In vitro stimulation of Na^+-K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill

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McCORMICK, STEPHEN D., AND HOWARD A. BERN. *In vitro stimulation of Na^+-K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill.* Am. J. Physiol. 256 (Regulatory Integrative Comp. Physiol. 25): R707–R715, 1989.—To investigate the hormonal control of gill Na^+-K^+-adenosinetriphosphatase (ATPase) (the sodium pump) in coho salmon, a technique for the culture of primary gill filaments for up to 4 days was developed. Trypan blue exclusion was >99.9%, histological appearance of the cells was normal, and total [Na^+], [K^+], and protein content of gill filaments cultured for 2–4 days was unchanged from initial levels (measured immediately after isolation). In fish with initially low gill Na^+-K^+-ATPase activity (presmolts), cortisol (0.1, 1.0, and 10.0 μg/ml) caused a significant dose-dependent increase in gill Na^+-K^+-ATPase activity over initial (41%) and control levels (45%) after 4 days in culture. In fish with initially high gill Na^+-K^+-ATPase activity (postsmolts), cortisol partially prevented the decline in activity that occurred during 4 days of culture. The relative ability of steroids to increase gill Na^+-K^+-ATPase activity was dexamethasone > cortisol = 11-deoxycortisol > cortisone. Insulin (0.1, 1.0, and 10.0 μg/ml), alone or in combination with cortisol, had no significant effect on gill Na^+-K^+-ATPase activity. Cortisol treatment significantly increased maximum binding capacity of [^{3}H]ouabain in gill tissue (from 2.92 to 5.22 pmol/mg dry wt) but had no significant effect on the dissociation constant. These results demonstrate that cortisol has direct effects on the osmoregulatory physiology of the teleost gill.

Oncorhynchus kisutch; parr-smolt transformation; osmoregulation; sodium pump; corticoids

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SODIUM-POTASSIUM-STIMULATED adenosinetriphosphatase (Na^+-K^+-ATPase, the sodium pump) is generally considered to be the major active mechanism for generating ionic and electrical gradients in transport epithelia such as the mammalian kidney and the teleost gill. In teleosts, gill Na^+-K^+-ATPase is located on the basolateral membrane of chloride cells (13) and plays a central role in current models of gill ion transport in hyperosmotic environments (31). Gill Na^+-K^+-ATPase activity and ouabain binding increase two- to fivefold after adaptation of most teleosts to seawater (14, 30). In many migratory salmonids, gill Na^+-K^+-ATPase activity and salinity tolerance increase in freshwater during the parr-smolt transformation, in preparation for seawater entry (18, 36).

Several in vivo studies have implicated the major corticosteroid of teleosts, cortisol, in regulating gill Na^+-K^+-ATPase activity. Cortisol treatment can increase gill Na^+-K^+-ATPase activity in hypophysectomized (2, 4, 19, 25) and intact teleosts (6, 10, 28). Growth hormone and prolactin have also been implicated in controlling gill Na^+-K^+-ATPase activity (20, 24, 27, 28). Despite the many studies examining the in vivo actions of cortisol, there is currently no evidence that cortisol has direct (in vitro) effects on any aspect of branchial transport.

To examine the possible direct effect of cortisol on gill Na^+-K^+-ATPase activity and ouabain binding, we have developed an organ culture system for the maintenance of gill tissue for up to 4 days. Naito and Ishikawa (21) have previously reported culture of dispersed gill cells. To our knowledge, long-term culture of intact gill tissue has not previously been reported, and the method used in this study represents a useful technique for endocrinological, biochemical, and physiological studies of the teleost gill.

MATERIALS AND METHODS

Animals. Coho salmon (*Oncorhynchus kisutch*) obtained from Iron Gate Hatchery (California Department of Fish and Game) were reared in 1-m-diam, 800-liter-capacity outdoor tanks in the Life Sciences Building courtyard at the University of California at Berkeley. Water temperatures were maintained between 10 and 13°C throughout the year. Fish were fed Oregon Moist pellet (Moore-Clarke, LaConner, WA) ad libitum twice daily. Presmolt coho salmon (10- to 11-month-old fish, sampled in January and February) weighed 20–30 g; postsmolts (16- to 18-month-old fish, sampled from July to September) weighed 60–100 g.

Gill organ culture. Blood was collected from the caudal vessels with a heparinized syringe, after which the tail was removed at the caudal peduncle resulting in partial exsanguination of the gills. The first gill arch from each side was removed and placed in minimal essential medium (MEM; described below) on ice. Primary filaments were severed just above the septum (approximately one-half their total length of 0.6–1.5 cm) so that they could be separated from one another. In all subsequent steps the primary filaments were moved with suction with the use of a positive displacement pipet, so that any tissue damage was limited to the proximal (severed) end. For each hormone treatment or control, two primary filaments (3–4 when presmolt salmon were used) were placed in 0.5 ml MEM with 500 U/ml penicillin and 250 μg/ml streptomycin in sterile 24-well culture plates. Gill fila-

ments were preincubated in this media at 14°C for 2–4 h with gentle shaking. Preincubation medium was then removed and replaced with 0.5 ml MEM containing 50 U/ml penicillin, 50 μg/ml streptomycin, and hormone or hormone vehicle. Gill filaments were incubated at 14°C with gentle shaking for 1–4 days. After culture, gill filaments were removed from culture wells with suction and either placed in 200 μl ice-cold SEI buffer (0.3 M sucrose, 0.02 M Na_2 EDTA, and 0.05 M imidazole, pH 7.3), frozen immediately on dry ice and stored for up to 10 days at −80°C, or subjected to other treatments as described below.

The culture medium was MEM (GIBCO, Hanks' salts) with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer to which 4 mg/ml bovine serum albumin (Sigma radioimmunoassay grade) and 292 μg/ml L-glutamine were added immediately before use and adjusted to pH 7.55 with NaOH (final osmolarity 298 mosM). Stock solutions of hormones were prepared in the following concentrations and solvents: corticoids (10 mg/ml) in ethanol and insulin (10 mg/ml) in 0.01 N HCl. All solutions, including control and intermediate doses, contained the same amount of hormone solvent as the maximum dose. Ethanol at this concentration had no significant effect on Na^+-K^+-ATPase activity during in vitro exposure.

Validation of gill organ culture. Before routine culture of gill tissue, several parameters were measured to determine the health of tissue after 2–4 days of culture. Gill histology was examined after fixation in Bouin's-Hollande solution and staining with Masson's trichrome. Trypan blue exclusion was measured by placing tissue in salmon Ringer solution (SR; see below) containing 0.05% trypan blue for 5 min followed by two rinses of 500 μl SR. The tissue was then examined under ×50 magnification, and the proportion of the stained gill surface was estimated: (number of respiratory lamellae stained × proportion of lamellar surface stained)/total number of respiratory lamellae. Because of the convoluted nature of gill tissue this estimate must be considered a relative measurement. Trypan blue did not enter the blood space over the short period of exposure.

To measure total [Na^+] and [K^+] content of gill tissue, primary filaments were rinsed quickly (2–3 s) in 5 ml deionized water and then placed in 1 ml 0.1 N HNO_3 for 24 h (14). The solution of extracted ions was then measured for [Na^+] and [K^+] with the use of atomic absorption spectrophotometry, and the filaments were dried to a constant weight at 60°C.

Protein content per unit dry weight was measured by drying filaments to a constant weight at 60°C, followed by homogenization in 60 μl 0.1% Na deoxycholate and measurement of protein content of the homogenate. Protein content per unit DNA was measured by homogenizing filaments in 100 μl 100 mM phosphate buffer followed by measurement of protein and DNA in the homogenate. Homogenates of each preparation were first centrifuged at 1,000 *g* for 1 min to remove nonsoluble (largely cartilaginous) tissue. Protein was measured by the method of Larson et al. (16) and DNA by the method of Hinegardner (11).

Measurement of Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity was measured by a modification of the method of Penefsky and Bruist (23). In this assay, ouabain-sensitive hydrolysis of ATP was coupled through the action of pyruvate kinase (PK) and lactate dehydrogenase (LDH) to the oxidation of NADH, which was measured spectrophotometrically. In this process ATP is regenerated and the accumulation of ADP is equimolar to the oxidation of NADH.

Five to 10 min before assay, tissue was rapidly thawed, removed from SEI buffer, and homogenized in 85 μl SEI buffer with 0.1% Na deoxycholate in a 200 μl-capacity ground glass homogenizer (Wheaton no. 357848). The homogenate was centrifuged at 5,000 *g* for 30 s to remove cartilage; the resultant pellet had no detectable Na^+-K^+-ATPase activity when resuspended and homogenized in SEI buffer with 0.1% Na deoxycholate. The supernatant was removed and assayed for Na^+-K^+-ATPase activity and protein content.

Details of optimization of assay conditions is presented in APPENDIX. Routinely, 15–25 μl of gill homogenate were placed in a final volume of 1 ml assay mixture containing 50 mM imidazole, 1 U/ml LDH, 2.5 U/ml PK, 2 mM phosphoenolpyruvate (PEP), 0.05 mM NADH, 0.5 mM ATP, 0.4 mM KCN, 45 mM NaCl, 2.5 mM $MgCl_2$, and 10 mM KCl (pH 7.5). A duplicate cuvette run simultaneously contained 0.5 mM ouabain. The assay mixture was quickly mixed and the change in absorbance (340 nm) recorded in a Perkin-Elmer recording spectrophotometer. The assay mixture was maintained at 25°C before assay, and the temperature of cuvettes was maintained at this temperature during the assay in a water-jacketed cuvette holder.

The rate of NADH oxidation (equimolar to ATP hydrolysis and ADP production) was calculated from the linear rate between 3 and 9 min after initiation of the reaction. The absolute level of ATP hydrolysis was determined from an experimentally determined ADP standard curve. Protein content of the homogenate was measured by the method of Larson et al. (16) with the use of bovine serum albumin as standard. Na^+-K^+-ATPase was calculated as the difference in ouabain-sensitive and -insensitive ATP hydrolysis and expressed as micromoles ADP per milligrams of protein per hour. The coefficient of variation (standard deviation per mean) of the Na^+-K^+-ATPase assay was 3.3% (5 replicate determinations from the same gill homogenate). The coefficient of variation among filaments from the same animal was 6.8% (homogenates of 5 pairs of primary filaments from the first gill arch).

Measurement of specific ouabain binding. Ouabain-binding experiments were performed by a modification of the method of Hootman and Ernst (12). Ouabain exposures were carried out in SR solution (in mM): 130 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 0.8 $MgSO_4$, 1.0 KH_2PO_4, 10 $NaHCO_3$, and 10 HEPES buffer adjusted to pH 7.6. [^{3}H] ouabain (26.9 Ci/mmol) and [^{14}C]sucrose (500 mCi/mmol) were obtained from New England Nuclear.

All solutions for ouabain exposure contained 0.2 μCi/ml [^{14}C]sucrose that was used as an extracellular space marker. At least four concentrations of ouabain between

4×10^{-8} and 6×10^{-5} M were used for each determination. Concentrations greater than 2×10^{-7} M [^{3}H]ouabain were made by adding appropriate amounts of cold ouabain. For each concentration of [^{3}H]ouabain, a duplicate assay containing saturating cold ouabain (1×10^{-3}M) was also performed. Assays at each level of [^{3}H]ouabain concentration were performed in duplicate or triplicate in a final volume of 0.25 ml.

After 4 days of culture in MEM with and without cortisol, primary filaments were removed and rinsed in SR. They were then minced into ~1-mm^3 pieces to facilitate entry of reagents into extracellular space. SR was then removed and minced gill tissue was incubated in culture wells containing [^{3}H]ouabain and [^{14}C]sucrose at 14°C for 3 h, an amount of time that was experimentally determined to permit equilibrium binding. At the end of this period, gill tissue was removed and placed in 0.5 ml microfuge tubes and centrifuged for 30 s at 4,000 *g*. After removal of the supernatant, the tissue was rinsed twice in SR and a final time in deionized water, followed each time by centrifugation and removal of supernatant. Tissue was then dried to a constant weight at 60°C, weighed, and then placed in a scintillation vial and rehydrated in 200 μl deionized water overnight with constant agitation. One milliliter of Protosol (New England Nuclear) was added and vials were again agitated overnight, followed by addition of 9 ml scintillant. Samples were counted on a Beckman LS 1801 scintillation counter with the use of standard double-isotope techniques. Samples of incubation media were also counted to determine the true level of [^{3}H]ouabain and [^{14}C]-sucrose.

The total binding of ouabain was determined by subtracting the [^{3}H]ouabain in the extracellular space, as determined by [^{14}C]sucrose. Scatchard analysis was performed with the use of an iterative program for nonlinear curve fitting (SCAFIT) as described by Rodbard et al. (29). Statistical analyses described in text and figures were performed with the use of CRISP (CRUNCH, Berkeley CA). Sample sizes are the number of individual fish from which gill tissue was obtained; tissue from every fish was tested at all sampling periods and doses.

RESULTS

Validation of organ culture technique. Immediately after dissection, trypan blue was taken up by ~0.5% (±0.2%) of the surface cells of individual primary gill filaments ($n = 4$). Uptake of the dye was located exclusively at the proximal (severed) end. After 1, 2, and 4 days in culture, there was no trypan blue uptake at the severed end and only occasional uptake throughout the tissue, estimated to be <0.1% of the total surface area ($n = 4$).

Histological appearance of all cell types (mucous, chloride, squamous pavement, and pillar) appeared normal throughout culture as judged by their relative size, appearance and coloration with Masson's trichrome (Fig. 1). There was, however, a change in the three-dimensional structure of the primary filament with time. After 2 days of culture there was a slight diminution of the length of secondary lamellae and a thickening of the area between the proximal end of the secondary lamellae and the cartilage of the primary filament (primary filament width). After 4 days in culture the secondary filaments were approximately one-half their original length and slightly wider (particularly at the base) and there was a further enlargement of the primary filament width.

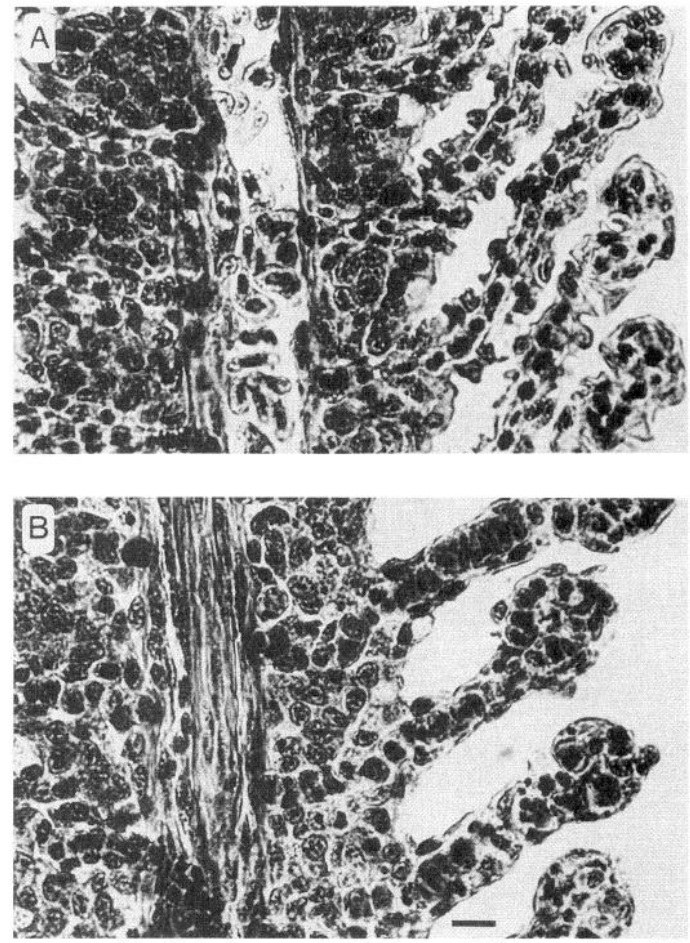

FIG. 1. Histological appearance of primary gill filaments before (*A*) and after (*B*) 2 days of organ culture. Tissue was stained with Masson's trichrome. —, 10 μm.

The total [Na^+] and [K^+] contents of primary gill filaments were measured initially (immediately after dissection) and after 1, 2, and 4 days in culture (Fig. 2). Despite variations in mean [Na^+] of up to 40%, changes in [Na^+] content of gill tissue were not statistically significant [$P = 0.15$, one-way analysis of variance (ANOVA)]. The large variation in [Na^+] content may have been due, in part, to variations in extracellular space. Gill [K^+] content varied only slightly within and among sampling times. There was a slight (13%) but significant ($P < 0.05$) increase in total [K^+] content after 2 days in culture.

Protein content of tissue, expressed on a per microgram DNA or a per microgram dry weight basis, was measured in primary filaments after 2 days in culture in the presence and absence of 10 μg/ml cortisol (Table 1). There was no significant change in gill protein content in culture, nor was there a significant effect of in vitro cortisol treatment ($P > 0.05$, one-way ANOVA).

Effect of cortisol on Na^+-K^+-ATPase activity. Gill tis-

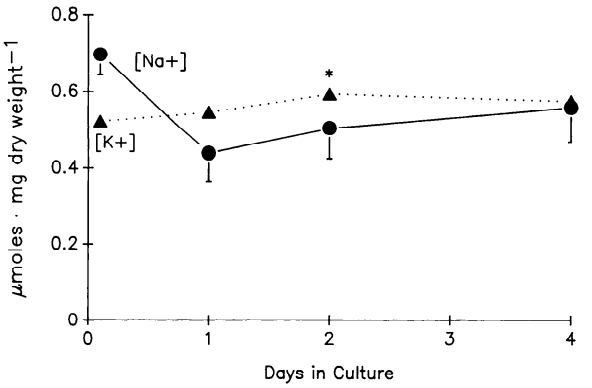

FIG. 2. Ion content of primary gill filaments during 4 days of culture. Values are means ± SE; $n = 5$ at each dose and sampling period. Samples at *time 0* are those taken directly from fish before culture. There was no significant change in [Na^+] over time [$P = 0.15$, one-way analysis of variance (ANOVA)]. * [K^+] content increased slightly (13%) but significantly after 2 days in culture ($P < 0.05$, one-way ANOVA, Student-Newman-Keuls test).

sue from freshwater-adapted, postsmolt coho salmon had initial Na^+-K^+-ATPase activity of 2.76 ± 0.27 μmol ADP·mg protein^{-1}·h^{-1} ($n = 6$) that declined to 0.95 ± 0.03 μmol ADP·mg protein^{-1}·h^{-1} after 4 days in culture, a decrease of 66% (Fig. 3). There was no statistically significant effect of cortisol (0.1, 1, and 10 μg/ml) on gill Na^+-K^+-ATPase activity after 1 day. After 2 and 4 days, cortisol resulted in a significant dose-dependent increase in gill Na^+-K^+-ATPase activity compared with control levels ($P < 0.05$, one-way ANOVA). At the highest dose (10 μg/ml), cortisol increased gill Na^+-K^+-ATPase activity by 28 and 44% over control levels after 2 and 4 days, respectively, in culture. Although gill Na^+-K^+-ATPase activity was lower than initial activity at all time points, cortisol had a partial sustaining effect on Na^+-K^+-ATPase activity in vitro.

Gill tissue from presmolts had initial Na^+-K^+-ATPase activity that was lower than that of postsmolts (1.01 ± 0.11 μmol ADP·mg protein^{-1}·h^{-1}, $n = 6$) and remained constant through 4 days of culture (0.91 ± 0.12 μmol ADP·mg protein^{-1}·h^{-1}; Fig. 4). Exposure to 10 μg/ml cortisol increased Na^+-K^+-ATPase activity to 1.22 ± 0.07 and 1.42 ± 0.12 μmol ADP·mg protein^{-1}·h^{-1} after 2 and 4 days, respectively. The increase in gill Na^+-K^+-ATPase activity occurring after 4 days exposure to 10 μg/ml was a significant increase over both initial (41%) and *day 4* controls (45%; $P < 0.05$, one-way ANOVA, Student-Newman-Keuls test). Intermediate increases (10–24%) in gill Na^+-K^+-ATPase activity occurred at the lower cortisol dose of 1 μg/ml after 2 and 4 days in culture (Fig. 4).

Effect of various corticoids and insulin on Na^+-K^+-ATPase activity. The ability of several corticoids to affect Na^+-K^+-ATPase activity was examined in gill tissue from postsmolts after 2 days exposure at doses of 1 and 10 μg/ml (Fig. 5). At 1 μg/ml, only dexamethasone significantly increased gill Na^+-K^+-ATPase activity above control levels ($P < 0.05$, one-way ANOVA, Student-Newman-Keuls test). At 10 μg/ml, both cortisol and 11-deoxycortisol significantly increased gill Na^+-K^+-ATPase activity ($P < 0.05$). Increasing dexamethasone from 1 to 10 μg/ml did not result in further increase in gill Na^+-K^+-ATPase activity, suggesting that the tissue is not capable of responding to higher doses of corticoids. Cortisone had no significant effect on gill Na^+-K^+-ATPase activity at any dose used. These results indicate that the ability of corticoids to increase gill Na^+-K^+-ATPase activity was dexamethasone > cortisol = 11-deoxycortisol > cortisone.

The effect of in vitro insulin exposure on gill Na^+-K^+-ATPase activity, either alone or in conjunction with cortisol exposure, was examined by exposing gill tissue from postsmolt coho salmon to 0, 0.1, 1, and 10 μg/ml insulin and 0, 1, and 10 μg/ml cortisol for 2 days (Table 2). As in previous experiments, cortisol resulted in a dose-dependent increase in gill Na^+-K^+-ATPase activity that was 20–40% greater than controls ($P < 0.001$, two-way ANOVA). Although there was some indication that insulin may have accentuated the ability of the tissue to respond to cortisol at the highest dose, there was no statistically significant effect of insulin ($P > 0.5$, two-way ANOVA). Because insulin was clearly not necessary for the tissue to respond to hormone treatment, it was not routinely included in the culture medium.

Effect of cortisol on ouabain binding. To determine whether cortisol-induced increases in gill Na^+-K^+-ATPase activity were due to increases in the number of pump sites, ouabain-binding experiments were performed on gill tissue from presmolt coho salmon. After 4 days exposure to 0 or 10 μg/ml cortisol, gill tissue was minced and exposed to [^{3}H]ouabain and [^{14}C]sucrose (the latter used as an extracellular space marker) and allowed to reach equilibrium binding after 3 h.

An example of Scatchard analysis of [^{3}H]ouabain binding in gill tissue from one fish, with and without cortisol exposure, is shown in Fig. 6. Cortisol exposure resulted in an increase in maximum binding capacity (B_{max}) from 2.92 to 5.22 pmol/mg dry weight ($n = 5$, $P = 0.05$, t test; Table 3), whereas the dissociation constant (K_d) was not significantly affected by cortisol treatment ($P = 0.24$; Table 3). The proportional increase in B_{max} (+40%) is nearly identical to the cortisol-induced increase in gill

TABLE 1. *Effect of culture and cortisol treatment on protein content of primary gill filaments*

	Initial	After 2 Days in Culture	
		Control	Cortisol (10 μg/ml)
Micrograms protein/micrograms DNA	24.0±3.8	28.4±0.1	27.0±1.5
Micrograms protein/micrograms dry weight	0.233±0.003	0.257±0.008	0.242±0.007

Values are means ± SE; $n = 4$ for micrograms protein/micrograms DNA and 5 for micrograms protein/micrograms dry weight. There was no significant effect of culture or cortisol treatment on either measure of protein content ($P > 0.05$, one-way analysis of variance).

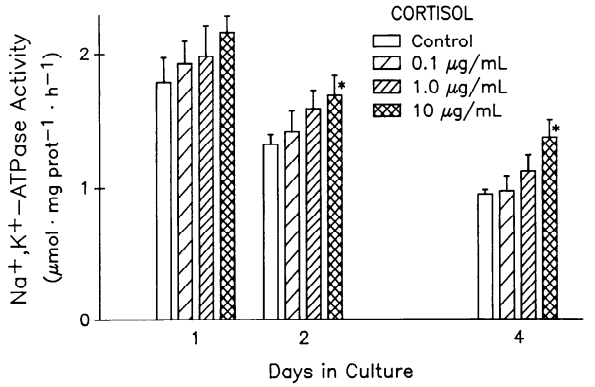

FIG. 3. Effect of in vitro cortisol exposure on Na^+-K^+-ATPase activity of gill tissue from freshwater-adapted postsmolt coho salmon. Values are means ± SE; $n = 6$ at each dose and sampling period. * Significantly different from controls at a given time point ($P < 0.05$, one-way analysis of variance, Student-Newman-Keuls test). Initial (*time 0*) gill Na^+-K^+-ATPase activity was 2.76 ± 0.27 μmol ADP·mg protein^{-1}·h^{-1}.

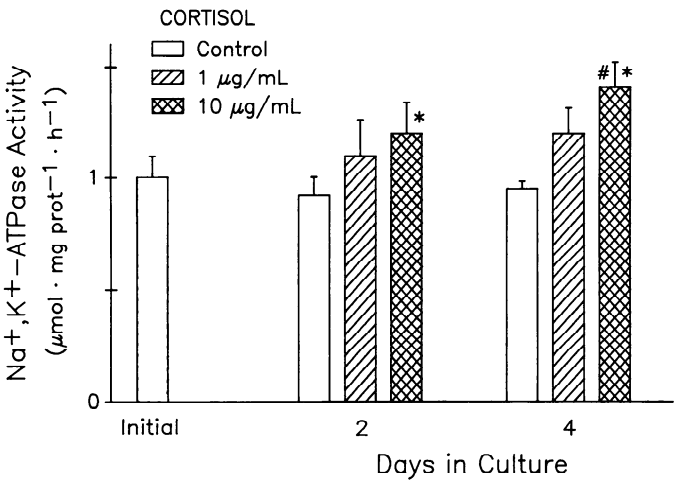

FIG. 4. Effect of in vitro cortisol exposure on Na^+-K^+-ATPase activity of gill tissue from freshwater-adapted presmolt coho salmon. Values are means ± SE; $n = 6$ at each dose and sampling period. # and * significantly different from initial (*time 0*) and control (*day 4*) values, respectively ($P < 0.05$, one-way analysis of variance, Student-Newman-Keuls test).

Na^+-K^+-ATPase activity in presmolts as reported above (45%).

[^{3}H]ouabain binding was also performed on fresh (noncultured) gill tissue from presmolt coho salmon. K_d was 0.34 ± 0.09 μM ($n = 4$), and B_{max} was 4.19 ± 0.93 pmol/mg dry wt.

DISCUSSION

Primary gill filaments placed in organ culture for up to 4 days had a low level of trypan blue uptake, maintained a normal histological appearance, and had relatively constant total [Na^+] and [K^+] content. In addition, protein content was also unchanged during culture and with cortisol exposure. Because measurements of Na^+-K^+-ATPase activity are expressed on a per milligram of protein basis, this latter result is important in establishing that increases in gill Na^+-K^+-ATPase activity are not due to the possible influence of cortisol on protein content of the tissue. Although Na^+-K^+-ATPase was stimulated by cortisol, this stimulation would not be detected by these measurements of total protein content, because Na^+-K^+-ATPase constitutes only a small fraction of the total protein content of the tissue.

Presmolt coho salmon have initially low levels of gill Na^+-K^+-ATPase activity, which increase during the course of the parr-smolt transformation and remain elevated afterward (3, 36, unpublished observations). Gill Na^+-K^+-ATPase activity measured in presmolts in the

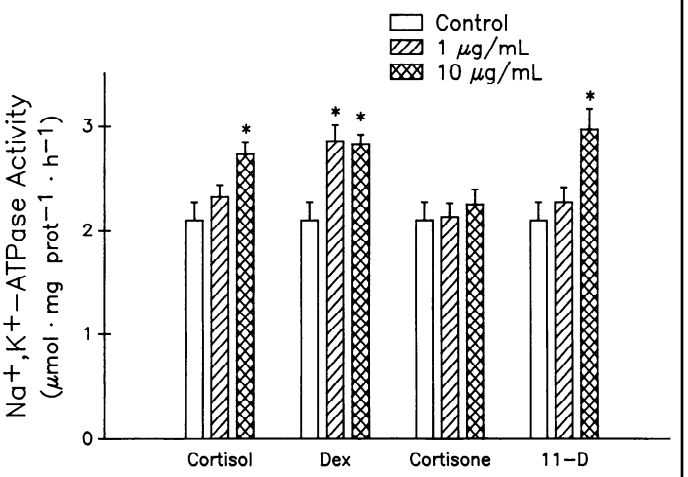

FIG. 5. Effect of in vitro corticoid exposure on Na^+-K^+-ATPase activity of gill tissue from freshwater-adapted postsmolt coho salmon after 2 days in culture. Corticoids used are cortisol, dexamethasone, cortisone, and 11-deoxycortisol. Values are means ± SE; $n = 6$ at each dose. * Significantly different from controls ($P < 0.05$, one-way analysis of variance, Student-Newman-Keuls test). Initial (*time 0*) gill Na^+-K^+-ATPase activity was 3.88 ± 0.25 μmol ADP·mg protein^{-1}·h^{-1}.

TABLE 2. *Effect of in vitro cortisol and insulin exposure on gill Na^+-K^+-ATPase activity*

Insulin, μg/ml	Cortisol, μg/ml		
	0	1.0	10.0
0	1.80±0.18	1.94±0.19	2.18±0.22
0.1	1.68±0.12	1.90±0.15	2.22±0.12
1.0	1.74±0.14	2.16±0.18	2.30±0.35
10.0	1.88±0.18	2.06±0.21	2.42±0.20

Values are means ± SE; $n = 5$ in each group. Exposure was for 2 days. Effect of cortisol was significant [$P < 0.001$, two-way analysis of variance (ANOVA) testing overall effect of cortisol], whereas effect of insulin was not ($P > 0.5$; two-way ANOVA).

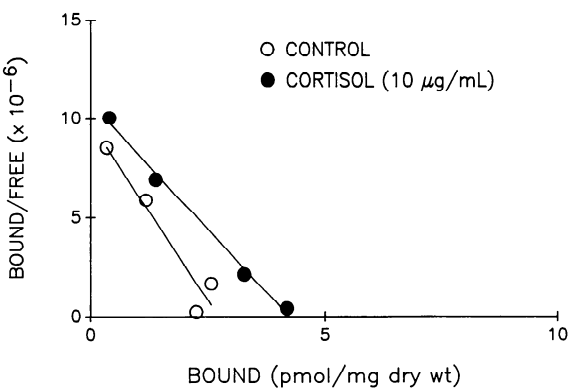

FIG. 6. Scatchard plot of [^{3}H]ouabain binding in gill tissue after in vitro cortisol exposure. Values are from gill tissue from a single individual with and without 4 days of in vitro cortisol exposure.

TABLE 3. *Effect of in vitro cortisol exposure on [^{3}H]ouabain binding in minced gill tissue*

	K_d, μM	B_{max}, pmol/mg dry weight
Control	0.42±0.13	2.92±0.36
Cortisol-exposed, 10 μg/ml	0.63±0.10	5.22±0.47*

Values are means ± SE; n = 5. K_d, dissociation constant; B_{max}, maximum binding capacity. * Significantly different from control levels (P = 0.005, t test).

present study (1.01 μmol ADP·mg protein^{-1}·h^{-1}) is similar in magnitude to gill Na$^+$-K$^+$-ATPase activity in hypophysectomized coho salmon (unpublished observations) and presumably represents a basal or unstimulated level. This basal activity declined by only 10% during gill organ culture, a further indication of the validity of the organ culture methodology.

Postsmolt coho salmon had initially elevated gill Na$^+$-K$^+$-ATPase activity that declined progressively through culture (Fig. 3). After 4 days in culture, gill Na$^+$-K$^+$-ATPase activity had declined by 66% to 0.95 μmol ADP·mg protein^{-1}·h^{-1}, similar to the basal level of presmolt coho salmon. This decline in gill Na$^+$-K$^+$-ATPase activity during culture, not seen in gill tissue with initially low Na$^+$-K$^+$-ATPase activity, is presumably due to the absence of hormones during organ culture.

In gill tissue from both pre- and postsmolt coho salmon, addition of cortisol to the culture media resulted in increases in gill Na$^+$-K$^+$-ATPase activity relative to controls. Increasing cortisol concentration in the medium resulted in increased gill Na$^+$-K$^+$-ATPase activity, indicating a dose-dependent cortisol effect. In gill tissue from postsmolts, cortisol partially prevents the in vitro decline in Na$^+$-K$^+$-ATPase activity. This effect could be interpreted as either a stimulation of gill Na$^+$-K$^+$-ATPase activity by cortisol or a protective action, in which cortisol prevents the degradation of Na$^+$-K$^+$-ATPase in culture. In gill tissue from presmolts, however, it is clear that cortisol acts to stimulate gill Na$^+$-K$^+$-ATPase activity, because exposure to 10 μg/ml cortisol for 4 days results in gill Na$^+$-K$^+$-ATPase activity that is significantly higher (41%) than initial (*day 0*) activity.

The in vitro cortisol concentrations used in the present study (0.1–10 μg/ml) include and surpass the normal circulating levels in this species, which range from 2 to 350 ng/ml (26, 35). It is perhaps not surprising that concentrations of cortisol higher than those present in plasma are necessary to elicit an in vitro response. In the culture method used in the present study, there is no efficient delivery of hormone through the blood that occurs in vivo. Proteins that may facilitate binding of hormone to receptors and increase the efficacy of a given hormone concentration are not present in vitro, although little is known concerning steroid binding proteins in teleosts. Finally, it is possible that the number of steroid receptors decline in culture (9), decreasing the ability of the tissue to respond.

In gill tissue from presmolts, exposure to 10 μg/ml cortisol resulted in a 41% increase in gill Na$^+$-K$^+$-ATPase activity over a 4-day period. If this proportional increase were extended to a 20-day period, Na$^+$-K$^+$-ATPase activity would increase fivefold over initial levels to 5.4 μmol ADP·mg protein^{-1}·h^{-1}. This is similar to the time course and magnitude of changes in gill Na$^+$-K$^+$-ATPase activity that occurs in salmonids during the parr-smolt transformation (18, 36, unpublished observations) and in response to increased environmental salinity (17). Although a time course of changes in gill Na$^+$-K$^+$-ATPase activity in response to in vivo cortisol treatment has not been published, the in vitro changes observed in the present study are consistent with the time required to observe changes in gill Na$^+$-K$^+$-ATPase activity after in vivo cortisol treatment in this species (2, 27, 28).

Analysis of the relative ability of several corticoids to elicit an increase in gill Na$^+$-K$^+$-ATPase activity indicates that the response is highly specific. Dexamethasone had the most potent effect, followed by cortisol, which was equipotent with 11-deoxycortisol. Cortisone did not effect gill Na$^+$-K$^+$-ATPase activity at any of the doses used. This hierarchy of response (dexamethasone > cortisol = 11-deoxycortisol > cortisone) is identical to the relative binding capacity of these corticoids in gill membrane preparations from the brook trout (*Salvelinus fontinalis*; Ref. 5) and American eel (*Anguilla anguilla*; Ref. 7). The correspondence of the relative ability of corticoids to elicit a biological response in gill tissue in vitro and to bind to gill corticoid receptor is expected if induction of gill Na$^+$-K$^+$-ATPase activity is a receptor-mediated event.

Weisbart and McGowan (34) and Patino et al. (22) found that the plasma of Atlantic and coho salmon, respectively, can contain as much as two to four times more cortisone than cortisol. These authors suggested that cortisone may have an as yet unknown physiological function in salmonids. In the present study, the ability of cortisol to stimulate gill Na$^+$-K$^+$-ATPase activity in vitro was at least an order of magnitude greater than that of cortisone. Although cortisone may be the major circulating corticoid in salmonids, our results indicate that cortisol is the probable physiological effector of the adrenal cortex homologue for gill tissue.

Insulin had no direct effect on gill Na$^+$-K$^+$-ATPase activity in the present study, and although there was some trend toward an increasing response to cortisol in the presence of insulin, it was not statistically significant. Insulin did not enhance the ability of in vitro cortisol treatment to increase gill Na$^+$-K$^+$-ATPase activity in tilapia (*Oreochromis mossambicus*; unpublished observations). Doneen and Bern (8) have found that insulin is not necessary for cortisol or prolactin to bring about in vitro changes in water permeability in the urinary bladder of the euryhaline marine teleost *Gillichthys mirabilis*. Although the number of in vitro studies on teleosts is limited, these results contrast with many studies on mammalian tissue in which insulin has been found to be a necessary component of culture media to elicit hormonal responses (33). There may be a difference between the in vitro requirements of organs from homeothermic and poikilothermic vertebrates.

The values for K_d and B_{max} of Na$^+$-K$^+$-ATPase in gill

tissue in the present study are in the range of those reported for [^{3}H]ouabain binding by mammalian and avian transport epithelia (12). Crude homogenates of gill tissue from the mullet, *Mugil cephalus*, have [^{3}H]-ouabain binding of 2–20 pmol/mg dry weight, which increased with increasing salinity (13a). Stagg and Shuttleworth (32) reported [^{3}H]ouabain binding of 14.6 pmol/mg dry weight in the perfused gill of freshwater-adapted flounder, *Platichthys flesus*, which like Na^+-K^+-ATPase activity was unaffected by salinity adaptation in this species. Sargent and Thomson (30) found that seawater adaptation increased gill Na^+-K^+-ATPase activity and [^{3}H]ouabain binding to the same extent (3.1- and 3.2-fold, respectively) in gill plasma membrane preparations from the eel (*Anguilla anguilla*). In the present study, in vitro cortisol exposure increased B_{max} of [^{3}H]ouabain and gill Na^+-K^+-ATPase activity to the same extent (40 and 45% after exposure to 10 μg/ml cortisol for 4 days). These results suggest that, as in seawater adaptation, in vitro cortisol exposure increases the number of sodium pump sites, or in enzymatic terms, the number of Na^+-K^+-ATPase molecules.

Several lines of evidence from the present study are consistent with a genomic action of cortisol in its effect on Na^+-K^+-ATPase: *1*) the time course of action (>1 day) is sufficient for the synthesis of new protein; *2*) the highly specific nature of the corticoid response is indicative of receptor mediation, which for steroids usually (although not always) results in a response at the genomic level; *3*) Na^+-K^+-ATPase activity is increased above initial levels (in the case of presmolts); and *4*) the number of ouabain binding sites (indicative of the total number of pump sites) is increased through cortisol exposure. Although some of these observations (particularly the latter two) could be explained by the unmasking or insertion of previously manufactured sodium pump sites through the action of cortisol, an increase in the number of pump sites through protein synthesis offers the best explanation of all of these observations. Experiments using inhibitors of transcription and translation would more directly address this issue.

In the present study, we are unable to determine whether cortisol-induced increases in gill Na^+-K^+-ATPase occur only in chloride cells or occur in all cell types of the gill, suggesting a general action of cortisol on branchial cellular physiology. However, judging from the localization of ouabain binding in teleost gill tissue primarily to chloride cells (13), the former seems most likely. In addition, in vivo cortisol treatment appears to increase the number of chloride cells in coho salmon (28). We are currently investigating whether morphological changes in chloride cells after seawater adaptation (such as increased number of mitochondria, proliferation of the tubular membrane system, and development of an apical crypt) can be induced by in vitro cortisol treatment.

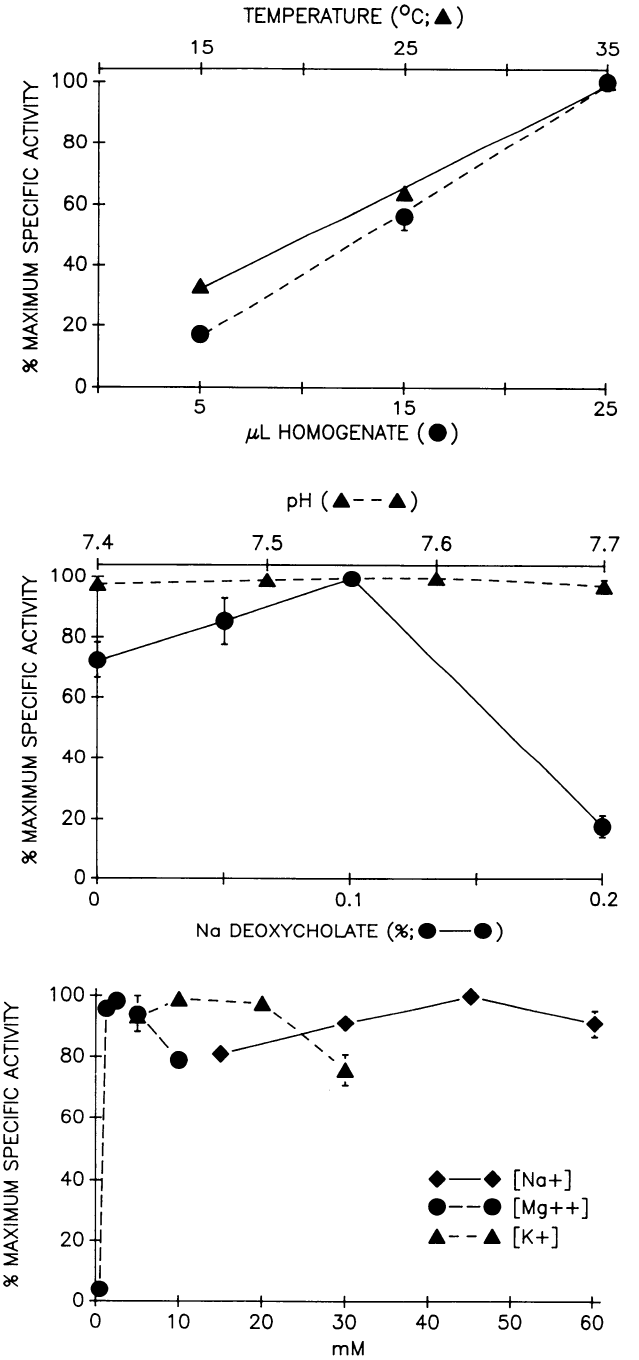

FIG. 7. Results of optimization procedures on gill Na^+-K^+-ATPase activity. Effect of ion concentration (*bottom*), pH and detergent concentration (*middle*), and temperature and amount of homogenate (*top*). Values are means ± SE of gill homogenates from at least 3 fish. During alteration of concentration of a given ion (*bottom*), concentrations of other 2 ions are kept constant ([Na^+], [K^+], and [Mg^{2+}] maintained at 45, 10, and 2.5 mM, respectively).

APPENDIX

Measurement of enzyme catalytic activity is method dependent and it is therefore important that measurements be carried out under well-defined conditions (1). In addition, the possibility that factors other than the enzyme concentration are affecting measurements of catalytic activity can be minimized by ensuring that an assay is performed under optimal conditions. These include substrate availability, cofactor and ion requirements, and homogenization techniques, including detergent solubilization. In this APPENDIX we report the results of several experiments designed to optimize the conditions for measurement of gill Na^+-K^+-ATPase activity by the method described in the accompanying paper.

Ion and substrate availability and pH. Unless otherwise

stated, activity measurements were performed on gill tissue from freshwater-adapted postsmolt coho salmon homogenized in SEI buffer with 0.1% Na deoxycholate as previously described. Preparations from four fish were examined for the effect of varying ion concentration between 0.5 and 10 mM [Mg^{2+}], 5 and 30 mM [K^+], and 15 and 60 mM [Na^+] (Fig. 7). Preliminary samples were run to determine the probable optimum concentration for a given ion, then the level of this ion was maintained constant while other ion concentrations were varied. Maximum Na^+-K^+-ATPase activity was achieved with 2.5 mM [Mg^{2+}], 10 mM [K^+], and 45 mM [Na^+]. Alterations of ion concentrations in this range had no effect on the measurement of ATP hydrolysis as judged by absorbance changes after addition of a known amount of ADP.

The concentration of each substrate (ATP, PEP, NADH), enzyme (LDH, PK), and ouabain were individually determined to be at saturating levels by doubling the normal assay concentration in a duplicate cuvette (while maintaining each of the other assay components at routine assay concentrations). There was no significant effect of increasing any substrate or inhibitor in this manner ($n = 3$).

With the use of the routine assay mixture of 2.5 mM [Mg^{2+}], 10 mM [K^+], and 45 mM [Na^+], homogenates from three fish were assayed for Na^+-K^+-ATPase activity at pH 7.4, 7.5, 7.6, and 7.7. Below pH 7.4 there is a decreased absorbance change following addition of a known quantity of ADP, presumably through an effect on LDH or PK (23). Catalytic activity of homogenates was only slightly affected by pH in the tested range, decreasing a maximum of 3% at pH 7.7. Maximum activity occurred at pH 7.5 and 7.6; pH 7.5 was used in the routine assay.

Effect of freezing and detergent solubilization. Duplicate samples of gill tissue from the same animal were either stored on ice in SEI buffer for 1 h or frozen on dry ice and stored for 1 wk. The samples were then homogenized in SEI buffer with 0.1% Na deoxycholate and assayed for Na^+-K^+-ATPase activity with the use of the routine assay procedure. Unfrozen samples were 27% lower ($P < 0.001$, one-way ANOVA) than those that had been frozen ($n = 3$).

Four samples of gill tissue from each of three fish were homogenized in SEI buffer with 0, 0.05, 0.1, or 0.2% Na deoxycholate. Catalytic activity in the routine assay mixture increased with increasing Na-deoxycholate concentration up to 0.1% and was dramatically reduced at 0.2% (Fig. 7). Na-deoxycholate was routinely used at the 0.1% level.

Effect of temperature and enzyme concentration. Gill tissue from three fish was homogenized in SEI buffer with 0.1% Na-deoxycholate and assayed at 15, 25, and 35°C. Assay mixture and cuvettes were preheated or precooled to the appropriate temperature before the addition of homogenate, and temperature was maintained constant during the assay with a water-jacketed cuvette holder. Catalytic activity increased with increasing temperature and was linear in the temperature range tested (Fig. 7).

Enzyme concentration was increased by increasing the amount of homogenate added to cuvettes (5, 15, and 25 μl). Homogenates of gill tissue from three fish had a protein content of 500–800 ng protein/μl homogenate (approximately double that used in routine assays in which 25 μl homogenate were added to cuvettes). Increasing amounts of homogenate resulted in a linear increase in catalytic activity in the range tested (Fig. 7). These results indicate that the assay is linear over a wider range of enzyme concentrations than was used in the present study and that substrates are not limiting in the assay.

The authors thank Brian Williams for skillful technical assistance, Dr. Katsuya Haya and Brenda Waiwood for advice on Na^+-K^+-ATPase activity measurements, and Drs. Graham Young and Richard Nishioka for help on various aspects of the study. Drs. Graham Young and Jeffery R. Demarest made many helpful comments in review of the manuscript. Dr. Seth R. Hootman made available an unpublished manuscript concerning [^{3}H]ouabain binding methods.

This work is a result of research sponsored in part by NOAA, National Sea Grant College Program, Department of Commerce, under grant number NA80AA-D-00120 through the California Sea Grant College Program, in part by the California State Resources Agency project number R/F-117, and in part by National Science Foundation Grant PCM 84-0549. The U.S. Government is authorized to reproduce and distribute for governmental purposes. S. D. McCormick is a National Research Service Award/National Institutes of Health postdoctoral fellow.

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Received 28 January 1988; accepted in final form 20 October 1988.

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