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5′-NUCLEOTIDASE AND ALKALINE PHOSPHODIESTERASE ACTIVITIES IN TROUT GILL LOCALIZE TO ENDOTHELIAL (PILLAR) CELLS

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Summary

Tissue homogenates from rainbow trout gill had three- to fivefold higher specific activity for 5′-nucleotidase (5′NT) and more than twofold greater alkaline phosphodiesterase (APD) activity than liver or kidney homogenates. In isolated plasma membranes, gill 5′NT activity was 3–5 times greater than that of the kidney or liver; gill and kidney plasma membranes had similar APD specific activities, both more than five times that of liver. 5′NT and APD activities were localized by histochemistry to the endothelial (pillar) cells of trout gill secondary lamellae. Staining was consistent with the concentration of both activities at the apical plasma membranes of pillar cells (i.e. at the lamellar microvascular surfaces). This localization may reflect a capacity for processing nucleotide metabolites circulating in the blood, perhaps relating to purinergic regulation of local lamellar hemodynamics. There was no histochemical evidence of either 5′NT or APD activity in the gill epithelial (pavement) cells that interface directly with the environment. In contrast, in trout kidney, both enzyme activities localized to the apical region of tubule epithelial cells. The absence of 5′NT and APD activity in pavement cells reinforces the unique structural and functional character of the gill–environment epithelial barrier. The results indicate that 5′NT and APD activities have particular potential application as markers in efforts to isolate and characterize specific gill plasma membrane fractions.

Key words: rainbow trout, *Oncorhynchus mykiss*, 5′-nucleotidase, alkaline phosphodiesterase, gill, pillar cell, secondary lamellae, plasma membrane, marker enzymes.

Introduction

The gill is the site of several important homeostatic processes in teleosts; gas exchange, iono/osmoregulation, acid–base regulation and the elimination of nitrogenous waste occur principally at the gill epithelium (Hughes and Morgan, 1973; Evans, 1993; Goss *et al.* 1992; Wilkie, 1997). This range of functions and the direct interfacial disposition of the gills with the ambient environment make this tissue particularly remarkable among physiologically active epithelia.

Amplification of exchange surface areas underlies gill function and is conferred morphologically by extensive epithelial outfolding from the gill filaments. The flattened gill epithelial (pavement) cells comprise more than 95 % of the ambient-facing gill surface area in freshwater fish (Isaia, 1984); chloride cells and mucous cells are also present in the gill epithelium (Laurent and Dunel, 1980). Within each thin epithelial leaflet, or secondary lamella (see Fig. 2B), a central sinusoidal network produces an extensive vascular surface with minimal blood-to-water diffusion distances (Hughes and Morgan, 1973). The secondary lamellar microvasculature is structurally supported and lined by unique endothelial cells termed pillar cells (Olson, 1991).

Revealing the structural basis for gill functions has contributed greatly towards a mechanistic appreciation of this physiologically dynamic tissue. For example, recent evidence localizing a vacuolar H^+-ATPase at the apical plasma membrane of pavement cells has significant impact on models of acid–base regulation and Na^+ uptake in freshwater fish (Lin *et al.* 1994; Sullivan *et al.* 1995). The significance of structure–function relationships extends to the gill circulatory system, where regulated adjustments in perfusion patterns influence gill function and components of the branchial vasculature interact with circulating bioactive molecules (Olson, 1991, 1998).

A number of enzymes, particularly those implicated in ion regulation (e.g. Na^+/K^+-ATPase or H^+-ATPase), have attracted significant attention in studies of gill physiology. However, the occurrence of other enzymes and their relationship to gill function remain largely unexplored. Recently, in reporting temperature-acclimation-associated changes in the cholesterol levels of plasma membranes isolated from different tissues of trout, we noted elevated levels of activity in gill for several marker enzymes specific to the apical domain of epithelial plasma membranes (Robertson and Hazel, 1995).

These data are intriguing because (i) high levels of enzyme activity suggest (teleologically) some role in gill function, and (ii) plasma-membrane-associated enzyme activities could be

useful as markers in efforts to isolate and characterize specific gill cell types or particular membrane fractions (e.g. the apical plasma membrane of gill epithelial cells, which constitutes the primary gill–environment barrier membrane). The morphology of the secondary lamellae accommodates two likely localizations for apical plasma membrane enzymes: at the pavement cell barrier membrane and/or at the pillar cell membrane lining the microvascular surface (see Fig. 2B).

Here, we investigate two plasma membrane marker enzyme activities found to be elevated in trout gill, 5′-nucleotidase (5′NT) and alkaline phosphodiesterase (APD). Continuous-time enzyme assays are utilized to confirm and extend prior observations concerning these activities in homogenates and isolated plasma membranes from the gill and other tissues of trout. In addition, APD and 5′NT activities are localized using histochemistry to the endothelial (pillar cell) compartment of the gill secondary lamellae. The implications of these findings are discussed in relation to gill function and their potential application in studies involving gill membrane isolation.

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)], obtained from the Alchesay National Fish Hatchery (Alchesay, AZ, USA), were housed in the aquaculture facilities of the Animal Resource Center at Arizona State University. Fish were maintained in fresh water at 20 °C under recirculating culture conditions; animals were held under a constant 12 h:12 h L:D cycle and were fed a commercial diet to satiation daily. The body masses of the trout used in this study ranged 100 to 300 g.

Tissue homogenate and plasma membrane preparations

Complete details of these procedures have been published (Robertson and Hazel, 1995). Briefly, after the trout had been killed by a blow to the head, the tissues were quickly excised for processing. Crude homogenates were prepared from gill (epithelial scrapings), liver and kidney (whole organs); samples for enzyme assays were stored at −70 °C in 250 mmol l^{-1} sucrose, 20 mmol l^{-1} Tris buffer (pH 7.4). Plasma membranes, isolated using further sucrose and Percoll density gradient centrifugations, were stored in 20 mmol l^{-1} Tris buffer (pH 7.4) at −70 °C until assayed. The protein content of preparations was measured using the bicinchoninic acid method (Smith *et al.* 1985).

5′-Nucleotidase enzyme assay

A coupled, continuous-time spectrophotometric assay was adapted (Pieri *et al.* 1992; Belfield and Goldberg, 1969; Ipata, 1967) to measure the specific activity of 5′NT (EC 3.1.3.5) in trout samples. In this assay, the production of inosine from adenosine (by adenosine deaminase) is consequent to the liberation of adenosine from AMP (by 5′NT). Final reaction mixtures (total volume 1.0 ml) contained 50 mmol l^{-1} Tris (pH 7.6), 15 mmol l^{-1} β-glycerophosphate, 1 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} AMP, 2.5 μg ml^{-1} (0.4 units ml^{-1}) adenosine deaminase and 25 μl of homogenate or membrane sample. Assays were run at room temperature (23 °C); the linear decrease in absorbance at 264 nm was followed over 12 min using a Hewlett Packard 8452A diode array spectrophotometer. Because inosine has a lower molar absorbance than AMP at 264 nm, enzyme-mediated conversion results in declining absorbance readings. 5′NT activity was quantified from the negative slope of absorbance over time using the difference between the extinction coefficients of AMP and inosine under the assay conditions (i.e. 8.1×10^3 cm^{-1} mol^{-1}).

Alkaline phosphodiesterase enzyme assay

The activity of APD (EC 3.1.4.1; also commonly referred to as phosphodiesterase I) was determined according to the continuous-time method of Razzell (1963). Reaction mixtures (1.0 ml final volume) contained 100 mmol l^{-1} Tris (pH 8.9), 0.5 mmol l^{-1} thymidine 5′-monophosphate *p*-nitrophenol ester (Sigma, St Louis, MO, USA) and 25 μl of homogenate or membrane sample. The linear increase in absorbance at 400 nm, due to enzymatic liberation of *p*-nitrophenol from substrate, was followed at room temperature over 12 min. APD activity was calculated using an extinction coefficient of 1.8×10^4 cm^{-1} mol^{-1} for *p*-nitrophenol. When included in reactions, $MgCl_2$ and EDTA were added as 25 μl samples of stock solutions prepared in assay buffer. Some assays were also carried out using an alternative substrate (1 mmol l^{-1} *p*-nitrophenol phenylphosphonate; Sigma) in 100 mmol l^{-1} Tris at pH 8.1 (Kelley *et al.* 1975).

Tissue preparation for histochemistry

The first three gill arches on both sides were quickly dissected free and gently blotted to remove any surface blood. Gill filaments were trimmed free from arches at the base of filaments and cut into approximately 1 cm panels; owing to the presence of the interfilament septum, panels remained as contiguous units. Tissue was immediately frozen by being placed for 1–2 min in hexane cooled to approximately −70 °C by immersion in a methanol/dry ice slurry. Frozen gill tissue was then kept on dry ice for cryosectioning the same day. For kidney tissue, segments of the whole organ 1–2 cm in length were carefully isolated from the posterior region of the body cavity and treated as above.

In perfusing the gills for histochemical studies, an isolated head method was used to clear the gill vasculature with Cortland's trout saline, as previously described (Robertson and Hazel, 1995). Effective perfusion was judged by uniform bleaching of filaments; perfused gills were subsequently processed for histochemistry as noted.

Cryosectioning and general histochemical procedures

Frozen tissue was sectioned at 10–12 μm on a cryostat. Sections were mounted on microscope slides and stored at −20 °C for up to 48 h. Immediately prior to histochemical reactions, sections were air-dried at room temperature (23 °C) for 10 min. Enzyme incubations were performed at room temperature by flooding tissue sections with reaction mixture. After enzyme reactions, sections were fixed in 4%

formaldehyde for 10 min, rinsed several times in distilled water, mounted in glycerol jelly and examined under a light microscope. The results described are based on an analysis of 2–5 tissue samples from at least three fish.

5′NT histochemistry

A modified lead salt capture method (Frederiks and Marx, 1988) was used to localize 5′NT activity. The basic reaction mixture contained 100 mmol l^{-1} Tris maleate buffer (pH 7.2), 10 mmol l^{-1} $MgCl_2$, 7.2 mmol l^{-1} $PbNO_3$, 17 % polyvinyl alcohol and 5 mmol l^{-1} AMP. Enzymatic hydrolysis of substrate (AMP) produces phosphate, which reacts with $PbNO_3$ to form a precipitate of lead phosphate at the site of 5′NT activity. The best results with trout tissue sections were obtained using 10–15 min incubations in this primary reaction mixture. Reactions were terminated by rinsing sections in distilled water. To visualize reaction product, lead phosphate was converted to lead sulfide by incubating the tissue in 1 % ammonium sulfide for 1 min.

Routine negative controls included the omission of substrate from the reaction mixture and the use of sections that had been heat-inactivated by immersion in distilled water at 100 °C for 5 min. Typically, negative controls were also incubated for at least twice as long as concurrent positive sections. To confirm the specificity of 5′NTase activity, other control reaction mixtures were employed as detailed in the Results section.

APD histochemistry

The activity of APD was localized using a direct azo dye coupling technique (Sierakowska and Shugar, 1963; Sierakowska *et al.* 1963). The reaction mixture was made up of 100 mmol l^{-1} Tris (pH 9.0) containing 2 mg ml^{-1} thymidine 5′-monophosphate *p*-naphthyl ester (Sigma) and 0.8 mg ml^{-1} Fast Red TR. Naphthol liberated from the substrate reacts with Fast Red TR to form an insoluble red product marking the location of APD activity. The optimal time for incubation of trout cryosections was found to be 20–30 min. Reactions were stopped by rinsing sections in distilled water. The omission of substrate from the incubation mixture and the use of heat-inactivated sections were employed as standard negative controls.

Results

Enzyme specific activities

In crude tissue homogenates, mean 5′NT specific activity in gill was five times higher than in kidney and more than three times that in liver (Table 1). Plasma membranes from each of these tissues were isolated by density gradient centrifugation; purified gill membranes had 3–5 times greater 5′NT specific activity than those from kidney and liver, respectively.

The mean specific activity of APD in crude homogenates was more than two times higher in gill than in liver or kidney (Table 1). Plasma membranes isolated from gill and kidney had similar APD activities, both more than five times that of liver membranes.

To our knowledge, APD activity in fish gills has not been examined previously. As a basis for comparing the activity present in trout gills with that characterized from other sources, we manipulated components of APD reaction mixtures. Although the addition of exogenous divalent cation was not required for activity, including increasing concentrations of $MgCl_2$ (up to 1.6 mmol l^{-1}) in reaction mixtures potentiated APD activity in plasma membranes isolated from gill (Fig. 1). The addition of a divalent cation chelator (EDTA) to the reaction mixtures abolished enzyme activity. Similar results (not shown) were obtained for trout liver and kidney plasma membrane APD activity. Assays employing an alternative substrate (*p*-nitrophenol phenylphosphonate) resulted in 10- to 100-fold lower APD specific activities in plasma membranes prepared from all three trout tissues (data not shown).

Enzyme histochemistry

The functional significance of elevated activities of 5′NT and APD in trout gills could well relate to their cellular localization. To examine this further, we used enzyme histochemistry to localize 5′NT and APD in trout gills. For histochemical studies, trout gills were rapidly frozen and cryosectioned along the long axis of filaments (Fig. 2A).

5′-Nucleotidase

The histochemical reaction product for 5′NT unambiguously localized to the core of the secondary lamellae in trout gills (Figs 3A,B, 4). This localization corresponds to the pillar

Table 1. *Specific activities of 5′-nucleotidase (5′NT) and alkaline phosphodiesterase (APD) in trout tissues measured using continuous-time spectrophotometric assays*

		Gill	Kidney	Liver
5′NT	Crude homogenates (nmol min^{-1} mg^{-1})	8.2±0.6	1.6±0.1	2.4±0.3
	Plasma membranes (nmol min^{-1} mg^{-1})	53.6±3.9	16.7±1.4	10.1±0.6
	Purification factor	6.5	10.4	4.2
APD	Crude homogenates (nmol min^{-1} mg^{-1})	8.4±0.5	3.7±0.2	3.3±0.3
	Plasma membranes (nmol min^{-1} mg^{-1})	100.6±8.0	118.9±7.1	18.4±3.8
	Purification factor	12	32.1	5.6

Values for crude homogenates and density-gradient-isolated plasma membranes are means ± S.E.M. of five samples.
The membrane purification factor is the ratio of the specific activity of plasma membranes to that of crude homogenates.

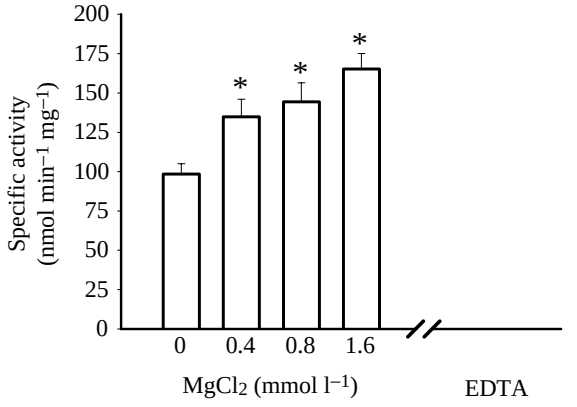

Fig. 1. Divalent cation requirements of alkaline phosphodiesterase (APD) in plasma membranes isolated from trout gill. Specific activity is potentiated by including increasing concentrations of $MgCl_2$ in reaction mixtures and is abolished in the presence of 0.5 mmol l^{-1} EDTA. Values are means + S.E.M. (N=5). An asterisk indicates a value significantly different from that in the absence of $MgCl_2$ (t-tests; P<0.05).

(endothelial) cells which form the lamellar microvascular network (Fig. 2B). Significantly, the overlying pavement (gill epithelial) cells showed no indication of reaction product, even in sections reacted three times longer than was found to produce an optimal signal in pillar cells.

In sections cut perpendicular to the plane of the secondary lamellae (i.e as in Figs 2A or 3), 5′NT reaction product was not homogeneously distributed. A pattern of intermittent, regularly spaced bands of intense staining was typical in these sections (Fig. 3A,B). In many instances, high concentrations of reaction product could clearly be seen to be associated with the margins of vascular spaces (Figs 3B, 4B). This pattern indicates that greatest 5′NT activity is located along the walls of secondary lamellar blood channels, which are formed by pillar cell apical plasma membranes (see Fig. 2B).

The folding of some respiratory leaflets resulted in sections cut parallel to the plane of the lamellae. In these cases, dense 5′NT reaction product was distributed in a pattern of rings within each lamellae (Fig. 4). These circular profiles again appear to correspond to the plasma membranes of the pillar cell trunks which delimit the secondary lamellar blood spaces (see Fig. 2B). This interpretation is further supported by the apparent presence of a central (pillar cell) nucleus within many of the rings.

To investigate the possible contribution of blood constituents to these results, we cleared trout gills by perfusion with Cortland's saline and then processed the tissue for histochemistry. An identical pattern of staining for 5′NT (and APD) activity was found in sections from perfused and non-perfused animals, indicating that humoral factors were unlikely to have been responsible for the observed localization of activity.

Cartilage and the vascular endothelium of gill filaments also stained for 5′NT (and APD) activity. However, our present interests concern the exchange surfaces associated with functions specific to the secondary lamellae. In addition, specific activity data were generated from gill epithelial scrapings, which are compositionally weighted towards respiratory lamellar elements. Thus, the localization described here is limited to the secondary lamellae.

Omitting substrate (AMP) from 5′NT reaction mixtures (Fig. 3C) or heat-treating sections at 100 °C for 5 min prior to incubation (not shown) resulted in a lack of formation of reaction product. To ensure that the results obtained using this histochemical method specifically reflected 5′NT activity, a number of other control conditions were used (data not shown). Incorporation of 50 mmol l^{-1} sodium fluoride, 10 mmol l^{-1} ADP or 10 mmol l^{-1} EDTA in the incubation mixtures eliminated the staining pattern seen when these inhibitors were not present.

Additionally, even though 5′NT histochemistry reactions were carried out under nearly neutral conditions (pH 7.2), the possible contribution of alkaline phosphatase to the observed results was tested. No effect on the pattern or intensity of reaction product formed was observed when an inhibitor of alkaline phosphatase (10 mmol l^{-1} tetramisole) was added to the reaction mixtures. Thus, the results obtained using this method in trout gill parallel those obtained for rat liver (Frederiks and Marx, 1988) and are consistent with specific histochemical localization of 5′NT.

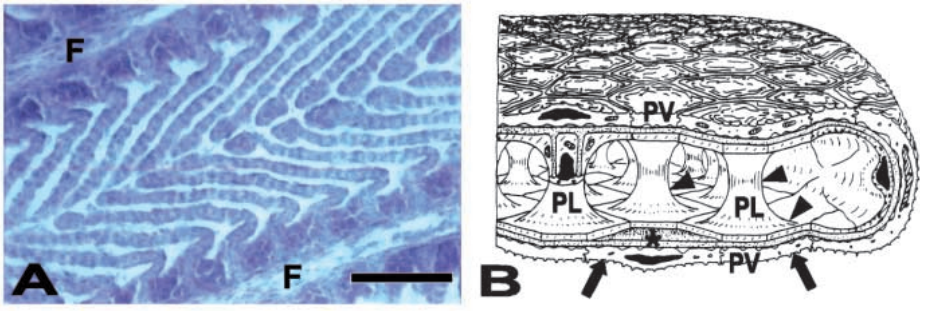

Fig. 2. Trout gill secondary lamellar structure. (A) In gill sectioned along the long axis of two adjacent filaments (F), many secondary lamellae project into the interfilament space. Here, lamellae are cut perpendicular to the plane of blood flow. Cryostat section (10 µm thick), stained with 0.6 % Toluidine Blue. Scale bar, 50 µm. (B) Schematic representation detailing the cytoarchitecture of a secondary lamella. Pavement cells (PV) lie on the gill surface, their apical plasma membranes representing the gill–environment barrier membrane (arrows). Pillar cells (PL) define the central vascular network of the lamellae; their apical plasma membranes form the lining of the blood space (arrowheads). At their basal plasma membranes, pavement and pillar cells share a basement membrane (*). Columns extending through the pillar cells provide a structural link between the basement membranes on opposing sides of the lamellae. Redrawn from Olson (1991) with permission.

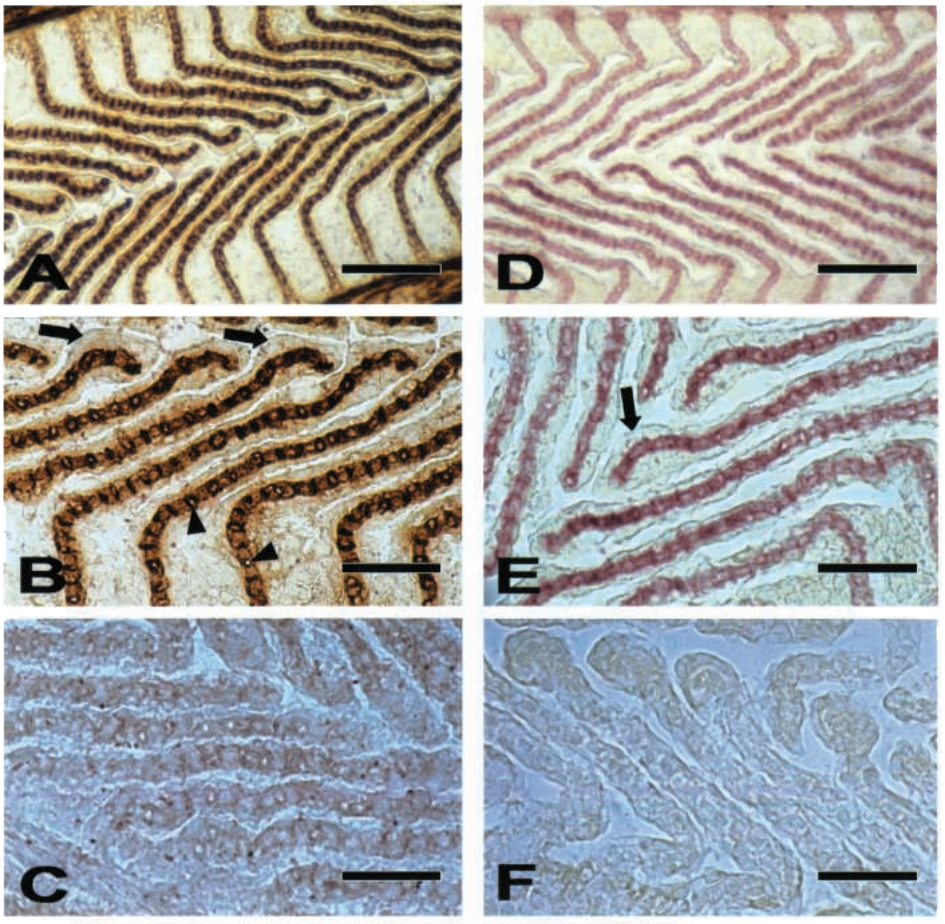

Fig. 3. Enzyme histochemistry of trout gill secondary lamellae. (A) Brown reaction product for 5′-nucleotidase (5′NT) histochemistry is localized to the core of the secondary lamellae. (B) At higher magnification, staining for 5′NT activity is clearly restricted to the pillar cells of the lamellae. The overlying pavement cells show no indication of enzyme activity (arrows). Reaction product is distributed in a banded pattern within the lamellar endothelium; in some cases, staining is clearly concentrated around the margins of vascular spaces (arrowheads). (C) No product formation is seen when substrate (AMP) is omitted from the 5′NT histochemistry reaction mixture. (D) Histochemistry for alkaline phosphodiesterase (APD) produces a red reaction product which is clearly restricted to the secondary lamellar endothelium. (E) Again, no APD activity is detectable in the pavement cells (arrow). (F) Omission of substrate during APD histochemistry incubations eliminates reaction product formation. Scale bars: A, D, 50 μm; B, C, E and F, 25 μm.

Alkaline phosphodiesterase

Preliminary studies indicated that an APD histochemistry method developed for rat tissues (Sierakowska and Shugar, 1963) could be effectively employed in trout gill sections with some modification. Specifically, it was necessary to reduce the concentration of capture dye (Fast Red TR) to 0.8 mg ml^{-1} (from 4 mg ml^{-1}) in reaction mixtures in order to achieve an optimal histochemical signal for APD activity in trout gills. Polyvinyl alcohol (PVA) has been reported to improve the histochemical localization of several enzyme activities (Frederiks and Marx, 1988). However, the inclusion of 17 % PVA in APD reaction mixtures was found not to affect the localization of reaction product; therefore, PVA was not used in APD histochemistry.

The results of APD histochemistry of trout gill cryosections were very similar to those for 5′NT. ADP reaction product was exclusively localized to the core endothelial compartment of the secondary lamellae (Fig. 3D,E). Again, there was no indication of APD activity associated with the pavement cells, even in sections exposed to extended incubation. As was found for 5′NT, the most intense staining for APD activity in gill sections was consistent with an apical plasma membrane (microvascular wall) pillar cell disposition.

Omitting substrate from APD incubation mixtures resulted in an absence of reaction product formation in gill sections (Fig. 3F). Negative results were also obtained when 10 mmol l^{-1} EDTA was included in the reaction mixtures or when sections were heat-treated (100 °C, 5 min) prior to incubation (not shown).

Kidney

In view of the apparent lack of 5′NT or APD activity in gill epithelial (pavement) cells, we wanted to verify the ability to resolve histochemically an apical epithelial localization in a trout tissue. On the basis of work in other vertebrates, 5′NT and APD are expected to be localized at the apical plasma membrane of epithelial cells in kidney tubules (Le Hir and Kaissling, 1993; Sierakowska *et al.* 1963). Enzyme histochemistry of trout kidney sections thus represents an additional level of internal control for our studies. Moreover, there appears to be a lack of available data demonstrating localization of these enzymes in the teleost kidney.

The reaction product for 5′NT was concentrated at the lumen of trout kidney tubules (Fig. 5A). Other than in apical regions, the epithelial cells comprising the tubules did not show evidence of histochemical staining. Diffusely distributed 5′NT reaction product was also seen in the interstitium around the tubules. Similarly, intense APD histochemical staining in trout kidney sections was restricted to the luminal region of tubules (Fig. 5B). These results are thus consistent with an apical plasma membrane localization for 5′NT and APD activities in trout kidney epithelial cells. For both 5′NT and APD, not all

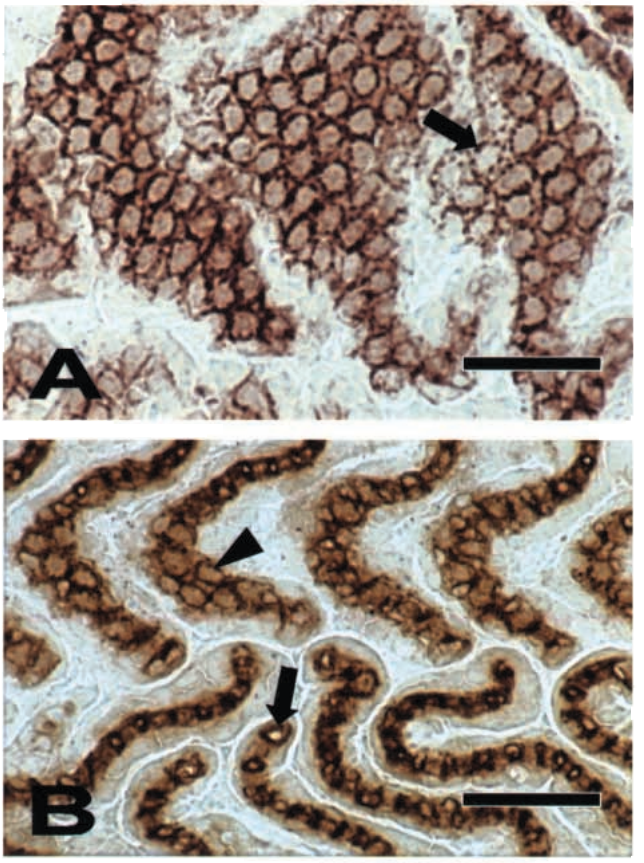

Fig. 4. 5′-Nucleotidase (5′NT) histochemistry of trout gill suggests a concentration of activity at the apical plasma membrane (microvascular lining) of pillar cells. (A) In this section cut parallel to the plane of the secondary lamellae, 5′NT reaction product is concentrated in a pattern of rings. This is consistent with localization at the peripheral margin of the trunk-like pillar cells (see Fig. 2B). In some instances, staining occurs in a punctate pattern along the outside edges of pillar cells (arrow). (B) Portions of two rows of secondary lamellae from adjacent filaments. The upper row is cut in a tangential plane, revealing some ring-like patterns of staining (arrowhead), as above. In the lower row, reaction product is most dense along the margins of blood spaces cut in cross section (arrow). Scale bars, 25 μm.

tubules in a given sections showed enzyme activity, probably reflecting functional segmentation and heterogeneity in enzyme distribution along the tubules.

Discussion

The specific activities of 5′NT and APD are elevated in tissue homogenates and isolated plasma membranes from the gill compared with the kidney or liver (Table 1). These data, obtained using more convenient and sensitive continuous-time enzyme assays (Ipata, 1967), are consistent with prior observations made using fixed-time analysis of these activities in trout tissues (Robertson and Hazel, 1995).

The magnitude of the 5′NT specific activities obtained using the continuous-time assay averaged approximately half the fixed-time values (Robertson and Hazel, 1995) both for homogenates and for isolated membranes of trout tissues. Thus, both measures of 5′NT activity yielded essentially the same purification factors (i.e. the ratio of the activity in the isolated membranes to that in the homogenates). Continuous-time values for APD activity averaged double (homogenates) or triple (membranes) the corresponding fixed-time data. This resulted in two- to threefold higher purification factors for kidney and gill, respectively, with the continuous procedure; liver membrane purifications were similar using both APD assays. The agreement in scope and direction between the two approaches supports the applicability of the more convenient continuous-time assays for trout membrane studies.

Elevated activities of 5′NT and APD raise the question of the role these enzymes may play in gill physiological function. Additionally, as apical plasma membrane ectoenzymes, 5′NT and APD potentially serve as useful markers for specific gill plasma membrane fractions. Our interest in exploring structure–function relationships in gill cellular membranes therefore motivated further investigations of these activities in trout gill.

Other studies of 5′NT and APD in fish gill are quite limited. Leray *et al.* (1979) did measure the activities of a number of enzymes of adenine nucleotide metabolism, including 5′NT, in trout gill tissue. 5′NT is a biochemically well-characterized protein, with the exoenzyme attached to the plasma membrane *via* a glycosylphosphatidylinositol (GPI) linkage (Zimmermann, 1992). Cytosolic forms of 5′NT have also been identified (Zimmermann, 1992; Yamazaki *et al.* 1991); on the basis of our results, the possible occurrence of cytosolic 5′NT in pillar cells cannot be dismissed. However, 5′NT activity in trout tissues was concentrated in membrane fractions purified by density gradient centrifugation, and these fractions were also specifically enriched with a variety of other plasma membrane markers (Robertson and Hazel, 1995). Additionally, the distribution of histochemical reaction product suggests a plasma membrane localization of 5′NT activity in gill pillar cells and kidney tubule epithelial cells. These observations thus support the presence of a significant plasma membrane-associated component of 5′NT activity in trout tissues.

No prior studies concerning APD in fish gill could be identified. Trout gill plasma membrane APD activity exhibited a divalent cation requirement and substrate preference consistent with those of APD characterized from other sources (Kelley *et al.* 1975; Razzell, 1968; Razzell and Khorana, 1959). Significantly, proteins with APD activity have also been found to exhibit nucleotide pyrophosphatase activity (Belli and Goding, 1994; Narita *et al.* 1994; Rebbe *et al.* 1993; Decker and Bischoff, 1972; Razzell, 1968). The best-characterized proteins with APD/nucleotide pyrophosphatase activity belong to the somatomedin/phosphodiesterase family of type II transmembrane proteins (Fuss *et al.* 1997); these include PC-1 (van Driel and Goding, 1987), PD-Iα (Narita *et al.* 1994) and B10 (Scott *et al.* 1997). Other reports have ascribed APD activity to a GPI-linked protein (Nakabayashi *et al.* 1993).

Thus, while the APD activity in trout gill shares characteristics with APD from other sources, the specific molecular identity of the gill enzyme remains an open question.

Using histochemistry, both 5′NT and APD activities localized to the pillar cells in trout gill secondary lamellae. Reaction product appears most heavily concentrated at the pillar cell regions delimiting the walls of the vascular spaces. This pattern corresponds with the localization of high activity at the apical plasma membrane of these unusually polarized cells (see Fig. 2B). The same result is seen in sections from gills cleared of blood by perfusion, making it unlikely that blood components contribute significantly to the histochemical staining. This is also consistent with the very low activities of 5′NT and APD found in isolated trout erythrocyte plasma membranes (J. C. Robertson, unpublished results).

As apical epithelial markers, 5′NT and APD might have been expected to be found on gill epithelial cell barrier membranes (i.e. the pavement cell apical plasma membrane). However, there was no histochemical indication of either activity in the pavement cells. In contrast, histochemical signals for both activities were clearly present at the apical region of epithelial cells in the kidney tubules, despite the kidney having 5′NT and APD specific activities lower than or similar to those of gill preparations. Therefore, the lack of evidence for activity in pavement cells would not appear to reflect a limitation in detecting an apical epithelial histochemical signal (due, for example, to selective elimination of reaction product from apical surfaces during rinsing).

The basal plasma membrane domains of the pavement and pillar cells oppose one another along a shared basement membrane (Fig. 2B). Reaction product formation which paralleled or demarcated the basement membrane was not commonly or consistently observed in trout gill sections. Thus, in accordance with the apical distribution in tissues of other vertebrates, the histochemical results suggest that 5′NT and APD activities are not associated with the basal plasma membranes of pavement or pillar cells.

In sections cut along the plane of the lamellae, 5′NT staining of pillar cells sometimes occurred in a punctate (rather than continuous) ring pattern (see Fig. 4A). This could be interpreted as indicating a concentration of activity near the basement membrane columns which extend through the pillar cell bodies (see Fig. 2B). While only observed in some instances, this suggestive pattern warrants further investigation, particularly in the light of the possible involvement of these collagenous columns in pillar cell structural dynamics (see below).

It is important to acknowledge that the histochemical methods employed in this study afford limited resolution. In view of technical considerations (e.g. antibody availability and reactivity), enzyme histochemistry was appealing as an initial, cornerstone approach. And the resulting localization of 5′NT and APD activities to pillar cells of trout gill secondary lamellae represents a novel and significant finding; evidence for an apical plasma membrane concentration of these activities in pillar cells is additionally provocative. However, more definitive subcellular localization (including the demonstration of the exofacial membrane disposition expected of ectoenzymes) will require ultrastructural studies or other approaches.

The results reported here obviously fuel interest in the physiological role that 5′NT and APD activities play in trout gill secondary lamellae. Assuming that their activities towards nucleotide substrates have biological relevance, speculation as to the function of these ectoenzymes has centered on (i) nucleotide salvage, and (ii) modulation of purinergic signaling.

The luminal disposition of 5′NT and APD in ducts and tubules has been interpreted as suggesting a role in the processing of nucleotides for (re)capture by cells (Le Hir and Kaissling, 1993). A similar salvage function would not strictly apply to the blood-facing, pillar cell location reported here. However, an emerging function of the gill vasculature involves the processing of circulating hormones and metabolites (Olson, 1998). For example, angiotensin-converting enzyme has been localized to pillar cells (Olson *et al.* 1989). A cytochrome P450 has also been detected in pillar cells, increasing in expression with exposure to environmental xenobiotics (Van Veld *et al.* 1997; Stegeman *et al.* 1991). Thus, a vascular wall localization for 5′NT and APD in gill secondary lamellae might reflect a mechanism for handling or clearing circulating nucleotides.

Another physiological role postulated for 5′NT and APD involves modulation of purinergic signaling (Dubyak and El-Moatassim, 1993; Zimmermann, 1992). This signaling pathway has been implicated in gill vascular regulation;

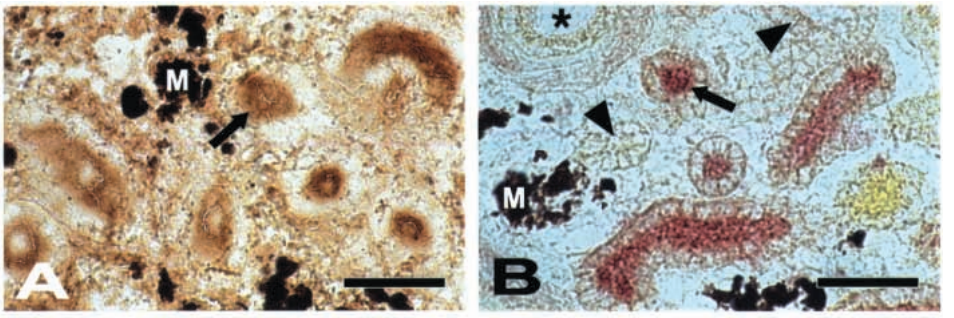

Fig. 5. Enzyme histochemistry of trout kidney. (A) Reaction product for 5′-nucleotidase (5′NT) is clearly concentrated in the lumen of kidney tubules (arrow). Other than in the apical region, epithelial cells comprising tubules show no indication of staining. Tissue around the tubules contains diffusely distributed reaction product and some melanin-rich pigment (M). (B) In tubules cut in cross (circular) or more longitudinal (elongate) section, red reaction product for alkaline phosphodiesterase (APD) also has an obvious luminal localization (arrow). However, not all the tubules show evidence of enzyme activity (arrowheads). A small blood vessel (*) and melanin pigment (M) are also visible. Scale bars, 25 μm.

adenosine is known to be a vasoconstrictive agent in fish gill (Sundin and Nilsson, 1996; Colin *et al.* 1979). While studies of gill vascular control have typically employed whole gills or arches and focused on the contributions of pre- and post-secondary lamellar vessels to flow characteristics, the pillar cells may also potentially influence lamellar hemodynamics. Localization of myosin and actin (Smith and Chamley-Campbell, 1981; Bettex-Galland and Hughes, 1973) around the basement membrane columns which extend through the pillar cell body (Fig. 2B) has led to speculation that these cells have a contractile capacity. Biochemical analysis of isolated plasma membranes (Poli *et al.* 1997) could identify purinoceptors in pillar cells.

Preliminary histochemical analysis of gills from several other species (including tilapia, *Oreochromis niloticus* and eel, *Anguilla rostrata*) indicates a similar pillar cell disposition of 5′NT and APD activities to that observed in trout (J. C. Robertson, unpublished results). These results thus suggest that any function 5′NT and APD may play in gill secondary lamellae may be conserved among teleosts.

Many of the important functional properties of gills are associated with the plasma membranes of different cell types; the characterization of these membranes is therefore of significant physiological interest. For example, the pavement cell apical plasma membranes of freshwater trout constitute an expansive surface across which a large osmotic gradient is maintained (Evans, 1993). Tissue-level studies reveal that cholesterol may be particularly important in restricting gill water permeability (Robertson and Hazel, 1995, 1998); more direct studies with isolated membranes could enhance our understanding of barrier membrane physiology.

The observations reported here should be valuable in demonstrating the isolation of specific gill plasma membrane fractions. The purification of secondary lamellar vascular surface membranes is clearly facilitated by the localization of both 5′NT and APD to pillar cell apical plasma membranes. Moreover, since the vascular surface area of the secondary lamellae in trout is greater than the environment-facing area (Olson, 1998), 5′NT and APD activities could be especially useful in confirming the effective separation of pillar and pavement cell plasma membranes during isolation procedures.

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References

BELFIELD, A. AND GOLDBERG, D. M. (1969). Inhibition of the nucleotidase effect of alkaline phosphatase by β-glycerophosphate. *Nature* **219**, 73–75.

BELLI, S. I. AND GODING, J. W. (1994). Biochemical characterization and human PC-1, an enzyme possessing alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. *Eur. J. Biochem.* **226**, 433–443.

BETTEX-GALLAND, M. AND HUGHES, G. M. (1973). Contractile filamentous material in the pillar cells of fish gills. *J. Cell Sci.* **13**, 359–370.

COLIN, D. A., KIRSCH, R. AND LERAY, C. (1979). Haemodynamic effects of adenosine on gills of the trout (*Salmo gairdneri*). *J. comp. Physiol.* **130**, 325–330.

DECKER, K. AND BISCHOFF, E. (1972). Purification and properties of nucleotide pyrophosphatase from rat liver plasma membranes. *FEBS Lett.* **21**, 95–98.

DUBYAK, G. R. AND EL-MOATASSIM, C. (1993). Signal transduction *via* P2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* **265**, C577–C606.

EVANS, D. H. (1993). Osmotic and ionic regulation. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 315–341. Boca Raton, FL: CRC Press.

FREDERIKS, W. M. AND MARX, F. (1988). A quantitative histochemical study of 5′-nucleotidase activity in rat liver using the lead salt method and polyvinyl alcohol. *Histochem. J.* **20**, 207–214.

FUSS, B., BABA, H., PHAN, T., TUOHY, V. K. AND MACKLIN, W. B. (1997). Phosphodiesterase I, a novel adhesion molecule and/or cytokine involved in oligodendrite function. *J. Neurosci.* **17**, 9095–9103.

GOSS, G. G., PERRY, S. F., WOOD, C. M. AND LAURENT, P. (1992). Mechanisms of ion and acid–base regulation at the gills of freshwater fish. *J. exp. Zool.* **263**, 143–159.

HUGHES, G. M. AND MORGAN, M. (1973). The structure of fish gills in relation to their respiratory function. *Biol. Rev.* **48**, 419–475.

IPATA, P. L. (1967). A coupled optical enzyme assay for 5′-nucleotidase. *Analyt. Biochem.* **20**, 30–36.

ISAIA, J. (1984). Water and non-electrolyte permeability. In *Fish Physiology*, vol. X (ed. W. S. Hoar and D. J. Randall), pp. 1–38. San Diego, CA: Academic Press.

KELLEY, S. J., DARDINGER, D. E. AND BUTLER, L. G. (1975). Hydrolysis of phosphonate esters catalyzed by 5′-nucleotide phosphodiesterase. *Biochemistry* **14**, 4983–4988.

LAURENT, P. AND DUNEL, S. (1980). Morphology of the gill epithelia in fish. *Am. J. Physiol.* **238**, R1147–R1159.

LE HIR, M. AND KAISSLING, B. (1993). Distribution and regulation of renal ecto-5′-nucleotidase: implications for physiological functions of adenosine. *Am. J. Physiol.* **264**, F377–F387.

LERAY, C., RAFFIN, J. P. AND WINNINGER, C. (1979). Aspects of purine metabolism in the gill epithelium of rainbow trout, *Salmo gairdneri* Richardson. *Comp. Biochem. Physiol.* **62**B, 31–40.

LIN, H., PFEIFFER, D. C., VOGL, A. W., PAN, J. AND RANDALL, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J. exp. Biol.* **195**, 169–183.

NAKABAYASHI, T., MATSUOKA, Y., TAGUCHI, R., IKEZAWA, H. AND KIMURA, Y. (1993). Proof of alkaline phosphodiesterase I as a phosphatidylinositol-anchor enzyme. *Int. J. Biochem.* **25**, 689–696.

NARITA, M., GOJI, J., NAKAMURA, H. AND SANO, K. (1994). Molecular cloning, expression and localization of a brain-specific phosphodiesterase I/nucleotide pyrophosphatase (PD-Iα) from rat brain. *J. biol. Chem.* **269**, 28235–28242.

OLSON, K. R. (1991). Vasculature of the fish gill: anatomical correlates of physiological functions. *J. Electron Microsc. Tech.* **19**, 389–405.

OLSON, K. R. (1998). Hormone metabolism by the fish gill. *Comp. Biochem. Physiol.* **119**A, 55–65.

OLSON, K. R., LIPKE, D. W., KULLMAN, D., EVAN, A. P. AND RYAN, J. W. (1989). Localization of angiotensin-converting enzyme in the trout gill. *J. exp. Zool.* **250**, 109–115.

PIERI, C., FALASCA, M., MARCHESELLI, F., RECCHIONI, R. AND MORONI, F. (1992). Lipid peroxidation causes an increase of lipid order and a decrease of 5′-nucleotidase activity in the liver plasma membrane. *Cell. molec. Biol.* **38**, 437–442.

POLI, A., PAVAN, B., LUCCHI, R., BORSAIO, P. G., FABBRI, E. AND ROSSI, R. (1997). Biochemical and pharmacological characterization of adenosine A1 receptors in eel (*Anguilla anguilla*) brain. *Fish Physiol. Biochem.* **16**, 19–27.

RAZZELL, W. E. (1963). Phosphodiesterases. In *Methods in Enzymology*, vol. VI (ed. S. P. Colwick and N. O. Kaplan), pp. 236–245. New York: Academic Press.

RAZZELL, W. E. (1968). Polynucleotidase activity of animal and plant tissue phosphodiesterases. *Can. J. Biochem.* **46**, 1–7.

RAZZELL, W. E. AND KHORANA, H. G. (1959). Studies on polynucleotides. III. Enzymatic degradation, substrate specificity and properties of snake venom phosphodiesterase. *J. biol. Chem.* **234**, 2105–2113.

REBBE, N. F., TONG, B. D. AND HICKMAN, S. (1993). Expression of nucleotide pyrophosphatase and alkaline phosphodiesterase I activities of PC-1, the murine plasma cell antigen PC-1. *Molec. Immunol.* **30**, 87–93.

ROBERTSON, J. C. AND HAZEL, J. R. (1995). Cholesterol content of trout plasma membranes varies with acclimation temperature. *Am. J. Physiol.* **269**, R1113–R1119.

ROBERTSON, J. C. AND HAZEL, J. R. (1998). Involvement of cholesterol in restricting the water permeability of trout gill barrier membranes. *Fedn Proc. Fedn Am. Socs exp. Biol.* **12**, A332.

SCOTT, L. J., DELAUTIER, D., MEERSON, N. R., TRUGNAN, G., GODING, J. W. AND MAURICE, M. (1997). Biochemical and molecular identification of distict forms of alkaline phosphodiesterase I expressed on the apical and basolateral plasma membrane surfaces of rat hepatocytes. *Hepatology* **26**, 995–1002.

SIERAKOWSKA, H. AND SHUGAR, D. (1963). Cytochemical localization of phosphodiesterase by the azo dye simultaneous coupling method. *Biochem. biophys. Res. Commun.* **11**, 70–74.

SIERAKOWSKA, H., SZEMPLINSKA, H. AND SHUGAR, D. (1963). Intracellular localization of phosphodiesterase by a cytochemical method. *Acta biochim. pol.* **10**, 399–411.

SMITH, D. G. AND CHAMLEY-CAMPBELL, J. (1981). Localization of smooth-muscle myosin in branchial pillar cells of snapper (*Chrysophys auratus*) by immunofluorescence histochemistry. *J. exp. Zool.* **215**, 121–124.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GRTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. AND KLENZ, D. C. (1985). Measure of protein using bicinchoninic acid. *Analyt. Biochem.* **150**, 76–85.

STEGEMAN, J. J., SMOLOWITZ, R. M. AND HAHN, M. E. (1991). Immunohistochemical localization of environmentally induced cytochrome P450IA1 in multiple organs of the marine teleost *Stenotomus chrysops* (scup). *Toxicol. appl. Pharmac.* **110**, 486–504.

SULLIVAN, G. V., FRYER, J. N. AND PERRY, S. F. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J. exp. Biol.* **198**, 2619–2629.

SUNDIN, L. AND NILSSON, G. E. (1996). Branchial and systemic roles of adenosine receptors in rainbow trout: an *in vivo* microscopy study. *Am. J. Physiol.* **271**, R661–R669.

VAN DRIEL, I. R. AND GODING, J. W. (1987). Plasma cell membrane glycoprotein PC-1. Primary structure deduced from cDNA clones. *J. biol. Chem.* **262**, 4882–4887.

VAN VELD, P. A., VOGELBEIN, W. K., COCHRAN, M. K., GOKSØYER, A. AND STEGMAN, J. J. (1997). Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicol. appl. Pharmac.* **142**, 348–359.

WILKIE, M. P. (1997). Mechanisms of ammonia excretion across fish gills. *Comp. Biochem. Physiol.* **118**A, 39–50.

YAMAZAKI, Y., TRUONG, V. L. AND LOWENSTEIN, J. M. (1991). 5′-Nucleotidase I from rabbit heart. *Biochemistry* **30**, 1503–1509.

ZIMMERMANN, H. (1992). 5′-Nucleotidase: molecular structure and functional aspects. *Biochem. J.* **285**, 345–365.