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Fish Gill Respiratory Cells in Culture: A New Model for Cl^--secreting Epithelia

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Abstract. Primary cultures of sea bass gill cells grown on permeable membranes form a confluent, polarized, functional tight epithelium as characterized by electron microscopy and electrophysiological and ion transport studies. Cultured with normal fetal bovine serum (FBS) and mounted in an Ussing chamber, the epithelium presents a small short-circuit current (I_{sc}: 1.4 ± 0.3 μA/cm^2), a transepithelial voltage (V_t) of 12.7 ± 2.7 mV (serosal positive) and a high transepithelial resistance (R_t: 12302 ± 2477 Ω × cm^2). A higher degree of differentiation and increased ion transport capacities are observed with cells cultured with sea bass serum: numerous, organized microridges characteristic of respiratory cells are present on the apical cell surface and there are increased I_{sc} (11.9 ± 2.5 μA/cm^2) and V_t (25.9 ± 1.7 mV) and reduced R_t (4271 ± 568 Ω × cm^2) as compared with FBS-treated cells. Apical amiloride addition (up to 100 μM) had no effect on I_{sc}. The I_{sc}, correlated with an active Cl^- secretion measured as the difference between $^{36}Cl^-$ unidirectional fluxes, was partly blocked by serosal ouabain, bumetanide, DIDS or apical DPC or NPPB and stimulated by serosal dB-cAMP. It is concluded that the chloride secretion is mediated by a $Na^+/K^+/2Cl^-$ cotransport and a Cl^-/HCO_3^- exchanger both responsible for Cl^- entry through the basolateral membrane and by apical cAMP-sensitive Cl^- channels. This study gives evidence of a functional, highly differentiated epithelium in cultures composed of fish gill respiratorylike cells, which could provide a useful preparation for studies on ion transport and their regulation. Furthermore, the chloride secretion through these cultures of respiratorylike cells makes it necessary to reconsider the previously accepted sea water model in which the chloride cells are given the unique role of ion transport through fish gills.

Key words: (Teleosts — *Dicentrarchus labrax* — Branchial epithelium — Ion transport — Cl^- secretion)

Introduction

Marine fish living in a hyperosmotic medium actively secrete salt through specialized epithelia (gill, operculum or rectal gland) to maintain their blood ionic levels and reabsorb water through the intestine to keep their osmotic pressure substantially below those of sea water (SW). The structural heterogeneity of the gill epithelium with both chloride and respiratory cells, together with the complex branchial circulatory system made it difficult to assess the contributions of each cell type to the various gill functions, particularly that of ion transport. Moreover, membrane potentials and intracellular ion concentrations, i.e., the electrochemical gradients of the principal secreted ions (Na^+ and Cl^-) through the basolateral and apical membranes have not been determined, nor have the ion transporters been clearly identified and characterized. Most of our knowledge in fact comes from *in vivo* studies (Forster, 1976) or from preparations of isolated perfused heads (Payan & Matty, 1975), isolated gill arches (Richards & Fromm, 1969), fish opercular membranes (composed essentially of chloride cells; Degnan, Karnaky & Zadunaisky, 1977) and another very specialized SW epithelium, that of the rectal gland (for review *see* Valentich, Karnaky & Moran, 1995). In all models the chloride cells were given the key role in ion secretion (Keys & Willmer 1932; Maetz, 1971; Karnaky, Degnan & Zadunaisky, 1977; Mayer-Gostan & Maetz, 1980; Foskett & Scheffey, 1982; Foskett et al., 1983).

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A decisive experiment came from the use of a vibrating probe to map the operculum and localize the ionic current (chloride secretion) in the chloride cells (Foskett & Scheffey, 1982).

Recently, we developed a primary cell culture of gills isolated from a SW fish, the sea bass *Dicentrarchus labrax* (Avella, Berhaut & Payan, 1994). Cells grown on impermeant supports (plastic culture dishes) formed an organized epitheliumlike structure and presented the morphological characteristics of respiratory cells. The primary cell culture of gills from a fresh-water (FW) rainbow trout (*Oncorhynchus mykiss*) developed by Pärt et al. (1993) was also composed of respiratory cells. However, since access is only possible to the apical side of cells grown on impermeant supports, ion and water transport studies are limited and in addition the normal functioning of such preparations is questionable. An attempt to develop a functional epithelium in culture was made by Pärt & Bergström (1995) who plated cells of *Oncorhynchus mykiss* on a permeant support to have access to both sides of the epithelium. They reported the formation of a monolayer presenting a large transepithelial resistance (characteristic of a tight epithelium) but did not furnish details about ion transport properties, including transepithelial voltage or short-circuit current.

In the present study, a primary culture of sea bass gills was developed on a permeant support. Morphological and ion transport studies demonstrate the high degree of differentiation and functionality of such a preparation composed of respiratorylike cells. These findings have to be considered with respect to previous described gill models of salt-secreting epithelium.

Materials and Methods

Animals

Sea bass (*Dicentrarchus labrax*) with an average weight of 40 g were obtained from a local sea farm (Sociéte 3A, Antibes, France). They were kept at ambient temperature (14° to 18°C) and with natural lighting in a semi-open circuit (tank water completely renewed every 6 hr) in 1-m^3 tanks containing Mediterranean SW (36 g/l salinity) and were fed daily with Aqualim pellets (Aquasarb, France) at 2.5% of their body weight.

Primary Cell Culture

Solutions

All solutions were prepared using tissue-culture quality chemicals. The solutions and culture conditions have been described in Avella et al. (1994). Washing medium: Leibovitz L15 (ICN Pharmaceuticals, France) supplemented with 20 mM NaCl; Fungizone 0.1 μg/ml; penicillin 200 UI/ml; streptomycin 200 μ/ml; gentamycin 400 μg/ml. All antibiotics were supplied by Sigma (St. Louis, MO). Culture medium: Leibovitz L15 supplemented with 10% fetal bovine serum FBS (Multiser, Cytosystems, New South Wales, Australia) or with 10% sea bass serum SBS (*see below for preparation*); 20 mM NaCl; penicillin 100 UI/ml; streptomycin 100 μg/ml; gentamycin 200 μg/ml. The final pH of all solutions was adjusted to 7.8.

Cell Culture Conditions

Before culture preparation, fish were held in a 10-liter tank of aerated SW containing antibiotics and fungicides: Furaltadone 0.02% (Sigma), Temerol 0.02% (Francodex, Carros, France). Fish were decapitated and the gills were excised. The gill arches were removed and the remaining filaments were dipped into washing medium (*see above*). They were washed under gentle automatic shaking (5 × 10 min, 100 agitations/min). Single cell suspensions were prepared by a technique modified from that of Pärt et al. (1993): the filaments were trypsinized twice (0.05% Trypsin 0.02% EDTA solution, Sigma) under 250 agitations/min at room temperature, the cell suspension filtered through 70 μm sterile filters (Falcon) into culture medium. The suspension was then centrifuged twice at 200 × *g* and resuspended in culture medium (*see above*). Cell counts were made with Trypan Blue exclusion (0.2%). Cells were seeded in Costar-Transwell 0.45 μm pore inserts (25 mm diameter, 4.8 cm^2 surface; Costar, MA) in 6-well Costar plates at a high density (2×10^6 cells/cm^2). Cells were maintained in a low-temperature incubator (Jouan, France) at 17°C, in a humidified atmosphere (i.e., atmospheric pCO_2). After 24 hr, cells reached confluency. The medium was changed every other day and the cells were used from days 6 to 9.

Preparation of Sea Bass Serum (SBS)

Blood was collected at the fish farm from the caudal vein of sea bass of 30- to 500-g weight and allowed to clot by standing overnight in closed tubes at 4°C. The serum was then separated from the clot, pooled, and centrifuged at 10,000 × *g* at 4°C for 1 hr. Finally, it was filtered directly through a 0.2 μm Corning filter, aliquoted in sterile tubes and frozen at −20°C for storage.

Electron Microscopy

Both apical and basal sides were rinsed with serum-free medium and fixed for 45 min at 4°C in 1.5% glutaraldehyde and 0.8% paraformaldehyde buffered with 0.05 M sodium cacodylate (pH 7.8). The epithelium was then rinsed 3 times in phosphate buffer saline and left at 4°C overnight.

For transmission electron microscopy (TEM), cells on filters were fixed for 1 hr in 1% osmium tetroxide (Euromedex, France) in the same buffer and dehydrated in serial ethanol concentrations. The filter was then directly embedded in Epon 812. Ultrathin sections (70 nm) were realized with a Reichert ultramicrotome, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined under a Phillips CM12 electron microscope (Eindhoven, Nederland).

For scanning electron microscopy (SEM), cells on filters were dehydrated in serial ethanol concentrations followed by 5-min incubation in 1,1,1,3,3,3,-Hexamethyldisilazan (Merck, Darmstadt, Germany) at room temperature and allowed to dry. The cells were mounted on aluminum stubs with conductive double adhesive tape and coated with gold/palladium for 3 min in an argon atmosphere in a vacuum evaporator. Cells were examined with a field transmission electron microscope (Jeol JSM 6300F) at two kV.

Electrical Measurements

The transepithelial electrical potential (V_t), short-circuit current (I_{sc}) and resistance (R_t) of epithelial cells were monitored in a modified

Ussing chamber (Ehrenfeld, Raschi & Brochiero, 1994) designed to fit the Transwell insert. The volumes of the apical and basolateral bathing solutions were 2 and 4 ml, respectively. The spontaneous V_t was measured through Agar-KCl salt bridges and was clamped at zero volts, through platinum electrodes, using an automatic voltage clamp (Model VC 600, Physiological Instruments, Houston, TX). The sign of the transepithelial potential was referred to as basal side *vs.* apical side (the latter taken as reference). The I_{sc} was continuously recorded on a chart paper recorder (SEFRAM, France) and additional pulses were applied (10 mV, 1 sec duration every 60 sec) to determine the epithelial resistance (R_t). In all experiments the cell culture medium (antibiotics and serum-free) was used on both sides to avoid possible intracellular ion and volume changes due to different experimental and culture media.

Kinetic Studies

Unidirectional transepithelial Cl^- fluxes (J_{in}: for mucosal to serosal side; J_{out}: for serosal to mucosal side), allowing J_{net} (J_{out}-J_{in}) determination, were successively performed on the same cell insert in a 6-well culture plate. ^{36}Cl (0.62 μCi/ml) was added to the appropriate side of the epithelium and four samples were taken on the other side at 20-min intervals by complete replacement with fresh medium. After intensive washing of the mucosal and serosal sides (3 × 5 min with 3 ml cold solution), the complete protocol was repeated for the opposite unidirectional flux measurement. Aliquots (samples of 10 μl taken in duplicate) were collected at the beginning and end of each period to determine the specific activity of the labeled solution. All samples were placed in counting vials supplemented with 10 ml of ACS (Amersham, England) for counting in a liquid scintillation counter (Packard Instruments). Fluxes were expressed as neq/h.cm^2.

Drugs and Chemicals

Ouabain, amiloride, diphenylamine-2-carboxylic acid (DPC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), bumetanide and dibutyryl 3′,5′-cyclic adenosine monophosphate (dB-cAMP) were purchased from Sigma Chemical (St Louis, MO). 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was a gift from Dr. R. Greger (Freiburg, Germany).

Statistics

Data variability is expressed as the standard error of the mean (SE). The Student's *t*-test (paired or unpaired, specified in the figures) was used for estimating the significance of differences between means.

Results

Morphological Observations

Our primary cell cultures revealed essentially one type of cell resembling the respiratory cell type, with few mucous cells and no chloride cells (the latter easily recognized by apical pits, cytoplasm rich in mitochondria and developed vesiculotubular system, Pisam & Rambourg, 1991). SEM observations revealed a regular epithelialike structure composed of a mosaic of polygonal epithelial cells (longest axis about 10 to 30 μm) presenting well-defined intercellular junctions (Fig. 1). The apical cell surface was formed of a complex system of individual microvilli (protrusions) or much convoluted microridges closely resembling those of respiratory (pavement) cells often described in teleost gills (Laurent & Dunel, 1980; Hughes, 1979). However, clear morphological differences corresponding to different stages of differentiation were found correlated with cell culture conditions, i.e., the nature of the supplemented serum. In cells cultured with fetal bovine serum (FBS), the apical surface presented irregular microridges (Fig. 1*a*) or reduced ridges which can be considered as microvilli (Fig. 1*b*); some cells even lacked any organization with an almost smooth surface. On the other hand, in sea bass serum (SBS)-treated cells, a high degree of cell differentiation was observed in the form of regular and concentric microridge arrangements (Fig. 1*c* and *d*). In some cells, the microridges were linked to each other by cross bridges (Fig. 1*e*); a few cells presented organized concentric ridges and grooves surrounding cell invaginations (Fig. 1*f*). Although the general features of respiratory cells were observed with both types of sera used, the morphology of the cells cultured with SBS resembled that of *in vivo* gill epithelium of sea bass more closely than did that of FBS-treated cells (for comparison, *see* SEM and TEM observations of sea bass gill structure *in vivo* in Avella et al., 1994).

TEM observations: SBS-treated cells grown on permeable filters presented a polarized structure, with tight junctions located at the apical side and numerous microridges at the cell surface as revealed by transversal sections (Fig. 2*a* and *e*) confirming SEM observations. Numerous desmosomes associated with a lateral, dense fibrilla meshwork of filaments linked the different cells (Fig. 2*b*). The nucleus was surrounded by a prominent Golgi apparatus consisting mainly of lamellar and vesicular components of various sizes (Fig. 2*c*). The cytoplasm contained cisternae from rough and smooth endoplasmic reticulum, a high number of ribosomes and few dispersed mitochondria (Fig. 2*d*). In fact, the epitheliumlike structure seems to be composed of two or three cell layers (Fig. 2*e* and *f*) as observed in the *in vivo* gill epithelium (Laurent, 1984). FBS-treated cells presented few, small microridges (Fig. 2*f*) and formed a thinner epithelium than SBS-treated cells (compare Fig. 2*e* and *f*).

Ion Transport Studies

Electrophysiological Approach

Due to the high seeding density, primary cultures of sea bass gill cells reached confluency quickly (24 hr) but electrical parameters (I_{sc}, V_t and R_t) were measured through cells in culture from days 6 to 9 (mean data reported in Table 1). FBS-treated cells grown on per-

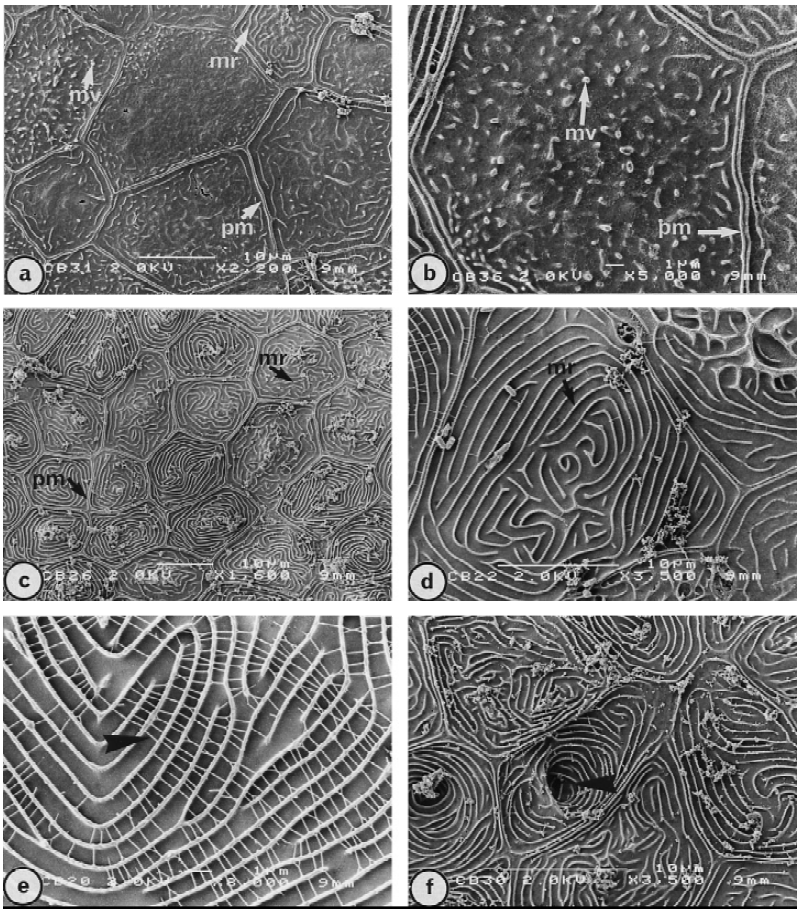

Fig. 1. Sea bass gill cells cultured on permeable membranes observed by SEM. The confluent cell culture is composed of contiguous epithelial polygonal cells. (*a*) Cells cultured in the presence of fetal bovine serum (FBS) show an irregular arrangement of microridges or microvilli and well-defined intercellular junctions. (*b*) High magnification of microvillilike projections in FBS-treated cells. (*c*) Cells cultured in the presence of sea bass serum (SBS) reveal a regular arrangement of convoluted microridges and well-defined intercellular junctions. The culture consists of a mosaic of respiratorylike cells (pavement cells) with surface microridges which are more concentric, numerous and dense than those of FBS-treated cells. (*d*) High magnification of surface microridges in SBS-treated cells. (*e*) Microridges of cells cultured with SBS are rarely connected to each other by cross bridges (arrow). (*f*) Invaginations surrounded by microridges were rarely seen in SBS-treated cells (arrow). *Abbreviations:* mr: microridge; mv: microvilli; pm: plasma membrane. Scale is given by bars: 10 μm for *a,c,d,f* and 1 μm for *b,e.*

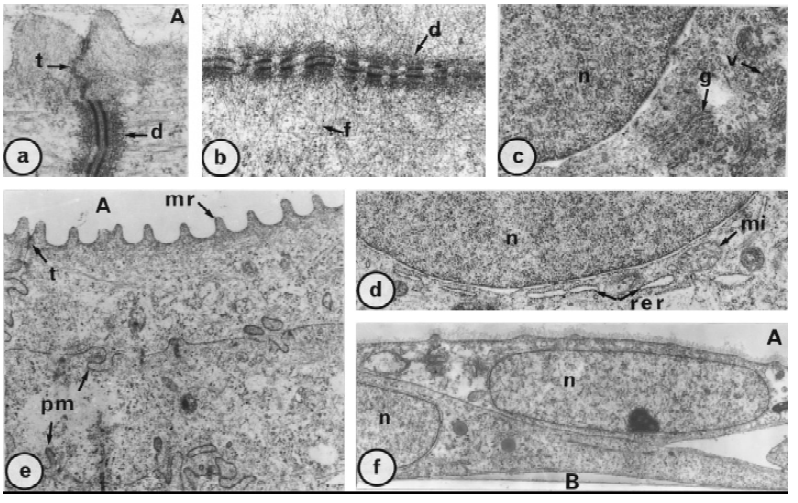

Fig. 2. Sea bass gills cultured on permeable membranes observed by TEM. (*a*) Cells cultured in the presence of SBS (transverse section). Detail of apical intercellular space: note the tight junction and desmosome (×24,574). (*b*) Detail of numerous desmosomes linking contiguous cells in culture with SBS (×25,422). (*c*) Intracellular organization of SBS-treated cells: detail of the developed Golgi apparatus with numerous vesicles surrounding the nucleus (×22,409). (*d*) Intracellular organization of SBS-treated cells: note the rough endoplamic reticulum and mitochondria (×10,746). (*e*) Transverse section of SBS-cultured cells on their permeable support: note the thickness of the epithelium (plurilayer) and the numerous and developed microridges (×11,176). (*f*) Transverse section of cells cultured in the presence of FBS (×8,727). *Abbreviations:* A: apical side; B: basal side; d: desmosome; f: filaments; g: Golgi apparatus; mi: mitochondria; mr: microridge; n: nucleus; pm; plasma membrane; rer: rough endoplamic reticulum; t: tight junction; v: vesicles.

meant filters presented a large R_t indicative of (i) a tight epithelium and (ii) a low ion transport capacity. This latter assumption was confirmed by the current which decreased slightly in the first 5–10 min to stabilize (I_{sc} = 1.4 ± 0.3 μA/cm^2) for long periods (hours) allowing for easy pharmacological treatments; the transepithelial electrical potential was 12.7 ± 2.7 mV, basal side positive. These parameters were different in cells cul-

Table 1. Electrophysiological characteristics of gill cells in culture with different sera

	I_{sc} (μA/cm²)	V_t (mV)	R_t (Ω × cm²)
FBS (n = 16)	1.4 ± 0.3	12.7 ± 2.7	12302 ± 2477
SBS (n = 39)	11.9 ± 2.5*	25.9 ± 1.7**	4271 ± 568**

Statistical comparisons (Student unpaired *t*-test, two tail): SBS data significantly different from FBS data with *P < 0.01 and **P < 0.001.

tured in the presence of SBS. R_t dropped to a value three times lower than that of the FBS-treated cells, while V_t was significantly increased twice and I_{sc} by 660% to reach 11.9 ± 2.5 μA/cm² (Table 1). Our data were analyzed in terms of a simple equivalent circuit as reported for the toad bladder (Yonath & Civan, 1971), assuming that the ion transport exists through cellular and paracellular pathways. Therefore, G_t was plotted as a function of I_{sc} in FBS- and SBS-treated cells (Fig. 3), the Y intercept representing the shunt conductance and the slope, the driving force of the system. A significant correlation (P < 0.0001, n = 39) was found in SBS-treated cells with a Y intercept of 0.132 ± 0.041 mS/cm² and a slope of 29 ± 2 mV. The cells presented a low shunt conductance (paracellular pathway), characteristic of a tight ion transporting epithelium. The plot of G_t *vs.* I_{sc} for FBS-treated cells gave a slope not significantly different from zero, the mean value of G_t was 0.128 ± 0.021 mS/cm² (n = 16), which is similar to the Y intercept of SBS-treated cells.

Unidirectional Chloride Fluxes

The I_{sc} developed in FBS- and SBS-treated cells could be due to transport of a cation from the mucosal to serosal side of the epithelium or to transport of an anion in the opposite direction. Since in preliminary experiments, amiloride up to 100 μM concentration had no effect on I_{sc} (*data not shown*), there was no evidence to postulate the presence of apical Na^+ channels. We therefore looked for the presence of a chloride secretion (serosa to mucosa) by measuring ^{36}Cl unidirectional fluxes in both culture conditions. Electrical parameters of the Transwell-inserts were first measured, followed by J_{in} and J_{out} determinations in open-circuit conditions (Fig. 4). In FBS-cultured cells, J_{out} (76 ± 18 neq/h.cm²) was approximately five times larger than J_{in} (15 ± 3 neq/h.cm²) resulting in a net Cl^- secretion of 60 ± 19 neq/h.cm². The short-circuit current (I_{sc}) was 2.90 ± 0.17 μA/cm², a value not significantly different from J_{net} when expressed in the same units (77 ± 4 neq/h.cm²), indicating that chloride secretion was the sole source of ion transport. With cells cultured in SBS-containing medium, both unidirectional fluxes were significantly higher than in FBS-treated cells; the mean J_{out} and J_{in} were 204 ± 7

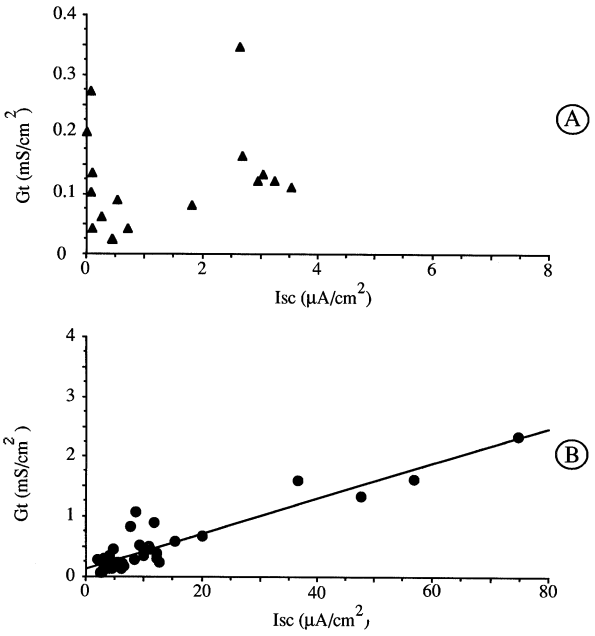

Fig. 3. Plot of the transepithelial conductance (G_t) *vs.* short-circuit current (I_{sc}). (*A*) Data from FBS-treated cells, correlation not significant (r = 0.19, n = 16). (*B*) Data from SBS-treated cells: G_t = (0.029 ± 0.002) I_{sc} ± (0.132 ± 0.041), n = 39, r = 0.91, P < 0.0001. Different scales are given for FBS- and SBS-treated cells.

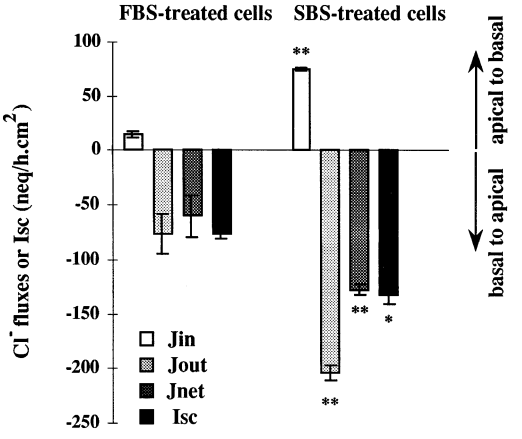

Fig. 4. Unidirectional and net Cl^- fluxes (J_{in}, J_{out} and J_{net}) and short-circuit current (I_{sc}) in FBS- and SBS-treated gill cells in culture. Statistical comparisons: Student unpaired *t*-test (one tail): the difference is significant with **P < 0.005 and *P < 0.05. No statistical difference was observed between J_{net} and I_{sc} within each group. n = 3 for FBS-treated cells; n = 5 for SBS-treated cells.

neq/h.cm² and 75 ± 1 neq/h.cm² respectively and the net chloride secretion was 128 ± 5 neq/h.cm² which matches the measured I_{sc} expressed in the same units (132 ± 9 neq/h.cm²); however, in this experimental group, the stimulatory effect of SBS addition on I_{sc} was not so pronounced (4.92 ± 0.37 μA/cm²; n = 5) when compared to the mean data of table 1 and this was due to an

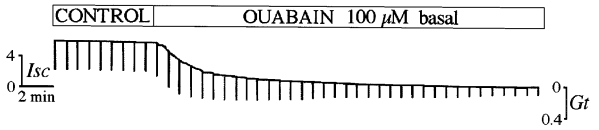

Fig. 5. Inhibitory effect of ouabain on I_{sc} ($\mu A/cm^2$) and G_t (mS/cm^2). A representative tracing is given. Ouabain was added to the serosal side at a final concentration of 100 μM. The transient deflections in current result from periodic V_t pulses (10 mV).

already significant current in FBS-treated cells (a variability was observed in the different batches of sea bass gill cell preparations). It can be concluded that Cl^- secretion accounts for the measured I_{sc} and that SBS treatment increases the ion transport capacity of the sea bass primary culture.

Effects of Pharmaceuticals and Chemicals

A pharmacological approach was chosen to determine the nature of the transporters located at the two opposite cellular membranes and involved in transepithelial chloride secretion. The stability of I_{sc} in the control period facilitated the study of drug effects. Active transports of most ions is energized directly or indirectly by Na/K-ATPase, and the complete inhibition of I_{sc} and G_t by serosal ouabain addition (100 μM) confirmed this assumption (Fig. 5, Table 2). We investigated the mechanism of Cl^- transport in the basolateral membrane and tested the possible involvement of $Na^+/K^+/2Cl^-$ and Cl^-/HCO_3^- transporters both of which could mediate Cl^- entry through this barrier. Bumetanide, a $Na^+/K^+/2Cl^-$ cotransporter blocker, and DIDS, a Cl^-/HCO_3^- exchanger blocker, were therefore used. Bumetanide (100 μM) was found to rapidly (<1 min) reduce I_{sc} ($n = 7$, control: 5.23 ± 0.92 $\mu A/cm^2$, bumetanide: 2.42 ± 0.81 $\mu A/cm^2$; Table 2) and V_t (control: 29.4 ± 6.6 mV, bumetanide: 10.7 ± 2.7 mV). An illustration of the bumetanide effect is given in Fig. 6*A*, DIDS application (100 μM) to bumetanide-treated cells inducing an additional decrease in Cl^- transport capacities (Fig. 6*A*, Table 2). When the order of administration of the drugs was reversed the results were similar (Fig. 6*B*), i.e., successive inhibitions of the Cl^- current.

We intended to identify the apical transporters, suspecting the presence of Cl^- channels on these membranes and for this purpose, NPPB and DPC, two well-known Cl^- channel blockers were used. Apical addition of 50 μM NPPB caused significant decreases in I_{sc} and G_t (Fig. 7*A*, Table 2). However, the responses to NPPB of the various culture cell preparations tested showed considerable variability (Fig. 7*B*), with a mean inhibitory effect on I_{sc} of 42.8 ± 10.1% ($n = 9$). Apical addition of DPC at a concentration of 500 μM, blocked the Cl^--current to an extent (55.8 ± 5.8%, $n = 6$) similar to that of a 10

Table 2. Effect of drugs on the electrical parameters of gill cell cultures

	I_{sc} ($\mu A/cm^2$)	V_t (mV)	G_t (mS/cm^2)
Ouabain ($n = 3$)	3.5 ± 0.2**	14.5 ± 6.9	0.19 ± 0.10*
Bumetanide ($n = 7$)	2.8 ± 0.3*	18.7 ± 5.9*	0.03 ± 0.01*
DIDS ($n = 4$)	1.7 ± 0.6*	5.8 ± 2.5*	0.03 ± 0.01*
NPPB ($n = 9$)	3.8 ± 1.1*	8.4 ± 2.9*	0.09 ± 0.04*
DPC ($n = 11$–12)	6.4 ± 1.1*	25.2 ± 5.1**	0.15 ± 0.04**

Mean of individual data differences before and after drug addition.
Note: DIDS was introduced after bumetanide addition.
Statistical comparisons: Student paired *t*-test: data significantly different from control period with $*P < 0.05$ (one tail) and $**P < 0.005$ (two tail).

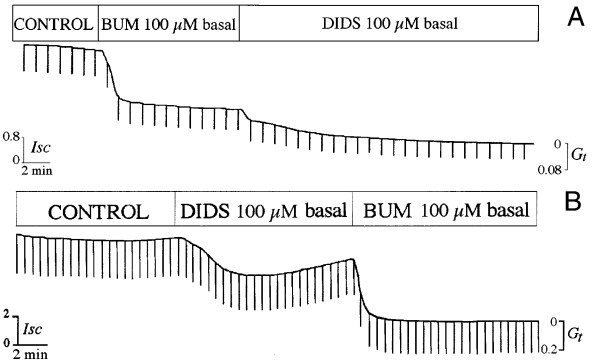

Fig. 6. *A* and *B*. Inhibitory effects of bumetanide and DIDS on I_{sc} ($\mu A/cm^2$) and G_t (mS/cm^2). Representative tracing are given. The two drugs were added successively to the serosal side at a final concentration of 100 μM. The transient deflections in current result from periodic V_t pulses (10 mV).

times lower NPPB concentration. A large inhibition of I_{sc} and G_t was observed with 10^{-3} M DPC (Fig. 8*A*). A DPC dose-response curve for I_{sc} indicated a half-maximal I_{sc} inhibition (IC_{50}) of 4×10^{-4} M (Fig. 8*B*).

Chloride secretion in many epithelia has been found to be regulated by the modulation of cAMP-dependent Cl^--channels. In our experiments, cAMP was used in its permeable form, i.e., dB-cAMP at a concentration of 10^{-3} M on the serosal side. Of the 7 cultured epithelia tested, 5 responded to this agent and 2 were not modified by cAMP addition. An illustration of the stimulation of Cl^- secretion is given in Fig. 9 which shows increases of I_{sc} and G_t. The mean stimulation for I_{sc} was 32.4% ± 10.6 ($n = 5$, $P < 0.05$), for V_t was 19.3% ± 6.5 ($P < 0.05$) and for G_t 14.5% ± 6.0.

Discussion

The principal innovation of this study is the development of a primary cell culture of sea bass gill epithelium

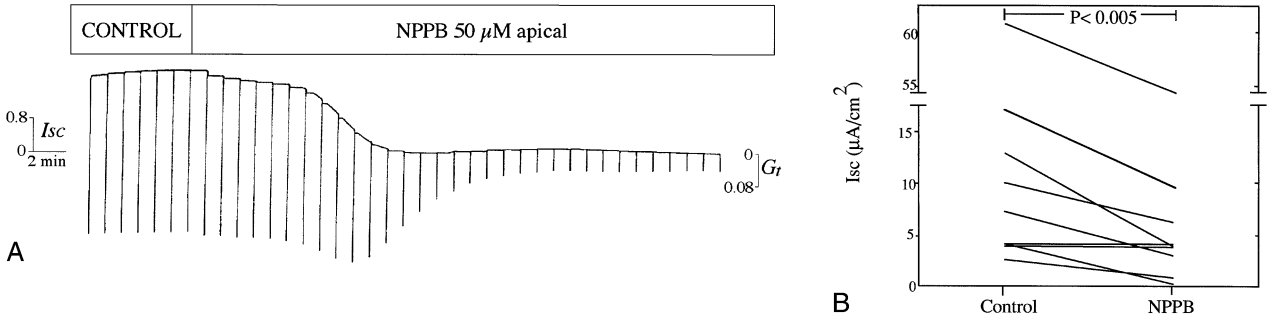

Fig. 7. (*A*) Inhibitory effect of NPPB on I_{sc} (μA/cm^2) and G_t (mS/cm^2). A representative tracing is given. NPPB was added to the mucosal side at a final concentration of 50 μM. The transient deflections in current result from periodic V_t pulses (10 mV). (*B*) Individual data of I_{sc} inhibition by NPPB addition (50 μM, mucosal side). Statistical comparisons: Student paired *t*-test (two tail): the difference is significant with $P < 0.005$.

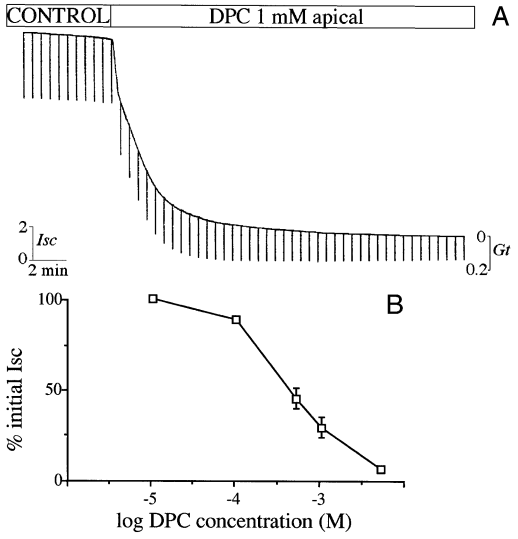

Fig. 8. (*A*) Inhibitory effect of DPC on I_{sc} (μA/cm^2) and G_t (mS/cm^2). DPC was added to the mucosal side at a final concentration of 1 mM. Representative tracing of I_{sc} and G_t inhibitions with time after DPC (10^{-3} M) apical addition. (*B*) Log-dose response of % initial I_{sc} against log DPC concentrations. Sample size was 6–12. Data shown are means ± SEM; when not shown the error bars were smaller than the symbol.

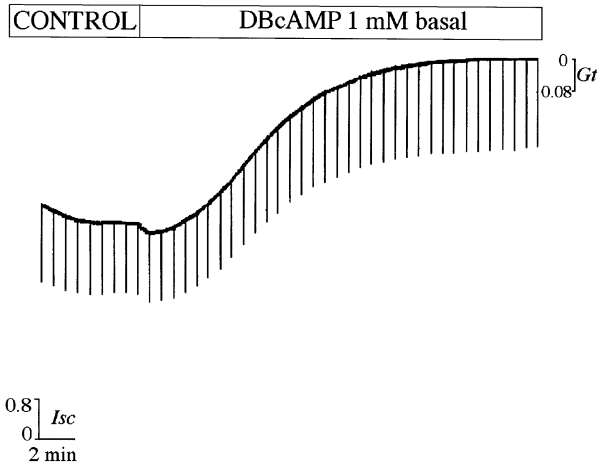

Fig. 9. Stimulatory effect of dB-cAMP on I_{sc} (μA/cm^2) and G_t (mS/cm^2). A representative tracing is given. dB-cAMP was added to serosal side at a final concentration of 1 mM.

which, when grown on permeable membranes, presents the morphological and functional characteristics of a Cl⁻-transporting epithelium.

A Highly Differentiated Epithelial Structure Formed of Respiratorylike Cells

The ultrastructure of the cultured cells revealed a high degree of differentiation of one cell type of the *in vivo* gill epithelium, the respiratory cells also called pavement cells (Hughes & Wright, 1970; Wright, 1973; Laurent & Dunel, 1980; for European sea bass, *see* Avella et al., 1994). It should be pointed out that specific structure such as cross bridges connecting microridges and surface invaginations (*see* Fig. 1) have also been observed in the respiratory surface of the gills of rainbow trout (Kendall & Dale, 1979). No chloride cells and few mucous cells were present in our preparations. On the basis of their characteristic surface microridges, Pärt et al. (1993) also only detected respiratory cells in cultured gills of a freshwater (FW) teleost. This is not surprising because the predominant cell population in the branchial epithelium consists of these pavement cells regardless of the salinity of the environment.

The origin of the culture serum determined the morphofunctional differentiation of the respiratory cell-like preparation. Branchial cells from sea bass cultured in SBS clearly presented a higher morphological degree of differentiation than those cultured in FBS medium. In the fish serum, surface microridges became more numerous, regular in shape and organization and structurally identical with *in vivo* gill respiratory cells. The morphological developments in SBS-treated cells were also accompanied by functional changes as measured by the evolution of their ion transporting capacity (Table 1).

A comparison of the transepithelial conductances of the two types of sera-treated cells (i.e., in SBS or FBS media) throws light on the difference existing between cellular and paracellular pathways. In SBS-treated cells, G_t was 0.48 ± 0.08 mS/cm^2, and in FBS-treated cells G_t was 0.128 ± 0.021 mS/cm^2. The plot of G_t *vs.* I_{sc} gave a shunt conductance value of 0.132 ± 0.041 mS/cm^2 which is not significantly different from the mean FBS-treated cells G_t value. This would indicate that in FBS-treated cells, the cellular conductance is negligible in agreement with a very low ion transport capacity. Fish serum was found to be essential for the expression of ion transporters implicated in the chloride transport through a cellular pathway as illustrated by I_{sc} and G_t increases. Fish serum has already been found to be an essential supplement for other fish cell cultures (Dickman & Renfro, 1986; Clark, Taylor & Tchen, 1987) and our results pose interesting questions about the factors including hormones responsible for this cellular differentiation and the way in which they function.

The sea bass gill cells in primary culture can be classified as a tight epitheliumlike structure (R_t mean value for SBS-treated cells 4271 Ω.cm^2), presenting a noticeable I_{sc} (mean value 11.9 μA/cm^2) and a relatively large transepithelial potential (mean value 25.9 mV). Its transepithelial resistance can be compared with the R_t values of cultured gill cells from FW trout in which a high tissue resistance (R_t = 3000–5000 Ω.cm^2) was also found with no indication of ion transport capacities (Pärt & Bergström, 1995). A comparison with other isolated SW epithelia such as those from the operculum and the rectal gland, which are also ion-secreting epithelia, is possible. The opercular membrane from the killifish or tilapia presented the characteristics of a comparably leaky epithelium (R_t ranging from 173–259 Ω.cm^2) with a large I_{sc} (53–138 μA/cm^2) and a V_t ranging between 8–21 mV (Zadunaisky, 1984) as did also the shark rectal gland (R_t = 29 Ω.cm^2; I_{sc} = 66 μA/cm^2 and V_t = 1.9 mV; Greger et al., 1984). In the cultured opercular epithelium, I_{sc} ranged from 150 to 300 μA/cm^2 (Marshall et al., 1995). On the other hand, the cultured cells of shark rectal gland presented much higher R_t levels (2000 Ω.cm^2) with low I_{sc} (0–10 μA/cm^2) and V_t (<1 mV) values under nonstimulated conditions but these increased with hormonal stimulation (Valentich & Forrest, 1991). Therefore, a wide variability in the R_t and I_{sc} occurs among the different models of SW ion transport and this could be due to the different (i) types of cell involved, (ii) hormonal states or, (iii) origin of the cells studied (native epithelium or cultured preparations).

Evidence of a Chloride-secreting Epithelium

The current measured in SBS-treated cells was identified as an active Cl^- secretion since (i) measurements of net Cl^- fluxes were not significantly different from I_{sc}, (ii) a correlation was found between the two parameters, and (iii) I_{sc} was blocked by Cl^- transport inhibitors. As already discussed, the presence of fish serum in the culture medium was important for the development of the Cl^--secreting capacity.

A pharmacological approach was used to identify the different transporters located at the opposite membranes to the sea bass gill epithelium and responsible for the active Cl^- secretion. Cl^- entry through the basolateral membranes (b.l.m.) is likely to be mediated by a $Na^+/K^+/2Cl^-$ cotransport in parallel with a Cl^-/HCO_3^- exchanger as suggested from the inhibitory effects of bumetanide and DIDS on I_{sc}. Both cotransport and exchange mechanisms account for Cl^- entry into the cell, since Cl^- excretion was almost totally inhibited when the two drugs were successively added. Na^+/K^+-ATPase was found to play a key role in the establishment of the net Cl^- secretion since addition of serosal ouabain completely blocked I_{sc}. This pump by keeping low cellular Na^+ and Cl^- levels provides the driving force for Cl^- entry through b.l.m. by the $Na^+/K^+/2Cl^-$ cotransport and the Cl^-/HCO_3^- exchanger. The blocking effect of ouabain must therefore be interpreted as an indirect effect on both electroneutral transporters. Chloride transport through the apical membrane is considered to be mediated by a conductive pathway in view of the presence of Cl^- channels sensitive to NPPB or DPC. Thus, cellular Cl^- accumulation by both electroneutral transporters above the Cl^- equilibrium potential will result in the Cl^- diffusion through the apical Cl^- channels. To allow such an electrodiffusion, the apical membrane potential (cell negative) must overcome the presumed unfavorable chemical gradient through this membrane. For example, a cell Cl^- concentration of 30 mM was found in A6 cells which are a renal cell line also forming a chloride-secreting epithelium with similar transporters and channels (Brocchiero et al., 1995). Membrane potentials and intracellular ion concentrations will have to be measured in the sea bass gill preparation to establish the precise electrochemical gradients and electromotive forces driving Cl^- through the apical membrane channels.

Since Cl^- secretion was stimulated in sea bass gill cells in culture by cAMP, by analogy with other Cl^- secreting epithelia we propose that apical membrane Cl^- channels are regulated by cAMP. A working model presented in Fig. 10 summarizes our main findings. This model of Cl^- secretion, proposed from our results with sea bass gill respiratory cells in culture, will be discussed in (i) the context of ion transport through the gills of SW fishes and (ii) in relation to other models of Cl^--secreting epithelia.

Although the osmoregulatory function of the SW fish gill has been clearly established, i.e., that of secreting monovalent ions such as Na^+ and Cl^- actively, a

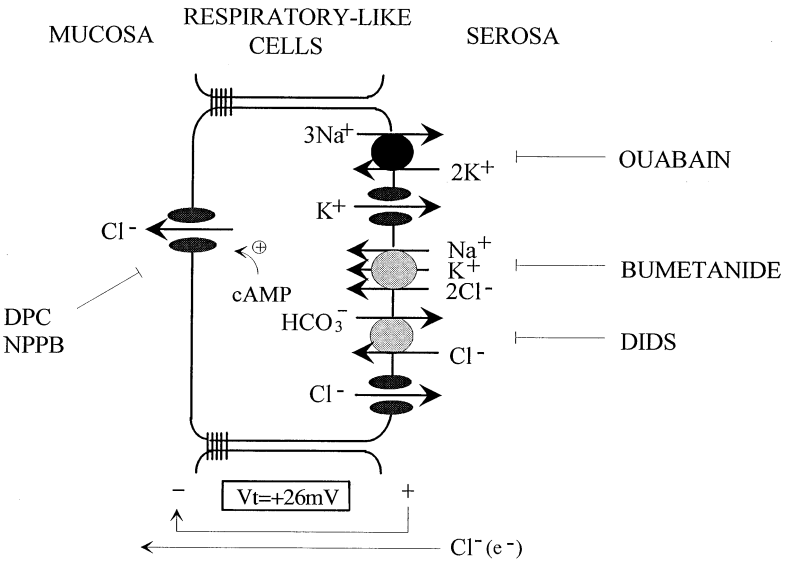

Fig. 10. Model for transepithelial Cl^- secretion in primary cultures of sea bass gill cells (respiratorylike cells): The electrogenic Na^+/K^+ ATPase pump, located on the basolateral membrane (b.l.m.) maintains low intracellular Na^+ and Cl^- activities and is therefore at the origin of an inwardly directed chemical gradient for these ions. Cl^- crosses the b.l.m. by two electroneutral transporters, a $Na^+/K^+/2Cl^-$ cotransporter (inhibited by bumetanide) and a HCO_3^-/Cl^- exchanger (inhibited by DIDS). Intracellular Cl^- is postulated therefore to be above equilibrium and may follow an outwardly directed diffusive pathway along its favorable electricochemical gradient through apical Cl^- channels, blocked by DPC and NPPB and stimulated by the intracellular cAMP levels. A cation, K^+ or more probably Na^+, has to follow Cl^- ions to satisfy the principle of electroneutrality; the nature of the cation involved and the pathway (cellular or paracellular) have yet to be determined.

model of the ion transport through this epithelium is still under discussion and several reported problems have not been solved. The transepithelial electrical potential through the gill is not precisely known, since attempts at its evaluation in SW teleosts *in vivo* have yielded results which should be considered with caution given the numerous technical problems linked to the complex structure of the gill (for reviews *see* Potts, 1984; Péqueux, Gilles & Marshall, 1988). The relative contributions to ion transport of the two main cell types i.e., the pavement cells (respiratory cells) which constitute more than 90% of the whole cell population and the chloride cells, have been established by indirect studies, as for instance by morpho-functional changes on adaptation (Avella et al., 1987; Laurent & Perry, 1990; Goss et al., 1992) but direct experimental support is lacking. Up to now, the generally accepted SW model is that proposed by Silva et al. (1977*a*,*b*) in which the presence of a Na^+/K^+ ATPase located on the b.l.m. of chloride cells (Karnaky, Kinter & Kinter, 1976) generates the ionic and electrical gradients enabling Na^+ and Cl^- to be excreted into SW. The so-called chloride cells have been given the main role in Cl^- secretion, Na^+ ions passing through leaky junctions by paracellular pathways (between chloride cells and accessory cells) (Sardet, Pisam & Maetz, 1979). This conclusion was derived by analogy with results from another chloride-secreting epithelium: that of the operculum of the killifish and tilapia, which is largely composed of chloride cells (Burns & Copeland, 1950; Karnaky & Kinter, 1977; Degnan et al., 1977; Foskett & Sheffey, 1982; Foskett et al., 1983). The sea bass gill cell culture used in our study was composed of respiratorylike cells which actively secrete chloride. The new and principal discovery of our study is therefore that the respiratory cells have an active role in Cl^- secretion and can thus also qualify ionocytes like the chloride cells. The Cl^- secretion capacity (12 $\mu A/cm^2$) of sea bass gill cells in culture is approximately 4.5 to 11 times lower than that of the opercular epithelium (for review, *see* Zadunaisky, 1984). However, considering the large number of respiratory cells in *in vivo* tissue, 90% of the total epithelial surface area, the Cl^- secretion through respiratory cells cannot be ignored. Our data certainly support the establishment of a new working model for ion transport in sea-water gills where both cell types (respiratory cells and chloride cells) are involved. It is relevant that in FW gills, the respiratory cells which were usually accorded a minor participation in ion transport, have recently been reevaluated relative to this function and given a key role in proton secretion (supported by the presence of V-ATPase) and Na^+ absorption (Lin et al. 1994; Goss, Laurent & Perry, 1994).

The model that we propose (Fig. 10) is similar to that established for the SW fish opercular epithelium (Marshall, 1995; Zadunaisky et al., 1995) and the shark rectal gland epithelium (Silva et al., 1977*b;* for review *see* Valentich, 1995) and the gill chloride cell (Silva et al., 1977*a*). The presence of a $Na^+/K^+/2Cl^-$ cotransporter is a general feature in Cl^-- secreting epithelia, and this transporter has been cloned for the first time in the shark rectal gland (Xu et al., 1994). A Cl^-/HCO_3^- exchanger was also found in the operculum (Zadunaisky et al., 1995) but it was not mentioned in shark cell cultures or SW fish gills. The presence of apical Cl^- channels sensitive to DPC and NPPB was also established in shark rectal gland (Greger, Schlatter & Gögelein, 1987) and in the opercular membrane of marine fishes (Marshall et al., 1995). In these epithelia, cAMP was found to increase Cl^- secretion (Greger et al., 1984, for rectal gland; Marshall et al., 1995, for opercular membrane) so it was not surprising to observe a similar effect in the sea bass gill preparation. Recently, Devor et al. (1995) studying cul-

tured cells from the elasmobranch rectal gland, found that the single channel conductance responsible for cAMP-mediated Cl^- secretion is a homologue of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) recently cloned from the shark rectal gland (Marshall et al., 1991). The analogy of CFTR to the low conductance anion channel described in primary culture of opercular epithelium cells was also noticed by Marshall et al. (1995). Direct patch-clamp experiments will be necessary to identify the channel (s), involved in Cl^- secretion through the sea bass gill cells in culture.

In conclusion, the cultured respiratory cells of the sea bass gill provide a model of the SW fish gill for studying ion and water transports. This preparation will be of particular interest for investigating the hormonal and cell signaling involved in the regulation of the channels and transporters concerned in Cl^- and Na^+ secretion.

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