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CHAPTER 20

Opercular membranes and skin

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I. Introduction

The opercular membrane is the inner (buccal) lining of the opercular bone. It is of special interest to fish physiologists because it is an area of scaleless skin that in some teleosts contains numerous mitochondria-rich chloride cells. As in gill tissue, chloride cells are responsible for monovalent ion secretion in seawater and are probably involved in ion uptake in fresh water. But unlike gill tissue, the opercular membrane is a flat epithelium, making the chloride cells more accessible for morphological examination and the study of their transport properties.

The presence of chloride cells in opercular epithelium was first observed by Burns and Copeland[5] in seawater-adapted mummichog (killifish, *Fundulus heteroclitus*). Degnan *et al.*[8] and Karnaky *et al.*[17] mounted the mummichog opercular membrane in an Ussing chamber and found net chloride secretion; since then the opercular membrane has been widely used to examine the mechanisms and control of ion secretion in teleosts. Using the vibrating probe technique, Foskett and Scheffey[13] localized chloride secretion (measured as change in current density) to chloride cells in the opercular membrane of seawater-adapted tilapia (*Oreochromis mossambicus*). More recently the opercular membrane has been used to examine *in vitro* ion transport in freshwater-adapted teleosts and the potential role of mitochondria-rich chloride cells in ion uptake[20,24].

Most of the research with opercular membranes has been conducted in only two species, the Mozambique tilapia and the mummichog. More species with opercular membranes rich in chloride cells probably exist but simply have not yet been examined. Chloride cells are found at relatively low density in the opercular

membrane of Atlantic salmon (*Salmo salar*) and American shad (*Alosa sapidissima*, personal observation). Other areas of scaleless skin such as that from the jaw of the longjaw mudsucker (*Gillichthys mirabilis*)[21,23], the abdomen of the shanny (*Blennius pholis*)[26] and the cleithrum of rainbow trout (*Oncorhynchus mykiss*)[20] contain chloride cells and have been profitably used for studies of ion transport; the methods outlined here for the opercular membrane should be applicable to most preparations of scaleless skin. Several excellent reviews of the transport physiology of the gill and opercular membrane have been published[7,10,11,17,30]; this contribution will concentrate on the practical aspects of opercular membrane preparations.

II. Membrane dissection and culture

Given that the opercular membrane is only a few cell layers thick (15–50 μm), it is not surprising that its dissection is "nontrivial". Isolation of the tilapia opercular membrane is made particularly difficult by the numerous fibrous connections between the basal membrane and the operculum. The opercular bone is cut from the fish and held with alligator clips at the dorsal (cut) end and suspended in a petri dish containing Ringer's balanced salt solution (with 5 mM glucose) appropriate for the species. The branchiostegal rays are cut at each end, and the skin connecting these to the operculum is cut. With the branchiostegal rays still attached and held by forceps, the opercular membrane is carefully pulled upward while severing the underlying fibers with micro-dissecting scissors (e.g. DeWecker style). For most purposes the membrane should be isolated free of the silvery material between the membrane and bone. Isolation of the opercular membrane of the mummichog is performed in a similar manner (see ref. 8).

Following dissection the membrane can be used for physiological or morphological studies, or cultured for a period of several days. To date, only the opercular membrane of tilapia has been subjected to organ culture[22]. In this method the isolated opercular membrane (0.5–1.0 cm^2) is attached to a collagen-coated disc of lens paper (soaked in liquid collagen for 20 s and dried on teflon under ultraviolet light) to which it adheres throughout the culture period. The primary purpose of the collagen is to promote adhesion of the membrane to the lens paper; failure to keep the membrane flat during culture results in its rapid deterioration. Individual membranes are placed in commercially available media such as Minimal Essential Medium (MEM, Hanks' balanced salt solution, including 5 mM glucose, 1.3 mM $CaCl_2$, 4.2 mM $NaHCO_3$ and 25 mM HEPES) with 4 mg/ml bovine serum albumin (RIA grade), 500 U/ml penicillin and 250 μg/ml streptomycin in sterile 12-well culture plates with 1 ml of medium and pre-incubated for 1–2 hours with gentle shaking. Pre-incubation medium is then removed and replaced with 2 ml incubation medium: MEM (Hanks' salts) with 4 mg/ml bovine serum albumin, 292 μg/ml L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamicin sulfate, and 5 μg/ml bovine insulin (although insulin is not strictly a requirement, it may potentiate some endocrine responses). Temperature should be constant and within the range normally experienced by the species being examined. Culture should take

place in humidified chambers under air or oxygen:carbon dioxide mixtures in the range of 99.7:0.3 to 99:1. The pH should be adjusted to remain constant between 7.5–7.8 after gassing. Incubation medium should be changed daily. Since this method has been utilized in only one species to date, its applicability to new species should be examined by testing cell viability (e.g. dye exclusion) and histological examination of the tissue.

III. Ion transport

1. Ussing chamber

The ability to place the opercular membrane in an Ussing chamber has been the most important advantage of this preparation over isolated gill preparations. It provides easy access to apical and basolateral surfaces, and permits isolation of secretory effects from possible hemodynamic (e.g. pressor) effects of pharmacological or endocrine agents. The Ussing chamber is designed so that the isolated membrane separates two fluid-filled chambers of equal volume.

Although many styles of Ussing chambers can be used (see Fig. 1 for one example), most commonly the opercular membrane is stretched over a 0.3–1.0 cm hole in the center of a rectangular piece of lucite or plexiglass (mounting plate). Small pins several mm outside the hole are used to maintain the membrane flat and stretch it to its original (undissected) dimensions. A circular mounting disc with a central hole (same size as that of the mounting plate) and small holes to accommodate the mounting pins is placed over the membrane. Modest amounts of silicone grease are placed on the inner edges of the mounting plate and disk that contact the membrane, prior to placement of the membrane. The mounting plate and disc are then placed into receiving grooves (edged with silicone grease) to separate the two halves of the chamber which generally contain 2–10 ml of fluid. Each chamber should be gently bubbled with air or a 99:1 (oxygen:carbon dioxide) gas mixture to maintain oxygen tension and fluid mixture. Magnetic stirrers may be used in lieu of bubbling.

The membrane and mounting plate must be well-sealed in order to prevent leaks. Leaks are a greater problem in membranes such as the jawskin of the longjaw mudsucker which contain convoluted basal infoldings. Although leaks are often obvious in tracer studies (large, nonlinear fluxes), this is not necessarily the case in measurements of transepithelial potential (TEP) or short-circuit current when Ringer's solution is on both sides of the membrane. Methods for detecting and correcting for "edge leaks" can be found in Kottra *et al.*[18].

The Ussing chamber is generally used for radiotracer studies of ion fluxes and electrophysiology of the membrane, often in combination. For radiotracer studies, paired membranes are used to examine influx and efflux of ions by placing radioisotopes in the serosal and mucosal sides, respectively, and measuring the accumulation of the isotope in the other half of the chamber. To date, transport studies have been conducted only for sodium, chloride and calcium; studies with other ions and nutrients seem practicable and desirable.

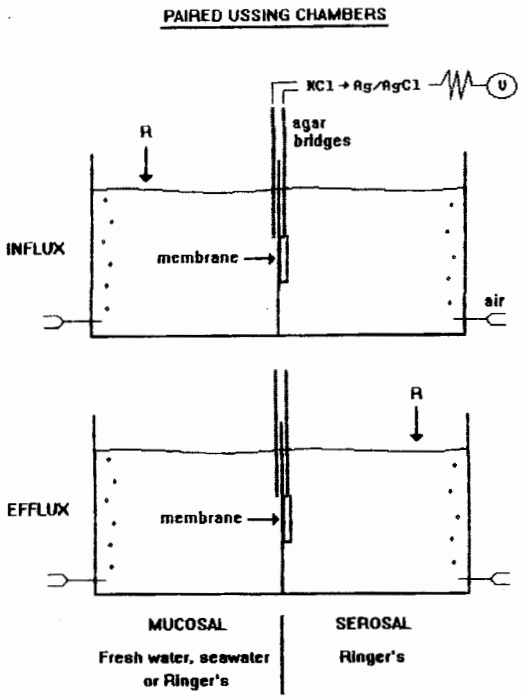

Fig. 1. Paired Ussing chambers used for measurement of ion fluxes and transepithelial potential. Inside dimensions of one-half of chamber are 1.5 × 2.2 × 2.5 cm ($l \times w \times h$). The membrane is placed over a central hole of 0.5 cm diameter and sealed between two pieces of plexiglass which divide the chamber in half. Efflux and influx of ions (radioisotope: R) is measured by periodic sampling of solution on the side opposite that to which radioisotope was added (an equal volume is removed from each side to keep fluid levels equal). If membrane is to be short-circuited (Ringer's solution on both sides of membrane), another set of paired agar bridges is added to either side of each chamber.

The TEP is measured with paired polyethylene 4% agar–3 M KCl bridges placed 2–3 mm from each side of the membrane, which terminate in 3 M KCl and are in turn connected to a voltmeter by Ag/AgCl or calomel electrodes. Current may be passed across the membrane via agar bridges in each chamber that are connected to Ag/AgCl electrodes and a current clamping device. Corrections for electrode asymmetry are made prior to placement of the membrane by placing electrodes in their normal positions in the Ussing chamber filled with Ringer's solution. Correction for junction potentials should be made when the serosal and mucosal solutions are different[3]; this is particularly important when the serosal solution is of low ionic strength.

Many studies have used Ringer's solution on both halves of the chamber, which allows the membrane to be short-circuited. In the opercular membrane of seawater-adapted tilapia and mummichog and the jawskin of the long-jawed mudsucker, the short-circuit current is strongly correlated with chloride secretion. This relationship should be established when new species are examined. Although the use of Ringer's

solution on the mucosal side of the membrane is important for establishing the electrophysiological properties of the membrane, it is not "physiological" in the sense that the animal would normally experience such a solution on its external surface (with the exception of an estuary of 10–12 ppt seawater, a relatively rare occurrence). To estimate the "normal" flux rates of the opercular membrane or skin, it may be more appropriate to utilize solutions approximating the composition of seawater or fresh water[9,20,24].

2. *Other methods in ion transport*

The accessibility of chloride cells in the opercular membrane makes this preparation useful in recently developed methods in transport physiology. Foskett and Scheffey[13] successfully used the vibrating probe to examine the characteristics and regulation of electrogenic transport in the seawater-adapted tilapia. In this method a metal microelectrode is rapidly vibrated between two points, allowing localization of the electric field and current density; short-circuit current and conductance can be measured for individual chloride cells. Using glass microelectrodes and equipment similar to that used in Ussing-chamber studies, Zadunaisky and colleagues[31] were able to record intracellular voltage in chloride cells of the mummichog opercular membrane.

Fluorescent dyes whose spectral characteristics are altered after binding to a specific ion (ion-sensitive dyes) have been used in studies of ion transport and intracellular signalling[14]. Development of new dyes and increased affordability of image analysis software (for providing ratio analysis necessary for resolving dye fluorescence) should result in even greater utilization of these methods by physiologists. Intracellular pH has been measured in chloride cells in the opercular membrane of mummichog using the pH-sensitive dye BCECF (W.S. Marshall, personal communication). To date, other ion and pH sensitive dyes have not been utilized in studies of teleost skin or opercular membrane.

IV. Morphology of chloride cells

Since its introduction by Karnaky and coworkers[16], DASPEI has been widely used to identify, quantify and measure chloride cells in the opercular membrane of teleosts. DASPEI (2-*p*-dimethylaminostyrylethylpyridiniumiodide; ICN Biomedicals Inc., Irvine, CA, USA) is a mitochondria-specific stain that presumably stains only active mitochondria[4]. From a stock solution of 0.5 mM in distilled water, DASPEI is diluted to a final concentration of 2 μM in an appropriate Ringer's solution. The tissue is incubated for 45 minutes at the acclimation temperature of the animal. Remove tissue, rinse several times in Ringer's solution, place on slide with a cover slip and examine under a fluorescent microscope with a 450–490 nm bandpass excitation filter, a 510 nm chromatic beam splitter, and a 520 nm longwave pass filter. Fluorescence will last for up to 1 hour. Mitochondria-rich cells will appear yellow or greenish-yellow. At least initially, duplicate tissue should be examined

without DASPEI staining to distinguish autofluorescence (which can be significant in some tissues) from "true" DASPEI staining. Cell numbers and size can be conveniently counted or measured through the microscope or after photography or digitization of the image.

Although determination of chloride cell density is straight-forward with this method, measurements of cell size can be problematic. In preparations where the chloride cells are tall and columnar, care must be taken to view the cells directly from above so that errors are not introduced in measurement of cross-sectional area or diameter; some cells or entire preparations may have to be eliminated.

A variety of other dyes and stains that utilize or explore the specialized properties of chloride cells have also been used. Champs-Maillet's fixative (0.2% osmic acid with saturated zinc powder and 25 mg/ml iodine) stains the extensive tubular system, and thus the entire chloride cell, black[2]. Anthroylouabain, a fluorescent analog of ouabain, has been used to localize and quantify cells rich in Na^+,K^+-ATPase[23], preferentially staining chloride cells in the opercular membrane of seawater-adapted (but not freshwater-adapted) tilapia. Na^+,K^+-ATPase can also be localized using autoradiography with [^{3}H]ouabain, K^+-dependent *p*-nitrophenyl phosphatase or immunocytochemistry[1], though none of these have yet been utilized in teleost skin. Histochemical methods have been used to localize carbonic anhydrase activity in several cell types of the opercular membrane of the mummichog[12,19]. Electron microscopy has also proven useful in examining the properties and location of chloride cells in the opercular membrane and skin of several teleosts.

V. Future research

The opercular membrane is similar to the gill in its direct connection to the external environment, and (in some teleosts) a large number of chloride cells. The two tissues are dissimilar in that the opercular epithelium is two-dimensional (or nearly so) with easy access to the serosal and mucosal surfaces of the membrane. It is this combination of similarities and distinctions that has made the opercular membrane an important investigative tool in our understanding of ion transport in fishes. Accessibility of chloride cells makes the opercular membrane useful for examining unresolved areas of chloride cell function and regulation. The functional relevance of the two (or more) types of chloride cells identified by their ultrastructural appearance[29] and the possible dual function (uptake and secretion) of chloride cells has yet to be determined. The opercular membrane should be suitable for receptor studies, particularly localization and quantitation of receptors and studies of hormone-receptor interactions. It should be possible to combine organ culture with Ussing chamber studies to examine long-term (several days) effects of hormones on ion transport. Many toxic chemicals, particularly heavy metals, exert their detrimental effects through disruption of normal ion transport. The opercular membrane and skin should prove useful in distinguishing direct and indirect effects, and determining the cellular and biochemical actions of toxic compounds (e.g. ref. 6). In some species, the opercular membrane is apparently

specialized for respiration[25], making preparations in these species potentially useful for *in vitro* studies of oxygen and carbon dioxide diffusion and transport.

Although the opercular membrane is widely used as a "model" for the gill, extrapolation of findings should be done conservatively, particularly when in conflict with results obtained in the gill. Moreover, the opercular membrane and skin are of interest in their own right, contributing significantly to the ion fluxes of the whole animal; up to 60% of chloride efflux in the shanny[26], and 50% of calcium uptake in trout[28]. The opercular membrane and skin may be especially important early in development when the gills are poorly developed[15]. Since only a small number of teleosts have been examined in this regard, the importance of skin to ion transport and other physiological functions may be greater than currently appreciated.

VI. References

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