Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3635–3638, April 1992
Physiology

Calcium uptake in the skin of a freshwater teleost

(tilapia/ion transport/opercular membrane/calcium metabolism)

S. D. McCormick*†‡, S. Hasegawa‡, and T. Hirano‡

‡Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164, Japan; and *Department of Integrative Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720

Communicated by Howard A. Bern, January 8, 1992 (received for review October 20, 1991)

ABSTRACT **The skin, particularly the opercular membrane of some teleosts, contains mitochondrion-rich "chloride" cells and has been widely used as a model to study branchial salt-extrusion mechanisms in seawater fish. Skin isolated from the operculum of the freshwater Nile tilapia (*Oreochromis niloticus*) can transport Ca^{2+} against an ionic and electrical gradient. Adaptation of Nile tilapia to a low-Ca^{2+} environment increased the capacity of the opercular membrane to transport Ca^{2+}. The density of mitochondrion-rich cells increased in parallel with Ca^{2+} transport capacity. The results demonstrate net Ca^{2+} uptake by vertebrate skin and strongly implicate mitochondrion-rich cells as the site of Ca^{2+} uptake in fresh water.**

Calcium is necessary for a variety of functions in animals, including bone and scale growth, muscle contraction, transmission of nerve impulses, hormone secretion, and intracellular signaling. In animals living in fresh water, calcium can be obtained from food or from the surrounding medium. Freshwater fish have the capacity to maintain normal blood Ca^{2+} levels (2–4 mM) in a wide range of external Ca^{2+} concentrations (<0.01 mM), even when food is withheld for long periods (1–3). Although the gills (4–12) and skin (5, 13) have both been implicated as sites of Ca^{2+} uptake in teleosts, net Ca^{2+} uptake in isolated skin or gill tissue has not, to our knowledge, previously been demonstrated. The present study was undertaken to determine whether net Ca^{2+} uptake occurs in fish skin *in vitro* and whether mitochondrion-rich (MR) cells are involved in Ca^{2+} uptake.

MATERIALS AND METHODS

The Nile tilapia *Oreochromis niloticus* is an Old World cichlid endemic to the freshwater lakes of the Rift Valley of Africa. Juvenile Nile tilapia, weighing 40–60 g and previously reared in fresh water, were kept in 33-liter tanks maintained at 24 ± 1°C with charcoal filtration and aeration. Fish were kept in either freshwater (FW: 0.77–1.04 mM Na^+/0.54–0.58 mM Ca^{2+}) or low-Ca^{2+} freshwater (LCFW: deionized water supplemented with NaCl, 0.50–0.97 mM Na^+, and 0.003–0.011 mM Ca^{2+}). Fish were maintained under experimental conditions for at least 2 weeks before sampling and were fed a maintenance ration of commercial trout chow (0.5% body weight per day). Water samples were taken intermittently from tanks containing fish to determine environmental [Na^+] and [Ca^{2+}]. Although LCFW solution contains normal, freshwater concentrations of Na^+ and Cl^-, it has low levels of other ions; we explicitly assume that the results obtained are due to decreased Ca^{2+}; absence of other divalent ions does not cause morphological changes in teleost gills (14). Environmental and total plasma [Na^+] and [Ca^{2+}] were measured by atomic absorption spectrophotometry. Two-thirds of the tank volume in each group was replaced every other day. Food was withheld for 24 hr before sampling of individual fish. Fish were killed by cranial concussion and pithing and were bled from the caudal vessels into heparinized syringes. Plasma was isolated by centrifugation and stored at −80°C.

Opercular membranes (the epithelium lining the buccal side of the opercular bone) were removed and placed in tilapia Ringer's solution (140 mM NaCl/10 mM $NaHCO_3$/4 mM KCl/2 mM NaH_2PO_4/1 mM $MgSO_4$/1 mM $CaCl_2$/5.5 mM glucose, pH 7.8), and isolated as described in ref. 15. Paired membranes (removed from both right and left sides) were used to avoid any variation among individuals. Membranes were attached to stainless steel pins on a circular Plexiglas ring that was then mounted on a Plexiglas chip and placed in the center of an Ussing-style chamber (described in detail in ref. 16). Seals were made watertight with silicone grease. The exposed membrane was 0.5 cm in diameter, and the total chamber volume was 14 ml. Tilapia Ringer's solution was added to the basal (blood) side, and artificial freshwater solution (1 mM NaCl/0.5 mM NaH_2PO_4/0.2 mM $CaCl_2$, pH 7.0) was added to the apical side. Two polyethylene 4% agar/3 M KCl bridges were placed 6 mm apart on either side of the membrane; each bridge terminated in 3 M KCl connected to a voltmeter by Ag/AgCl electrodes. Electrode asymmetry was nullified, and liquid junction potentials were corrected (17) immediately before each experiment. After a stable transepithelial potential (TEP) was achieved (change of <0.2 mV/min), $^{45}CaCl_2$ (New England Nuclear) was added to the apical side of one chamber and the basal side of the other chamber to attain a final activity of 2 μCi/ml (1 Ci = 37 GBq). Air was bubbled into each side of the chamber to promote mixing and oxygenation. After a 45-min isotope equilibration period, samples of 250–500 μl from each side of the chambers were removed every 45–90 min. Scintillation fluid was added to samples from the unlabeled bath at each interval, and radioactivity was counted for 20 min. An initial and a final sample from the labeled side was measured to verify the specific activity. Unidirectional fluxes were calculated from the linear rate of $^{45}Ca^{2+}$ movement (45–225 min) based on the known specific activity with appropriate corrections for volume changes. After each experiment, leakage across the membrane was tested by removing the solution from one side of the chamber; if fluid movement into the empty half of the chamber was detected, the preparation was considered leaky, and the results were discarded.

In an effort to determine the site of Ca^{2+} transport, we examined the skin for MR cells, which are known to be the sites of active ion transport in a variety of transport epithelia in fish. After dissection, opercular membranes were affixed to lens paper and placed in tilapia Ringer's solution with 2 μM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FW, freshwater solution; LCFW, low-calcium FW solution; MR, mitochondrion rich; TEP, transepithelial potential; DASEPI, 2-(4-dimethylaminostyryl)-1-ethylpyridium iodide.

†To whom reprint requests should be addressed at: Anadromous Fish Research Center, U.S. Fish and Wildlife Service, P. O. Box 796, Turners Falls, MA 01376.

2-(4-dimethylaminostyryl)-1-ethylpyridium iodide (DASEPI, K & K Laboratories), a mitochondrion-specific stain, which stains only active mitochondria (18–20). After 1 hr the tissue was rinsed several times with tilapia Ringer's solution and then examined with an epifluorescent microscope (Nikon Diaphot) with a 100-W mercury light source containing a 450–490 bandpass excitation filter, a 510 chromatic beam splitter, and a 520 longwave pass filter. Images were recorded with a silicon intensified target camera (Hamamatsu Photonics, Hamamatsu, Japan) and a video cassette recorder (Sony, Tokyo; 30 frames per s) by using several fields from each preparation. MR cell density was measured by counting the number of DASEPI-positive cells in at least 10 fields of 0.46 mm^2 for each of five individuals per group. MR cell size was determined by measuring the minimum cross-sectional distance (cell diameter) from video cassette recorder still frames. Use of the minimum cross-sectional distance minimized errors introduced by cells that were not perpendicular to the focal plain. Measurements were made only on images in sharp focus that had clear distinctions in cell outline. Video cassette recorder images were averaged over a 3.3-s period, and relative fluorescent (DASEPI) intensity per unit area was measured in the central portion (excluding cell boundaries) of MR cells.

RESULTS

Plasma $[Na^+]$ (FW tilapia: 159 ± 2 mM, LCFW tilapia: 157 ± 2 mM, n = 13) and plasma $[Ca^{2+}]$ (FW tilapia: 3.1 ± 0.1 mM, LCFW tilapia: 3.0 ± 0.1 mM, n = 14) were not affected by exposure to low environmental calcium ($P > 0.2$, Mann–Whitney U test).

TEP of opercular membranes was serosal-side positive and not significantly affected by low environmental calcium (FW tilapia: +10.5 ± 0.8 mV, n = 8; LCFW tilapia: +11.5 ± 0.8 mV, n = 8; P = 0.3). Under these conditions net Ca^{2+} uptake would have to occur against both an electrical and chemical gradient (5-fold higher $[Ca^{2+}]$ on serosal side, serosal-side positive).

In all preparations, Ca^{2+} influx and efflux were linear throughout the 3-hr period of measurement (see Fig. 1). Ca^{2+} influx of paired membranes was seven to eight times greater than Ca^{2+} efflux (Figs. 1 and 2), indicating that net uptake occurred under the imposed conditions.

Ca^{2+} influx was >2-fold higher in opercular membranes of tilapia from LCFW relative to those in FW ($P < 0.05$). Although mean Ca^{2+} efflux was higher in the LCFW group, there was no statistically significant difference between the groups. Net Ca^{2+} uptake was 2-fold higher in fish adapted to LCFW ($P < 0.05$, Fig. 2).

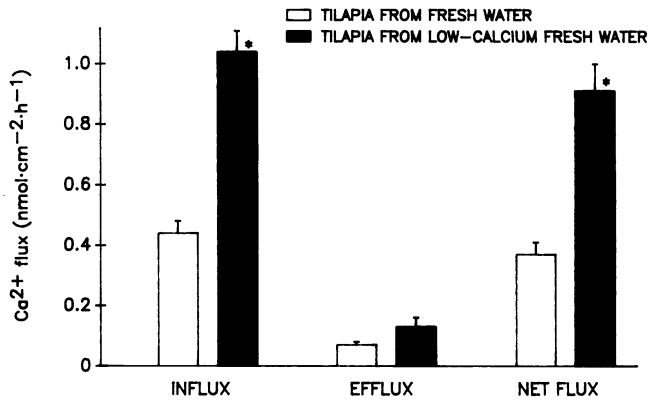

FIG. 2. Ca^{2+} fluxes in isolated opercular membrane of Nile tilapia. Open bars represent opercular membranes from fish adapted to normal-Ca FW, filled bars represent opercular membranes from fish adapted to LCFW. Four paired membranes were used to measure Ca^{2+} fluxes in each group. Values are expressed as mean ± SEM. *, Significant difference from normal-Ca FW group ($P < 0.05$, Mann–Whitney U test).

MR cell density of the opercular membrane increased 2-fold from adaptation to LCFW ($P < 0.01$, Table 1, Fig. 3). MR cell size (diameter) ranged between 6 and 12 μm; adaptation of fish to LCFW shifted MR cell-size distribution (Fig. 4) and slightly (6%) but significantly increased average MR cell size relative to fish in FW (Table 1). Relative fluorescence (per unit area) of MR cells also increased slightly (8%) but significantly after adaptation to LCFW ($P <$ 0.05, Table 1).

DISCUSSION

Results of the present study indicate that the skin (opercular membrane) of Nile tilapia is capable of Ca^{2+} uptake against an ionic and electrical gradient, thus demonstrating a net Ca^{2+} uptake in isolated epithelia of a freshwater vertebrate. The kinetics of Ca^{2+} uptake were examined in trout gill by using the perfused head technique (5); however, limitations in this method have prevented measurement of Ca^{2+} efflux and net flux. Net Ca^{2+} uptake has been demonstrated in the isolated intestine of a marine teleost (21). Ca^{2+} uptake was suggested to occur in the skin of frogs (22) but was not supported by subsequent studies (23, 24).

The isolated opercular membrane of Nile tilapia in fresh water could transport Ca^{2+} against an ionic and electrical

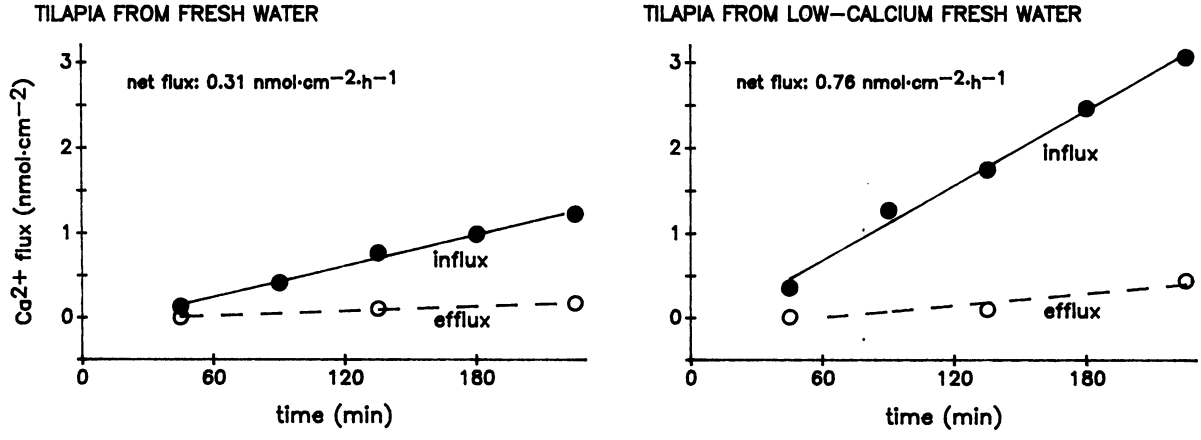

FIG. 1. Linear Ca^{2+} fluxes in isolated, paired opercular membranes of Nile tilapia adapted to normal-calcium FW or LCFW. Measurements began after a 45-min isotope equilibration period.

Table 1. Effect of adaptation of Nile tilapia to LCFW on MR cell density, size (diameter), and fluorescence intensity

	Adaptation medium	
	Normal Ca^{2+}	Low Ca^{2+}
Cell density, cells per cm^2	7,598 ± 1274	17,588* ± 3662
Cell size, μm	8.4 ± 0.1	8.9* ± 0.1
Fluorescence intensity, units	1.92 ± 0.03	2.08* ± 0.04

Preparations of opercular membrane were stained with DASEPI and examined as detailed in text. MR cell size and relative fluorescent intensity were measured on 20 cells in preparations from five individuals in each group (n = 100 per group). Values are mean ± SEM.

*Significant difference from fish adapted to normal-Ca^{2+} FW ($P < 0.05$, Mann–Whitney U test).

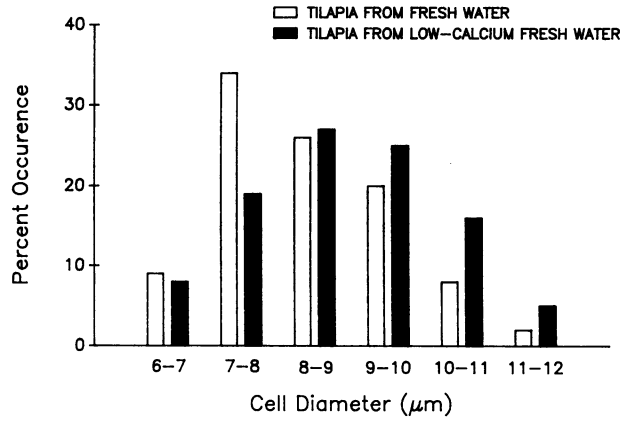

FIG. 4. MR cell size distribution in isolated opercular membranes of Nile tilapia adapted to normal-calcium FW and LCFW. Preparations of opercular membrane were stained with DASEPI and examined as detailed in text. MR cell size and relative fluorescent intensity were measured on 20 cells in preparations from five individuals in each group (n = 100 per group). The density of MR cells was >2-fold greater in opercular membranes of Nile tilapia adapted to LCFW.

gradient, even when the external Ca^{2+} concentration *in vitro* (0.2 mM) was lower than the levels the tilapia had experienced *in vivo* (0.5 mM). Adaptation of Nile tilapia to LCFW resulted in a >2-fold increase in net influx of Ca^{2+} by the opercular membrane. The present study supports the idea that increases in net Ca^{2+} uptake in fish under conditions of low environmental Ca^{2+} (25) are the result of increases in the capacity of the gills and skin to actively transport Ca^{2+}.

Estimates of whole-animal net Ca^{2+} uptake in teleosts in fresh water range between 3 and 20 $\mu mol \cdot h^{-1} \cdot kg^{-1}$ (25, 26), indicating that the basal Ca^{2+} uptake by the opercular membrane might contribute 1–7% to the total net Ca^{2+} uptake (calculations are based on an average 8 cm^2 opercular membrane in a 50-g fish). This estimate represents a minimum contribution of the opercular membrane, as the measurements in the present study represent a basal, unstimulated level of Ca^{2+} uptake. Chemical mediators (e.g., hormones) present in the whole animal are likely to increase substantially the *in vivo* uptake of the opercular membrane. In some teleosts MR cells are located throughout the skin, particularly in larvae and juveniles (27), indicating a potentially greater overall contribution of the skin to total Ca^{2+} uptake than that estimated solely from the opercular membrane. Based on whole-animal $^{45}Ca^{2+}$ exposures, Perry and Wood (5) deter-

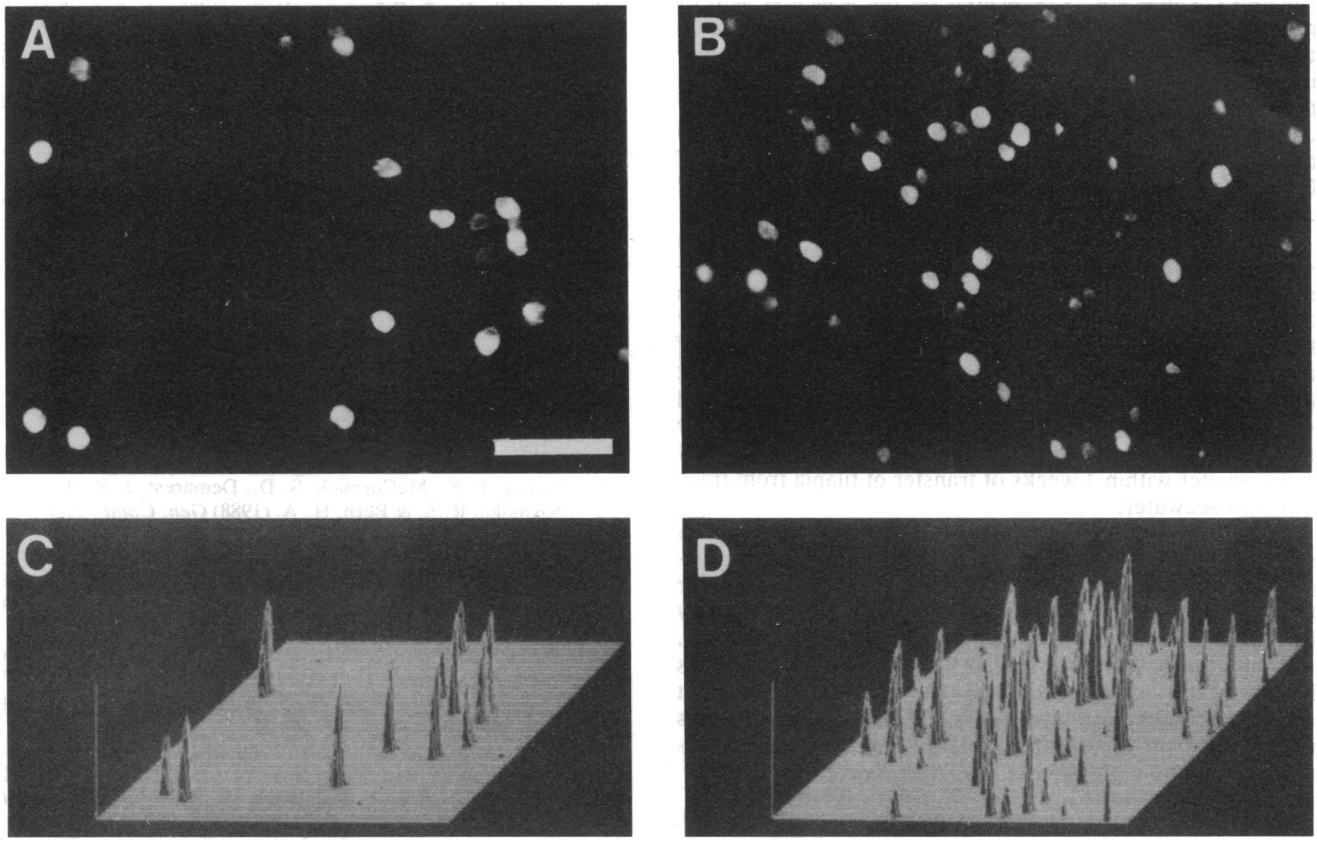

FIG. 3. MR cells in opercular membranes of Nile tilapia adapted to normal-calcium freshwater (*A* and *C*) and low-calcium freshwater (*B* and *D*). (*A* and *B*) Photomicrograph of isolated opercular membrane stained with the mitochondrion-specific fluorescent dye DASEPI. (Bar = 50 μm.) (*C* and *D*) Three-dimensional image of corresponding fluorescent image (*A* and *B*, respectively) showing fluorescent intensity as a function of the planar image area.

mined that up to 50% of nondietary Ca^{2+} uptake occurs through the skin of rainbow trout (*Oncorhynchus mykiss*).

The TEP of fish in fresh water can be slightly positive or slightly negative (28), whereas euryhaline fish adapted to sea water are usually positive relative to the external medium, with increasing salinity causing a more positive TEP (28–30). Young *et al.* (30) reported that the whole-animal TEP in the closely related tilapia *Oreochromis mossambicus* in fresh water was −1 to +10 mV. In the present study, TEP of the isolated opercular membrane under "physiological" conditions ranged between 8 and 13 mV and was not affected by prior adaption to LCFW solution. The TEP of the isolated head of rainbow trout increased from −10.2 to +1.2 when subjected to an increase in environmental calcium from 0.05 to 2.52 mM (5). As in the present study, increased Ca^{2+} uptake could not be accounted for by changes in the transepithelial potential.

The "chloride secretory cell" was originally described by Keys and Willmer (31) in the gills of seawater-adapted teleosts. These cells are MR and have been shown to be the site of Cl^- secretion in the gill and skin of seawater-adapted teleosts (32–34). However, MR cells are often termed "chloride cells", even when found in freshwater-adapted teleost, where a Cl^- secretory function is unlikely and a Cl^- uptake function is uncertain (for reviews of MR cell structure and function, see refs. 35–37). Using the isolated, perfused head of rainbow trout, Payan *et al.* (4) implicated MR cells as the site of Ca^{2+} uptake in the gill. Morphological studies have also provided indirect evidence that MR cells are involved in Ca^{2+} uptake in the gill of freshwater teleosts (6, 38), although conflicting evidence exists (14). Perry and Wood (5) found that Ca^{2+} influx and "chloride" cell density increased in parallel in the gills of rainbow trout adapted to low external calcium (25 mM Ca^{2+}). Research on the scaleless skin of rainbow trout (W. S. Marshall and C. M. Wood, personal communication) indicates that the magnitude of Ca^{2+} influx is related to the density of MR cells. Along with these studies, the present findings that Ca^{2+} uptake and MR cell density double after adaptation of the Nile tilapia to low environmental calcium strongly implicates MR cells as the site of Ca^{2+} uptake in teleost skin. In fresh water, the "chloride" cell may be a "calcium" cell.

In addition to increases in MR cell density, there was a slight increase in MR cell size after adaptation to low environmental calcium, which was the result of a shift in the size distribution of MR cells. Though slight, this increase may functionally relate to the increased Ca^{2+} transport capacity of the opercular membrane. It should be noted, however, that this hypertrophy is small relative to the increase in MR cell size that occurs after adaptation of teleosts to seawater. For example, Foskett *et al.* (39) reported a 60% increase in MR cell diameter within 3 weeks of transfer of tilapia from fresh water to seawater.

Net Ca^{2+} uptake in the skin occurred against both an electrical and ionic gradient, suggesting an active, energy-dependent transport system. Biochemical and vesicular transport properties of gill tissue suggest that a high-affinity Ca^{2+}-ATPase is involved in Ca^{2+} uptake in this tissue (6–12), although the possibility of other active transport pathways has not been ruled out. Although the present study does not address the mechanism(s) of active Ca^{2+} uptake, the methods used should prove useful in determining both the mechanism(s) and the regulation of Ca^{2+} transport.

We thank H. A. Bern and A. Urano for their interest and encouragement during this study. S.D.M. was a Research Fellow of the University of Tokyo on leave from the University of California at Berkeley during the conduct of these studies. This study was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education and from the Fisheries Agency, Japan, and also by the National Sea Grant College Program, Department of Commerce, under Grant NA80AA-D-00120, through the California Sea Grant College Program, and the California State Resources Agency, Project R/F-117.

1. Ogino, C. & Takeda, H. (1976) *Bull. Jpn. Soc. Sci. Fish.* **42,** 793–799.
2. Ichii, T. & Mugiya, Y. (1983) *Comp. Biochem. Physiol. A Comp. Physiol.* **74,** 259–262.
3. Rodgers, D. W. (1984) *Can. J. Fish. Aquat. Sci.* **41,** 1774–1780.
4. Payan, P., Mayer-Gostan, N. & Pang, P. K. T. (1981) *J. Exp. Zool.* **216,** 345–347.
5. Perry, S. F. & Wood, C. M. (1985) *J. Exp. Biol.* **116,** 411–433.
6. Perry, S. F. & Flik, G. (1988) *Am. J. Physiol.* **254,** R491–R498.
7. Doneen, B. A. (1981) *J. Cell. Physiol.* **145,** 51–61.
8. Flik, G., Wendelaar Bonga, S. E. & Fenwick, J. C. (1984) *Comp. Biochem. Physiol. B Comp. Biochem.* **79,** 9–16.
9. Flik, G., van Rijs, J. H. & Wendelaar Bonga, S. E. (1985) *J. Exp. Biol.* **119,** 335–347.
10. Flik, G., Wendelaar Bonga, S. E. & Fenwick, J. C. (1991) *Biol. Cell* **55,** 265–272.
11. Naon, R. & Mayer-Gostan, N. (1989) *Am. J. Physiol.* **256,** R313–R322.
12. Flik, G. & Perry, S. F. (1989) *J. Endocrinol.* **120,** 75–82.
13. Mashiko, K. & Jozuka, K. (1964) *Annot. Zool. Jpn.* **37,** 41–50.
14. Laurent, P., Hobe, H. & Dunel-Erb, S. (1985) *Cell Tissue Res.* **240,** 675–692.
15. McCormick, S. D. (1990) *Am. J. Physiol.* **259,** R857–R863.
16. Foskett, J. K., Machen, T. E. & Bern, H. A. (1982) *Am. J. Physiol.* **242** Suppl., R380–R389.
17. Barry, P. H. & Diamond, J. M. (1970) *J. Membr. Biol.* **3,** 93–122.
18. Bereiter-Hahn, J. (1976) *Biochim. Biophys. Acta* **423,** 1–14.
19. Karnaky, K. J., Degnan, K. J., Garretson, L. T. & Zadunaisky, J. A. (1984) *Am. J. Physiol.* **246,** R770–R775.
20. McCormick, S. D. (1990) *Cell Tissue Res.* **260,** 529–533.
21. Sundell, K. & Björnsson, B. T. (1988) *J. Exp. Biol.* **140,** 171–186.
22. Watlington, C. O., Burke, P. K. & Estep, H. L. (1968) *Proc. Soc. Exp. Biol. Med.* **222,** 853–856.
23. Zadunaisky, J. A. & Lande, M. A. (1972) *Am. J. Physiol.* **222,** 1309–1315.
24. Baldwin, G. F. & Bentley, P. J. (1981) *Comp. Biochem. Physiol.* **68,** 181–185.
25. Fenwick, J. C. (1987) in *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, eds. Pang, P. K. T., Schreibman, M. P. & Sawyer, W. H. (Academic, New York), Vol. 2, pp. 319–342.
26. Flik, G., Fenwick, J. C., Kolar, Z., Mayer-Gostan, N. & Wendelaar Bonga, S. E. (1985) *Am. J. Physiol.* **249,** R432–R437.
27. Hwang, P. P. (1989) *J. Morphol.* **200,** 1–8.
28. Potts, W. T. W. (1984) in *Fish Physiology*, eds. Hoar, W. S. & Randall, D. J. (Academic, New York), Vol. XB, pp. 105–128.
29. Dharmamba, M., Bornancin, M. & Maetz, J. (1975) *J. Physiol.* **70,** 627–636.
30. Young, P. S., McCormick, S. D., Demarest, J. R., Lin, R. J., Nishioka, R. S. & Bern, H. A. (1988) *Gen. Comp. Endocrinol.* **71,** 389–397.
31. Keys, A. & Willmer, E. N. (1932) *J. Physiol.* **76,** 368–378.
32. Burns, J. & Copeland, D. E. (1950) *Biol. Bull.* **99,** 381–385.
33. Karnaky, K. J., Degnan, K. J. & Zadunaisky, J. A. (1977) *Science* **195,** 203–205.
34. Foskett, J. K. & Scheffey, C. (1982) *Science* **215,** 164–166.
35. Payan, P., Girard, J. P. & Mayer-Gostan, N. (1984) in *Fish Physiology*, eds. Hoar, W. S. & Randall, D. J. (Academic, New York), Vol. XB, pp. 39–63.
36. Zadunaisky, J. (1984) in *Fish Physiology*, eds. Hoar, W. S. & Randall, D. J. (Academic, New York), Vol. XB, pp. 129–176.
37. Pequex, A., Gilles, R. & Marshall, W. S. (1988) in *Comparative and Environmental Physiology Vol. 1: NaCl Transport in Epithelia*, ed. Greger, R. (Springer, Berlin), pp. 1–73.
38. Ishihara, A. & Mugiya, Y. (1987) *J. Exp. Zool.* **242,** 121–129.
39. Foskett, J. K., Logsdon, C. D., Turner, T., Machen, T. E. & Bern, H. A. (1981) *J. Exp. Biol.* **93,** 209–224.