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Ultrastructural localisation of acid phosphatase in intestinal eosinophilic granule cells (EGC) of rainbow trout (*Oncorhynchus mykiss*) following degranulation with capsaicin

Mark D. Powell, Heather A. Briand, Glenda M. Wright and John F. Burka
Department of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada

Summary. Enzyme cytochemistry was used to investigate possible lysosome involvement in capsaicin induced degranulation of the eosinophilic granule cell (EGC) of the rainbow trout intestine. Three adult rainbow trout *(Oncorhynchus mykiss)* were injected intraperitoneally with capsaicin in a saline vehicle (0.5 $\mu g.g^{-1}$ body weight). Following a 2 hour period of incubation, the fish were killed, and a mid portion of the intestine was dissected and fixed in cold glutaraldehyde buffered with sodium cacodylate. Vibratome sections were incubated in either reaction medium containing ß-glycerophosphate and cerium chloride in acetate buffer or substrate (ß-glycerophosphate) deficient control medium. Sections were then refixed in osmium tetroxide and processed for electron microscopy. Acid phosphatase was found to be localised within lysosomes. The enzyme was not found in the large cytoplasmic granules under normal or capsaicin-stimulated conditions. EGCs which had migrated to the lamina propria in response to the capsaicin stimulation had a distinct multivesicular granule morphology. These multivesicular granules did not contain acid phosphatase suggesting that this form of EGC degranulation is not a lysosomally mediated event.

Key words: Acid phosphatase, Lysosomes, Trout, EGC, Degranulation

Introduction

The eosinophilic granule cell (EGC) of the rainbow trout intestine has many histochemical characteristics similar to mammalian mast cells (Ellis, 1977). Capsaicin and substance P induce degranulation of EGCs results in the multivesicular subdivision of EGC granules in the lamina propria and reticulation of the granules of those EGCs confined to the stratum compactum (Powell et al., 1991). This multivesicular granule substructure is reminiscent of the multivesicular bodies of autophagic and crinophagic cells (Holtzman, 1976). Crinophagy involves the controlled autolysis of stored materials in neuroendocrine and neurosecretory cells (Marzella and Glaumann, 1987).

The presence of lysosomes is usually associated with phagocytic or autolytic activities and acid phosphatase is used as an enzymatic marker for the identification of lysosomes in cells (Schwartz and Austen, 1980). Mammalian eosinophil granules are reported to function as lysosomes: they contain acid phosphatase as well as other hydrolytic enzymes (eg., arylsulfatase) and peroxidase associated with phagocytosis (Venge, 1990). The presence of acid phosphatase in EGC granules is ambiguous. Previous studies (Ezeasor and Stokoe, 1980; Powell et al., 1991) have suggested that acid phosphatase might not be a normal constituent of EGC granules but rather a feature of their degradation. The present study was designed to determine whether lysosomes were involved in the crinophagic-like degranulation observed in capsaicin-stimulated EGCs (Powell et al., 1991) by using acid phosphatase as a cytochemical marker.

Materials and methods

Capsaicin (0.5 $\mu g.g^{-1}$ body weight) was systemically administered by intraperitoneal injection to three yearling rainbow trout *(Oncorhynchus mykiss)* which had been held at 10 ± 0.5° C in the Fish Health Unit of the Atlantic Veterinary College and fed ad libitum to satiation on a commercial pelleted feed. Following injection, fish were held for a two hour incubation period prior to euthanasia by spinal severance. A one

Offprint requests to: Mark D. Powell, Department of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada

cm piece of intestine immediately posterior to the spleen was promptly excised and fixed overnight in cold 3% glutaraldehyede in 0.1 M sodium cacodylate buffer with 7% sucrose (pH 7.2). Tissue sections (150 µm) were cut on a vibratome (Technical Products Inc., Series 1000) and washed in 0.1 M sodium cacodylate buffer (+ 7% sucrose) followed by 0.1 M acetate buffer (pH 5.0) with 7% sucrose added. The tissue sections were then incubated in excess reaction medium (1mM ß-glycerophosphate, 2mM cerium chloride ($CeCl_3$) (Sigma Chemical Company, Mo., USA) in a 0.1 M acetate buffer (pH 5.0) (+ 7% sucrose) or a substrate deficient control medium for two hours at 37° C. After one hour incubation, the reaction and control buffers were drained and replaced with fresh media. After a total of two hours incubation, the tissues were washed in cold (4° C) 0.1 M acetate buffer (+ 7% sucrose) in order to stop the reaction and then washed in 0.1 M sodium cacodylate buffer (+ 7% sucrose). The tissue slices were then refixed in 3% glutaraldehyde in 0.1 M cacodylate buffer with 7% sucrose for one hour and washed overnight in excess cacodylate buffer. The following day the tissue was post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (+ 7% sucrose) (pH 7.2) and dehydrated in ascending grades of ethanol. Tissue was then cleared in propylene oxide and embedded in Epon/Araldite resin.

Negative controls consisted of gut slices incubated in ß-glycerophosphate-deficient reaction medium. Three additional fish were not injected with capsaicin to serve as experimental controls to illustrate the localisation of acid phosphatase in unstimulated fish.

Ultrathin (70 nm) sections were supported on formvar (J.B. E.M. Services, Montreal, P.Q., Canada) coated 200 mesh copper grids and viewed unstained with a Hitachi H600 electron microscope at 75kV.

The localisation of acid phosphatase was identified by the presence of an electron dense deposit of cerium phosphate in cellular structures where the enzyme was present. Cerium has distinct advantages as a capturing agent for phosphate ions released by acid phosphatase because it produces a finer, more uniform deposit with fewer nonspecific deposits of the reaction products as compared to the more traditionally used lead (Robinson and Karnovsky, 1983).

Results

Control fish

EGCs in the stratum compactum of control fish appeared similar to those previously described in detail (Ezeasor and Stokoe, 1980; Powell et al., 1991). Perigranular halos were frequently seen around the granules of EGCs located in the stratum compactum. Cerium phosphate deposits were seen localised in occasional (eg., 1-2 per cell in 4-5 cells per section) lysosome-like structures (220 - 350 nm in diameter) in the EGCs in the stratum compactum. These could easily be distinguished from small granules by the absence of the homogenous granule matrix that is seen in all EGC granules. There was no evidence of cerium deposits in any of the granules (Fig. 1). Similarly, acid phosphatase was identified in lysosomes within ensheathing cells associated with the EGCs of the stratum compactum. All experimental tissues (ie., trout gut incubated with ß-glycerophosphate medium) exhibited a positive reaction for acid phosphatase in lysosomes. No acid phosphatase was detected in the negative control tissues.

Only occasionally were EGCs observed in the lamina propria of uninjected fish (2 - 4 cells per section). They appeared similar to those in the stratum

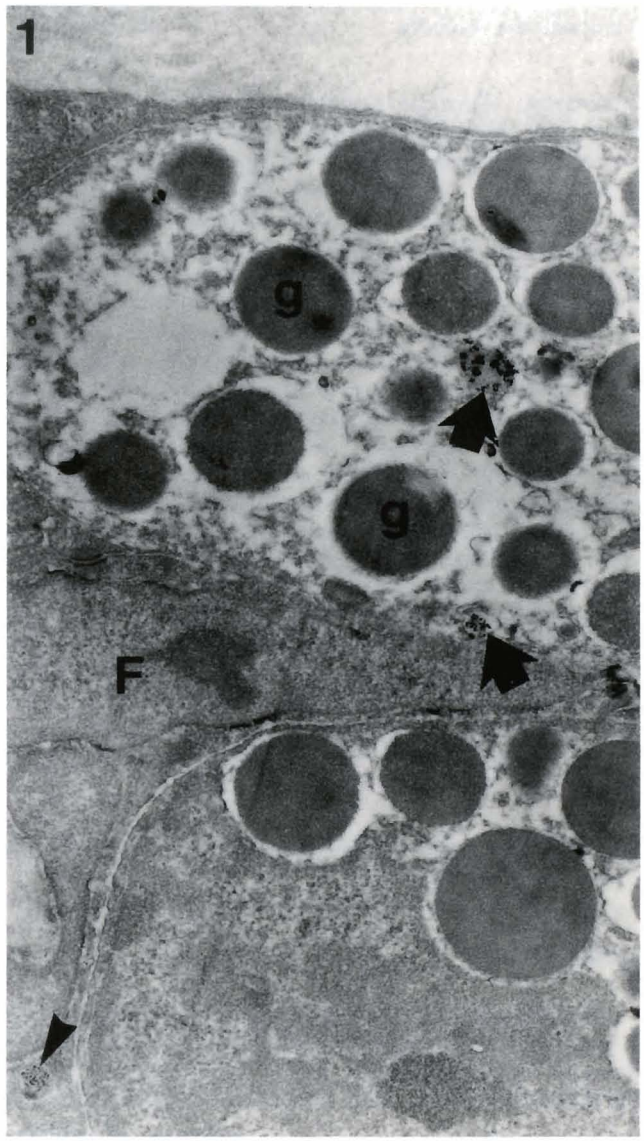

Fig. 1. Lysosomes (arrows) in an EGC and ensheathing cell (arrowhead) in the stratum compactum of an unstimulated fish. F = Ensheathing cell nucleus, g = EGC granules. × 17,500

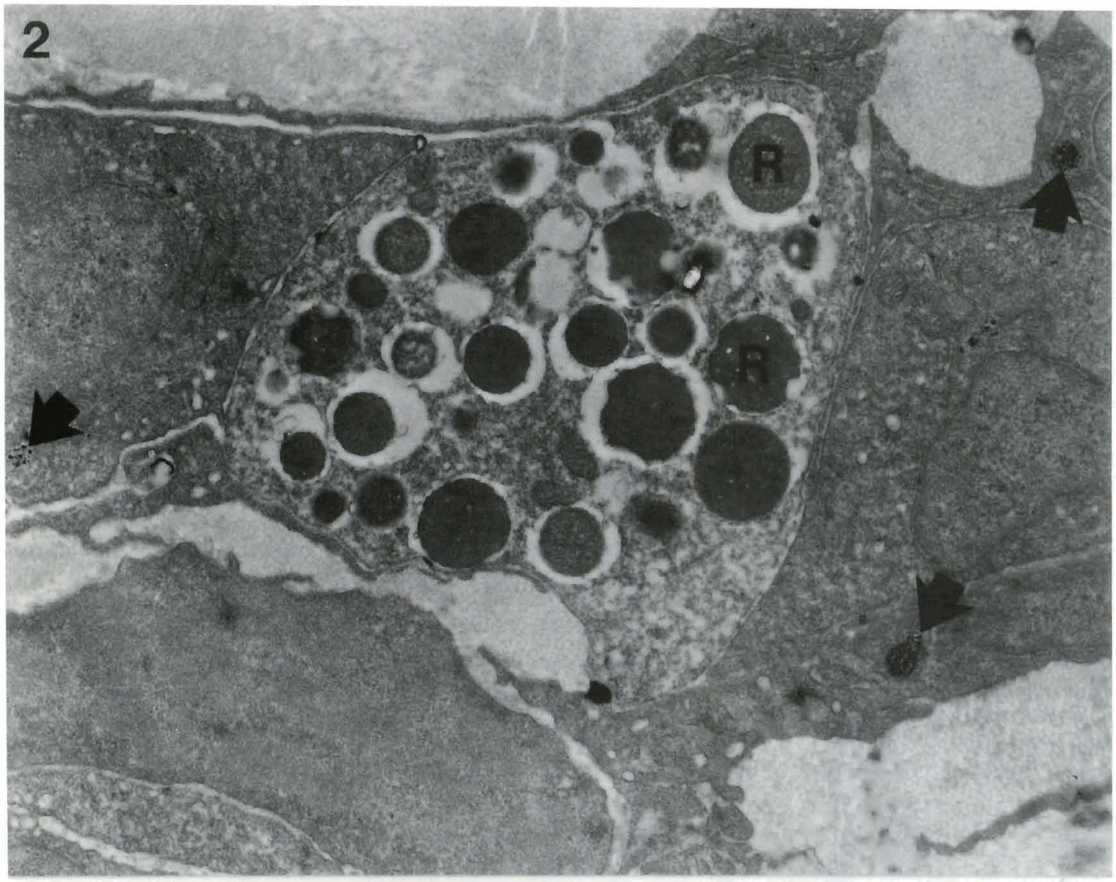

Fig. 2. Lysosomes (arrows) in the ensheathing cells around an EGC in the stratum compactum of a capsaicin-injected fish. Note the reticulated EGC granules (R). × 12,500

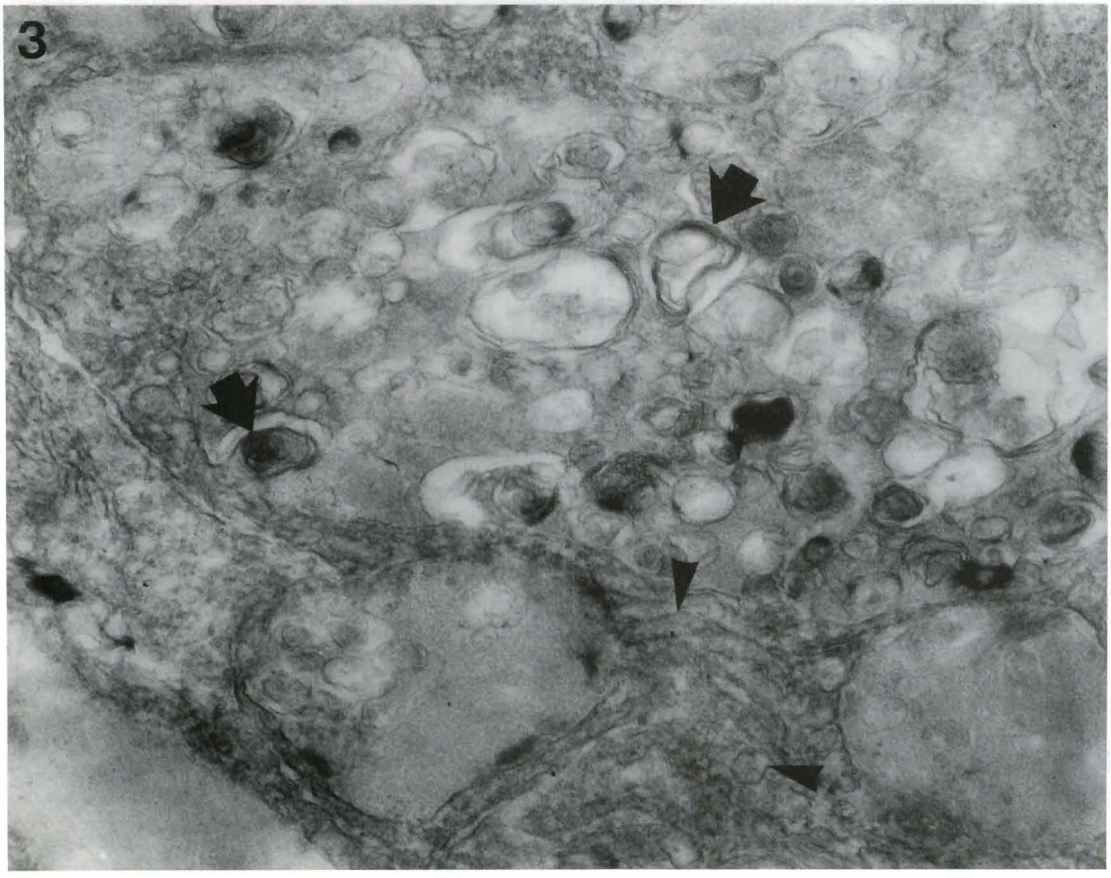

Fig. 3. Multivesicular substructure of an EGC granule following exposure to capsaicin showing multiple myelin-like figures (arrows) and smooth walled tubular structures in the cytoplasm (arrowheads). × 50,000

compactum but were not associated with ensheathing cells. EGCs in this region showed no evidence of the complex granule substructure previously observed in EGCs of the lamina propria of stimulated fish (Powell et al., 1991).

Capsaicin-injected fish

The EGCs of the stratum compactum exhibited a similar morphology to those we have described previously (Powell et al., 1991). Extensive perigranular halos and reticulated granules were evident (Fig. 2). A few lysosomes (1 - 2 per cell in 4 - 5 cells per section), identified by the localised reaction product, were present in the cytoplasm of these cells. The incidence of lysosomes was not different from that in the same region in control fish. Acid phosphatase was not detected in association with any of the granules whether they were intact, haloed or reticulated. Acid phosphatase was not detected in any cells of the negative control tissues. As in the controls a positive acid phosphatase reaction was evident in the ensheathing cells. Acid phosphatase was observed in occasional cisternae of rough endoplasmic reticulum and lysosomes of these cells.

Granules of EGCs in the lamina propria exhibited a multivesicular morphology similar to that described previously (Powell et al., 1991). No evidence of acid phosphatase was observed in association with the multivesicular granules or the smooth tubulo-vesicular cytoplasmic structures (Fig. 3). Acid phosphatase was localised in lysosomal structures within epithelial cells which flank the lamina propria and in occasional fibroblast-like cells in the lamina propria. Acid phosphatase reaction was not observed in epithelial or fibroblast-like cells in any of the negative control tissues.

Discussion

Acid phosphatase was localised cytochemically in lysosomal structures of EGCs but not within the granules. Lysosomes within the associated ensheathing cells and epithelial cells were also reactive for acid phosphatase. Consequently, it can be concluded that acid phosphatase is not a constituent of EGC granules. This absence of acid phosphatase from the EGC granules is interesting as it apparently contradicts a previous study on intestinal EGCs from untreated rainbow trout where a weak positive reaction for acid phosphatase was present in the periphery of a few granules (Ezeasor and Stokoe, 1980). Similarly, no reference was made in the earlier study of acid phosphatase reactions being observed in lysosomes and rough endoplasmic reticulum within ensheathing cells in the stratum compactum. Ezeasor and Stokoe (1980) did not report the presence of lysosomes in EGCs. In our study the enzymatic reaction, although non-existent in the EGC granules, was very strong in lysosome-like structures. These discrepancies may be explained by differences in experimental methods. Ezeasor and Stokoe (1980) used the Gomori's method of acid phosphatase localisation which uses a lead containing medium. It has been shown that lead as a capture agent can give an artifactual deposition of reaction product (Essner, 1973). Cerium on the other hand does not cause the non-specific precipitation (Robinson and Karnovsky, 1983).

The subdivision of granules observed in the EGCs of the lamina propria following stimulation with capsaicin corroborate data obtained by us under slightly different experimental conditions (Powell et al., 1991). Interestingly, acid phosphatase was not detected in the multivesicular-like EGC granules, which would discount them from being multivesicular lysosome bodies which are seen in crinophagic neuroendocrine and neurosecretory cells (Pfeifer, 1987). The absence of acid phosphatase in the multivesicular-like granules coupled with the lack of cytological changes such as swollen mitochondria and disrupted nuclei dispels any suggestion that the cells are undergoing autolysis or cell death as defined by Wyllie (1981). The changes in granule morphology observed in the EGCs of the lamina propria in capsaicin-injected fish does not therefore appear to be lysosomally mediated. However, these changes may be coincident with a proteolytic degradation brought about by enzymes other than acid phosphatase which are localised in the granule. This situation is somewhat similar to that seen in mammalian mast cells where acid phosphatase is primarily localised in lysosomes and not normally in the granule (Jamur and Vugman, 1988). Non-lysosomal mechanisms for protein degradation involves the targeting of susceptible proteins by ubiquitin and their subsequent hydrolysis in the protesome (Hershko, 1988; Dunn, 1990). While it is not known whether such mechanisms exist in EGCs, it would appear that granule breakdown of these cells in response to stimulation with capsaicin is not under lysosomal control. Rather degranulation is probably due to an as yet undetermined non-lysosomal mechanism. Mammalian mast cell granules do not contain acid phosphatase but, the enzyme is localised within lysosomes (Schwartz and Austen, 1980). Mammalian mast cell granules do however, contain a variety of enzymes such as alkaline phosphatase and arylsulfatase (Schwartz and Austen, 1980). EGC granules have also been reported to contain alkaline phosphatase (Smith, 1975; Ezeasor and Stokoe, 1980) as well as arylsulfatase (Ezeasor and Stokoe, 1980). This suggests that perhaps mast cell degranulation and capsaicin-induced EGC degranulation may be forms of non-lysosomal protein breakdown. The localisation of acid phosphatase in EGC lysosomes, and not the granules, provides further support to the theory that the piscine EGC may be analogous to the mammalian mast cell.

It appears that the multivesicular degranulation observed in trout intestinal EGCs following systemic exposure to capsaicin is not an autophagic response.

Rather it is a form of degranulation involving non-lysosomal mechanisms for the possible breakdown and release of the granule contents.

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