Sphingomyelin Metabolism Is Linked to Salt Transport in the Gills of Euryhaline Fish

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ABSTRACT: By *in vivo* and *in vitro* studies of L-(3-^{3}H)serine and [9,10(n)-^{3}H]palmitic acid incorporation into phospholipids, we show a change in the renewal of the ceramide moiety of sphingomyelin in the gills of euryhaline fish (sea bass and eels) when the animals were subjected to abrupt alterations in environmental salinity. *In vivo*, decrease of the salinity from sea water (salinity 3.7%) to diluted sea water (salinity 1%) induced an increase of label incorporation into gill sphingomyelin. The same was true when gills from sea water-adapted sea bass or sea water-adapted eels were incubated in diluted sea water. A decrease in free ceramides synthesis was also observed in the gills of sea water-adapted sea bass when the salinity of the incubation medium was reduced. Direct inhibition of Na^+/K^+-ATPase activity with ouabain decreased the sphingomyelin synthesis in the gills of sea bass during *in vitro* incubation in diluted sea water, whereas treatment with furosemide stimulated sphingomyelin synthesis in the same gills incubated in sea water. These findings indicate that changes in Na^+ fluxes modify the sphingomyelin turnover and control the production of free ceramides and sphingosine in gill cells of euryhaline fish. In view of the well-known effects of these sphingomyelin degradation products on isolated tumor cell differentiation, we suggest that they play a very important role in modulating chloride cell distribution and metabolism of fish gills during abrupt changes in environmental salinity.

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Sphingomyelin (SPH) is found in all fish and crab tissues as a component of cell membranes (1–4). There is a greater percentage of SPH in gill phospholipids than in those of other tissues (2,4), and sea water (SW) adaptation increases this percentage in the gills of euryhaline crabs (5). The biosynthetic pathway for SPH appears to be very active in the gills of teleosts and crustaceae (6,7). This pathway involves ceramide synthesis from long-chain bases followed by transfer of a phosphocholine moiety catalyzed by phosphatidylcholine (PC)-ceramide cholinephosphotransferase (8). Factors influencing SPH turnover include serine and acyl-CoA availability, and the relative activities of key enzymes in the synthesis and degradation pathways. The first step in degradation involves the removal of the head-group by sphingomyelinase, an enzyme found in gill cells (2).

In addition to their respiratory function, the gills of euryhaline fish and crabs participate in osmoregulation (9,10). The gills are the site of active NaCl transport and the direction of active salt transport is correlated with external salinity. In gills of *Anguilla anguilla* and *Platichthys flesus*, two euryhaline teleosts, active NaCl absorption begins during adaptation to fresh water (FW) (11,12). The branchial epithelium of SW-adapted fish show a much larger population of chloride cells than that of FW-adapted fish (13). These cells are the site of chloride transport and possess enzymatic characteristics of electrolyte-transporting epithelia (13,14). In gill epithelium, most of the Na^+/K^+-ATPase activity is found in chloride cells, and cell density depends directly on the external salinity (13,14). In both teleosts and crabs, changes in chloride cell density are accompanied by an alteration of phospholipid metabolism (5,15). It has been reported (8) that SPH concentration is different in gills of FW- and SW-adapted crabs (*Eriocheir sinensis*). Changes in the concentration of this major phospholipid may reflect modification of its turnover rate. Moreover, SPH catabolism liberates ceramide and/or free sphingosine, potential second messengers which could modulate cell function (16,17). The recent discovery that these intermediates play an important role in controlling cell growth, differentiation and oncogenesis (17–20) has motivated this study of SPH metabolism during abrupt salinity changes in fish gills.

MATERIALS AND METHODS

Two different species of euryhaline fish were used in this study, a marine fish: sea bass (*Dicentrarchus labrax*) and a freshwater fish: yellow European eel (*A. anguilla*). Sea bass (*D. labrax*) averaging 75 g were originally obtained from a sea farm (Centre de production aquacole IFREMER, Palavas les Flots, France) and were kept in indoor tanks with continuously flowing Mediterranean SW (salinity 3.7%) at 14–15°C for 4 wk. Eels averaging 100 g were originally obtained from the Rhône river and were acclimated to continuously flowing

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Abbreviations : Amiloride, N-amidino-3,5-diamino-6-chloropyrazinecarboxamide; furosemide, 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid; FW, fresh water; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; SW, sea water; TLC, thin-layer chromatography; TPA, 12-0-tetradecanoylphorbol 13-acetate.

FW or SW at 14–15°C for 4 wk. Sea bass were fed a standard diet and eels were starved.

In vivo *L-3(^{3}H)-serine incorporation.* Sea bass acclimated to SW received 100 μCi of L-3(^{3}H)-serine (29 Ci /mmol; Amersham, Les Ulis, France) per 30 g wet weight by intraperitoneal injection and were randomly divided in two groups: (i) SW (salinity 3.7%) and (ii) diluted SW (salinity 1%) at a controlled temperature of 17°C. After 16, 24, and 36 h, the animals were killed by a blow on the head, and gills were isolated and weighed. Lipid extraction and purification were performed on the fresh tissue by the method of Folch *et al.* (22), modified by Chapelle *et al.* (23). The phospholipid classes were resolved by two-dimensional thin-layer chromatography (TLC) on precoated silica-gel G-60 plates (Merck, Darmstadt, Germany) as previously described by Portoukalian *et al.* (24). Phospholipids were detected with a specific spray reagent (25) and quantified by determining the phosphorus content in each spot as previously described (5,24). For the radioactive determination, phospholipid spots revealed on the TLC chromatograms were counted in the presence of silica gel, by scraping into a scintillation vial and resuspending in 3 mL water/ethanol (1:1, vol/vol) and 8 mL Picofluor 30 (Packard, Downers Grove, IL) (26).

In vitro *L-3(^{3}H)-serine incorporation.* Gills from SW-adapted sea bass were cut at their bases, and each pair transferred to ultra-filtered Mediterranean SW or diluted SW, rinsed and blotted on paper. Then, one gill of each pair was placed in SW and the other in diluted SW containing 0.3 μM L-3(^{3}H)-serine (29 Ci/mmol; Amersham) or 0.2 μM [9,10(n)-^{3}H]-palmitic acid (40–60 Ci/mmol; Amersham) and incubated at 15°C for the indicated time. Lipids were extracted and analyzed as described above.

Analysis of long-chain bases in SPH. Phospholipids were separated by one dimensional TLC on precoated silica-gel G-60 plates in chloroform/methanol/water (65:35:6, by vol). The SPH spots, visualized with iodine, were scraped into tubes, eluted with a mixture of chloroform/methanol/acetic acid/water (50:39:1:10, by vol) and washed as described by Arvidson (27). Some lipid extracts were subjected to mild alkaline methanolysis (28) before purification of SPH by TLC. SPH was first enzymatically hydrolyzed using 10 units/mL phospholipase C (Type 1 from *Clostridium perfringens*; Sigma, St. Louis, MO) in 1 mL of Tris pH 7.4, 30 mM $CaCl_2$ mixed with 1 mL diisopropyl ether for 18 h at 20–25°C to obtain the ceramides (29). Diisopropyl ether was evaporated in a stream of nitrogen before the addition of 4 mL chloroform/methanol (1:1, vol/vol) and 1.8 mL water. Ceramides were extracted in the chloroform phase, followed by centrifugation. Aliquots of the water and chloroform soluble extracts were counted. Under these conditions of hydrolysis, TLC of the chloroform extract on silica gel in chloroform/methanol/water (65:35:6, by vol) showed the total disappearance of SPH. The ceramides were acid hydrolyzed in teflon-lined screw-capped tubes in concentrated HCl/water/methanol (1:2:6, by vol) for 18 h at 80°C (30). The samples were taken to dryness with added methanol and benzene to eliminate HCl and water. The residues, redissolved in 0.5 mL of chloroform/methanol (2:1, vol/vol) and diluted with standards (mixture of SPH, ceramides, sphingosine, fatty acids, and esters), were separated by one-dimensional TLC on precoated 10 x 5 cm TLC plates. The plates were first developed to 5 cm in chloroform/methanol/water (100:42:6, by vol) and then, after drying, in chloroform/methanol (50:3, vol/vol) to the 10-cm length. The fatty acids and esters, ceramides, sphingosine, and SPH spots were visualized with iodine and scraped into scintillation-counting vials and counted. Under these conditions, all of the radioactivity initially found in the ceramide was distributed between sphingosine and fatty acids, according to the radioactive precursor.

Determination of radioactivity in free ceramides of gills. For free ceramide measurements, lipids were subjected to mild alkaline hydrolysis (28) in 0.4 M methanolic NaOH at 37°C for 1 h. Concentrated HCl was added to obtain a final concentration of 0.4 N acid, and hydrolysis was continued for 1 h at the same temperature. After extraction with chloroform, the resulting extracts were diluted with solutions of purified ceramides and separated by one-dimensional TLC on 10 x 5 cm precoated silica-gel plates by two successive developments, as described above. SPH and ceramides were visualized with iodine, scraped, and counted. The ceramide radioactivity in tissue, in dpm/100 mg wet gills, was calculated from SPH radioactivity found on the plate.

Statistics. Means for individual phospholipid radioactivity were compared using one-way analysis of variance. Percentages were arcsine transformed. If test for normality failed, then a nonparametric Kruskal-Wallis one-way analysis of variance on ranks was used. All values presented are means ± SEM.

RESULTS

In vivo incorporation of L-(^{3}H)serine into gill lipids of the sea bass during abrupt change in salinity from SW (3.7%) to diluted SW (1.0%) is summarized in Table 1. During this 36-h experimental period, 90% of lipid radioactivity in the gills was present in phospholipids, and this stayed relatively constant [from 7318 ± 293 (4) dpm/100 mg wet gills at 16 h to 8031 ± 250 (4) at 36 h in SW and 6143 ± 288 (4) to 7655 ± 774 (4) in diluted SW]. When the distribution of the radiolabel among lipid species was determined (see Table 1), radioactivity was significantly lower ($P < 0.01$) in SPH of the gills of fish in SW than in those of fish transferred to diluted SW. No major variations for the other lipids were observed. The gross distribution of radioactivity in PC, phosphatidylserine (PS), and phosphatidylethanolamine (PE) appeared to be independent of salinity. In these same experiments, phospholipid composition showed the same general pattern in the gills of both animals, with SPH representing 11.5 ± 0.2% of total lipid phosphorus [PC: 46.0 ± 0.3%, PS: 6.4 ± 0.2%, phosphatidylinositol (PI): 4.4 ± 0.2%, and PE: 17.2 ± 0.5%]. To determine whether the differences in SPH metabolism during the abrupt decrease in salinity resulted from a direct effect on

TABLE 1

***In vivo* Distribution of L(3-^{3}H)Serine into Gill Lipids of the Sea Bass During an Abrupt Change from Sea Water (SW, salinity 3.7%) to Diluted Sea Water (salinity 1%)[a]**

Lipid	SW	Diluted SW
SPH	9.00 ± 0.17[b]	11.12 ± 0.10[c]
PC	18.16 ± 0.57	18.51 ± 0.42
PS	38.54 ± 0.81	37.74 ± 0.55
PE	14.64 ± 0.28	15.78 ± 0.33
NL	12.53 ± 0.50	10.71 ± 0.44

[a]Twenty-four fish adapted to SW for at least 6 wk received 100 µCi each of L-(3-^{3})serine by intraperitoneal injection and were kept in aerated SW or diluted SW tanks at a controlled temperature of 17°C; four were killed 16, 24, and 36 h after the initial injection. The gill lipids were extracted and analyzed as described in the Materials and Methods section.
[b]Values are expressed as dpm% of the total lipid extracted from the gills and are the means ± SEM of 12 animals.
[c]Values statistically different, $P < 0.01$, between SW and diluted SW; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; NL, neutral lipids.

gill SPH turnover, we have carried out *in vitro* experiments, looking at the effect of medium salinity on the incorporation of L-(^{3}H)serine into phospholipids of isolated gills from SW-adapted fish. As shown in Table 2, there was a marked increase (+43%) in SPH radioactivity (from 11.6 to 16.4%) in phospholipids extracted from gills incubated in diluted SW. Low salinity resulted in a decrease (−13%) in PS radioactivity without modifying PC and PE label percentage.

To confirm the effect of abrupt change in external salinity on gill SPH metabolism in another euryhaline fish, we have analyzed the *in vitro* incorporation of L-(^{3}H)serine into the phospholipids of gills obtained from SW- and FW-adapted eels. In these experiments, gills were incubated in a synthetic medium with an osmotic pressure and ionic composition similar to eel plasma. There was a large difference between the incorporation of the radiolabel into SPH of gills from SW- or FW-adapted eels (Fig. 1). There were no major differences between the other phospholipids, except for a small increase of the label in PC of gills from SW-adapted eels.

The distribution of the label in SPH isolated from gills after *in vivo* radioactive serine incorporation revealed that

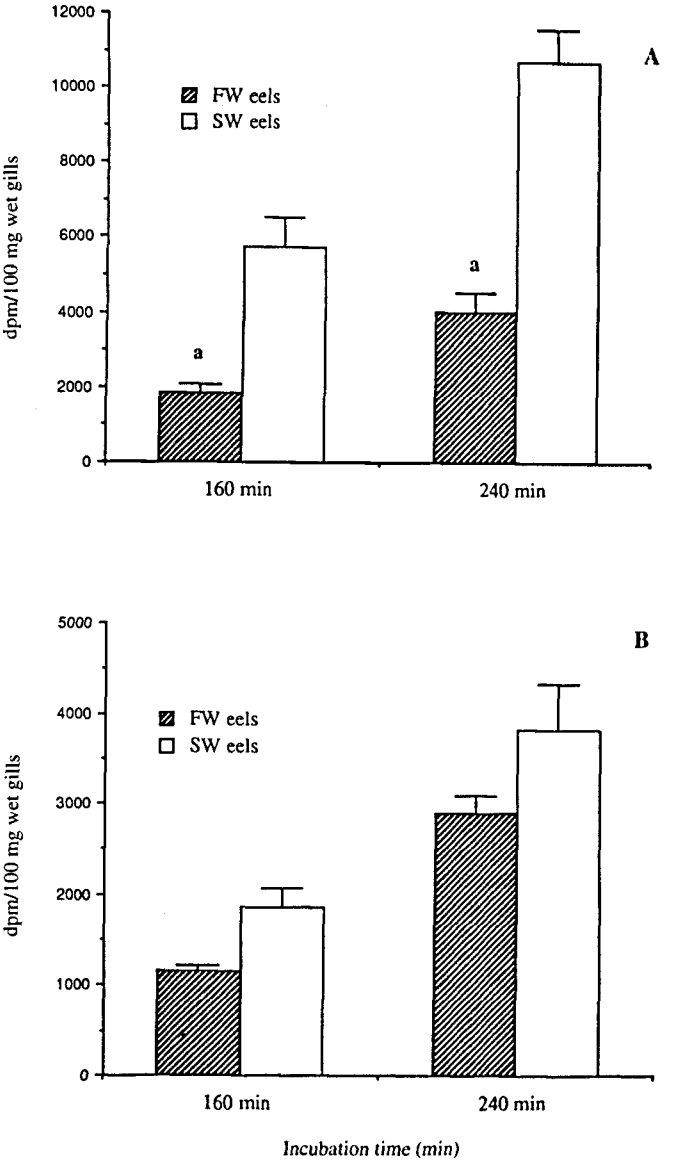

FIG. 1. *In vitro* incorporation of L-(U^{14}C)serine into gill sphingomyelin (A) and phosphatidylcholine (B) of salt water (SW)- and fresh water (FW)-adapted eels. Gills from SW- or FW-adapted eels were incubated for 160 and 240 min at 17°C in a synthetic medium of NaCl, 147 mM; KCl, 2.66 mM; $CaCl_2$, 2.17 mM; $MgCl_2$, 2.34 mM; HNa_2PO_4, 8.1 mM; and H_2NaPO_4, 1.9 mM, containing 0.35 µCi L-(U^{14}C)serine/mL. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between gills from SW- and FW adapted eels.

TABLE 2

Influence of the Salinity of *in vitro* Incubation Medium on the Distribution of L-(3-^{3}H)Serine into Gill Phospholipids of Sea Bass[a]

Lipid	Incubation medium	
	SW	Diluted SW
SPH	11.56 ± 0.19[b]	16.40 ± 0.06[c]
PC	4.48 ± 0.27	5.20 ± 0.06
PS	48.03 ± 1.32	41.58 ± 0.79[d]
PE	9.87 ± 0.76	10.79 ± 0.35

[a]Gills from SW-adapted sea bass were incubated for 4 h at 17°C in SW (salinity 3.7%) or diluted SW (salinity 1%) containing 5 µCi L-(3-^{3}H)serine/mL.
[b]Values are expressed as dpm% of the total dpm in the lipid extracted from the gills and are the means ± SEM of six animals. Values statistically different: [c]$P < 0.01$, [d]$P < 0.05$, between SW and diluted SW. See Table 1 for abbreviations.

about 80–85% of total radioactivity was found in the sphingosine moiety. The fatty acids and choline isolated during enzymatic and acid treatments contained 10–15% of the radioactivity. After *in vitro* incubation, 90–95% of the radioactive label was found in the sphingosine moiety of the purified SPH. These results were consistent with the sphingosine biosynthetic pathway, in which the first stage is a condensation of serine and palmitoyl-CoA followed by an acylation to ceramide. To form SPH, the phosphorylcholine head-group seems to be transferred from PC to ceramide (6). To determine if the rate of the acylation step in the SPH biosynthesis was altered by the salinity change, we have measured the incorporation of (^{3}H)palmitic acid into gill SPH under the same

in vitro conditions. Results of these experiments with gills from sea bass are summarized in Figure 2. The salinity of the incubation medium had a large effect on the rate of radioactive palmitic acid incorporation into gill SPH. For the other lipids, no differences between SW and diluted SW were observed except after 2 h of incubation in PC. In these experiments, 27% of the radiolabel was located in sphingosine and 73% in the acyl moiety of the SPH isolated after SW or diluted SW incubation.

The above results demonstrate an effect of external salinity on the rate of SPH synthesis in gills of sea bass and eels. However, they give only an imperfect picture of the biochemical changes due to salinity variations. For this reason, it was of interest to measure radioactivity incorporated into free ceramide, an intermediate of SPH metabolism. Figure 3 shows the activity of SPH and ceramides in the gills of sea bass after 4 h of incubation in diluted SW or SW containing 15 µCi (^{3}H)serine/mL. We observed an effect of external salinity on the incorporation of radiolabel into free ceramides and SPH. Diluted SW caused a decrease in label incorporation into tissue free ceramides (essentially with nonhydroxylated fatty acids) together with an increase in incorporation into SPH.

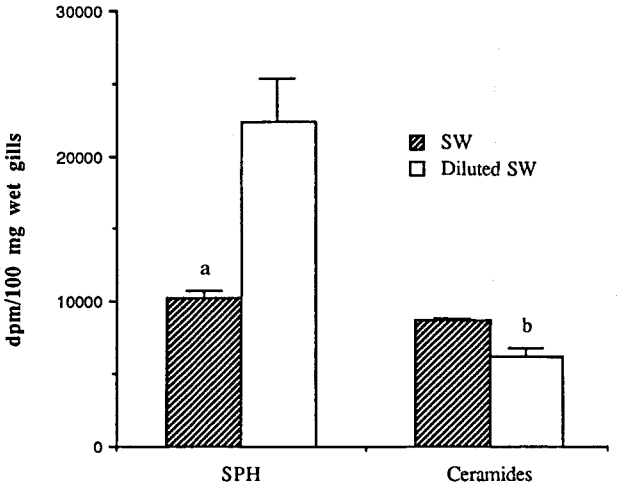

FIG. 3. Sphingomyelin and ceramide radioactivities in gills from SW-adapted sea bass after 4 h of incubation at 17°C in SW or diluted SW containing 15 µCi (^{3}H)serine/mL. Values are means ± SEM of four animals. Values statistically different: a, $P < 0.01$ and b, $P < 0.05$, between gills from SW and diluted SW incubation media; SPH, sphingomyelin. See Figure 1 for other abbreviation.

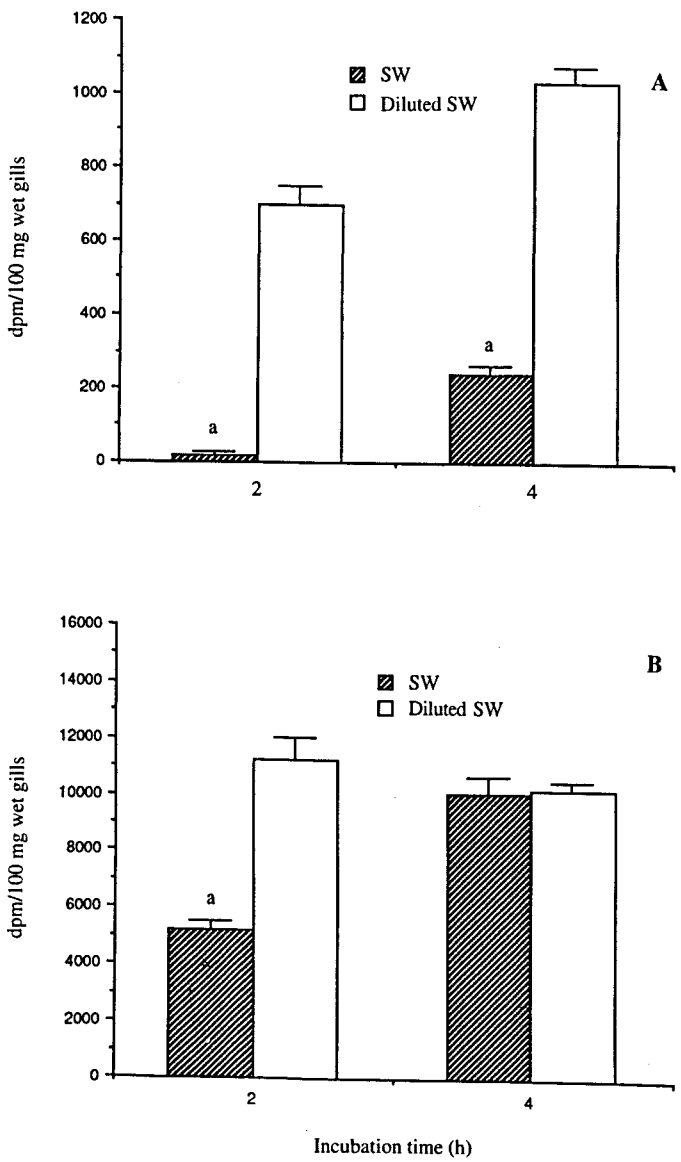

FIG. 2. Influence of medium salinity on the incorporation of [9,10(n)-^{3}H]palmitic acid into (A) gill sphingomyelin and (B) phosphatidylcholine of sea bass. Gills from SW-adapted sea bass were incubated for 2 and 4 h at 17°C in a SW or diluted SW medium containing 5 µCi (^{3}H)palmitic acid/mL. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between gills from SW (salinity 3.7%) and diluted SW (salinity 1%) incubation medium. See Figure 1 for abbreviation.

To test the hypothesis that the increase in SPH biosynthesis is linked with the change of Na^+ flux in branchial cells during salinity changes, we have studied the effect of Na^+ transport modulators on *in vitro* incorporation of L-(^{3}H)serine into SPH of sea bass gills. The effect of ouabain on L-(^{3}H)serine incorporation into gill phospholipids of sea bass incubated in diluted SW is summarized in Figure 4. Ouabain (1 mM) decreased the incorporation of the label into gill SPH (from 16.4 ± 0.5 to 11.9 ± 0.9%) after 4 h of incubation. In the same medium, neither 1 mM N-amidino-3,5-diamino-6-chloropyrazinecaarboxamide (amiloride), nor 1 mM 5-[aminosulfonyl]-4-chloro-2-[2-furanylmethyl)-amino] benzoic acid (furosemide) affected the SPH synthesis. Under the same conditions, however, furosemide strongly increased the incorporation of the label into PC (see Fig. 5). During incubation in SW, only furosemide increased the incorporation of the radiolabel into SPH from 7.6 ± 0.3 to 10.6 ± 0.5%; the other Na^+ modulators had no effect (Fig. 5).

DISCUSSION

This study shows that an abrupt decrease in salinity causes a marked increase in SPH turnover in the gills of euryhaline fish. However, this effect is probably transient because maximal changes appear to occur within 6–48 h. The same pattern

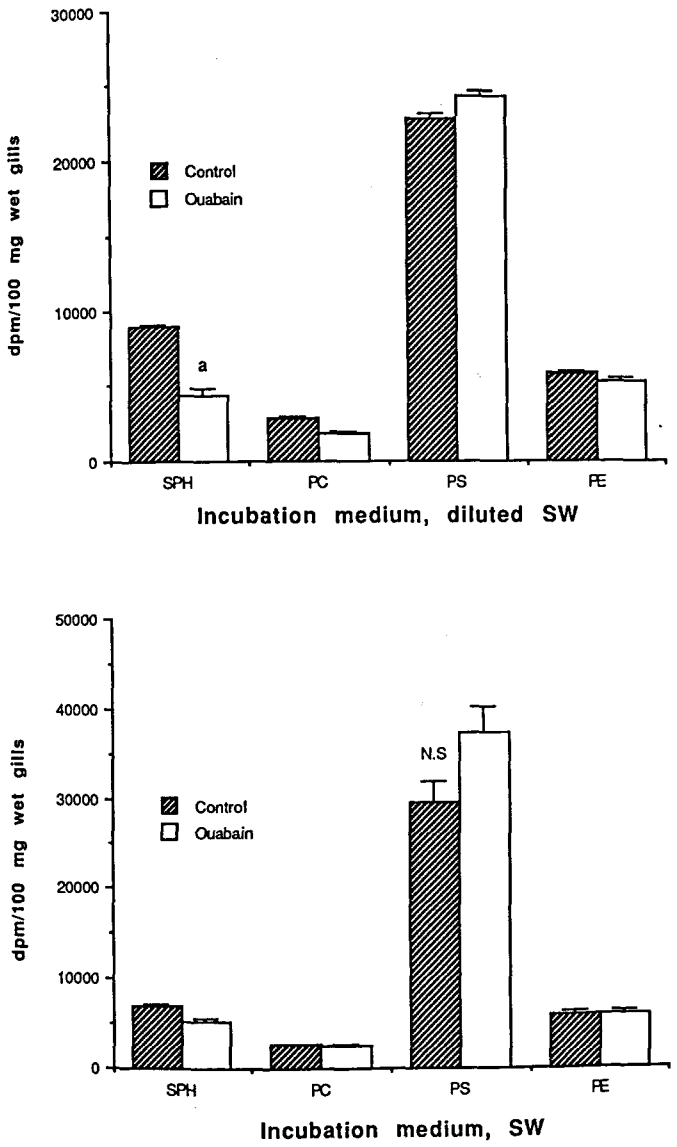

FIG. 4. Effect of 1 mM ouabain on L-(3-^{3}H)serine incorporation into gill phospholipids of sea bass during a 4-h *in vitro* incubation in diluted SW. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between control and ouabain-treated gills; N.S., not significant. See Figure 1 for other abbreviation.

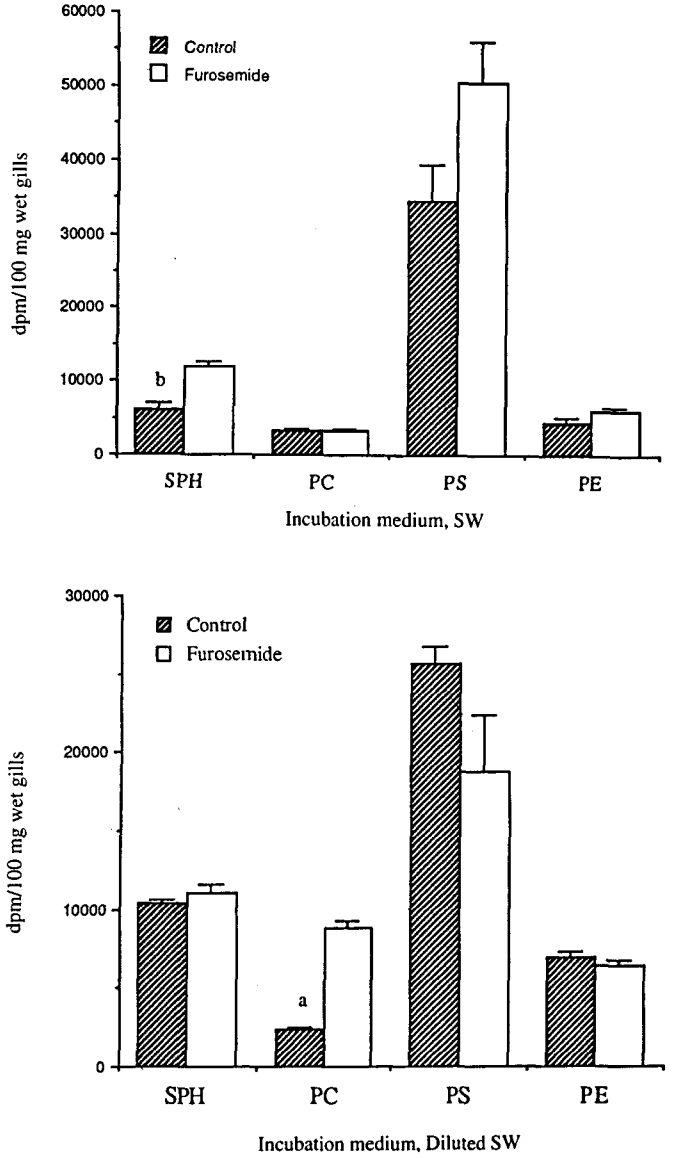

FIG. 5. Effect of 1 mM 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid (furosemide) on L-(3-^{3}H)serine incorporation into gill phospholipids of sea bass during a 4-h *in vitro* incubation in SW or diluted SW. Values are means ± SEM of four animals. Values statistically different: a, $P < 0.01$ and b, $P < 0.05$, between control and furosemide-treated gills; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine. See Figures 1 and 3 for other abbreviations.

is observed in SW- or FW-adapted eels (2). However, 16–36 h after the transfer of SW-adapted sea bass to diluted SW, the percentage of radioactivity incorporated into SPH increases by 24%. Four hours after the salinity change *in vitro*, radioactivity incorporation increases by 110% for SPH and decreases by 36% for free ceramide, an intermediate metabolite. These results demonstrate that an alteration in ceramide metabolism induced by an abrupt change of salinity takes place in the gills of euryhaline fish and that it may mediate some of the cellular rearrangement known to occur after an osmotic shock. Indeed, the number of chloride cells in the gills of euryhaline fish transferred from FW to SW and of hyperosmotic crab transferred from SW to FW increases (13,31).

Relatively little is known about the control of proliferation and distribution of the mitochondria-rich chloride-transporting cells in gills. The induction of the differentiation program of these cells is started by an unknown mechanism. In murine erythroleukemia cells, Kiss *et al.* (32) have shown that treatment with phorbol esters at concentrations sufficient to induce cell differentiation significantly increased the rate of SPH synthesis. In the same cells, the drop of intracellular Na^+ induced by dimethylsulfoxide appears as an early event of cell differentiation (33), and the decrease in Na^+/K^+ pump activity measured by Mayer and Bernstein (34) is probably one of the multiple factors inducing the differentiation program. Similarly, amiloride (a specific inhibitor of Na^+/H^+ exchange) interferes with 12-0-tetradecanoylphorbol 13-acetate (TPA)-induced differentiation of HL-60 cells (35).

We have shown that SPH biosynthesis *in vitro* by gills from SW-adapted sea bass, can be rapidly elevated by incubation in diluted SW. A similar effect has been noted by Girard *et al.* (36) for chloride cells from the gills of SW- compared with FW-adapted eels, in a study of the effect of adrenalin on ^{32}P incorporation into phosphatidic acid and PI.

Under our experimental conditions, the use of different inhibitors of Na^+ transport allowed additional observations. In FW or diluted SW, the pump transporting K^+ from the extracellular space into the cell is ATP dependent and Na^+ coupled (37). This Na^+/K^+ pump is inhibited by ouabain which acts on the basolateral membrane of the transporting cells (38,39). This ouabain-inhibited Na^+/K^+-ATPase is present in the gills of fish, and its activity is strongly enhanced by adaptation to SW in euryhaline fish (40). On the other hand, in FW, the active Na^+ uptake in trout gills is also inhibited by amiloride or furosemide, a second group of Na^+ transport modulators (41). These diuretics inhibit the active transfer of Na^+ on the apical membrane of the ion-transporting cells (42). With SW-adapted sea bass, 1 mM amiloride has no effect on the activation of SPH synthesis observed during *in vitro* incubation in diluted SW medium. It is interesting to note that amiloride has practically no effect on the cellular sodium movements under these conditions (42). Concentration of the Na/K pump inhibitor ouabain, which causes a reduction in sodium efflux (38), has an inhibitory effect on the radiolabel incorporation. Furosemide, another loop diuretic, activates the SPH synthesis in SW but has no effect in diluted SW.

The transient increase in SPH synthesis observed in the gills of euryhaline animals during a sudden decrease of salinity probably corresponds to the increased turnover of a second messenger provided by SPH metabolism in some specialized cells of the gills. This is the first time that the modulation of SPH metabolism in a specialized organ has been linked directly with environmental changes.

Lavie *et al.* (43) have shown in various cultured cell types that the replacement of cell-conditioned medium by HEPES-buffered saline causes a rapidly increase in the levels of endogenous sphingoid bases. In both isolated fish gills and cultured cells, activation or inhibition of the SPH cycle may also be triggered pharmacologically or by changing medium conditions. At least three components of this cycle act as biological signals in cells: ceramides, sphingosine, and sphingosine-1-phosphate (44). The biochemical targets of these intermediates in synthesis or degradation pathways of SPH are unknown; however, ceramides and sphingoid bases act as modulators of protein kinase C activity (45), and these compounds have been shown to regulate cell growth, cell differentiation, programmed cell death and eicosanoid production (17,18).

SPH turnover is influenced by the availability of precursors and by the relative activities of key enzymes in the synthesis and degradation pathway. The synthesis is dependent on the activity of sphingomyelin synthase which transfers the phosphorylcholine head group from PC to ceramide, yielding SPH and diacylglycerol (6,46). The degradation pathway is influenced by the activity of pH-sensitive sphingomyelinase, an enzyme found in plasma membranes and in lysosomes (47), which catalyzes the removal of the SPH head-group to give ceramides. In view of our results, we can hypothesize that the activity of these enzymes in the gills of euryhaline fish may be modulated by changes in intracellular Na^+ or pH induced by variation in external salinity. If this is the case, the increase of SPH turnover (caused by the activation of SPH synthase and/or sphingomyelinase during transfer to diluted SW) may control the levels of intermediate compounds such as sphingoid bases, ceramides or diacylglycerol. In turn, these intermediates would act as signals controlling protein kinase C activity, thereby initiating the activation or inhibition of chloride cell differentiation. The report by Kolesnick (48), in which sphingomyelinase prevents the morphologic changes associated with TPA-induced conversion to the macrophage phenotype in HL-60 cells, supports this hypothesis. However, in HL-60 sensitive-cells, Okazaki *et al.* (49) found that 1-α,25-dihydroxyvitamin D_3 activates a sphingomyelinase that increases SPH turnover and causes the formation of intracellular ceramides, which then induce cell differentiation to the monocytic phenotype. These observations do not allow identification of the intermediate of the SPH pathway, which controls chloride cell proliferation in the gills of euryhaline fish during abrupt changes in salinity.

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REFERENCES

1. Whitney, J.O. (1974) The Effect of External Salinity upon Lipid Synthesis in the Blue Crab *Callinectes sapidus* Rathbun and the Spider Crab *Libinia emarginata* Leech, *Comp. Biochem. Physiol. 49A*, 433–440.
2. Zwingelstein, G. (1982) Les Lipides Amphiphiles du Poisson, in *Nutrition du Poisson* (Fontaine, M., ed.) pp. 249–262, Editions du CNRS, Paris.
3. Chapelle, S. (1984) Contribution à l'Etude du Métabolisme des Phospholipides chez les Crustacés, Ph.D. Thesis, Université de Liége, Liège, Belgium, pp. 23–87.
4. Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J., and Tocher, D.R. (1993) The Metabolism of Phospholipids and Polyunsaturated Fatty Acids in Fish, in *Aquaculture* (Lalhou, B., and Vitiello, P., eds.) pp. 103–124, American Geophysical Union, Washington.
5. Chapelle, S., Dandrifosse, G., and Zwingelstein, G. (1976) Metabolism of Phospholipids of Anterior or Posterior Gills of the Crab *Eriocheir sinensis* M. Edw. During the Adaptation of This Animal to Media of Various Salinities, *Int. J. Biochem. 7*, 343–351.
6. Zwingelstein, G. (1979–1980) Les Effets de l'Adaptation à l'Eau de Mer sur le Métabolisme Lipidique du Poisson, *Oceanis 5*, 117–130.
7. Chapelle, S., and Zwingelstein, G. (1984) Phospholipid Composition and Metabolism of Crustacean Gills as Related to Changes in Environmental Salinities: Relationship Between

Na^+/K^+ ATPase Activity and Phospholipids, *Comp. Biochem. Physiol. 78B*, 363–372.
8. Merrill, A.H., and Jones, D.D. (1990) An Update of the Enzymology and Regulation of Sphingomyelin Metabolism, *Biochim. Biophys. Acta 1044*, 1–12.
9. Payan, P., Girard, J.P., and Mayer-Gostan, N. (1984) in *Fish Physiology* (Hoar, W.S., Randall, D.J., and Brett, J.R., eds.) pp. 241–283, Academic Press, London.
10. Péqueux, A., and Gilles, R. (1981) Na^+ Fluxes Across Isolated Perfused Gills of Chinese Crab *Eriocheir sinensis, J. Exp. Zool. 92*, 173–186.
11. Krogh, A. (1939) *Osmotic Regulation in Aquatic Animals*, Cambridge University Press, London, New York.
12. Maetz, J. (1974) in *Biochemical and Biophysical Perspectives in Marine Biology* (Malins, D.C., and Sargent, J.R., eds.) Vol.1, pp. 1–167, Academic Press, London.
13. Zadunaisky, J.A. (1984) The Chloride Cells: Active Transport of Chloride and the Paracellular Pathways, in *Fish Physiology*, (Hoar, W.S., Randall D.J., and Brett, J.R., eds.) pp. 13–176, Academic Press, London.
14. Foskett, J.K., and Scheffey, C. (1982) The Chloride Cells: Definitive Identification as the Salt-Secretory Cells in Teleosts, *Science 215*, 164–166.
15. Neufeld, G.J., Holliday, C.W., and Pritchard, J.B. (1980) Salinity Adaptation of Gill Na,K-ATPase in the Blue Crab, *Callinectes sapidus, J. Exp. Zool. 211*, 215–224.
16. Brichon, G. (1984) Régulation Physiologique du Métabolisme des Glycérophospholipides Azotés chez l'Anguille Européenne. Effets de l'Adaptation en Eau Douce ou en Eau de Mer à Deux Températures (12 et 22°C), Thèse es-sciences naturelles, Universite Claude Bernard, Lyon, p. 200.
17. Hakomori, S-I. (1981) Glycosphingolipids in Cellular Interaction, Differentiation, and Oncogenesis, *Ann. Rev. Biochem. 50*, 733–764.
18. Hannun, Y.A., and Bell, R.M. (1989) Functions of Sphingolipids and Sphingolipid Breakdown Products in Cellular Regulation, *Science 243*, 500–507.
19. Merrill, A.H., Serini, A.M., Stevens, V.L., Hannun, Y.A., Bell, R.M., and Kinkade Jr., J.M. (1986) Inhibition of Phorbol Ester-Dependent Differentiation of Human Promyelocytic Leukemic (HL-60) Cells by Sphinganine and Other Long-Chain Bases, *J. Biol. Chem. 261*, 12610–12615.
20. Kim M-Y., Linardic C.M., Karolak L.A., and Hannun, Y.A. (1991) Identification of Sphingomyelin Turnover as an Effector Mechanism for the Action of Tumor Necrosis Factor α and γ-Interferon, *J. Biol. Chem. 266*, 484–489.
21. Obeid L.M., Linardic, C.M., Karolac, L.A., and Hannun, Y.A. (1993) Programmed Cell Death Induced by Ceramide, *Science 259*, 1769–1771.
22. Folch, J., Lees, M., and Stanley, G.H.S. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
23. Chapelle, S., Zwingelstein, G., Meister, R., and Brichon, G. (1979) The Influence of Acclimation Temperature on the Phospholipid Metabolism of an Aquatic Crustacea (*Carcinus maenas*), *J. Exp. Zool. 210*, 371–380.
24. Portoukalian, J., Meister, R., and Zwingelstein, G. (1978) Improved Two-Dimensional Solvent System for Thin-Layer Chromatographic Analysis of Polar Lipids on Silica Gel 60 Precoated Plates, *J. Chromatogr. 152*, 569–574.
25. Dittmer, J.C., and Lester, R.L. (1964) A Simple Specific Spray for the Detection of Phospholipids on Thin-Layer Chromatograms, *J. Lipid Res. 5*, 126–127.
26. Pyrovolakis, J.A., Harry, D.S., Martin, M.J., and McIntyre, N. (1974) A Simple Method for Liquid Scintillation Counting of Weak β-Emitting Labelled Lipids after Separation by Thin-Layer Chromatography, *Clin. Chim. Acta 50*, 441–444.
27. Arvidson, G.A.E. (1968) Structural and Metabolic Heterogeneity of Rat Liver Glycerophosphatides, *Eur. J. Biochem. 4*, 478–486.
28. Vance, D.E., and Sweeley, C.C. (1967) Quantitative Determination of the Neutral Glycosyl Ceramides in Human Blood, *J. Lipid Res. 8*, 621–630.
29. Karlsson, K.A. (1968) Enzymatic Hydrolysis of Sphingomyelins: Use in Structure Analysis, *Acta Chem. Scand. 22*, 3050–3052.
30. Gaver, R.C., and Sweeley, C.C. (1965) Methods for Methanolysis of Sphingolipids and Direct Determination of Long-Chain Bases by Gas Chromatography, *J. Am. Oil Chem. Soc. 42*, 294–298.
31. Gilles, R., and Péqueux, A. (1985) Ion Transport in Crustacean Gills: Physiological and Ultrastructural Approaches, in *Transport Processes, Iono- and Osmoregulation: Current Comparative Appraches* (Gilles, R., and Gilles-Baillien, M., eds.) pp. 137–158, Springer Verlag, Heidelberg.
32. Kiss, Z., Deli, E., and Kuo, J.F. (1988) Phorbol Ester Stimulation of Sphingomyelin Synthesis in Human Leukemic HL60 Cells, *Arch. Biochem. Biophys. 265*, 38–42.
33. Lannigan, D.A., and Knauf, P.A. (1985) Decreased Intracellular Na^+ Concentration is an Early Event in Murine Erythroleukemic Cell Differentiation, *J. Biol. Chem. 260*, 7322–7324.
34. Mayer, D., and Bernstein, A. (1978) Early Transport Changes during Erythroid Differentiation of Friend Leukemic Cells, *J. Cell. Physiol. 94*, 275–285.
35. Besterman, J.M., May Jr., W.S., LeVine, H., Cragoe Jr., E.J., and Cuatrecasas, P. (1985) Amiloride Inhibits Phorbol Ester-Stimulated Na^+/H^+ Exchange and Protein Kinase C. An Amiloride Analog Selectively Inhibits Na^+/H^+ Exchange, *J. Biol.Chem. 260*, 1155–1159.
36. Girard, J-P., Thomson, A.J., and Sargent, J.R. (1977) Adrenalin Induced Turnover of Phosphatidic Acid and Phosphatidyl Inositol in Chloride Cells from the Gills of *Anguilla anguilla*, *FEBS Lett. 73*, 267–270.
37. Pressley, T.A., Graves, J.S., and Krall, A.R. (1981) Amiloride-Sensitive Ammonium and Sodium Ion Transport in the Blue Crab, *Am. J. Physiol. 241*, R370–378.
38. Glynn, I.M., and Karlish, S.J.D. (1975) The Sodium Pump, *Ann. Rev. Physiol. 37*, 13–56.
39. Payan, P., Matty, A.J., and Maetz, J. (1975) A Study of the Sodium Pump in the Perfused Head Preparation of the Trout *Salmo gairdneri* in Fresh Water, *J. Comp. Physiol. 104*, 33–48.
40. Kamiya, M., and Utida, S. (1969) Sodium-Potassium-Activated Adenosinetriposphatase Activity in Gills of Fresh-Water, Marine and Euryhaline Teleosts, *Comp. Biochem. Physiol. 31*, 671–674.
41. Kirschner, L.B., Greenwald, L., and Kerstetter, T.H. (1973) Effect of Amiloride on Sodium Transport Across Body Surfaces of Fresh Water Animals, *Am. J. Physiol. 224*, 832–837.
42. Greenwald, L., and Kirschner, L.B. (1976) The Effect of Poly-L-Lysine, Amiloride and Methyl-L-Lysine on Gill Ion Transport and Permeability in the Rainbow Trout, *J. Memb. Biol. 26*, 371–383.
43. Lavie, Y., Blusztajn, J.K., and Liscovitch, M. (1994) Formation of Endogenous Free Sphingoid Bases in Cells Induced by Changing Medium Conditions, *Biochim. Biophys. Acta 1220*, 232–238.
44. Hannun, Y.A., and Linardic, C.M. (1993) Sphingolipid Breakdown Products: Anti-Proliferative and Tumor-Suppressor Lipids, *Biochim. Biophys. Acta 1154*, 223–236.
45. Hannun, Y.A., Loomis, C.R., Merrill Jr., A.H., and Bell, R.M. (1986) Sphingosine Inhibition of Protein Kinase C Activity and Phorbol Dibutyrate Binding *in vitro* in Human Platelets, *J. Biol. Chem. 261*, 12604–12609.

46. Hampton, R.Y., and Morand, O.H. (1989) Sphingomyelin Synthase and PKC Activation, *Science 246*, 1050.
47. Barnholz, Y., Roitman, A., and Gatt, S. (1966) Enzymatic Hydrolysis of Sphingolipids. II. Hydrolysis of Sphingomyelin by an Enzyme from Rat Brain, *J. Biol. Chem. 241*, 3731–3737.
48. Kolesnick, R.N. (1989) Sphingomyelinase Action Inhibits Phorbol Ester-Induced Differentiation of Human Promyelocytic Leukemic (HL-60) Cells, *J. Biol. Chem. 264*, 7617–7623.
49. Okazaki, T., Bielawska, A., Bell, R.M., and Hannun, Y.A. (1990) Role of Ceramides as a Lipid Mediator of 1α,25-Dihydroxyvitamin D_3-Induced HL-60 Cell Differentiation, *J. Biol. Chem.* 265, 15823–15831.

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