β-Cyclodextrin and permeability to water in the bladder of *Bufo arenarum*

G. Orce, G. Castillo, and Y. Chanampa

Abstract: We measured the effect of β-cyclodextrin (BCD, a cholesterol scavenger) on water flow across the isolated toad bladder exposed to an osmotic gradient (J_w) by a gravimetric technique. BCD, when present in the solution bathing the apical side of the bladder, inhibited the increase in J_w caused by nystatin, a polyene antibiotic that acts by directly binding apical membrane cholesterol. When present in the basolateral bath, BCD inhibited the increase in J_w caused by basolateral exposure to oxytocin (which binds membrane receptors and stimulates the synthesis of cAMP), but did not alter the response to theophylline (which inhibits hydrolysis of cAMP by cyclic nucleotide phosphodiesterase). The present data are consistent with the notion that agents that increase J_w by interacting with membrane receptors, which appear to be clustered in cholesterol-rich domains of the basolateral membrane, are altered by cholesterol depletion, whereas agents that do not interact with receptors or other basolateral membrane components are not affected by this treatment. In either case, cholesterol depletion of the apical membrane does not affect the increase in J_w brought about by an increase in intracellular cAMP concentration.

Key words: β-cyclodextrin, water flow, epithelial permeability.

Résumé : Nous avons mesuré l'effet de la β-cyclodextrine (BCD, un piégeur de cholestérol) sur le flux d'eau à travers la vessie isolée du crapaud exposée à un gradient osmotique (J_w), en utilisant une technique gravimétrique. Dans la solution baignant le côté apical de la vessie, la BCD a inhibé l'augmentation de J_w induite par la nystatine, un antibiotique polyène qui agit en fixant directement le cholestérol sur la membrane apicale. Dans la solution basolatérale, la BCD a inhibé l'augmentation de J_w causée par l'exposition basolatérale à l'oxytocine (qui fixe les récepteurs membranaires et stimule la synthèse de l'AMPc), mais n'a pas modifié la réponse à la théophylline (qui inhibe l'hydrolyse de l'AMPc par la nucléotide cyclique phosphodiestérase). Les présents résultats sont en accord avec la notion que les agents qui augmentent J_w en interagissant avec les récepteurs membranaires, qui semblent être regroupés dans les domaines riches en cholestérol de la membrane basolatérale, sont modifiés par la déplétion en cholestérol, alors que les agents qui n'interagissent pas avec les récepteurs ou avec d'autres composants de la membrane basolatérale ne répondent pas à ce traitement. Dans un cas comme dans l'autre, la déplétion de la membrane apicale en cholestérol n'influe pas sur l'augmentation de J_w provoquée par l'augmentation de la concentration d'AMPc intracellulaire.

Mots-clés : β-cyclodextrine, flux d'eau, perméabilité épithéliale.

[Traduit par la Rédaction]

Introduction

Cholesterol (CHOL) is an important integral component of the cell membrane required for normal cell function. In eukaryotic cells, between 65% and 90% of unesterified CHOL may reside in the plasma membrane (Liscum and Munn 1999). Lipids are distributed in a nonhomogeneous manner in the layers of the membrane, and lipid structures called "rafts" — small and very dynamic membrane microdomains rich in CHOL and glycosphingolipids — have been recognized (Simons and van Meer 1988). These rafts are often linked with hormone receptor or molecules functionally related to intracellular signaling cascades (α subunits of the G protein, adenylyl cyclase, etc.) (Anderson 1998; Burger et al. 2000).

CHOL has been shown to be involved in the regulation of the function of some receptors to hormones, including peptides and catecholamines (Klein et al. 1995; Burger et al. 2000). However, studies of the influence of CHOL on membrane transport functions, particularly water permeability, are few and performed mostly in mammalian tissues.

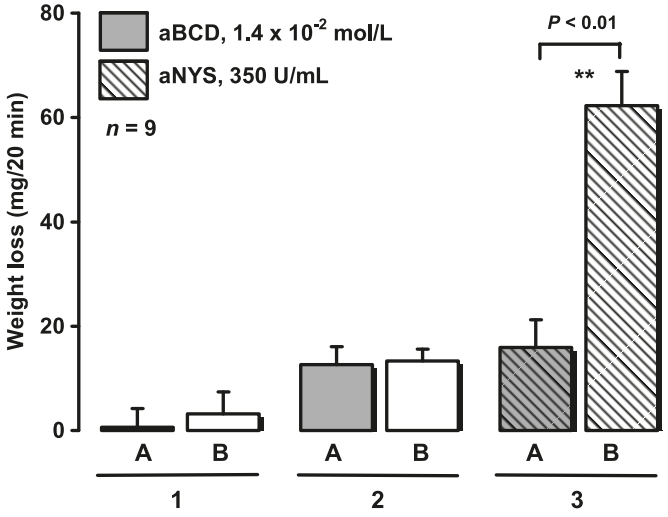
The isolated toad bladder is considered a good functional model of the distal tubule of the mammalian kidney (Leaf 1982). Functionally, it allows for the hormonal control of water reabsorption at a postrenal stage by an animal whose kidney lacks the capacity to concentrate tubular fluid and does so using essentially the same mediators and mechanisms found in the mammalian distal nephron. From an experimental point of view, it allows ready access to either the apical or the basolateral membranes separately, thus making it possible to study the effects of different agents in either surface of the organ using a simple technique. The permeability of the bladders's basolateral membrane is relatively high, the water-tight

Received 18 January 2011. Accepted 26 April 2011. Published at www.nrcresearchpress.com/cjpp on .

G. Orce, G. Castillo, and Y. Chanampa. School of Medicine, National University of Tucumán; and Department of Physiology and Neuroscience, INSIBIO (UNT–CONICET), Junín 1229, 4000 Tucumán, Argentina.

Corresponding author: G. Orce (e-mail: orce.gabriel@gmail.com).

Fig. 1. Effect of β-cyclodextrin present in the apical bath (aBCD) on the hydroosmotic response to nystatin (NYS). The preparations were mounted with the basolateral side (b) bathed in Ringer's solution and the apical side (a) in Ringer's solution diluted 1:5 with water. After the baseline period (block 1), BCD (1.4×10^{-2} mol/L) was added to the apical bath (aBCD) of one preparation (block 2), and 40 min later nystatin (350 U/mL) was added to the apical bath of both preparations (aNYS) and left there for 80 min (block 3). The paired columns represent baseline weight loss of the preparations (block 1) and maximal changes during exposure to aBCD (block 2) and aNYS (block 3). **, $P < 0.01$: a significant difference from the previous column for the same preparation.



apical membrane being the most important factor limiting the passage of water (Kristensen and Ussing 1985).

Water flow across the bladder exposed to a transepithelial osmotic gradient (J_w) is physiologically mediated by an increase in intracellular cAMP concentration. This increase can be brought about by hydroosmotic agents such as oxytocin (OT), which binds to membrane receptors and stimulates adenylyl cyclase, or theophylline, which acts as an inhibitor of nucleotide phosphodiesterase, without significantly interacting with membrane components. In either case, the hydroosmotic response involves the insertion in the apical membrane of aquaporins, true "water channels" preexisting in intracellular vesicles (Agre et al. 1998). Apical membrane permeability to water can also be increased by the polyene antibiotic nystatin. Although nystatin, which binds to plasma membrane CHOL, is recognized primarily as a Na^+/K^+ ionophore, it also increases J_w via a mechanism involving neither cAMP or aquaporins (Orce et al. 2004).

In addition, a paracellular pathway exists that has low permeability to water, limited mainly by the tight junctions existing between the cells at the boundary that separates the apical and basolateral membranes. The usually very low permeability of the paracellular pathway to water can be increased by exposure of the outer (apical) face of the epithelium to hypertonic solutions (apical hypertonicity), which causes the tight junctions between cells to open, significantly increasing J_w. Under those conditions, the orientation of the imposed osmotic gradient — being reversed as compared to physiological conditions — causes the induced

Fig. 2. Effect of β-cyclodextrin in the apical bath (aBCD) on the hydroosmotic response to oxytocin (OT). The preparations were mounted with the basolateral side (b) bathed in Ringer's solution and the apical side (a) in Ringer's solution diluted 1:5 with water. After the baseline period (block 1), BCD (1.4×10^{-2} mol/L) was added to the apical bath (aBCD) of one preparation (block 2), and 40 min later OT (50 mU/mL) was added to the apical bath of both preparations (aOT) and left there for 80 min (block 3). The paired columns represent baseline weight loss of the preparations (block 1) and maximal changes during exposure to aBCD (block 2) and aOT (block 3). **, $P < 0.01$: significant difference from the previous column for the same preparation.

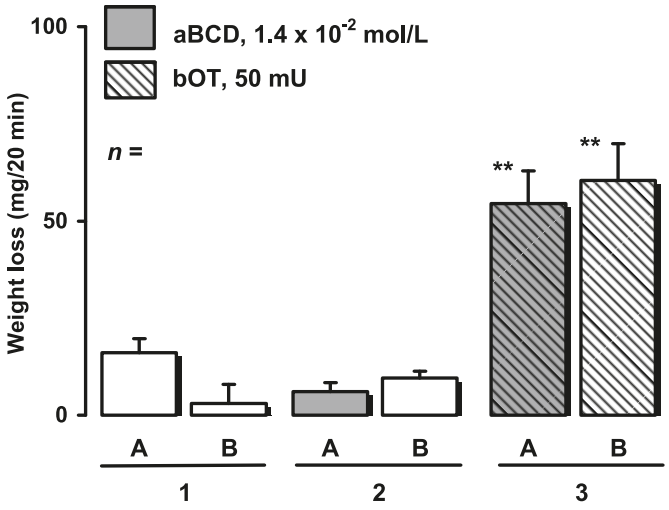


Fig. 3. Effect of β-cyclodextrin in the basolateral bath (bBCD) on the hydroosmotic response to oxytocin (OT). The preparations were mounted with the basolateral side (b) bathed in Ringer's solution and the apical side (b) in Ringer's solution diluted 1:5 with water. After the baseline period (block 1), BCD (1.4×10^{-2} mol/L) was added to the basolateral bath (bBCD) of one preparation (block 2), and 40 min later OT (50 mU/mL) was added to the basolateral bath of both preparations (bOT) and left there for 80 min (block 3). The paired columns represent baseline weight loss of the preparations (block 1) and maximal changes during exposure to bBCD (block 2) and bOT (block 3). **, $P < 0.01$: significant difference from the previous column for the same preparation.

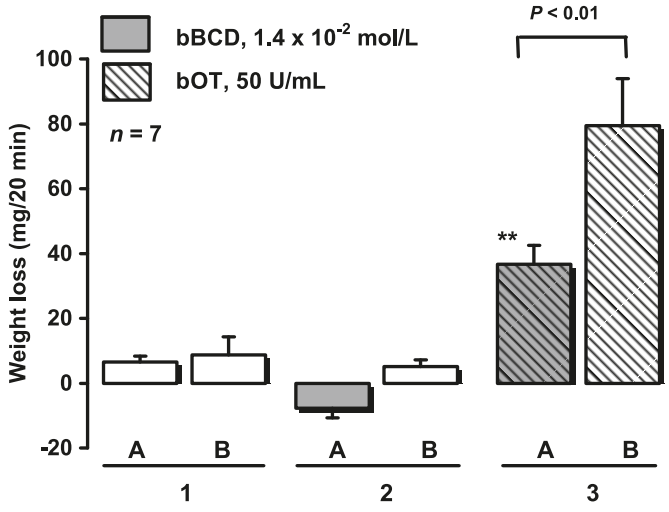
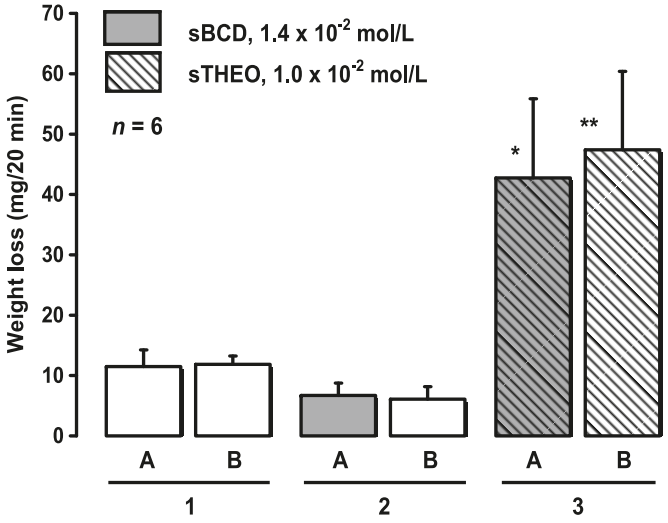


Fig. 4. Effect of β-cyclodextrin in the basolateral bath (bBCD) on the hydroosmotic response to theophylline (THEO). The preparations were mounted with the basolateral side (b) bathed in Ringer's solution and the apical side (a) in Ringer's solution diluted 1:5 with water. After the baseline period (block 1), BCD (1.4×10^{-2} mol/L) was added to the basolateral bath (bBCD) of one preparation (block 2), and 40 min later THEO (1.0×10^{-2} mol/L) was added to the basolateral bath of both preparations (bTHEO) and left there for 80 min (block 3). The paired columns represent baseline weight loss of the preparations (block 1) and maximal changes during exposure to aBCD (block 2) and bTHEO (block 3). *, $P < 0.05$; **, $P < 0.01$: significant difference from the previous column for the same preparation.



J_w to proceed counter to its usual direction and the preparation to gain weight.

In the context of the transport functions of the epithelial membrane, the use of agents capable of modifying the CHOL content of the membrane offers a means to record the effects of this change on the various mechanisms involved in regulating water permeability. Cyclodextrins are water-soluble cyclic oligosaccharides that increase the water solubility of nonpolar substances such as lipids, incorporating them within their hydrophobic cavity (Burger et al. 2000). β-Cyclodextrin (BCD) is an oligomer made of 7 glucose molecules that exhibits greater affinity for CHOL than other membrane lipids and can be used to selectively deplete the membrane of the compound (Ohtani et al. 1989). This paper describes the effects of BCD on water permeability in the isolated bladder of the toad.

Materials and methods

Bufo arenarum toads of both sexes were obtained locally and kept on wet sand. The animals were hydrated for 18–24 h before being sacrificed by destruction of the central nervous system. Osmotically induced water flow (J_w) across the isolated urinary bladder was measured by a gravimetric technique (Orce and Castillo 1986). Briefly, the 2 hemibladders (one of which was used as control) were mounted separately at one end of glass tubes open at both ends, with the apical side lining the inside of the resulting bag, which was filled with 1 mL of hypotonic solution prepared by diluting amphibian Ringer's solution 1:5 with distilled water. The preparations were suspended and immersed in 5 mL of Ringer solution (isotonic with plasma). At the end of each period, the preparations were patted with moist gauze to eliminate excess solution and weighed. The apical solution was then discarded and renewed or substituted with the appropriate solution for the experiment in progress.

The Ringer's solution used in contact with the basolateral side contained: 105.0 mmol/L NaCl; 2.0 mmol/L KCl; 1.0 mmol/L $MgSO_4$; 25.0 mmol/L Tris–HCl buffer, pH 7.5; 1.0 mmol/L $CaCl_2$; and 6.0 mmol/L glucose. The osmolality of the Ringer's solution was 230–235 mOsmol/kg H_2O. In some experiments the apical bath was made hypertonic by adding enough mannitol to bring the osmolality of the solution to twice the value of the Ringer solution.

Changes in weight of the preparation due to the concentration gradient established between the apical and basolateral solutions were measured by weighing the preparation every 20 min with a precision of 0.1 mg.

The solution in contact with the basolateral side of the preparation was oxygenated and stirred by bubbling with atmospheric air. The preparations were allowed to rest for 1 h, changing the bath twice in that period, before starting the experimental period proper. Baseline weight changes were recorded for 20–40 min before exposing the preparations to drugs or experimental maneuvers. Exposure of preparations to drugs was done by substituting either the basolateral or the apical bath with fresh solutions containing the agent under study.

Mannitol, norepinephrine, and BCD were purchased from Sigma Chemical Co. (St. Louis, Mo., USA); OT (Syntocinon, Novartis) was donated by Novartis Argentina SA (Buenos Aires, Argentina). The remaining drugs used were of analytical grade and obtained commercially. All drugs were dissolved or diluted in the appropriate solution immediately before use and added directly to the solution used in the experiment in progress.

Experiments were performed at room temperature (20–24 °C). Results are expressed as means ± SE. Statistical analysis was performed using a Student's t test, and $P < 0.05$ was considered the criterion for significance.

The experimental animals were treated humanely at all times in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Results

Effect of exposure to BCD on the response to nystatin

The hydroosmotic response to nystatin (350 U/mL) in the apical bath (aNYS) was significantly inhibited in preparations preexposed for 40 min to BCD (1.4×10^{-2} mol/L) in the same bath (aBCD) (Fig. 1).

Effect of exposure to BCD on the response to OT

A 40-min exposure to BCD (1.4×10^{-2} mol/L) in the apical bath (aBCD) did not affect the hydroosmotic response to OT (50 mU/mL) in the basolateral bath (bOT) (Fig. 2). A similar exposure to BCD in the basolateral bath (bBCD) brought about a significant inhibition of the hydroosmotic response to bOT (Fig. 3).

Table 1. Effect of β-cyclodextrin (BCD) on the increase in osmotic water flow (J_w) caused by exposure of the apical side of the bladder to a hypertonic solution.

(*i*) Effect of adding BCD to the basolateral bath.					
	J_w (mg/20 min)			*P*	*P*diff
	Baseline	bBCD	aM		
Treated	5.1±2.2	13.6±2.9	–22.3±5.4	<0.01	ns
Control	8.6±2.7	15.3±8.0	–24.6±7.9	<0.01	
(*ii*) Effect of adding BCD to the apical bath.					
	J_w (mg/20 min)				
	Baseline	aBCD	aM	*P*	*P*diff
Treated	16.8±2.6	18.6±3.9	–27.6±8.7	<0.01	ns
Control	16.5±3.6	19.3±5.0	–23.2±6.5	<0.01	

Note: The preparations were mounted with the basolateral side (b) bathed in Ringer's solution and the apical side (a) in Ringer's solution diluted 1:5 with water. After the baseline period, BCD (1.4×10^{-2} mol/L) was added to the basolateral (bBCD) or the apical bath (aBCD) of one preparation, and 40 min later mannitol (3.5×10^{-1} mol/L) was added to the apical bath of both preparations (aM). Weighing continued for 80 min after addition of mannitol. Values shown are means ± SE of the maxima or minima in each period, and P_{diff} is the *P* value of the difference between the response to aM in the treated and control preparations ($n = 6$ in treated and control groups). ns, not significant.

Effect of exposure to BCD on the response to theophylline

In contrast, a 40-min exposure to BCD (1.4×10^{-2} mol/L) in the basolateral bath (bBCD) had no effect on the hydrosmotic response to theophylline (1.0×10^{-2} mol/L) in the same bath (bTHEO) (Fig. 4).

Response to apical hypertonicity

Increased J_w by addition of mannitol (3.1×10^{-1} mol/L) to the apical bath was not altered by preexposure to BCD (1.4×10^{-2} mol/L) either in the apical (aBCD) or the basolateral bath (bBCD) during the 40 min period (Table 1).

Discussion

Exposure of isolated toad bladder to the BCD did not per se consistently modify the values of basal water permeability. Inhibition of the hydroosmotic response to nystatin present in the external bath following exposure of the same side of the tissue to BCD (Fig. 1), however, was very evident and consistent with the removal and sequestration of CHOL from the membrane. The simplest explanation for this inhibition implies that a decreased amount of CHOL in the membrane translates as a decrease in the available binding sites for nystatin and thus a fall in magnitude of the response.

A similar exposure of the apical membrane to aBCD did not alter the response to bOT stimulation (Fig. 2), suggesting that the presence of a high amount of CHOL in this membrane is not critical for the response. In addition, this result suggests that a 40-min apical exposure to BCD does not affect aquaporin function, neither by direct action nor by modifying the lipid environment of aquaporins.

On the other hand, exposure of the basolateral side of the bladder to BCD significantly inhibited the response to stimulation by the peptide (Fig. 3).

In contrast, the hydroosmotic response to bTHEO (which increases J_w without interacting with receptors or other membrane components) was not modified by the same BCD treatment (Fig. 4).

These results strongly suggest that the integrity of the lipid environment surrounding the receptors involved in this effect is crucial to the development of the response. A modulating action of BCD on the OT receptor and other structures, such as the nicotinic acetylcholine receptors and related G protein (Burger et al. 2000; Becher and McIlhinney 2005), has been described in other tissues. In the case of the OT receptor in the myometrium of guinea pigs, changes in the binding affinity of the peptide following exposure to a BCD derivative appear to involve more than just a modification of the lipid environment surrounding the receptor and may imply a direct interaction of CHOL with the receptor itself (Klein et al. 1995).

The receptor that mediates the hydroosmotic effect in the bladder of the toad is not a specific receptor for OT, as its native agonist is the peptide hormone vasotocin. The agreement between our present results and previous observations reported in guinea pig uterus and other tissues, however, shows that there may be great similarities between the receptors involved, despite the results being from animal species that are relatively distant from an evolutionary point of view (mammals and amphibians).

In contrast to observations made in other polarized epithelia, the lack of effect of exposure to BCD on the J_w increase caused by apical exposure to hypertonic solution (Table 1) is a strong indication that changes in the lipid structure of the membrane have little impact on the process of permeabilization brought about by opening the tight junctions.

The information presented here adds to the conclusion that, as in other tissues, membrane CHOL removal by BCD alters the organ's function. It also points out that in epithelia performing vectorial transport — and thus exhibiting significant functional differences between its apical and basolateral membranes — the effects depend to a great extent on the membrane that is depleted. This evidence also adds weight to the notion that membrane CHOL has great physiological relevance, considering the primary function of water permeability in animal life.

References

Agre, P., Bonhivers, M., and Borgnia, M.J. 1998. The aquaporins, blueprints for cellular plumbing systems. J. Biol. Chem. **273**(24): 14659–14662. doi:10.1074/jbc.273.24.14659. PMID:9614059.

Anderson, R.G.W. 1998. The caveolae membrane system. Annu. Rev.

Biochem. **67**(1): 199–225. doi:10.1146/annurev.biochem.67.1.199. PMID:9759488.

Becher, A., and McIlhinney, R.A. 2005. Consequences of lipid raft association on G-protein-coupled receptor function. Biochem. Soc. Symp. **72**(72): 151–164. PMID:15649139.

Burger, K., Gimpl, G., and Fahrenholz, F. 2000. Regulation of receptor function by cholesterol. Cell. Mol. Life Sci. **57**(11): 1577–1592. doi:10.1007/PL00000643. PMID:11092453.

Klein, U., Gimpl, G., and Fahrenholz, F. 1995. Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry, **34**(42): 13784–13793. doi:10.1021/bi00042a009. PMID:7577971.

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**(2): F103–F111. PMID:7039340.

Liscum, L., and Munn, N.J. 1999. Intracellular cholesterol transport. Biochim. Biophys. Acta, **1438**(1): 19–37. PMID:10216277.

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., and Pitha, J. 1989. Differential effects of α-, β-and γ-cyclodextrins on human erythrocytes. Eur. J. Biochem. **186**(1–2): 17–22. doi:10.1111/j.1432-1033.1989.tb15171.x. PMID:2598927.

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. C, **84**(1): 139–144. PMID: 2873944.

Orce, G., Castillo, G., Chanampa, Y., and Bellomio, A. 2004. Permeability to water in a tight epithelium: possible modulating action of gap junctions. Can. J. Physiol. Pharmacol. **82**(6): 417–421. doi:10.1139/y04-037. PMID:15381966.

Simons, K., and van Meer, G. 1988. Lipid sorting in epithelial cells. Biochemistry, **27**(17): 6197–6202. doi:10.1021/bi00417a001. PMID:3064805.