-Tear.

to be incorrect, we found an unexpected effect of Cl^- on the water permeability of the isolated frog corneal epithelium.

Unidirectional Water Fluxes

Table 1 shows unidirectional water fluxes across 80 individual corneal epithelia. While opposite fluxes were similar for the same type of bathing solution, fluxes across corneas bathed in the Cl^--rich solutions were about 60% larger than in Cl^--free solutions. Initially we reasoned that Cl^- and water may be sharing the same channel and only when Cl^- was diffusing out of the cell across the apical membrane would water move through the channel. However, although Table 1 shows that water moved equally in either direction, Cl^- movement (recorded as a short-circuit current, I_{sc}) was in the stroma-to-tear direction. Furthermore, addition of Cl^- secretagogues such as epinephrine, forskolin and the Ca^{2+} ionophore A23187 produced the typical 40–80% stimulation of the I_{sc} and Cl^- conductance without any effect on water fluxes (*results not shown*). Thus, it seemed that it was the presence of Cl^-, rather than its movement, that was influencing the movement of water. With this in mind, experiments were then done in which Cl^- was differentially added or removed from each bathing solution (isosmotic replacement with SO_4^{2-} or gluconate). The results, which are shown in Table 2, point to the presence of Cl^- on the apical-side solution as the factor responsible for the increases in water permeability. Various protocols were then completed to collectively demonstrate that it was, indeed, the addition or removal of Cl^- from the apical bathing solution that changed the water permeability irrespective of the presence of Cl^- in the basolateral bathing solution.

We have previously shown that amphotericin B creates ion channels on the apical side of the corneal epithelium (Candia, Bentley & Cook, 1974) that are also permeable to water (Parisi et al., 1980). After amphotericin B treatment, the basolateral membrane becomes rate limiting to the movement of ions, and possibly to water. If the latter were true and if apical Cl^- was indeed increasing apical water permeability, Cl^- should have little or no effect after amphotericin B and *vice versa.* Table 3 shows the combined effects of amphotericin B and Cl^- on water permeability. The top panel shows the typical effect of amphotericin B on corneal epithelial water permeability in Cl^--free solutions. The center panel shows that when the corneas are bathed in Cl^--rich solutions, the control fluxes are higher and amphotericin B has no further effect. The bottom panel reiterates the lack of an amphotericin B effect on water permeability after Cl^- stimulation and also shows the reverse effect, a lack of a Cl^--induced permeability increase after amphotericin B. A simple interpretation, consistent with these results is that amphotericin B- and Cl^--induced water permeability reside in parallel at the apical membrane. If either channel is operational, or activated, the basolateral side becomes rate limiting and activation of an apically resident additional channel is superfluous. We then studied the activation of the water permeability as the concentration of Cl^- on the apical-side solution was increased in a stepwise fashion. Figure 2 is the representation of these experiments. A small activation appears at 35 mM and seems to reach a maximum at about 60 mM [Cl^-]. We also wanted to study the time course of the activation. Typically, during water flux experiments, samples were taken every 15 min, and the increase in water flux was observed in the first 15-min period after the addition of apical Cl^-. It was possible, therefore, that the effect of Cl^- was considerably faster. We performed several experiments in which sampling was made every minute immediately prior to and following the change from a Cl^--free to a Cl^--rich condition. The results, shown in Fig. 3, indicate that the apical water permeability is activated within a minute, or possibly less, of Cl^- addition. The points in Fig. 3 represent the average value at the middle of a period between two consecutive samples. Spacing of the sampling did not affect the

Table 2. Sidedness of the Cl effect on the unidirectional water fluxes across the isolated frog corneal epithelium

	Cl-free (both sides)	Cl-rich (apical)	Cl-rich (basolateral)	Cl-rich (both sides)
Mean	9.3	15.2[a]		
± SE	0.47	0.78		
(n = 19)				
Mean	10.8		10.5	15.8[a]
±SE	0.84		0.99	0.77
(n = 4)				
	Cl-rich (both sides)	Cl-free (apical)	Cl-free (basolateral)	Cl-free (both sides)
Mean	17.8	10.6[b]		
± SE	0.75	0.83		
(n = 15)				
Mean	17.2		16.6	10.2[b]
± SE	1.12		1.18	0.81
(n = 5)				

[a] Significantly larger than corresponding Cl-free control. $P < 0.01$ as paired data.
[b] Significantly smaller than corresponding Cl-rich control. $P < 0.01$ as paired data.
Fluxes are in $\mu l \cdot min^{-1} \cdot cm^{-2}$

steady state of the flux values, giving support to the observed quick increase in flux. Once the Cl^- effect was clearly established, we tested the effect of another halogen, Br^-. As shown in Table 4, the addition of 75 mM Br^- to the apical side produced a stimulation of the water fluxes identical to, if not slightly larger than, that produced by Cl^- addition.

Mercurials are known to block water channels in many systems (Verkman, 1989). Thus, we tested the effect of 0.5 mM $HgCl_2$ on water fluxes across the corneal epithelium. As shown in Table 5, $HgCl_2$ addition reduced the Cl^--stimulated water flux from 14.5 $\mu l \cdot min^{-1} \cdot cm^{-2}$ to a value normally observed in the absence of Cl^-. After amphotericin B, $HgCl_2$ produced less of a reduction, as if the amphotericin B-induced channels were not affected by Hg^{2+}.

The effect of $HgCl_2$ on the I_{sc} was variable, but in most cases it increased. On the other hand, Hg^{2+} produced a substantial reduction of the transepithelial electrical resistance from 1.95 ± 0.27 to 0.23 ± 0.20 $K\Omega \cdot cm^2$ ($n = 16$; $P < 0.001$). Such a large decrease in resistance (with a moderate increase in I_{sc}) probably represents an increase in paracellular conductance.

Net Water Flux

Once we established that apical Cl^- increased the diffusional water permeability of the corneal epithelium in a few experiments, we examined whether or not the osmotic permeability, P_f, was also increased by Cl^-. For these experiments, the closed-chamber device described in Materials and Methods was utilized. Figures 4–6

Table 3. Effects of Cl and amphotericin B on unidirectional water fluxes across the isolated frog corneal epithelium

	Cl-free Ringer			
	Tear to Stroma (n = 22)		Stroma to Tear (n = 11)	
	Control	Amph. B	Control	Amph. B
Mean	11.5	15.8[a]	11.8	15.3[a]
± SE	0.37	0.48	1.50	1.17
	Cl-rich Ringer			
	Tear to Stroma (n = 6)		Stroma to Tear (n = 4)	
	Control	Amph. B	Control	Amph. B
Mean	17.1	15.9	20.5	19.8
± SE	1.21	0.98	1.39	1.49
	Consecutive effects of Cl and Amphotericin B			
	Control (Cl-free)	Cl-rich	Amph. B	Cl-rich
Mean	8.3	13.4[b]	14.7[b]	
± SE	0.76	1.15	1.37	
(n = 7)				
Mean	9.5		12.6[b]	12.6[b]
± SE	0.79		0.49	0.70
(n = 9)				

[a] Significantly larger than its respective control. $P < 0.01$ as paired data.
[b] Significantly larger than its respective control. $P < 0.01$ as paired data.
All fluxes are in $\mu l \cdot min^{-1} \cdot cm^{-2}$. Amph. B: apical amphotericin B at 10^{-5} M.

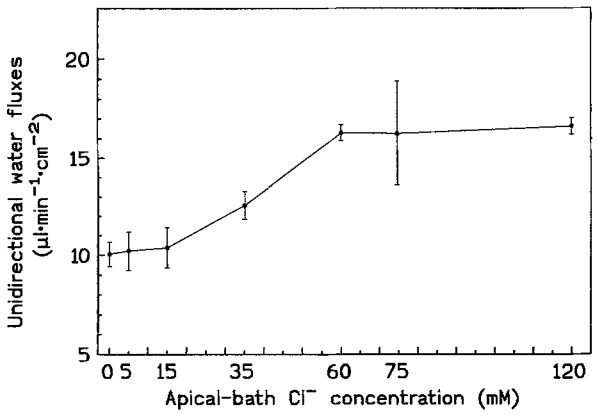

Fig. 2. Unidirectional water fluxes (in either direction) as a function of apical-bath Cl^- concentration. Each point is the average of four to eight determinations. Individual corneas were used to measure water fluxes at two or three different Cl^- concentrations. All changes in Cl^- were isosmotic.

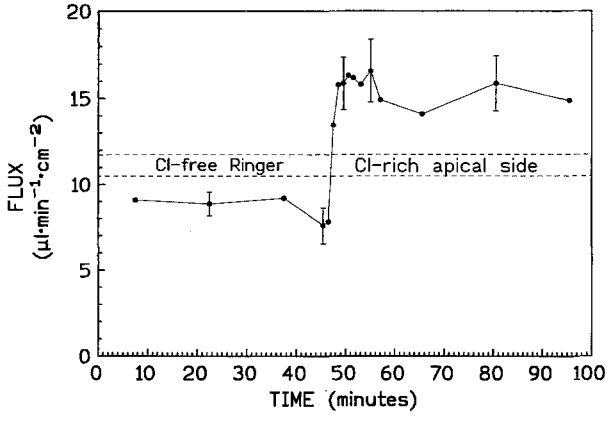

Fig. 3. Unidirectional water fluxes as a function of time when Cl^- (75 mM) was suddenly added to the apical bath. Changing the sampling intervals from 15 to 10 and 1 min before adding Cl^- did not increase the flux. After Cl^- addition prolonging the sampling interval did not affect the Cl^--increased water flux. Points are means of four experiments. Some error bars were omitted for clarity.

show typical records. The experiment in Fig. 4 was done in Cl^--free solution. The stromal side (the closed compartment in contact with the stroma and connected to the capillary cylinder) contained a solution with an osmolarity of about 288 mOsM. The tear sides of the two corneas were bathed with a 24 mOsM solution, so that there was about a 264 mOsM difference across each individual cornea with the driving force for water in the tear-to-stroma direction. In control conditions, there was no water flux for about 2 hr despite the osmotic gradient. After addition of amphotericin B, a flux of about 0.4 µl/min was elicited. This flux spontaneously slowed down and ceased when the osmolarity of the tear side was made equal to that of the stromal side. Figure 5 shows another

Table 4. Effect of 75 mM apical bromide on unidirectional water fluxes across the isolated frog corneal epithelium

	Cl-free Ringer	
	Control	Bromide
Mean	10.0	16.8*
± SE	0.76	1.51
(n = 4)		

* Significantly larger than control. $P < 0.01$ as paired data.
Fluxes are in $\mu l \cdot min^{-1} \cdot cm^{-2}$.

Table 5. Effect of 0.5 mM apical mercuric chloride on unidirectional water fluxes across the isolated frog corneal epithelium

	Cl-rich Ringer		
	Control	Amph. B	$HgCl_2$
Mean	14.5		10.0*
± SE	1.20		1.58
(n = 4)			
Mean	13.3	13.9	11.8
± SE	0.85	0.84	0.72
(n = 14)			

* Statistically significant decrease. $P < 0.01$.
Fluxes are in $\mu l \cdot min^{-1} \cdot cm^{-2}$.

experiment done in Cl^--rich solutions. Initially the same Ringer's bathed both sides of the corneas. After a period of stabilization there was no fluid movement during 75 min. At this point, the tear-side solutions were diluted to one-half so that an osmotic gradient of about 120 mOsM was established for water movement from tear-to-stroma. A stable flow of about 0.2 µl/min was observed for almost 3 hr. Amphotericin B had little effect on this flux, which was totally inhibited by 0.5 mM $HgCl_2$. In the final experiment, shown in Fig. 6, the basolateral side was bathed with a physiological Ringer's solution. Initially, there was an isoosmolar Cl^--free Ringer's on the apical sides and little fluid flow was observed. Then the tear sides were made hypertonic, which induced a flow from stroma-to-tear. Upon addition of Cl^- to the tear-side solution (maintaining osmolarity), the flow more than doubled, and it was reverted to the Cl^--free value upon $HgCl_2$ addition.

Discussion

Because of the demonstrated importance of fluid movement across the endothelium to the maintenance of corneal dehydration and transparency (Maurice, 1972; Fischbarg & Lim, 1974; Hodson & Miller, 1976), the permeability to water of the other limiting layer, the corneal

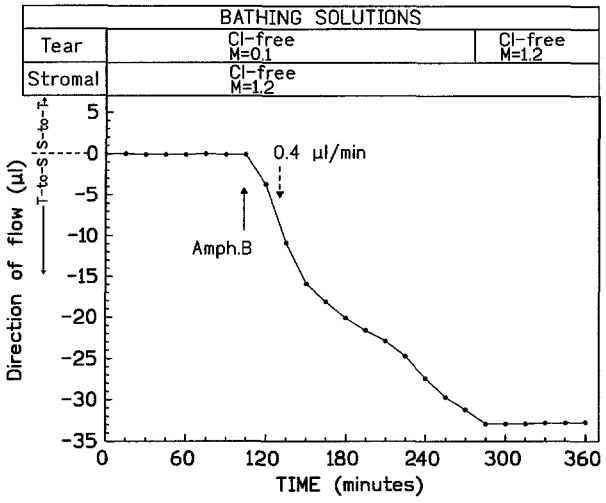

Fig. 4. Volume flow as a function of time between the center compartment and outer compartments across two corneas mounted in parallel in a volume flow chamber (chamber "b" in Materials and Methods; *see* Fig. 1). M is the fractional osmolarity of the solution relative to 240 mOsM (i.e., M = 0.1 represents 24 mOsM and M = 1.2 indicates 288 mOsM). Points are readings from the capillary, which were plotted to generate this representative trace of three similarly performed experiments. In Cl^--free solutions there was no noticeable flow despite a 264 mOsM gradient. Addition of amphotericin B (10^{-5} M) to the tear side of both corneas induced a flow of 0.4 µl/min in the tear-to-stroma direction that attenuated to a stable lower value. The flow was halted when the osmolarities of the tear sides were increased to match that in the central stromal compartment.

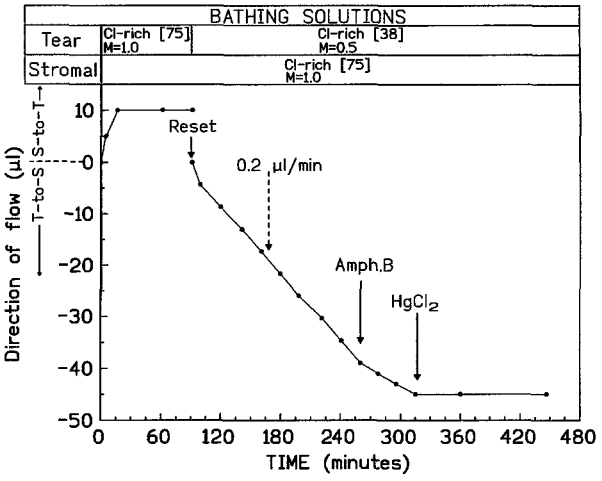

Fig. 5. A representative trace of transcorneal volume flow as a function of time (n = 3). Methods and parameters as described in the legend to Fig. 4. Numbers in brackets indicate Cl^- concentrations in mM. Initially the corneas were bathed bilaterally with an identical Cl^--rich solution. With this protocol there is usually a short-lived, spontaneous, stroma-to-tear fluid secretion that is possibly driven by the natural NaCl secretory activity of the corneas. At 90 min, the osmolarities of the tear sides were reduced by 50% and the [Cl^-] were lowered to 38 mM. This maneuver immediately elicited a tear-to-stroma fluid flow. Amphotericin B did not have a significant effect on the flow, which was inhibited upon 0.5 mM $HgCl_2$ addition to the apical sides.

epithelium, has received limited attention. One report (Fischbarg & Montoreano, 1982) showed a relatively high P_f for the rabbit corneal epithelium although the authors did not elaborate on the findings. Another report in the isolated toad cornea (Parisi et al., 1980) showed that the epithelium had a relatively high P_{dw} for a tissue thought to have limited fluid transport properties. Only recently the possibility that the epithelium could transport fluid outwardly was carefully considered based on published data (Fischbarg, 1992). These considerations as well as the possibility that the Cl^- channels that exist in the apical membrane of the frog corneal epithelium could function as water channels, prompted us to reexamine water movement across this membrane in vitro. We used two conventional approaches for the measurement of water movement, diffusional flow with tritiated water, and the net volumetric water flow elicited by an osmotic gradient. We did not attempt to measure the spontaneous water flow elicited by the apical secretion of NaCl.

Diffusional Water Flow

When the cornea is mounted in an Ussing-type chamber between two relatively large volume compartments the

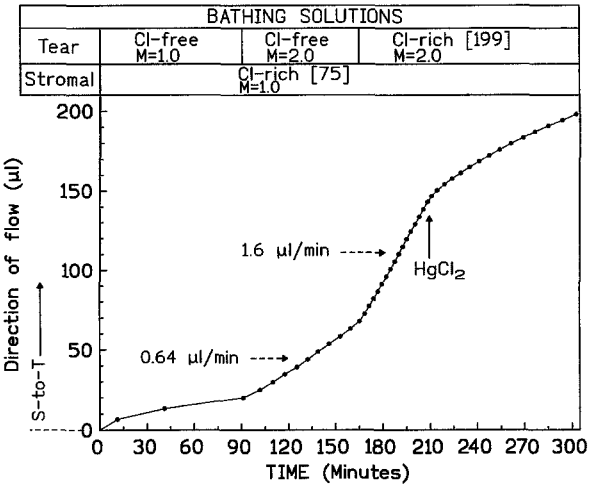

Fig. 6. A representative trace of transcorneal volume flow as a function of time (n = 6). Methods and parameters similar to the descriptions in legends of Figs. 4 and 5. Corneas were initially bathed bilaterally with solutions of the same osmolarity, but the apical baths lacked Cl^-. A spontaneous flow (possibly driven by basolateral-to-apical NaCl secretion) was observed. Increasing the osmolarities of the apical sides (240 mOsM gradient) increased the flow substantially. Adding Cl^- to the apical sides (without changing the osmotic gradient) more than doubled the flow, which was in turn reduced by 0.5 mM $HgCl_2$ addition to the apical sides. In general, larger water flows were elicited in the stroma-to-tear direction than in the opposite direction by equivalent gradients and conditions suggesting a degree of rectification.

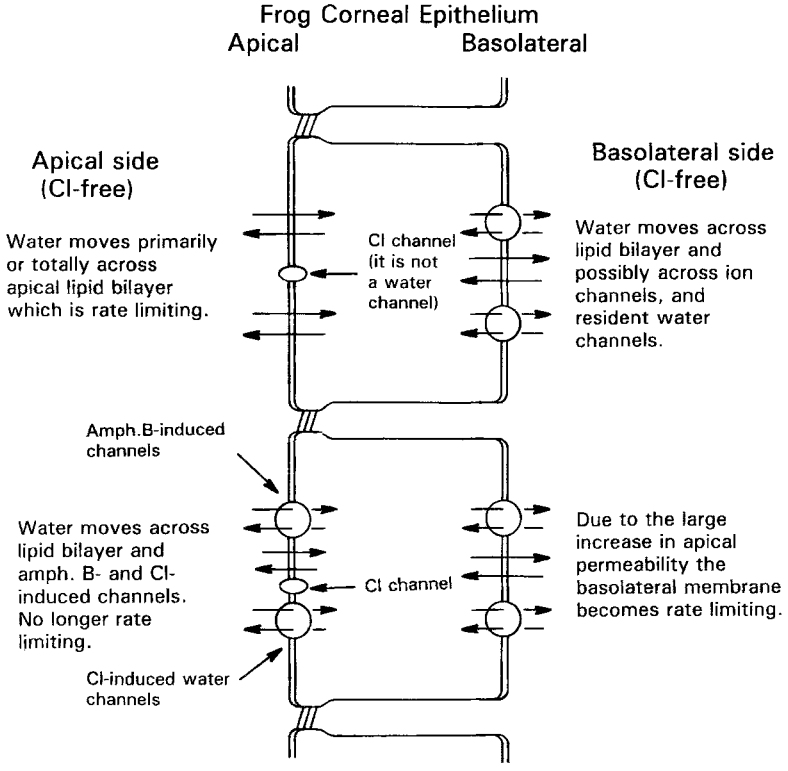

Fig. 7. Diagram of experimental results suggesting that Cl^- or Br^- can change, by an allosteric effect, the conformation of transmembrane proteins to render them water permeable.

composition and osmolarity of the bathing solutions are independent of transport processes across the tissue. Thus, it was not unexpected that the unidirectional ^3H_2O fluxes were essentially equal in the two opposite directions (Table 1). A notion that water may utilize Cl^- channels for its movement across the apical membrane was initially bolstered by the finding that water fluxes were as much as 70% larger in Cl^--rich compared to Cl^--free solutions. This idea was not sustained since stimulants of Cl^- secretion (forskolin, calcium ionophore A23187 and epinephrine), and an inhibitor of such (bumetanide), were found to have only minimal effects on water fluxes (*results not shown*). Furthermore, isotonic removal of Cl^- from only the apical side has no appreciable effect on the basolateral-to-apical Cl^--originated I_{sc} (Candia, 1984), while the water fluxes in either direction were inhibited by about 60% (Table 1). Therefore, one can conclude that the effect is due to the contact of Cl^- with the external side of the apical membrane, because intracellular Cl^- has no influence on water fluxes (i.e., under conditions in which Cl^- is limited to the basolateral side, cell Cl^- is maintained by Na–Cl cotransport, sustaining its efflux across the apical membrane, and the I_{sc}, but no water permeability increase is observed). Presumably, intracellular Cl^- would be in contact with the internal face of the apical membrane, an ineffectual sidedness from the standpoint of water movement.

It is well established that treatment with the channel-forming ionophore amphotericin B increases the cationic permeability of the apical membrane of the corneal epithelium markedly (Candia et al., 1974). Similar permeability increases have also been demonstrated in other epithelia (Lichtenstein & Leaf, 1965; Bentley & Candia, 1975). In addition, in the isolated toad cornea, amphotericin B also increased the transcorneal water permeability (Parisi et al., 1980), a finding repeated here with the frog cornea. In other experiments, Holz & Finkelstein (1970) showed water permeability inductions in lipid membranes upon amphotericin B exposure. Thus, the effect of amphotericin B on water fluxes can be logically explained as a consequence of an increased water permeability at the apical membrane. As detailed by Parisi et al. (1980), the 50–60% increase in water permeability represents a minimal value. When corrections are made for the restrictions of the stroma, and possibly the basolateral cell membrane (both of which are in series with the apical membrane), the actual increase in apical water permeability can be estimated to be 100% or more. It is interesting to note that the increase in permeability produced by apical Cl^- is similar to that produced by amphotericin B in the absence of apical Cl^- (Tables 2 and 3). Furthermore, the effects of apical Cl^- and amphotericin B were not additive (Table 3), strongly suggesting that they were creating parallel water pathways in the same cell membrane. The effect of apical Cl^- was relatively fast (within one minute and possibly less, Fig. 3) and immediately reversible (Table 2) suggesting a

direct effect, possibly allosteric, without entailing a cellular signaling system.

The Cl^--induced water permeability pathway was blocked in the presence of $HgCl_2$ (Table 5; Figs. 5 and 6), suggesting that it is a water channel similar to the other recently described aquoporins (Neilsen et al., 1992; Preston et al., 1992; Van Hoek & Verkman, 1992) that had been modulated by Cl^-. Bromide addition also activated the water permeability of the corneal epithelium (Table 4). At this point we can only speculate that the mechanism by which these halogens act cannot differentiate between the two anions. One characteristic of epithelia is that they allow little net water flow across the cellular lipid bilayer even in the presence of a considerable osmotic gradient. Once water channels are activated or inserted in the cell membrane, the osmotic permeability (P_f) increases many fold and a net water flow can be easily detected, as is the case with vasopressin-stimulated water flow in the toad bladder (Hays & Leaf, 1962; Finkelstein, 1976). A similar type of result was observed in our measurements of the net water flux under the influence of a large osmotic gradient (264 mOsM) across the corneal epithelium. No net water flux could be detected in Cl^--free solution. A flow of 0.2–0.4 μl/min was elicited by apical addition of either amphotericin B or Cl^-. The volume flow was reduced by $HgCl_2$ (Figs. 5 and 6).

Our results are collectively summarized in the diagram of Fig. 7. In Cl^--free solutions, the movement of water is only across the lipid bilayer, which is rate limiting. The movement of water across the extensive basolateral membrane is postulated to occur through normally present channels. Apical Cl^- activates normally present but inactive mercuric-sensitive water channels in the apical membrane. Amphotericin B induces a water permeability distinct from that activated by Cl^-; this permeability is not inhibited by $HgCl_2$. The Cl^- channels, themselves, do not seem to allow for any measurable water movement. The mechanism(s) by which Cl^- activates the postulated water channels is unknown. As a tentative hypothesis, we suggest that Cl^- (or Br^-) can change, by an allosteric effect, the conformation of transmembrane proteins to render them water permeable.

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References

Bentley, P.J., Candia, O.A. 1975. Effects of amphotericin B on ionic transport and sodium permeability of the toad lens. *Am. J. Physiol.* **229:**1520–1525

Candia, O.A. 1972. Ouabain and sodium effects on chloride fluxes across the isolated bullfrog cornea. *Am. J. Physiol.* **223:**1053–1057

Candia, O.A. 1973. Short-circuit current related to active transport of chloride in frog cornea: effects of furosemide and ethacrynic acid. *Biochim. Biophys. Acta* **298:**1011–1014

Candia, O.A. 1976. Fluid and chloride transport by the epithelium of the isolated frog cornea. *Fed. Proc.* **35**(Part 1):703

Candia, O.A. 1984. Secretion of chloride by the frog cornea. *In:* Chloride Transport Coupling in Biological Membranes and Epithelia .G.A. Gerencser, editor. pp. 393–414. Elsevier, North-Holland

Candia, O.A., Bentley, P.J., Cook, P.I. 1974. Stimulation by amphotericin B of active Na transport across amphibian cornea. *Am. J. Physiol.* **226:**1438–1444

Candia, O.A., Mia, A.J., Yorio, T. 1993. Influence of filter supports on transport characteristics of cultured A6 kidney cells. *Am. J. Physiol.* **265:**C1479–C1488

Candia, O.A., Montoreano, R., Podos, S.M. 1977. Effect of the ionophore A23187 on chloride transport across isolated frog cornea. *Am. J. Physiol.* **233:**F94–F101

Chalfie, M., Neufeld, A.H., Zadunaisky, J.A. 1972. Action of epinephrine and other cyclic AMP-mediated agents on the chloride transport of the frog cornea. *Invest. Ophthalmol.* **11:**644–650

Clarke, L.L., Grubb, B.R., Gabriel, S.E., Smithies, O., Koller, B.H., Boucher, R.C. 1992. Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science* **257:**1125–1128

Finkelstein, A. 1976. Nature of the water permeability increase induced by antidiuretic hormone (ADH) in toad urinary bladder and related tissues. *J. Gen. Physiol.* **68:**137–143

Fischbarg, J. 1992. The biology of the cornea. *In:* Fundamentals of medical cell biology. Vol. 6. E.E. Bittar, editor. p. 151. JAI Press

Fischbarg, J., Lim, J.J. 1974. Role of cations, anions and carbonic anhydrase in fluid transport across rabbit corneal endothelium. *J. Physiol.* **241:**647–675

Fischbarg, J., Montoreano, R. 1982. Osmotic permeabilities across corneal endothelium and antidiuretic hormone-stimulated toad urinary bladder structures. *Biochim. Biophys. Acta* **690:**207–214

Hasegawa, H., Skach, W., Baker, O., Calayag, M.C., Lingappa, V., Verkman, A.S. 1992. A multifunctional aqueous channel formed by CFTR. *Science* **258:**1477–1479

Hays, R.M., Leaf, A. 1962. Studies on the movement of water through the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45:**905–914

Hodson, S., Miller, F. 1976. The bicarbonate ion pump in the endothelium which regulates the hydration of the rabbit cornea. *J. Physiol.* **263:**271–302

Holz, R., Finkelstein, A. 1970. The water and nonelectrolyte permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* **56:**125–145

Klyce, S.D. 1975. Transport of Na^+, Cl^-, and water by the rabbit corneal epithelium at resting potential. *Am. J. Physiol.* **228:**1446–1452

Lichtenstein, N.S., Leaf, A. 1965. Effect of amphotericin B on the permeability of the toad bladder. *J. Clin. Invest.* **44:**1328–1342

Maurice, D.M. 1972. The localization of the fluid pump in the cornea. *J. Physiol.* **221:**43–54

Neilsen, S., Smith, B.L., Christenson, E.I., Knepper, M.A., Agre, P. 1992. CHIP28 water channels are localized in constitutively water permeable segments of the nephron. *J. Cell Biol.* **120:**371–383

Parisi, M., Candia, O.A., Alvarez, L. 1980. Water permeability of the toad corneal epithelium: the effects of pH and amphotericin B. *Pfluegers Arch.* **383:**131–136

Preston, G.M., Carrol, T.P., Guggino, W.B., Agre, P. 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* **256:**385–387

Van Hoek, A.N., Verkman, A.S. 1992. Functional reconstitution of the isolated erythrocyte water channel CHIP28. *J. Biol. Chem.* **267:**18267–18269

Verkman, A.S. 1989. Mechanisms and regulation of water permeability in renal epithelia. *Am. J. Physiol.* **257:**C837–C850

Zadunaisky, J.A. 1966. Active transport of chloride in frog cornea. *Am. J. Physiol.* **211:**506–512