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Contribution of Mucosal Chloride to Chloride in Toad Bladder Epithelial Cells

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Summary. Epithelial cells were scraped from the bladders of toads of the species *Bufo marinus* obtained from the Dominican Republic. These epithelial cells exchanged their chloride virtually completely with ^{36}Cl in the medium within 60 min. Of this chloride, about 93% came from the serosal medium. The approximately 20 mmole/kg dry wt of chloride which equilibrates with ^{36}Cl in the mucosal medium was still present when choline replaced sodium in the medium in the presence of amiloride (10^{-4} M) and was almost all readily removed by rapid washing of the mucosal surface immediately prior to analysis. These observations suggest that little chloride of mucosal origin is truly intracellular. This conclusion is supported by the fact that after vasopressin the increased cellular chloride was not of mucosal origin.

Many epithelia transport sodium from the medium bathing their outer surfaces to that bathing their inner surfaces by an active energy-dependent process. To preserve electroneutrality, this net movement of sodium must be accompanied either by an equal net movement of negatively charged ions in the same direction or of positively charged ions in the opposite direction or by some combination of such movements. These ion movements may be a passive consequence of the electrical gradient generated by the movement of sodium. Alternatively, they might also result from primary active ion transport. In many epithelia the pathways which ions traverse remain to be established. Since primary active transport requires the coupling of metabolic energy to the ion movement, active transport should require movements of ions through the cells. However, passive ion movements could occur either through the cells, between the cells through the tight junctions and lateral intercellular spaces, or by both these routes.

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The mucosal membrane of the toad bladder epithelial cell seems to be highly selective. While sodium can cross this membrane (Leaf, 1965; Macknight, Civan & Leaf, 1975) other cations, e.g., choline (Macknight *et al.*, 1975) and potassium (Gatzy, 1971; Finn & Nellans, 1972; Robinson & Macknight, 1976) cannot. Much less is known of the permeability of the mucosal cell membrane to chloride. In the open-circuited bladder *in vitro* there is a net movement of chloride from the mucosal to serosal medium in response to the electrical gradient generated by sodium transport, but whether this chloride moves through or between the cells or by both routes remains debatable.

The purpose of the present experiments was to investigate the exchangeability of chloride in the epithelial cells with chloride in the mucosal and serosal media. The evidence obtained suggests that the mucosal membrane permeability to chloride is low and it seems likely that at least most of the chloride crossing the toad bladder must pass between rather than through the cells.

Materials and Methods

The sodium Ringer's solution contained (mM): 117 Na^+, 3.5 K^+, 1.0 Ca^{2+}, 117 Cl^-, and 10 glucose, buffered at pH 7.8 by 2 mM HPO^{2-}. Sodium-free choline Ringer's solution contained (mM): 117 choline, 4.0 K^+, 1.0 Ca^{2+}, 117 Cl^-, and 10 glucose, buffered at pH 7.8 by 2 mM HPO^{2-}.

Inulin-carboxyl-^{14}C and inulin-methoxy-^3H were obtained from New England Nuclear Corporation as was $H^{36}Cl$ which was neutralized with NaOH before use. Amiloride was the gift of Merk, Sharp and Dohme.

The methods used here were essentially those described by Macknight, DiBona, Leaf & Civan (1971*a*), Macknight, Leaf & Civan (1971*b*) and Macknight *et al.* (1975). Toads of the species *Bufo marinus*, obtained from the Dominican Republic (National Reagents, Bridgeport, Conn.) were doubly pithed and their hearts immediately perfused with sodium Ringer's to deblood the bladders.

Paired hemibladders were mounted in chambers of area 8.04 cm^2. Initially, both mucosal and serosal surfaces were bathed with sodium Ringer's and the open-circuit potential difference recorded. They were then continuously short-circuited. Air was bubbled through both mucosal and serosal solutions throughout the experiments.

Once the short-circuit current (SCC) had stabilized, the chambers were drained and refilled with the appropriate solutions as indicated. Whenever it was desired to bathe the mucosal surface of the hemibladder with sodium-free solution, the mucosal chamber was drained, then filled and drained five times using fresh choline Ringer's before filling with choline Ringer's. This procedure allowed washout of sodium from the tissue and chamber and prevented any appreciable concentration of sodium in the final solution during the course of the experiment. The sodium contents of these solutions were checked by flame photometry at the end of each experiment. Both the mucosal and serosal solutions contained radioactive inulin for at least 60 min before the end of each experiment. Since there is no measurable flux of inulin across the bladder, the use of ^3H-inulin in one

Table 1. Labelling of epithelial chloride

Incubated	1 hr	Δ	p	2 hr	Δ	p
Epithelial tissue (mmole/kg dry wt)						
Cl	584±31	8±6	>0.10	551±10	3±5	>0.50
^{36}Cl	576±32			548±12		
Cellular composition (mmole/kg dry wt)						
Cl	182±6	8±5	>0.10	160±5	4±5	>0.50
^{36}Cl	174±5			156±9		
% cellular Cl labelled with ^{36}Cl						
	1 hr		95.6±2.9			
	2 hr		97.6±3.4			
n=8						

Hemibladders from 4 toads were cut in half and incubated in NaR containing ^{3}H inulin and ^{36}Cl in beakers bubbled with air. After either 55–65 min or 115–125 min, quarter bladders were removed, blotted, scraped and the scrapings analyzed. The experiment was so designed that the mean values obtained at 1 hr and 2 hr were both derived from quarter bladders from the same hemibladders.

solution and ^{14}C inulin in the other allowed separate correction of tissue composition for any mucosal and serosal extracellular fluid in the sample. At the conclusion of each experiment, the chambers were drained and the portion of the hemibladder exposed in the chambers removed.

In the initial experiment to examine exchangeability of cellular chloride (Table 1), hemibladders were cut in two and incubated in beakers aerated with air as described by Robinson and Macknight (1976). When removed from the beakers these quarterbladders were handled in the same way as hemibladders removed from chambers.

All tissues were blotted several times on Whatman filter paper No. 542 until no visible moisture was transferred to the paper. They were then placed mucosal surface up on a Pyrex petri dish and the epithelial cells were removed by scraping with a glass slide. It has been shown that this procedure removes over 90% of the epithelial cells from the underlying tissue without contamination by other tissue elements (Macknight *et al.*, 1971*a*). The epithelial cell scrapings were then transferred to tared Pyrex tubes.

Scrapings were weighed, dried to constant weight at 105 °C in a hot air oven, cooled at room temperature for 40 min, and reweighed. The water content of the scraped tissue was taken to be equal to the loss of weight upon drying.

In all experiments, tissue was extracted for 7–10 days in 10 ml of 0.1 M nitric acid. Sodium and potassium were measured with an EEL flame photometer and chloride with a Cotlove titrator.

Inulin-^{14}C, inulin-^{3}H and ^{36}Cl were determined by adding 17 ml scintillation solution to 2 ml tissue extract. The samples were then counted in a three-channel Packard Tri-Carb Liquid Scintillation counter. Samples of medium, 0.1 ml, were diluted by the addition of 3 ml 0.1 M nitric acid and 2 ml of this was then counted in the same way. For counting the three β emitters simultaneously, the counter was set up so that no ^{3}H or ^{14}C counts appeared in the ^{36}Cl channel. The only spillover of counts which required correction was spillover from ^{36}Cl to ^{14}C (14% of the ^{36}Cl counts appeared in the ^{14}C channel) and from ^{14}C to ^{3}H (5.3% of the total ^{14}C counts appeared in the ^{3}H channel).

Corrections were further minimized by using ^{3}H-inulin and ^{36}Cl in the same (the mucosal) medium.

The tissue values of water and ions were corrected for contamination with extracellular fluid using the assumption that isotopically labeled inulin equilibrated in the extracellular space and that the ions in this space were at the same concentration as in the bulk of the medium. Recent work (McIver & Macknight, 1974) confirms that inulin is as suitable an extracellular marker as any for toad bladder epithelial cells under these experimental conditions.

The intracellular water content is expressed as kg H_2O/kg dry wt. The ion contents of the cells are shown in mmoles/kg dry wt. Their concentrations in mmoles/kg intracellular water can be derived by dividing the ion content by the cellular water content.

The intracellular Cl content determined by ^{36}Cl was calculated in the following way:

$$\text{I.C. Cl content} = \frac{(\text{intracellular counts})(\text{Cl}_m) \times 10^3}{(\text{medium counts})(\text{tissue dry wt})}$$

where I.C. Cl content = mmoles of chloride/kg tissue dry weight from ^{36}Cl, Cl_m = μmoles of chloride/ml of mucosal medium, intracellular counts = total tissue counts/min corrected for ^{36}Cl in inulin space of the mucosal and serosal surfaces, medium counts = counts/min/ml mucosal medium, and tissue dry wt = tissue dry weight in mg. In experiments in beakers where media containing ^{36}Cl bathed both mucosal and serosal surfaces, Cl_m = μmoles of chloride/ml in the medium and medium counts represent counts in the medium.

Values presented in text and Tables represent the mean ± SEM where appropriate, of the number of observations shown. Means of paired analyses are presented with their difference and its SE. Significances of differences between means have been evaluated using Student's *t* test.

Results

Exchangeability of Epithelial Chloride

Before entry of chloride to the epithelial cells from the mucosal medium could be investigated, it was important to investigate exchangeability of cellular chloride. Table 1 shows the results of simultaneously labelling chloride for 1 or 2 hr from both mucosal and serosal media. The results for total scrapings (epithelial cells plus extracellular fluid) and for cellular chloride (chloride outside of the inulin space) are shown. The differences in both cases for both 1 and 2 hr between chloride measured by potentiometric titration and by ^{36}Cl are trivial and it seems that all cellular chloride exchanges with medium chloride within 60 min. Subsequent experiments were therefore designed to allow at least 60 min of exposure of cells to ^{36}Cl before scraping and analysis.

Does Chloride Enter Cells from the Mucosal Medium?

Experiments examining the sodium content of epithelial cells have revealed that of the total content, 30 – 40 mmole/kg dry wt representing

Table 2. The effect of mucosal choline Ringer's and amiloride on ^{36}Cl uptake from the mucosal medium

	H_2O	Na	K	Cl	^{36}Cl
	kg/kg dry wt	mmole/kg dry wt			
NaR	4.48±0.09	252±12	513±8	334±11	21.4±2.1
Choline R	4.26±0.08	192±6	498±8	291±5	21.9±2.6
Δ	0.22±0.08	60±10	15±15	43±12	0.5±3.7
p	>0.05	<0.001	>0.30	<0.01	>0.80
n=8					

Paired hemibladders mounted in chambers were exposed on both surfaces to NaR. Once SSC stabilized, the chambers were drained. Both mucosal surfaces were washed thoroughly 2 to 3 times over 40 min with either NaR or Choline R, while NaR bathed the serosa. The mucosal medium was then replaced with either NaR, or Choline R containing ^{3}H inulin and ^{36}Cl. Choline R also contained amiloride, 10^{-4} M. The serosal medium was NaR + ^{14}C inulin. After 60 min hemibladders were removed, blotted, scraped and the scrapings analyzed.

20–25% equilibrates with ^{24}Na in the mucosal medium (Macknight *et al.*, 1975). Of this about one-third to one-half is still there after incubation with amiloride (10^{-4}–10^{-3} M) in the mucosal medium, is removed by rapid washing of the mucosal surface before analysis, and may therefore not be truly intracellular (Macknight *et al.*, 1975).

Incubation of hemibladders for 60 min in sodium Ringer's with ^{36}Cl in the mucosal medium resulted in a labelling of 21 mmole/kg dry wt representing only about 7% of the total cellular chloride (Table 2).[1] Moreover, with mucosal sodium replaced by choline, and amiloride, 10^{-4} M, to prevent any uptake of sodium either diffusing from serosal to mucosal medium or remaining in the mucosal chamber after washing, the same total amount of cellular chloride was labelled with mucosal ^{36}Cl (Table 2), in spite of the fact that cellular sodium decreased as expected (Macknight *et al.*, 1975). Since choline does not enter cells from the mucosal medium cellular chloride decreases also to preserve electroneutrality (Macknight *et al.*, 1975). Despite this decrease in cellular chloride, the fact that the cellular chloride equilibrated with mucosal ^{36}Cl did not change suggests that much of this chloride lying outside of the mucosal inulin space may nevertheless not be truly intracellular.

1 The data presented here, and in Tables 3 and 4, was obtained at a different time of year and on a different batch of toads from those used for the experiments reported in Table 1. The cellular chloride contents in Tables 2–4 are considerably higher than those in Table 1. It is our experience that cellular water, sodium and chloride show such fluctuations from time to time, for reasons which remain to be clarified. Since both control and experimental observations were made on hemibladders from the same toads these differences should not affect the conclusions drawn from the results.

Table 3. Effect of washing of the mucosal surface, on ^{36}Cl in epithelial cells

	H_2O	Na	K	Cl	^{36}Cl
	kg/kg dry wt	mmole/kg dry wt			
Control	4.38±0.10	258±10	489±10	329±8	24.3±3.1
Washed	4.41±0.16	245±15	532±20	314±15	3.1±1.8
Δ	0.03±0.10	13±10	43±20	15±11	21.2±3.0
p	>0.70	>0.20	>0.50	>0.20	<0.001
n=8					

Paired hemibladders mounted in chambers were exposed on both surfaces to NaR. Once SCC stabilized, the chambers were drained. The mucosal surface was then bathed with NaR containing ^{3}H inulin and ^{36}Cl, the serosal surface with NaR and ^{14}C inulin. After 60 min one hemibladder (control) was removed, blotted and scraped, the other was briefly washed 5× over 30–45 sec with K_2SO_4 made isosmotic with sucrose, before removal, blotting and scraping.

To examine this further, the experiments, the results of which are shown in Table 3, were performed. Rapid washing of the mucosal surface of the hemibladder at the end of the experiment before its removal from the chamber removes a significant fraction of noninulin space sodium (Macknight *et al.*, 1975) and potassium (Robinson & Macknight, 1976). As shown in Table 3 it also removes most of the ^{36}Cl. Washing was performed with K_2SO_4 made isosmotic with sucrose. Neither potassium, SO_4, nor sucrose readily penetrates the mucosal cellular membranes. These solutes should not therefore displace chloride from the cells. Note that total cellular chloride fell to the same extent as ^{36}Cl, as did sodium. However, the scatter in the measurements of these ions by chemical analysis is too great for these small changes to reach the level of statistical significance. (The increased potassium content and the associated increase in its SEM reflect the fact that the mucosal surface was washed with K_2SO_4). These results therefore suggest that little if any mucosal medium chloride actually enters the epithelial cells under normal conditions.

The Effect of Vasopressin on Cellular Chloride

It has been shown previously (Macknight *et al.*, 1971*b*) that epithelial cells scraped from hemibladders mounted in chambers have gained water, sodium and chloride after exposure to vasopressin and that the source of this increased sodium is the mucosal medium (Macknight *et al.*, 1971*b*). Table 4 summarizes the results of experiments to identify the source of the increased cellular chloride which accompanies this uptake

Table 4. Effect of vasopressin on cellular ^{36}Cl in toad bladder

	H_2O	Na	K	Cl	^{36}Cl
	kg/kg dry wt	mmole/kg dry wt			
Control	3.63±0.23	221±21	457±15	277±18	29.0±2.8
+vasopressin	4.06±0.18	266±18	437±13	338±12	34.0±4.3
Δ	0.43±0.10	45±9	20±12	61±13	5±4
p	<0.001	<0.001	>0.05	<0.001	>0.20
n=15					

Paired hemibladders mounted in chambers were exposed on both surfaces to NaR. Once SCC stabilized, the chambers were drained. The mucosal surface was then bathed in NaR containing ^{3}H inulin and ^{36}Cl, the serosal surface with NaR+^{14}C inulin. After 50 min, vasopressin (100 mU/ml) was added to the serosal medium bathing one of each pair of hemibladders. Once SCC had risen and stabilized in the vasopressin-treated tissue, hemibladders were removed, blotted, scraped, and the scrapings analyzed.

of sodium. The increased cellular water, sodium and chloride, seen in Table 4, after vasopressin, confirm the previous results. However, unlike sodium, the increased cellular chloride has not come from the mucosal medium, for cellular chloride equilibrated with ^{36}Cl in the mucosal medium has not increased (Δ 5±4) though total cellular chloride has increased by 61±13 mmole/kg dry wt. Thus the chloride accompanying the increased sodium after vasopressin must have come from the serosal medium.

Discussion

The results presented here suggest that epithelial cellular chloride is obtained largely, if not entirely, from the serosal not the mucosal medium, both under normal conditions, and after vasopressin. This conclusion is based upon (i) the small fraction of total noninulin space chloride which equilibrates with ^{36}Cl in the mucosal medium (Table 2), (ii) the fact that this fraction is unaltered when sodium Ringer's is replaced on the mucosal side by choline Ringer's containing sufficient amiloride to completely block sodium entry to the epithelial cells, even though cellular chloride falls substantially as expected (Table 2), (iii) the fact that rapid washing of the mucosal surface with isosmotic K_2SO_4 containing sucrose removes almost all of the chloride equilibrated with ^{36}Cl in the mucosal medium (Table 3), and (iv) the observation that, though cellular chloride increases markedly after vasopressin, the amount equilibrated with mucosal ^{36}Cl is unchanged (Table 4).

The location of that chloride of mucosal origin which lies outside the mucosal inulin space but which, because it is rapidly removed by

washing and is unaffected by amiloride, seems not to be truly intracellular, is conjectural. Since the spaces occupied by inulin, and by the much smaller molecule mannitol, on the mucosal surface are very similar (Macknight *et al.*, 1975), the problem seems not simply to be one of inadequate measurement of the extracellular space. Both cations, including sodium (Macknight *et al.*, 1975), potassium (Robinson & Macknight, 1976) and choline (A.D.C. Macknight, *unpublished observations*), and now the anion chloride, may all be removed to some extent from the mucosal surface by rapid washing. Are ions associated with the glycoprotein which lies in close apposition to the mucosal cellular membrane? Are ions associated with a mucus layer over the epithelial cell surface? Alternatively, are some ions removed, by rapid washing, from the tight junctions? Only further experimental work can answer these questions.

From these results it is concluded that the mucosal cellular membrane in epithelial cells from bladders of Dominican toads is quite impermeable to chloride which would pass predominantly through the tight junctions and lateral intercellular spaces from mucosal to serosal media rather than through the cells. Thus the positive charge on cellular sodium in the transepithelial transport pool would be counterbalanced by chloride of serosal, not mucosal, origin. When the transport pool increased in size, as after vasopressin, more chloride would be required to enter cells to maintain electroneutrality, and this chloride would come from the serosal medium (Table 4).

It is important to note that the experiments reported here were performed on toads of the species *Bufo marinus* obtained from the Dominican Republic. Unlike toads of the same species obtained from Colombia (Finn, Handler & Orloff, 1967) and some species of frogs (Kristensen, 1972; Alvarado, Dietz & Mullen, 1975; Schneider, 1975; Watlington & Jessee, 1975), these Dominican Republic toads do not appear to transport chloride actively from mucosal to serosal solutions (Davies, Martin & Sharp, 1968). Thus the conclusion presented here, that little or no chloride enters the cells from the mucosal medium, might not apply to epithelia transporting chloride.

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