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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; Lewinson 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 Romeu 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 cloride 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; Lewinson 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 (Zaccone 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.

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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; Lewinson 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 methosulphate (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-indoxyl-phosphate, 1 m*M* $MgSO_4$, and 20–40 mg tetranitroblue tetrazolium (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 m*M* 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 m*M Tris*-HCl buffer (pH 9), *p*-nitrophenylphosphate (*Tris* or Na-salt), 20 m*M* KCl, 30 m*M* $MgCl_2$, and 20 m*M* $SrCl_2$, the final pH being 9.0. Controls were performed by omitting the substrate or adding ouabain at a final concentration of 1–10 m*M*.

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% H_2O_2 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 immunoglobulin, 1:100-diluted peroxidase-antiperoxidase (PAP) complex (Sigma), and the 3,3-diaminobenzidine/H_2O_2 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 pricipitate 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, 6). 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 exogeneous electron carrier (100 m*M* 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 m*M* 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 m*M* 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 m*M* was used. Also,

Fig. 1a–c. Succinate dehydrogenase. Reaction product is visible in the apical poles of epidermal flask cells (*f*) of *A. tigrinum* (**a**), *X. laevis* (**b**) and *B. marinus* (**c**). Large deposits are present in epithelial cells (*e*) of the intermediate and outer cell layers. × 640

Fig. 2a, b. Isocitrate dehydrogenase. Heavy deposition of reaction product is visible in the apical poles of epidermal flask cells (*f*) of *A. tigrinum* (**a**) and *X. laevis* (**b**), but a marked reaction is also visible in epithelial cells (*e*) of the middle and outer cell layers. *bl*, basal layer. × 640

Fig. 3a–c. Glucose-6-phosphate dehydrogenase. Large amounts of fine reaction products are visible in the apical poles of epidermal flask cells (*f*) of *A. tigrinum* (**a**) and *Rana pipiens* (**c**), as well as around the basal pole of these cells in *X. laevis* (**b**). Heavy deposition of reaction product is visible in epithelial cells (*e*) of the intermediate and outer epidermis. The corneous layer, which is seen sloughing off from the underlying epidermis, shows artifactual staining due to lipid adsorption of the dye (**a, b**). *bl*, basal layer. × 640

Fig. 4a–c. 3-hydroxybutyrate dehydrogenase. Enzyme activity marks the apical poles of epidermal flask cells (*f*) of *A. tigrinum* (**a**), *X. laevis* (**b**) and *Rana pipiens* (**c**), but the reaction extends throughout the full depth of the epidermis (**b, c**). Note the unstained corneous layer (*arrows*) in **b** and **c**. The granules (*arrows*) present in the keratinizing region of **a** are non-specific dye deposits. × 640

Fig. 5. Glycerol-3-phosphate dehydrogenase. The apical pole of an epidermal flask cell (*f*) of *A. tigrinum* contains dense reaction product. × 640

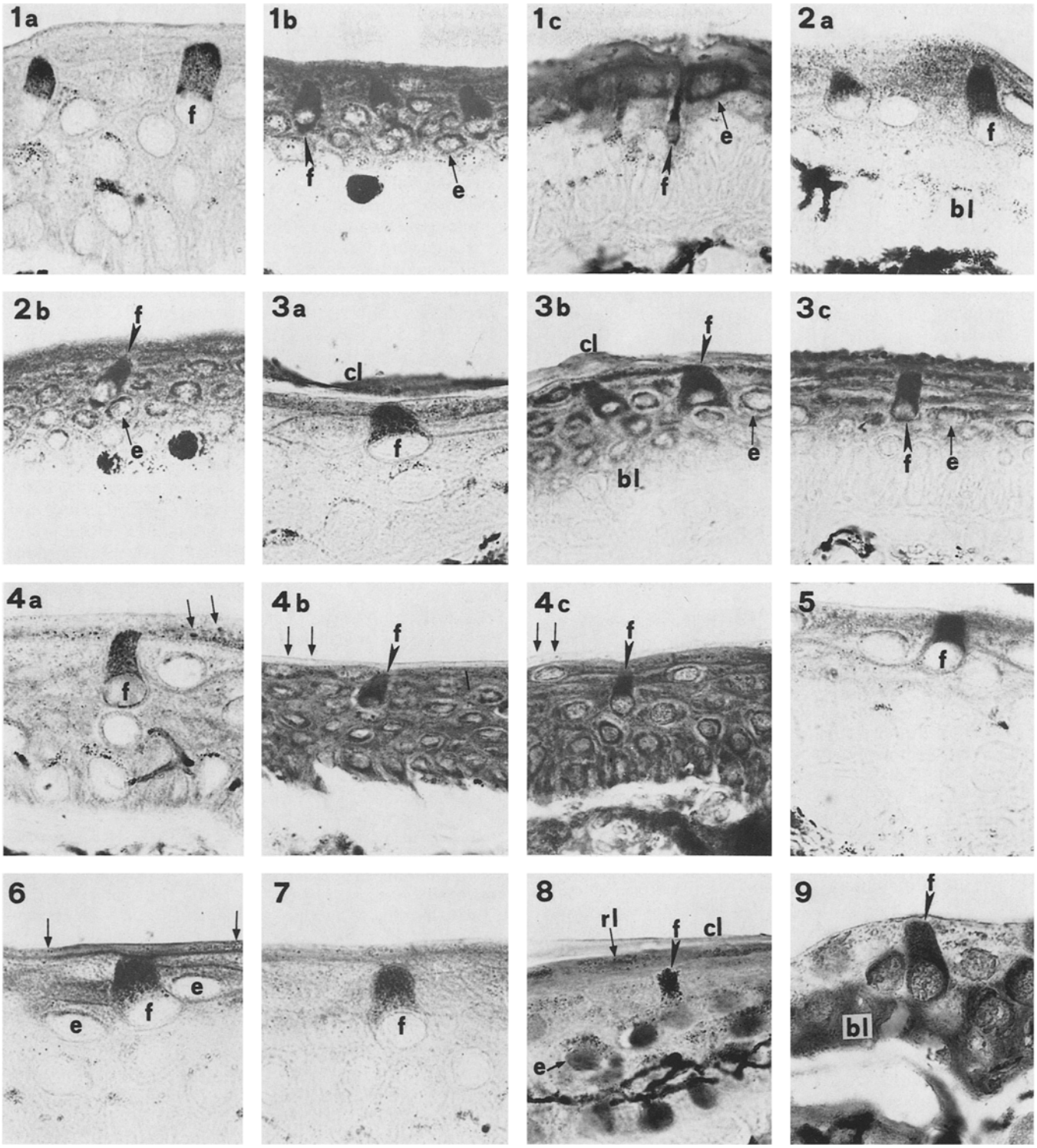

Fig. 6. Alkaline phosphatase. The apical pole of an epidermal flask cell (*f*) of *A. tigrinum* shows a granular pattern of intense staining for this enzyme. The presence of reaction product is also visible in some epithelial cells (*e*) of the replacement layer. The granules (*arrows*) of the corneous layer are only unspecific formazan-adsorption artifacts. × 640

Fig. 7. K^+-dependent NPPase. Dense reaction products indicating sites of the enzyme activity are clearly visible in the apical pole of an epidermal flask cell (*f*) of *A. tigrinum*. × 640

Fig. 8. Section of Carnoy-fixed epidermis of *A. tigrinum*. Strong CA immunoreaction is visible in the apical region (*arrow*) of a flask cell (*f*) which reaches the outer layer. PAP staining with anti-HCA-I serum (dilution 1:50), and brief counterstaining with haematoxylin. *rl*, replacement layer; *cl*, corneous layer; *e*, epithelial cell. × 640

Fig. 9. CA-II immunoenzymatic staining of the apical pole of a flask cell (*f*) in the epidermis of *A. laterale*. PAP staining with anti-RCA-II serum (dilution 1:100). *bl*, basal layer. × 640

no significant amount of reaction product was found when the substrate, *p*-nitrophenylphosphate, was omitted from the incubation medium.

The reaction pattern of the cytochemical staining of CA-I and CA-II correlated well with the cytochemical localization of the activity of the various dehydrogenases, ALPase and K^+-*p*-NPPase. The localization of material immunoreactive with AHPO15 reaction product revealed the presence of CA-I in the apical poles of flask cells as well as in the cytoplasm of epithelial cells of the intermediate and basal epidermis. CA-I immunostaining was seen in a fair number of above mentioned reacting cells (Fig. 8).

The strong immunostaining obtained with anti-RCA-II serum showed CA-II to be localized both in flask cells and in epithelial cells of the lower epidermis (Fig. 9); however, it was difficult to identify flask cells labelled using the PAP method, because moderate background staining was present throughout the epithelium. These immunocytochemical results indicate that both sheep and rat antisera contain antibodies that are cross-reactive with amphibian CA isozymes.

No indications of immunospecific reactivity were detected in control sections exposed to nonimmune sera instead of anti-CA sera. When one component of the immunostaining sequence was omitted, no CA activity was detectable.

Discussion

The characteristic distribution patterns of high activities of oxidative enzymes, ALPase and nitrophenylphosphatase found in the epidermal flask cells of the species studied were similar to those of the same series of the enzymes in the ionocytes of fish epidermis (Zaccone 1983; Zaccone et al.1984). Although there is insufficient information available to enable one to accept or reject any of the current models of ion transport, the present findings may serve as a useful tool in further studies of the morphology and function of flask cells, which are widely thought to be osmoregulatory units in the amphibian epidermis.

The use of immunocytochemistry to localize CA in flask cells also has the advantage of identifying individual isozymes of CA. This cytochemical approach indicates that the studied enzymes are located in the apical poles of cells; the reaction product observed in the apical cell region also may indicate enzyme function with respect to ion transport.

In the transporting epithelia of vertebrates, the enzyme Na^+-K^+-ATPase is responsible for the maintenance of cellular homeostasis. The high content of K^+-*p*-NPPase in flask cells, whose activity is considered to be equivalent to the dephosphorylation step of the Na^+-K^+-ATPase reaction (Ernst and Hootman 1981), may explain the presence of the Na^+-K^+ pump in these cells; this feature has also been found in the epidermis and urinary bladder of some anurans (Mills 1981; De Bortoli et al. 1982). In addition, there is evidence that Na-pumping sites are associated with the plasma membrane of all living cells layers of the frog epidermis, as shown by exposure to ^{3}H-ouabain (Mills 1981). This indicates that ion transport via the middle and lower epithelium may be driven by the Na^+-K^+-ATPase pump. The simultaneous occurrence of this transport system in both epithelial and flask cells is interesting. However, in order to reveal the intracellular localization of transport enzymes, both ultracytochemical and electron-microscopic immunocytochemical studies are necessary (G. Zaccone, S. Fasulo, A. Licata, P. Lo Cascio, unpublished results).

It is therefore of central importance to ascertain the subcellular routes by which specific ionic species are driven by K^+-*p*-NPPase within flask cells. An extensive membranous network of tubules fills almost the entire cytoplasmic space of these cells (Lewinson et al. 1984). Fish ionocytes, which have an important role in the transport of ions, also have a tubulovesicular system, and the enzyme Na^+-K^+-ATPase is localized on the cytoplasmic surfaces of these tubules (Hootman and Philpott 1979; Zaccone et al. 1984). It is noteworthy that this enzyme, which was observed in flask cells of the species studied, shows a reaction pattern similar to that of succinate and isocitrate dehydrogenases, thus suggesting that these enzymes are indicators of the level of ATP synthesis. Recent studies have provided evidence indicating that flask cells are specialized for chloride transport (Katz and Larsen 1984). A possible feature of this ion-transport route may be the involvement of CA isozymes contained in these cells. The function of CA in the catalyzation of the reversible hydration of CO_2 concurs with a role in the export and exchange of H^+ or HCO_3. It is possible that HCO_3 ions may influence an ATPase (similar in its general properties to Na^+-K^+-ATPase) specific for ion transport. An anion-stimulated ATPase, which is activated by chloride (Cl^-/HCO_3^-/ATPase), is located in the microsomal fraction of teleost chloride cells (Bornancin et al. 1980). A similar situation may also exist in flask cells of the amphibian epidermis, in which these cells serve as a site of passive Cl^- absorption (Katz and Larsen 1984). This possibility is currently being investigated in our laboratory.

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