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Rapid Activation of Gill Na^+,K^+-ATPase in the Euryhaline Teleost *Fundulus heteroclitus*

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ABSTRACT The rapid activation of gill Na^+,K^+-ATPase was analyzed in the mummichog (*Fundulus heteroclitus*) and Atlantic salmon (*Salmo salar*) transferred from low salinity (0.1 ppt) to high salinity (25–35 ppt). In parr and presmolt, *Salmo salar* gill Na^+,K^+-ATPase activity started to increase 3 days after transfer. Exposure of *Fundulus heteroclitus* to 35 ppt seawater (SW) induced a rise in gill Na^+,K^+-ATPase activity 3 hr after transfer. After 12 hr, the values dropped to initial levels but showed a second significant increase 3 days after transfer. The absence of detergent in the enzyme assay resulted in lower values of gill Na^+,K^+-ATPase, and the rapid increase after transfer to SW was not observed. Na^+,K^+-ATPase activity of gill filaments in vitro for 3 hr increased proportionally to the osmolality of the culture medium (600 mosm/kg > 500 mosm/kg > 300 mosm/kg). Osmolality of 800 mosm/kg resulted in lower gill Na^+,K^+-ATPase activity relative to 600 mosm/kg. Increasing medium osmolality to 600 mosm/kg with mannitol also increased gill Na^+,K^+-ATPase. Cycloheximide inhibited the increase in gill Na^+,K^+-ATPase activity observed in hyperosmotic medium in a dose-dependent manner (10^{-4} M > 10^{-5} M > 10^{-6} M). Actinomycin D or bumetanide in the culture (doses of 10^{-4} M, 10^{-5} M, and 10^{-6} M) did not affect gill Na^+,K^+-ATPase. Injection of fish with actinomycin D prior to gill organ culture, however, prevented the increase in gill Na^+,K^+-ATPase activity in hyperosmotic media. The results show a very rapid and transitory increase in gill Na^+,K^+-ATPase activity in the first hours after the transfer of *Fundulus heteroclitus* to SW that is dependent on translational and transcriptional processes. *J. Exp. Zool. 287:263–274, 2000.*

Acclimation to changing environmental salinity requires preexisting mechanisms and the ability to respond to changing conditions. Activation of gill chloride cells and regulation of gill Na^+,K^+-ATPase are very important for the acclimation of fish from freshwater (FW) to seawater (SW) (Epstein et al., '67; De Renzis and Bornancin, '84; Zadunaisky, '84; Karnaky, '86). In numerous euryhaline species, an increase in gill Na^+,K^+-ATPase activity occurs 2–3 days after transfer from hypoosmotic to hyperosmotic conditions (*Anguilla rostrata*: Forrest et al., '73; *Anguilla anguilla*: Bornancin and De Renzis, '72; *Dormitator maculatus*: Evans and Mallery, '75; *Fundulus heteroclitus*: Jacob and Taylor,'83). Anadromous species show a similar if somewhat delayed pattern of gill Na^+,K^+-ATPase activation, with increases at 3–7 days after transfer to SW (*Oncorhynchus kisutch*: Boeuf et al., '78; *Salvelinus fontinalis*: McCormick and Naiman, '85; *Salmo gairdneri*: Madsen and Naamansen, '89; *Salmo salar*: Berge et al., '95; *Dicentrarchus labrax*: Jensen et al., '98).

There are, however, several instances where more rapid activation of gill Na^+,K^+-ATPase have been reported. Using a gill microsome preparation, Towle et al. ('77) found a rapid increase in enzyme activity during the first 30 min after transfer of *Fundulus heteroclitus* from FW to SW. However, in the same species Marshall et al. ('99) did not observe any change in gill Na^+,K^+-ATPase activity following exposure to SW. In *Mugil cephalus*, transfer from 10 to 45 ppt induced an increase in Na^+,K^+-ATPase content, as measured by ouabain binding, 3 hr after transfer (Hossler, '80). In tilapia, *Oreochromis mossambicus*, direct transfer from FW to SW does not induce rapid changes in gill Na^+,K^+-ATPase. However, when the

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fish were acclimated to 20 ppt for 24 hr and then transferred to 30 ppt, gill Na^+,K^+-ATPase increased 3 hr after transfer (Hwang et al., '89).

The physiological significance and cellular mechanism for this rapid increase in Na^+,K^+-ATPase activity in the gill chloride cell is not clear. Short-term activation may involve phosphorylation of the sodium pump catalytic subunit or changes in the subcellular distribution of pump units without reflecting changes in its biosynthetic rate (Berterello and Katz, '93; Ewart and Klip, '95). The general model for salt secretion in the gill and opercular chloride cells in fish and the shark rectal gland postulates a bumetanide-sensitive $Na^+/K^+/Cl^-$ on the basolateral membrane (Silva et al., '77; for review see Wood and Shuttleworth, '95, Marshall and Bryson, '98). Activation of the $Na^+/K^+/Cl^-$ cotransporter is necessary for chloride secretion, leading to an increase in intracellular Na^+ concentration, which would activate Na^+,K^+-ATPase in chloride cells. In dogfish (*Scyliorhinus canicula*) rectal gland epithelium culture, a rapid and transient cycloheximide-sensitive rise in Na^+,K^+-ATPase activity has been observed following increased medium osmolality (Cutler et al., '96). The use of actinomycin D, an inhibitor of RNA transcription, and cycloheximide, an RNA translation inhibitor, have shown that the rapid activation of Na^+,K^+-ATPase activity is dependent on transcription and translation, although this depends on the biological model used (Verrey et al., '82; Doucet et al., '86).

Fundulus heteroclitus is an estuarine euryhaline fish with the capacity to live in a wide range of environmental salinities. In its intertidal habitat the fish is subjected to large and often rapid changes in environmental salinity. In part due to the existence of chloride cells on the opercular membrane, *Fundulus heteroclitus* has been widely used as a model to study structure and function of chloride cells and activation of gill Na^+,K^+-ATPase (Zadunaisky, '84; Karnaky, '86; Wood and Marshall, '94; Marshall et al., '97, '99; Mancera and McCormick, '98). The aim of this study was to analyze the potential mechanisms of rapid activation of gill Na^+,K^+-ATPase in the euryhaline teleost *Fundulus heteroclitus* transferred from low-salinity water (LSW; 0.1 ppt salinity) to SW. For comparison, the pattern of gill Na^+,K^+-ATPase activation was analyzed in an anadromous fish, the Atlantic salmon (*Salmo salar*). Rapid activation in vitro was examined in *Fundulus heteroclitus* using different osmolalities in gill organ culture. The influence of several inhibitors (actinomycin D, cycloheximide, and bumetanide) was also analyzed to determine the mechanisms involved in rapid activation of gill Na^+,K^+-ATPase.

MATERIALS AND METHODS

Experimental animals

Fundulus heteroclitus (2–4 g body weight) were collected in the Connecticut River estuary and transferred to the Conte Anadromous Fish Research Center, Turners Falls, MA. Fish were acclimated for at least 2 weeks to LSW (Instant Ocean, 0.1 ppt salinity) under simulated natural photoperiod and constant temperature (15°C). They were maintained in a 60-litre aquarium, and 50% of the water was changed every 3 days. Fish were fed daily with commercial fish food (Tetramix, Tetrawerke, Germany). They were fasted for 24 hr prior to and throughout the experiment. Experiments in vivo and in vitro were conducted between April and June and September and November of 1995 and 1996.

Juvenile Atlantic salmon (*Salmo salar*) were obtained from the White River National Fish Hatchery (Bethel, VT) and brought to the Conte Anadromous Fish Research Center. Fish were maintained under simulated natural photoperiod in circular 1,000-litre tanks with filtered (particle and activated charcoal filtration) recirculating chilled (10°C) FW and continuous aeration. They were fasted for 24 hr prior to and throughout the experiment. Experiments were conducted between April and June (smolts) and September and November (parr) of 1995.

Experiments in vivo

Experiment 1

Fundulus heteroclitus acclimated to LSW were transferred to SW (35 ppt) or LSW and sampled at different times. The size of these fish did not allow us to obtain sufficient plasma for determination of osmolality and/or ion concentrations during the transfer.

Experiment 1A

Fish were saple at 0 hr (LSW-acclimated fish), 3 hr, 6 hr, 12 hr, 24 hr, 3 days, and 7 days after transfer to SW.

Experiment 1B

Fish were sampled at 0 hr (LSW-acclimated fish), 1 hr, 2 hr, 3 hr, and 6 hr after transfer to SW.

Experiment 1C

Fish were acclimated to LSW and transferred to LSW and sampled at 3 hr and 6 hr after transfer. This experiment was performed for testing the influence of handling and transfer stress on gill Na^+,K^+-ATPase activity.

Experiment 2

Salmo salar acclimated to FW were transferred to increased salinity and sampled at 0 hr (FW-acclimated fish), 3 hr, 6 hr, 12 hr, 1 day, 3 days, 7 days, and 14 days after transfer. Two different experiments were performed.

Experiment 2A

Salmon parr were transferred to SW (25 ppt). Reduced salinity was used in this experiment because of the lower salinity tolerance of parr.

Experiment 2B

Salmon smolts were transferred to SW (30 ppt). Fish were anesthetized (100 mg litre^{-1} MS-222, pH 7.0), weighed, and a gill biopsy removed and placed in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at –80°C. Sample size was 6–7 at each time point.

Experiments in vitro

Experiment 3

To examine the influence of osmolality changes on gill Na^+,K^+-ATPase, gill filaments were cultured in media with 300 mosm/kg, 500 mosm/kg, 600 mosm/kg, or 800 mosm/kg. Samples were taken at 0 hr, 3 hr, and 6 hr of culture.

Experiment 4

To examine the time course of changes in gill Na^+,K^+-ATPase in vitro in response to hyperosmotic conditions, gill filaments were cultured in isoosmotic (300 mosm/kg) and hyperosmotic (600 mosm/kg) media. Samples were taken at 0 hr, 1 hr, 2 hr, 3 hr, and 6 hr of culture.

Experiment 5

To examine the influence of salt vs. osmolality, gill filaments were cultured in media with 300 mosm/kg, 600 mosm/kg by addition of NaCl, and 600 mosm/kg by addition of mannitol. Samples were taken at 3 hr of culture.

Experiment 6

Gill culture in hyperosmotic medium (600 mosm/kg) containing cyclohexamide (Sigma C-0934) and bumetanide (Sigma B-3023) at doses of 10^{-4} M, 10^{-5} M, and 10^{-6} M (Musch et al., '82; Doucet et al., '86).

Experiment 7

Gill culture in hyperosmotic medium (600 mosm/kg) containing actinomycin D at doses of 10^{-4} M, 10^{-5} M, and 10^{-6} M (Doucet et al., '86). The antibiotic was dissolved in preincubation and incubation media to the working doses.

Experiment 8

Gill culture in isoosmotic (300 mosm/kg) and hyperosmotic (600 mosm/kg) media using gill filaments of LSW-adapted fish injected with a single dose of actinomycin D (0.75 µg g^{-1} body weight, Sigma A-1410) 3 days before gill culture. Fish received intraperitoneal injections of 10 µl g^{-1} body weight of actinomycin D in saline solution. A dose of 1 µg g^{-1} body weight induced a significant decrease in total body Na^+ turnover and an increase in total body Na^+ in *Fundulus kansae* (Nichols and Fleming, '90). In a preliminary experiment we observed that a dose of 1 µg/g body weight was lethal in 2 days for *Fundulus heteroclitus* in LSW. At 0.75 µg g^{-1} body weight, fish survived for at least 5 days.

Gill organ culture

The method of McCormick and Bern ('89) was used to culture primary gill filaments. Briefly, fish were anesthetized, bled, and gill arches removed. Primary filaments were severed above the septum and 4–6 filaments were placed in individual wells with preincubation medium in a 24-well microplate. After 0.5–1 hr the preincubation medium was replaced with incubation medium. In experiments using cycloheximide and actinomycin D, these drugs were present in both the preincubation and incubation media. Gill filaments were incubated at 15°C under 99 O_2:1 CO_2 atmosphere with gentle shaking. Samples, in duplicate for each fish, were taken at the time of dissection (0 hr) and at different time points of culture and placed in 100 µl of SEI and frozen until measurement of Na^+,K^+-ATPase activity.

The preincubation medium was Minimal Essential Medium (MEM, GIBCO) with Hanks' salt, 25 mM HEPES buffer, and 4 mg ml^{-1} bovine serum albumin (Sigma RIA grade); 250 U ml^{-1} penicillin G and 250 µg g^{-1} streptomycin sulfate were added immediately prior to use, and the medium was adjusted to pH 7.55 with NaOH (final osmolality, 300 mOms/kg). The incubation medium was MEM with Earle's salts, 4 mg ml^{-1} bovine serum albu-

min, 292 μg ml^{-1} L-glutamine, 50 U ml^{-1} penicillin G sulfate, and 50 μg ml^{-1} streptomycin sulfate adjusted to pH 7.6 when saturated with 99 O_2:1 CO_2. The standard osmolality of incubation medium was 300 mosm/kg; increased osmolality was obtained by addition of a sterile solution of NaCl 17.5% (Tocher et al., '94).

Analytical techniques

Na^+,K^+-ATPase activities were determined using the microassay method of McCormick ('93). Gill tissue was homogenized in 125 μl of SEID (SEI buffer with 0.1% deoxycholic acid), then centrifuged at 3000*g* for 30 sec. Duplicate 10 μl homogenate samples were added to 200 μl assay mixture with and without 0.5 mM ouabain in 96-well microplates at 25°C and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as μmol ADP/mg protein/hour. The Pierce BCA Protein kit (Pierce, Rockford, IL) was used with bovine albumin as standard. Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA).

Statistics

Significant differences among groups were tested by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Significant differences between groups at the same time were tested by *t*-test. Two-way ANOVA and Student-Newman-Keuls multiple comparison test were used to test the significance of time and detergent treatment. Results were considered significantly different at $P < 0.05$.

RESULTS

Experiments in vivo

The transfer of *Fundulus heteroclitus* from 0.1 to 35 ppt induced a 70% increase in gill Na^+,K^+-ATPase activity 3 hr after transfer. After 12 hr the values dropped to initial levels. A second significant increase occurred 3 days after transfer, with only slightly higher levels after 7 days (Fig. 1). The time course of changes in gill Na^+,K^+-ATPase indicate that activation occurred 3 hr after transfer but not before (Fig. 2). In fish transferred from LSW to LSW, there was no change in gill Na^+,K^+-ATPase at any time (data not shown). In the absence of detergent (0.1% deoxycholic acid) in the gill homogenate, Na^+,K^+-ATPase activity was 15–22% lower than with detergent, and the rapid increase in the gill Na^+,K^+-ATPase 3 hr after transfer was not observed (Figs. 1 and 2).

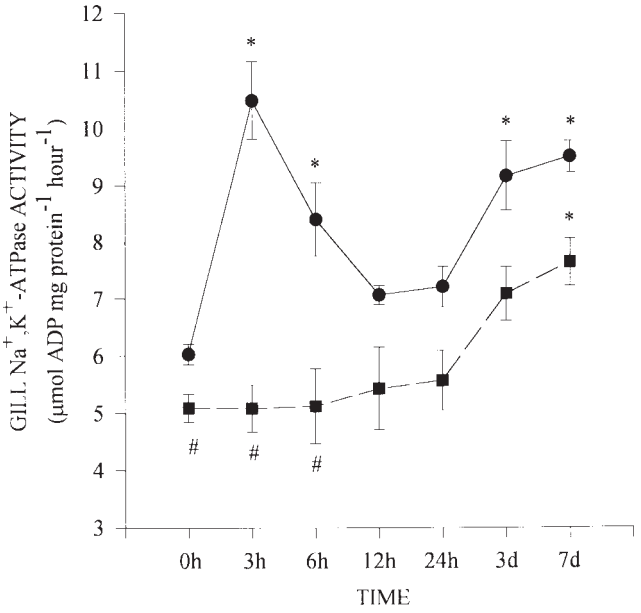

Fig. 1. Changes in gill Na^+,K^+-ATPase activity after transfer of *Fundulus heteroclitus* from LSW (0 hr) to SW (35 ppt salinity). Gill Na^+,K^+-ATPase activity was measured with detergent (0.1% deoxycholic acid; circles) and without detergent (squares). Each point represents mean ± standard error (n = 6–7 fish). *Indicates significant difference from time 0 hr ($P < 0.05$, two-way ANOVA test and Student-Newman-Keuls multiple comparison test). #Indicates significant difference between groups at the same time ($P < 0.05$, *t*-test).

Gill Na^+,K^+-ATPase activity of Atlantic salmon parr transferred from FW to SW (25 ppt) increased significantly 3 days after transfer and continued to rise at day 7 (Fig. 3A). Similar changes were seen in smolts transferred to 30 ppt (Fig. 3B). There was no significant change in enzyme activity in either group in the first 24 hr after transfer. In the absence of detergent, gill Na^+,K^+-ATPase was lower than with detergent (Fig. 3).

Experiments in vitro

Gill Na^+,K^+-ATPase activity remained constant during the first 6 hr of incubation when gill tissue was maintained in culture medium of 300 mosm/kg. With increased osmolality in the culture medium, gill Na^+,K^+-ATPase activity increased at 3 hr and decreased to initial levels at 6 hr (Figs. 4 and 5). The increase in gill Na^+,K^+-ATPase at 3 hr was dependent on medium osmolality (600 mosm/kg > 500 mosm/kg > 300m Osm/kg; Fig. 4). Osmolality of 800 mosm/kg resulted in 15% decreases of gill Na^+,K^+-ATPase activity relative to 600 mosm/kg, suggesting physiological

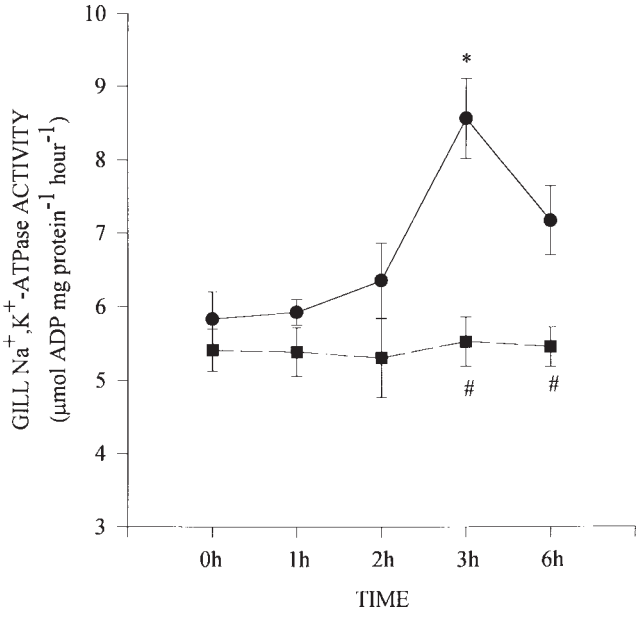

Fig. 2. Time course in gill Na^+,K^+-ATPase activity during the first hours after transfer of *Fundulus heteroclitus* from LSW (0 hr) to SW (35 ppt salinity). Gill Na^+,K^+-ATPase activity was measured with detergent (0.1 % deoxycholic acid; circles) and without detergent (squares). Each point represents mean ± standard error (n = 6–7 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, two-way ANOVA test and Student-Newman-Keuls multiple comparison test). #Indicates significant difference between groups at the same time ($P < 0.05$, *t*-test).

impairment of the tissue at this high osmolality. Use of mannitol to increase medium osmolality to 600 mosm/kg induced a 33% increase in gill Na^+,K^+-ATPase activity, similar to the 42% increase seen when NaCl was used (Fig. 6). The increase in gill Na^+,K^+-ATPase with hyperosmotic medium was not observed if detergent (0.1% deoxycholic acid) was absent in the homogenate preparation (data not shown).

Cycloheximide in vitro prevented the increase in gill Na^+,K^+-ATPase activity observed in hyperosmotic medium (doses of 10^{-4} M and 10^{-5} M). This inhibition was dose dependent (Fig. 7). Bumetanide at doses of 10^{-4} M, 10^{-5} M, and 10^{-6} M had no effect on Na^+,K^+-ATPase activity of gill filaments cultured in a hyperosmotic medium (Fig. 7).

The addition of actinomycin D to the culture media (doses of 10^{-4} M, 10^{-5} M, and 10^{-6} M) did not affect the increase in gill Na^+,K^+-ATPase observed in hyperosmotic medium (600 mosm/kg) (Fig. 8). However, gill filaments from fish previously treated with actinomycin D and cultured under hyperosmotic conditions did not show an increase in Na^+,K^+-ATPase (Fig. 9).

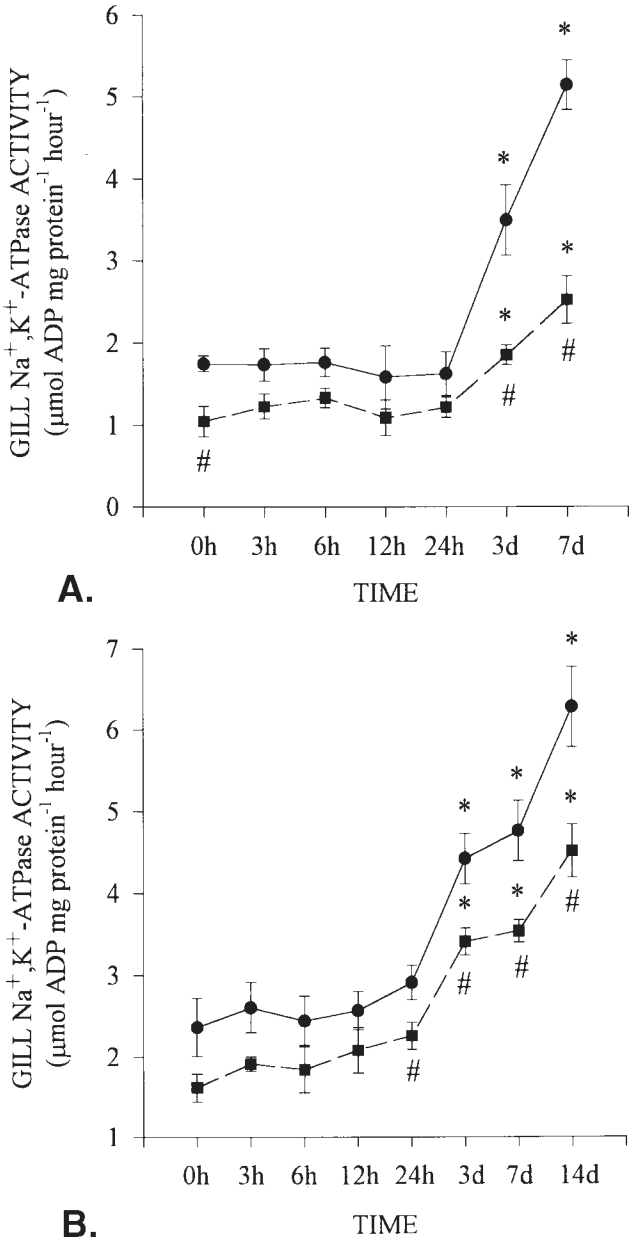

Fig. 3. Changes in gill Na^+,K^+-ATPase activity after transfer of *Salmo salar* parr from FW (0 hr) to SW (25 ppt salinity) (**A**) and of *Salmo salar* presmolt from FW (0 hr) to SW (30 ppt salinity) (**B**). Gill Na^+,K^+-ATPase activity was measured with detergent (0.1 % deoxycholic acid; circles) and without detergent (squares). Each point represents mean ± standard error (n = 6–7 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, two-way ANOVA test and Student-Newman-Keuls multiple comparison test). #Indicates significant difference between groups at the same time ($P < 0.05$, *t*-test).

DISCUSSION

The acclimation of *Fundulus heteroclitus* and other euryhaline fish to SW seems to occur in two phases: a first phase where there is a rapid in-

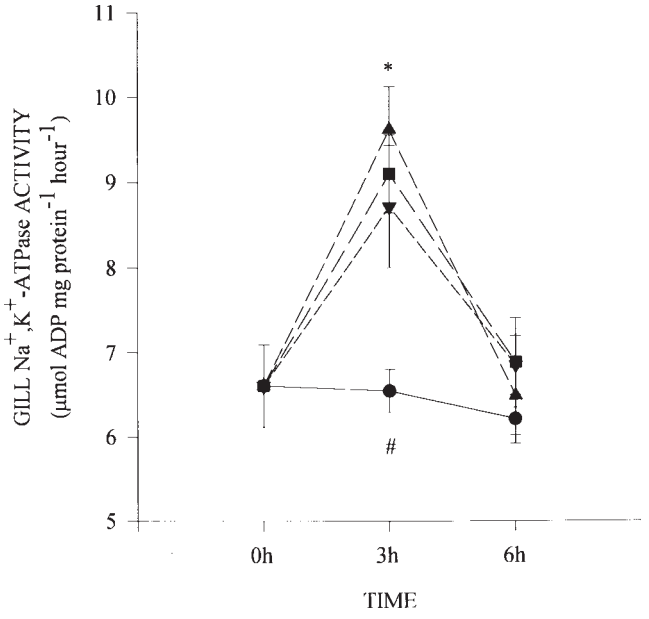

Fig. 4. Changes in gill Na^+,K^+-ATPase activity in organ culture using different osmolality media (circles: 300 mosm/kg; squares: 500 mosm/kg; triangles: 600 mosm/kg; inverted triangles: 800 mosm/kg). Values are means ± standard error (n = 4–5 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test). #Indicates significant difference between groups at the same time ($P < 0.05$, Student-Newman-Keuls multiple comparison test).

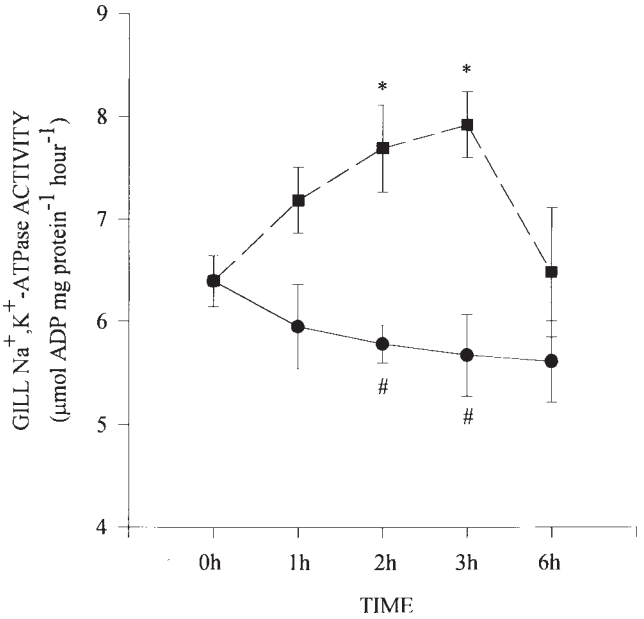

Fig. 5. Time course of gill Na^+,K^+-ATPase activity in vitro using isoosmotic (300 mosm/kg; circles) or hyperosmotic (600 mosm/kg; squares) culture medium. Values are means ± standard error (n = 4–5 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test). #Indicates significant difference between groups at the same time ($P < 0.05$, *t*-test).

crease in gill ion permeability and an increase in sodium and chloride secretion accompanied by moderate increases in plasma ions, and a second phase, several days post-transfer, where an increase in gill Na^+,K^+-ATPase activity with a proliferation and/or development of chloride cells occurs, net sodium and chloride efflux increases, and plasma ion balances are restored (De Renzis and Bornancin, '84; Zadunaisky, '84; Karnaky, '86; Pequeux et al., '88; Wood and Marshall, '94). Our in vivo results demonstrate that in the euryhaline teleost *Fundulus heteroclitus,* there is a rapid increase in gill Na^+,K^+-ATPase during the first phase of SW acclimation that could drive the typical chloride secretion of this phase. The rapid increase in activity is not likely due to an increase in the number and/or size of chloride cells because of the speed of these changes. Our in vitro results indicate that increased osmolality itself can induce rapid activation of gill Na^+,K^+-ATPase. This finding suggests that this rapid activation is independent of the endocrine system, although paracrine and autocrine mechanisms may play a role.

In this study we found rapid activation of gill Na^+,K^+-ATPase activity in an intertidal euryhaline species (*Fundulus heteroclitus*) but not in the anadromous Atlantic salmon. Most salmonids make the transition from FW to SW in spring and undergo a relatively slow smolting process that prepares the fish for life in SW. Rapid activation was found in the intertidal *Fundulus heteroclitus* perhaps because this species is often exposed to rapid changes in environmental salinity throughout its life cycle. It will be necessary to examine more species to determine if rapid activation of gill Na^+,K^+-ATPase is a common characteristic of intertidal teleosts.

The magnitude and timing of changes in gill Na^+,K^+-ATPase activity during the first phase of SW acclimation of *Fundulus heteroclitus* vary among published studies. Using a gill microsome preparation, Towle et al. ('77) found an increase in gill Na^+,K^+-ATPase one-half hour after transfer of *Fundulus heteroclitus* from FW to SW (30 ppt). Our results using a simple homogenate preparation indicate an increase in gill Na^+,K^+-ATPase 3 hr post-transfer but not before, and that use of detergent is necessary to observe this increase. Using the same Na^+,K^+-ATPase assay used in the present study, Marshall et al. ('99) did not observe a change in gill Na^+,K^+-ATPase during the first phase (1 hr,

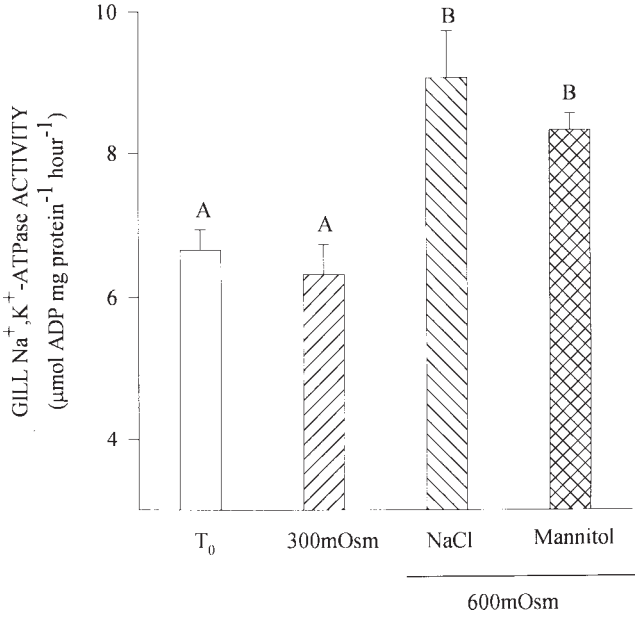

Fig. 6. Changes in gill Na^+,K^+-ATPase activity after 3 hr in organ culture using hyperosmotic media obtained by addition of NaCl or mannitol. Values are means ± standard error (n = 4–5 fish). Same letters indicate no significant differences among groups ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test).

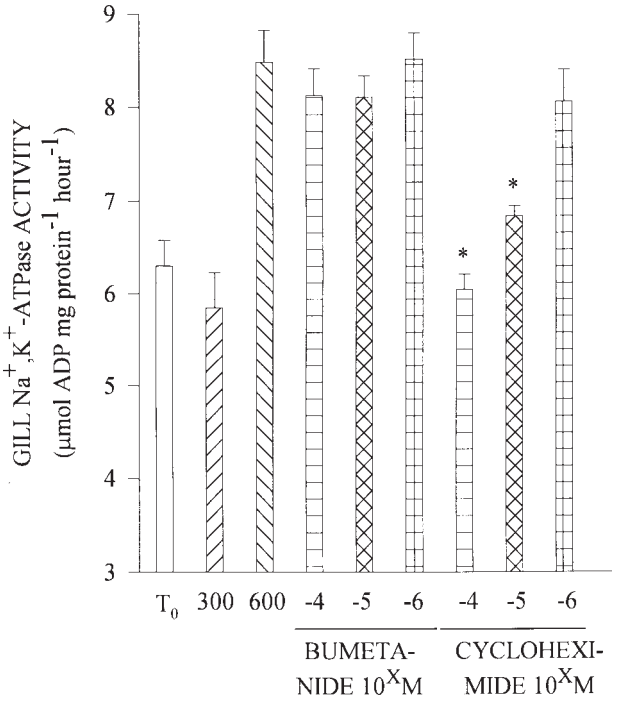

Fig. 7. Effect of bumetanide and cycloheximide on gill Na^+,K^+-ATPase activity after 3 hr in culture using hyperosmotic medium (600 mosm/kg). Values are means ± standard error (n=4–5 fish). *Indicates significant difference compared with hyperosmotic control medium ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test).

3 hr, and 8 hr post-transfer) or second phase (1 day, 2 days, 7 days, 14 days, and 30 days post-transfer) of SW acclimation, perhaps because of the lower salinity used (30 ppt vs 35 ppt). Increases in gill Na^+,K^+-ATPase during the first phase of SW acclimation have been found in other teleosts. In *Mugil cephalus* transferred from brackish water (10 ppt) to SW (45 ppt), there is an increase in gill Na^+,K^+-ATPase content (ouabain binding) 3 hr after transfer (Hossler, '80). A similar rapid (3-hr) increase in gill Na^+,K^+-ATPase has been reported in *Oreochromis mossambicus* acclimated to 20 ppt and transferred to 30 ppt (Hwang et al., '89).

In *Fundulus heteroclitus,* lower levels of gill Na^+,K^+-ATPase activity have been found in fish acclimated to intermediate salinity relative to fish in FW or SW (Towle et al., '77). Similar salinity dependence of gill Na^+,K^+-ATPase activity has been found in the europea sea bass *Dicentrarchus labrax* (Jensen et al., '98). Given these reduced levels under isoosmotic conditions, it will be interesting to study the dynamics of gill Na^+,K^+-ATPase activity after transfer from intermediate salinity to both FW and SW. In addition, it will be useful to examine the dynamics of other pumps, such as H^+-ATPase, that may be involved in ion uptake (Lin and Randall, '95).

In a previous study, Marshall et al. ('99) transferred *Fundulus heteroclitus* from FW to SW (30 ppt) and evaluated plasma osmolality and Na^+ concentration. They observed an increase in plasma osmolality and Na^+ concentration 1 hr after transfer, a decrease at 3 hr, and a subsequent increase at 8 hr. Our results showed that gill Na^+,K^+-ATPase increased 3 hr after transfer and decreased at 6–12 hr. These results suggest that 3 hr after exposure to SW, increased gill Na^+,K^+-ATPase may contribute to the reduction of high osmolality and plasma Na^+ concentration. The subsequent decrease in gill Na^+,K^+-ATPase at 6–12 hr could be related to the increase in plasma osmolality and Na^+ observed at this time by Marshall et al. ('99). The inverse correlation between gill Na^+,K^+-ATPase activity and plasma ion levels following SW transfer is indicative of the role of the sodium pump in sodium transport by the gill.

Several studies have shown that actinomycin D, an inhibitor of mRNA synthesis, perturbs the whole-body ion flux adjustments during transfer to different osmotic environments (Maetz et al.,

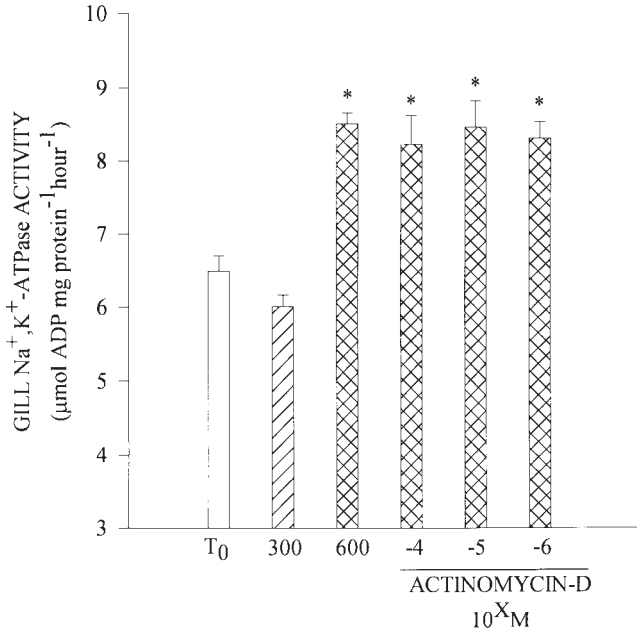

Fig. 8. Effect of actinomycin D on gill Na^+,K^+-ATPase activity after 3 hr in culture using hyperosmotic medium (600 mosm/kg). Values are means ± standard error (n = 4–5 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test).

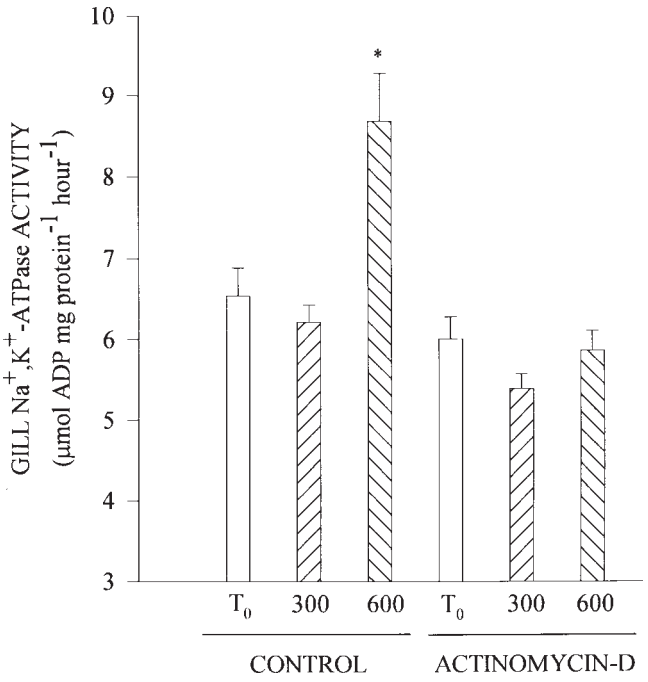

Fig. 9. Gill Na^+,K^+-ATPase activity after 3 hr in isoosmotic (300 mosm/kg) or hyperosmotic (600 mosm/kg) culture medium. FW-acclimated fish were injected with saline solution or with actinomycin D (0.75 μg g^{-1} body weight) 3 days before organ culture. Values are means ± standard error (n = 4–5 fish). *Indicates significant difference relative to time 0 hr ($P < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test).

'69; Nichols and Fleming, '90). Actinomycin D reduced gill Na^+,K^+-ATPase in SW-acclimated fish and fish transferred from FW to SW, but it was less effective on gill Na^+,K^+-ATPase in FW-acclimated fish (*Anguilla anguilla*: Motais, '70; *Chelon labrosus*: Gallis et al., '79; *Oncorhynchus tshawytscha*: Beckman and Zaugg, '90). A preliminary experiment with *Fundulus heteroclitus* indicated that actinomycin D injection reduced gill Na^+,K^+-ATPase after 3 days in SW- but not LSW-acclimated fish (data not shown). Using an in vitro approach, the addition of this inhibitor had no effect on rapid activation of gill Na^+,K^+-ATPase observed in a hyperosmotic medium. However, use of gill filaments of actinomycin D–injected fish for culture in hyperosmotic media prevented the typical increase in Na^+,K^+-ATPase observed under hyperosmotic conditions. This apparent conflict between in vivo and in vitro effects of actinomycin D could indicate that a longer time is necessary to observe the effects of this drug on mRNA synthesis (3 days in vivo vs. 6 hours in vitro) or that actinomycin D is not effective in organ culture. Rapid stimulation of gill Na^+,K^+-ATPase activity may be dependent on an existing pool of mRNA that is utilized during exposure to high osmolality. In vivo treatment with actinomicin-D could decrease this pool, thus affecting the ability to respond to high osmolality; while in vitro treatment has no effect on this pool. In *Anguilla anguilla* an initial transient increase in gill Na^+,K^+-ATPase without increase of α and β-subunit mRNA expression has been observed 6-12 h after transfer from FW to SW (Cutler et al., '96). Similarly *Oreochromis mossambicus* showed a 5.4-fold increase of gill Na^+,K^+-ATPase protein but only 2.6-fold increase in Na^+,K^+-ATPase mRNA in SW-acclimated relative to FW-acclimated fish (Hwang et al., '98). This difference between the increase in level or timing of changes between mRNA and protein Na^+,K^+-ATPase has been reported in several tissues under different physiological situations and may indicate changes in translational or posttranslational kinetics (Ohara et al., '93; Lee et al., '95).

Cycloheximide is a translation inhibitor that also affects ion fluxes of fish transferred from one environmental salinity to another (*Anguilla anguilla*: Maetz et al., '69; *Fundulus kansae*: Nichols and Fleming, '90). In vitro studies have shown that use of cycloheximide inhibits stimu-

lation of Na^+,K^+-ATPase in renal thick ascending limb by dexamethasone (Doucet et al., '86) and in cultured A6 cells from kidneys of *Xenopus laevis* by aldosterone (Verrey et al., '87). In primary cultures of rectal gland cells of *Scyliorhinus canicula*, a transient and cycloheximide-sensitive increase in Na^+,K^+-ATPase has been observed after increasing medium osmolality (Cutler et al., '96). Our in vitro results, using gill filament culture under hyperosmotic media, show that the increase in gill Na^+,K^+-ATPase activity is cycloheximide-sensitive in a dose-dependent manner and suggests that rapid activation is dependent on translation.

The modifications implicated in the rapid activation of Na^+,K^+-ATPase molecules may involve phosphorylation of the pump catalytic subunit (see Berterello and Katz, '93; Ewart and Klip, '95). Towle et al. ('77) suggested that in *Fundulus heteroclitus,* short-term acclimation from FW to SW involves modifications in catalytic rate rather than number of Na^+,K^+-ATPase molecules. The rapid increase in Na^+,K^+-ATPase activity observed in our experiments also could be ascribed to changes in catalytic rate of Na^+,K^+-ATPase molecules. Synthesis of proteins involved in phosphorylation or dephosphorylation could explain our results using actinomycin D and cycloheximide in gill culture that suggest the necessity of translation and transcription for the rapid increase of Na^+,K^+-ATPase activity.

The rapid increase in gill Na^+,K^+-ATPase activity could also involve changes in the subcellular distribution of pump units (see Berterello and Katz, '93; Ewart and Klip, '95). The existence of a latent (e.g., cytoplasmatic) pool of Na^+,K^+-ATPase molecules in gill chloride cells ready to use when it is necessary could explain these results. Upon transfer from LSW to SW, this pool could be mobilized into the plasma membrane resulting in increased Na^+,K^+-ATPase activity. The typical increase in gill Na^+,K^+-ATPase activity observed during the first hours after transfer from LSW to SW and after in vitro exposure to hyperosmotic media is not detected if detergent is absent in the assay. This effect of detergent may relate to activation of a latent pool of sodium pumps that are only observed if detergent is used in the assay. Use of detergents solubilizes intrinsic membrane proteins and increasing access to substrates, thereby increasing enzymatic activity (Gupte and Lane, '85; Reynolds et al., '85; Omatsu-Kanbe and Kitasato, '87). The use of detergent may have induced appropriate rearrangement of catalytic subunits of Na^+,K^+-ATPase pumps to give the results observed in our experiments.

Our in vivo and in vitro results indicate that increase in environmental osmolality using NaCl induced activation of Na^+,K^+-ATPase in gill chloride cells. The use of mannitol for increasing osmolality of in vitro culture medium without increasing external Na^+ levels also induced a rapid activation of Na^+,K^+-ATPase activity. Activation of the sodium pump may be related to cell shrinkage induced by hyperosmotic conditions. Increased Na^+,K^+-ATPase activity under hyperosmotic conditions also has been shown in different cell types and has been linked to changes in cell volume (canine kidney: Bowen, '92; bovine renal: Ferrer-Martinez et al., '96; dogfish rectal gland: Cutler et al., '96). Zadunaisky et al. ('95) studied chloride transport in chloride cell opercular epithelium of *Fundulus heteroclitus* during rapid changes in osmolality. This isolated epithelium showed an increase in chloride secretion 5-10 minutes after increasing medium osmolaity with mannitol. The authors concluded that "the rapid signal for adaptation to higher salinities is an increased tonicity of the plasma that induces chloride cell shrinkage, increased chloride secretion with activation of the $Na^+K^+/C1^-$ cotransporter." In primary cultures of *Scyliorhinus canicula* rectal gland cells, the increase observed in Na^+,K^+-ATPase activity after increasing NaCl concentration is also prevented by bumetanide, suggesting that activation of the $Na^+/K^+/Cl^-$ cotransporter is necessary for rapid stimulation of Na^+,K^+-ATPase activity (Cutler et al., '96). In our gill culture system, the use of the bumetanide did not affect rapid activation of Na^+,K^+-ATPase, indicating that stimulation of the Na+/K+/Cl^- cotransporter is not necessary for rapid activation of Na^+,K^+-ATPase in *Fundulus heteroclitus*. We cannot rule out the possibility, however, that in the present study the inhibitor did not reach the basolateral side of gill chloride cells, where the $Na^+/K^+/Cl^-$ cotransporter is located.

Towle et al. ('77) reported that in *Fundulus heteroclitus* transferred from FW to SW, gill Na^+,K^+-ATPase showed similar levels at 30 min, 60 min, and 3 days post-transfer, but there is no information about the time course of Na^+-K^+-ATPase activity between 1 hr and 3 days. Our in vivo and in vitro results indicate a decrease in gill Na^+,K^+-ATPase 6–12 hr after exposure to hyperosmotic conditions. The reason of this time course observed in our experiment is not known. The initial increase of gill Na^+,K^+-ATPase could be ascribed to the existence of a cytoplasmatic/membranal pool of Na^+,K^+-ATPase pumps stimulated by the transfer to high environmental salinity, enzyme phosphorylation, or

to an increase in translation/posttranslational kinetics of Na^+,K^+-ATPase. The subsequent decrease observed in Na^+,K^+-ATPase could indicate a decrease in the number of Na^+,K^+-ATPase pumps. The reason could be ascribed to a shorter half-life of Na^+,K^+-ATPase pumps during acute stimulation or exposure to stressful hyperosmotic conditions. Although the turnover rates of Na^+,K^+-ATPase have not been examined in fish, the turnover of chloride cells is greater in high salinity (Uchida and Kaneko, '96).

An important question that arises from our results is whether differences in gill Na^+,K^+-ATPase measured biochemically represent sodium pumps that are functionally active in the whole animal. This question can be approached by measuring ouabain binding or ouabain-sensitive ion fluxes. If the gill Na^+,K^+-ATPase activity measured in the present study is functionally active, increased ouabain binding or ouabain-sensitive fluxes should be observed in the first hours after transfer, and a secondary decrease will also occur.

The important physiological role of gill chloride cells for acclimation to hyperosmotic environments, with Na^+,K^+-ATPase activity as the pump used for driving the excess of sodium and chloride ions, is widely accepted (see McCormick, '95; Marshall and Bryson, '98). The increase in gill Na^+,K^+-ATPase observed in *Fundulus heteroclitus* and *Salmo salar* during the second phase of acclimation (3–4 days after transfer) is in agreement with several previous studies on these species (e.g., Jacob and Taylor., '83; McCormick et al., '89). These long-term increases in gill Na^+,K^+-ATPase activity probably reflect an increase in the number and size of chloride cells, as has been reported for these and a number of other teleosts species (Bornancin and De Renzis, '72; Forrest et al., '73; Evans and Mallery, '75; Boeuf et al., '78; Madsen and Naamansen, '89; Jensen et al., '98).

In conclusion, the results of the present paper show a rapid and transitory increase in gill Na^+,K^+-ATPase activity during the first hours after transfer of *Fundulus heteroclitus* (an intertidal teleost) but not *Salmo salar* (an anadromous salmonid) from FW to SW. Similar results are obtained in vitro using a gill culture system after increasing medium osmolality. The origin of this increase in gill Na^+,K^+-ATPase activity could involve modifications of pump catalytic subunits, changes in the subcellular distribution of pump units, or increases in translational or posttranslational kinetics. The results obtained with actinomycin D and cycloheximide suggest that the rapid activation of gill Na^+,K^+-ATPase activity in *Fundulus heteroclitus* is dependent on transcriptional and translational processes.

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