Enzyme cytochemical and immunocytochemical studies of flask cells in the amphibian epidermis

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Summary. The localization of oxidoreductases and transport enzymes in flask cells of the amphibian epidermis was studied at the light-microscopic level. In these cells, the deposition of cytochemical reaction products was very similar to that found in fish epidermal ionocytes, thus demonstrating histochemical similarities between these two types of cells. The present histochemical results revealed high levels of activity of alkaline phosphatase (ALPase), potassium-dependent nitrophenylphosphatase (K⁺⁺-p-NPPase) and carbonic-anhydrase isozymes (CA-I and CA-II) in the apical region of the flask cells, indicating that enzyme zonation may be the main site of the ion pumping.

Introduction

Flask cells in the skin of young and adult axolotls as well as other adult caecilians and anurans have been extensively studied (Lodi 1971; Guardabassi et al. 1972; Lavker 1972; Budtz and Larsen 1975; Whitear 1975; Greven 1980; Brown et al. 1981; Fox 1983a, b; Levinson et al. 1982, 1984). It has been suggested that they are involved in ion-transport processes and play a role in moulting (Budtz and Larsen 1973; Whitear 1977; Masoni and Romeo 1979). Most previous studies concerning the transport capacity of flask cells have focused on their active transport of sodium (Voûte et al. 1975), a mechanism which is activated by the effect of aldosterone (Maetz et al. 1958; Crabbé 1964). Very recently, it has been suggested that these cells contain the cellular pathway for chloride in amphibian skin (Katz and Larsen 1984), rather like the mitochondria-rich chloride cells present in teleost skin (Marshall and Nishioka 1980).

Most investigators have attempted to correlate the physiological functions of flask cells with the biochemical parameters observed in fish chloride cells (ionocytes; Levinson et al. 1984). The recent demonstration of the presence of potassium-dependent nitrophenylphosphatase (K⁺⁺-p-NPPase) activity of the Na⁺⁺-K⁺⁺-ATPase complex (the enzymatic equivalent of the sodium pump) in the epidermal ionocytes of fish (Zacone et al. 1984) and flask cell ultrastructure consistent with a transport role, further supports the view that both of these cell types have an osmoregulatory function.

The exact role of flask cells is, however, not completely understood. They are known to contain carbonic anhydrase (Lodi 1971; Guardabassi et al. 1972; Rosen and Friedley 1973; Levinson et al. 1982, 1984), but little is known about their cytochemistry. Furthermore the validity of some previously presented results is questionable, and many studies have been limited to the newt epidermis. The exact location of the operative enzymes and the enzyme mechanisms connected with ion transport in flask cells remain to be determined. The aim of this preliminary study was to describe the enzyme cytochemical activities of several dehydrogenases, alkaline phosphatase (ALPase) and K⁺⁺-p-NPPase, as well as the immunocytochemical localization of carbonic anhydrase isozymes I and II (Ca-I and CA-II) in the flask cells of amphibian epidermis. We also attempted to correlate the significance of the present cytochemical results with reference to current hypotheses of the function of ion transport across lower vertebrate skin.

Materials and methods

Tissues. Skin obtained from adult salamanders (Ambystoma tigrinum and Ambystoma laterale) and anurans (Xenopus laevis, Rana pipiens, and Bufo marinus) was used. Specimens were taken from different regions of the body and rapidly frozen with liquid-nitrogen-cooled isopentane.

Enzyme cytochemistry

Serial sections were cut at a thickness of 6 μm in a cryostat at −25°C and incubated at 37°C. The fixation was performed when appropriate to the technique used.

Dehydrogenase enzyme cytochemistry. The activity of succinate dehydrogenase (EC 1.3.99.1) was demonstrated using the method of Nachlas et al. (1957), but with the addition of 50 mg/0.1 ml phenazine methosulphate. Glycerol-3-phosphate/menadione-oxidoreductase (EC 1.1.99.5) activity was demonstrated using a modification of the method of Wattenberg and Leong (1960), in which the succinate substrate was replaced by an equimolar concentration of glycerol-3-phosphate. The method described by Barka and Anderson (1963) was employed to demonstrate the activity of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30). The activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and isocitrate/NADP⁺-oxidoreductase (decarboxylating; EC 1.1.1.42) was demonstrated using the method of Meijer and de Vries (1974, 1975). The specificity of the dehydrogenase enzyme reactions was tested by different control experiments in which cryostat sections were incubated in...
media without substrate, without coenzyme, or without the electron carrier phenazine methosulfate (PMS).

For lipid extraction, aliquots of fixed and unfixed cryostat sections were passed through 100% acetone for 7 min (at +22°C) or 10 min (at -40°C), respectively (Kugler and Wrobel 1978).

ALPase cytochemistry. To demonstrate ALPase (EC 3.1.3.1) activity, both unfixed and 2% glutaraldehyde-fixed sections were immersed in incubation medium for 5–30 min at room temperature or 37°C. This medium (McGadey 1970) consisted of 0.1 M Tris-HCl buffer (pH 9.2), 16 mg 5-bromo-4-chloro-3-indolyl-phosphate, 1 mM MgSO4, and 20–40 mg tetrathylthiourea (TNBT) the final pH being 9.0. Control media were obtained by omitting the substrate from the standard incubation medium containing TNBT or by adding 2.5 mM l-p-bromo-tetramisole oxalate (BTO), an inhibitor of ALPase activity.

K+ -p-NPPase cytochemistry. After fixation in a mixture of 2% formaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), frozen sections were immersed in incubation medium for 25 min at room temperature or 37°C. The incubation medium (Mayahara et al. 1980) consisted of 250 mM Tris-HCl buffer (pH 9), p-anisidophenyl phosphate (Tris or Na-salt), 20 mM KCl, 30 mM MgCl2, and 20 mM SrCl2, the final pH being 9.0. Controls were performed by omitting the substrate or adding ouabain at a final concentration of 1–10 mM.

Immunocytochemistry

Skin specimens were fixed for 3 h in Carnoy’s fluid (ethanol/chloroform/glacial acetic acid, 6:3:1) and embedded in low-melting-point paraffin. Serial sections (8 µm) were deparaffinized and rehydrated, and the slides were kept in phosphate-buffered saline (PBS) at 4°C until stained. The immunoperoxidase staining method of Sternberger (1979) was modified according to principles of the immunocytochemical procedure described by Kumpulainen (1984). Sections were pretreated for 10 min with 0.5% H2O2 and then sequentially incubated in the following: 1:40-diluted normal goat serum, 1:40 to 1:400-diluted polyclonal sheep antiserum to human carbonic anhydrase I (anti-HCA-I, AHPO15, lot Z767B; Serotec, UK) and 1:100-diluted anti-rat carbonic anhydrase-II serum (anti-RCA-II; a generous gift from Dr. T. Kumpulainen, Department of Anatomy, University of Oulu, Finland), 1:20-diluted swine anti-rabbit serum immuno globulin, 1:100-diluted peroxidase-antiperoxidase (PAP) complex (Sigma), and the 3,3-diaminobenzidine/H2O2 substrate medium for peroxidase, to reveal the antigenic sites of CA isozymes.

In control experiments, normal (nonimmune) sera were used as primary antisera in dilutions comparable to those of the CA-specific sera. In addition, the specificity of the sera was tested by omitting a component of the staining sequence.

Results

All of the dehydrogenases tested were cytochemically demonstrated in the flask cells of the epidermis of the urodeles and anurans studied (Figs. 1a–c, 2a, b, 3a–c, 4a–c, 5).

Cells whose apical pole was in contact with the external layer were labelled with a granular precipitate (formazan) that marked the site of the enzyme.

The delineation of flask cells by the reaction product of various oxidoreductases (i.e. succinate dehydrogenase, isocitrate dehydrogenase, 3-hydroxybutyrate dehydrogenase, glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase) was particularly striking, because the precipitate was much more abundant than that observed in epithelial cells of the outer, intermediate and basal cell layers (Figs. 2a, b, 3a–c, 4a–c, 5). These reaction products also provided an outline of the nuclei of flask cells, and some cells exhibited dense reaction products in the basal pole level with the intermediate cell layer.

The dehydrogenase enzyme reactions were almost completely absent when the specific substrate was omitted from the incubation mixture. Control sections incubated in the absence of the exogenous electron carrier (100 mM PMS) or coenzyme did not exhibit any stained structures. Pretreatment of cryostat sections with acetone for 10 min did not affect the activity of any of the dehydrogenases in flask cells of the species studied. This procedure, however, resulted in a decrease in the unspecific adsorption of formazan at the level of the corneous layer. A great reduction in adsorption artifacts was also seen in the keratinized layer when sections were pretreated in acetone for 10 min and then incubated in the medium used for the cytochemical demonstration of ALPase activity. The reaction product indicating ALPase activity accumulated in the apical poles of flask cells (Fig. 6), rather like the reaction product indicating the cytochemical localization of K+ -p-NPPase activity (Fig. 7).

The intense ALPase reaction observed within the cytoplasm of flask cells was significantly reduced when 2.5 mM BTO was added to the control media; similar results were obtained when medium without substrate but containing TNBT was used.

In control experiments, the addition of 10 mM ouabain to the standard media reduced the amount of K+ -p-NPPase activity. The inhibitory effect of ouabain was much less evident when a concentration of 1 mM was used. Also,