General and Comparative Endocrinology **123,** 280–294 (2001)
doi:10.1006/gcen.2001.7679, available online at http://www.idealibrary.com on IDEAL®

The Physiological Effects of 3,5′,3′-Triiodo-L-thyronine Alone or Combined with Cortisol on Cultured Pavement Cell Epithelia from Freshwater Rainbow Trout Gills

Scott P. Kelly and Chris M. Wood

Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

Accepted May 17, 2001

The effects of 3,5′,3′-triiodo-L-thyronine (T_3; 10 or 100 ng ml^{-1}), alone or combined with cortisol (500 ng ml^{-1}), on the physiological properties of cultured pavement cell epithelia from freshwater rainbow trout gills were assessed. T_3 had dose-dependent effects on electrophysiological, biochemical, and ion transporting properties of cultured epithelia in both the absence and the presence of cortisol. These included reduced transepithelial resistance (TER), increased net Na^+ and Cl^- movement (basolateral to apical) under asymmetrical culture conditions (freshwater apical/L15 media basolateral), and elevated Na^+–K^+-ATPase activity. However, paracellular permeability was elevated only in high-dose T_3-treated preparations. In T_3 + cortisol-treated epithelia, similar T_3-induced alterations in TER, net Na^+ and Cl^- movement, and paracellular permeability were observed, whereas the activity of Na^+–K^+-ATPase was further elevated. Under symmetrical culture conditions (L15 medium apical/L15 medium basolateral), T_3 had no effect on transepithelial Na^+ and Cl^- transport, which was passive. However, T_3 + cortisol treatment resulted in active Na^+ extrusion (basolateral to apical). Under asymmetrical conditions, hormone treatment did not change the pattern of ion movement (active Na^+ extrusion, active Cl^- uptake). These experiments demonstrate that cultured pavement cell epithelia from freshwater rainbow trout are T_3-responsive and provide evidence for the direct action of T_3 and the interaction of T_3 and cortisol on the physiology of this preparation. © 2001 Academic Press

INTRODUCTION

Recently, techniques for the primary culture of gill cells on permeable supports have provided a promising new direction in the development of an *in vitro* model for the freshwater (FW) fish gill (Wood and Pärt, 1997; Wood *et al.,* 1998; Gilmour *et al.,* 1998; Fletcher *et al.,* 2000; Kelly and Wood, 2001). Certain procedures have allowed for the *in vitro* "reconstruction" of a flat epithelium composed exclusively of gill pavement cells that mimics many of the passive transport and electrophysiological characteristics of the intact gill (Wood and Pärt, 1997; Wood *et al.,* 1998). However, cultured gill epithelia do not yet fully simulate the active ion transport processes observed in the intact freshwater fish gill (Wood and Pärt, 1997; Wood *et al.,* 1998). In this regard, an important avenue for further development of cultured gill epithelia is the potential use of ionoregulatory hormones. Recently we have demonstrated that cortisol can promote active Na^+ and Cl^- transport in the correct vectorial direction across cultured pavement cell epithelia when preparations are bathed on both sides with media (Kelly and Wood, 2001). However, when freshwater was introduced to the apical side of cortisol-supplemented epithelia, the pattern of ion transport was not significantly different from that of untreated preparations. Clearly, further investigation into the potential uses of other ionoregulatory hormones is warranted.

0016-6480/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

The reported effects of thyroid hormones, thyroxine (T_4) and 3,5′,3′-triiodo-l-thyronine (T_3), on gill function *in vivo* are contradictory. For example, in various species, T_3 or T_4 have been reported to have stimulatory (Subash Peter *et al.,* 2000; Madsen and Korsgaard, 1989), inhibitory (Omelanjiuk and Eales, 1986), or no (Dangé, 1986, Bjornsson *et al.,* 1987, Madsen, 1990) effects on gill Na^+–K^+-ATPase activity. However, through interaction with other endocrine axes, a more consistent response has been observed. Na^+–K^+-ATPase increased after combined T_4 and cortisol treatment in tilapia (Dangé, 1986) and combined T_4 and growth hormone treatment in amago salmon (Miwa and Inui, 1985). These observations, together with findings of increased interrenal sensitivity to ACTH (Young and Lin, 1988) and increased pituitary growth hormone production (Moav and McKeown, 1992) in the presence of thyroid hormones, have led to general agreement that thyroid hormones play an important role in teleost osmoregulation (for review see McCormick, 1995). However, we are unaware of any studies that have investigated the actions of thyroid hormones, alone or in combination with other hormones, on the physiology of gill pavement cells or on the physiology of cultured gill epithelia *in vitro.* Recent evidence suggests that the inward movement of Na^+ occurs, at least in part, across the pavement cell (for review see Perry, 1997). Therefore, the combined actions of T_3, the biologically active derivative of T_4 (Eales and Brown, 1993), and cortisol on pavement cell function are of interest as simultaneous thyroid hormone/cortisol treatment appears to be more effective at increasing Na^+–K^+-ATPase activity in the gills of a freshwater fish *in vivo* than is either hormone alone (Dangé, 1986).

The goal of the present study was to investigate the potential effects of T_3 on the physiology of gill epithelia composed exclusively of pavement cells in primary culture. Principally we sought to investigate whether the actions of T_3 would promote transepithelial ion transport. Some of our early observations suggested that T_3 stimulated pavement cell Na^+–K^+-ATPase activity but reduced transepithelial resistance (TER) across pavement cell epithelia. As we have previously demonstrated that cortisol alone did not stimulate Na^+–K^+-ATPase in this preparation but did reduce TER and paracellular permeability (Kelly and Wood, 2001), the second goal of the current study was to investigate the combined actions of T_3 and cortisol on pavement cell epithelia. We hypothesized that a T_3-induced increased Na^+–K^+-ATPase combined with a cortisol-induced increase in TER (reduction in paracellular permeability) may promote the active transport of Na^+ and Cl^- under conditions of asymmetrical exposure (apical freshwater/basolateral media).

MATERIALS AND METHODS

Preparation of Cultured Branchial Epithelia

All gill cell cultures were derived from locally obtained stocks of rainbow trout (*Oncorhynchus mykiss*) (80 – 175 g) held in dechlorinated running tapwater (composition: $[Na^+] = 0.55$, $[Cl^-] = 0.70$, $[Ca^{2+}] = 1.00$, $[Mg^{2+}] = 0.15$, $[K^+] = 0.05$ mM, pH 7.8–8.0) at seasonal temperatures (13–17°). All procedures for gill cell isolation and culture were conducted in a laminar flow hood with sterile techniques according to methods originally developed by Pärt *et al.* (1993), with modifications described by Wood and Pärt (1997). Full details of these methods have been described by Kelly *et al.* (2000). Briefly, fish were stunned by a blow to the head and decapitated, and gill filaments were excised. Gill cells were isolated by two consecutive cycles of tryptic digestion (Gibco BRL Life Technologies; 0.05% trypsin in phosphate-buffered saline (PBS), with 5.5 mmol L^{-1} EDTA) and resuspended in culture medium (Leibovitz's L-15 supplemented with 2 mmol L^{-1} glutamine, 5–6% foetal bovine serum (FBS), 100 i.u. ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 200 $\mu g\ ml^{-1}$ gentamycin). Appropriate volumes of cell suspension were added to 25-cm^2 culture flasks (Falcon) to give a seeding density of 520,000 cells cm^{-2}. Flasks were held in an air atmosphere at 18°. After 24 and 96 h, nonadherent cells were removed by changing the media (L-15 plus 2 mmol L^{-1} glutamine, 6% FBS, and antibiotics; see above) and from 96 h onward, all media used in flasks and inserts were antibiotic-free. After a further 48–72 h (total time in flask culture = 144–168 h), the harvesting and reseeding of cells onto permeable Falcon culture inserts (Cyclopore polyethylene terephthalate "filters," Becton–Dickinson, Franklin Lakes, NJ; pore density: 1.6×10^6 pores · cm^{-2}; pore size: 0.45 μm; growth surface: 0.9 cm^2) were conducted by the

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

removal and replacement of medium with trypsin solution (see above). Cell detachment was confirmed via visual inspection under a phase-contrast microscope (Zeiss) and trypsination was terminated by the addition of suspended cells to a "stop" solution (10% FBS in PBS, pH 7.7). The "stop" solution was centrifuged at low speed to obtain a cell pellet and cells were resuspended in media and seeded onto culture inserts at a density of 700,000–800,000 cells cm^{-2}. Inserts were held in 12-well companion plates (Falcon) under incubation conditions identical to those stated above. Initially, inserts (apical side) and companion wells (basolateral side) contained 0.8 and 1.0 ml medium, respectively, and bathing solutions were topped-up to 1.5 and 2.0 ml at 24 h. A complete change of media was conducted every 48 h thereafter. When asymmetrical conditions were tested, temperature-equilibrated (18°) freshwater (Acrodisc sterilized, 0.2-μm pore size, chemical composition same as original holding water) was added to the apical side of the insert after several rinses to ensure removal of any residual media.

Hormone Treatment

Single-use aliquots of stock hormone solutions were prepared by dissolving either T_3 (Sigma) or cortisol (hydrocortisone hemisuccinate; Sigma) in PBS (pH 7.7) to achieve a final concentration of 0.5 mg ml^{-1}. A few drops of 5 M NaOH was used to dissolve T_3. This had no effect on the pH of medium to which T_3 was added due to large dilution factors. Aliquots of stock hormone solutions were stored at −20° until use. When ready for use, stock hormone solutions were thawed and diluted in L-15 medium (L-15 plus 2 mmol L^{-1} glutamine, 6% FBS with or without antibiotics as appropriate; see above) so as to be added fresh on each medium change. Two concentrations of T_3 (10 and 100 ng ml^{-1}) and one concentration of cortisol (500 ng ml^{-1}) were used. Care was taken to retain physiological significance by including physiologically relevant doses of hormone (i.e., 10 ng ml^{-1} of T_3 compared to *in vivo* levels of ≈1–8 ng ml^{-1}; Redding *et al.,* 1984; Leatherland, 1985; and 500 ng ml^{-1} of cortisol compared to *in vivo* levels that can reach ≈500 ng ml^{-1}; Barton and Iwama, 1991); however, 100 ng ml^{-1} T_3 could be considered pharmacological. Treatment of the cells with each appropriate dose of hormone commenced immediately after first seeding of the cells into culture flasks. After the cells were reseeded onto cell culture inserts, hormones were supplemented on the basolateral side of the insert only. Cells and epithelia bathed in media without the addition of supplemental hormonal treatment were assigned as controls, or in the case of one experiment, the assigned controls were supplemented with cortisol (500 ng ml^{-1}) only.

Electrophysiological Measurements

Chopstick electrodes (STX-2) connected to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL) were used to monitor transepithelial resistance. All values for TER were expressed relative to blank corrections with vacant inserts containing appropriate solutions. Daily measurements of TER across filter inserts under symmetrical conditions (i.e., with culture media on both sides) were made 48 h after the initial seeding of inserts. Under asymmetrical conditions (i.e., freshwater added to the apical side) TER and/or transepithelial potential (TEP) measurements were conducted at appropriate time intervals (see Results). TEP measurements were recorded using agar–salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes (World Precision Instruments) to a high-impedence electrometer (Radiometer pHM 84, Copenhagen, Denmark). After correction for junction potential, TEP measurements were expressed relative to the apical side as 0 mV.

Na^+–K^+-ATPase Activity and Epithelium Protein Content

Na^+–K^+-ATPase activity in individual inserts was measured as described by McCormick (1993), with slight modifications. After mild trypsination (≈2 min) to obtain a cell suspension, trypsination was terminated as previously described and the cell suspension was centrifuged (Beckman J-21C; 0–4°) for 10 min at 500*g* to obtain a cell pellet. The cell pellet was resuspended and washed in PBS (pH 7.7) and centrifuged again for a further 10 min at 500*g*. After removal of the PBS, SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3, 0–4°) was added to the cell pellet and the preparation was quick frozen in liquid nitrogen. Cells were stored at −70° until further analysis.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

Prior to analysis, samples were thawed, and a volume of SEID buffer (0.5 g sodium deoxycholate in 100 ml of SEI) was added, resulting in a final SEI to SEID ratio of 4:1. Cells were sonicated (Branson; Sonifier 450) on ice for 30 s and centrifuged (Eppendorf 5415C; 0–4°) at 5000 *g* for 1 min, and the resulting supernatant was immediately analyzed for Na^+–K^+-ATPase activity. Due to the low amount of protein available, the assay was run for 40 min at 24°; however, the enzyme activity was linear throughout this period. The protein content of supernatants was measured by the Bradford method (Sigma) with bovine serum albumin (Sigma) as a standard. All enzyme activities are expressed as protein specific activities. Protein content for individual epithelia (μg $epithelia^{-1}$) was determined after correction for the volume of buffer used for epithelia/cell sonication.

[^{3}H]PEG-4000 Permeability

Under both symmetrical and asymmetrical conditions, permeability of the cultured epithelium preparation was measured with a paracellular permeability marker, [^{3}H]polyethylene glycol (molecular mass 4000 Da; "PEG-4000;" NEN-Dupont) according to the methods and equations previously outlined by Wood *et al.* (1998), Gilmour *et al.* (1998), and Kelly and Wood (2000). After the addition of 1 μCi PEG-4000 to basolateral culture medium, permeability was determined in the efflux direction (basolateral to apical) only. The appearance of PEG-4000 in the apical compartment was determined at 12-h (symmetrical) and at 12- to 24-h (asymmetrical) consecutive intervals. During flux periods, TER was closely monitored at time intervals ranging from 1 to 6 h.

Net and Unidirectional Ion Flux Measurements

The effect of hormonal treatment on net ion (Na^+ and Cl^-) flux rates under asymmetrical conditions (directly measured $J_{net}^{Na^+}$ and $J_{net}^{Cl^-}$ without the use of isotopes) was assessed with two series of epithelia over a 12- to 24-h period, with methods and calculations identical to those outlined by Wood *et al.* (1998) and Kelly and Wood (2001).

In several series of control (no hormone treatment) and hormone-treated epithelia, unidirectional ion flux rates (employing radiotracers) were determined according to the methods and calculations of Wood *et al.* (1998). For both influx (apical to basolateral) and efflux (basolateral to apical) studies, 1 μCi of isotope ($^{22}Na^+$ or $^{36}Cl^-$) was added to the apical or basolateral side of the culture and the appearance monitored on the "cold" side. Under symmetrical conditions, ion flux rates were first recorded in the basolateral to apical direction across the inserts, after an incubation period of 6 h, and then in the basolateral to apical direction after a second 6-h incubation period. Between influx and efflux measurements, inserts were washed out for a period of 2–3 h with "cold" medium. Measurements of TER and TEP were recorded at time 0 and at the end of each flux period (6 h). With this approach, each insert could be used as a single individual for calculations of the Ussing flux ratio criterion (see below), provided that electrophysiological measurements did not change. Under asymmetrical conditions, inserts were used for either influx or efflux measurements only and matched for calculations of the Ussing flux ratio criterion based on criteria of electrophysiological similarity (see Fletcher *et al.,* 2000). Each insert was used only once in the pairing procedure. Measurements of TER and TEP were taken at the beginning, middle (3 h), and end of each flux period (6 h).

Ussing Flux Ratio Criterion

Disagreement of the measured flux ratio (J_{in}/J_{out}) with the prediction of the Ussing flux ratio was employed as an indication of active transport (Kirschner, 1970). The predicted Ussing flux ratio was calculated as

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap} \cdot e^{-(zFV/RT)}}{A_{Bl}} \quad (1)$$

where A_{Ap} and A_{Bl} are the activities of the ions (Na^+ and Cl^-) on the apical and basolateral sides, z is the ionic valence, V is the measured TEP in volts (average for matched inserts), and F, R, and T have their usual thermodynamic values. In media, A_{Na} and A_{Cl} were 75% of measured concentrations (cf. Wood *et al.,* 1998). Under asymmetrical conditions, A_{Na} and A_{Cl} in apical freshwater were taken as equal to the measured concentrations.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

Statistical Analysis

All data are expressed as mean values ± SE (n), where n represents the number of filter inserts. For comparisons between varying groups, data were subjected to either a repeated one-way analysis of variance or a two-way analysis of variance as appropriate. Significant differences between groups were detected with either a Student–Newman–Keuls test or a Student unpaired or paired t tests as appropriate. Sigmastat software (Jandel Scientific) was employed throughout. The level of statistical significance for all analyses was $P < 0.05$.

RESULTS

Microscopy

Examination of cells, in flasks and inserts, by phase-contrast microscopy did not reveal any evidence of mitochondria-rich cells in culture (cf., Fletcher *et al.,* 2000; Kelly *et al.,* 2000).

Treatment with 3,5′,3′-Triiodo-L-thyronine Alone

TER measurements and [^{3}H]PEG permeability under symmetrical culture conditions. Transepithelial resistance of the epithelium increased over the 7-day period according to the typical sigmoidal pattern reported in earlier studies (Wood and Part, 1997; Fletcher *et al.,* 2000). The addition of T_3 to culture media, at concentrations of 10 and 100 ng ml^{-1}, resulted in a dose-dependent decrease in TER evident on all days after day 2. After 7 days in culture, control (0 ng ml^{-1} T_3) epithelia exhibited a typical plateau in TER at 18.2 ± 2.7 kΩ cm^2 (cf. Fletcher *et al.,* 2000; Kelly *et al.,* 2000), whereas the TERs of epithelia treated with 10 and 100 ng ml^{-1} T_3 were 11.0 ± 2.1 and 2.6 ± 0.1 kΩ cm^2, respectively (Fig. 1a). Experimental manipulations were performed following day 7. Under symmetrical culture conditions, the paracellular permeability of epithelia treated with 100 ng ml^{-1} T_3 was significantly greater than that measured across control epithelia (0 ng ml^{-1} T_3), whereas 10 ng ml^{-1} T_3 had no significant effect (Fig. 1b).

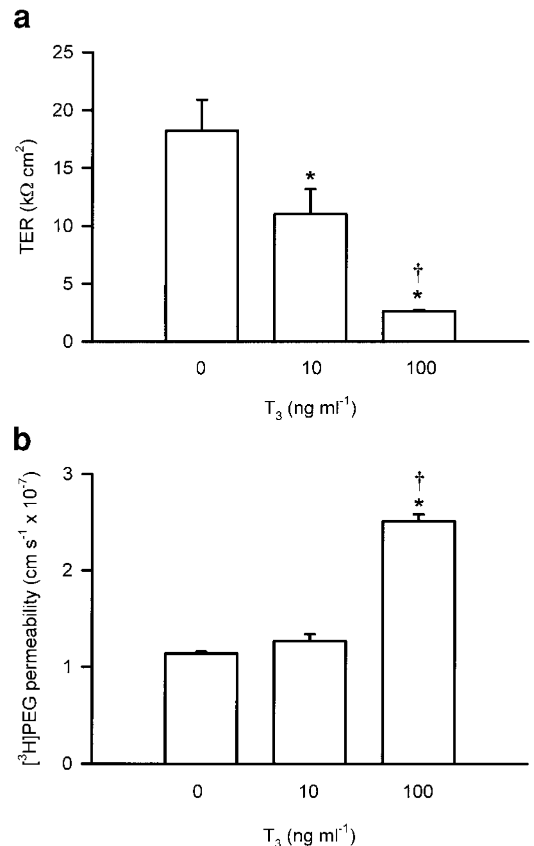

FIG. 1. Effect of 3,5′,3′-triiodo-l-thyronine (T_3) on (a) transepithelial resistance (TER) of cultured pavement cell epithelia after 7 days under symmetrical (L15 apical/L15 basolateral) culture conditions and (b) [^{3}H]PEG permeability under symmetrical culture conditions. Data are expressed as mean values ± SE (n = 3–5). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng ml^{-1} T_3) and T_3-treated epithelia (10 or 100 ng ml^{-1}); a dagger denotes significant difference ($P < 0.05$) between 10 and 100 ng ml^{-1} T_3-treated epithelia.

TER measurements and [^{3}H]PEG permeability under asymmetrical culture conditions. The replacement of medium with freshwater in the apical compartment of the insert resulted in an immediate increase in the TER of all preparations. In control epithelia, resistance increased approximately 1.7-fold, from ≈18 kΩ cm^2 under symmetrical conditions to a peak of ≈31 kΩ cm^2 immediately after the introduction of freshwater (Fig. 2a). In epithelia treated with 10 or 100 ng ml^{-1} T_3, peak resistance values occurred at 1 and 3 h after freshwater exposure, respectively (Fig. 2a). Peak TER values under asymmetrical conditions for preparations treated with 10 or 100 ng ml^{-1} T_3 were

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

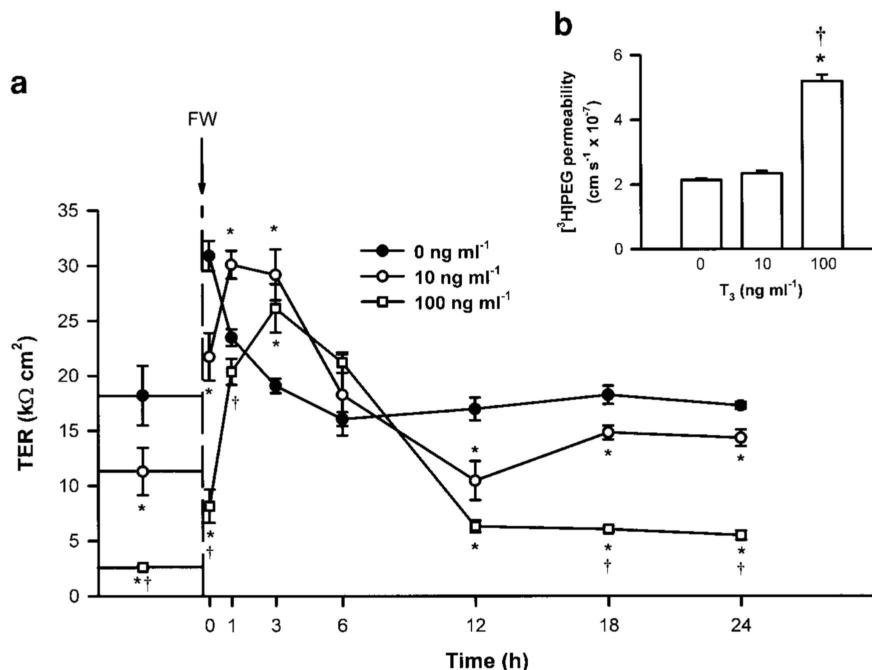

FIG. 2. Effect of 3,5′,3′-triiodo-l-thyronine (T_3) on (a) changes in transepithelial resistance (TER) over time under asymmetrical (FW apical/L15 basolateral) culture conditions and (b) [^{3}H]PEG permeability under asymmetrical culture conditions. Data are expressed as mean values ± SE (n = 3–5). In (a) an asterisk denotes significant difference ($P < 0.05$) between control and T_3-treated epithelia, whereas † denotes significant difference ($P < 0.05$) between 10 and 100 ng ml^{-1} T_3-treated epithelia. In (b) an asterisk denotes significant difference ($P < 0.05$) between control (0 ng ml^{-1} T_3) and T_3-treated epithelia (10 or 100 ng ml^{-1}), a dagger denotes significant difference ($P < 0.05$) between 10 and 100 ng ml^{-1} T_3-treated epithelia.

approximately 2.6- and 10-fold higher than those under symmetrical conditions. After reaching peak resistance, all preparations exhibited a general decline in TER over the first 12 h of asymmetrical exposure and thereafter were relatively stable for the second 12 h (Fig. 2a). After 24 h of asymmetrical conditions, control epithelia continued to exhibit a TER significantly greater than T_3-treated preparations, whereas epithelia treated with a lower dose of 10 ng ml^{-1} T_3 exhibited a significantly greater TER than those treated with 100 ng ml^{-1} T_3. Paracellular permeability was significantly greater across epithelia exposed to asymmetrical conditions than those exposed to symmetrical conditions (Figs. 1b and 2b). Under asymmetrical conditions, the paracellular permeability of epithelia treated with 100 ng ml^{-1} T_3 was again significantly greater than that measured across control epithelia (0 ng ml^{-1} T_3), whereas 10 ng ml^{-1} T_3 had no significant effect (Fig. 2b).

Direct measurement of net Na^+ and Cl^- flux rates under asymmetrical conditions. Net Na^+ and Cl^- flux rates, from the basolateral media to the apical freshwater, were directly measured from samples of apical freshwater taken after 24 h of exposure. Epithelia treated with 10 and 100 ng ml^{-1} T_3 exhibited net Na^+ flux rates of ~164 to ~262 nmol cm^{-2} h^{-1}, respectively. These were significantly greater than control rates of ~127 nmol cm^{-2} h^{-1} (Fig. 3a). Net Cl^- flux rates were found to exhibit a similar pattern in response to varying T_3 levels (Fig. 3b).

Treatment with 3,5′,3′-Triiodo-L-thyronine + Cortisol

Transepithelial resistance measurements under symmetrical and asymmetrical culture conditions. In this series of preparations, TER of epithelia treated with both cortisol and T_3 was significantly lower than the TER measured across preparations treated with cortisol only. After 7 days in culture, the TERs of epithelia treated with cortisol only, 10 ng ml^{-1} T_3 + cortisol, or 100 ng ml^{-1} T_3 + cortisol were 31.3 ± 0.6, 24.0 ± 1.8, and 27.3 ± 1.2 kΩ cm^2, respectively.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

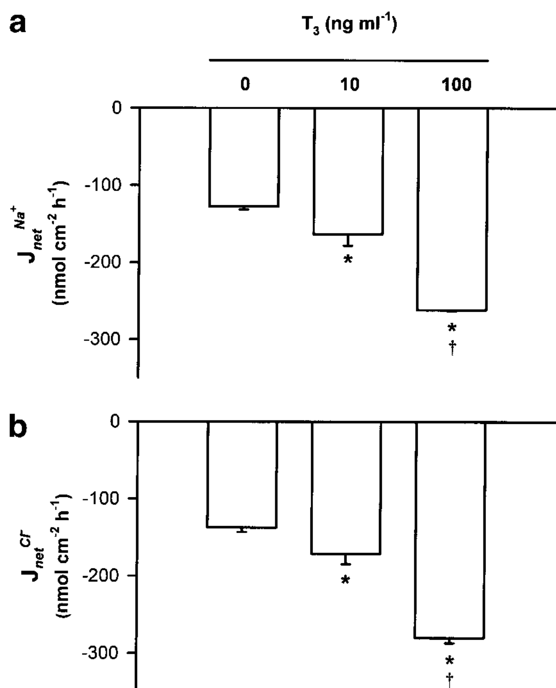

FIG. 3. J_{net} values of (a) Na^+ and (b) Cl^- (i.e. basolateral to apical fluxes) in control (0 ng ml^{-1} T_3) and T_3-treated (10–100 ng ml^{-1}) cultured epithelia exposed to apical FW for 24 h. Data are expressed as mean values ± SE (n = 3–5). An asterisk denotes significant difference ($P < 0.05$) from control values; a dagger denotes significant difference ($P < 0.05$) between 10 and 100 ng ml^{-1} T_3-treated epithelia.

In epithelia treated with either cortisol or T_3 + cortisol, the response to apical FW was similar to that of other preparations (Fig. 4). Initial exposure led to a rapid increase in TER that, in all groups, peaked after 1–3 h. However, during the first 6–12 h of asymmetrical exposure, epithelia treated with 10 ng ml^{-1} T_3 + 500 ng ml^{-1} cortisol exhibited a degree of TER stability that was not observed in other preparations from the same series.

[^{3}H]PEG-4000 permeability. Under both symmetrical and asymmetrical conditions, no significant difference in paracellular permeability was observed between groups treated with either cortisol alone or T_3 + cortisol (Fig. 5). Exposure to asymmetrical conditions did, however, result in significantly elevated paracellular permeability in all groups over a 0- to 12-h flux period and further elevations in PEG permeability were observed during a 12- to 24-h flux (Fig. 5).

Direct measurement of net Na^+ and Cl^- flux rates under asymmetrical conditions. Net Na^+ and Cl^- flux rates, from the basolateral media to the apical freshwater, were directly measured from samples of apical freshwater taken after either 12 or 24 h of exposure. Net Na^+ and Cl^- flux rates were low in epithelia treated either with cortisol alone or with a combined T_3 and cortisol regime (Table 1). In these epithelia, net Na^+ and Cl^- flux rates during the first 12 h of asymmetrical exposure were always significantly lower than flux rates observed during 12–24 h of asymmetrical exposure (Table 1). During this 12- to 24-h period, epithelia treated with lower doses of T_3 (10 ng ml^{-1}) + cortisol generally exhibited lower Na^+ and Cl^- flux rates than those treated with either cortisol alone or higher doses of T_3 (100 ng ml^{-1}) + cortisol (Table 1).

Na^+–K^+-ATPase Activity

The activity of Na^+–K^+-ATPase in epithelia was significantly elevated in preparations treated with T_3 and further elevated by T_3 + cortisol treatment (Fig. 6). Na^+–K^+-ATPase activity in cultured epithelia was not significantly affected by cortisol treatment alone. Furthermore, the elevated enzyme activity was always greater in preparations treated with lower doses of T_3 (10 ng ml^{-1}). This phenomenon could also be observed when inserts were treated with both low doses of T_3 and cortisol combined.

Epithelial Protein Content

The soluble protein content of epithelia was found to vary significantly between groups treated with different hormonal regimes. The highest epithelial protein content was found in inserts treated with T_3 alone and these were significantly greater than either control, cortisol-treated, or T_3 + cortisol-treated preparations. The epithelial protein contents (μg epithelia^{-1}) of the various groups were as follows; control = 84.0 ± 1.6; T_3 (10 ng ml^{-1}) = 110.1 ± 3.8; T_3 (100 ng ml^{-1}) = 99.9 ± 2.4; cortisol (500 ng ml^{-1}) = 76.1 ± 1.8; T_3 + cortisol (10 ng ml^{-1} + 500 ng ml^{-1}) = 75.8 ± 1.3; T_3 + cortisol (100 ng ml^{-1} + 500 ng ml^{-1}) = 76.7 ± 1.9.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

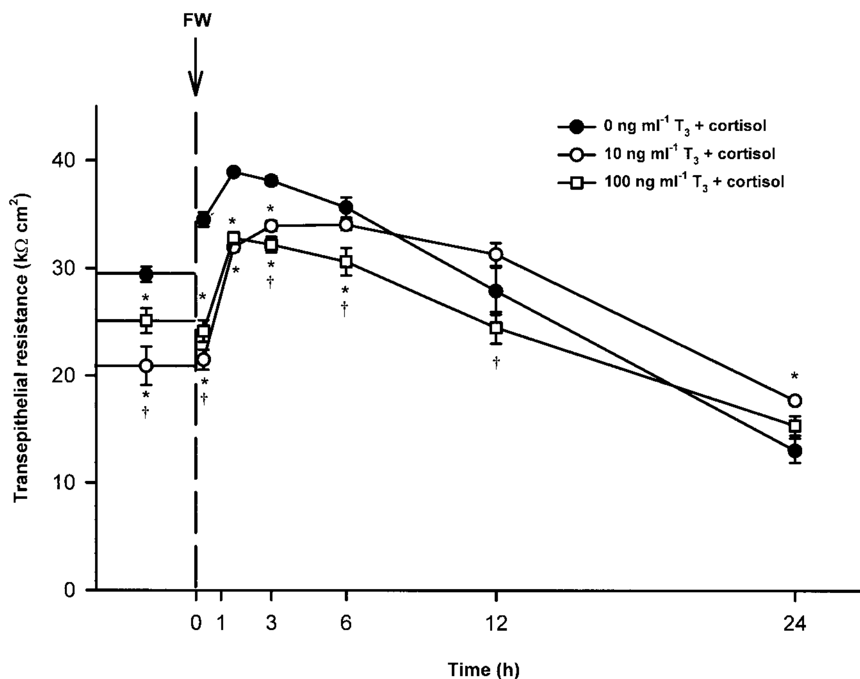

FIG. 4. Effect of either cortisol (500 ng ml^{-1}) or 3,5′,3′-triiodo-l-thyronine (T_3) + cortisol (10 ng ml^{-1} T_3 or 100 ng ml^{-1} T_3 + 500 ng ml^{-1} cortisol) on changes in transepithelial resistance (TER) of cultured branchial epithelia over time under asymmetrical (FW apical/L15 basolateral) culture conditions. Data are expressed as mean values ± SE (n = 6–7). An asterisk denotes significant difference ($P < 0.05$) between control (500 ng ml^{-1} cortisol) and T_3 + cortisol-treated epithelia, a dagger denotes significant difference ($P < 0.05$) between 10 and 100 ng ml^{-1} T_3 + cortisol-treated epithelia.

Symmetrical Unidirectional Na^+ and Cl^- Flux Rates and the Ussing Flux Ratio Criterion

Based on our observations in the above experiments, epithelia used for radiotracer studies were either untreated controls (no hormone treatment), treated with low doses of T_3 (10 ng ml^{-1}), or treated with low doses of T_3 (10 ng ml^{-1}) + cortisol (500 ng ml^{-1}). Under symmetrical conditions all preparations responded to the hormonal treatment in a manner similar to that previously observed. The plateau TERs of control, T_3, and T_3 + cortisol-treated inserts were 14.2 ± 1.2 kΩ cm^2 (n = 13), 9.2 ± 1.7 kΩ cm^2 (n = 6), and 23.5 ± 0.8 kΩ cm^2 (n = 8), respectively. Under control conditions, radioisotopically measured unidirectional Na^+ and Cl^- fluxes revealed approximately equal movement of ions in both directions (Fig. 7). A TEP of +2.77 ± 0.18 mV was observed during the flux period (Table 2). Application of the flux ratio criterion indicated no significant active transport of either Na^+ or Cl^- (Table 2). In T_3-treated preparations, both the influx and the efflux components of Na^+ and Cl^- were

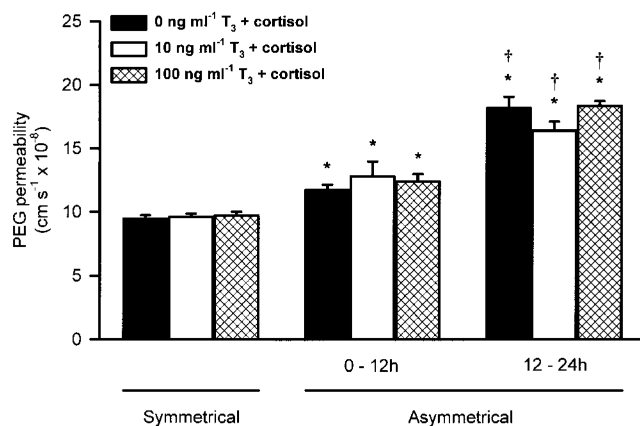

FIG. 5. Changes in [^{3}H]PEG-4000 permeability across cortisol-treated (500 ng ml^{-1} cortisol) and T_3 (10–100 ng ml^{-1}) + cortisol (500 ng ml^{-1})-treated cultured epithelia under both symmetrical (L15 apical/L15 basolateral) and asymmetrical (FW apical/L15 basolateral) culture conditions. Data are expressed as mean values ± SE (n = 6–7). An asterisk denotes significant difference ($P < 0.05$) between culture conditions (symmetrical, asymmetrical); a dagger denotes significant difference ($P < 0.05$) between time 0–12 h and 12–24 h under asymmetrical conditions. No significant difference was detected between any of the hormone treatments ($P > 0.05$).

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

TABLE 1

J_{net} for Na^+ and Cl^- (i.e., Basolateral to Apical Net Fluxes) in Cortisol-Treated (500 ng ml^{-1}) and T_3 (10–100 ng ml^{-1}) + Cortisol (500 ng ml^{-1})-Treated Epithelia over 12–24 h Exposure to Asymmetrical (FW Apical/L15 Basolateral) Culture Conditions

	J_{net} (nmol cm^{-2} h^{-1})			
	Na^+		Cl^-	
	0–12 h	12–24 h	0–12 h	12–24 h
Cortisol (500 ng ml^{-1})	37.0 ± 3.3	126.6 ± 10.4*	62.3 ± 4.1	159.5 ± 11.9*
T_3 + cortisol (10 ng ml^{-1} + 500 ng ml^{-1})	42.4 ± 3.7	99.0 ± 3.6†*	70.2 ± 4.1	129.8 ± 3.9†*
T_3 + cortisol (100 ng ml^{-1} + 500 ng ml^{-1})	58.4 ± 3.9†‡	127.4 ± 8.9‡*	94.6 ± 6.1†‡	147.4 ± 10.8*

Note. Data are expressed as mean values ± SE (n = 6–7).
* Significance between 0–12 h and 12–24 h flux measurements within a hormonal treatment.
† Significantly different from cortisol (500 ng ml^{-1}) within a time period.
‡ Significantly different from T_3 + cortisol (10 ng ml^{-1} + 500 ng ml^{-1}) within a time period.

significantly elevated compared to those of untreated controls (Fig. 7). However, similar to control conditions, TEP was +1.94 ± 0.26 mV and net Na^+ and Cl^- flux were close to zero. Application of the flux ratio criterion again revealed no significant active transport of either Na^+ or Cl^- (Table 2). Under symmetrical conditions, the unidirectional movements of ions across epithelia treated with T_3 + cortisol were significantly lower than those observed in either control or T_3-treated preparations. The movement of Cl^- was again approximately equal in both directions; however, net Na^+ flux was in the outward direction and was significantly greater than that observed in control epithelia (Fig. 7). The TEP across epithelia treated with

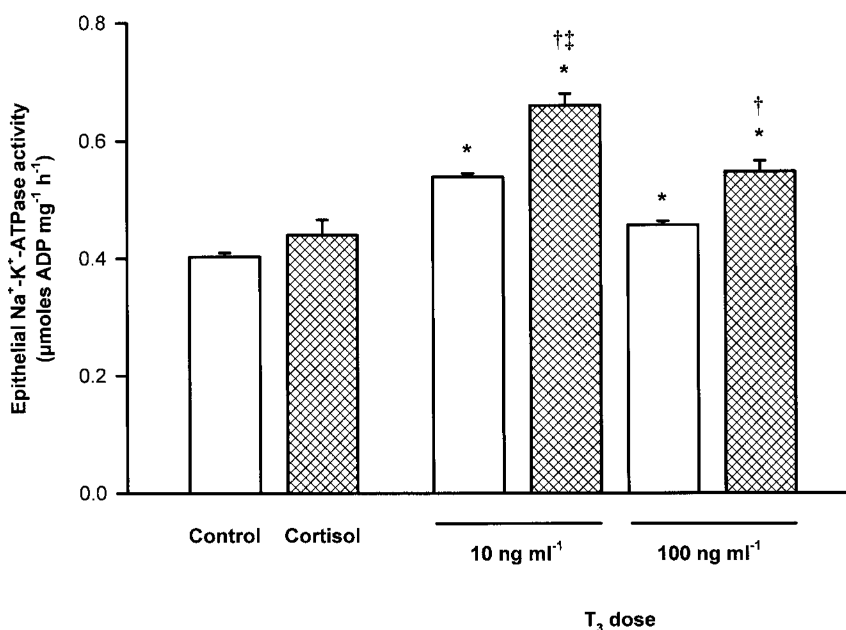

FIG. 6. Na^+–K^+-ATPase activity in cultured pavement cell epithelia after 24 h asymmetrical (FW apical/L15 basolateral) exposure. Data are expressed as mean values ± SE (n = 4–15). An asterisk denotes significant difference ($P < 0.05$) between control (no hormonal treatment) and T_3-treated epithelia or between cortisol control (500 ng ml^{-1} cortisol) and T_3 + cortisol-treated epithelia. Significant difference ($P < 0.05$) between T_3- and T_3 + cortisol-treated epithelia, at a specific dose of T_3 (either 10 or 100 ng ml^{-1} T_3), is denoted by †; ‡ denotes significant difference ($P < 0.05$) between T_3 (10 ng ml^{-1}) + cortisol-treated and T_3 (100 ng ml^{-1}) + cortisol-treated preparations.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

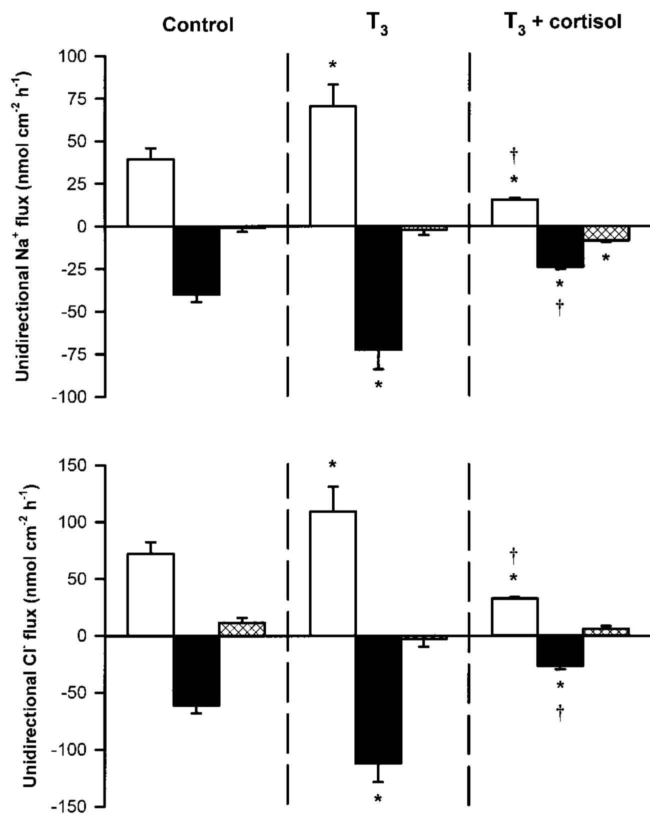

FIG. 7. Unidirectional fluxes of (top) Na^+ and (bottom) Cl^- in control (no hormone treatment), T_3-treated (10 ng ml^{-1}), and T_3 (10 ng ml^{-1}) + cortisol (500 ng ml^{-1})-treated epithelia under symmetrical culture conditions (L15 apical/L15 basolateral). Open, solid, and hatched bars represent ion influx, efflux, and net flux, respectively. Data are expressed as mean values ± SE (n = 6–13). An asterisk denotes significant difference ($P < 0.05$) between control and hormone-treated preparations; a dagger denotes significant difference ($P < 0.05$) between T_3− and T_3 + cortisol-treated preparations.

both T_3 and cortisol was +3.17 ± 0.19 mV and was significantly greater than the TEP measured across T_3-treated epithelia (Table 2). According to the Ussing flux ratio criterion, no active Cl^- transport was apparent; however, active Na^+ transport occurred in the outward direction (basolateral to apical).

Asymmetrical Unidirectional Na^+ and Cl^- Flux Rates and the Ussing Flux Ratio Criterion

Prior to the introduction of apical freshwater (under symmetrical conditions), matched preparations used for asymmetrical radiotracer studies exhibited an average TER of 16.8 ± 1.7 kΩ cm^2 (control, n = 10), 15.9 ± 1.6 kΩ cm^2 (10 ng ml^{-1} T_3, n = 11), and 23.3 ± 1.2 kΩ cm^2 (10 ng ml^{-1} T_3 + 500 ng ml^{-1} cortisol, n = 6). During the 6-h asymmetrical flux period, TER across the same epithelia was significantly elevated in all groups at 23.0 ± 2.0 kΩ cm^2 (control, n = 10), 24.1 ± 1.2 kΩ cm^2(10 ng ml^{-1} T_3, n = 11), and 31.0 ± 0.6 kΩ cm^2 (10 ng ml^{-1} T_3 + 500 ng ml^{-1} cortisol, n = 6). Under asymmetrical conditions, control, T_3−, and T_3 + cortisol-treated epithelia responded similarly. Na^+ and Cl^- efflux rates increased by about 50% relative to efflux rates found under symmetrical conditions, whereas ion influx rates greatly diminished to just a few percent of efflux rates, in approximate proportion to the reduction in apical Na^+ and Cl^- concentrations (Fig. 8). This resulted in net flux rates that were only slightly different from efflux rates. However, the net movement of Na^+ and Cl^- from the basolateral to apical compartment of the insert was significantly lower in T_3 + cortisol-treated epithelia (Fig. 8).

In control, T_3-treated, and T_3 + cortisol-treated epithelia, TEP over the 6-h flux period averaged −11.20 ± 0.79, −11.70 ± 0.54, and −15.60 ± 0.70 mV, respectively, and disagreement between predicted and observed flux ratios indicated active Cl^- uptake and Na^+ extrusion (Table 2). No differences in the pattern of ion movement across epithelia treated with either T_3 or T_3 + cortisol were observed.

DISCUSSION

Thyroid hormone receptors have been found in the gills of several teleost species, including rainbow trout (Bres and Eales, 1988; Lebel and Leloup, 1989); however, only a few studies have specifically investigated the effects of thyroid hormones on gill function (Subash Peter *et al.,* 2000; Dangé, 1986; Omeljaniuk and Eales, 1986; Madsen, 1990) and no previous studies have investigated the potential effects of thyroid hormones on gill pavement cell function. Conditions under which T_3 levels are naturally elevated in fish often involve ionoregulatory disturbance. These include smoltification (McCormick *et al.,* 1987), seawater entry (Redding *et al.,* 1984), and exposure to sublethal stressors (Waring and Brown, 1997). Notably, all of these

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

TABLE 2

Comparison between Ussing Flux Ratios (J_{in}/J_{out}) for Na^+ and Cl^- and Transepithelial Potential (TEP) in Control (No Hormones), T_3-Treated (10 ng ml^{-1}), and T_3 (10 ng ml^{-1}) + Cortisol (500 ng ml^{-1})-Treated Epithelia under Symmetrical (L15 Apical/L15 Basolateral) and Asymmetrical (FW Apical/L15 Basolateral) Culture Conditions

	Control		T_3		T_3 + cortisol	
Symmetrical	Predicted ratio	Observed ratio	Predicted ratio	Observed ratio	Predicted ratio	Observed ratio
Na^+	0.893 ± 0.009	0.934 ± 0.041	0.901 ± 0.014	0.953 ± 0.050	0.867 ± 0.007	0.642 ± 0.021*
Cl^-	1.098 ± 0.009	1.191 ± 0.058	1.072 ± 0.013	0.947 ± 0.062	1.111 ± 0.011	1.303 ± 0.113
	(n = 13)	(n = 13)	(n = 6)	(n = 6)	(n = 8)	(n = 8)
TEP (mV)	+2.77 ± 0.18 (n = 13)		+1.94 ± 0.26† (n = 6)		+3.17 ± 0.19‡ (n = 8)	
Asymmetrical	Predicted ratio (× 10^{-3})	Observed ratio (× 10^{-3})	Predicted ratio (× 10^{-3})	Observed ratio (× 10^{-3})	Predicted ratio (× 10^{-3})	Observed ratio (× 10^{-3})
Na^+	19.55 ± 0.57	8.98 ± 0.55*	17.70 ± 0.96	9.90 ± 0.47*	21.78 ± 0.63	7.68 ± 0.66*
Cl^-	8.47 ± 0.41	17.47 ± 1.33*	8.04 ± 0.24	16.15 ± 1.03*	6.64 ± 0.17	14.29 ± 1.30*
	(n = 10)	(n = 10)	(n = 11)	(n = 11)	(n = 6)	(n = 6)
TEP (mV)	−11.22 ± 0.79 (n = 10)		−11.75 ± 0.54 (n = 11)		−15.61 ± 0.69†‡ (n = 6)	

* Significant difference between predicted and observed flux ratio.
† Significant difference between TEPs of hormone-treated and control inserts within a culture regime (L15/L15 or FW/L15).
‡ Significant difference between TEPs of T_3− and T_3 + cortisol-treated inserts within a culture regime (L15/L15 or FW/L15).

conditions are also likely to result in an elevation in circulating cortisol levels in fish (Redding *et al.*, 1984; Waring and Brown, 1997). Furthermore, evidence suggests that thyroid hormones, either alone or in combination with cortisol, have a positive effect on gill ionoregulatory function *in vivo* (Subash Peter *et al.*, 2000; Dangé, 1986). This is the first study to address the potential effects of thyroid hormones and combined effects of thyroid hormone and cortisol on gill pavement cell function.

After 6–7 days in culture, all untreated control preparations in the present set of experiments exhibited resistance values in the range of 14–18 kΩ cm^2. Although these values were not atypical (see Fletcher *et al.*, 2000; Kelly *et al.*, 2000), variation in TER in different batches of inserts is an inherent trait of this model system (Wood *et al.*, 1998; Fletcher *et al.*, 2000; Kelly *et al.*, 2000). We do not know the proximate causes of these variations, though they appear to be related to season. Therefore, performing experiments within a relatively short time frame normally excludes any problems associated with comparing epithelial response. The same suite of experiments have not yet been employed in epithelia exhibiting lower plateau TER values (e.g. $<$5 kΩ cm^2), which generally occur in warmer seasons. Therefore, it remains to be seen whether T_3 would have the same physiological effects on preparations with lower baseline resistances.

In the current study, the first notable T_3-induced alteration in epithelial status was reduced TER under symmetrical culture conditions. This occurred both in the presence and in the absence of cortisol, a hormone which has previously been demonstrated to increase TER, principally via reduced paracellular permeability, across pavement cell epithelia under identical culture conditions (Kelly and Wood, 2001). In all epithelial preparations, with the exception of high-dose T_3-treated inserts (100 ng ml^{-1} T_3 only), no significant alteration was observed in paracellular permeability in control groups (either no hormone treatment or cortisol treatment alone) and T_3 − or T_3 + cortisol-treated groups, although preparations exposed to cortisol exhibited characteristically lower paracellular permeability. This would suggest that T_3 caused a reduction in the TER of most preparations, including those in which cortisol was present, by increasing the transcellular permeability of epithelia. Evidence demonstrating a T_3-induced increase in the passive permeability of plasma membranes of T_3-responsive cell lines derived from mammalian tissues has previously

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

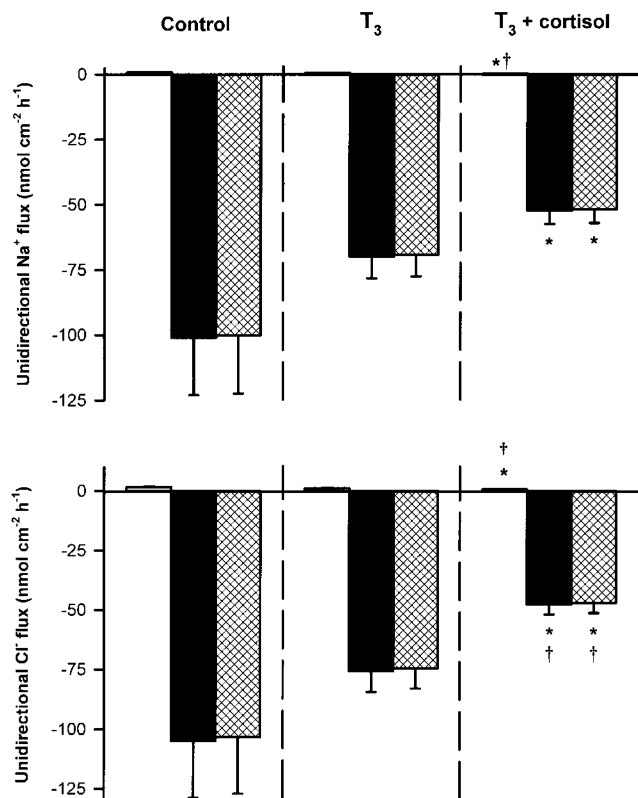

FIG. 8. Unidirectional fluxes of (top) Na^+ and (bottom) Cl^- in control (no hormone treatment), T_3-treated (10 ng ml^{-1}), and T_3 (10 ng ml^{-1}) + cortisol (500 ng ml^{-1})-treated epithelia under asymmetrical culture conditions (FW apical/L15 basolateral). Open, solid, and hatched bars represent ion influx, efflux, and net flux, respectively. Data are expressed as mean values ± SE (n = 6–11). An asterisk denotes significant difference ($P < 0.05$) between control and hormone-treated preparations; a dagger denotes significant difference ($P < 0.05$) between T_3− and T_3+ cortisol-treated preparations.

been reported (Kinsella and Sacktor, 1985; Ismail-Beigi *et al.,* 1986; Haber *et al.,* 1988) and is thought to be related to increased passive Na^+ influx and K^+ efflux. A similar T_3-induced response in the current preparation would be consistent with these observations.

Despite lower symmetrical TER measurements in T_3− and T_3 + cortisol-treated epithelia, the introduction of asymmetrical conditions resulted in elevated TER across all hormone-treated preparations, and in the case of T_3 (10 ng ml^{-1}) + cortisol-treated epithelia, greater stability during the first 12 h of exposure. Recently we have demonstrated that "clamping" the osmotic gradient (at typical plasma osmotic pressure levels) across epithelia exposed to asymmetrical conditions elicits a greater initial increase in TER and sustained TER stability (S. P. Kelly, M. Grosell, H. J. M. Hansen and C. M. Wood, unpublished observations), suggesting that the mechanics of epithelial integrity are compromised by the rapid introduction of low osmotic pressure freshwater on the apical side. This phenomenon may be due to differences in cell volume regulation between osmotically "clamped" epithelia and those exposed to freshwater only. Similarities between the early stage response of T_3− and T_3 + cortisol-treated epithelia under asymmetrical conditions and osmotically clamped preparations are notable, indicating that T_3 treatment may enhance cell volume regulation. In addition, the T_3-induced increase in Na^+–K^+-ATPase activity observed here would likely favor this response as the enzyme's involvement in "housekeeping" and central control of cellular volume are crucial for the maintenance of low intracellular [Na^+] and high intracellular [K^+]. This response is in line with reports on T_3-induced alterations in thyroid-responsive mammalian tissues where thyroid hormones are a major determinant in the steady state activity of Na^+–K^+-ATPase (for reviews see Ismail-Beigi, 1993; Ewart and Klip, 1995). During asymmetrical exposure, paracellular permeability increased in all treatments. This has been demonstrated previously and discussed in detail by Wood *et al.* (1998) and Gilmour *et al.* (1998).

In control epithelia, Na^+–K^+-ATPase activity (≈0.40 μmol ADP mg^{-1} h^{-1}) was ≈50–60% lower than gill activities reported for freshwater-adapted salmonids (McCormick and Bern, 1989). These observations are consistent with the distribution of Na^+–K^+-ATPase activity in freshwater fish gill cells (Sargent *et al.,* 1975; Kültz and Jürss, 1993) and are therefore not unexpected for an epithelial preparation lacking chloride cells. Cortisol alone did not significantly alter the Na^+–K^+-ATPase activity of cultured epithelia, consistent with our previous observations (Kelly and Wood, 2001). However, the current study demonstrated for the first time that T_3 elevates Na^+–K^+-ATPase activity in pavement cells, particularly at lower doses of 10 ng ml^{-1}. Furthermore, treatment of epithelia with T_3 + cortisol further elevated enzyme activity in a manner that, again, seemed dependent on T_3 dose. Generally, the exogenous administration of thyroid hormones alone has not been reported to elevate gill Na^+–K^+-ATPase activity in fish (Dangé, 1986; Omeljaniuk and

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

Eales, 1986; Madsen, 1990). However, in line with our observations, Subash Peter *et al.* (2000) have recently reported that T_3, when used at low doses *in vivo*, stimulates branchial Na^+–K^+-ATPase activity in freshwater tilapia. Furthermore, combined thyroxine and cortisol administration has also been reported to elevate Na^+–K^+-ATPase activity in freshwater tilapia (Dangé, 1986). The significance of the current observations, however, is that elevated Na^+–K^+-ATPase activity did not appear to alter transepithelial ion transport, further emphasizing a possible role in cell volume regulation. This area has been largely overlooked in gill tissues, most likely due to the focus on Na^+–K^+-ATPase as the primary driving force behind ion extrusion or uptake across branchial epithelia.

The movement of Na^+ and Cl^- across untreated epithelia under symmetrical conditions was entirely passive. Wood *et al.* (1998) and Fletcher *et al.* (2000) have previously reported the same situation in cultured fish gill epithelia without hormonal support. In control epithelia exposed to asymmetrical conditions (apical freshwater), the Ussing flux ratio analysis indicated active transport of Cl^- in the inward direction and Na^+ in the outward direction. Again, similar patterns have previously been observed by Wood *et al.* (1998), Fletcher *et al.* (2000), and Kelly and Wood (2001).

In T_3-treated preparations under symmetrical conditions, the movement of Na^+ and Cl^- across epithelia was slightly greater than that observed in controls, which would be expected given the lower TER, hence, greater permeability of these epithelia. However, no disagreement was found between the predicted and the observed flux ratio indicating, again, that Na^+ and Cl^- movement across these epithelia was passive. Under asymmetrical conditions, no significant differences were observed in the pattern of Na^+ and Cl^- movement, although the efflux of both ions tended to be slightly lower.

The treatment of epithelia with T_3 + cortisol under symmetrical culture conditions greatly reduced the movement of Na^+ and Cl^- in both directions across epithelia. However, whereas the movement of Cl^- was approximately balanced in both directions, net Na^+ flux tended toward the outward direction. These trends were reflected in the flux ratio analysis, in which Cl^- movement was found to be passive (no disagreement between the observed and the predicted ratio) and Na^+ movement was found to be active in the outward direction (extrusion, basolateral to apical). This is particularly interesting as we have recently demonstrated that the treatment of cultured pavement cell epithelia with cortisol alone (under symmetrical culture conditions) resulted in the active uptake of Na^+ and Cl^- (Kelly and Wood, 2001). This suggests that the treatment of epithelia with T_3 + cortisol, as opposed to cortisol alone, markedly altered the transport characteristics of the preparation despite similarities in basic electrophysiological characteristics (i.e., both cortisol alone and T_3 + cortisol cause increased TER and TEP relative to controls). The results clearly demonstrate that the actions of a single hormone can be markedly altered by the presence of another. A similar response to T_3 or combined mineralocorticoid–T_3 treatment has been described in toad urinary bladders, in which the mineralocorticoid aldosterone increased net reabsorptive Na^+ flux from the urinary to the extracellular compartment (Rossier *et al.,* 1979). Whereas T_3 treatment alone had no effect on Na^+ movement, the addition of T_3 to aldosterone-treated bladder preparations inhibited the actions of the mineralocorticoid. Furthermore, T_3 has also been reported to have an inhibitory effect on the antinatriuretic actions of aldosterone in adrenalectomized, euthyroid rats (Diezi *et al.,* 1980). Taken together, these reports and the current data suggest a common mechanism by which antagonism between thyroid and mineralocorticoid hormones occurs in divergent animal groups.

Under asymmetrical conditions, Na^+ and Cl^- efflux was reduced in epithelia treated with T_3 + cortisol relative to control and T_3 treatment alone. This is in line with the higher TER measurements and low permeability of these preparations. It is likely that cortisol is the principal agent causing reduced ion movement. Cortisol alone has previously been demonstrated to cause dose-dependent reductions in paracellular permeability and Na^+ and Cl^- efflux, and increases in TER across cultured pavement cell epithelia (Kelly and Wood, 2001), whereas T_3 alone, at lower doses, resulted in reduced TER and unaltered paracellular permeability. Despite these changes, the active transport component of T_3 + cortisol-treated preparations was similar to that of the untreated control, in which we again saw active Cl^- uptake and Na^+ extrusion. Thus, we were unable to sustain our original hypoth-

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

esis that combined T_3 and cortisol treatment would promote active ion uptake under these conditions.

Nevertheless, all epithelia exposed to apical freshwater, regardless of hormone treatment or not, exhibited active Cl^- uptake at least. Currently popular models of ion transport across freshwater fish gills suggest that active Cl^- uptake occurs across chloride cells in association with Cl^-/HCO_3^- exchange (Perry, 1997). Thus, the finding of active chloride uptake across pavement cell epithelia suggests either that current theories on Cl^- transport across fish gills are incomplete or that pavement cells in primary culture exhibit altered transport characteristics. In this regard, the transport characteristics of an analogous cultured epithelium, composed exclusively of pavement cells derived from the sea bass (*Dicentrarchus labrax*) gill (Avella and Ehrenfeld, 1997), are of interest. Sea bass pavement cells in primary culture exhibit active Cl^- extrusion and, as such, contradict popular models of Cl^- movement across the seawater fish gill, which attribute Cl^- movement exclusively to the chloride cells (Marshall, 1995). Therefore, both freshwater and seawater cultured pavement cell epithelia exhibit unexpected transport properties.

In conclusion, cultured pavement cell epithelia derived from freshwater rainbow trout are T_3-responsive tissues. Alterations in the physiology of these preparations after treatment with either T_3 alone or T_3 + cortisol appeared to be in line with the response of cultured cells and/or tissues derived from mammalian or other lower vertebrate sources. The current results suggest that T_3 alone may not promote active transepithelial ion transport across pavement cell epithelia but that T_3 may be important in pavement cell volume regulation via increased cellular permeability and elevated Na^+–K^+-ATPase activity. As conditions during which T_3 naturally elevates are often associated with ionoregulatory disturbance, fish would benefit from enhanced gill volume regulatory function and this is an avenue worthy of further study. The potentiating effects of cortisol (i.e., reduced paracellular permeability and further increased Na^+–K^+-ATPase activity) would undoubtedly emphasize these benefits. The current experiments suggest that the combined actions of T_3 and cortisol do not promote active transepithelial ion transport in cultured pavement cell epithelia under conditions of asymmetrical exposure. However, we have yet to investigate the effects of these hormones on cultured gill cell preparations that contain both pavement cells and mitochondria-rich cells (cf., Fletcher *et al.,* 2000), which will constitute another avenue worthy of study.

ACKNOWLEDGMENTS

This study was supported by an NSERC Research Grant to C.M.W. All procedures conformed to the guidelines of the Canadian Council of Animal Care. We thank Angel Sing for her excellent technical assistance.

REFERENCES

Avella, M., and Ehrenfeld, J. (1997). Fish gill respiratory cells in culture: A model for Cl^- secreting epithelia. *J. Membr. Biol.* **156,** 87–97.

Barton, B. A., and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1,** 3–26.

Björnsson, B. T., Yamauchi, K., Nishioka, R. S., Deftos, L. J., and Bern, H. A. (1987). Effects of hypophysectomy and subsequent hormonal replacement therapy on the hormonal and osmoregulatory status of coho salmon, *Oncorhynchus kisutch. Gen. Comp. Endocrinol.* **68,** 421–430.

Bres, O., and Eales, J. G. (1988). High-affinity, limited capacity triiodothyronine-binding sites in nuclei from various tissues of the rainbow trout (*Salmo gairdneri*). *Gen. Com. Endocrinol.* **69,** 71–79.

Dangé, A. D. (1986). Branchial Na^+–K^+–ATPase activity in freshwater or saltwater acclimated tilapia, *Oreochromis* (*Sarotherodon*) *mossambicus*: Effects of cortisol and thyroxine. *Gen. Comp. Endocrinol.* **62,** 341–343.

Diezi, J., Nenniger, M., and Rossier, B. C. (1980). Inhibition of the anti-natriuretic action of aldosterone by thyroid hormone in the rat. *Pfluegers Arch. Eur. J. Physiol.* **385,** 91–93.

Eales, J. G., and Brown, S. B. (1993). Measurement and regulation of thyroidal status in teleost fish. *Rev. Fish Biol. Fish.* **3,** 299–347.

Ewart, H. S., and Klip, A. (1995). Hormonal regulation of the Na^+-K^+-ATPase: Mechanisms underlying rapid and sustained changes in pump activity. *Am. J. Physiol.* **269,** C295–C311.

Fletcher, M., Kelly, S. P., Pärt, P., O'Donnell, M. J., and Wood, C. M. (2000). Transport properties of cultured branchial epithelia from freshwater rainbow trout: A novel preparation with mitochondria-rich cells. *J. Exp. Biol.* **203,** 1523–1537.

Gilmour, K. M., Pärt, P., Prunet, P., Pisam, M., McDonald, D. G., and Wood, C. M. (1998). Permeability and morphology of a cultured epithelium from the rainbow trout during prolonged apical exposure to freshwater. *J. Exp. Zool.* **281,** 531–545.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

Haber, R. S., Ismail-Beigi, F., and Loeb, J. N. (1988). Time course of Na,K transport and other metabolic responses to thyroid hormone in Clone 9 cells. *Endocrinology* **123,** 238–247.

Ismail-Beigi, F. (1993). Thyroid hormone regulation of Na,K-ATPase expression. *Trend. Endocrinol. Metab.* **4,** 152–155.

Ismail-Beigi, F., Haber, R. S., and Loeb, J. N. (1986). Stimulation of active Na^+ and K^+ transport by thyroid hormone in a rat liver cell line: Role of enhanced Na^+ entry. *Endocrinology* **119,** 2527–2536.

Kelly, S. P., and Wood, C. M. (2001). Effect of cortisol on the physiological properties of a cultured pavement cell epithelium from freshwater rainbow trout gills *Am. J. Physiol.* in press.

Kelly, S. P., Fletcher, M., Pärt, P., and Wood, C. M. (2000). Procedures for the preparation and culture of "reconstructed" rainbow trout branchial epithelia. *Meth. Cell Sci.* **22,** 153–163.

Kinsella, J., and Sacktor, B. (1985). Thyroid hormone increases Na^+-H^+ exchange activity in renal brush border membranes. *Proc. Natl. Acad. Sci. USA* **82,** 3606–3610.

Kirschner, L. B. (1970). The study of NaCl transport in aquatic animals. *Am. Zool.* **10,** 365–375.

Kültz, D., and Jürss, K. (1993). Biochemical characterization of isolated branchial mitochondria-rich cells of *Oreochromis mossambicus* acclimated to fresh water or hyperhaline sea water. *J Comp. Physiol. B* **163,** 406–412.

Leatherland, J. F. (1985). Studies of the correlation between stress-response, osmoregulation and thyroid physiology in rainbow trout, *Salmo gairdnerii* (Richardson). *Comp. Biochem. Physiol.* **80A,** 523–531.

Lebel, J. M., and Leloup, J. (1989). Triiodothyronine binding to putative solubilized nuclear thyroid hormone receptors in liver and gill of the brown trout (*Salmo trutta*) and the European eel (*Anguilla anguilla*). *Gen. Comp. Endocrinol.* **75,** 301–309.

Madsen, S. S. (1990). Effect of repetitive cortisol and thyroxine injections on chloride cell number and Na^+/K^+ -ATPase activity in gills of freshwater acclimated rainbow trout, *Salmo gairdneri. Comp. Biochem. Physiol.* **95A,** 171–175.

Madsen, S. S., and Korsgaard, B. (1989). Time course effects of repetitive estradiol-17β and thyroxine injections on the natural spring molting of Atlantic salmon, *Salmo salar L. J. Fish Biol.* **35,** 119–128.

Marshall, W. S. (1995). Transport processes in isolated teleost epithelia: Opercular epithelium and urinary bladder. *In* "Fish Physiology," Vol 14, "Cellular and Molecular Approaches to Fish Ionic Regulation" (C.M. Wood and T.J. Shuttleworth, Eds.), pp. 1–23. Academic Press, San Diego.

McCormick, S. D. (1995). Hormonal control of gill Na^+ -K^+ -ATPase and chloride cell function. *In* "Fish Physiology," Vol 14, "Cellular and Molecular Approaches to Fish Ionic Regulation" (C. M. Wood and T. J. Shuttleworth, Eds.), pp. 285–315. Academic Press, San Diego.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na^+–K^+-ATPase activity. *Can. J. Fish Aquatic Sci.* **50,** 656–658.

McCormick, S. D., and Bern, H. A. (1989). *In vitro* stimulation of Na^+-K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill. *Am. J. Physiol.* **256,** R707–R715.

McCormick, S. D., Saunders, R. L., Henderson, E. B., and Harmon, P. R. (1987). Photoperiod control of parr–smolt transformation in Atlantic salmon (*Salmo salar*): Changes in salinity tolerance, gill Na^+-K^+-ATPase activity, and plasma thyroid hormones. *Can. J. Fish. Aquat. Sci.* **44,** 1462–1468.

Miwa, S., and Inui, Y. (1985). Effects of l-thyroxine and ovine growth hormone on smoltification of amago salmon (*Oncorhynchus kisutch*). *Gen. Comp. Endocrinol.* **58,** 436–442.

Moav, B., and McKeown, B. A. (1992). Thyroid hormone increases transcription of growth hormone mRNA in rainbow trout pituitary. *Horm. Metab. Res.* **24,** 10–14.

Omeljaniuk, R. J., and Eales, J. G. (1986). The effect of 3,5,3′-triiodo-l-thyronine on gill Na^+/K^+–ATPase of rainbow trout *Salmo gairdneri*, in freshwater. *Comp. Biochem. Physiol.* **84A,** 427–429.

Pärt, P., Norrgren, L., Bergstrom, E., and Sjoberg, P. (1993). Primary cultures of epithelial cells from rainbow trout gills. *J. Exp. Biol.* **175,** 219–232.

Perry, S. F. (1997). The chloride cell: Structure and function in the gills of freshwater fish. *Annu. Rev. Physiol.* **59,** 325–347.

Redding, J. M., Schreck, C. B., Birks, E. K., and Ewing, R. D. (1984). Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch. Gen. Comp. Endocrinol.* **56,** 146–155.

Rossier, B. C., Gäggeler, H-P., Brunner, D. B., Keller, I., and Rossier, M. (1979). Thyroid hormone–aldosterone interaction on Na^+ transport in toad bladder. *Am. J. Physiol.* **236,** C125–C131.

Sargent, J. R., Thomson, A. J., and Bornancin, M. (1975). Activities and localization of succinic dehydrogenase and Na^+/K^+ -activated adenosine triphosphatase in the gills of fresh water and sea water eels (*Anguilla anguilla*). *Comp. Biochem. Physiol.* **51B,** 75–79.

Subash Peter, M. C., Lock, R. A., and Wendelaar Bonga, S. E. (2000). Evidence for an osmoregulatory role of thyroid hormones in freshwater Mozambique tilapia. *Gen. Comp. Endocrinol.* **120,** 157–167.

Waring, C. P., and Brown, J. A. (1997). Plasma and tissue thyroxine and triiodothyronine contents in sublethally stressed, aluminum-exposed brown trout (*Salmo trutta*). *Gen. Comp. Endocrinol.* **106,** 120–126.

Wood, C. M., Gilmour, K. M., and Pärt, P. (1998). Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **119A,** 87–96.

Wood, C. M., and Pärt, P. (1997). Cultured branchial epithelia from freshwater fish gills. *J. Exp. Biol.* **200,** 1047–1059.

Young, G., and Lin, R. (1988). Response of the interrenal to adrenocorticotropic hormone after short-term thyroxine treatment of coho salmon (*Oncorhynchus kisutch*). *J. Exp. Zool.* **245,** 53–58.

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.