J. Membrane Biol. 143, 207–217 (1995)

The Journal of
Membrane
Biology

Chloride Transport Activation by Plasma Osmolarity During Rapid Adaptation to High Salinity of *Fundulus heteroclitus*

J.A. Zadunaisky,[1,2,3] S. Cardona,[1,2] L. Au,[1,2] D.M. Roberts,[2] E. Fisher,[1,2] B. Lowenstein,[2] E.J. Cragoe, Jr., K.R. Spring[3]

[1]Departments of Physiology and Biophysics and of Ophthalmology, New York University Medical Center, 550 First Avenue, New York, NY 10016

[2]Mount Desert Island Biological Laboratory, Salsbury Cove, Maine 04672

[3]Laboratory of Kidney and Electrolyte, Metabolism, National Institutes of Health, Bethesda, Maryland 20892

Received: 7 June 1994/Revised: 23 September 1994

Abstract. Transition from low salt water to sea water of the euryhaline fish, *Fundulus heteroclitus,* involves a rapid signal that induces salt secretion by the gill chloride cells. An increase of 65 mOsm in plasma osmolarity was found during the transition. The isolated, chloride-cell-rich opercular epithelium of sea-water-adapted *Fundulus* exposed to 50 mOsm mannitol on the basolateral side showed a 100% increase in chloride secretion, which was inhibited by bumetanide 10^{-4} M and 10^{-4} M DPC (*N*-Phenylanthranilic acid). No effect of these drugs was found on apical side exposure. A Na^+/H^+ exchanger, demonstrated by NH_4Cl exposure, was inhibited by amiloride and its analogues and stimulated by IBMX, phorbol esters, and epithelial growth factor (EGF). Inhibition of the Na^+/H^+ exchanger blocks the chloride secretion increase due to basolateral hypertonicity. A Cl^-/HCO_3^- exchanger was also found in the chloride cells, inhibited by 10^{-4} M DIDS but not involved in the hyperosmotic response. Ca^{2+} concentration in the medium was critical for the stimulation of Cl^- secretion to occur. Chloride cell volume shrinks in response to hypertonicity of the basolateral side in sea-water-adapted operculi; no effect was found on the apical side. Fresh-water-adapted fish chloride cells show increased water permeability of the apical side. It is concluded that the rapid signal for adaptation to higher salinities is an increased tonicity of the plasma that induces chloride cell shrinkage, increased chloride secretion with activation of the $Na^+K^+2Cl^-$ cotransporter, the Na^+/H^+ exchanger and opening of Cl^- channels.

Key words: Gill chloride cell — Cl^- secretion — *Fundulus heteroclitus* — Cell volume regulation — Na^+/H^+ exchanger — Cl^-/HCO_3^- exchanger

Correspondence to: J.A. Zadunaisky

Introduction

The isolated opercular epithelium of the killifish, *Fundulus heteroclitus,* is an excellent model to study chloride secretion because of the presence of abundant chloride cells. When mounted as a membrane it produces a current carried only by chloride ions from the basolateral to the apical side (*see* Zadunaisky, 1984; Karnaky, 1992). In the present study, this preparation was used to understand the mechanisms of the rapid signal detected by the chloride cells to secrete chloride when euryhaline fish move from low salinity to sea water. Simultaneously, the effect of cell volume shrinkage on membrane transporters was studied.

The adaptation from low to high salinities of euryhaline fish as in the case of *Fundulus* occurs in two distinct stages. There is a quick adaptation that consists of a rapid increase of chloride permeability by the gill in general (Maetz & Bornacin, 1975). The second adaptation is a long-term one taking several days or weeks and manifested by an increase in the electrical resistance and formation of new junctional strands in the tight junction (Sardet, Pisam & Maetz, 1979). There is also an increase in the number of chloride cells, and the presence of more sites for ouabain binding in the chloride cells, an index of a greater number of molecules of the Na^+K^+ATPase (Karnaky et al., 1976; Sardet et al., 1979). However, the mechanism concerning the rapid changes that permit the fish to accomplish the quick transition from a few milliosmoles in the surrounding medium to the 1,000 plus milliosmoles in sea water is not known.

It is clear that during adaptation to higher salinities euryhaline fish drink sea water, absorb salts through the intestine and secrete them through the gills, utilizing this organ in preference to the kidney (Smith, 1930). The changes in osmolarity then occur not only in the outside or apical side of the chloride secretory cells but also in the plasma, that is, in the basolateral side of the epithelium. In fact, there is a transient increase in the concentration of NaCl in the plasma of euryhaline fish that can reach 50 mM above the normal level and last for many hours (Holmes & Donaldson, 1969). The gill secretory epithelium is then activated and starts to transport NaCl to the apical side, while the concentration of salt in the plasma and therefore its osmolarity is reduced. The second phase is controlled by hormones, possibly cortisol (Foskett et al., 1983) when the necessary changes in protein synthesis and cell proliferation for the prolonged, fully adapted stage take place.

We utilized isolated opercular epithelia (Degnan, Karnaky & Zadunaisky, 1977) of *F. heteroclitus* to test if osmolarity and therefore cell volume changes are involved in the rapid process of adaptation. Besides the opercular epithelium (Zadunaisky, 1984), we have found chloride secretion in the frog skin (Zadunaisky, Candia & Chiarandini, 1963), the frog cornea (Zadunaisky, 1966), the rabbit cornea (Zadunaisky et al., 1973), the retinal pigment epithelium (Wiederholt & Zadunaisky, 1984), and the ciliary epithelium of the eye (Wiederholt & Zadunaisky, 1986). Updates on epithelial chloride transport can be found in Zadunaisky (1984) and more recently in Zadunaisky (1992).

The present findings indeed indicate that increases in basolateral osmolarity produce rapid stimulation of the chloride secretion of the isolated opercular epithelium. This stimulation requires the presence of the Na^+/H^+ exchanger, the $Na^+K^+2Cl^-$ cotransporter but does not involve the Cl^-/HCO_3^- exchanger. The characterization of these membrane proteins and their involvement in the osmotic response of the chloride cells of the opercular epithelium are herein described.

Materials and Methods

F. heteroclitus, male and female, were trapped in fluvial estuaries draining into Frenchman's Bay in Maine near Bar Harbor during late June, July and August. Fish were kept for a minimum of one week in running sea water before use and fed twice daily commercial fish food or frozen bloodworms. Adaptation to fresh water was done in 3–4 stages reducing the salt content gradually during 4–5 days. This new method of adaptation to fresh water differs from the one utillized by Degnan et al. (1977). The slow adaptation utilized here renders opercular preparations that do not show an electrical potential difference or short-circuit current, in contrast to our previous observation. Also, specimens were utilized at least one month after being in full fresh water. Survival after one month in tap fresh water was 60%. In New York and Bethesda, fish were kept in artificial static sea water tanks, at a room temperature of 20°C and bubbled with air.

Tissue Preparation

Specimens were immobilized by pithing and the epithelium lining the inside of the opercular flaps was dissected out as originally described in this laboratory (Degnan et al., 1977). Essentially, the epithelium was gently teased out away from the bone of the operculum using microdissection forceps or "ad hoc" glass tips. Then the inner opercular sheet was placed over the aperture of a plastic disc that was then mounted in a highly modified Ussing chamber.

Recently, we have departed from the "pinning" method of securing the operculum to the disc to a new strategy. On one side of the plastic disc a groove was machined around the aperture for the tissue. The opercular epithelium was extended over the aperture of the disc reaching over the groove. Then a flat Teflon washer machined to fit tightly in the groove was placed over and pressed in with the tissue. A metal rod 3.5 inches long and a tip diameter equal to the diameter of the ring or washer was used to press the washer and tissue into the groove. In this method no Sylgard is used; however, the electrical resistances of the preparations are comparable to the ones obtained with the original "pinning" method. However, the potential differences and short-circuit currents are 20% lower with the new method, but the ring mounting is rapid, simpler and more effective than the old method.

Electrical Measurements

The potential difference of the opercular epithelia mounted in the chamber with Ringer solutions on both sides was monitored with calomel electrodes connected via agar-Ringer bridges to a voltage clamp unit (University of Iowa 710 C-1). Current was measured by short-circuiting the voltage to O automatically. Tissue conductance was monitored continuously by passing a bidirectional pulse of 1–2 mV with a duration of 1 sec every 50 sec.

Solutions and Gases

The basic solution used contained (in mM) 135 NaCl; 16 $NaHCO_3$; 2.5 KCl; 1.0 $CaCl_2$ and 1.0 $MgCl_2$ and 5.5 dextrose. All solutions were adjusted to a pH of 7.4. The basic preparation consisted in an operculum from a sea-water-adapted fish bathed in the above Ringer and gassed with 1% CO_2 99% O_2.

For studies of the Na^+/H^+ and Cl^-/HCO_3^- exchangers in CO_2 and HCO_3^- free conditions, the gas used was air and the Ringer solution contained 25 mM HEPES buffer substituting for the bicarbonate. The pH was adjusted to 7.4.

Chemicals and Drugs

All chemicals and drugs were reagent grade, purchased from Sigma Chemical. The amiloride analogues HMA (3-amino-6-*N*-diaminomethylene)-5-(1-homopiperidyl)pyrazinecarboxamide) and EIPA(3-amino-6-chloro-5-(*N*-ethyl-*N*-isopropylamino)-*N*-(diaminomethylene)pyrazine-carboxamide) were synthesized specifically for this study (Cragoe et al., 1967) and purchased from Mr. E.J. Cragoe, Jr. The relative potency of HMA and EIPA are documented in a study by Simchowitz, Kleyman and Cragoe (1992). Drugs were added to the hemichambers containing Ringer solution in volumes ranging from 50 to 250 μl dissolved in either the basic solution or DMSO. Tests for the effects of DMSO alone were performed in each case that it was used.

Plasma Samples

Osmolarity was measured in plasma samples of 50 μl obtained from the pooled blood of seven specimens of *Fundulus*. A sharp section of the tail was performed and blood allowed to free flow into small heparinized test tubes. Attempts to determine osmolarity in smaller samples produced erratic results. The pooled plasma gives a reliable number for the osmolarity and its changes; however, it did not permit a classical statistical analysis. The differences in plasma osmolarity, as seen in the section of Results on Fig. 1 are self-explanatory.

Quantitative Microscopy

Quantitative microscopy was used to measure chloride cell area and volume utilizing computer programs based on planimetry. Imaging was performed with an inverted microscope (Leica Diavert) fitted with differential interference contrast optics (Furlong & Spring, 1990). A high resolution television camera (Video Scope 2000 N) was mounted on the microscope and connected to a video monitor. Images were stored on computer disks for later morphometric analysis (Image1), Universal Image, Media, PA). The opercular epithelia were secured as described above with the flat washer and groove arrangement and sealed in a special flat chamber with separate perfusion of both apical and basolateral sides. The tissue was centered in the optical axis of the microscope and chloride cell images were obtained. The complexity of the tissue, including the presence of other cells types, required some training to distinguish the chloride cells. However, after some practice they could be studied with these methods.

Results

Plasma Osmolarity during the Transition from Fresh to Sea Water

Fundulus fully adapted to fresh water were transferred to sea water. Plasma samples were obtained immediately before the transfer and at regular intervals thereafter up to 84 hr. Osmolarity was measured and plotted against time. The results are shown in Fig. 1, where it can be observed that from a control value of 290 mOsm the osmolarity of plasma increased to 355 mOsm in 10–15 hr. The difference of 65 mOsm represents a 22.4% increase in tonicity and in terms of a salt such as NaCl corresponds to a 32.5 mM increase in the plasma. Slowly afterwards, the plasma levels drop to normal while the fish are still in sea water. These results clearly indicate that the regulation is there and stimulated the search for osmotic effects "in vitro" on the chloride-cell-rich opercular epithelium mounted as a membrane.

Response of Isolated Opercular Epithelia to Osmolarity Increases Induced by Mannitol in the Basolateral Side

Sea-water-adapted operculi were mounted in regular Ringer solution and 50 mOsm of mannitol was tested on the chloride current.

The result of one typical experiment is shown in Fig. 2*A*. The addition of mannitol produced a rapid increase in the short-circuit current when added to the basolateral

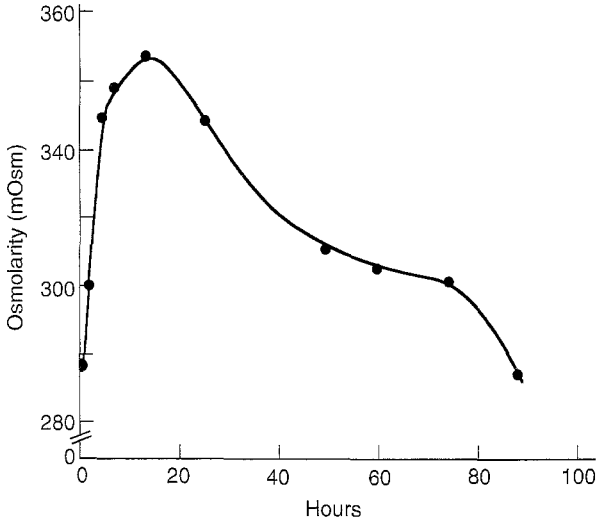


Fig. 1. Plasma osmolarity of *F. heteroclitus* transferred at 0 time from fresh water to sea water. Each point corresponds to the osmolarity of a plasma sample pooled from the blood of seven fish. Note the increase of 65 mOsm at the peak and the gradual return to normal plasma values.

side of the preparation, but no response was observed when added to the apical side. The increase is sustained and it slowly tends to decrease in several hours. Washouts of the basolateral side containing the added mannitol with regular Ringer solution induced a very slow return to basal current. Therefore, one dose of mannitol only was used in each experiment.

In Fig. 2*B* a dose-response curve for six experiments for each osmolarity is shown. The increase in current is presented as a percentage increase above the basal, spontaneous current of the tissues. Addition of 50 mOsm of mannitol in the basolateral side produced close to a 100% increase in chloride current, without significant changes in electrical resistance. Maximal effects were observed starting at 200 mOsm of mannitol. The basic salt solution had an osmolarity of 300 mOsm, therefore the addition of 50 mOsm represents a 16.6% change in total osmolarity and 300 mOsm equals 200%. The sensitivity of the cells to small increases in the osmolarity of the basolateral side solution is also appreciated in Fig. 2*B* where an increase of 4.1% in total osmolarity produced by the addition of 12.5 mOsm of mannitol induces an increase of more than 20% in the chloride current output.

Therefore, the sensitive side is the basolateral or plasma side of the tissue and the effect most probably occurs as a consequence of shrinkage of the chloride cells, as shown in the results of the imaging experiments described further on.

The activation of the chloride secretory system by the osmotic shock implies opening of the Cl channel, activation of the $Na^+K^+2Cl^-$ cotransporter, and possibly the activation of the Na^+/H^+ and/or the Cl^-/HCO_3^- exchangers. The effect of 50 mOsm of mannitol to produce

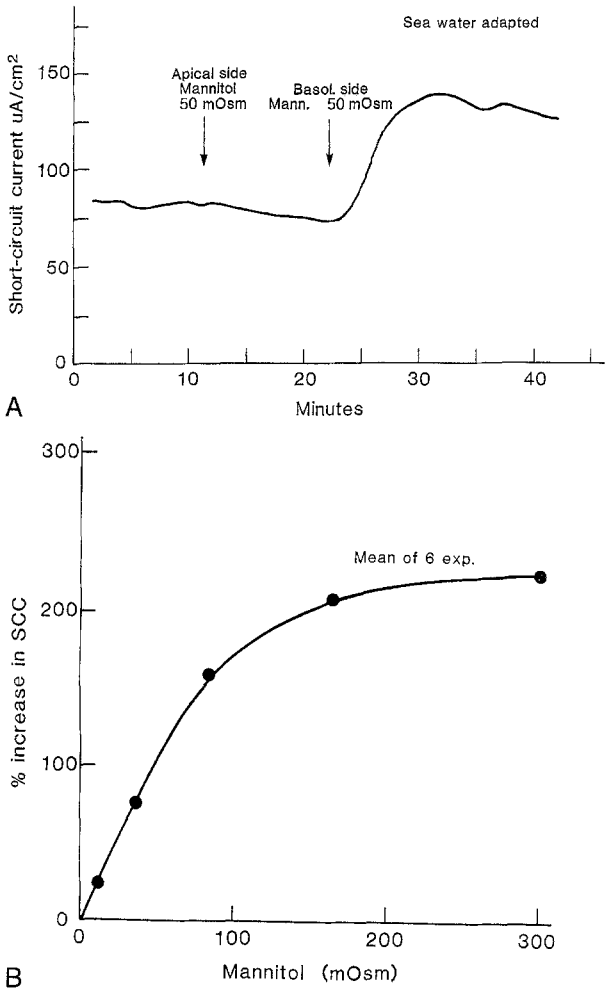


Fig. 2. (*A*) Increase of chloride secretion produced by 50 mOsm of mannitol in an isolated operculum of *F. heteroclitus*. No effect is found on the apical side, but shrinkage of the chloride cells induced on the basolateral side produce rapid current stimulation. (*B*) Dose-response curve of chloride current *vs.* increased osmolarity of the basolateral side. Each point is the mean of six experiments. The system is extremely sensitive to hypertonicity; 12.5 mOsm induced more than a 20% increase in current output. Maximal increases in chloride secretion are observed above 200 mOsm.

approximately a doubling of the chloride current was tested after blocking each of these mechanisms with drugs that are known to have some inhibitory specificity.

In the case of the Na^+/H^+ and Cl^-/HCO_3^- exchangers, it was pertinent to demonstrate their presence in these preparations before testing any involvement in the osmotic activation of Cl^- secretion.

Lack of Response to 50 mOsm of Mannitol When Blocking the $Na^+K^+2Cl^-$ Cotransporter with Bumetanide or the Chloride Channels with DPC

The effect of 50 mOsm of mannitol was tested after the chloride current was inhibited by bumetanide 10^{-4} M added to the tissues on the basolateral side. Loop diuretics also reduce drastically the chloride current in this preparation (Zadunaisky, 1984). Figure 3*A* shows that after the addition of bumetanide there is no increase in the short-circuit current when the osmolarity of the basolateral side is increased with mannitol. In a total of eight other preparations the response was blocked after the addition of bumetanide. In some cases when the current had not dropped below 50% there was a small response, but even if some residual current was left below 50% there was no response to the mannitol, indicating that either the full integrity of the $Na^+K^+2Cl^-$ cotransporter is needed or bumetanide has some other effects including some action on the chloride channels of the apical membrane.

DPC is a blocker of chloride channels in many chloride secretory preparations (Scheide et al., 1988). On the opercular epithelium maximal effects were obtained with 10^{-4} M added to the basolateral side in four preparations; one of the results is shown in Fig. 3*B*. The current was reduced in 5 min after the application of DPC and in 40–50 min the chloride secretion was reduced to zero. The sensitivity of the opercular epithelium chloride cells appears to be greater than other chloride secretory tissues such as the corneal epithelium (Scheide et al., 1988). Mannitol was added at 50% inhibition of the current and no response was observed to this osmotic increase. The inhibition therefore of chloride secretion with bumetanide or DPC blocks the activation produced by the increased osmolarity in the basolateral side.

Presence of the Na^+/H^+ Exchanger, Its Activation, Inhibition and Participation in the Osmotic Response of the Chloride Cells

The Na^+/H^+ exchanger (Aronson, 1985) is known to be activated by shrinkage in diverse cell types (Hoffmann & Simonsen, 1989 and together with the Cl^-/HCO_3^- exchanger plays a role in regulatory volume increase. To test if the Na^+/H^+ exchanger is activated during the response to hypertonicity of the basolateral side of the chloride cells of the isolated opercular epithelium, it was necessary to reveal its presence in the tissues. We have utilized the fact that the short-circuit current follows closely the changes in intracellular pH to examine the presence of the Na^+/H^+ exchanger. In the frog skin epithelium intracellular pH measurements have indicated that the current is an excellent parameter for studies of cell pH (Harvey, Thomas & Ehrenfeld, 1988). The short-circuit current is decreased when the intracellular pH becomes acid and increased as the pH becomes alkaline.

The tests for the presence of the Na^+/H^+ exchanger consisted in acidification with 15 mM NH_4Cl added to the basolateral side of the preparations. There was a

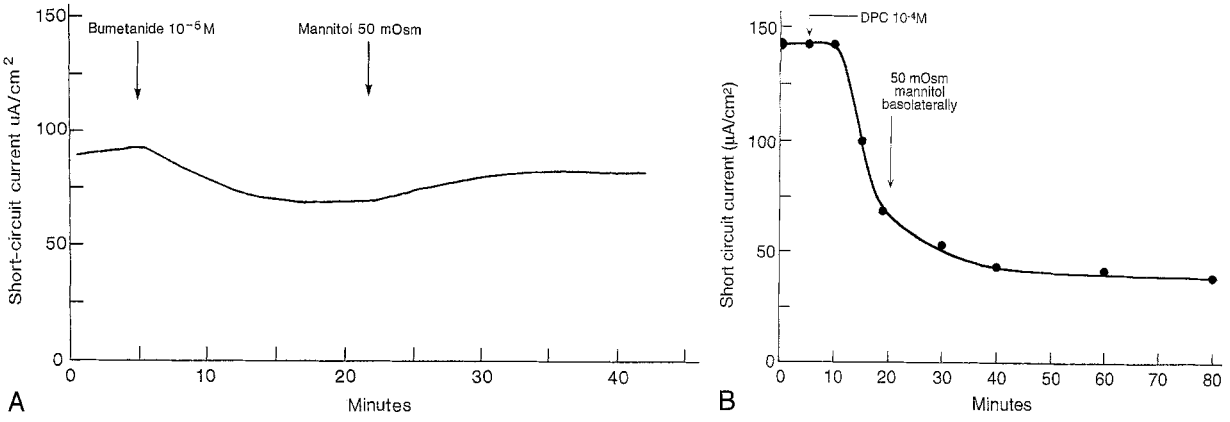


Fig. 3. (*A*) Lack of response to basolateral hypertonicity of 50 mOsm induced with mannitol, after addition of 10^{-4} M bumetanide to the basolateral side. The $Na^+K^+2Cl^-$ cotransporter is most probably involved in the activation by cell shrinkage. (*B*) Inhibitory action of the chloride channel blocker DPC at 10^{-4} M on the chloride current of an operculum and lack of response to basolateral hypertonicity with 50 mOsm mannitol. The chloride channel efficacy is needed for the osmolarity to induce its shrinking and stimulatory effect of chloride secretion.

transient increase in current as the cell briefly undergoes alkalinization. The current then decreased to a minimum during acidification, at which time the solution of the basolateral side was replaced with fresh HEPES Ringer without NH_4Cl. This results in a restoration of the normal intracellular pH and the current returns to its initial resting values as the intracellular pH becomes more alkaline. This method introduced by Boron and De Weer (1976) produces a sequence of intracellular pH displacements that activate the Na^+/H^+ exchanger.

The addition of NH_4Cl to the apical side did not produce changes in the short-circuit current, but when added to the basolateral side, a rapid drop in the current was observed. Therefore, the addition of NH_4Cl and drugs affecting the Na^+/H^+ exchanger were performed only on the basolateral side of the preparation.

The quantification of the activity of the Na^+/H^+ exchanger was measured as the velocity of recovery of the current after the elimination of the NH_4Cl from the chamber. The slope of the current rise produced a very reliable number that permitted the detection of inhibition or stimulation of the Na^+/H^+ exchanger.

In Fig. 4*A* the cycle of pH change in control conditions can be observed. Figure 4*B* presents the recovery of the current under the action of factors that are known in other tissues to activate this exchanger (Grinstein, 1988). Epidermal growth factor at 10^{-5} M produced an acceleration of the recovery of 155% in seven experiments, phorbol ester (PMA) at a concentration of 10^{-3} produced a stimulation of 165% in seven experiments and IBMX at 10^{-4} produced stimulation of 216% in eight experiments. Therefore, the Na^+/H^+ exchanger is present in the basolateral side of the chloride cells.

The addition of epidermal growth factor, or phorbolmeristoil acetate at the concentrations used in these experiments did not produce any modification of the

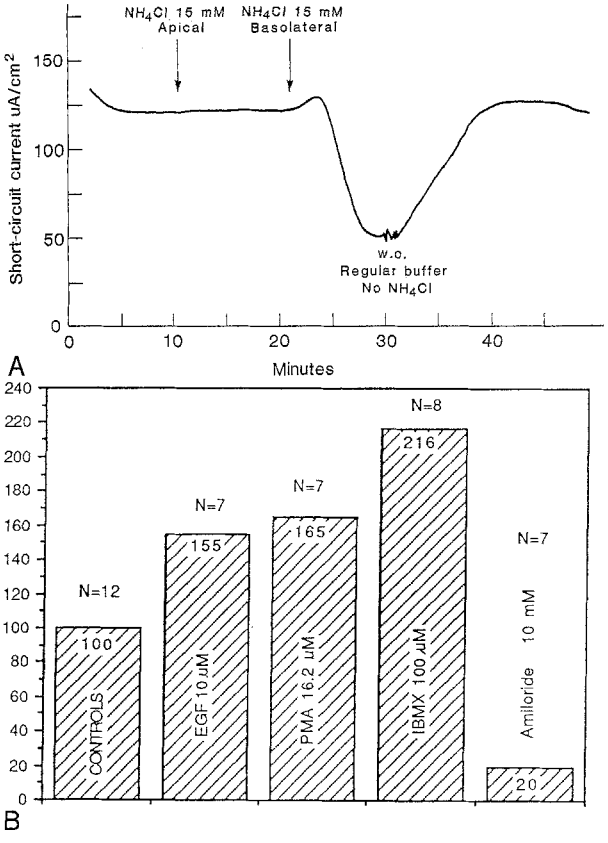


Fig. 4. (*A*) Na^+/H^+ exchanger. Action of NH_4Cl on the chloride current of isolated chloride-cell-rich opercular epithelium. Acidification of intracellular pH and return to normal produce parallel effects on the chloride current. The influence was only found on the basolateral side. (*B*) Stimulatory effects on the recovery of the chloride current after washout of NH_4Cl from the basolateral side. IBMX, phorbol ester, and EGF have stimulatory effect while amiloride inhibits the recovery. A Na^+/H^+ exchanger is present and activated or blocked from the basolateral side.

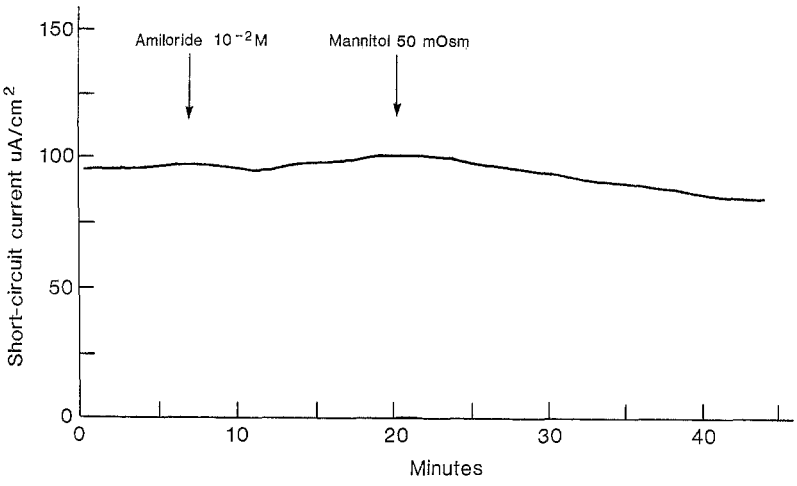


Fig. 5. Amiloride block of the chloride current increase expected with hypertonicity of the basolateral side. The Na^+/H^+ exchanger is activated or participates in the hypertonic response of the chloride secretion.

short-circuit current of opercular preparation in Ringer in both sides. IBMX produces a very slow, graded increase in current that did not affect the rapid rise seen here after acidification of chloride cells.

In Fig. 5 it is shown that amiloride at high concentration blocks the response to 50 mOsm of mannitol in the basolateral side. We must therefore conclude that blocking the Na^+/H^+ exchanger eliminates the osmotic response of the chloride secretory epithelium. In Fig. 6*A* the relative inhibitory effect of different doses of amiloride is shown and Fig. 6*B* shows the action of somewhat more potent analogues of amiloride. It is clear that there is a dose-dependent inhibition by amiloride of the response to an osmotic load of 50 mOsm of mannitol basolaterally and that analogues of this agent have a greater effect than amiloride at the same dose utilized of 10^{-4} M.

Presence of the Cl^-/HCO_3^- Exchanger and Lack of Involvement in the Activation of Cl^- Secretion by Hypertonicity of the Basolateral Side

From the initial publications on the preparation of the opercular epithelium from our laboratory, we were aware that bicarbonate was essential for the development of chloride secretion (Degnan et al., 1977; Zadunaisky, 1984). It is now clear that HCO_3^- is required because of the presence of the Cl^-/HCO_3^- exchanger. In Fig. 7*A* it is shown that switching from 16 mM $NaHCO_3$ in the Ringer to completely bicarbonate free and low CO_2 solutions, using HEPES buffer to keep the pH at 7.4, leads to a drastic reduction in the Cl^- secretion, but not to zero. On returning to the bicarbonate-rich medium the chloride current recovers to its initial values. In Fig. 7*B* it is shown that the same degree of inhibition of the chloride secretion is accomplished with 10^{-4} M DIDS added basolaterally. DIDS is known to inhibit the Cl^-/HCO_3^- exchanger. In eight experiments the reduction of current due to bicarbonate-free conditions was 88%. In seven experiments with DIDS the inhibition was 92% of the current.

The Cl^-/HCO_3^- exchanger is not involved in the response to hypertonicity in the basolateral side. When DIDS was added to the basolateral side of the opercular epithelia in regular Ringer, inhibition of the chloride current occurred. Mannitol was then added at 50 mOsm to the basolateral side. Figure 8 shows that the response to mannitol is not altered. In 10 experiments the current was activated by 50 mOsm mannitol after inhibition with DIDS.

Calcium Involvement in the Response to Hypertonicity

The concentration of calcium in the medium bathing the operculi was reduced from 1.0 mM to 100 μM and "0" μM, the latter being the concentration of Ca^{2+} in Ringer prepared simply without $CaCl_2$. The response to hypertonicity induced by several concentrations of mannitol on the basolateral side was tested under these low calcium conditions. The results were compared to the control curve of mannitol dose *vs.* current response in 1 mM Ca^{2+} already shown in Fig. 2. In Fig. 9 it is observed that a reduction in Ca^{2+} in the Ringer lowers the response to hypertonic mannitol in the basolateral side of the operculum. With the lowest Ca^{2+} concentration of "0" the effects are drastic and the results most probably indicate that Ca^{2+} entry from the outside medium is important for the activation of the current through shrinkage of the chloride cells.

Chloride Cell Volume Response to Tonicity

The response of the cell volume of chloride cells of isolated operculi to hypertonicity, both on the apical and

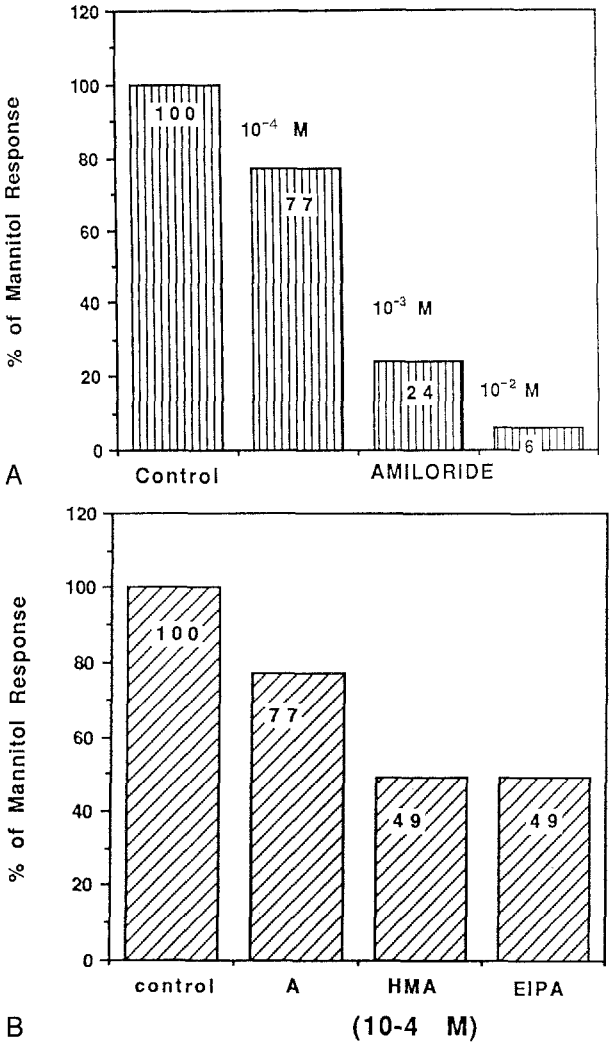


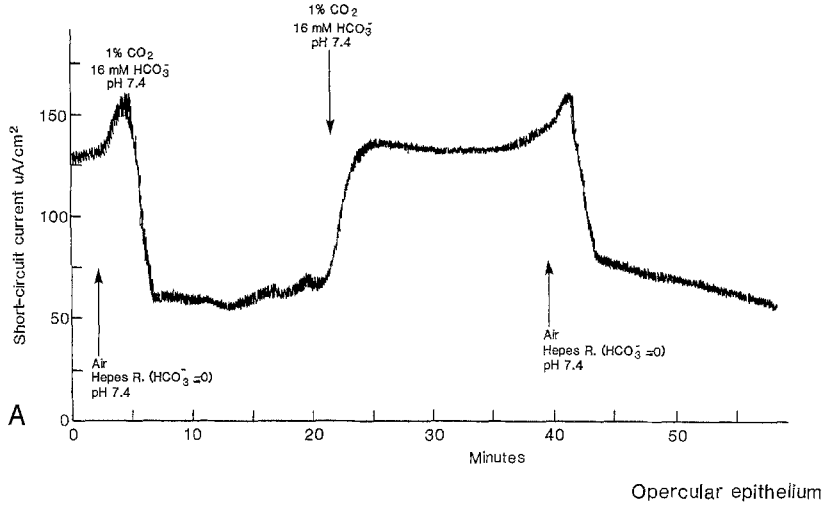
Fig. 6. (*A*) Relative inhibition of the osmotic response found with different concentrations of amiloride in isolated opercular epithelia. Blocking the Na^+/H^+ eliminates the increase of the chloride current induced by basolateral hypertonicity. Bars are mean of seven experiments each. The numbers inside the bars indicate the percentage of the response remaining after treatment. (*B*) Relative inhibition of the current increase produced with mannitol by amiloride and its analogues HMA and EIPA at 10^{-4} M concentration. Bars are mean of seven experiments each. Numbers inside them indicate percentage of current stimulation remaining after drugs.

basolateral side, was tested with quantitative optical microscopy and imaging techniques described in Materials and Methods. Both sea-water and fresh-water-adapted *Fundulus* were used. As shown in the Table in the sea-water-adapted case, the response to an increase of 20% of osmolarity obtained by the addition of 60 mOsm mannitol added to the medium perfusing the basolateral side induced a 21% decrease in the computed volume of chloride cells in different opercular preparations. Similar exposure in separate experiments to the same hypertonicity on the apical side produced no response in the cell volume.

In operculi from fresh-water-adapted fish, the situation was different for the apical side. The response of the basolateral side was a reduction of 19.2% to a tonicity increase of 20%. However, the apical side of these chloride cells from fresh-water-adapted fish was also permeable to water because the apical side responded with an 18.6% reduction in cell volume when the tonicity was increased 20% with mannitol. There is, therefore, a drastic difference in the water permeability of fresh water and sea water chloride cells. In the sea water adapted, the apical membrane is impermeable to water and no changes can be induced by increasing the osmolarity of the apical side medium. In fresh-water-adapted opercular cells, the hypertonicity produced cell shrinkage, as much as when it was increased in the basolateral side. Changes in water permeability of fish in general and particularly of the gills and skin are known. However, this difference in chloride cell apical membrane adaptation to the low salinities is striking.

Discussion

The evidence presented here tends to show that the signal immediately recognized by euryhaline fish during their transition from a lower to higher salinity, is a change in osmolarity, most probably the increase in plasma tonicity arising from the obligatory drinking of sea water. The evidence for this statement is based on the following results presented here: (i) During rapid adaptation to sea water the plasma osmolarity increased more than 50 mOsm due to higher salt content; (ii) The test for any increase in chloride secretion that could be triggered by the higher salinity indicates that the basolateral or plasma side of the chloride-cell-rich operculum is the place where 50 mOsm of the nonpenetrating sugar, mannitol, induces a 100% increase of the chloride secretory rate; (iii) The effect is related to the total increase in osmolarity with a maximal effect of more than a 200% increase above control level at twice the tonicity of the medium-imitating fish plasma. The extrapolation from intact fish to the isolated preparation is not far-fetched. The chloride cells of the opercular epithelium have identical properties to the ones in the gill secretory epithelium. The only difference could be that in the opercular epithelium the tight junctions controlling the permeability of the tissue are most probably located between the cells of the surface epithelium, while in the gill epithelium they are located between chloride cells (Karnaky, 1992). This anatomical situation, however, does not dismiss the extrapolation, especially the lack of response from the apical side to hypertonicity. The chloride cell pits are directly exposed to the sea water and therefore what we witnesss in the experiment is the actual low



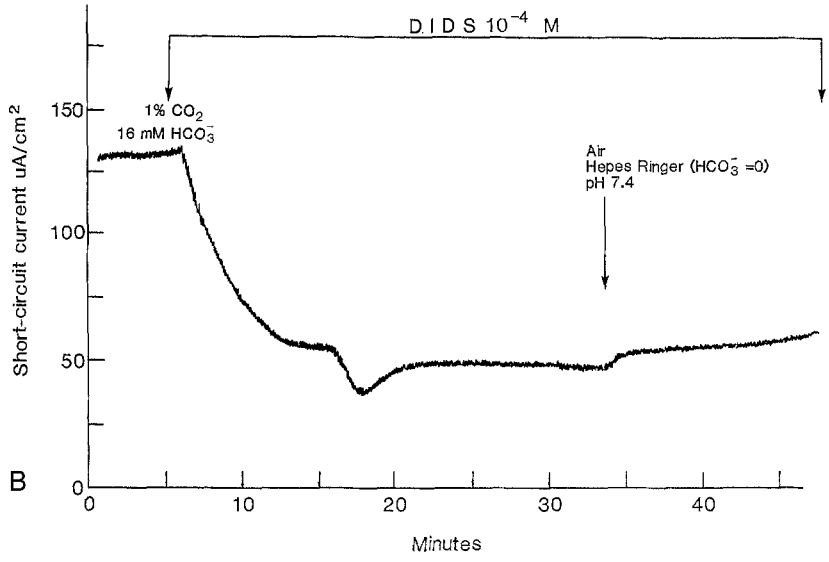


Fig. 7. Cl^-/HCO_3^- exchanger. (*A*) The removal of HCO_3^- and CO_2 keeping the pH of the bathing solutions at 7.4 with HEPES buffer, produced remarkable reduction of chloride current. Recovery occurs when HCO_3^-, CO_2 rich medium is readmitted. The first part of the sequence is repeated. (*B*) Inhibition with DIDS of the chloride current in the presence of HCO_3^- and CO_2. Note that HEPES Ringer, free of HCO_3^- or CO_2 do not elicit further reduction of the current. The effects of DIDS or the removal of bicarbonate had proportionally similar inhibitory action.

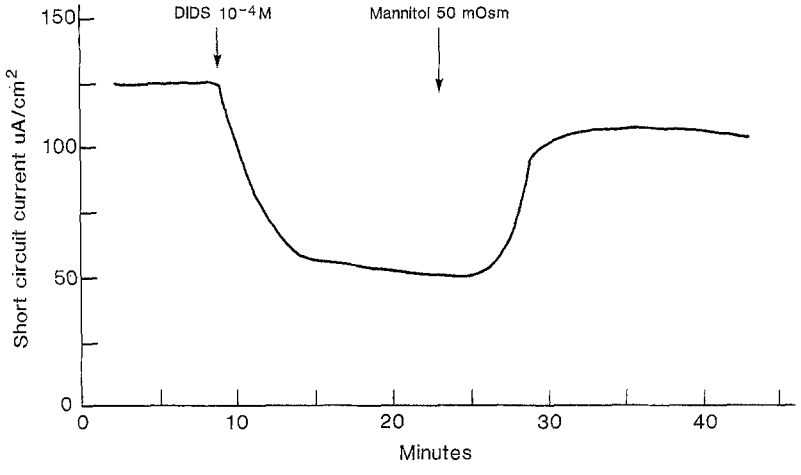


Fig. 8. Blocking the Cl^-/HCO_3^- exchanger with DIDS did not affect the increase in chloride current expected when the tonicity of the basolateral side of isolated opercular epithelium is increased with mannitol. The Cl^-/HCO_3^- exchanger does not participate in the hypertonic response.

water permeability of the apical membrane of the chloride cells of sea-water-adapted fish.

The opercular chloride cells of sea-water-adapted fish shrink readily when exposed to hypertonicity from the basolateral side, but do not show volume changes when hypertonic medium is added to their apical side. This is demonstrated by the results obtained when computing cell volume by quantitative microscopy. The

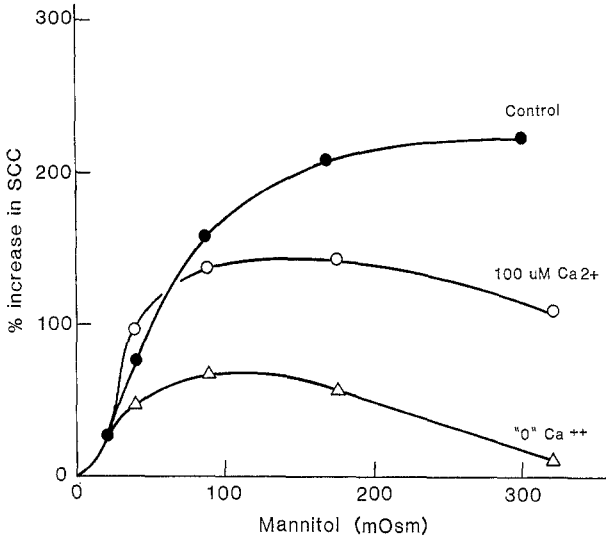


Fig. 9. Ca^{2+} requirement. Reduction in Ca^{2+} concentration in solutions bathing both sides of isolated operculi has drastic effects on the hypertonic response of the basolateral side elicited with mannitol; 100 μM and "0" Ca in the media drastically change the maximal current at the 100 mOsm and above osmolarities tested.

cells responded as osmometers; that is, they shrank predictably when the hypertonic solution was on the basolateral side.

A search for the mechanisms involved in this stimulation of chloride secretion during a change in cell volume should follow the evidence for other cells in the field of cell volume regulation (Hoffmann & Simonsen, 1989). Here, the main difference with previous descriptions for other cells is that the induction of shrinkage produces a vectorial increase in the existing chloride secretion. Apart from this characteristic, the effects studied have similarities with the response to induced volume changes of many other cells: (i) The effect is osmolarity dependent and saturable; (ii) the shrinkage activates the $Na^+K^+2Cl^-$ cotransporter, since blockage with the loop diuretic bumetanide eliminates the response; (iii) the Cl^- channels in the apical membrane must be functional for this events to occur, because when they are blocked with DPC the response is lost. These channels have been inferred (Scheide et al., 1988) from the depolarization of the apical membrane of opercular chloride cells with isoproterenol. (iv) The hypertonicity activates the Na^+/H^+ exchanger that we have now described in the chloride cells. In other cell types this is also part of the response to hypertonicity (Grinstein, 1988). (v) Finally, the Ca^{2+} concentration in the bathing medium appears to be critical for the activation of the chloride secretion by the hypertonicity in the basolateral side.

Chloride cell pH is apparently extremely important for the adaptation process. The NH_4Cl experiments shown here indicate that a Na^+/H^+ exchanger accessible from the inside, or basolateral side, exists in the chloride cells. The inhibition with high doses of amiloride or lower, more reasonable doses of its analogues permit the conclusion of its presence. The activation by IBMX, phorbol esters, and EGF only attests to the characteristics this Na^+/H^+ exchanger has in common with the one found in other tissues or cells (Grinstein, 1988).

The activation of the Na^+/H^+ exchanger during shrinkage most likely produces a sudden change in intracellular pH. In turn the pH change activates chloride secretion by producing an effect either on the Cl^- channels or on the mechanisms of phosphorylation of the channels. Another possibility is a contribution of the pH change to the solubility of Ca^{2+} and its mobilization from and to cellular storage. Further experimentation could provide answers to these questions. However, our observation on cell pH is indirect and confirmation with the appropriate intracellular reporters would be desirable.

The presence of a Cl^-/HCO_3^- exchanger in these tissues is also demonstrated here by the importance of bicarbonate for the chloride secretion as well as the inhibition with DIDS. The lack of participation of the Cl^-/HCO_3^- exchanger in the hypertonic response is most probably a characteristic of the tissue. In preliminary work we have found that this exchanger is involved in the effect of hypotonicity in the basolateral side. Lowering the osmolarity of the basolateral side indicates in this preliminary work the need for the integrity of the Cl^-/HCO_3^- exchanger.

In the measurements of volume of the chloride cells, we find that in fully fresh-water-adapted specimens hypertonicity on the apical membrane side produces predictable osmometer-like shrinkage of chloride cells. This simply means that the permeability to water is high. This observation contrasted with the lack of response of the apical membrane in cells obtained from sea-water-adapted fish. In operculi of fresh-water-adapted fish, mounted in the Ussing chamber, the voltage and current are zero (Zadunaisky, 1984). There is probably a profound change in the membrane properties of the chloride cells during the transition that is naturally manifested more drastically when fish are adapted to the extremes of fresh water and sea water. In physiological conditions probably a lower degree of change exists because *F. heteroclitus* habits involve transitions from brackish, partly salted water to full sea water. Probably, experiments with operculi of fish adapted to partly salted water would be of interest. The high water permeability of the apical membrane of the chloride cells in operculi of fish fully adapted to fresh water opens the question of how much does the salinity of the apical side contribute to the activation of chloride transport. This possibility will have to be examined in future research.

In Fig. 10 "an up-to-date" model of the chloride cells is presented, including the newly described Na^+/H^+ and Cl^-/HCO_3^- exchangers.

Table. Effects of 20 mOsm of mannitol on the cell volume of chloride cells

Adaptation	Side	Cell volume μm^3	Minimal volume μm^3	%	Shrinkage % of control
Fresh Water	Apical $N = 6$	1,625 ± 189	1,299 ± 118	81.4* ± 3.8	19.9 ± 5.1
	Basol. $N = 5$	1,750 ± 208	1,414 ± 165	80.8* ± 4.0	17.8 ± 4.9
Sea Water	Apical $N = 10$	1,537 ± 222	1,570 ± 248	103 ± 6.0	1.2 ± 3.0
	Basol. $N = 5$	1,660 ± 191	1,328 ± 131	80.0* ± 3.6	21.0 ± 6.1

Mannitol was added to the apical or the basolateral side perfusion of sea water or fresh water adapted operculi of *F. heteroclitus*.
Mean cell volumes are followed by ±SE. *N* indicates number of cells in which the volume was measured by planimetric methods on a minimum of six and maximum of nine optical sections obtained at intervals of 2.5 μm. (*) Statistically significant differences. The exposure of the apical membrane of sea-water-adapted cells did not produce significant cell volume change indicating low water permeability. Apical membrane of fresh water and basolateral of both sea-water and fresh-water-adapted produced expected volume shrinkages of approximately 20%. Data available for the return from hypertonic to isotonic solutions produced results that permit a similar interpretation.

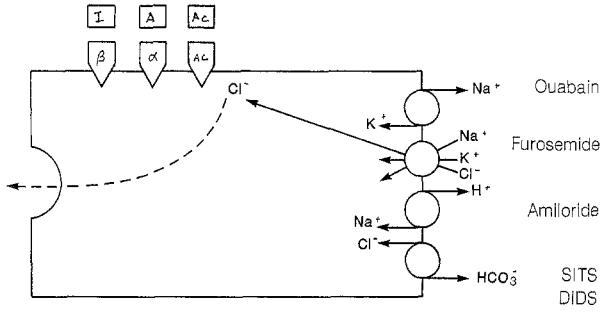


Fig. 10. The diagram above presents an up-to-date picture of the main elements of the cell membrane of chloride cells involved in the active transport of chloride, its regulation and the osmotic response observed from the basolateral side. *I*, and *A* represent alpha and beta catecholamine receptors and *M*, a muscarinic acetylcholine receptor.

The work was supported by the National Institutes of Health, Research Grant EY01340 to J.A.Z. Part of this research was performed while Dr. Zadunaisky was a Scholar In Residence at the Fogarty International Center of The National Institutes of Health in Bethesda, Maryland. Ms. Dawn Roberts was a fellow of the Grass Foundation and Pew Foundation during this work. Grants from the National Science Foundation and the National Institutes of Health to the Mount Desert Island Biological Laboratory also provided assistance for this research.

References

Aronson, P.S. 1985. Kinetic properties of the plasma membrane Na^+/H^+ exchanger. *Ann. Rev. Physiol.* **47:**545–560

Boron, W.F., De Weer, P. 1976. Intracellular pH transients in squid giant axon caused by CO_2, NH_3 and metabolic inhibitors. *J. Gen. Physiol.* **67:**91–112

Cragoe, E.J., Jr., Woltersdorf, O.W., Jr., Bicking, J.B., Kwong, S.F., Jones, J.H. 1967. Pyrazine diuretics. II,N-Amidino-3-amino-5-substituted 6-halpyrazinecarboxamides. *J. Med. Chem.* **10:**66–75

Degnan, K.J., Karnaky, K.J., Zadunaisky, J.A. 1977. Active chloride transport in the *in vitro* opercular skin of a teleost (*Fundulus heteroclitus*) a gill-like epithelium rich in chloride cells. *J. Physiol.* **294:**155–191

Foskett, J.K., Bern, H.A., Machen, T.E., Conner, M. 1983. Chloride cells and the hormonal control of teleost osmoregulation. *J. Exp. Biol.* **106:**255–272

Furlong, T.J., Spring, K.R. 1990. Mechanisms underlying volume regulatory decrease by *Necturus* gallbladder epithelium. *Am. J. Physiol.* **258:**c1016–c1024

Grinstein, S., editor 1988. Na^+/H^+ Exchange, CRC, New York

Harvey, B.J., Thomas, S.R., Ehrenfeld, J. 1988. Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. *J. Gen. Physiol.* **92:**767–791

Hoffmann, E.K., Simonsen, L.O. 1989. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69:**315–382

Holmes, W.N., Donaldson, E.M. 1969. Body compartments and distribution of electrolytes. *In:* Fish Physiology, I. W.S. Hoard, and D.J. Randall, editors. pp. 1–89. Academic, New York

Karnaky, K.J. 1992. Teleost osmoregulation: changes in the tight junction in response to salinity of the environment. *In:* Tight Junctions. M. Cereijido, editor. pp. 175–201. CRC, Boca Raton, FL

Karnaky, K.J., Kinter, L.B., Kinter, W.B., Stirling, C.E. 1976. Teleost chloride cell. II. Autoradiographic localization of gill NaKATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments. *J. Cell. Biol.* **70:**157–177

Maetz, J., Bornacin, M. 1975. Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. *Fortschr. Zool.* **23:**322–362

Sardet, C., Pisam, M., Maetz, J. 1979. The surface epithelium of teleostian fish gills. Cellular and juctional adaptations of the chloride cell in relation to salt adaptation. *J. Cell Biol.* **80:**96–112

Scheide, J.I., Curci, S., Schettino, T., Zadunaisky, J.A. 1988. Intracellular voltage recordings in the opercular epithelium of *Fundulus heteroclitus*. *J. Exp. Zool.* **246:**126–130

Simchowitz, L., Kleyman, T.R., Cragoe, E.J., Jr. 1992. An overview of structure-activity relationships, in amiloride and its analogs: Unique cation inhibitors. E.J. Cragoe, Jr., T. Kleyman and L. Somchowitz, editors. pp. 12–14. VCH Publishers, Deerfield Beach, FL

Smith, H.W. 1930. The absorption and excretion of water and salts by marine teleosts. *Am. J. Physiol.* **93:**485–505

Wiederholt, M., Zadunaisky, J.A. 1984. Decreased intracellular chloride activity by furosemide in frog retinal pigment epithelium. *Curr. Eye Res.* **3:**673–675

Wiederholt, M., Zadunaisky, J.A. 1986. Membrane potentials and intracellular chloride activity in ciliary body of the shark. *Pfluegers Arch.* **407:**S112–115

Zadunaisky, J.A. 1966. Active transport of chloride in frog cornea. *Am. J. Physiol.* **211:**506–5

Zadunaisky, J.A. 1984. The chloride cell: The active transport of chloride and the paracellular pathways. *In:* Fish Physiology, XB. W.S. Hoard, and D.J. Randall, editors. pp. 129–176. Academic, New York

Zadunaisky, J.A. 1992. Membrane transport in ocular epithelia. *In:* Barriers and Fluids of the Eye and Brain. M. Segal, editor. pp. 1–15. CRC, London

Zadunaisky, J.A., Candia, O.A., Chiarandini, D. 1963. The origin of the short circuit current in the isolated skin of the South American frog *Leptodactylus ocelatus. J. Gen. Physiol.* **47:**393–402

Zadunaisky, J.A., Lande, M.A., Chalfie, M., Neufeld, A.H. 1973. Ion pumps in the cornea and their stimulation by epinephrine and cyclic-AMP. *Exp. Eye Res.* **15:**577–584