BRIEF REPORT / RAPPORT BREF

Permeability to water in a tight epithelium: possible modulating action of gap junctions

Gabriel Orce, Graciela Castillo, Yolanda Chanampa, and Alejandra Bellomio

Abstract: Osmotic water flow (Jw) across tight distal nephron epithelial membranes increases upon exposure to vasopressin: following binding of the hormone to its receptors, intracellular cyclic AMP concentration increases, leading to insertion of aquaporins in the apical membrane. The involvement of intercellular communication in the process, however, has not been adequately explored. Octanol, 1.2×10^{-3} M, a gap junction inhibitor, significantly reduced Jw (expressed as mg·20 min^{-1}) in isolated toad urinary bladders (a model of the distal nephron) subjected to a transepithelial osmotic gradient and exposed to agents mimicking the vasopressin-triggered mechanism: oxytocin, 50 mIU·mL^{-1} (from 185.3 ± 28.0, $P < 0.001$, to 69.0 ± 23.6, $P < 0.05$; Pdiff < 0.01, $n = 6$), and cyclic AMP, 2.5×10^{-3} M (from 98.0 ± 32.6, $P < 0.02$, to 31.0 ± 13.9, NS; Pdiff < 0.05, $n = 12$), without altering the effect of nystatin, 450 U·mL^{-1}, which increases Jw via a mechanism unrelated to apical aquaporin insertion (163.2 ± 16.3, $P < 0.001$, in controls vs. 150.3 ± 10.4, $P < 0.001$, in octanol-treated bladders; Pdiff: NS, $n = 6$). Another gap junction blocker, carbenoxolone, 2.0×10^{-4} M (CBX), exerted similar effects on the responses to oxytocin, 100 mIU·mL^{-1}, reducing the response from 256.7 ± 33.6, $P < 0.001$, to 102.7 ± 10.4, $P < 0.001$; Pdiff < 0.01, $n = 6$) and nystatin, which was unaffected (95.0 ± 20.9, $P < 0.01$, vs. 132.0 ± 27.0, $P < 0.01$; Pdiff: NS, $n = 6$). Our results suggest that either gap junctions or, alternatively, unapposed gap junction hemichannels, may be important in the regulation of Jw in the isolated toad bladder, by modulating a step in the physiological process leading to increased apical membrane permeability.

Key words: Bufo arenarum, toad urinary bladder, water flow, epithelial permeability, n-octanol, carbenoxolone.

Résumé : Le débit d'eau (Jw) osmotique à travers les membranes épithéliales du néphron distal augmente de lors l'exposition à la vasopressine : après la fixation de l'hormone à ses récepteurs, la concentration d'AMP cyclique intracellulaire augmente, favorisant l'insertion d'aquaporines dans la membrane apicale. Toutefois, le rôle de la communication intercellulaire dans le processus est encore mal connu. L'octanol, $1{,}2 \times 10^{-3}$ M, un inhibiteur de jonctions lacunaires, a significativement réduit Jw (exprimé en mg·20 min^{-1}) dans les vessies urinaires isolées de crapauds (un modèle de néphron distal) soumises à un gradient osmotique transépithélial et exposées à des agents reproduisant le mécanisme déclenché par la vasopressine : l'ocytocine, 50 mUI·mL^{-1} (de 185,3 ± 28,0, $P < 0{,}001$, à 69,0 ± 23,6, $P < 0{,}05$; Pdiff < 0,01, $n = 6$), et l'AMP cyclique, $2{,}5 \times 10^{-3}$ M (de 98,0 ± 32,6, $P < 0{,}02$, à 31,0 ± 13,9, NS; Pdiff < 0,05, $n = 12$) sans modifier l'effet de la nystatine, 450 U·mL^{-1}, qui augmente Jw par un mécanisme indépendant de l'insertion apicale des aquaporines (163,2 ± 16,3, $P < 0{,}001$, dans les vessies témoins, vs 150,3 ± 10,4, $P < 0{,}001$, dans les vessies traitées à l'octanol; Pdiff : NS, $n = 6$). Une autre bloqueur de jonctions lacunaires, carbenoloxone, $2{,}0 \times 10^{-4}$ M (CBX), a eu des effets similaires sur les réponses à l'ocytocine, 100 mUI·mL^{-1}, réduisant la réponse de 256,7 ± 33,6, $P < 0{,}001$, à 102,7 ± 10,4, $P < 0{,}001$; Pdiff < 0,01, $n = 6$) et à la nystatine, qui n'a pas été affectée (95,0 ± 20,9, $P < 0{,}01$, vs 132,0 ± 27,0, $P < 0{,}01$; Pdiff: NS, $n = 6$). Nos résultats donnent à penser que les jonctions lacunaires, ou des demi-canaux non jonctionnels, pourraient jouer un rôle important dans la régulation du Jw dans la vessie isolée du crapaud, en modulant une étape dans le processus physiologique favorisant une augmentation de la perméabilité de la membrane apicale.

Mots clés : Bufo arenarum, vessie urinaire de crapaud, débit d'eau, perméabilité épithéliale, n-octanol, carbenoloxone.

[Traduit par la Rédaction]

Received 27 October 2003. Published on the NRC Research Press Web site at http://cjpp.nrc.ca on 26 July 2004.

G. Orce[1], G. Castillo, Y. Chanampa, and A. Bellomio. Departamento de Fisiología y Neurociencia, INSIBIO (UNT-CONICET), Instituto de Fisiología – Facultad de Medicina, Avda. Roca 2200-4000 Tucumán, Argentina.

[1]Corresponding author (e-mail: orcegap@yahoo.com)

 doi: 10.1139/Y04-037

Introduction

The exchange of matter and energy between living beings and the environment, and between body fluid compartments, often takes place across epithelial membranes.

Epithelial cells consist of an apical (external) membrane, which exhibits selective water and solute permeability, normally limiting their passage, and a basolateral (internal) membrane, in contact with the animal's *internal milieu* (Kristensen and Ussing 1985). They are separated by the tight junction, which forms a continuous seal around each cell, constituting the entrance to the paracellular pathway of passage (Mitic and Anderson 1988).

Depending primarily on the tightness of its tight junctions, epithelia are classified as leaky (highly permeable to water and ions, as in the proximal renal tubule) or tight (as in the distal tubule and collecting duct).

Regulated water flow (Jw) across tight epithelia is crucial for maintaining volumes and solute concentrations in organic compartments within narrow limits. This regulation (usually under hormonal control) is critical to survival, and has been widely studied (Knepper 1997, Nielsen et al. 2002).

The kidney, particularly the collecting duct, is centrally involved in the control of the volume and osmolality of the different compartments of the body.

Skin and bladder from toads have been intensively used extensively as models of the renal collecting duct (Leaf 1982). In the absence of the native antidiuretic hormone (vasotocin), these tissues exhibit low water permeability. With exposure to vasotocin or its analogues, such as vasopressin or oxytocin, permeability increases (the hydrosmotic effect). The effect is mediated by cyclic AMP (cAMP) and ultimately involves the insertion in the apical membrane of aquaporins, "water channels" pre-existing in intracellular vesicles (Chrispeels and Agre 1994, Deen and van Os 1998).

The exogenous addition of cAMP, which bypasses the receptor-binding and enzyme-activation steps, brings about the same response.

Apical water permeability is also increased by agents acting directly on the membrane, completely bypassing the process leading to aquaporin insertion. Nystatin, a polyene antibiotic, binds to membrane sterols, generating pores that act as water channels. The mechanism involves none of the steps described above (Zager 2000), and has been used in the past to functionally "remove" the apical membrane (Orce et al. 1981; Garty 1984).

Gap junctions are ubicuitous structures involved in intercellular communication. They are formed by the apposition of connexons, which are formed by an ordered clustering of connexins (i.e., tubular structures harboring channels) and are inserted across the membrane, between neighboring cells. These intercellular channels allow cells to exchange small molecules.

The participation of gap junctions has been demonstrated in the maintenance of epithelial tissue integrity and function (Iwata et al. 1998; Edelman et al. 1994), in processes related to the functions of mammalian renal tissues (Yaoita et al. 2002), including tubular epithelia of animals (Guo et al. 1998) and humans (Hillis et al. 1997), and of transport epithelia isolated from amphibians (Shahin and Blankenmeyer 1989).

More recently, the independent existence of hemichannels, unapposed connexons in nonjunctional portions of the membrane, has been recognized (Ebihara 2003). These structures are large conductance channels permeable to hydrophilic molecules up to 1 kDa; opening these channels can significantly alter cell homeostasis (Vergara et al. 2003).

Despite the fact that the syncitial behavior of the principal cells in this type of epithelium (Rick et al. 1978) is well-known, the influence on epithelial transport processes of direct cell communication with other cells or the environment has not been well studied.

We used octanol and carbenoxolone, agents known to close channels within the gap junctions (Weingart and Bukauskas 1998; Burghardt 1995), to explore the influence of these structures on the regulation of transcellular water permeability. Our results suggest that gap junctions and (or) hemichannels may be important in increasing Jw in the isolated bladder, probably by modulating one or more steps of the process of apical membrane activation.

Methods

Bufo arenarum toads were obtained locally, kept on wet sand, and hydrated for 18–24 h before use. They were pithed, and Jw was determined in the isolated urinary bladder using a gravimetric technique (Orce and Castillo 1986). Briefly, both hemibladders (one of which was used as a control) were mounted as bags, mucosal side inside; this was achieved by tying each hemibladder to the end of a glass tube that was open at both ends. Each hemibladder was filled with 1 mL of a hypo-osmotic solution and suspended in 5 mL of amphibian Ringer's solution, establishing an osmotic gradient across its wall.

The hemibladder preparations were left to stand for 60 min; baths were changed once during that period. To determine Jw, the hemibladders were weighed every 20 min; the apical solution was discarded at the end of each 20-min period, and either renewed or substituted with the solution to be used in the following period. Typically, baseline Jw was determined during two 20-min periods in drug-free solutions, after which the bladders not in the control group were exposed to one or more of the experimental drugs; this was achieved by replacing one or both baths with solutions containing the study drugs.

Concentration of the Ringer's solution bathing the serosal border of the bladder was as follows (in mmol/L): NaCl, 105.0; KCl, 2.0; $MgSO_4$, 1.0; Tris–HCl buffer, 25.0 (pH 7.5); $CaCl_2$, 1.0; and glucose, 6.0. Osmolality was 230–235 mOsm/Kg H_2O. The solution was agitated and aerated with air. The hypo-osmotic solution used on the apical border of the bladder was Ringer's solution diluted with distilled water, in a ratio of 1:5.

Oxytocin (Syntocinon, 5 IU/mL) was generously donated by Novartis Argentina (Buenos Aires, Argentina), octanol (Merck, Darmstadt), nystatin, and carbenoxolone (Sigma, St. Louis, Mo.) were commercially obtained. All drugs were dissolved/diluted immediately before use, and added directly to the bath. Stock solutions of octanol (diluted 1:10) and nystatin (6.5×10^{-3} mol/L) were prepared in methanol, and control preparations were given an equal volume of the sol-

vent; the volume added was never greater than 0.5% of the bath volume.

The animals used in the study were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Experiments were performed at room temperature (20–24 °C); results are expressed as mean ± S.E.M. Results were statistically evaluated by Student's *t* test; $P < 0.05$ was considered significant.

Results

Effect of octanol on the viability of the isolated bladder

To check for deleterious effects of prolonged exposure to octanol, a gap-junction inhibitor, bladders were bathed in Ringer's solution containing 1.2×10^{-3} mol/L octanol for 120 min. After being rinsed in a drug-free solution for 80 min, the bladders were challenged with 50 mIU·mL^{-1} oxytocin, and Jw was recorded during a 100-min period. The Jw response in the octanol-treated bladders was 79.1 ± 23.1 mg·20 min^{-1} ($P < 0.01$) vs. 76.0 ± 12.2 ($P < 0.001$) in the preparations not exposed to the inhibitor ($n = 12$).

Effect of octanol on the response to oxytocin

Exposure of both sides of the bladder to octanol did not *per se* change the pattern of weight loss. However, the response to exposure of the serosal border of the preparations to oxytocin (OT) was 69.0 ± 23.6 mg·20 min^{-1} ($P < 0.05$) in preparations exposed to octanol, whereas those exposed only to OT increased by 185.3 ± 28.0 mg·20 min^{-1} ($P < 0.001$); the P value of the difference (Pdiff) was < 0.01) (Fig. 1).

Effect of octanol on the response to cAMP

Similarly, the bladder's Jw response to 2.5×10^{-3} mol/L cAMP added to the serosal bath, was reduced from 98.0 ± 32.6 mg·20 min^{-1} ($P < 0.02$) in control preparations to 31.0 ± 13.9 mg·20 min^{-1} (nonsignificant) in octanol-treated bladders (Pdiff < 0.05) (Fig. 2)

Effect of octanol on the response to nystatin

In contrast, the Jw response to 450 U·mL^{-1} (NYS) added to the mucosal bath was not affected by prior exposure to octanol: the hydrosmotic response to the peptide was 163.2 ± 16.3 mg·20 min^{-1} ($P < 0.001$) in bladders treated with OT only vs. 150.3 ± 10.4 mg·20 min^{-1} ($P < 0.001$) in preparations also exposed to octanol (Pdiff: NS) (Fig. 3).

Effects of carbenoxolone on the response to oxytocin or nystatin

Although exposure of the bladder to carbenoxolone caused no effect *per se*, the Jw response of the preparations to serosal oxytocin decreased, without altering the response to mucosal NYS (Table 1).

Discussion

The mechanisms of the Jw increase brought about by vasopressin in tight distal nephron epithelia are thoroughly understood, and bear a strong similarity to the mechanisms at work in the isolated toad bladder. Little is known, however, about the influence of intercellular communication on transepithelial water flow.

Fig. 1. Effect of octanol on the hydrosmotic response of the isolated bladder to oxytocin. After measuring the baseline osmotic water flow (Jw) (bars 1 and 2, left panel), 1.2×10^{-3} mol/L octanol (OcOH) was added to both the serosal and mucosal baths of one preparation (bars 3 and 5). Controls (bars 4 and 6) received the same volume of vehicle, methanol (middle panel). Oxytocin (OT), 50 mIU/mL, was added to the serosal bath of all preparations 80 min later, and Jw was measured for 120 min (right panel). Bars show the maximum weight loss of the preparations. + and ++: $P < 0.05$ and < 0.01 vs previous values for the same preparations, respectively.

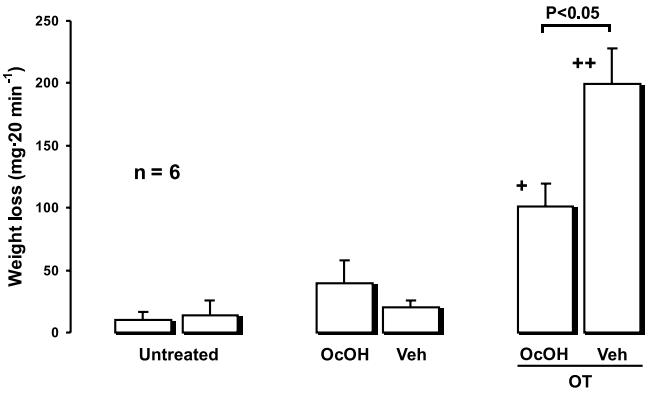
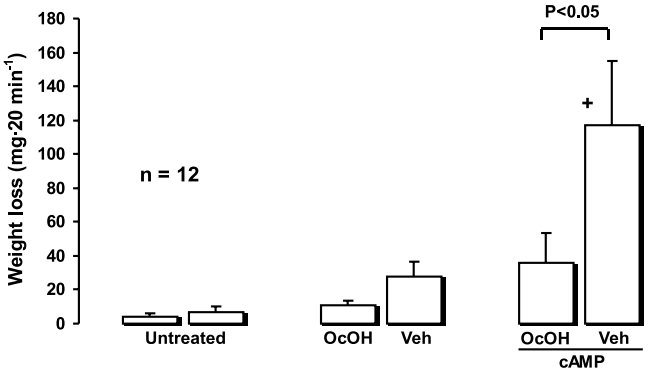


Fig. 2. Effect of octanol on the hydrosmotic response of the isolated bladder to cAMP. The experimental protocol was as described in Fig. 1, except that 2.5×10^{-3} mol/L cAMP (cAMP), was used instead of oxytocin, and water flow (Jw) was measured for 160 min after the addition of cAMP. + $P < 0.05$ vs the previous value for the same preparations.



Octanol, a known blocker of gap-junction channels, did not affect the viability of the isolated toad bladder; the Jw response to oxytocin was not altered in bladders transiently exposed to the inhibitor and rinsed.

We found that octanol and carbenoxolone inhibited the hydrosmotic response of the bladder to oxytocin, without altering the permeabilizing effect of nystatin on the apical membrane. Furthermore, inhibition of the effect of cAMP by octanol indicates that the inhibitor exerts its action after generation of the mediator. Our results also show that neither inhibitor affected the passage of water across the cell, once the apical barrier was traversed.

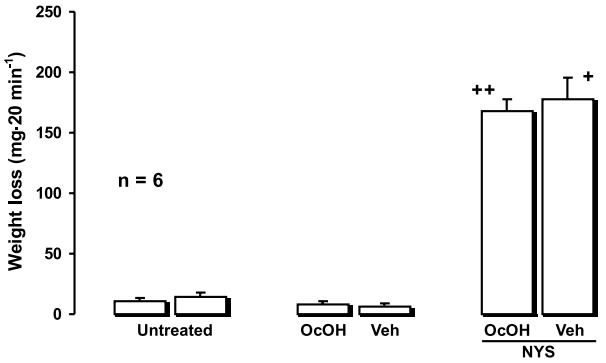
Taken together, these data strongly suggest that channels existing within the connexon are involved, in some manner, in the full development of the hydrosmotic response, proba-

Table 1. Effect of carbenoxolone (CBX) on the Jw response to hydrosmotic agents in the toad bladder.

Preparation	1	2	3	4	5
	Baseline	s&mCBX	P	serosal OT	P
CBX + OT	–6.2±5.8	–4.7±5.0	NS	114.8±12.1	< 0.001
Control	2.7±4.3	–4.8±2.2	NS	259.2±32.3	< 0.001
Diff				–154.0±32.3	< 0.01
	Baseline	s&mCBX	P	mucosal NYS	P
CBX + NYS	7.0±3.6	10.0±6.4	NS	136.1±29.0	< 0.01
Control	3.8±7.4	12.0±7.1	NS	128.7±23.7	< 0.001
Diff				7.4±38.3	

Note: Bladders were mounted and their baseline Jw measured (column 1). CBX preparations were exposed to 2.0×10^{-4} mol/L carbenoxolone in both serosal and mucosal baths (s&mCBX) for 80 min; the values reached are reported in column 2, and the P values of the responses in column 3. Both CBX and control preparations were then exposed to the agent indicated (either 100 mIU·mL^{-1} oxytocin (OT) or 450 U·mL^{-1} nystatin (NYS)), and peak values (column 4) and P values of the responses shown in (column 5) were recorded. For treated and control groups, $n = 6$.

Fig. 3. Effect of octanol on the hydrosmotic response of the isolated bladder to nystatin. The experimental protocol was as described in Fig. 1, except that 450 U/mL nystatin (NYS), was used in the mucosal bath as the water flow (Jw)-stimulating agent, and Jw continued to be measured for 160 min after the addition of nystatin. + and ++: $P < 0.05$ and < 0.01 vs previous values for the same preparations, respectively.



bly at a step prior to, or affecting, the insertion of aquaporins in the apical membrane.

Because gap-junction inhibitors also block hemichannel function, our results do not distinguish the exchange of materials between cells from exchange between cells and the environment. The precise modulating function performed by these structures in the process leading to aquaporin activation/membrane insertion is hard to imagine at the present time. It is possible that either the synchronizing capacity afforded by the sincytial nature of the epithelium, or the uptake or discharge of a substance or substances are important for the full development of the permeabilization process.

Further study is required, however, to more precisely describe the site and mode in which modulation takes place.

References

Burghardt, R.C., Barhoumi, R., Sewall, T.C., and Bowen, J.A. 1995. Cyclic AMP induces rapid increases in gap junction permeability and changes in the cellular distribution of connexin 43. J. Membr. Biol. **148**: 243–253.

Chrispeels, M.J., and Agre, P. 1994. Aquaporins: water channel proteins of plant and animal cells. Trends Biochem. Sci. **19**: 421–425.

Deen, P.M.T., and van Os, C.H. 1998. Epithelial aquaporins. Curr. Opin. Cell. Biol. **10**: 435–442.

Ebihara, L. 2003. New roles for connexons. News Physiol. Sci. **18**: 100–103.

Edelman, J.L., Sachs, G., and, Adorante, J.S. 1994. Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells. Am. J. Physiol. **266**: C1210–C1221.

Garty, H. 1984. Current-voltage relations at the basolateral membrane in tight amphibian epithelia: use of nystatin to depolarize the apical membrane. J. Membr. Biol. **77**: 213–222.

Guo, R., Liu, L., and Barajas, L. 1998. RT-PCR study of the distribution of connexin 43 mRNA in the glomerulus and renal tubular segments. Am. J. Physiol. **275**: R439–R447.

Hillis, G.S., Duthie, L.A., Mlynski, R., McKay, N.G., Mistry, S., MacLeod, A.M., Simpson, J.G., and Haites, N.E. 1997. The expression of connexin 43 in human kidney and cultured renal cells. Nephron, **75**: 458–63.

Iwata, F., Joh, T., Ueda, F., Yokoyama, Y., and Itoh, M. 1998. Role of gap junctions in inhibiting ischemia-reperfusion injury of rat gastric mucosa. Am. J. Physiol. **275**: G883–G888.

Knepper, M.A. 1997. Molecular physiology of urinary concentrating mechanism: regulation of aquaporin water channels by vasopressin. Am. J. Physiol. **272**: F3–F12.

Kristensen, P., and Ussing, H.H. 1985. Epithelial organization. *In* The kidney: physiology and pathophysiology. *Edited by* D.W. Seldin and G. Giebisch, Raven Press, New York, pp. 173–188.

Leaf, A. 1982. From toad bladder to kidney. Am. J. Physiol. **242**: F103–F111.

Mitic, L.L., and Anderson, J.M. 1988. Molecular architecture of tight junctions. Annu. Rev. Physiol. **60**: 121–142.

Nielsen, S., Froklær, J., Marples, D., Kwon, T-H., Agre, P., Knepper., M.A. 2002. Aquaporins in the kidney: from molecules to medicine. Physiol. Rev. **82**: 205–244.

Orce, G.G., and Castillo, G.A. 1986. A role for the kallikrein-kinin system in the regulation of osmotic water permeability in the toad bladder. Comp. Biochem. Physiol. **84C**: 139–144.

Orce, G.G., Castillo, G.A., and Margolius, H. S. 1981. Kallikrein inhibitors decrease short-circuit current in the toad bladder by inhibiting sodium uptake. Hypertens. Suppl. **3**: II-92 – II-95.

Rick, R., Dörge, A., von Arnim, E., and Thurau, K. 1978. Electron microprobe analysis of frog skin epithelium: evidence for a

syncitial sodium transport compartment. J. Membr. Biol. **39**: 313–331.

Shahin, S.H., and Blankenmeyer, J.T. 1989. Demonstration of gap junctions in the frog skin epithelium. Am. J. Physiol. **257**: C658–C664.

Vergara, L., Bao, X., Cooper, M., Bello-Reuss, E., Reuss, L. 2003. Gap-junctional hemichannels are activated by ATP depletion in human renal proximal tubule cells. J Membr Biol. **196**: 173–184.

Weingart, R., and Bukauskas, F.F. 1998. Long-chain n-alkanols and arachidonic acid interfere with the Vm-sensitive gating mechanism of gap junction channels. Pfluegers Arch. **435**: 310–319.

Yaoita, E., Yao, J., Yoshida, Y., Morioka, T., Nameta, M., Takata, T., Kamiie, J., Fujinaka, H., Oite, T., and Yamamoto, T. 2002. Up-regulation of connexin 43 in glomerular podocytes in response to injury. Am. J. Pathol. **161**: 1597–1606.

Zager, R.A. 2000. Polyene antibiotics: relative degrees of in vitro cytotoxicity and potential effects on tubule phospholipid and ceramide content. Am. J. Kidney Dis. **36**: 238–249.