IN VITRO STUDIES OF EOSINOPHILIC GRANULE CELLS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) SMALL INTESTINE

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ABSTRACT

Eosinophilic granule cells (EGCs) have been identified in various tissues of salmonids and other species of teleosts. Although the function of EGCs is not clearly understood, their morphology and location have caused them to be likened to mammalian mast cells. *In vivo* studies have demonstrated that EGCs will degranulate following the administration of compound 48/80 and A23187, two agents that traditionally cause mast cell degranulation. EGCs have also been observed to proliferate and/or degranulate when exposed to specific bacterial extracellular proteins and parasitic diseases. In addition, these cells degranulate following exposure to the neuropeptide, substance P, and neurotoxin, capsaicin, two agents known to cause mast cells to degranulate. For these reasons, EGCs are thought to play a part in the nonspecific defence mechanism of fish, a role similar to mast cells in mammals. In mammals, this defence mechanism is mediated by the release of biogenic amines such as serotonin and histamine.

In this study, an attempt was made to induce the degranulation of EGCs from rainbow trout (*Oncorhynchus mykiss*) small intestine *in vitro* using the mast cell secretagogues compound 48/80 (100 μg/ml) and A23187 (10 μg/ml). Evaluation of EGC degranulation was done at both a histological and biochemical level.

Cell counting at the light microscope level was used to determine if exposure to compound 48/80 or A23187 would result in a significant decrease in the number of intact EGCs in the *stratum compactum* of the trout intestine. Such a decrease would be indicative of an explosive degranulation previously demonstrated in both mammalian mast cell and *in vivo* EGC studies. Tissues ($n=4$) were exposed for 30 minutes to one of the above agents or its respective control. Evidence of a significant decrease in the number of intact EGCs in the treatment tissues was not observed ($p>0.05$). This was due in part to regions of explosive degranulation evident in both control and treatment tissues. This nonspecific degranulation may have masked any observable effects of in vitro exposure to compound 48/80 or A23187.

High performance liquid chromatography (HPLC) was used to determine if exposure to either compound 48/80 or A23187 could result in the release of the mast cell mediator serotonin. Tissue bath concentrations of serotonin were measured before and 30 minutes after exposure to both drugs ($n=12$). Serotonin and its metabolite, 5-hydroxyindole acetic acid (5-HIAA), were quantified using reversed-phase (C18) HPLC with electrochemical detection. Results obtained indicate that baseline concentrations of serotonin and 5-HIAA did not increase significantly following exposure to either compound 48/80 or A23187 ($p>0.05$). Further research is required to determine the actual source of both the detected serotonin and 5-HIAA.

The results of this study neither confirm nor contradict the supposed similarity of function between the teleost EGC and the mammalian mast cell. Continued research, especially with regards to *in vitro* and cell culture work is required in order for the function of the EGC to be clearly understood.

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ABBREVIATIONS

Abbreviation	Term
ACTH	adrenocorticotropic hormone
A. salmonicida	*Aeromonas salmonicida*
Ca^{++}	calcium
Cm	circular smooth muscle
cm	centimeter
Cmp. 48/80	compound 48/80
CO_2	carbon dioxide
CTMC	connective tissue mast cell
E	epithelium
ECD	electrochemical detection
ECP	extracellular products
EGC	eosinophilic granule cell
EM	electron microscopy
ETOH	ethanol
Gc	Golgi stack
Gg	giant granule
Gr	released granule
GTP	guanosine triphosphate
5-HIAA	5-hydroxyindole acetic acid
hr	hour
HPLC	high performance liquid chromatography
IgE	immunoglobulin E
IgM	immunoglobulin M
IP	intraperitoneal
IP_3	inositol triphosphate
KHS	Krebs-Henseleit physiological solution
Kv	kilovolt
LM	light microscopy
Lm	longitudinal smooth muscle
Lp	lamina propria
M	molar
m	meter
µg	microgram
µl	microlitre
µm	micrometer
mg	milligram

Abbreviation	Term
ml	millilitre
min	minute
MMC	mucosal mast cell
NANC	nonadrenergic-noncholinergic nervous system
NCC	natural cytotoxic cells
ND	not detectable
ng	nanogram
Ng	normal sized granule
NK	natural killer cells
nm	nanometre
Nu	nucleus
O_2	oxygen
PAS-GL	periodic acid-Schiff granular leucocyte
pg	picogram
PREINC	preincubation
rpm	rotations per minute
S	serosa
Sc	stratum compactum
SEM	standard error of the mean
Sm	smooth muscle
V. anguillarum	*Vibrio anguillarum*

CHAPTER 1. LITERATURE REVIEW

1.1. Eosinophilic granule cells

The term eosinophilic granule cell (EGC) was first introduced by Roberts *et al.*, (1971) to describe a mononucleated granular cell found in the epidermis of the European plaice (*Pleuronectes platessa*). Although highly prevalent in many teleost species, the function of this cell remains unknown. Histological and *in vivo* research indicates a possible functional similarity to the mammalian mast cell, and yet this supposition remains unconfirmed. The objective of this study is to investigate *in vitro* EGCs from rainbow trout intestine, and to correlate these results with those that have previously been demonstrated *in vivo*.

1.1.1. General morphology of the EGC

Morphologically, the EGC is similar to the mammalian tissue mast cell (Ellis, 1977). Under normal conditions, EGCs are round to oval shaped, and vary in size, averaging 10 to 15 μm in diameter. Within the cells are numerous spherical granules, which ultrastructurally appear to be membrane bound, homogeneous and electron dense. These granules range in size from 0.1 to 1.3 μm in diameter (Ezeasor and Stokoe,

1980). A narrow perigranular halo is commonly observed, separating the granule matrix and its membrane (Ezeasor and Stokoe, 1980; Vallejo and Ellis, 1989; Powell *et al.*, 1993B). Reticulated granules, those that are spotted with irregular lucent patches, are occasionally observed. These granules are thought to be in the process of breaking down, or having just released their granule contents (Smith, 1975; Ezeasor and Stokoe, 1980; Powell *et al.*, 1993B).

The nucleus of the EGC is typically oval and eccentrically located. The nuclear envelope may display shallow depressions in its outline, which commonly house the cytoplasmic granules. The nuclei of EGCs contain equal amounts of hetero- and euchromatin, the denser heterochromatin being distributed against the nuclear periphery (Smith, 1975; Ezeasor and Stokoe, 1980).

Other ultrastructural characteristics include the prevalence of Golgi complex and low numbers of mitochondria (Roberts *et al.*, 1971; Vallejo and Ellis, 1989). The amount of rough endoplasmic reticulum appears to decrease as the cells become mature, concomitant with the formation of the stratum granulosum (Bergeron and Woodward, 1983). Ezeasor and Stokoe (1980) also observed portions of the cytoplasm of EGCs that were generally devoid of organelles other than free ribosomes. These regions were commonly located at one pole of the cell. The organelle free zones occasionally took the form of pseudopodia, which may play a role in a suggested capacity for migration in the EGC (Powell *et al.* 1990; Lamas *et*

al., 1991).

1.1.2. Histology of the EGC

The cytochemical properties of the EGC are contradictory, sharing properties intermediate between mammalian eosinophils and mast cells. Similar to mammalian eosinophil granules, those of the EGC stain intensely with acidic dyes such as Giemsa and eosin due to the presence of basic proteins (Roberts *et al.*, 1971; Smith, 1975; Ezeasor and Stokoe, 1980). EGC and mast cell granules, unlike eosinophil granules, do not contain acid phosphatase, an enzyme associated with autolytic and phagocytic activity (Ezeasor and Stokoe, 1980; Metcalfe, 1984; Powell *et al.*, 1992). However, mast cell granules do contain the enzymes alkaline phosphatase and aryl sulfatase (Metcalfe, 1984). Both these enzymes have been located in the granules of EGCs (Smith, 1975; Ezeasor and Stokoe, 1980).

The granules of both mast cells and EGCs stain intensely with toluidine blue in ethanol. Unlike mast cells, there is no metachromatic reaction, indicating the absence of acid proteoglycans, such as heparin. The granules are thought to be periodic acid-Schiff negative, as a result of an absence of neutral hexose sugars and/or sialic acids (Smith, 1975; Ezeasor and Stokoe, 1980), but Bergeron (1982) demonstrated a weak positive reaction to this stain. Added to the inconsistency, EGCs stained both positively (Ezeasor and Stokoe, 1980) and negatively (Smith, 1975) with aldehyde fuchsin, a stain used for mast cells.

1.1.3. Prevalence in teleosts

To date, most of the research on the EGC has been completed using salmonid species. Early histological work on the intestine of Atlantic salmon (*Salmo salar*), king salmon (*Oncorhynchus tschawytscha*) and sockeye salmon (*Oncorhynchus nerka*) describes the presence of this cell type (Bolton, 1933).

Similar to the mammalian mast cell, EGCs are commonly distributed in tissues exposed to the external environment. In addition to the gastrointestinal tract, EGCs have been identified in the nares (Roberts, 1972; Smith, 1975), gills (Smith, 1975; Powell *et al.*, 1990), epidermis (Blackstock and Pickering, 1980), heart tissue, and in close proximity and in the lumens of capillaries (Smith, 1975, Amin and Trasti, 1989; Powell *et al.*, 1990). They have also been observed in the kidney (Lamas *et al.*, 1991) as well as the meninges of the brain (Smith, 1975) of various salmonid species.

In salmonids, EGCs appear to be most numerous in the gastrointestinal tract. Although they appear to be relatively scattered in the oesophageal wall, EGCs tend to form a continuous layer in the stomach, pyloric caeca, and the anterior to posterior intestine. This concentrated layer of cells is referred to as the stratum granulosum and is closely associated with the collagenous network of the stratum compactum. Large numbers of EGCs have been noted in the lamina propria of the mucosa, and a few can be observed in the muscularis as well as in close association with the walls of lymphatics and blood vessels (Smith, 1975; Ezeasor and Stokoe,

1980, Bergeron and Woodward, 1983).

Bergeron and Woodward (1982; 1983) observed the ontogeny of EGC formation in the stratum granulosum of rainbow trout small intestine. Although apparent in a few fish by 16 weeks, they were not observed in all fish until 28 weeks post-hatch. No precursor cells were noted prior to and during the first appearance of the EGC. As EGCs have been observed in the intestinal capillaries and lymphatics, Bergeron and Woodward suggested that they reach the intestine through the vasculature. They postulated that early stages of EGC development may occur in some other unidentified organ. However, to date no evidence of EGCs in blood smears of noninfected teleosts exists (Powell *et al.*, 1990; Lamas *et al.*, 1991).

EGCs have also been identified in a variety of other teleost species and tissues, including peritoneal exudates from the Australian eel, *Anguilla australis* (Hine and Wain, 1989), intestine of the sergeant major fish, *Abudefduf saxatilis* (Reimschuessel *et al.*, 1987), skin of the common carp, *Cyprinus carpio* (Cross and Matthews, 1991), and the posterior intestine of the traira, *Hoplias malabaricus* (Chiariani-Garcia and Ferreira, 1992).

1.1.4. Periodic acid-Schiff granular leucocyte

The periodic acid-Schiff positive granular leucocyte, or PAS-GL, has been identified in the blood and connective tissues of species including the common white sucker

(*Catostomus commersonnii*). This cell type has not been identified in salmonid species. Although morphologically similar to the EGC, this cell stains positively to periodic acid-Schiff stain, indicating the presence of a neutral polysaccharide within the granules. These granules are thought to contain a protoheparan, as they have been demonstrated to stain metachromatically following experimental sulphation (Barber and Mills Westermann, 1975; 1978A).

1.1.5. Mammalian mast cell

Several factors exist that demonstrate a similarity between EGCs and PAS-GLs to the mammalian connective tissue mast cell. These include their morphology, location, some cytochemical properties and the ability to degranulate *in vivo* following exposure to specific agents. For this reason, researchers have postulated that the function of the EGC and PAS-GL in teleosts could be similar to that of the mast cell in mammals (Barber and Mills Westermann, 1978A; Ezeasor and Stokoe, 1980; Vallejo and Ellis, 1989; Powell *et al.*, 1991).

Mast cells are derived from the bone marrow and enter their tissue locations as precursor cells which show only a slight similarity to the mature mast cell (Theoharides, 1990). Two different populations of mast cells are known to exist in the tissues of mammals. This heterogeneity is based upon locational, morphological, histochemical and fixational characteristics. Heterogeneity also exists in relation to the cell's granule mediator content and sensitivity to agents that induce activation

and mediator release. On the basis of these differences, the connective tissue (CTMC) and mucosal (MMC) mast cell have been described (for review: Bienenstock, 1988; Irani and Schwartz, 1989; Barrett and Pearce, 1993). It has been suggested that this heterogeneity of mast cell phenotypes could be influenced by microenvironmental factors such as cell growth factors (Galli, 1993).

In mammals, mast cells play a pivotal role in the initiation of anaphylactic reactions. Anaphylaxis is an immediate (Type I) allergic reaction. This reaction is a consequence of specific antigen binding to two adjacent IgE antibodies that are bound to FcεRI receptors on the external surface of the mast cell plasma membrane. The sequence of events that follows the binding of antigen to IgE leading to mediator synthesis and/or release has not yet been fully determined (Landry *et al.*, 1992; Alber and Metzger, 1993).

A second pathway of mast cell activation has also been identified. This pathway occurs only in CTMCs, and is unrelated to antigens and cell-bound IgE. This non-antigenic pathway involves the activation of trimeric GTP-binding proteins (G proteins) through a receptor independent membrane-assisted mechanism (Landry *et al.*, 1992; Foreman, 1993). The common characteristic shared by the triggers of the non-antigenic pathway is their cationic amphiphilic nature. These triggers include peptides from insect venoms such as mastoparan, neuropeptides such as substance P, peptidic hormones such as adrenocorticotropic hormone (ACTH) and natural and

synthetic polyamines such as spermine and compound 48/80. Anaphylatoxins, C3a and C5a complement fragments, also induce CTMCs to degranulate in this manner (Landry *et al.*, 1992; Foreman, 1993).

Ionophores have been demonstrated to induce mast cell degranulation and mediator release. The pathway of activation can be characterised as being non-antigenic and also G-protein independent. Ionophores form lipid soluble complexes with cations and directly transport these ions across hydrophobic barriers, for instance the plasma membrane (Pearce, 1986). Considerable interest has been focused upon those ionophores that differentially transport Ca^{++}. This is due to the fact that regardless of the mode of activation, mast cell degranulation and mediator release is a Ca^{++} dependent reaction (Hoth *et al.*, 1993). An example of an ionophore frequently used to degranulate mast cells is the antibiotic A23187 (Pearce, 1986; Foreman, 1993).

Antigenic and non-antigenic activation or degranulation of mast cells is accompanied by a partial or complete release of granule associated-mediators into the extracellular matrix (Theoharides, 1990). Biological mediators that are stored and released from mast cell granules include heparin or chondroitin sulphate, neutrophil and eosinophil chemotactic factors, phospholipases, proteolytic enzymes, cytokines and biogenic amines such as histamine and serotonin (Metcalfe, 1984; Galli, 1993). There are also a number of mediators that are generated in association with mast cell degranulation.

These mediators include arachidonic acid metabolites (leukotrienes and prostaglandins), lipoxins and platelet activating factor (Metcalfe, 1984).

The mast cell mediators have a wide range of acute inflammatory effects, including increased vascular permeability, chemotactic activity and parasitic expulsion through muscle contraction. They also participate in more persistent immunogenic inflammatory responses, rather than solely the initiation of acute hypersensitivity. This prolonged defence involves releasing factors that enhance leucocytic infiltration to the site of inflammation (Tizard, 1988; Cotran *et al.*, 1989). This infiltration is controlled by a variety of substances released by mast cell granules, including cytokines, a group of messenger proteins also involved in cell mediated immunity (Galli, 1993). The infiltration of leucocytes in turn promote sequelae such as granuloma formation, angiogenesis and tissue fibrosis. These events are typical of a more chronic inflammatory response (Tizard, 1988).

It is interesting to note that mast cells play an integral role in defence against the infiltration of intestinal parasites in mammals. Both MMCs and CTMCs have been observed to proliferate in intestinal tissues following exposure to specific nematodes (Askenase, 1980; Marzio *et al.*, 1992). The release of mast cell biogenic amines, such as serotonin and histamine, contribute to this resistance by several means. These include activating effector cells, creating an inhospitable environment and aiding in the expulsion of the parasite through a series of violent contractions of the intestinal

smooth muscle (Askenase, 1980).

1.1.6. Prevalence of EGCs in teleost diseases

Fish pathologists often observe an increased prevalence and/or degranulation of the EGCs in various tissues following specific parasitic and viral infections. EGCs have also been noted to proliferate in non-infectious conditions. Although several of these cases have been published, most often they are not formally recorded. This is due in part to the uncertainty of the cells' specific function.

EGCs have been located within cyst tissue of rainbow trout intestine with naturally acquired infections of the parasites *Diphyllobothrium dendriticum* and *D. ditremum*. Large numbers of these EGCs were degranulated, having released their granules into the extracellular matrix (Sharp *et al.*, 1989).

Parasitic diseases of nonsalmonid species have also demonstrated a proliferation of EGCs into the site of infection. Following a microsporidian infection of *Glugea* sp. in the sergeant major fish (*Abudefduf saxatilis*), an infiltration of EGCs was noted in the intestinal lamina propria. The EGCs have also been observed to form a capsule, up to 3 cells thick, around the xenoma of the microsporidian (Reimschuessel *et al.*, 1987). Diffuse populations of granulocytes, including EGCs, have been noted in sites vacated by mature trophonts of the holotrich ciliate *Ichthyophthirius multifiliis* in carp (*C. carpio*) epidermis (Cross and Matthews, 1992).

EGC proliferation and degranulation have been associated with three salmonid viral diseases. Post-smolt Atlantic salmon clinically infected with infectious pancreatic necrosis virus were found to have an increased number of EGCs within the lamina propria of the intestine (Smail *et al.*, 1992). A pathognomonic sign of infectious haematopoietic necrosis virus infection, is a characteristic degranulation of the EGCs of the intestinal stratum granulosum followed by a necrosis of the EGCs (Yasutake and Amend, 1972; Wolf, 1988). A second salmonid rhabdovirus, viral haemorrhagic septicemia, has also been observed to produce degranulation of EGCs in infected fish (personal communication, David Speare, University of Prince Edward Island).

Large numbers of EGCs have been noted in putative neoplastic lesions of the gastrointestinal tract of coho salmon, *Oncorhynchus kisutch*. These lesions infiltrated all layers of the intestine. Affected fish also displayed a proliferation of EGCs in the liver, kidney, spleen and pancreatic tissues. Granules from EGC lesions were observed to be present in the extracellular matrix of the affected tissues (Kent *et al.*, 1993). A proliferation of EGCs in the sensory epithelium of the nares has also been described in several salmonid species infected with ulcerative dermal necrosis (Roberts, 1972), the aetiology of which, has never been resolved (Roberts, 1989).

In addition, an abundance of EGCs have been observed in the wound area of chronic tagging lesions in Atlantic salmon (Roberts *et al.*, 1973). A similar increase in epidermal EGCs was noted following surface irritation due to repeated formalin

treatment (Blackstock and Pickering, 1980).

1.2. *In vivo* degranulation of eosinophil granule cells

In addition to the morphological and locational similarities, *in vivo* studies have demonstrated that EGCs will degranulate when exposed to those agents that degranulate mammalian mast cells *in vivo*. These agents include bacterial exotoxins, neuropeptides and neurotoxins, and mast cell secretagogues. A review of the studies investigating the *in vivo* effects of these agents upon EGCs follows.

1.2.1. Bacterial exotoxins

Mammalian CTMCs have been demonstrated to degranulate following *in vivo* exposure to *Vibrio cholorae* exotoxin (Tsuchiya *et al.*, 1972). The following research illustrates the response of EGCs to *in vivo* exposure to the extracellular products (ECPs) of two commercially significant bacterial diseases of farmed salmonids, namely furunculosis (*Aeromonas salmonicida)* and vibriosis (*Vibrio anguillarum).*

Following *in vivo* exposure to the ECP of *A. salmonicida*, Ellis (1985) was able to reproduce the gross pathological signs of furunculosis. This was concomitant with a marked degranulation of the intestinal EGCs. This degranulation was characterised by an extrusion of free granules into the extracellular matrix of the stratum

compactum by the EGCs. The release of granules was similar to the anaphylactic extrusion of mammalian mast cell granules (Röhlich *et al.*, 1971). In Ellis's study, as with other later investigations, the release of EGC granules was followed by an observable reduction in the number of granules within the cells as well as a marked decrease in the number of EGCs. This reduction in the number of EGCs in the stratum compactum was due in part to a mobilization of EGCs into the lamina propria and muscle tissue of the intestine. This mobilization was often associated with a disintegration of the stratum compactum (Ellis, 1985; Vallejo and Ellis, 1989; Powell *et al.*, 1993A).

Ultrastructurally, *in vivo* exposure to *A. salmonicida* ECP resulted in a swelling of the granule and widening of the perigranular halo. This was combined with a fusion of granule membranes resulting in the formation of labyrinthine channels. With certain ECPs, myelin-like figures were noted in the granule matrix, as well as a multivesiculated substructuring and planar subfractionation (Vallejo and Ellis, 1989; Powell *et al.*, 1993A).

Vallejo and Ellis (1989) believed that degranulation as a result of *in vivo* exposure to the ECP of *A. salmonicida* was a non-cytotoxic event and the EGCs were capable of regeneration. Evidence of EGC regeneration included a noticeable increase in cellular activity following the loss of most of the granules. This increase in cellular activity was characterised by an increase in the number of Golgi apparatus and the

presence of rough endoplasmic reticulum.

In a separate study, EGCs were observed to degranulate in the gills of Atlantic salmon following *in vivo* exposure to a major cytolysin of *A. salmonicida*. Cytolysins are toxins capable of causing cell lysis that can be isolated from ECP. Interestingly, this *A. salmonicida* cytolysin did not produce the marked degranulation of intestinal EGCs as characterised by ECP exposure, indicating a possible difference in the mechanisms of stimulation between gill and intestinal EGC populations (Lee and Ellis, 1991).

Lamas *et al.* (1991) investigated the effects of ECP isolated from *V. anguillarum* on EGC populations. In this study, *in vivo* exposure to *V. anguillarum* ECP was demonstrated to cause a mobilization and possible diapedesis of the EGCs. This migratory capacity resulted in the presence of EGCs in the blood, and an accumulation of EGCs in the kidney and spleen of treated fish. A reduction of EGCs in the intestinal stratum compactum was not observed, however EGCs appeared to be more prevalent in other regions of the gut, including the lamina propria. Many of the EGCs from treated fish observed in this study had a ruffled "active" plasma membrane, indicative of some form of granule release. And yet, free granules were not observed in the extracellular matrix (Lamas *et al.*, 1991).

In a separate *in vivo* study using *V. anguillarum* ECP, Powell *et al.* (1993A) demonstrated a histological and ultrastructural degranulation of intestinal EGCs similar to that outlined above for *A. salmonicida*. There was a marked decrease in the number of EGCs in the stratum compactum and an increase in EGCs in the lamina propria. Free granules were also observed in the extracellular matrix. Ultrastructurally, the granules were frequently multivesicular and had a pattern of crystalline-like sub-fractionation.

1.2.2. Neurotoxins and neurotransmitters

Substance P is a putative neurotransmitter of the nonadrenergic-noncholinergic nervous system (NANC) of both mammals (Shanahan *et al.*, 1985) and teleosts (Holmgren *et al.*, 1985). Capsaicin, a neurotoxin, is known to cause the release of substance P from NANC excitatory nerve endings (Buck and Burks, 1986). Both of these pharmacological agents are known to stimulate , and in the case of capsaicin, enhance mast cell degranulation in mammals (Mousli *et al.*, 1989; Nilsson *et al.*, 1990).

Following intraperitoneal exposure to both substance P and capsaicin, EGCs in the stratum compactum demonstrated limited degranulation, although there is also a significant decrease in the number of EGCs in the stratum compactum. The prevalence of EGCs in the lamina propria increased post-injection, and the granules of these cells exhibited a multivesicular appearance (Powell *et al.*, 1991; Powell *et al.*,

1993B). This multivesicular degranulation is comparable to an observed form of mast cell degranulation (Dvorak *et al.*, 1985).

Following the apparent migration to and degranulation of the EGCs in the lamina propria, there was a noted increase in the presence of EGC-like cells with an abundance of small granules. These cells were later observed to have migrated to the stratum compactum. This was postulated to be evidence of regranulation and possible maturation of the EGCs in the stratum compactum (Powell *et al.*, 1993B).

1.2.3. Mast cell secretagogues: compound 48/80 and A23187

Compound 48/80 and A23187 are two pharmacological agents that produce marked degranulation of mast cells in mammals (Röhlich *et al.*, 1971; Behrendt *et al.*, 1978; Pearce, 1986; Foreman, 1993). *In vivo* exposure to both these agents has been demonstrated to degranulate intestinal EGCs (Vallejo and Ellis, 1989; Powell, 1991).

Compound 48/80 is a synthetic polyamine produced by the acid-catalased condensation of formaldehyde and p-methoxy-N-methylphenethylamine (Pearce *et al.*, 1985). Compound 48/80 is often described as a classical mast cell degranulating agent as it has been used extensively in degranulation studies involving mammalian mast cells. It acts specifically upon connective tissue mast cells, resulting in an anaphylactic-like extrusion of the mast cell granules (Röhlich *et al.*, 1971; Pearce *et al.*, 1985; Landry *et al.*, 1992).

Following *in vivo* exposure to compound 48/80, EGCs have demonstrated an anaphylactic-like extrusion of their granules similar to CTMCs. This extrusion was coupled with varying degrees of granule vacuolation and loss of electron density (Vallejo and Ellis, 1989). The PAS positive granular leucocyte has also been demonstrated to degranulate following *in vivo* exposure to compound 48/80. This exposure resulted in a release of free granules of PAS-GLs into the extracellular matrix causing an observable reduction in the number of intracellular granules. Within 12 hrs post-injection, a regeneration of granules was apparent (Barber and Mills Westermann, 1978B).

A23187 is an ionophore capable of forming lipid soluble complexes with metal divalent cations such as calcium (Ca^{++}). This facilitates the transport of these ions across the hydrophobic plasma membrane. Similar to compound 48/80, A23187 is able to produce a marked degranulation and mediator release in mammalian CTMCs (Behrendt *et al.*, 1978; Pearce, 1986). Intraperitoneal exposure of rainbow trout intestinal EGCs to A23187 results in an anaphylactic-like extrusion of the EGC granules into the extracellular matrix (Powell, 1991).

1.3. Nonspecific defence in the gastrointestinal tract of teleosts

The gastrointestinal tract of teleosts is a major site of exposure to toxins and pathogens from the external environment. For this reason, it is the site of many nonspecific defence mechanisms. Nonspecific defence can be characterised as the natural, innate, genetically caused reaction mechanisms of the host. These host reactions constitute host resistance to exogenic noxae and disease pathogens. In the teleost intestine, as in other higher vertebrates, nonspecific defence of the gastro-intestinal tract can be characterised by physical, chemical, cellular and humoral defensive factors (Ellis, 1989; Schäperclaus, 1991).

The entire integument of teleosts and the luminal surface of the gastrointestinal tract, are covered by a continuously sloughed off and replenished layer of mucus. This mucous layer represents an effective physical barrier against the infiltration of toxins and micro-organisms into the underlying tissue (Ellis, 1989).

The mucous layer also acts as an effective chemical barrier inhibiting colonisation and/or invasion of infectious pathogens. Gastrointestinal mucus is known to contain lysozyme, proteolytic enzymes and C-reactive proteins as well as neutralizing, bacteriocidal and fungicidal components (Alexander and Ingram, 1992). Chemical factors typically secreted into the lumen of the teleost stomach and intestine also contribute to the hostile environment offered to potential pathogens. These factors

include stomach acids, digestive enzymes and bile (Ellis, 1989).

Once a pathogen or toxic agent penetrates through the physical and chemical barriers of the gastrointestinal tract, humoral and cellular defence mechanisms are activated (Schäperclaus, 1991).

Humoral factors are noncellular soluble substances found in the body fluids of vertebrates, including the teleosts (Ellis, 1989). These factors protect the teleost host through various means, including inhibiting the growth of the invading micro-organism (nutritional immunity) and producing lysis of the pathogen. An example of a humoral micro-organism growth inhibitor in teleosts and other vertebrates is transferrin. Transferrin binds free iron in the host, thereby depriving this essential element from the invading pathogen (bacteria, fungus, or parasite). Other humoral factors include lysins, enzymes capable of pathogen cell lysis. They can be either a single substance, for instance lysozyme, or a group of enzymes, such as the complement cascade (Alexander and Ingram, 1992).

Cellular nonspecific immunity is typically characterised in teleosts as phagocytosis. Phagocytosis involves the removal, by uptake mechanisms, of substances from the circulation and tissues by the hosts cell's, termed phagocytes. Similar to mammals, this role is performed by granulocytes (particularly neutrophils) and mononuclear cells (tissue macrophages and circulating monocytes). The nonresident tissue

phagocytes migrate to sites of infection and/or injury in the gastrointestinal tract as part of the teleost inflammatory response. The gastrointestinal tissues have a rich supply of blood vessels, which aid in the infiltration of these cells. Studies have indicated that these phagocytes possess both potent bactericidal and antiparasitic activity, thereby reducing the risk of further invasion of pathogens (Schäperclaus, 1991; Secombes and Fletcher, 1992). Recent evidence has suggested that EGCs are capable of some level of phagocytosis. In these studies, it was demonstrated that intestinal EGCs from rainbow trout were capable of endocytosing and degrading antigenic proteins both *in vivo* (Le Bail *et al.*, 1989; Dorin *et al.*, 1993B) and *in vitro* (Dorin *et al.*, 1993A).

Natural cytotoxic cells (NCC), that are similar in function to the mammalian natural killer (NK) cell have also been identified as a component of nonspecific cellular defence in teleosts (Ellis, 1989). In mammals, NKs are granular nonphagocytic lymphocytes that bind and destroy tumour cells, viral infected cells and some bacteria and fungi (Tizard, 1988). In teleosts, NCCs resemble monocytes and have been demonstrated to have cytotoxic effects on cells infected with viruses and parasites (Ellis, 1989).

The nonspecific defence function of granular leucocytes (heterophils, eosinophils, basophils) that morphologically resemble their mammalian counterparts is not clearly defined. This is due in part to both their variability in existence and histochemistry

in teleosts (Ellis, 1971; Ellis, 1989).

1.3.1. Hypersensitivity in teleosts

Inflammation is a protective response of the host to injury. It results in specific morphological and chemical changes in cells and tissues. Evidence of both acute and chronic inflammatory responses have been demonstrated in fish. These responses have the same general characteristics as in higher vertebrates, for instance, vasodilation and increased vascular permeability, leucocyte migration and infiltration, and finally resolution and healing (Tizard, 1988; Schäperclaus, 1991, Suzuki and Iida, 1992).

A reaction similar to Type I immediate hypersensitivity, or anaphylaxis, in mammals, has been demonstrated in several species of fish. The typical physiological and behavioral "shock" responses that have been noted include increased opercular movement and gasping, disorientation and loss of equilibrium, increased defecation and, in some instances, death (Ellis, 1982; Ellis, 1985; Jurd, 1987). Ellis (1985) observed typical signs of anaphylaxis in rainbow trout injected with ECP from *A. salmonicida* as well as a rapid degranulation of the intestinal EGCs. This reaction was concomitant with a decrease in the intestinal histamine levels and a rise in blood histamine levels. The response of the EGCs was very similar to that of mammalian mast cells undergoing a non-antigenic anaphylaxis-like reaction.

In another study, antigen-specific sensitization was transferred to unsensitized fish by serum from a sensitized individual (Goven *et al.*, 1980). The role of antigen as an initiating factor and the mechanism of the response that has been elicited remains unresolved. And yet, it has been theorized that EGCs and PAS-positive granular leucocytes may play a role similar to the mammalian mast cell in the initiation of the antigenic pathway of inflammation in teleosts (Ellis, 1982; Ellis, 1985; Jurd, 1987). Fish lack IgE, the immunoglobulin isotype responsible for inducing mast-cell hypersensitivity degranulation. The only immunoglobulin isotype present in fish is IgM, and some have suggested that it may mediate anaphylactic responses in fish (Goven *et al.*, 1980; Ellis, 1985).

1.4. Research objectives

The objective of this study was to evaluate both morphologically and biochemically the effects of *in vitro* exposure of Compound 48/80 and A23187 on intestinal EGCs of the rainbow trout. It was hypothesized that convergent results could be established between these *in vitro* studies and those previously undertaken *in vivo* for EGCs, thereby potentially strengthening the similarity between teleost EGCs and mammalian CTMCs.

CHAPTER 2. HISTOLOGICAL EVALUATION OF EGC DEGRANULATION INDUCED BY *IN VITRO* EXPOSURE TO COMPOUND 48/80 AND A23187

2.1. Introduction

EGCs in salmonids and other teleost species have been demonstrated to have distributional and morphological similarities to the mammalian connective tissue mast cell (CTMC). Similar to the CTMC, EGCs are found largely in the connective tissue of certain teleost species. The two cell types are mononucleated and both accommodate an abundance of cytoplasmic granules that are relatively homogenous with regards to their shape and density (Smith, 1975; Ezeasor and Stokoe, 1980; Irani and Schwartz, 1989; Barrett and Pearce, 1993).

Histochemical analogies between EGCs and mast cells have been less direct. This is due in part to the eosinophilic nature of the EGC granules. Mast cell granules are strongly basophilic. EGC granules, unlike those of the CTMC, do not contain sulphated glycosaminoglycans, such as heparin (Smith, 1975; Ezeasor and Stokoe, 1980). Evidence regarding the presence of EGC derived biogenic amines, similar to those found in mast cell granules, is not conclusive (Ellis, 1982; Ellis, 1985; Beorlegui

et al., 1992).

An increase in the prevalence of EGCs has been observed in several teleost species, including salmonids, that have acquired parasitic infections. This proliferation of EGCs is sometimes paired with an anaphylactic-like degranulation of the cells (Reimschuessel *et al.*, 1987; Sharp *et al.*, 1989; Cross and Matthews, 1992). Mammalian mast cells are also known to proliferate and degranulate following exposure to intestinal helminths, such as nematodes. In mammals, the infiltration of these parasites is hindered, often through violent muscle contractions that cause the parasite to be expelled into the luminal cavity (Askenase, 1980). Anaphylactic-like degranulation as well as an increase in EGC numbers has also been noted in specific salmonid viral diseases (Yasutake and Amend, 1972; Wolf, 1988; Smail *et al.*, 1992).

In an effort to further understand the function of the EGC and its similarity to the mammalian mast cell, *in vivo* studies have tried to manipulate EGC degranulation using agents that have been demonstrated to result in CTMC degranulation. These agents include bacterial exotoxins, neuropeptides and neurotoxins, and mast cell secretagogues (Ellis, 1985; Vallejo and Ellis, 1989; Powell, 1991). To date, experimental studies involving EGCs have largely involved salmonid species.

Based on histochemical data, intraperitoneal (IP) injection of the extracellular products (ECPs) of *A. salmonicida* and *V. anguillarum* is followed by degranulation

of EGCs that is characterised by an anaphylactic-like release of free granules into the extracellular matrix. Tsuchiya *et al.* (1972) noted a similar release in mast cells exposed to *V. cholera* exotoxin. Frequently, this expulsion of granules is coupled with a marked decrease in the number of observable EGCs in the stratum compactum of the intestine of rainbow trout (Ellis, 1985; Vallejo and Ellis, 1989; Powell *et al.*, 1993A). Quantification of the decline in EGC numbers in treated fish, through cell counting at the light microscopy (LM) level, demonstrated that this reduction of EGCs was statistically significant (Powell *et al.*, 1993A).

In other studies, EGCs from the intestine of rainbow trout were exposed through IP injection to the neuropeptide, substance P and the neurotoxin, capsaicin. Both agents were injected IP into fish. In both cases, there was a significant decrease in the number of observable intact EGCs at the LM level. However, an expulsion of granules into the extracellular matrix was not apparent (Powell *et al.*, 1991; 1993B).

In two earlier studies, granules expelled from both EGCs and PAS-GLs were observed in the extracellular matrix following *in vivo* exposure to the classical mast cell secretagogue compound 48/80 (Barber and Mills Westermann, 1978B; Vallejo and Ellis, 1989). An observable reduction in PAS-GLs in the haemopoietic tissue of the kidney was noted by Barber and Mills Westermann (1978B). A similar release of granules from intestinal EGCs has also been observed following *in vivo* exposure to the mast cell secretagogue A23187 (Powell, 1991).

In this study, intestinal tissue of rainbow trout was exposed *in vitro* to the mast cell secretagogues, compound 48/80 and A23187. Compound 48/80, similar to other traditional non-antigenic triggers, activates the pertussis toxin-sensitive G proteins located in the CTMC plasma membrane. This results in an activation of phospholipase C which promotes an increase in the production of inositol 1,4,5-trisphosphate (IP_3). IP_3 mobilizes Ca^{++} in mast cells from the endoplasmic reticulum, resulting in a rise in cytosolic Ca^{++} (Landry *et al.*, 1992, Hoth *et al.*, 1993). The ionophore A23187 also causes an increase in cytosolic Ca^{++} levels. Rather than activating G proteins, A23187 preferentially forms lipid soluble complexes with the divalent cation Ca^{++}. This facilitates the direct transportation of the Ca^{++} ions across the plasma membrane and into the cytosol (Pearce, 1986).

The mast cell secretagogues A23187 and compound 48/80 were chosen for this study for two reasons. Firstly, in mammalian systems both drugs have been demonstrated to trigger IgE-independent pathways of mast cell activation (Pearce, 1986; Landry *et al.*, 1992; Foreman, 1993). Secondly, *in vivo* studies have demonstrated that exposure to either compound 48/80 or A23187 cause intestinal EGCs from rainbow trout to degranulate (Vallejo and Ellis, 1989; Powell, 1991). The extent of intestinal EGC degranulation was to be measured at the LM level through cell counting. A significant decrease in the number of observable intact EGCs in those tissues exposed to either compound 48/80 or A23187 was considered indicative of EGC degranulation.

2.2. Methods

2.2.1. Fish holding

Rainbow trout were purchased from Integrated Water Systems (Brookvale, Prince Edward Island) and housed at the Atlantic Veterinary College's Fish Health Unit holding facility. The fish were held in a 1 m circular tank with a flow rate of 20 l/min of well water. The temperature was maintained at 10.5 -11°C and there was a 12 hr light / 12 hr dark photoperiod. Care and utilization of the fish followed the guidelines outlined by the Canadian Council on Animal Care (1984).

2.2.2. Drug preparation

Fresh stock solutions of Krebs-Henseleit physiological solution (KHS) (Kitchen, 1984) were prepared for each set of experiments (Appendix 1). The buffer (pH 7.45-7.55) was then chilled to 4°C and aerated with 95% O_2 : 5% CO_2 prior to using. Compound 48/40 in KHS was prepared as a 1.0mg/ml stock solution prior to each experiment. The treatment tissue was exposed to compound 48/80 at a concentration of 100µg/ml. This concentration is comparable to those used in *in vitro* mammalian mast cell studies (Pearce *et al.*, 1985). Vehicle controls for the compound 48/80 experiments consisted of an equal volume of KHS.

Stock solutions of A23187 in ethanol were prepared at a concentration of 1mg/ml and kept in the dark at -5°C. These solutions were then diluted in KHS to a

concentration of 10 μg/ml for the treatment solution. Similar concentrations of A23187 have been used in *in vitro* human lung mast cell studies (Church *et al.*, 1982). One percent ethanol in KHS was used as the vehicle control for these experiments.

2.2.3. Challenge protocol

Fish were killed by cervical dislocation. A 3 cm section of the proximal intestine in the vicinity of the spleen was removed. Extraneous fat was carefully trimmed from the surface of the section. Intestinal contents were flushed from the intestine with cold aerated KHS using a Pasteur pipette. The intact intestine was then cut along its longitudinal axis. The resulting flat piece of intestine was once again cut down the longitudinal axis to create two equal sized strips of intestine suitable for paired studies.

The two sections were randomly assigned as being either control or treatment. The strips of gut were then suspended in organ baths filled with 10 ml of aerated (95% 0_2 : 5% CO_2) KHS, which was maintained at 10°C. The tissues were allowed to equilibrate for 30 minutes before the experiment. Upon equilibration of the tissue, the appropriate test or vehicle control solution was added to the organ bath. The test exposure time was 30 minutes.

2.2.4. Tissue preparation

Following incubation, the tissue was removed from the organ baths and placed in chilled 3% glutaraldehyde in 0.13M Millonig's phosphate buffer (Appendix) for a minimum of 2 hours at 4°C before further manipulation. The tissue was then trimmed at its borders. The remaining section of tissue was subsequently cut in cross sections to produce 1 mm transverse sections. These sections were then placed in fresh fixative for at least 12 hours at 4°C. Following the primary glutaraldehyde fixation, the tissue was post-fixed in 1% osmium tetroxide in distilled water for 1 hour at 4°C.

Later the tissue was washed with distilled water and dehydrated using an ascending series of ethanols. Following dehydration, the sections were cleared with propylene oxide and infiltrated using a series of epon resin:propylene oxide mixtures (45 min in 50:50, 45 min in 75:25, 90 min pure epon). Ten tissue segments for the control and for the treatment per fish were then orientated in flat LKB Easy Molds (JB EM) and embedded in fresh Epon resin. The blocks were then polymerized overnight at 65-70°C.

Semi-thin (500nm) and thin (90nm) sections were cut on a Reichert-Jung Ultracut E ultramicrotome using glass knives made on an LKB Bromma 218 knifemaker. Semi-thin sections mounted on glass slides were stained with 1% toluidine blue in a 1% aqueous sodium borate solution (Appendix) and examined using an Olympus

BH-2 light microscope. Light micrographs were produced using a Zeiss III photomicroscope with Kodacolour 100 ASA film.

Thin sections for electron microscopy were placed on uncoated 200 mesh copper grids and double stained with a saturated solution of uranyl acetate in 50% ethanol and lead stain (Appendix). The thin sections were examined and photographed on both a Hitachi H600 and a H7000 series electron microscope operated at 75 kV. Electron micrographs were printed on Kodak Polycontrast III RC paper using a Kodak Ektamatic processor.

2.2.5. Cell counting criteria

EGCs were counted using light microscopy employing a final magnification of 1000x (oil immersion objective lens). Intact EGCs in the stratum compactum were counted in three individual tissue sections from each strip of intestine. The average number of cells/mm of stratum compactum was then calculated for each strip. EGCs defined as being "intact" at the light microscope (LM) level had the following criteria: the cells were nucleated, ovoid and had > 5 uniformly sized granules/cell. EGCs that contained extremely large granules (> 4μm in diameter) were not included in the count, as they clearly did not have the typical morphology of a normal non-degranulated EGC.

Several steps were taken in an effort to reduce variability of the number of EGCs

counted from one section to the next. Cells were only counted if the tissue was orientated in the correct plain. Correct orientation was determined when the circular muscle immediately below the stratum compactum was in a longitudinal plain and the longitudinal muscle was in a cross section. Areas lying beneath the villi were not counted as the stratum compactum in these areas tended to vary with regards to width. In addition, the number of villi/length of tissue varied from one strip of tissue to another. The origin of the tissue (ie. treatment or control) was withheld from the person counting the cells to eliminate any bias while counting.

2.2.6. Validation of cell counting criteria

Prior to commencement of cell counting, a preliminary study was established to determine if findings at the LM level with regards to physical state of the EGCs could be confirmed using electron microscopy. This preliminary study was also used to validate the ability to accurately resolve at the LM level extracellular granules that had been released from the EGCs. Thick sections from two randomly chosen tissue blocks representing a vehicle control (plain KHS) and treatment (100μg/ml compound 48/80) were observed using LM at 1000x. These sections had been cut from blocks that were prepared for subsequent thin sectioning. All EGCs from the stratum compactum from these sections were hand drawn and their physical state (intact or disrupted) was indicated. The sections were then thin sectioned; care was taken to ensure that the thin section was taken from a depth as close to the previously observed thick section as possible. The thin sections were then observed

at the electron microscope (EM) level, and the state of the EGCs was compared to that formerly observed at the LM level.

2.2.7. Testing of experimental design

During the course of the experiment problems relating to inconsistencies in the control tissues became apparent. Areas of regional explosive degranulation of the stratum compactum EGCs were evident in not only the test tissues, but also in the controls. This degranulation will be expanded upon in the Results section. This phenomena was originally attributed to problems relating to experimental design. For this reason, several smaller experiments were undertaken in an effort to try and determine the cause of the degranulation in the control tissues. In these experiments, an unbiased observer simply looked for evidence of degranulation in unmarked tissue samples (cell counting was not performed). Two factors were identified as being possible causes of degranulation in the controls: 1) time delays between removing the tissue from the fish and placing the sections into the chilled KHS, and 2) cutting the intestine into longitudinal strips. In both studies, free EGC granules within the extracellular matrix were considered to be evidence of a cell's degranulation. The following outlines briefly how these factors were tested as probable causes for explosive degranulation in the controls:

An experiment was established to determine if post mortem changes resulting from time delays prior to placing the intestine in the chilled KHS was the cause of the

nonspecific degranulation in the control tissues. Four fish were killed by spinal severance. A 3 cm section of proximal intestine was removed and cut open along the longitudinal axis and then divided into 3 equal cross sections. Immediately, at time 0, one section was placed in chilled fixative. The remaining two sections were laid on paper towels at room temperature for 10 and 30 minutes respectively prior to the initiation of fixation. Tissue preparation followed the protocol previously outlined in 2.2.4.

In an effort to determine if the cutting of the intestine along the longitudinal axis may have resulted in the degranulation of EGCs in the stratum compactum, a second experiment was established. Three fish were killed by spinal severance and a 3 cm section of proximal intestine was removed from each fish. The tissue was divided on a cross section and one section (in a tubular form) was placed immediately in chilled fixative. The second portion of intestine was carefully cut on its longitudinal axis to open up the intestinal ring prior to fixing. Tissue preparation then followed the protocol previously outlined in 2.2.4.

2.2.8. Statistical analysis

A paired Student-Newman Keuls t-test (Glantz, 1987) was used to test for significance ($p<0.05$) in the number of EGCs between the treatments (Compound 48/80 and A23187) and their appropriate controls.

2.3. Results

2.3.1. Control fish

The tissue sections at the LM level showed no evidence of mechanical damage; the epithelium and the brush border were both intact. Toluidine blue stained the EGCs orthochromatically, the granules staining homogeneously a dense blue. The majority of EGCs were evident in the collagenous network of the stratum compactum, which is nestled between the lamina propria of the mucosa and the circular smooth muscle of the muscularis (Figure 2.1). At this site, the EGCs comprised a continuous layer of cells (Figure 2.2).

Ultrastructurally, the EGCs of the control fish were similar to those described previously by Smith (1975) and Ezeasor and Stokoe (1980) (Figure 2.3). The mononucleated cells were predominately round to oval shaped, ranging in size from 10 to 15 μm in diameter. The membrane bound granules ranged in size from 0.1 to 1.3 μm in diameter. The granule matrix was largely homogenous and electron dense. However, reticulated granules were observed in some cells. Cytoplasmic organelles included stacks of Golgi saccules, rough endoplasmic reticulum, and a few mitochondria.

Although most EGCs appeared to be intact both at the LM and EM level, discrete regions of explosive EGC degranulation were commonly observed in sections of the

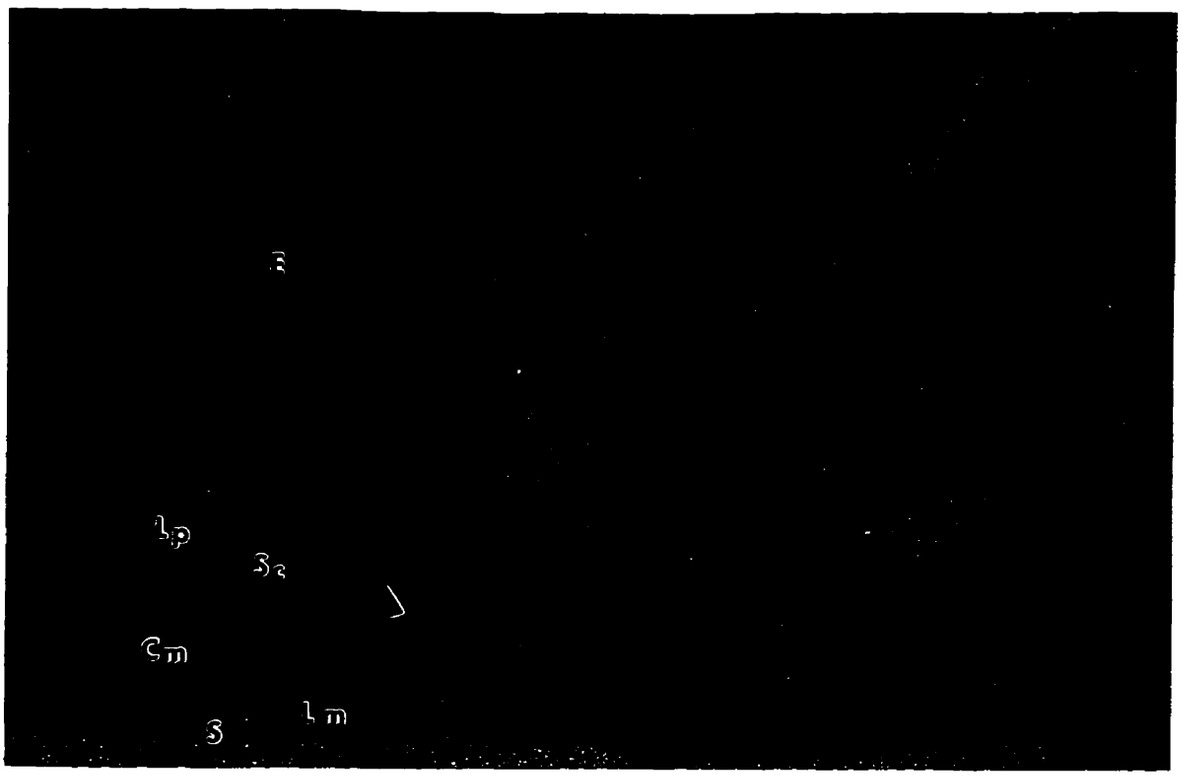

Figure 2.1. Cross section through the wall of control small intestine of rainbow trout. E= epithelium; Lp= lamina propria; Sc= stratum compactum; arrowhead= EGC; Cm= circular smooth muscle; Lm= longitudinal smooth muscle; S= Serosa. Tissue section was stained with toluidine blue. (x250)

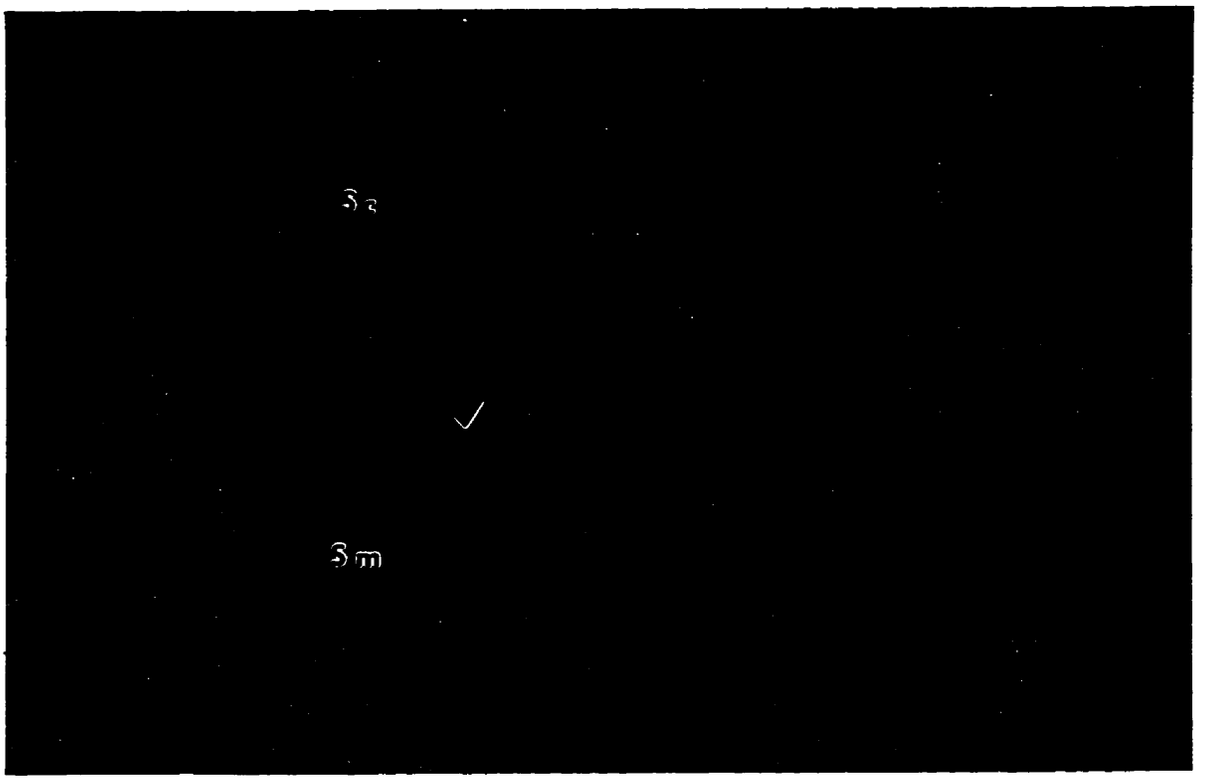

Figure 2.2. Intact EGCs forming a continuous layer in the stratum compactum of control rainbow trout small intestine. Arrowhead= EGC; Sc= stratum compactum; Sm= smooth muscle. (x1000)

Figure 2.3. Electron micrograph of an intact EGC from control rainbow trout small intestine. G= membrane bound granules; Nu= nucleus; Gc= Golgi stack; arrowhead= plasma membrane. (x 11520)

vehicle control tissues. In these regions, free, densely stained granules were evident throughout the collagenous extracellular matrix (Figure 2.4). At the EM level, it was observed that other cellular organelles had been released, for instance, mitochondria (Figure 2.5). It is interesting to note that the tissue layers above and below these regions of degranulation displayed no signs of mechanical damage (Figure 2.4).

2.3.2. Treatment fish

At the LM level, EGCs of the fish intestine exposed to both compound 48/80 and A23187 were histologically similar to those of the vehicle controls. EGCs from the stratum compactum from both the treatment and control tissues demonstrated areas of intact cells that were intermittently interspersed with regions of explosive degranulation. At the EM level it was noted that both the treatment (compound 48/80 and A23187) and control tissues displayed EGCs that were intact, as well as those that had expelled their granules into the extracellular matrix. Several sections from the control and treatment groups possessed intact EGCs that contained giant granules (>4μm in diameter). These giant granules were obvious at both the LM (Figure 2.6) and EM (Figure 2.7) level.

2.3.3. Validation of cell counting criteria

In both sections (control and treatment) over 50 cells were mapped out at the LM level. It should be noted that some difficulty existed with regards to locating these cells under the electron microscope due to the presence of grid bars and stain

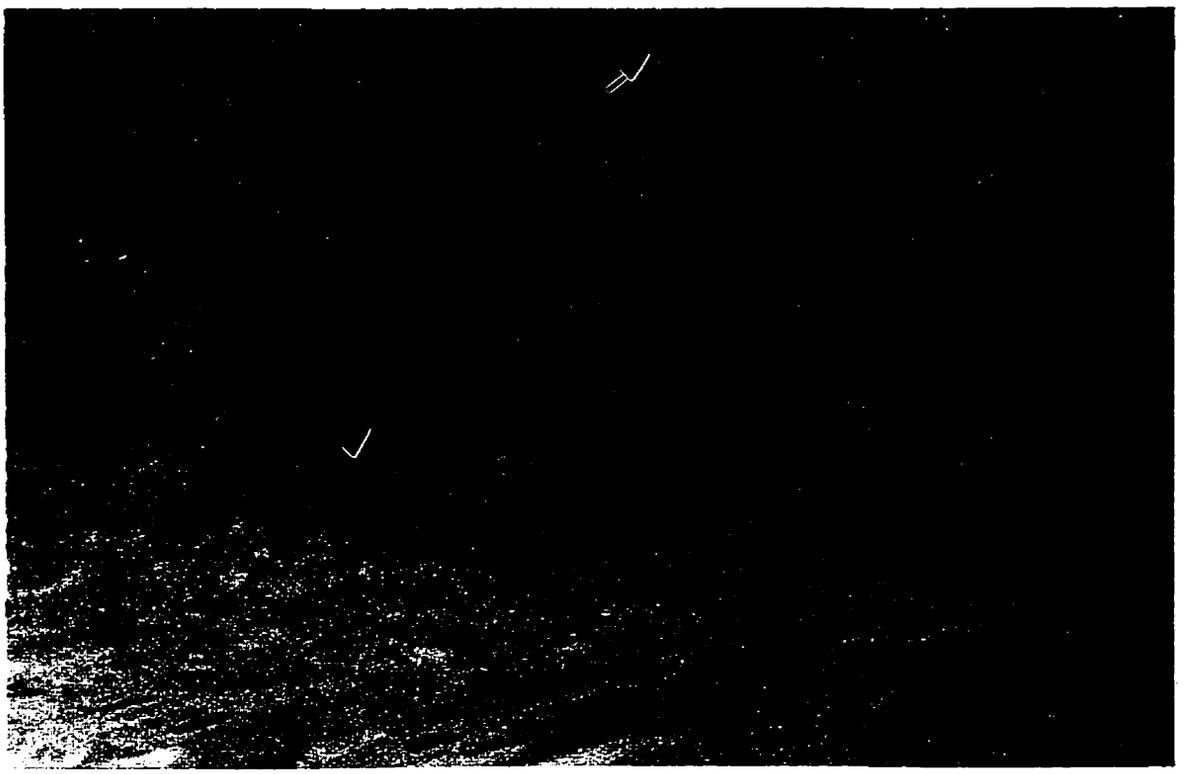

Figure 2.4. Degranulated EGCs with released granules within the extracellular matrix of control tissue. Arrowhead= released granules; arrow= intact epithelial brush border. (x630)

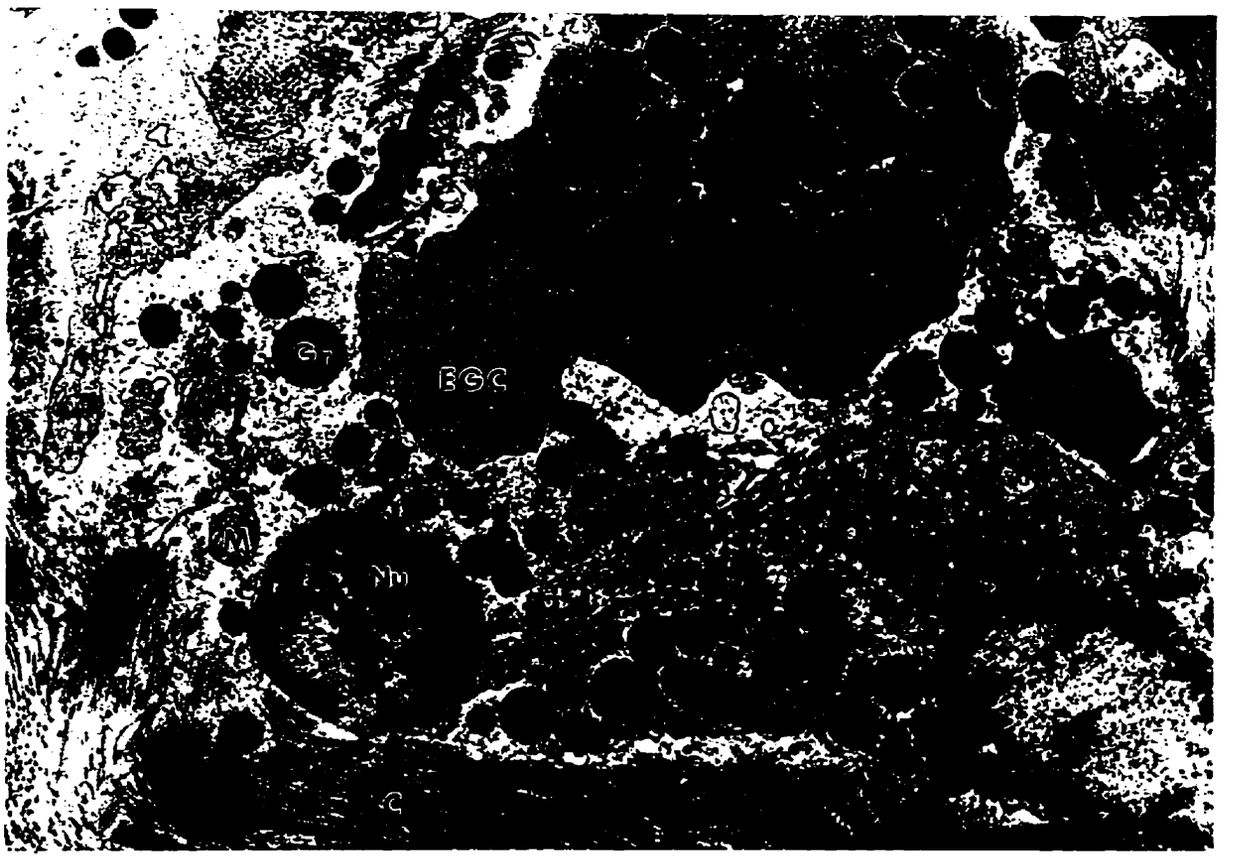

Figure 2.5. Ultrastructural evidence of degranulated EGCs from control rainbow trout small intestine. EGC= intact EGC, Gr= granules released into the extracellular matrix. Nu= released nucleus; M= released vacuolated mitochondria; C= collagen fibrils of the extracellular matrix. (x7680)

Figure 2.6. Evidence of giant granules within EGCs of the stratum compactum of control rainbow trout small intestine. G= giant granules; arrowhead= intact EGC. (x1000)

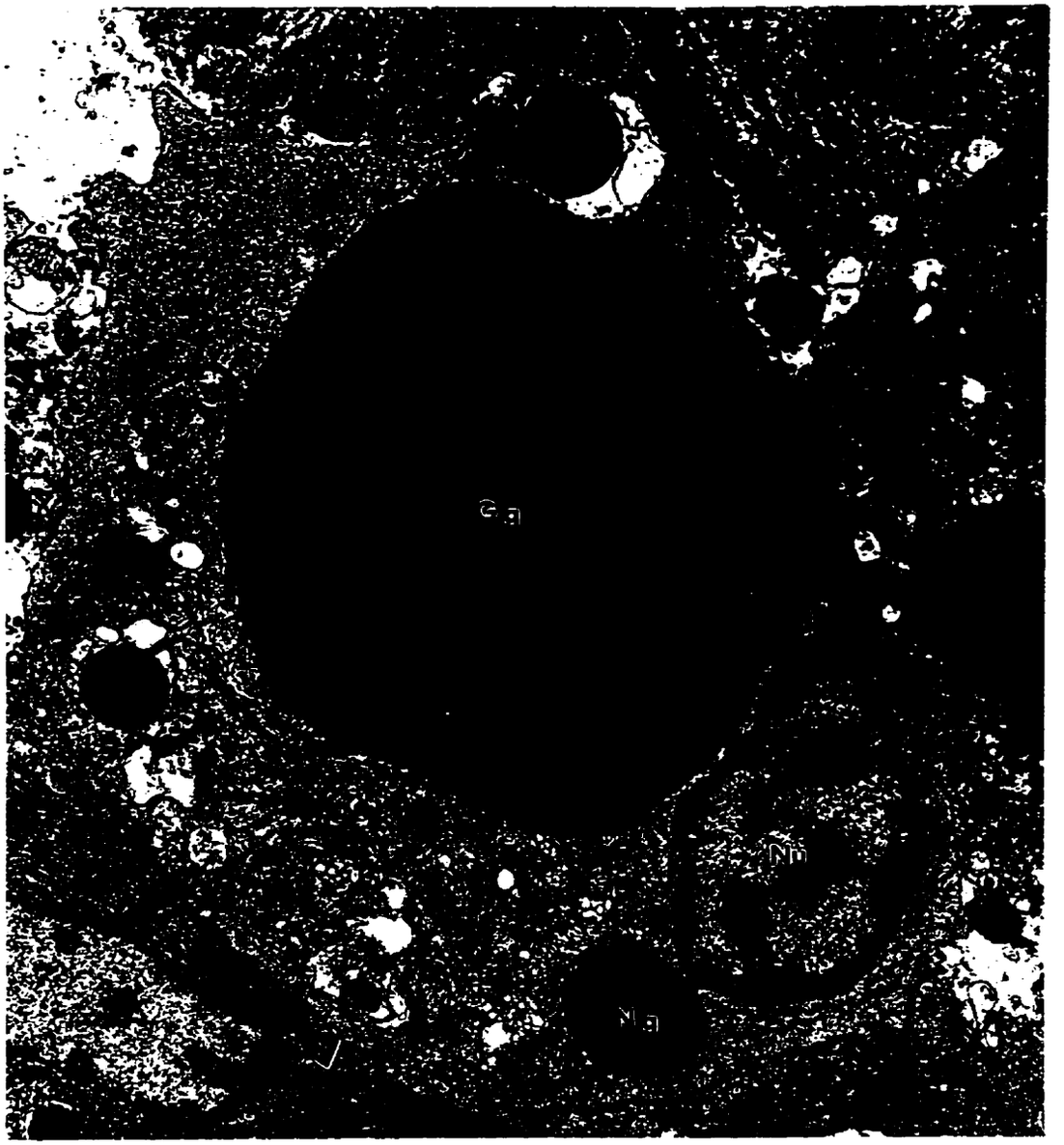

Figure 2.7: Ultrastructural evidence of an intact EGC with a giant membrane bound granule from control tissue. Gg= giant granule; Ng= normal sized granule; Nu= Nucleus; arrowhead= intact plasma membrane. (x 12000)

particles. For this reason, in both sections, the first 15 LM defined intact cells that could be located under the electron microscope were included in this study. At the LM level, intact cells were defined as being nucleated, ovoid and had > 5 uniformly sized granules/cell. Under the electron microscope, the intactness of the cell was validated by the presence of a complete plasma membrane surrounding the cell in question. In both the treatment and control sections, the 15 cells defined as being intact at the LM level were in fact intact at the EM level. This in effect established a consistency in the counting criteria used in the succeeding study. It was also interesting to note that, although not quantified, regions of free granules could be located accurately both at the LM and EM level.

2.3.4. Cell counting

With regards to the original hypothesis, *in vitro* exposure to compound 48/80 or A23187 did not significantly ($p > 0.05$) reduce the number of EGCs present in the stratum compactum (Table 2.1). Interestingly, the p-value for the compound 48/80 set of experiments was relatively low ($p = 0.08$). This resulted from a higher number of intact cells in the treatment tissues compared to the control tissues. Any difference that may have been present could have been masked by the effects of the regional explosive degranulation which produced large variations in cell counts. The large variation in the number of EGCs are reflected by the high standard errors associated with the counts.

Table 2.1. Summary of EGC counts from the stratum compactum of rainbow trout intestine following *in vitro* exposure to 100 μg/ml Compound 48/80 and 10 μg/ml A23187 and respective controls.

AGENT	N	EGCs/mm ± SEM CONTROL	EGCs/mm ± SEM TREATMENT	P VALUE
48/80	4	24.7 ± 6.0	38.6 ± 4.6	0.08
A23187	4	65.8 ± 11.9	72.7 ± 8.9	0.28

2.3.5. Testing of experimental design

Two individual tests were designed in an effort to determine the possible cause of the degranulation of EGCs in the control tissues. The first test was designed to test if post mortem autolysis contributed to control EGC degranulation. Evidence of degranulation was apparent at all time intervals devised for this experiment (0, 10, 30 minutes) (Figure 2.8 a,b,c). The second study was designed to determine if cutting the intestine along its longitudinal axis contributed to the degranulation in the control tissues. This did not appear to be the case, as there was evidence of degranulation in the control "non-cut" tissues in addition to those that had been cut (Figures 2.9 a,b).

2.4 Discussion

To date, most *in vivo* studies that have tried to manipulate EGC degranulation with mast cell secretagogues or bacterial exotoxins have been assessed morphologically. In these earlier studies, assessment of the degranulation of EGC populations both at the LM and EM level has been largely qualitative in nature. At the LM level, the expulsion of free granules into the extracellular matrix was typically considered to be indicative of EGC degranulation. In these studies, ultrastructural changes in the cells, for instance fusion of the granule membranes to form labyrinthine channels, were noted at the EM level (Ellis, 1985; Vallejo and Ellis, 1989; Powell, 1991; Powell

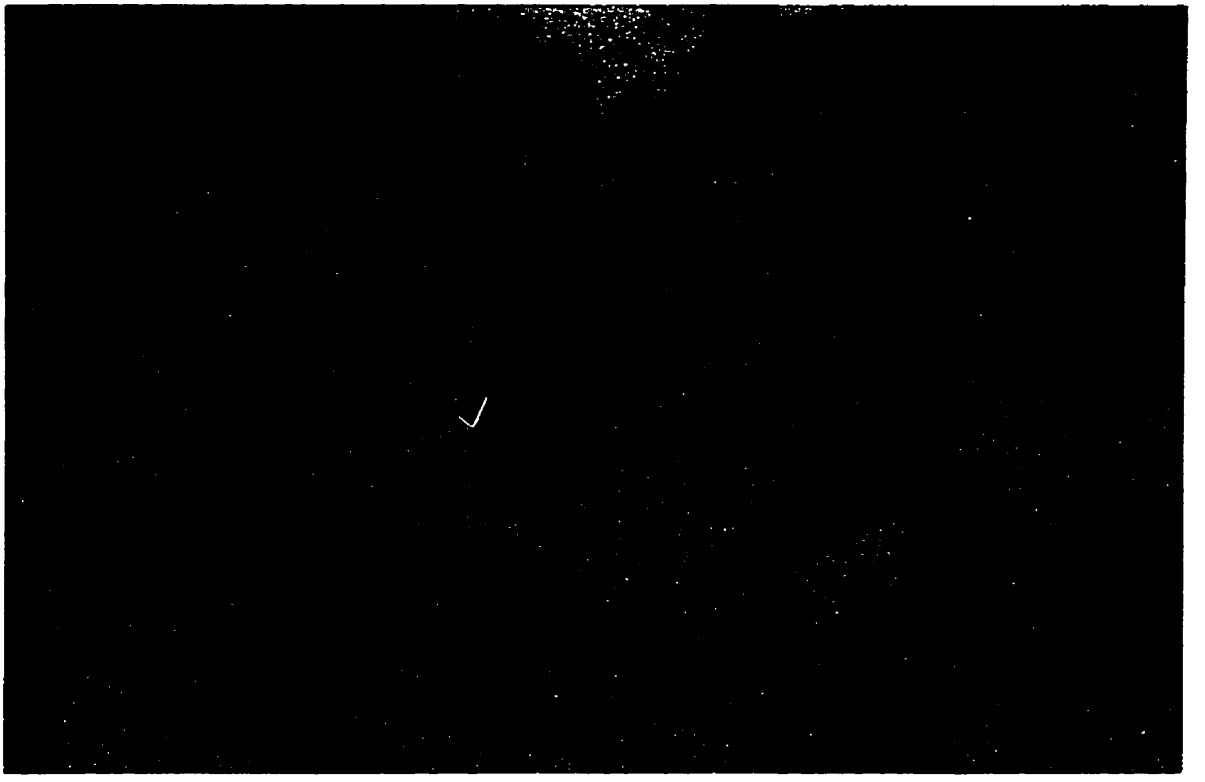

Figure 2.8.a. LM evidence of EGC degranulation following a time interval of 0 minutes. Arrowhead= evidence of released EGC granules into the extracellular matrix. (x1000)

Figure 2.8.b. LM evidence of EGC degranulation following a time interval of 10 minutes. Arrowhead= evidence of released EGC granules into the extracellular matrix. (x1000)

Figure 2.8.c. LM evidence of EGC degranulation following a time interval of 30 minutes. Arrowhead= evidence of released EGC granules into the extracellular matrix. (x1000)

Figure 2.9.a. Evidence of EGC degranulation, in the form of released granules into the extracellular matrix, in non-cut sections of tissue. Arrowhead = released granules. (x1000)

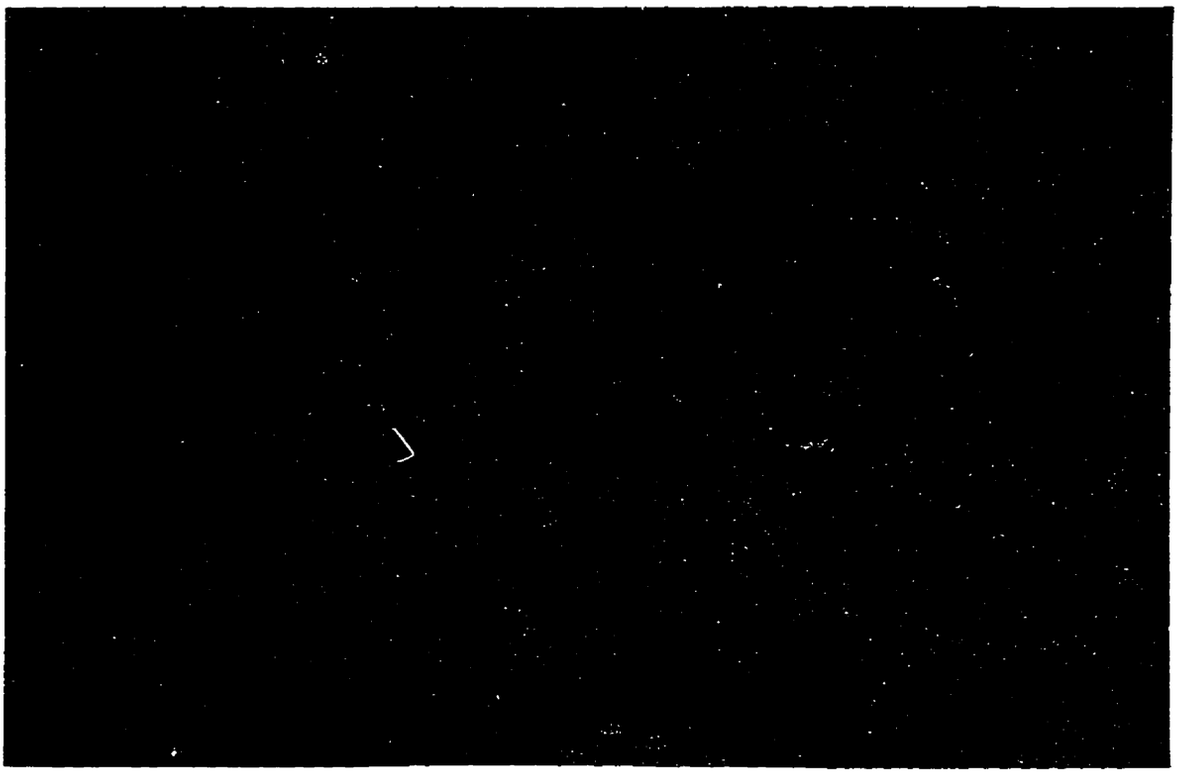

Figure 2.9.b. Evidence of EGC degranulation, in the form of released granules into the extracellular matrix, in dissected sections of tissue. Arrowhead= released granules. (1000x)

et al., 1993A). It is interesting to note that exposure to the neurotransmitter, substance P, orthe neurotoxin, capsaicin, did not cause the expulsion of free granules into the extracellular matrix, but did produce distinctive ultrastructural changes in the different EGC populations of the intestine (Powell *et al.*, 1991; Powell *et al.*, 1993B). In most of these studies, this EGC degranulation was marked by a reduction in the number of observable EGCs in the stratum compactum of the intestinal tissue. This reduction was frequently matched with a concomitant increase in the number of EGCs in the lamina propria, as a result of a possible migration (Ellis, 1985; Vallejo and Ellis, 1989; Powell *et al.*, 1991, 1993B; Powell *et al.*, 1993A). A few studies have used cell counting as a means of quantifying this phenomena, thereby assessing degranulation by a means that is not purely descriptive (Powell *et al.*, 1993A; Powell *et al.*, 1993B). In these studies, EGC degranulation is marked by a decrease in the number of visible EGCs at the light microscope level. This reduction could be the result of either A) a decrease in the overall visibility of the EGCs due to the loss of cytoplasmic granules into the extracellular matrix or B) a migration of the EGCs from the well-defined stratum compactum into the overlying lamina propria and less frequently into the muscle tissues lying below.

It is interesting to note that mammalian mast cell degranulation is typically assessed either qualitatively at an ultrastructural level (Rohlich, 1971; Behrendt *et al.*, 1978; Dvorak *et al.*, 1985) or quantitatively by measuring the release of mast cell mediators (Theoharides *et al.*, 1982; Jippo-Kanemoto *et al.*, 1993). Mast cell degranulation has

been quantified successfully although less frequently using cell counting at the LM level (Pesci *et al.*, 1993).

In this study, the results are inconclusive with respect to the occurrence of degranulation. However, it can be said that there was no significant change in the number of EGCs in the stratum compactum between the controls and the treatments. It was assumed that degranulation of EGCs would result in a decrease in the number of intact cells at the LM level. This assumption precluded the possibility that degranulation was in fact a subtle event, which may be better evaluated at the EM level.

It has not been conclusively established whether or not EGC degranulation requires the intervention of the central nervous system. For this reason, degranulation of EGCs may not be able to be manipulated *in vitro*, as it can be *in vivo*. An earlier study demonstrated that up to 32% of rainbow trout intestinal EGCs are found in close apposition with non-myelinated nerves (Powell *et al.*, 1991). It was suggested that this nerve-EGC relationship may provide an alternate physiological pathway of degranulation. This pathway may explain the *in vivo* degranulatory effects of capsaicin, a neurotoxin that causes the release of putative neurotransmitter substance P. Alternatively, substance P may not have acted directly upon the EGCs, but instead upon a spinal nerve reflex (Powell *et al.*, 1991; 1993B). Recent evidence also suggests a close association between mast cells and neurons. The neurons have been

implicated in possibly providing an alternate, more physiological pathway of mast cell degranulation in the surrounding tissues. This association may be an important link between the immune and nervous systems (Shanahan *et al.*, 1985; Theoharides, 1990). The regional degranulation was a concern as it created controls that were inconsistent, some having high and others having low cell counts. This phenomenon, which had not been reported in earlier *in vivo* studies was first attributed to experimental design. The experimental protocol was later validated when areas degranulation were in fact observed in tissues that had been immediately removed from the fish and placed in chilled fixative. Although this phenomenon had not been reported in the control tissues of earlier *in vivo* studies (Ellis, 1985; Powell, 1991), it has been observed previously. Smith (1975) noted areas of EGC degranulation in her control tissues in a histochemical study of salmonid EGCs. It is also interesting to note that researchers had difficulty isolating these cells due to degranulation (personal communication, Lucy Lee, University of Saskatchewan).

In the present study, intact EGCs with very large granules (>4μm in diameter) were observed in the control and treatment tissues both at the EM and LM level. Although the presence of giant cytoplasmic granules in EGCs has not been previously recorded, a more obvious fusion of smaller granules has been noted in Coho salmon with possible neoplastic lesions (Kent *et al.*, 1993). A similar occurrence has recently been observed in mast cells from beige rats with the autosomal recessive genetic disease Chédiak-Higashi syndrome. This syndrome has been characterized as a

generalized increase in the fusion of cytoplasmic granules and was originally recognized in melanocytes, neutrophils and lymphocytes. Interestingly, the level of histamine release is significantly higher from beige rat mast cells than those of normal rats. This supernormal histamine release may be the result of the giant granules found in beige rat mast cells (Jippo-Kanemoto *et al.*, 1993). This may be further evidence linking the EGC to an immunological function.

In conclusion, the observations generated from this study are inconclusive regarding the similarity of the EGC to the CTMC when exposed to specific agents *in vitro*. At this time, a morphological assessment of the effect of *in vitro* exposure of compound 48/80 and A23187 upon the EGC remains unestablished. For this reason, a second approach, attempting to quantify possible mediator release from the EGCs was considered.

CHAPTER 3. BIOCHEMICAL ANALYSIS OF MEDIATOR RELEASE FROM RAINBOW TROUT INTESTINE EXPOSED *IN VITRO* TO MAST CELL SECRETAGOGUES COMPOUND 48/80 AND A23187

3.1. Introduction

Eosinophilic granule cells (EGCs) are typically located in regions of antigen-encounter, for instance the gut, gills and the skin (Smith, 1975; Ezeasor and Stokoe, 1980; Powell *et al.*, 1990). Their location and morphology have caused them to be likened to the mammalian connective tissue mast cell (CTMC) (Ezeasor and Stokoe, 1980; Vallejo and Ellis, 1989; Powell *et al.*, 1991). In this study, *in vitro* experimentation was undertaken to determine if EGCs from rainbow trout intestine, similar to intestinal mammalian mast cells (Bienenstock, 1988; Irani and Schwartz, 1989), would secrete the biogenic amine serotonin following pharmacological manipulation.

The mammalian mast cell has a recognized pathophysiologic role as an effector cell in immediate hypersensitivity reactions (Cotran *et al.*, 1989). Mast cell activation by either immunological or non-immunological stimuli results in the release of pharmacologically active agents into the extracellular milieu (Irani and Schwartz,

1989; Foreman, 1993). These agents are typically referred to as mediators. In mast cells, mediators are divided into two major classes: preformed mediators which are stored in the granules and released upon activation of the cell, and those that are synthesised *de novo* by the mast cells and surrounding tissues at the time of activation (Barrett and Pearce, 1993). The *de novo* mediators are typically lipid in nature, being products of arachidonic acid metabolism. The generation of the *de novo* mediators is largely the consequence of the action of specific preformed mediators, such as histamine (Metcalfe, 1984).

Some preformed granule-associated mediators are rapidly released from the mast cells at the time of activation, whereas others remain firmly associated with the granule matrix following their discharge. The biogenic amines, serotonin and histamine, are both released rapidly into the extracellular milieu following mast cell activation (Metcalfe, 1984). Mast cell heterogeneity strongly reflects both the qualitative and quantitative aspects of the release of these two mediators. In those mammalian species studied to date, this heterogeneity is considered to be both intra and inter-specific (Irani and Schwartz, 1989; Barrett and Pearce, 1993).

Histamine is present in all mast cells, as well as basophils, enteroendocrine cells, and platelets (Metcalfe, 1984). The level of histamine located in mast cells varies according to the cell subtype although it is generally agreed that CTMCs have higher levels of this compound than their mucosal counterparts (1-30 vs 0.1-2 pg/cell) (Irani

and Schwartz, 1989). Levels of serotonin in mast cells are considerably more variable; in rat and mouse mast cells the concentration is 0.8-1.3 pg/cell. Serotonin is also found in platelets and enteroendocrine cells of the mucosal layer of the gastrointestinal tract as well as nervous tissue (Metcalfe, 1984). Theoharides *et al.* (1982) has demonstrated the differential release of serotonin and histamine and suggests that the release of serotonin from mast cells may not always originate from secretory granules.

Biogenic amines released from mast cells have been demonstrated to play an important role in the initiation of Type 1 hypersensitivity or anaphylactic reactions in mammals (Cotran et al., 1989). Anaphylactic "shock" type reactions have also been characterised in *Ictalurus punctatus* and *Carassius auratus*. In such cases the fish displayed behavioral patterns such as disorientation, increased ocular movement, gasping, and loss of equilibrium (Goven *et al.*, 1980; Ellis, 1985; Jurd, 1987). Suzuki and Iida (1992) reviewed the common cellular responses of inflammation in both fish and mammals including increased vasodilation and vascular permeability, followed by leucocytic migration. These responses are much slower in fish than in mammals, as they are typically temperature-dependent (Finn and Nielson, 1971). Following intraperitoneal (IP) injection to *A. salmonicida* extracellular protein (ECP), Ellis (1985) observed systemic signs of shock and explosive degranulation of EGCs from the intestine of rainbow trout. In addition, he demonstrated a concurrent decrease in the gut histamine levels and the occurrence of histamine in the blood as well as

a degranulation of EGCs in the intestinal tissue, all within 45 minutes post-injection. This would support the similarity of EGCs and CTMCs with respect to morphological, histochemical, and *in vivo* degranulatory characteristics similar to the CTMC.

Evidence of serotonin in the teleost gastrointestinal tract has been associated directly with enteric excitatory neurons (Watson, 1979; Anderson, 1983; Kitazawa, 1989) and enteroendocrine cells located in the mucosal folds (Anderson and Campbell, 1988; Burkhardt-Holm and Holmgren, 1989; Beorlegui *et al.*, 1992). On the other hand, the presence of histamine in the gastrointestinal tract of teleosts is somewhat contradictory. Earlier studies using fluorometric techniques detected histamine in the tissue preparations but at very low concentrations. These concentrations were determined to be typically 100 fold less than those found in mammals (Reite, 1965; 1969; Lorenz *et al.*, 1973). Ellis (1982) employing similar techniques determined the concentration of histamine in the rainbow trout intestine to be 2.5 μg/g of tissue. He later correlated decreases in this histamine level to the onset of exotoxin-induced anaphylaxis and EGC degranulation (Ellis, 1985). Hakanson *et al.* (1986), using the more specific technique of immunocytochemistry, described histamine reactivity associated with gastric enteroendocrine cells. But, similar to Beorlegui *et al.* (1992) who worked with rainbow trout, he found no evidence of histamine in the intestine of several fish species.

In this study, the release of serotonin was quantified using high performance liquid chromatography (HPLC) with electrochemical detection (ECD) following exposure to the mast cell secretagogues compound 48/80 and A23187. It was hoped that a correlation between the release of this biogenic amine and *in vitro* exposure to these pharmacological agents could be established.

3.2. Methods

3.2.1. Animal holding

Rainbow trout were purchased from Integrated Water Systems (Prince Edward Island) and kept at the Atlantic Veterinary College's Fish Health Unit holding facility. The fish were held in a 1 m circular tank with a flow rate of 20 l/min of well water. The fish were retained at 10.5-11°C under a 12 h light/dark photoperiod.

Eight week old Zucker lean rats were obtained from Charles River Laboratories (St. Constant, Quebec). The rats were housed in an artificially lit room with a 12 h light/dark cycle at a temperature of 22°C.

The care and utilization of the above animals followed the guidelines outlined by the Canadian Council on Animal Care (1984).

3.2.2. Drug preparation

Fresh stock solutions of Krebs-Henseleit physiological buffer (KHS) were prepared for each set of experiments (Kitchen, 1984). The buffer was chilled to 4°C and aerated in 95% O_2 / 5% CO_2 prior to using.

Treatment stock solutions of 1 mg/ml compound 48/40 (Sigma, St. Louis, Mo.) dissolved in KHS were prepared prior to each experiment. The treatment tissue was exposed to a compound 48/80 concentration of 100µg/ml. This concentration is similar to that used in mammalian *in vitro* mast cell studies (Pearce *et al.*, 1985). Controls consisted of plain KHS.

Stock solutions of A23187 (Sigma) in absolute ethanol (ETOH) were prepared at a concentration of 1 mg/ml. These solutions were then diluted in KHS. Treatment tissues were exposed to a concentration of 10 µg/ml A23187. Comparable concentrations of A23187 have been used in *in vitro* human lung mast cell studies (Church *et al.*, 1982). One percent absolute ETOH in KHS was used as the vehicle control for these experiments.

Stock solutions of 100 ng/ml serotonin and its metabolite, 5-hydroxyindole acetic acid (5-HIAA) in KHS were used as internal controls for the freeze drying process as well as standard nonfreeze dried controls for the electrochemical detector.

3.2.3. Experimental protocol for the electrochemical detection of serotonin

Fish were killed by cervical dislocation. A 4 cm section of the mid-intestine, in the vicinity of the spleen, was removed. Extraneous fat was carefully trimmed from the surface of the tubular section. A 5 mm cross segment was then removed from the centre of the section and placed immediately into Bouin's fixative. The intestinal contents of the remaining two sections (approximately 2 cm in length) were flushed out with chilled KHS using a Pasteur pipette. The sections of intestine were placed in chilled aerated KHS prior to the start of the experiment.

The two sections were randomly assigned as being either control or treatment. Each section was placed in a capped Falcon Blue Max graduated conical polypropylene tube (Becton Dickinson) with 10 ml oxygenated KHS (10°C) for preincubation. The tubes were rotated at 200 rpm on a Labline orbit shaker at 10°C for 30 minutes. The KHS was then removed and placed into chilled 25 ml polypropylene flasks. Nine ml of fresh KHS was quickly replaced into the polypropylene tubes holding the tissues. One ml of the above treatment solutions and their corresponding controls were added to the treatment and control tubes respectively. The treatment exposure lasted for 30 minutes. Once again, the buffer was removed, and the remaining intestine was weighed and placed into Bouin's for fixation.

Following the removal of the buffer after both the preincubation and treatment periods, the vial solutions were quickly pipetted at a volume of 1 ml into eight 5 ml

amber serum vials (Wheaton Glass Company). One ml of the internal control stock solutions was also placed into four identical vials. All the serum vials were capped with rubber flange stoppers and stored at -20°C.

Once the samples had frozen, the material was freeze dried using a Labconco Freeze Dry System. During this process the samples undergo prefreezing followed by primary and secondary drying. The vials were than stoppered under vacuum.

The contents from each of the eight vials were then reconstituted with 1 ml of deionized water. One ml of deionized water was added to each of the four internal control vials. All solutions were then filtered through a MSI Cameo 3N nylon 0.22 μm filter prior to injection onto the HPLC column. Once the sample had been injected, it was stored at -20°C.

The HPLC system used in these experiments consisted of a Beckman 110B Delivery Module, Beckman Altex 210A valve injector with a 20 μl injection loop, a Beckman Ultrasphere ODS C18 column, a Shimadzu ECD-6A electrochemical detector, and a Shimadzu C-R6A chromatopac integrator.

The mobile phase for the serotonin assay comprised (by volume) of 85% 0.15 M sodium acetate (pH 4.0), 10% HPLC grade methanol and 5% HPLC grade reagent alcohol (90.7% ETOH, 4.7% propanol, 4.6% methanol) (modified from Flachaire *et*

al., 1990). This buffer solution was degassed through vacuum filtration using a Millipore aqueous/solvent 0.22 μm filter. In an effort to maximize the assay's ability to detect serotonin, the optimum electrical potential was determined to be 750 mV with a flow rate of 1 ml/min.

3.2.4. Tissue histology

The tissue samples were fixed for 24 hours in Bouin's fixative and then rinsed and placed in 70% ethanol for a further 24 hours. Prior to being processed, the "curled" inverted end-portions of the intestine were removed. Only central segments of the intestinal sections were retained for further processing.

The intestine was processed using a Fisher Histomatic Tissue Processor under vacuum and at room temperature. In this system, the tissue is placed in buffered neutral 10% formalin for 2 hours, and 1 hour in the following series of reagents: two rinses of 70% ETOH, 3 rinses of 95% ETOH, 2 rinses of absolute ETOH, 2 rinses of xylene. The tissues were then penetrated with Tissue Prep II (Fisher) paraffin wax over a three hour period at 60°C.

A Brinkman Embedding Centre was used for final embedding in the above mentioned paraffin wax. The blocks were then trimmed and sectioned on a Reichert 820 Histostat Rotary Microtome. Section thickness was set at 5 μm. Sections were placed on glass slides and then dried on a Shandon Lipshaw drying oven for 30

minutes at 70°C.

The sections were deparaffinized to distilled water prior to staining with 0.1% toluidine blue in distilled water. Staining was for 20 seconds, followed by rinsing with distilled water. The sections were then taken down to xylene through two rinses of 95% ETOH, absolute ETOH and xylene.

3.2.5. Controls

Prior to injecting experimental samples onto the HPLC, nonfreeze dried standard controls of varying concentrations (100, 80, 50, 20, 10, 5 ng/ml) were used to establish a mass injected (2.0, 1.6, 1.0, 0.4, 0.2, 0.1 ng respectively) to peak area ratio for the serotonin and 5-HIAA. The 100 ng/ml freeze dried internal controls were also compared to these standards to establish the efficacy of the freeze drying and reconstitution protocol.

In an effort to determine whether the experimental design was capable of amassing and detecting mast cell derived serotonin following *in vitro* exposure to a specific pharmacological agents, proximal sections of rat intestine were utilized as protocol controls.

Two 8 week old lean Zucker rats were anaesthetized with 65 mg sodium pentobarbital/ml at a dose of 0.1ml/100g of body weight. The diaphragms of the rats were cut, and approximately 5cm of proximal small intestine was removed. The experimental protocol used was similar to that described above (section 3.2.3.), with three modifications: The drug dosage was 10μg/ml compound 48/80 for the treatment tissues, the tissues were incubated at 37°C and preincubation periods were not conducted.

3.2.6. Data analysis

Regression equations were derived for standard concentration curves for both serotonin and 5-HIAA for each series of runs. These were used to convert the experimental results of mediator release into ng/g of tissue. This data was tabulated on a spread sheet (Quattro Pro 4.0) and analysed for significance ($P<0.05$) using analysis of variance (Glantz, 1987).

3.3. RESULTS

3.3.1. Serotonin assay

Prior to running the experiment, the protocol was tested with regards to its efficiency in determining released serotonin and 5-HIAA levels. No significant difference was observed between 100 ng/ml standard controls ($n=4$) that were divided in two, half being kept at 4°C and injected unfiltered onto the column and the other half being

put through the freeze drying and filtering protocol ($p < 0.05$).

3.3.2. Rat intestine control

The absence of serotonergic neurons and the presence of serotonin containing CTMCs make rat intestine an ideal model for determining the ability of this protocol to measure serotonin following *in vitro* exposure to a secretagogue, such as compound 48/80. Detection for serotonin release was conducted at an attenuation (sensitivity) level of 8. At this attenuation, a serotonin peak was detected and quantified for only the two treatment tissues (Table 3.1). Even when the sensitivity level was doubled (attenuation of 4), a serotonin peak was not detected for the control tissues.

3.3.3. Detection of serotonin following *in vitro* exposure to compound 48/80 and A23187

Levels of released serotonin were quantified in all the fish control and test tissues for both compound 48/80 and A23187 (Table 3.2). These levels were generally relatively low being at the level of 5ng/g of tissue weight. For both the compound 48/80 and the A23187 set of experiments, one of the twelve pieces of intestine randomly assigned as being a test control tissue released extremely high amounts of serotonin into the baths. This resulted in a higher mean for the serotonin released with a correspondingly high standard error of the mean (SEM) for the test controls for both the compound 48/80 and the A23187 sets of experiments. There was no

TABLE 3.1: Mean serotonin release (μg/g of tissue) from rat proximal intestine following *in vitro* exposure of treatment tissue to 10 μg/ml compound 48/80.

RAT	CONTROL	TREATMENT Mean* Serotonin Release (μg/g of tissue)
RAT 1	ND	0.86
RAT 2	ND	0.43

ND: Not detectable
*: Mean of two replicate samples

TABLE 3.2: Mean serotonin release (ng/g of tissue) from rainbow trout small intestine following *in vitro* exposure to 100 μg/ml compound 48/80 or 10 μg/ml A23187, where n=12.

AGENT	PREINC. CONTROL ± SEM	PREINC. TREATMENT ± SEM	TEST CONTROL ± SEM	TEST TREATMENT ± SEM
CMP.48/80	4.6±0.6	5.1±0.9	29.4±21.9	6.3±1.6
A23187	9.9±3.5	4.8±0.7	28.5±22.0	4.5±0.6

CMP. 48/80: compound 48/80
PREINC: preincubation
SEM: standard error of the mean

significant difference in the mean amount of serotonin released in the control or treatment tissues in either the preincubation or the test period for compound 48/80 ($p=.322$) or A23187 ($p=.386$).

The mammalian serotonin metabolite, 5-HIAA was detected at low levels in the treatment and control tissues during both the preincubation and test periods (Table 3.3). These levels were generally higher than those for the released serotonin. Once again, there was no significant difference in the amount of serotonin detected in the control or treatment tissues in either the preincubation or test period for compound 48/80 ($p=0.831$) or A23187 ($p=0.062$). The relatively low p-value for A23187 is the result of the very high level of 5-HIAA that was detected in 2 of the 12 test control assays.

3.3.4. Tissue histology

The mucosal layer of the rainbow trout intestine appeared to be undamaged from the tissue protocol. An abundance of EGCs were also apparent beneath the mucosa in the stratum compactum. The resolution offered by the paraffin sections of the intestinal tract was very poor. For this reason an analysis of the state of intactness of the EGCs of the stratum compactum was hard to establish with any sense of confidence.

TABLE 3.3: Mean 5-HIAA release (ng/g of tissue) from rainbow trout small intestine following *in vitro* exposure to 100 μg/ml compound 48/80 or 10 μg/ml A23187, where n=12.

AGENT	INC. CONTROL ± SEM	INC. TREATMENT ± SEM	TEST CONTROL ± SEM	TEST TREATMENT ± SEM
CMP.48/80	15.4±2.6	13.1±2.1	12.3±2.6	13.5±2.5
A23187	10.4±2.0	11.7±2.2	24.2±7.1	11.6±2.2

CMP. 48/80: compound 48/80
PREINC: preincubation
SEM: standard error of the mean

3.4. Discussion

From this study it was clearly demonstrated that serotonin was released from the intestinal tract of the rainbow trout, and this release could successfully be quantified using HPLC with electrochemical detection. This demonstration of serotonin in the mid-intestine of rainbow trout is in accordance with other studies of the teleost gastrointestinal tract. These studies used various techniques including fluorescence histochemistry (Watson, 1979; Anderson, 1983) and immunocytochemistry (Holmgren *et al.*, 1985; Anderson and Campbell, 1988; Burkhardt-Holm and Holmgren, 1989; Beorlegui *et al.*, 1992) to confirm the presence of this biogenic amine in the gut. This study also identified the presence of the "mammalian" metabolite of serotonin, 5-HIAA, in the assay material.

A significant increase in the release of serotonin was not observed following administration of the known mast cell degranulators, compound 48/80 and A23187. Both these agents are considered to be effective "non-immunological" pharmacological tools that cause the release of mast cell mediators, such as serotonin and histamine, both *in vivo* and *in vitro* (Johnson and Moran, 1969; Foreman *et al.*, 1973; for review Foreman, 1993).

Histological evidence exists that *in vivo* exposure to compound 48/80 and A23187 results in degranulation of rainbow trout intestinal EGCs (Vallejo and Ellis, 1989;

Powell, 1991). Evidence of biogenic amine release correlating to concurrent degranulation of intestinal EGCs was demonstrated earlier in rainbow trout by Ellis (1985). In his study, Ellis noted a coincidental decrease in the histamine content of the gut, an appearance of histamine in the blood and a noticeable degranulation of EGCs in the intestinal wall within 45 minutes *in vivo* exposure to bacterial exotoxins. In discussing his results, Ellis alluded to the nonspecific nature of his orthophthalaldehyde fluorometric methodologies, suggesting that it was reasonable to assume that another biogenic amine type mediator was actually being measured. The possibility that it may have been serotonin that was released rather than histamine was strengthened by a later study using immunocytochemistry. In this second study, although there was no evidence of histamine, there was found to be an abundance of serotonin in the intestine of rainbow trout (Beorlegui *et al.*, 1992). For this reason, we decided that it may be possible to correlate EGC degranulation with a significant increase in the release of serotonin following exposure to either compound 48/80 or A23187.

Mast cell populations are considered to be highly heterogenous. This heterogeneity is manifested both within and between species and affects the morphological, biochemical and functional properties of these cells (Bienenstock, 1988; Irani and Schwartz, 1989; Barrett and Pearce, 1993). In relation to this study, it is of importance to note that different classes of mast cells display not only variations in their susceptibility to various secretagogues (including compound 48/80) but also

differential release of mediators (for review Barrett and Pearce, 1993). With such a high degree of heterogeneity among the mammalian classes of mast cells, surely one can expect to find some appreciable differences when comparing the teleost EGC to its possible mammalian counterpart.

From the previously presented *in vitro* histological work of this study, it was demonstrated that discrete regions of explosive degranulation were observed in the control tissue. Whether or not this phenomenon occurred in the study presented in this chapter is not known. This is due to the poor resolution offered by the paraffin sections that were cut. It may be possible that some of the released serotonin from the control tissues could have originated from the EGCs of the stratum compactum, as a result of this previously observed non-pharmacologically induced degranulation discussed in Chapter 2.

Although compound 48/80 and A23187 have been demonstrated to induce observable EGC degranulation *in vivo* (Vallejo and Ellis, 1989; Powell, 1991), their effects *in vitro* are still not known. It is conceivable that these drugs do not have a mediator releasing effect on the intestinal EGCs of the rainbow trout. Or else, the level of pharmacologically induced serotonin release may be too low to be significantly detected. In mammalian systems, it has been demonstrated that the amount of serotonin released to be effective need only be very low (Theoharides *et al.*, 1982). Finally, a more likely conclusion is that serotonin was released from either

the abundant serotonergic rich nerves or the enteroendocrine cells located in the gastrointestinal tract, and not from the EGCs (Watson 1979, Anderson, 1983; Holmgren *et al.*, 1985; Anderson and Campbell, 1988; Burkhardt-Holm and Holmgren, 1989, Beorlegui *et al.*, 1992).

Similar to mammals, there is evidence of a well developed ganglionated myenteric plexus between the outer longitudinal and inner circular smooth muscle of the gastrointestinal tract of teleosts, including salmonids. Innervation of the outer layer is sparse, but the inner layer of muscle appears to have an abundance of these serotonergic- rich nerve fibres (Watson, 1979; Anderson, 1983; Burkhart-Holm and Holmgren, 1989; Beorlegui *et al.*, 1992). These non-cholinergic non-adrenergic (NANC) nerves are considered to be excitatory in nature (Holmgren and Nilsson, 1981; Kitazawa, 1989), and have been found to release serotonin upon exposure to the neuropeptide Substance P (Holmgren *et al.*, 1985). Substance P is also considered to be a putative neurotransmitter of the NANC nervous system in the gastrointestinal tract, and has in fact been demonstrated to cause *in vivo* degranulation of mast cells (Mousli *et al.*, 1989) and EGCs (Powell *et al.*, 1992; Powell *et al.*, 1993B). As in mammals (Wood, 1993), this enteric nervous system is thought to have a role in gastrointestinal motility and immunophysiology (Fänge and Grove, 1979; Powell *et al.*, 1993B).

Serotonin containing enteroendocrine cells have also been observed through immunocytochemistry (Anderson and Campbell, 1988; Burkhart-Holm and Holmgren, 1989, Beorlegui, 1992). These cells are located in the mucosal folds of the intestinal epithelium and their secretions play a role in the motility and secretatory ability of the gastrointestinal tract (Burkhart-Holm and Holmgren, 1989).

In conclusion, this study has been successful in developing a simple yet highly sensitive assay for the detection of serotonin and its metabolite 5-HIAA from rainbow trout intestinal secretions. Future research may identify the definitive source of these compounds.

CHAPTER 4. GENERAL DISCUSSION

The objective of this study was to investigate *in vitro* both the morphological and the biochemical effect of compound 48/80 and A23187 on EGCs of rainbow trout intestine. In an attempt to further characterize the function of the EGC, the results of this study were compared to those previously established. To date, all studies regarding EGCs have either involved observations of fish collected from the wild or have occurred *in vivo* in a laboratory setting. On the whole, these studies have tended to infer a probable immunological function for the EGC, similar to the CTMC (Ezeasor and Stokoe, 1980; Vallejo and Ellis, 1989, Powell *et al.*, 1991).

A number of studies have demonstrated the increase in prevalence and/or degranulation of EGCs in wild fish specimens which have been exposed to a pathogenic insult, thereby inferring a strong immunological connection. Studies utilizing wild fish specimens have demonstrated that this effect not only occurs in a variety of teleosts, including salmonids, but also in a variety of tissues. For instance, parasitic diseases such as *Diphyllobothrium dendriticum* and *D. ditremum* have been observed to result in a degranulation of EGCs in the rainbow trout intestine (Sharp *et al.*, 1989). The microsporidian *Glugea* sp. causes both an infiltration and encapsulation by the EGCs around the pathogen in the intestine of the sergeant major fish (Reimschuessel *et al.*, 1987). EGCs have also been shown to infiltrate

sites vacated by parasites, for instance *I. multifiliis*, in the epidermis of the carp (Cross and Matthews, 1992). With regards to viral diseases, EGCs have been observed to proliferate in the intestine of Atlantic salmon infected with infectious pancreatic necrosis (Smail *et al.*, 1992) and degranulate and necrose in salmonids infected with infectious haematopoietic necrosis (Yasutake and Amend, 1972; Wolf, 1988). Large numbers of EGCs were noted to have infiltrated and degranulated in the intestine, kidney, spleen and pancreatic tissues of Coho salmon infected with putative neoplastic lesions (Kent *et al.*, 1993). A similar proliferation was observed in the nares of several salmonid species infected with ulcerative dermal necrosis (Roberts, 1972).

In addition to pathological insults, EGCs have been observed to proliferate in wild salmonids following exposure to nonpathologic insults. Repeated formalin treatments (Blackstock and Pickering, 1980) and tagging lesions (Roberts *et al.*, 1973) have been shown to induce an increase in the number of EGCs in affected tissues.

In vivo studies of EGCs have utilized specific agents such as mast cell secretagogues, bacterial exotoxins, neurotransmitters and neurotoxins in an effort to determine the function of the EGC and its possible relationship to the CTMC. Compound 48/80 and A23187, two pharmacological agents that produce marked degranulation in CTMCs (Johnson and Moran, 1969; Röhlich *et al.*, 1971; Foreman *et al.*, 1973; Pearce, 1986; Foreman, 1993) have also been demonstrated to cause a similar

anaphylactic-like degranulation in EGCs (Vallejo and Ellis, 1989; Powell, 1991). Degranulation and mobilization of intestinal EGCs following IP injection to ECP of *A. salmonicida* has been demonstrated in a number of studies (Ellis, 1985; Vallejo and Ellis, 1989; Powell *et al.*, 1993A). Mobilization, diapedesis (Lamas *et al.*, 1991) and degranulation (Powell *et al.*, 1993A) has been demonstrated using *V. anguillarum* ECP. Mammalian mast cells are also known to degranulate following exposure to *V. cholera* exotoxin (Tsuchiya *et al.*, 1972). Work by Powell (1991; *et al.*, 1991; *et al.*, 1993B) demonstrated a limited crinophagic-like degranulation and marked decrease in the number of EGC of the stratum compactum of rainbow trout intestine following IP injection of both the neuropeptide, substance P, and the neurotoxin, capsaicin. Smaller EGC-like cells were later observed in the stratum compactum, suggesting a possible regranulation process. Both substance P and capsaicin are known to stimulate, and in the case of capsaicin, enhance mast cell degranulation in mammals (Mousli *et al.*, 1990; Nilsson *et al.*, 1989).

From this research, no conclusive morphological or biochemical evidence was generated regarding the *in vitro* action of the known mast cell secretagogues compound 48/80 and A23187 on intestinal EGCs of the rainbow trout. For the morphological research, this was due in part to the lack of a control with consistently intact EGCs. Without an effective control, any significant difference with regards to a decline in cell numbers for the treatment was probably concealed. For this reason, it was not demonstrated whether an effect on EGCs following *in vitro* exposure to

compound 48/80 and A23187 could be manifested by a decrease in cell number or morphology at the LM level.

It should also be noted that the exposure time and concentrations of compound 48/80 and A23187 utilized in this study were extrapolated from previous *in vitro* mammalian mast cell studies (Church *et al.*, 1982; Pearce *et al.*, 1985). These extrapolations are considered to be a starting point for future *in vitro* EGC work. In fact, these values may not have been appropriate for *in vitro* EGC studies. This may be due in part to fundamental differences in the physiology and pharmacokinetics between mammals and fish. For this reason, further research is required regarding different doses and exposure times before it can be conclusively established if compound 48/80 and A23187 really have an effect on EGCs in fish.

Another important consideration regarding the outcome of this work is that the fish were not exposed to anaesthetics prior to being used in this study. However, anaesthetics have been used in most of the previous morphological and *in vivo* studies on EGCs (Smith, 1975; Ellis, 1985; Vallejo and Ellis, 1989; Lamas *et al.*, 1991; Powell, 1991; Powell *et al.*, 1993 A,B). These anaesthetics include ethyl p-aminobenzoate (benzocaine) and more commonly, tricaine methanesulphonate (MS 222). The pharmacological effects of anaesthetic agents in fish are not clearly understood (Brown, 1993). And yet, in mammals they typically function by decreasing the permeability of cell membranes to ions, most notably sodium. This

in turn may act to stabilize the affected cells (Allison, 1980). Whether or not such an effect occurred on the EGCs in the earlier mentioned studies is not known. It is possible that the use of anaesthetics may be accountable for the reduced amount of degranulation in the control tissues of these earlier studies.

In relation to mediator release, this study was able to demonstrate serotonin release from the intestinal sections using HPLC. Unfortunately, the biogenic amine was released in both the control and the treatment tissues. Serotonin release from the tissues was not significantly affected by *in vitro* exposure to either compound 48/80 or A23187. This would seem to suggest that A) similar to some mast cells (Bienenstock, 1988; Irani and Schwartz, 1989; Barrett and Pearce, 1993) serotonin is not present in the granules of EGCs, B) at the concentration and exposure time utilized in this study, the mast cell secretagogues compound 48/80 and A23187 do not have an effect on EGC granule mediator release, or C) release of serotonin resulting from exposure to these agents was at a level too low for significant changes to be detected. The source of both the detected serotonin and its metabolite, 5-HIAA, may have been the result of spontaneous degranulation of the EGCs and/or from alternate stores in the intestine. These alternate stores include the serotonergic neurons and enteroendocrine cells that are found in the rainbow trout intestine (Watson *et al.*, 1979; Anderson, 1983; Holmgren *et al.*, 1985; Anderson and Campbell, 1988; Burkhardt-Holm and Holmgren, 1989; Beorlegui *et al.*, 1992).

At this time, previous studies involving the EGC would appear to indicate a probable immunological function, similar to that of the CTMC. The results presented in this work appear to neither confirm nor contradict this hypothesis. It is recognized that more research is required in this area, especially with regards to further *in vitro* and cell culture work. Until this work is conducted, the true function of the EGC will remain unsolved.

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APPENDIX 1

Krebs-Henseleit (KHS) Physiological Solution:

In distilled water add:
118 mM sodium chloride
24.9 mM sodium bicarbonate
11.1 mM d-glucose
4.7 mM potassium chloride
2.2 mM calcium chloride (predissolved)
1.2 mM potassium phosphate monobasic
1.2 mM magnesium sulphate

Aerate the solution with 95% O_2 / 5% CO_2
The pH of the solution should range from 7.45 to 7.55.

Millonig's Buffer:

In 100ml water add:
4.52g monosodium phosphate (solution A)

In 100ml water add:
1.01g sodium hydroxide (solution B)

Add 83ml solution A to 17ml of solution B. The buffer should have a pH of 7.3.

Primary Glutaraldehyde Fixative:
(3% glutaraldehyde in 0.13M Millonig's phosphate buffer)

Add to 33ml water:
37.5ml Millonig's phosphate buffer
4.5ml 50% glutaraldehyde

Toluidine Blue:
(1% toluidine blue in a 1% sodium borate solution)

Mix:
1g sodium tetraborate
100ml distilled water

Add:
1g toluidine blue

Shake, then filter prior to using.

Uranyl Acetate:

In a 15ml centrifuge tube prepare a saturated solution of about 5% uranyl acetate in 50% ethanol.

Shake up pellet and centrifuge for 10 minutes at 2500 rpm.

Store in centrifuge tube covered with aluminum foil as the uranyl acetate is light sensitive.

Lead Stain (Sato):

Heat 90ml of distilled water to 40°C.

Add:
1.5g lead nitrate. Mix until completely dissolved.
1.5g lead acetate. Mix until completely dissolved.
1.5g lead citrate. Mix for 1 minute (will not dissolve).
3.0g sodium citrate. Mix for 1 minute.
24ml freshly prepared 1N NaOH. Discard solution if not completely clear.
40ml distilled water.

Cover beaker and let settle in fridge for 10 minutes. Stain can be stored in plastic syringes in fridge for up to 1 year.

APPENDIX 2

Materials	Sources
A23187	Sigma (St. Louis, Mo.)
acetic acid (glacial)	Fisher Scientific (Montreal, Que.)
araldite 502 resin	JBS Supplies (Dorval, Que.)
calcium chloride	Fisher Scientific
compound 48/80	Sigma (Halifax, NS.)
DMP-30 hardener	Marivac
dodecenyl succinic anhydride (DDSA)	JBS Supplies
ethanol	BDH (Halifax, NS.)
D-glucose anhydrous	Fisher Scientific
50% glutaraldehyde	JBS Supplies
5-hydroxyindole acetic acid	Fisher Scientific
5-hydroxytryptamine creatinine sulphate	Fisher Scientific
Jembed 812 resin	JBS Supplies
lead acetate	JBS Supplies
lead citrate	JBS Supplies
lead nitrate	BDH
magnesium sulphate	Fisher Scientific
methanol (HPLC grade)	BDH
95% O_2/ 5%CO_2	Island Oxygen (Charlottetown, PEI)
osmium tetroxide	Marivac
potassium chloride	Fisher Scientific
potassium phosphate monobasic	Fisher Scientific
reagent alcohol (HPLC grade)	Fisher Scientific
sodium acetate (HPLC grade)	Fisher Scientific
sodium borate	Fisher Scientific
sodium bicarbonate	Fisher Scientific
sodium chloride	Fisher Scientific
sodium citrate	JBS Supplies
sodium hydroxide	Fisher Scientific
sodium phosphate monobasic	Fisher Scientific
toluidine blue	Fisher Scientific
uranyl acetate	Fisher Scientific
rainbow trout	Integrated Water Systems (Brookvale, PEI)
rats	Charles River Laboratories(Quebec)