CONTROL MECHANISMS OF Na^+-K^+-ATPase - MEDIATED BRANCHIAL ION EXCHANGE IN COD *Gadus morhua*

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"Each of us carries in our veins a salty stream in which the elements sodium, potassium and calcium are combined in almost the same proportions as in sea water. This is our inheritance from the day, untold millions of years ago, when a remote ancestor, having progressed from the one-celled to the many-celled stage, first developed a circulatory system in which the fluid was merely the water of the sea."

Rachel Carson.

Declaration

I hereby declare that this thesis has been totally my own work and that it has not been presented in any other previous application for a degree.

Except where specifically acknowledged, all the work has been carried out by myself and where other information has been cited, this has also been specifically acknowledged by means of a reference.

Hazel J. Crombe

Acknowledgements

I should first like to thank my two supervisors Dr. Michael V. Bell (NERC Unit of Aquatic Biochemistry) and Dr. Peter Tytler (Biology Department) for their help, encouragement and enthusiasm throughout my three years of research training and for their extremely helpful critisism and discussion of my work. Their excellent supervision has been of crucial importance to the completion of this thesis. I am indebted to Prof. J. R. Sargent for allowing me to use the facilities of the NERC Unit of Aquatic Biochemistry where most of the work for this thesis was carried out.

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Finally, and most importantly, I must thank my family, husband David and sons David and Jamie for their patience and encouragement. Special thanks are also due to my parents who have helped me immensely throughout this PhD and before.

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Abstract

The cellular mechanisms involved in the control of sodium transport across the gills of a seawater stenohaline fish *Gadus morhua* were investigated with particular emphasis on the role of diacylglycerol (DAG) as a second messenger. A whole-body perfusion system identified several agents that induced changes in sodium efflux across the gills. The effects of these agents on Na^+-K^+-ATPase activity and phospholipid metabolism in isolated chloride cells were then investigated in an attempt to identify the second messengers involved in receptor-mediated stimulation of salt secretion in chloride cells.

The hormone atrial natriuretic factor (ANF) was found to reduce the sodium efflux in perfused gills and reduce Na^+-K^+-ATPase activity in homogenates of chloride cells. Thus its action appears to be at least partly due to an effect on active sodium transport by Na^+-K^+-ATPase. The mechanism by which ANF brings about a change in Na^+-K^+-ATPase activity was not via a receptor-mediated phospholipase C hydrolysis of phosphatidylinositides or indeed phospholipase C mediated hydrolysis of any other phospholipid. However receptor-mediated phosphatidylcholine (PtdCho) metabolism was probably involved and this may be accomplished by changes in cytidyltransferase activity.

The phosphatidylinositol cycle in unstimulated chloride cells and in cells treated with ANF was relatively inactive. Radioactive tracer studies revealed a high proportion of radio-incorporation into the phospholipid phosphatidyl-ethanolamine and relatively low amounts into the phosphoinositides unlike the

situation in other salt transporting epithelia or in cod brain. The significance of this in terms of signal transduction in the chloride cells of cod remains to be determined.

Diacylglycerol appears to have a role as a second messenger in the control of sodium efflux and Na^+-K^+-ATPase activity in the gills of cod. Both sodium efflux and Na^+-K^+-ATPase activity were reduced on administration of phorbol ester or a synthetic DAG analogue. The exact mechanism by which this inactivation was mediated remains unclear though it seems probable that it is mediated via activation of protein kinase C. A physiological effector for this pathway remains to be identified. Attempts to link the action of the hormone ANF with production of the second messenger DAG and the subsequent activation of PKC proved unsuccessful.

Abbreviations

ANF	Atrial natriuretic factor
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
AVT	Arginine vasotocin
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
cAMP	Cyclic 3', 5'-adenosine monophosphate
CDP-choline	Cytidine diphosphate-choline
cGMP	Cyclic 3', 5'-guanosine monophosphate
CL	Cardiolipin
CT	CTP:phosphocholine cytidyltransferase
CTP	Cytidine 5'-triphosphate
DAG	Diacylglycerol
DASPMI	2-(*p*-Dimethylaminostyryl)-1-methyl-pyridinum iodide
DCF	2, 7-dichlorofluroescein
DHA	Docosahexaenoic acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FDA	Fluorescein diacetate
GLC	Gas-liquid chromatography

GPC	Glycerophosphocholine
GPE	Glycerophosphoethanolamine
G-protein	Guanine nucleotide binding protein
GTP	Guanosine 5'-triphosphate
G3P	Glycerol-3-phosphate
H-7	1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
Ins 1P	Inositol 1-phosphate
Ins 2P	Inositol 2-phosphate
Ins $1,3P_2$	Inositol 1, 3-bisphosphate
Ins $1,4P_2$	Inositol 1, 4-bisphosphate
Ins $3,4P_2$	Inositol 3, 4-bisphosphate
Ins $1,3,4P_3$	Inositol 1, 3, 4-trisphosphate
Ins $1,4,5P_3$	Inositol 1, 4, 5-trisphosphate
Ins $1,3,4,5P_4$	Inositol 1, 3, 4, 5-tetrakisphosphate
Ins $1,3,5,6P_4$	Inositol 1, 3, 5, 6-tetrakisphosphate
Ins $1,4,5,6P_4$	Inositol 1, 4, 5, 6-tetrakisphosphate
Ins $3,4,5,6P_4$	Inositol 3, 4, 5, 6-tetrakisphosphate
Ins $1,3,4,5,6P_5$	Inositol 1, 3, 4, 5, 6-hexakisphosphate
Ins $1,2,3,4,5,6P_6$	Inositol 1, 2, 3, 4, 5, 6-pentakisphosphate

K_m	Michaelis constant
MS222	3 - Aminobenzoic acid ethyl ester
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MUFA	Monounsaturated fatty acid
Na^+-K^+-ATPase	Sodium - plus - potassium - dependent adenosine triphosphatase
OAG	1-oleoyl-2-acetyl glycerol
PAF	Platelet activating factor (1-*O* -alkyl-2-acetyl-*sn* -glycero-3-phosphocholine)
PAP	Phosphatidic acid phosphohydrolase
PDB	Phorbol 12, 13-dibutyrate
PEH	Phenylephrine hydrochloride
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGF_4	Prostaglandin F_4
Pi	Inorganic phosphate
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PtdA	Phosphatidic acid
PtdCho	Phosphatidylcholine
PtdEth	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdIns 3,P	Phosphatidylinositol 3-phosphate

PtdIns 4,P	Phosphatidylinositol 4-phosphate
PtdIns $3,4P_2$	Phosphatidylinositol 3,4-bisphosphate
PtdIns $4,5P_2$	Phosphatidylinositol 4,5-bisphosphate
PtdIns $3,4,5P_3$	Phosphatidylinositol 3,4,5-trisphosphate
Ptd Ser	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RAS	Renin-angiotensin system
SFA	Saturated fatty acid
SM	Sphingomyelin
TEP	Transepithelial potential
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
TPA	Tetradecanoyl-phorbol acetate
VIP	Vasointestinal peptide

Materials

Animals

Atlantic cod, *Gadus morhua*, were obtained from the University Marine Biological Station, Millport, Isle of Cumbrae, transported live to Stirling and maintained within a recirculating seawater system in 3000 litre tanks for up to 3 months. The temperature was maintained at 10-15°C and the fish fed a diet of chopped squid.

Chemicals

The phospholipid standards phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-monophosphate, phosphatidylcholine and phosphatidylethanolamine were obtained from Sigma Chemical Company Ltd., Poole, Dorset, UK as were phorbol 12,13-dibutyrate, 1-oleoyl-2-acetyl glycerol, bovine serum albumin, butylated hydroxytoluene, dimethylsulphoxide, MTT, fluorescein diacetate, HEPES, MS222, heparin, ATP, Janus Green, Trypan Blue, phytate hydrolysate, adrenaline, phenylephrine hydrochloride and collagenase, type IV (*Clostridium histolyticum*). [Ile3]-vasopressin, [Arg8]-vasotocin, staurosporine, H-7 and atrial natriuretic factor were purchased from Calbiochem-Nova biochem (UK) Ltd., Nottingham, UK. 2-(*p*- Dimethyl-aminostyryl)-1-methylpyridinium iodide was purchased from Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K. The 98% air/2% carbon dioxide gas mixture was purchased from British Oxygen Carriers, Cumbernauld, UK. High-performance TLC plates coated with silica gel 60 were from Merck, Darmstadt, Germany. Amberlite resin (IR-120, Analar) was obtained from

BDH Ltd., Poole, Dorset, U.K. Sodium orthovanadate, AnalaR-grade acetic acid and propan-1-ol were also purchased from BDH Ltd., and all other solvents of HPLC grade were obtained from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, U.K. Ecoscint A was from B.S.&S. (Scotland) Ltd., Portobello Industrial Estate, Edinburgh UK. Konika A2 X-ray film was supplied by MAS Stirling, Crieff, U.K. Millipore filters were obtained from Millipore (UK) Ltd., Harrow, Middlesex, UK. Whatman 1PS phase separation paper and Whatman No1 chromatograghy paper were purchased from Whatman Scientific Ltd., Maidstone, Kent, UK. All other chemicals were purchased from Sigma and were of 'Analar' grade.

Radiochemicals

Carrier free [^{32}P]-orthophosphate, [^{22}Na]-sodium chloride and [methyl-^{3}H]-choline chloride were purchased from Amersham International plc, Buckinghamshire, UK.

Contents

Looking for the answer

You hunt it,

you catch it,

you fool yourself;

the answer,

is always,

a step ahead.

Jens C. Skou

CHAPTER 1

GENERAL INTRODUCTION

1.1 OSMOREGULATION IN TELEOST FISH

1.2 THE ROLE OF THE GILLS

1.3 CONTROL MECHANISMS

1.4 OBJECTIVES OF THE PRESENT STUDY

1.1 OSMOREGULATION IN TELEOST FISH

One of the greatest challenges an organism faces is the need to maintain and regulate its body in dynamic equilibrium with respect to its environment and to be able to modify this state in response to any environmental change that it may encounter. Homeostasis is a particular problem for many marine fish which are subject to severe osmotic stress due to the high salinity of the sea. The mechanisms by which fish maintain osmotic homeostasis have been the subject of considerable study (reviewed by Maetz, 1974; Evans, 1979; 1980a) and the means by which these animals maintain the ionic composition and osmolarity of their body fluids with respect to the surroundings have been extensively studied at the physiological, cellular and biochemical level. In order to gain a full understanding of the process of osmoregulation it is important to encompass all these approaches.

At the beginning of this century Fredericq discovered that the osmotic pressure of the body fluids of the teleost fishes, whether in freshwater, seawater or migrating between the two media, is relatively constant (Fredericq, 1901). Later the chemical models proposed by Homer Smith in 1930 and August Krogh in 1939 outlined the special osmoregulatory mechanisms of the teleost fishes (Smith, 1930; Krogh, 1939). Most teleost fish maintain the ionic composition and osmolarity of their body fluids at levels significantly different from the external medium. The body fluids of freshwater species are markedly hypertonic and those of seawater species markedly hypotonic with respect to the external media (reviewed by Maetz, 1974; Kirschner, 1979; Evans, 1979; 1980a; Foskett *et al.* 1983).

Osmoregulation in teleost fish is largely the integrated transport activities of the gut, gill and renal systems of the organism (reviewed by Evans, 1980a). Homer Smith

first localised the head region of fish as the site of electrolyte secretion to the external medium and the gills have since been shown to be the site of large exchanges of sodium and chloride in seawater teleost species (Motais, 1967; Girard, 1976). Marine teleosts must constantly compensate for the osmotic loss of water and the influx of ions to maintain a constant internal milieu of around 370mOsm compared to the external seawater which is around 1000mOsm (reviewed by Foskett *et al.* 1983). Even in stenohaline fish there is a requirement for an adaptive capacity in order that salt secretion might be altered, for example, as a result of salt loading produced by feeding. Seawater teleosts osmoregulate by drinking water and actively secreting ions against a concentration gradient (Maetz, 1974; Kirschner, 1979; Evans, 1979; 1980a). The gut absorbs water, a process of cotransport with the active uptake of salt thereby adding to the salt burden (Kirsch *et al.* 1982; 1984). Monovalent ions are excreted almost entirely via the gills in marine teleosts and indeed the gills are the most important site of ionic regulation in these fish (Maetz, 1974; Kirschner, 1979; Evans, 1979; 1980a). The ingested sodium and sodium which has entered passively across epithelial surfaces is actively secreted across the gill to the external medium. The kidney is also involved in osmoregulation. In seawater, although filtration rates are low to conserve water, the kidney is the principal site of divalent ion excretion (Hickman & Trump, 1969; Evans, 1979).

In contrast, freshwater species experience water influx and ion efflux. Ionic regulation involves the active uptake of salts by the gills and guts and salt retention in the kidneys. Freshwater fish tend to drink less than seawater species (reviewed by Evans, 1980a) and water balance is brought about by the production of a copious dilute urine (see Evans, 1979 for relevant literature). Consequently they manage to maintain a blood osmolarity of around 340mOsm which is much higher

than that of the surrounding water which is equivalent to 1mM NaCl.

1.2 THE ROLE OF THE GILLS

The teleost gill is well adapted to the function of osmoregulation and the other functions of oxygen uptake and waste excretion that it serves. This complex structure contains both respiratory and osmoregulatory tissue in close proximity, has a very large surface area and is supplied with blood through an elaborate system of blood vessels.

In the teleost fish there are four gill arches on each side of the head with access to the external medium via the opercular openings. Each gill has two hemibranchs i.e. two rows of tapered gill filaments lying parallel to one another and perpendicular to the gill arch. The secondary lamellae extend from each side of the filament above and between the plane of the filament (Figure 1.1).

The gill epithelium belongs to the general class of tissues known as salt transporting epithelia found in other osmoregulatory organs such as the avian nasal salt gland, the elasmobranch rectal gland and the mammalian kidney and intestine. These epithelia form the boundary between the internal environment of the animal and the external medium. They facilitate movements of salt in both directions, moving salt inwards in the kidney of fresh water teleosts and frog skin and moving salt outwards in seawater teleost gills, avian nasal salt gland and elasmobranch rectal gland (reviewed by Hoffman, 1978).

The gill epithelium consists of four main cell types. The flat epithelial cells of the

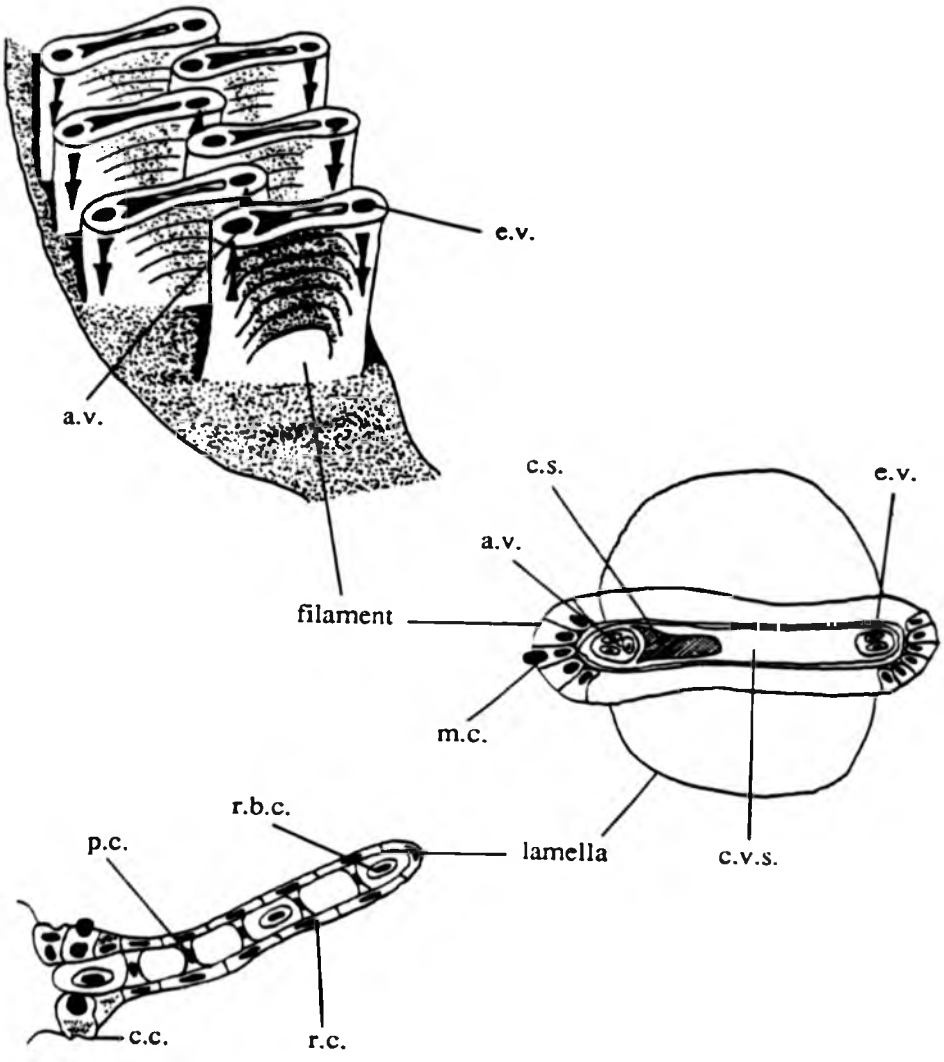

Figure 1.1. Structural features of the teleost gill. Each gill arch has two rows of filaments which project and diverge downstream from one another, perpendicular to the gill arch. The secondary lamellae extend from each side of the filament. a.v. afferent blood vessel; e.v. efferent blood vessel; c.s. cartilagenous spine supporting the filament; c.v.s. central venous space or compartment; c.c. chloride cell; m.c. mucous cell; r.c. respiratory cell; p.c. pillar cell; r.b.c. erythrocyte. The arrows indicate the direction of blood flow. Figure redrawn from Maetz (1971).

lamellae which are 3-5μm thick and involved in gas exchange are known as the respiratory cells. The two lamellar faces are separated by contractile pillar cells and in the interlamellar regions are found the mucous-secreting cells and the specialised salt secreting cells, the chloride cells (epithelial structure reviewed by Bettex-Galland & Hughes, 1971; Sargent *et al.* 1977; 1980; Payan & Girard, 1984). The chloride cells which were first characterised in seawater-adapted eel gills and named by Keys and Willmer (1932) can be found in many of the other aforementioned osmoregulatory epithelia. Chloride cells are characterised by the presence of large numbers of well developed mitochondria in close association with a tubular system formed from extensively infolded basal and lateral plasma membranes (reviewed by Philpott, 1980). Chloride cells are also present in freshwater gills where their role appears to be a reversal of that found in seawater gills (Maetz & Garcia-Romeu, 1964) i.e. to retain sodium lost by passive diffusion down its concentration gradient.

It has been demonstrated that chloride cells are much more abundant and better developed in marine or seawater-adapted species of fish (Newstead, 1967; Shira & Utida, 1970) where it is necessary to secrete large amounts of ions to maintain homeostasis. During seawater-adaptation of euryhaline species the levels of branchial salt excretion rises and this is accompanied by an increase in the size and/or the numbers of chloride cells present in the gill and a rearrangement of these cells within the epithelia. Liu (1942) was one of the first authors to report these observations and they have been confirmed by many other workers since (e.g. Doyle & Epstein, 1972; Thomson & Sargent, 1977; Sardet *et al.* 1979, 1980; Foskett *et al.* 1981; Pisam, 1981)

Chloride cells have been shown indirectly to be the site of branchial NaCl secretion

by the use of various ultrastructural, enzymatic and kinetic studies (Maetz & Bornancin, 1975; Kirschner, 1977; Karnaky, 1980; Philpott, 1980) and more directly using a vibrating probe technique by Foskett and Schreffrey (1982). The role of the chloride cell in gill osmoregulation is discussed in more detail in Chapter 3.

Chloride cells contain large amounts of the enzyme sodium - plus potassium - dependent adenosine triphosphatase (Na^+-K^+-ATPase) (Bonting, 1966). This enzyme was discovered by Skou (1957; 1960) and it is now widely accepted that Na^+-K^+-ATPase is the biochemical equivalent of the physiological 'sodium pump' involved in maintaining high potassium and low sodium intracellular concentrations thereby creating electrochemical gradients across the plasma membrane that are essential to the proper functioning of animal cells. These gradients allow cells to perform their general and special functions such as action potentials in nerve and muscle, transport of nutrients such as glucose, amino acids and phosphate and homeostatic mechanisms including osmoregulation and pH balance. In fish gills Na^+-K^+-ATPase has been localized to the basolateral membranes of the chloride cells using autoradiographic and cytochemical studies (Karnaky *et al.* 1976; Hootman & Philpott, 1979) and high levels of Na^+-K^+-ATPase activity have been detected in isolated chloride cells, particularly those of seawater-adapted species (Kamiya, 1972; Sargent *et al.* 1975). The mechanisms by which Na^+-K^+-ATPase is involved in osmoregulation are dealt with in more detail in Chapter 4.

1.3 CONTROL MECHANISMS

Much of the work carried out on osmoregulation has focussed on organ studies of

gills, cellular studies of chloride cells and enzymatic studies of Na^+-K^+-ATPase. Attempts have also been made to understand the underlying biochemical mechanisms which control sodium homeostasis and the search for physiological effectors has been extensive. Studies which have attempted to elucidate the cellular control mechanisms involved in a complex process like osmoregulation have been wide and varied and although some progress has been made in this field there are still vast gaps in our knowledge.

All cells must engage in some form of communication even if it is only a rudimentary mechanism which provides the cell with the facility to detect and respond to environmental stimuli. When individual cells become organised together into functional groups to form a complex organism it is even more evident that the individual cells need to be able to sense the general status of the whole organism in relation to its environment and also the particular functional status of other cells. An essential component of the evolutionary process of multicellular organisms is the development of appropriate intercellular communication systems. Some of the mechanisms that cells use to influence one another involve direct physical contacts i.e. gap junctions, but direct contact communication systems suffer from several disadvantages including the local nature of the response and the rather slow rate of information flow between cells. Other mechanisms of intercellular signalling which are rapidly propagated and which can reach widely distributed tissues have evolved and these encompass both the nervous and endocrine systems.

The molecular details of signal recognition and transduction processes can be very similar for cells constituting very different functional tissues and the distinction between hormonal and nervous inputs to a system is difficult since cells use similar mechanisms to transmit both types of signal. Indeed many of the molecules

involved in signal transduction can be found in both nervous and endocrine systems in mammals. For example, small peptides such as somatostatin and the catecholamines, adrenaline and noradrenaline, act as both hormones and neurotransmitters.

A hormone may be defined as a molecule released into the circulation which specifically modifies the metabolism of remote target cells. Hormones are very diverse both in chemical structure and in their mechanisms of action. They range from small molecules, like the aforementioned catecholamines, to relatively large polypeptides like growth hormone. Additionally hormones may cause quite different responses in different target cells. This may reflect the metabolism of the cell but may also reflect the fact that a single hormone may have more than one mechanism of action resulting from differential binding to different receptors. The time scale over which the effects of a hormone become apparent also varies from a few seconds to several hours or days.

Many different hormones have been implicated in the control of osmoregulation and various aspects of hormonal control in fish have been reviewed (Dharmamba, 1979; Lahlou, 1980; Foskett *et al.* 1983; Pang, 1983; Evans, 1984; 1990; Rankin & Bolis, 1984; Takei, 1993). In fish these hormones include predominantly prolactin and arginine vasotocin in freshwater species and cortisol and the renin-angiotensin system (RAS) in seawater species (reviewed by Evans, 1990; Takei, 1993). Other agents shown to stimulate salt excretion by the gill epithelium include ß-adrenergic agonists (Degnan *et al.* 1977; Degnan & Zadunaisky, 1979), vasointestinal peptide (VIP), glucagon (Foskett *et al.* 1982), and growth hormone (Bern & Madsen, 1992; Takei, 1993). They may all have a role in teleost osmoregulation in seawater.

Since the demonstration of the production of a massive and rapid diuresis and natriuresis in rats upon injection of atrial extracts (De Bold *et al.* 1981) there has been a great deal of interest in the role of the cardiac hormone, atrial natriuretic factor (ANF), in mammalian osmoregulation. The hormone has been isolated, sequenced and synthesised and its gene has been isolated, cloned and mapped on chromosome 1 in humans. There have already been numerous reviews of various aspects of ANF and its control (Cantin & Genest, 1985; Atlas, 1986; Sagnella & MacGregor, 1986; Buckalew *et al.* (1987), Trippodo, 1987; Baxter *et al.* 1988; Genest & Cantin, 1988; Goetz, 1988; Kramer, 1988; Samson, 1992).

In the past 6-7 years there has been an emergence of reports which strongly indicate that fish hearts and brains also produce a vasoactive and natriuretic factor. Atrial natriuretic factor has been found in cardiac tissue from hagfish (Reinecke *et al.* 1987a), teleosts (Westenfelder *et al.* 1988) and elasmobranchs (Solomon *et al.* 1985) and ANF binding sites have been identified in the kidney and aorta of the hagfish (Kloas *et al.* 1988). It is likely that the putative fish ANF has structural homology with mammalian ANF since immunoreactivity with antibodies to the mammalian hormone has been shown in all of these fish groups (Chapeau *et al.* 1985; Reinecke *et al.* 1985; 1987a; 1987b).

Longer term osmoregulatory control mechanisms usually associated with, for example, adaptation to different salinities may involve changes in the number and distribution of chloride cells and the up or down regulation of sodium pumps within the membrane. The hormones cortisol and prolactin have been shown to be important in the changes to chloride cells which occur during adaptation to different salinities. Cortisol is involved in the proliferation and differentiation of chloride cells whereas prolactin is involved with the dedifferentiation of chloride cells,

changes which can take several days to complete (reviewed by Foskett *et al.* 1983). On the other hand it is likely that shorter term control mechanisms might be utilised to cope with, for example, salt loading incurred by feeding and this may involve the switching on and off of existing sodium pumps i.e. the modulation of Na^+-K^+-ATPase activity. Much less is known about the hormones involved in this type of control.

There are two basic mechanisms for the transfer of information across the plasma membrane from the extracellular space to the cell interior. Firstly the hormone itself enters the cell and binds to a recognition site on an intracellular receptor protein, e.g. steroid hormones, or secondly, the hormone binds to a recognition site on the surface of the cell and brings about a series of events leading to the release of intracellular messengers which then bring about or initiate the appropriate cellular response, e.g. catecholamines. This second messenger hypothesis was first defined by Sutherland & Rall (1960).

Perhaps the simplest and best understood signal transduction system is that which utilises cyclic 3', 5'-adenosine monophosphate (cAMP) as a second messenger. Upon hormone binding to specific cell receptors on the extracellular membrane a conformational change results which leads to the activation of adenylate cyclase via a guanine nucleotide binding protein (G-protein), a ubiquitous component of signal transduction mechanisms. The active site of adenylate cyclase is located on the inner surface of the plasma membrane so that cytoplasmic ATP is converted to cAMP which then acts as an effector to modulate cell metabolism by the activation of protein phosphorylation by cAMP-dependent protein kinase. Hormone-sensitive adenylate cyclases have been found in almost all vertebrate cell types, (the major exception being the red blood cell), and a large number of hormones and other

effectors use this mechanism. The role of cAMP in signal transduction processes has been reviewed by Robison *et al.* (1968), Ross & Gilman (1980) and Schramm & Selinger (1984).

It is apparent that cAMP-dependent protein kinase is a general protein kinase in that it phosphorylates a number of different proteins. Protein phosphorylation and dephosphorylation is a very widespread mechanism for the operation of control processes and cells are known to contain many different protein kinases and phosphatases controlled in a variety of ways (reviewed by Cohen, 1982; 1985; 1989; Nestler *et al.* 1984; Alexander, 1990). Protein kinases can be separated into those that phosphorylate proteins on tyrosine residues (protein tyrosine kinases) and those that phosphorylate serine/threonine residues (protein serine/threonine kinases). There is also evidence emerging for the existence of dual specificity protein kinases (Linberg *et al.* 1992). Protein kinases that have been identified include the aforementioned cAMP-dependent and tyrosine protein kinases, calmodulin-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C (PKC) (reviewed by Nishizuka, 1984; 1986; Cuatrecasas, 1986; Blackshear *et al.* 1988), the latter being particularily relevant to this study. Protein kinase C, first reported by Inoue *et al.* in 1977, phosphorylates a number of proteins *in vivo* and *in vitro* (Woodgett *et al.* 1987; Farooqui *et al.* 1988) including Na^+-K^+-ATPase from the nerve of diabetic rat (Greene & Lattimer, 1986). Protein kinase C requires Ca^{2+}, phosphatidylserine (PtdSer), and diacylglycerol (DAG) for activity, the presence of the two lipid cofactors lowering the affinity for Ca^{2+} so that the enzyme is fully active at normal, resting cellular concentrations of Ca^{2+} (0.1μm) (Nishizuka, 1984; Kikkawa & Nishizuka, 1986; Berridge, 1987a; Parker *et al.* 1989; Bazzi & Nelsestuen, 1990).

Since the emergence of the cAMP story more than 30 years ago the search for other putative second messengers has ensued and has uncovered many other factors involved in signal transduction, e.g. cGMP, inositol phosphates, Ca^{2+}, eicosanoids, lymphokines, diacylglycerol and sphingosine, all of which have been found to have central roles in controlling a wide variety of cellular processes such as differentiation, secretion and metabolic regulation (reviewed by Berridge, 1987a; 1987b; Hannun & Bell, 1987; 1989; Merrill & Stevens, 1989; Michell *et al.* 1989; Pelech & Vance 1989; Sachs & Muallem, 1989; Billah & Anthes, 1990; Downes & MacPhee, 1990; Guy *et al.* 1990; Merrill & Jones, 1990; Rana & Hokin, 1990; Shimizu & Wolfe, 1990; Yamamoto & Kanaide, 1990). In particular certain lipids have emerged as being very potent and diverse modulators of cellular functions. Phospholipids provide the backbone of the cell membrane forming the matrix in which membrane-associated proteins are embedded, including enzymes and cell surface receptors. In addition many phospholipids also participate directly in signal transduction across the membrane. New roles for membrane lipids are continually being uncovered, some involving the complex lipids directly while others involve the cleavage of the lipids to yield other substances. Most of the products of lipid signal transduction are obtained by the cleavage of complex membrane lipids by various lipases. Figure 1.2 shows the sites of action of several phospholipases on a typical phospholipid substrate and Table 1.1 lists the products of cleavage by these phospholipases and some of the responses brought about by them.

The receptor-stimulated cleavage of phosphatidylinositol bisphosphate (PtdIns $4,5P_2$), to yield the second messengers inositol trisphosphate (Ins $3,4,5P_3$) and diacylglycerol (DAG) has been of particular interest. Receptor-mediated phosphatidylinositol (PtdIns) metabolism was discovered by Hokin & Hokin (1953) and was subsequently characterised by the same workers during the

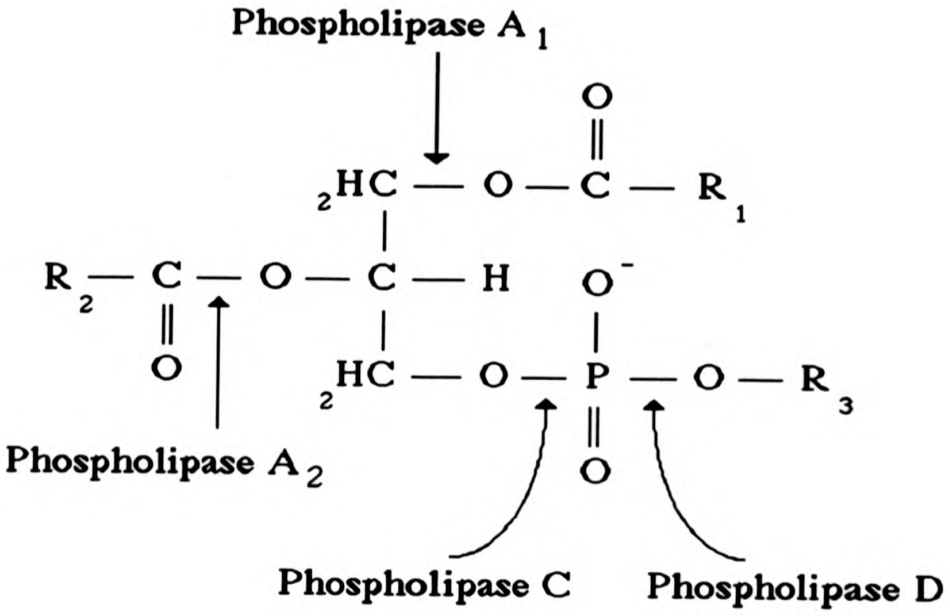

Figure 1.2. The sites of action of phospholipases A, B and C on a typical phospholipid molecule. R_1 and R_2 represent fatty acid chains and R_3 represents the base (choline, ethanolamine, serine or inositol).

Table 1.1. Putative second messengers derived from the action of phospholipases on phosphoglycerides.

PHOSPHOLIPASE	PRODUCTS	RESPONSE
A	Arachidonic acid	Production of prostaglandins, leukotrienes, etc.
		(+) Protein kinase C
	Lysophospholipids	(+/-) Protein kinase C
	1-Alkyl-lysoPC	Platelet activating factor
C	Diacylglycerol	(+) Protein kinase C
	Alkylacylglycerol	(-) Protein kinase C
	Ins1,4,5P_3	(+) Cytosolic calcium
	Inositol-P-glucan	Enzyme release, insulin action?
	Ceramide (from sphingomyelin)	Sphingosine (after ceramidase)
		(-) Protein kinase C
D	Phosphatidic acid	Increase cytosolic calcium
	Inositol glycan	Enzyme release, insulin action?

The products of cleavage of both simple phosphoglycerides (e.g. PtdCho and PtdIns) and more complex species (i.e. PtdIns glycan-linked proteins and sphingomyelin) are listed. (+) = stimulation, (-) = inhibition. For more details of the putative second messenger systems listed refer to Merrill (1989). Table adapted from Merrill (1989).

following years (Hawthorne, 1960 for review). The function of the response was not clear until Michell (1975) realised that it was responsible for the receptor-stimulated elevation in cytoplasmic calcium concentration. In 1979 Nishizuka and co-workers discovered that 1,2-diacylglycerol released during receptor-activated inositol lipid hydrolysis could also serve as a second messenger by activating protein kinase C (PKC). Between 1981 and 1984 it was recognised that PtdIns $4,5P_2$ is the major or only inositol lipid hydrolysed as a direct result of receptor stimulation (Michell *et al.* 1981) and that the inositol trisphosphate released causes the liberation of Ca^{2+} from an intracellular pool into the cytosol (Berridge & Irvine, 1984; 1989). These processes have been reviewed by Downes & Michell (1985), Nishizuka (1986), Berridge (1987a; 1987b), Berridge & Michell (1988) and Berridge (1993). The emerging picture of receptor-mediated phosphatidylinositol metabolism is much more complex than was originally proposed and more and more metabolites of PtdIns are being identified as cellular messengers, especially the inositol phosphates (reviewed by Berridge & Irvine, 1989; Shears 1989a; 1989b; Downes & MacPhee, 1990). The principle features of agonist-dependent PtdIns $4,5P_2$ hydrolysis are summarised in Figure 1.3. Such mechanisms form the cornerstone of an ubiquitous transduction mechanism now known to regulate a large number of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation (Berridge & Irvine, 1984; Hokin, 1985; Abdel-Latif, 1986; Berridge, 1987a; Berridge & Michell, 1988; Michell *et al.* 1989; Rana & Hokin, 1990), and some studies have already suggested that there may be a link between phosphatidylinositol metabolism and osmoregulation (Hokin & Hokin, 1960; 1967; Girard *et al.* 1977; Simpson & Sargent, 1985).

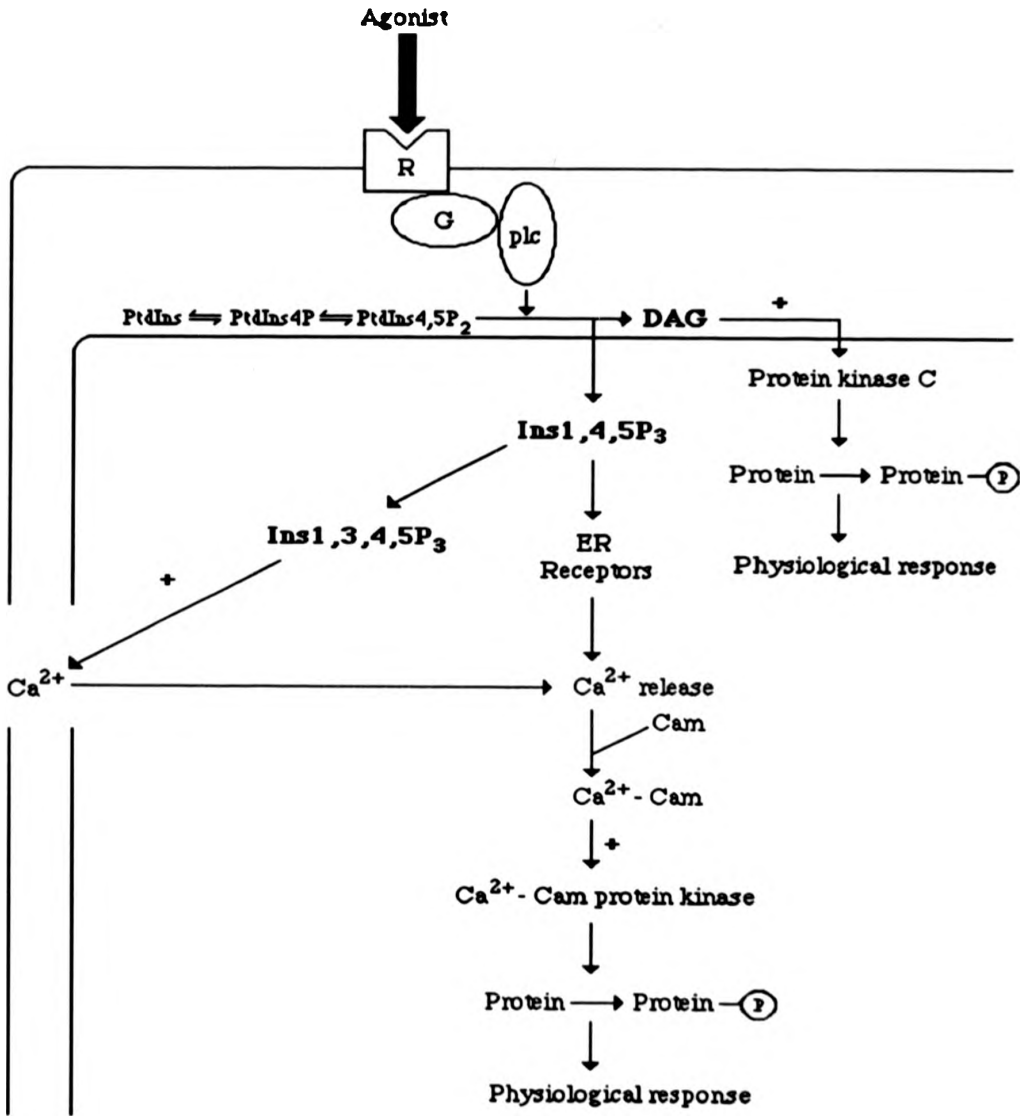

Figure 1.3. Principle features of agonist-stimulated phosphatidylinositol hydrolysis. The interaction of an agonist with its cell surface receptor (R) stimulates PtdIns $4,5P_2$ hydrolysis by phospholipase C (plc) via coupling to a guanine nucleotide-dependent protein (G). Both products of phospholipase C activity are second messengers. DAG remains within the membrane where it activates protein kinase C; Ins $1,4,5P_3$ stimulates calcium release from the endoplasmic reticulum (ER) and its metabolites can result in the influx of extracellular calcium. Both pathways ultimately lead to the phosphorylation of proteins which elicits a physiological response. Cam = calmodulin.

In the field of osmoregulation interest has switched to those subcellular events which occur following an environmental stimulus which ultimately brings about a change in the activity of Na^+-K^+-ATPase. A hypothesis explaining the steps which might be involved in the control of salt efflux across the gills of a marine teleost are shown in Figure 1.4. Just as in the control of other osmoregulatory systems such as the elasmobranch rectal gland and the avian nasal salt gland (Peaker & Linzell, 1975) it is possible that changes in blood salt levels in marine fish are detected by osmoreceptors. This response could elicit a release of hormone(s) which could in turn initiate changes in the target organ, the gills. The events which occur between the postulated hormone-receptor interaction on the chloride cell membrane and the change in the activity of the membrane bound Na^+-K^+-ATPase in these cells remain to be determined.

1.4 OBJECTIVES OF THE PRESENT STUDY

Marine fish whose body fluids are hypotonic with respect to their environment require a mechanism for the excretion of excess salt against an electrochemical gradient and a mechanism for maintaining body osmolarity. Salt balance is carried out primarily by the gills. Osmoregulation by the gills of marine fish is complex, dependent upon the integrated function of all of the aforementioned components. In particular, the structure of the gill filaments (including gill vascularization), the arrangement of different cell types in the epithelia, the salt secreting chloride cells and the enzyme Na^+-K^+-ATPase are able to respond to environmental stimuli. Most osmoregulatory studies to date have been carried out on freshwater or euryhaline species of fish with very few on stenohaline seawater species.

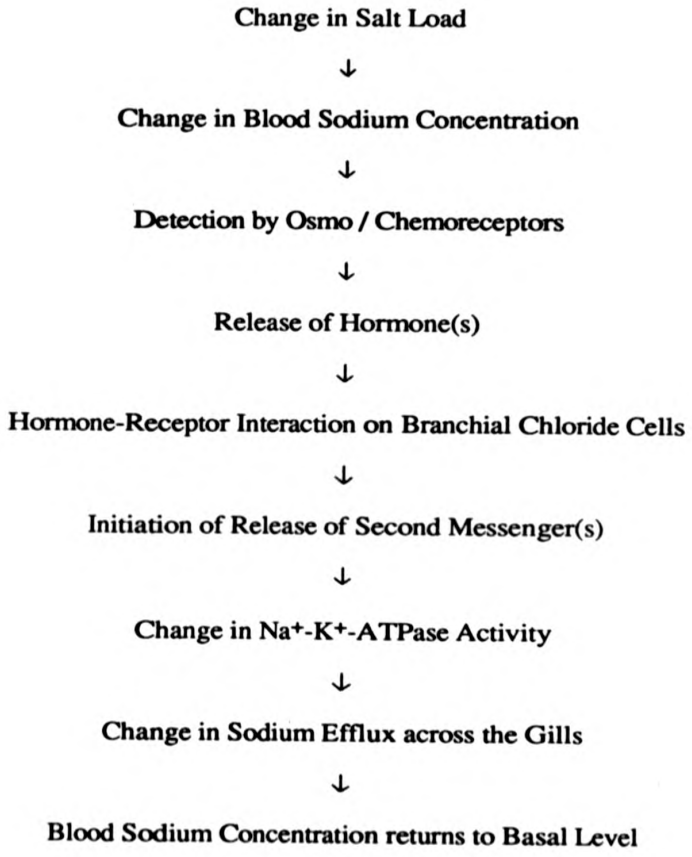

Figure 1.4. Hypothetical model of the mechanisms involved in osmoregulation by the gills of the marine teleost

The Atlantic cod *Gadus morhua* (see Plate 1.1) was chosen as the organism of study since it was relatively easy to obtain and keep specimens. The cod is particularily well suited for perfusion work and many haemodynamic investigations have been carried out on this species (Pettersson & Nilsson, 1979; 1980; Wahlquist, 1980; Wahlquist & Nilsson, 1980; Pettersson & Johansen, 1982) though there have been far fewer osmoregulatory studies (Fletcher, 1978). A variety of experimental approaches were employed including organ, cellular and biochemical studies leading ultimately to an investigation of putative effectors and second messengers that may be involved in the system. Chloride cells are especially abundant in the gills of marine fish and therefore the use of a species such as cod facilitated the preparation of chloride cells and Na^+-K^+-ATPase.

Work by Bell & Sargent (1987) had previously shown that the rectal gland from dogfish (*Scyliorhinus canicula*) is rich in protein kinase C (PKC). Diacylglycerol produced by phospholipase C digestion of phosphoglycerides, is a known activator of PKC (Takai *et al.* 1979) and is therefore a likely candidate as a second messenger in this system. Furthermore, work in this laboratory by Stenhouse, Bell &Tytler (1988, unpublished) suggested that phorbol ester, a DAG mimetic, affected the flux of ions across the gills of freshwater-adapted rainbow trout. Earlier work by Girard *et al.* (1977) on seawater-adapted eels provided evidence that phosphatidylinositol metabolism might play an important role in the control of osmoregulation in the teleost gill as is true for the shark rectal gland (Simpson & Sargent, 1985). The ultimate aim of the present study was to investigate the role of the inositol lipids, and especially DAG, in the control of osmoregulation in the gills of a marine teleost, the cod.

Initially, a whole body preparation was developed for the measurement of sodium

Plate 1.1. *Gadus morhua*, the Atlantic cod

efflux across the gills using radioisotopes to allow identification of effector(s) which could be used in subsequent experiments at the cellular and biochemical level. It was not intended to carry out a systematic search for putative hormones. The system allowed control of the various haemodynamic parameters affecting gill function and also any neuronal and thus endocrinological inputs to the system. Hormones that were known to be effectors in other systems were investigated in an attempt to identify the primary signal in the gills of cod. Chloride cells from the gills were then isolated to focus in on the events at the subcellular level involved in controlling sodium efflux and Na^+-K^+-ATPase activity and to study the role of various putative second messengers.

CHAPTER 2

SODIUM EFFLUX ACROSS THE GILLS OF *Gadus morhua*

2.1 INTRODUCTION

2.2 METHODS

2.2.1 Whole body perfusion

2.3 RESULTS

2.3.1 Validation of the perfusion technique employed

2.3.2 Relationship between sodium efflux and perfusion pressure

2.3.3 The effects of various agonists and hormones upon sodium efflux and perfusion pressure

2.4 DISCUSSION

2.1 INTRODUCTION

In 1930 Homer Smith identified the head region of *Anguilla rostrata* as the source of electrolyte exchange with the external medium (Smith, 1930). Investigations of possible mechanisms of electrolyte secretion began when Krogh (1939) demonstrated that Na^+ and Cl^- ions were taken up from freshwater by the head end of goldfish, *Carassius auratus*. He proposed that there were ionic exchange systems involved in maintaining electroneutrality and that sodium uptake might be coupled to NH_4^+ extrusion and Cl^- uptake to HCO_3^- efflux. The availability of radioisotopes of the principle ions in the post war period offered new opportunities for the investigation of ion flux across the gills and represented a significant step forward from previous studies which relied on chemical analysis of net fluxes. Radiotracer studies by Maetz & Garcia Romeu (1964) supported the work of Krogh demonstrating that Na^+ and Cl^- uptake could be stimulated independently of each other by injecting either NH_4^+ or HCO_3^- into the blood of the goldfish. Addition of these substances to the external medium had the reverse effect of inhibiting Na^+ and Cl^- uptake respectively. The gills were identified as the site of large exchanges of Na^+ and Cl^- in seawater species (Girard, 1976) and have subsequently been shown to be the most important site of monovalent ion secretion and ionic regulation in these fish (Maetz, 1974; Kirschner, 1979; Evans, 1979; 1980a).

It was discovered that the branchial epithelium of marine fish contained significant amounts of Na^+-K^+-ATPase located in the basolateral membranes of chloride cells. (Kamiya, 1972; Sargent & Thomson, 1974; Sargent *et al.* 1975). The mitochondrial rich chloride cells of the fish gill epithelium play an important role in osmoregulation (see Chapter 3 for details of the role of chloride cells). Burns and

Copeland (1950) showed that these chloride cells are widely distributed throughout the head region of the killifish *Fundulus heteroclitus* and it has been subsequently shown that the opercular membrane of this species is composed of 50-70% chloride cells identical to those of the fish gill (Karnaky *et al.* 1976, Karnaky & Kinter, 1977).

The Na^+-K^+-ATPase enzyme was known to mediate Na^+/K^+ exchange in a variety of tissues and enzyme activity levels were shown to be greater in marine compared to freshwater species of fish (Epstein *et al.* 1967). It was discovered that the efflux of radioisotopes from the eel *Anguilla anguilla* was sensitive to the external, seawater concentration of K^+ (Maetz, 1969) and thus it appeared that in the marine fish extrusion of Na^+ in exchange for K^+ involved the enzyme Na^+-K^+-ATPase (Maetz, 1971). The fat sleeper *Dormitator maculatus* also appeared to possess a K^+ sensitive ATPase identical to that of extracted gill Na^+-K^+-ATPase (Evans *et al.* 1973). The time course of activation of this enzyme corresponded with the time course of activation of K^+ sensitive Na^+ efflux when the fat sleeper was transferred from freshwater to seawater (Evans & Mallery, 1975). Effluxes of Na^+ and Cl^- were both shown to be inhibited by the Na^+-K^+-ATPase inhibitor ouabain when it was injected into the blood of *Anguilla rostrata* (Silva *et al.*, 1977) and since the enzyme was located on the basolateral plasma membrane of chloride cells (Karnaky *et al.* 1976) it was proposed that the branchial chloride cells secrete Cl^- via a basolateral cotransport of Na^+ and Cl^- energised by the movement of Na^+ down its electrochemical gradient maintained by Na^+-K^+-ATPase and followed by movement of Cl^- down its electrochemical gradient from cell to seawater (Ellis *et al.* 1977; Kyte, 1978; Sargent *et al.* 1980).

Although in these earlier experiments the use of intact animals ensured that proper perfusion and irrigation of branchial epithelium took place and that both neural and hormonal control systems were operational, it also meant that experimental manipulation of the control mechanism was difficult. Substantial alterations of the ionic composition of the blood cannot be easily made in intact fish. Also injection of known inhibitors and inducers of ionic regulator (effector) cells into intact fish can produce secondary changes via cardiovascular effects which could alter functional area or permeability and, consequently, branchial diffusional exchange. Furthermore, experimental manipulation of intact fish has been shown to stress fish (Eddy, 1981; Pickering, 1981) with associated endocrine changes which also produce secondary effects on branchial ion exchange. New approaches have been developed to allow closer investigation of the function and control of the branchial effector cells.

As early as 1931 a preparation in which the serosal and mucosal solutions bathing the gill epithelium could be manipulated and controlled was described (Keys, 1931), the so-called heart-gill preparation using *Anguilla anguilla*. This preparation had one major undesirable attribute in that the effects of agents administered to the preparation upon the gills or the heart could not be separated. Subsequently the isolated, perfused head preparation was developed by Payan and Matty (1975) using the rainbow trout and appeared to be a much more effective and viable procedure for the study of osmoregulation and haemodynamics in gills. This preparation allows the partitioning of the efferent perfusate into dorsal arterial and venous components if required (Girard & Payan, 1976; Claiborne & Evans, 1980). Work on the rainbow trout using this method was reviewed by Girard and Payan (1980). Girard (1976) used the isolated perfused head preparation of the seawater-adapted rainbow trout (*Oncorhynchus mykiss*) to examine various

aspects of Na^+ and Cl^- extrusion and found that the effluxes of Na^+ were near to that measured *in vivo* and that effluxes of Na^+ and Cl^- were stimulated by addition of K^+ to the external medium.

Most isolated perfused head preparations studied maintained Na^+ and Cl^- fluxes significantly below *in vivo* levels, for example, the work of Kelly *et al.* (1981) and that of Epstein *et al.* (1973) can be compared. The lower ion fluxes in head perfusions could be due to incomplete perfusion of the gill vasculature in the isolated head preparation or due to the lack of circulating, stimulatory hormones normally found in the *in vivo* state. The isolated perfused head preparation allows the investigator to alter the ionic and chemical composition of the perfusate or the external medium and monitor the effects upon fluxes. It has also been an invaluable technique in the study of drug administration. Thus Kelly *et al.* (1981) found that 10^{-4}M ouabain inhibited Na^+ and Cl^- efflux in the isolated perfused head of the eel *Anguilla anguilla* with no effect on afferent perfusion pressure or the efflux of tritiated water.

The majority of ion transport studies have utilised the trout head which suffers from serious haemodynamic degradation in a relatively short period of time (Girard, 1976). To delay this degradation adrenalin is often added to the perfusate (Payan, 1978) but it is clear that this hormone stimulates Na^+ uptake in freshwater and inhibits it in seawater (Girard, 1976, Payan, 1978, Shuttleworth, 1978). The degradation of the isolated head preparation is however less pronounced in other species, for example, sculpin (*Myoxocephalus octodecimspinosus*) (Claiborne & Evans, 1980) and dogfish (*Squalus acanthias*) (Evans & Claiborne, 1982).

The parameter which is least often controlled in the isolated perfused head preparation is the ratio of perfusate inflow to outflow which is a direct measure of the integrity of the system. Even a slight leakage of the perfusate into the external medium or the tissues of the animal will lead to erroneous determinations of ion fluxes. These leak pathways can be substantial and can obscure the importance of some ion flux pathways. Again this problem appears to be species specific.

An alternative to the isolated head preparation is the isolated perfused gill preparation which like the perfused head has been used extensively to study patterns of blood flow and hormonally mediated vasoactivity of the gill (Richards & Fromm, 1969; Rankin & Maetz, 1971; Bergman *et al.* 1974; Smith, 1977; Colin *et al.* 1979; Holbert *et al.* 1979; Petterson & Nilsson, 1979) but rather less to study ion transport systems. This is probably due to the difficulty of maintaining proper irrigation of isolated gill preparations despite the fact that perfusion is sound (Bergman *et al.* 1974; Holbert *et al.* 1979). Again studies of ion fluxes using this technique gave values well below those obtained using *in vivo* studies (Shuttleworth & Freeman 1974, Farmer & Evans 1981). Despite this the method has provided invaluable information otherwise unavailable using other techniques.

The transepithelial potential (TEP) across an epithelium is the result of interplay between active ion transport and passive diffusion (Potts, 1984). Whole animal TEP is a reflection of the contributions made by all the epithelia including the gills, gut, kidney and skin though most transepithelial ion transport occurs across the branchial epithelium (Potts, 1984). Transepithelial potentials are easily measured and have been used to provide important information on osmoregulatory abilities in a number of fish species (Shuttleworth *et al.* 1974; Iwata & Bern, 1985; Iwata *et al.* 1987; Young *et al.* 1988). Generally, it was thought that stenohaline species

maintained low potentials by excreting sodium and chloride ions and that euryhaline species maintained salt balance by means of chloride excretion, which produced a TEP higher than the Nernst potential for sodium (Potts, 1984). In a more recent study the TEP of a wide variety of species of marine fishes were found to lie within the range 20-24mV positive and it was concluded that these fish maintain salt balance by chloride secretion only (Potts & Hedges, 1991). The TEP of cod was 21mV positive which is higher than that recorded by Fletcher (1978).

The perfused gill was used to study salt uptake by freshwater fish and studies supported the idea that basolateral Na^+/K^+ exchange mediated via Na^+-K^+-ATPase played a role in Na^+ uptake in freshwater (Richards & Frömm, 1969; 1970; Shuttleworth & Freeman, 1974). Many studies had failed to measure transepithelial potentials so chemical versus electrical coupling could not be determined. Work by House (1963) and Evans (1969) had suggested that Na^+ was in electrochemical equilibrium and that therefore the net salt gain in seawater was not NaCl but only Cl^-. Other species were shown to have transepithelial potentials below the equilibrium potential for Na^+ (Evans, 1980b) which left any comprehensive theories on transport across marine gill epithelia inconclusive.

The flat epithelium of the opercular membrane which could be dissected free and mounted in "Ussing Chambers" (Ussing, 1960) proved to be a very suitable alternative to the extremely complex branchial epithelium with which to study the role of chloride cells in the process of ion transport. The ionic composition of both the serosal and mucosal solutions bathing the epithelium can be controlled, net movements of ions across the tissue can be measured and the electrical and chemical components determined. Additionally short-circuit currents can be quantified and resistances can be measured. By studying the voltage dependancy of the

unidirectional flux of an ion across an epithelium one can predict whether the ion flow is conductive or electroneutral and whether the pathway is cellular or paracellular (Frizzell & Schultz 1972; Mandel & Curran 1972). The isolated opercular membrane from seawater-adapted teleosts has been used to define the ionic transport properties of chloride cells but much less work has been done on freshwater ion regulation using this method.

In 1988, in this laboratory, an honours project student used a whole body perfusion technique to investigate the effects of phorbol ester upon gill function in the trout (Stenhouse 1988, unpublished). The results indicated that phorbol ester and thus diacylglycerol may have a role to play in the control of ion transport across the fish gill and these results partly prompted the present study.

The whole body perfusion technique which was again used in the present study to investigate the effects of various different putative agonists and hormones upon sodium efflux is based on the work of Daxboeck and is described by Davie *et al* (1982). The technique was modified and developed so that experimental conditions were such that they mimicked the *in vivo* state of the cod as closely as possible and most of the problems encountered by other authors using a variety of perfusion techniques were largely overcome. The technique was employed in an initial physiological approach to the study of gill osmoregulation and used as a window to determine which of the putative agents under examination might be worthy of further investigation in subsequent cellular and biochemical studies.

2.2 METHODS

2.2.1 Whole body perfusion

Fish were anaesthetised in 0.05% (w/v) 3-aminobenzoic acid ethyl ester (MS222, Sandoz), in seawater which was neutralised by addition of sodium bicarbonate. Heparin (1000 USP K-1 units, Sigma) was injected intraperitoneally and the fish left 20 minutes to allow the heparin to circulate. The fish was pithed, the pith-hole was sealed with blutac (Bostik) and the fish laid supine ventral side up in a specially moulded fibre-glass fish holder (Figure 2.1). The gills were immediately irrigated with aerated seawater which was pumped by a centrifugal pump (Eheim) at physiological flow rates of between 500 and 1000 ml min^{-1} (Johansen, 1972) from a reservoir containing 1 litre of seawater.

An incision was made along the linea alba to expose the heart cavity. A small incision was made in the bulbus arteriosus and a heat-flared PVC catheter (Portex, 1.4mm inside diameter) was inserted anteriorly into the ventral aorta and secured with a silk thread ligature. This arterial catheter was connected to a primed system whereby perfusion medium from a 200ml reservoir was pumped by a rodent heart pump (Harvard Apparatus 1407) around the fish's circulatory system taking great care to avoid the introduction of air. The pulse rate of pumping was in the range of 40-45 beats min^{-1} and the stroke volume was in the range of 0.5-0.8ml giving a simulated cardiac output of around 20ml min^{-1} for an 300g fish. The perfusion medium (NaCl 142mM; KCl 3.4mM; $CaCl_2.2H_2O$ 1.3mM; $NaHCO_3$ 19.5mM; $(NH_4)_2SO_4$ 0.4mM; $MgSO_4.7H_2O$ 4.1mM; Glucose 5.6mM; PVP 4%; HEPES

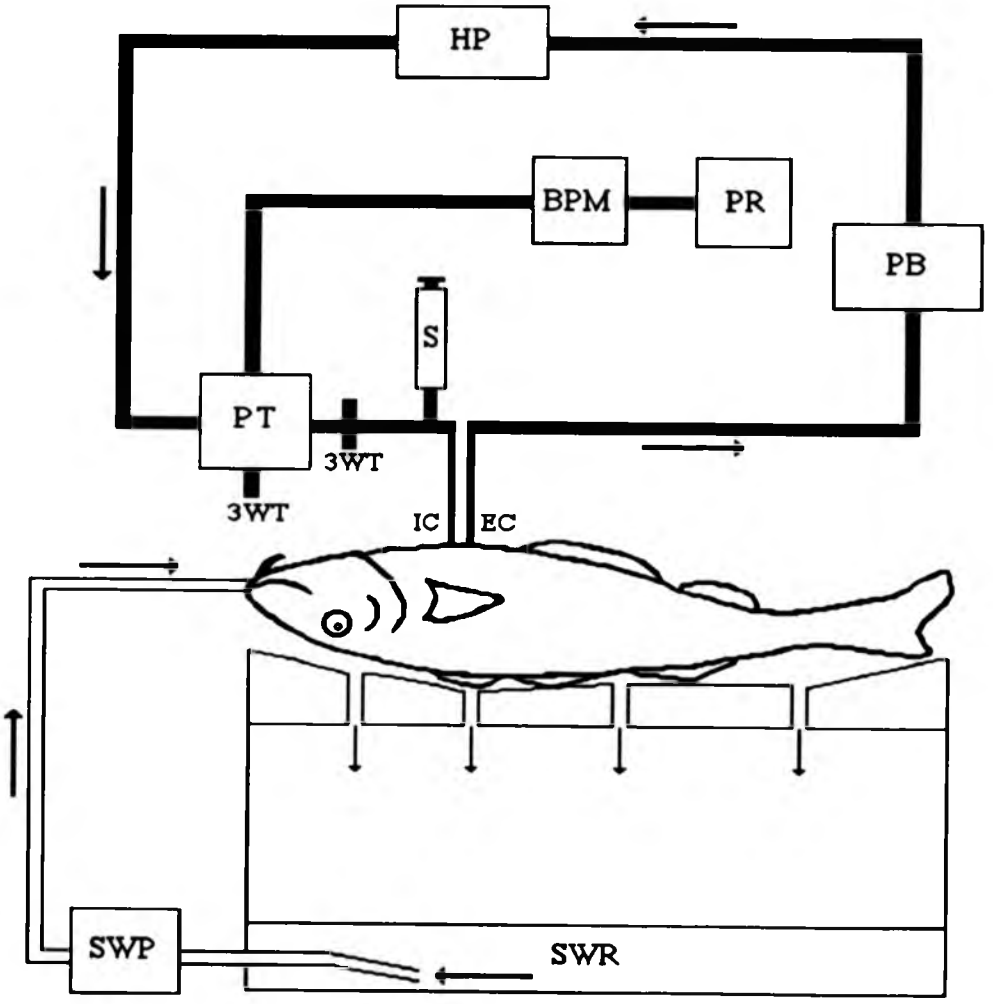

Figure 2.1. Diagrammatic representation of the apparatus used in perfusion experiments to measure sodium efflux and perfusion pressures in the gills of cod. SWR, sea-water reservoir; SWP, sea-water pump; HP, heart pump; PB, perfusion buffer; IC, input catheter; EC, efferent catheter; S, 10ml syringe; 3WT, three-way tap; PT, pressure transducer; BPM, blood pressure monitor; PR, pen recorder.

5.1mM; Heparin 10,000 USP Units l^{-1}; pH7.4, filtered through 3μM and 0.2μM Millipore filters and stored at 4°C) was a physiological, buffered solution, a modification of the saline described by Rankin & Maetz (1971). Evan's blue was included in the perfusion buffer in order that leakage could be detected. Adrenaline was not routinely used in the perfusion buffer as recommended by Pärt *et al.* (1982) because of the possible complications that could arise when administering other agonists. Also cod gills seemed less susceptible to lamellar rupture than trout and the preparation remained intact and viable for several hours without added adrenaline. The perfusate was continuously aerated with a 98% air/2% carbon dioxide gas mixture.

The perfusate was pumped through the circulation system and out through the aperture cut in the bulbus arteriosus. A second heat-flared PVC catheter was inserted, after complete exsanguination, into the bulbus towards the ventricle and secured by a silk thread ligature. A closed artificial circulatory system was thus formed. A pressure transducer was positioned at a point just before entry of the afferent catheter and was connected to a blood pressure monitor (CFB) and pen recorder oscillograph (George Washington), allowing the afferent perfusion pressure to be monitored throughout. Perfusion pressures were normally in the range of 30-45mmHg which were within normal physiological levels (Wahlquist & Nilsson, 1980; Nilsson & Pettersson, 1981). The perfusion was allowed to run for approximately 15-20 minutes to check for leaks of perfusate into the seawater (indicated by the appearance of Evan's blue in the surrounding medium) and also to ensure that the perfusion pressure was maintained. Only preparations with an intact artificial circulation were used.

Having ascertained that the system was intact sodium-22 was added to the

perfusion medium in the form of sodium chloride solution (40µl, 8µCi). The time course of changes in $^{22}Na^{+}$ activity in the seawater was monitored by taking a 2ml sample of seawater every 2.5 minutes and assaying for radioactivity using a Packard 500C gamma-counter. Changes in radioactivity in the perfusate were monitored by taking 10µl samples of perfusate medium at regular intervals of 15min. The transbranchial concentration gradient did not change significantly throughout the course of the measurement. Count rates were converted into µmol sodium and the rate of efflux was calculated as the gradient of the regression line describing the time course of sodium efflux. Efflux of sodium across the gills was expressed as µmol Na^{+} h^{-1} 100g $fish^{-1}$.

All experiments were carried out in a constant temperature room at 10±3°C (variation over all runs). Statistical analysis of results was by Student's paired t-test with results expressed as the means and standard deviations for (n) experiments.

2.3 RESULTS

2.3.1 Validation of the whole body perfusion technique

Initial experiments were performed to investigate the integrity of the perfusion technique. Once an individual experiment had been set up, the preparation was allowed to continue for around 15-20 minutes to ensure that the preparation was intact and that there were no leakages into the heart cavity or into the surrounding seawater. Radioactive sodium was then added to the perfusion medium and the perfusion was allowed to continue for up to 2.5 hours. Throughout this period the afferent perfusion pressure was monitored as was the appearance of radioactivity in the surrounding seawater. The rate of sodium efflux fell by between 5 and 12% over an initial period of 90min though this was not statistically significant and there was no change in perfusion pressure throughout this period. The rate of sodium efflux decreased significantly ($p<0.05$) between 90 and 150 minutes and it was therefore decided to conclude any future experiments within the 90min time period. Figure 2.2 shows the results of a typical control experiment. The perfusion pressure was relatively constant throughout the experiment and the sodium efflux formed a very good linear relationship with time ($R^2>0.99$).

Several agents were used to test the responsiveness of the perfusion technique and the results were compared to those reported in the literature. Figures 2.3-2.5 show the typical effects of administration of adrenaline (10^{-5}M), phenylephrine hydrochloride (10^{-4}M), orthovanadate (10^{-5}M) and arginine vasotocin (10^{-6}M) on sodium efflux and perfusion pressure. Administration of 10^{-5}M adrenaline (Figure 2.3) resulted consistently in an immediate, small but insignificant ($p>0.05$) decrease in mean perfusion pressure, a fall from 37±10 to 34±4 mmHg. This was

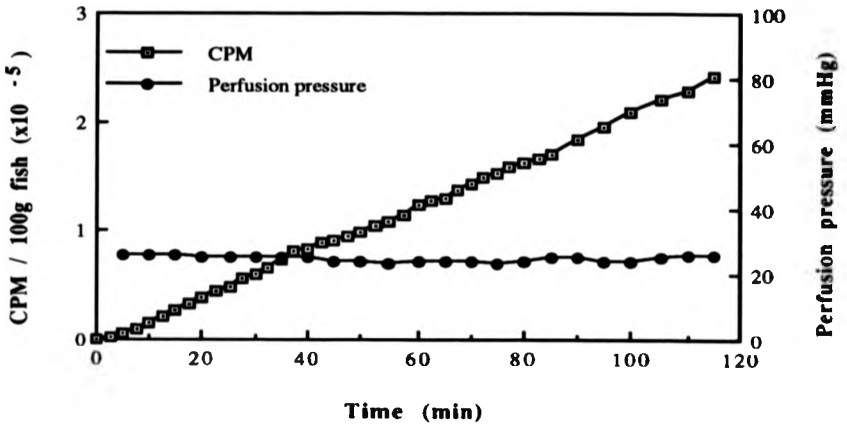

Figure 2.2. The data obtained from a typical perfusion experiment performed under control conditions (see Section 2.2 for details). The sodium efflux in this experiment was 309 μmol h^{-1} 100g $fish^{-1}$ and shows a good linear relationship with time (the R^2 value of the regression was 0.999). The perfusion pressure remained relatively constant throughout the experiment.

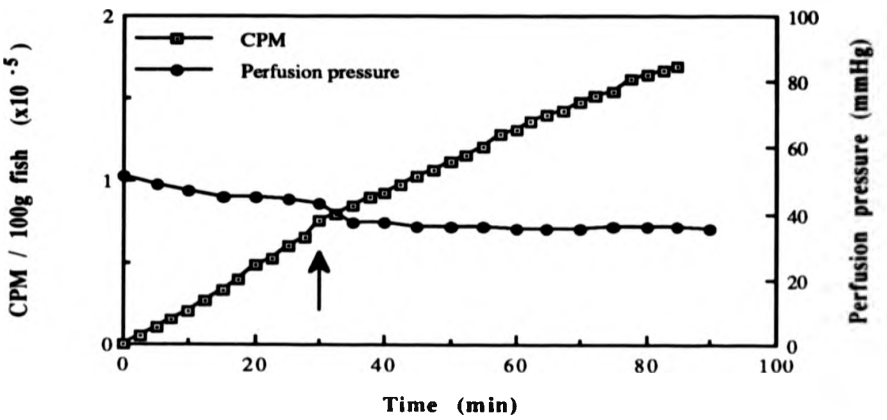

Figure 2.3. The effects of adrenaline (10^{-5}M) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the hormone 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

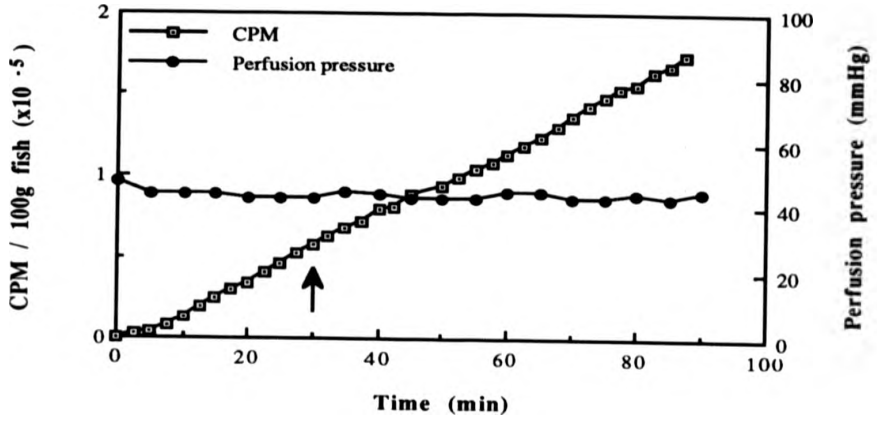

Figure 2.4. The effects of phenylephrine (10^{-4}M) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the alpha-adrenaline agonist 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

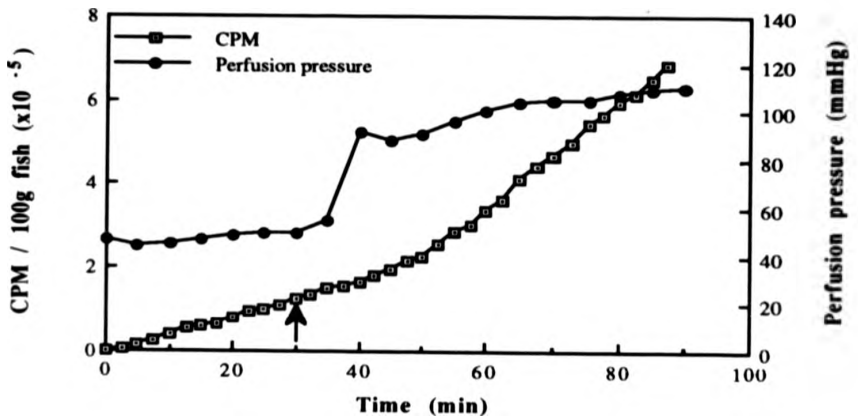

Figure 2.5. The effects of orthovanadate (10^{-5}M) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the Na^+-K^+-ATPase inhibitor 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

accompanied by a fall in sodium efflux from 363±7 to 268±2 μmol h^{-1} 100g $fish^{-1}$ (Table 2.1). The fall in sodium efflux was equivalent to a fall of 26% below the control level. The Student's paired t-test could not be used to determine the significance of this result due to the small sample number (n=2).

Administration of the alpha-adrenaline agonist phenylephrine hydrochloride at a concentration of 10^{-4}M (Figure 2.4) resulted in no significant changes in either perfusion pressure or sodium efflux (Table 2.1).

The administration of 10^{-5}M sodium orthovanadate (Figure 2.5) resulted in an immediate large significant increase of 69% ($p<0.01$) in mean perfusion pressure from 45±2 mmHg to 76±6mmHg (Table 2.1). There was also an increase in the mean rate of sodium efflux from 283±9 to 386±30 μmol h^{-1} 100g $fish^{-1}$. In all preparations the increase was consistently large (~40%), however, the significance of the change could not be determined due to the small sample number (Table 2.1).

Administration of AVT 10^{-6}M provided an interesting pattern of changes in Na^+ efflux and perfusion pressure. A typical result obtained upon administration of AVT can be seen in Figure 2.6. In this instance, a small but significant fall in sodium efflux was recorded. The mean perfusion pressure also changed significantly rising immediately after administration of the hormone and peaking 4-5 minutes later. Mean peak perfusion pressures were equivalent to a rise of around 58% above control levels, however, this rise was short lived and the perfusion pressure returned to the basal level approximately 15 minutes after the time of administration. The mean perfusion pressure over the 30 minutes following administration of AVT was increased by 26% ($p<0.01$) (Table 2.1).

Table 2.1. Summary of the effects of the agents used to characterise the perfusion technique on sodium efflux and mean afferent perfusion pressure in the gills of cod.

	Sodium efflux (μmol h^{-1} 100g $fish^{-1}$)		**Perfusion pressure** (mmHg)	
	Pre-treatment	**Post-treatment**	**Pre-treatment**	**Post-treatment**
No addition	328± 81 (4)	288± 58 (4) n.s.	32±10 (4)	31± 9 (4) n.s.
AVT (10^{-6}M)	289±106 (5)	254±121 (5) p<0.05	44± 4 (5)	55± 5 (5) p<0.01
Orthovanadate (10^{-5}M)	283± 9 (2)	386± 30 (2) n.d.	45± 2 (3)	76± 6 (3) p<0.01
Adrenaline (10^{-5}M)	363± 7 (2)	203±112 (2) n.d.	37±10 (3)	34± 4 (3) n.s.
Phenylephrine (10^{-4}M)	296± 27 (3)	250± 14 (3) n.s.	41± 7 (3)	43± 1 (3) n.s.

Pre-treatment data was obtained during the first 30 minutes of perfusion. Agents were administered 30 minutes after the start of perfusion and any changes were determined from data obtained during the subsequent 30 minutes. Data are expressed as the means and standard deviations of the number of experiments in parenthesis. The Student's paired t-test was used to determine the significance of the changes observed. n.s. = not significant; n.d. = not determined.

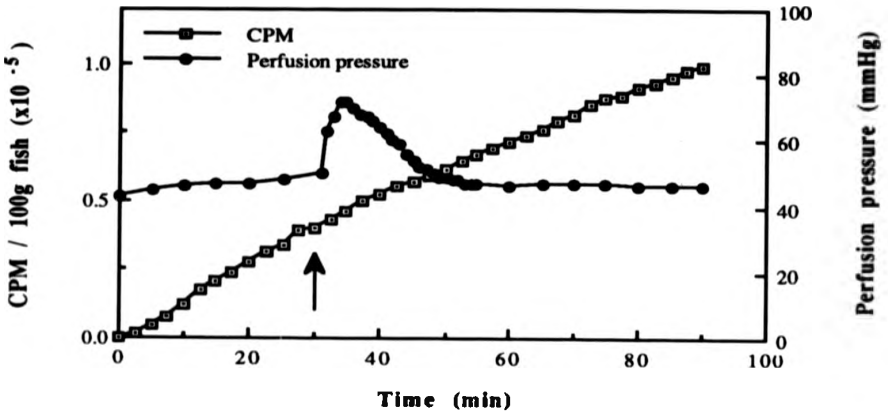

Figure 2.6. The effect of arginine vasotocin (10^{-6}M) on sodium efflux and perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the hormone 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes and any changes were calculated from the data obtained during the following 30 minute period.

Further evidence for the validity of the method was obtained from examination of the individual gill filaments at frequent time intervals throughout the time scale of perfusion. The gills were examined using a light microscope after perfusion for 0.5, 1.0, 1.5, 2.0 and 2.5 hours. The morphology of the gills was seen to be normal up to around 2 hours after which time the secondary filaments began to look slightly distorted. Scanning electron microscopic examination carried out by Ian Morgan in a study of caesium uptake across the gills of the rainbow trout showed no rupture of secondary lamellae in control and similarly perfused specimens (Morgan, 1992).

2.3.2 Relationship between sodium efflux and perfusion pressure

The possible effect of perfusion pressure upon sodium efflux across the gills was investigated. Figure 2.7 shows a plot of sodium efflux against the corresponding mean perfusion pressure for each experiment obtained over the first 30 minutes of perfusion. The plot incorporates the results of 40 individual experiments. There was a wide distribution in perfusion pressure (25-50 mmHg) and sodium efflux (100-700 μmol $h^{-1}100g$ $fish^{-1}$) though most fell within physiologically recorded ranges. Some of the higher or lower values were rejected for further studies because the viability of these preparations was in doubt. The correlation between the sodium efflux and perfusion pressure was low ($R^2 = 0.081$) indicating that only 8% of the variation in sodium efflux can be explained by perfusion pressure. Higher pressures may be required to elicit a significant response.

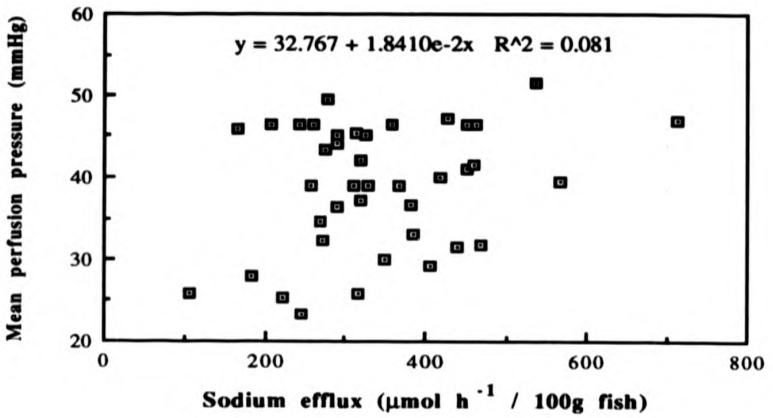

Figure 2.7. The relationship between sodium efflux and mean perfusion pressure in the gills of *Gadus morhua*. Measurements of sodium efflux and perfusion pressures were carried out under control experimental conditions (see text for details). The data represents the results of 40 experiments.

2.3.3 The effects of various agonists and hormones upon sodium efflux and perfusion pressure

In order to investigate the role of the second messenger diacylglycerol (DAG) and the associated protein kinase C (PKC) cascade in gill ionic regulation (refer to Sections 1.3 and 5.1 for more detail), the effects of the two compounds which mimic the action of DAG, namely the phorbol ester, phorbol 12, 13-dibutyrate (PDB) and 1-oleoyl-2-acetyl glycerol (OAG) were examined, as were the effects of the PKC inhibitor staurosporine. Since it was necessary to use dimethylsulphoxide (DMSO) as a solvent for some of these putative agonists an initial investigation of its effects upon the system was carried out. No significant changes in either perfusion pressures or sodium efflux were recorded following the addition of DMSO to the perfusate (Table 2.2).

Figures 2.8 and 2.9 show the typical results obtained upon administration of PDB at two different concentrations, 10^{-6}M and $3x10^{-6}$M. In each case of phorbol ester administration, the sodium efflux was significantly reduced ($p<0.05$ and $p<0.01$ respectively) and was accompanied by a significant increase in mean perfusion pressures ($p<0.01$ and $p<0.005$ respectively) (Table 2.2). On the application of PDB at a concentration of 10^{-6}M the mean sodium efflux was reduced by 22% and the mean perfusion pressure rose by 26%. These effects were more pronounced upon administration of PDB at the higher concentration of $3x10^{-6}$M with the sodium efflux falling by 23% and the mean perfusion pressure rising by 51%. Sometimes a third phase was seen in the time course of sodium efflux upon administration of PDB. This occured at around 70-80 minutes after the start of perfusion when there was a sharp increase in sodium efflux. This may have been due to the very high sustained perfusion pressures, which were well above the

Table 2.2. Summary of the effects of atrial natriuretic factor and various diacylglycerol investigative agents on sodium efflux and mean afferent perfusion pressures in the gills of cod.

	Sodium efflux (μmol h^{-1} 100g $fish^{-1}$)		**Perfusion pressure** (mmHg)	
	Pre-treatment	**Post-treatment**	**Pre-treatment**	**Post-treatment**
ANF (10^{-7}M)	374± 50 (3)	250± 31 (3) p<0.05	37± 4 (3)	34± 4 (3) n.s.
PDB (10^{-6}M)	448±142 (5)	253±106 (5) p<0.05	44± 6 (5)	56± 6 (5) p<0.001
PDB ($3x10^{-6}$M)	333± 68 (5)	212± 31 (5) p<0.01	34± 4 (5)	51± 7 (5) p<0.005
OAG ($4x10^{-6}$M)	419± 79 (3)	385± 88 (3) n.s.	38± 5 (3)	42± 6 (3) n.s.
Staurosporine ($3x10^{-9}$M)	345± 72 (3)	268± 89 (3) n.s.	42± 5 (3)	42± 7 (3) n.s.
DMSO ($5x10^{-5}$M)	323±142 (5)	269±117 (5) n.s.	36±10 (5)	35± 6 (5) n.s.

Pre-treatment data was obtained during the first 30 minutes of perfusion. Agonists were administered 30 minutes after the start of perfusion and any changes were determined from data obtained during the subsequent 30 minutes. Data are expressed as the means and standard deviations of the number of experiments in parenthesis. The Student's paired t-test was used to determine the significance of the changes observed. n.s. = not significant; n.d. = not determined.

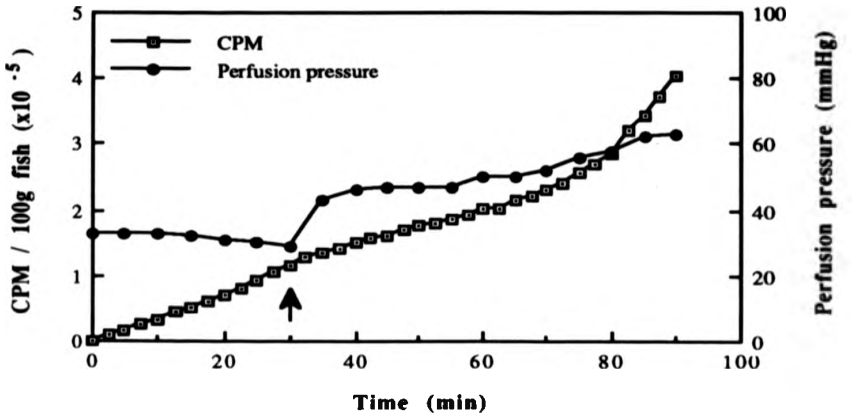

Figure 2.8. The effects of phorbol 12, 13-dibutyrate (10^{-6}M) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained when the phorbol ester was administered 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

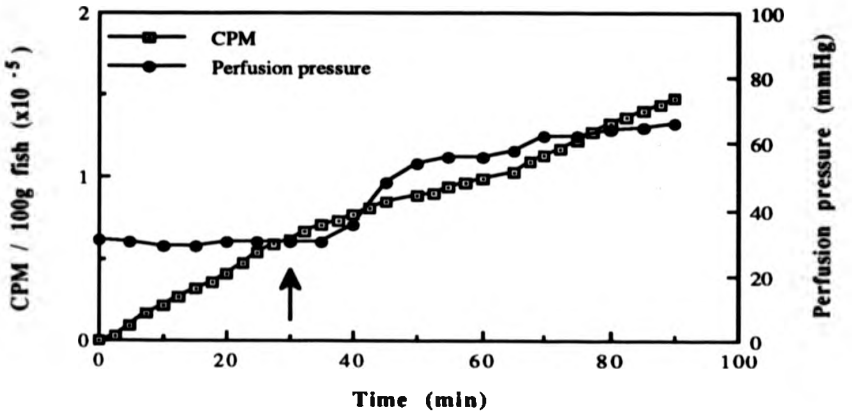

Figure 2.9. The effects of phorbol 12, 13-dibutyrate ($3x10^{-6}M$) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained when the phorbol ester was administered 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

normal physiological levels in the fish (and those used in the experiments depicted in Figure 2.7) causing the mechanical deterioration of the lamellar epithelium and a pressure driven efflux of ions out into the surrounding seawater. Examination of the gill filaments using the light microscope showed distortion of the filaments in these specimens at this stage of the experiment indicating a loss in the integrity of the secondary lamellae.

Administration of OAG ($4x10^{-6}M$) produced no significant changes in the means of either perfusion pressure or sodium efflux before and after treatment though there did appear to be a trend towards an increase in perfusion pressure on administration (~12%). Figure 2.10 shows the typical effect of OAG administration and Table 2.2 summarises the results. Application of the PKC inhibitor staurosporine ($3x10^{-9}M$) (Figure 2.11) resulted in a small but insignificant decrease in sodium efflux, equivalent to 12%, but no accompanying change in perfusion pressure (Table 2.2).

The effects of the physiological hormone atrial natriuretic factor (ANF) was also studied. The typical effects of ANF $10^{-7}M$ on sodium efflux and perfusion pressure can be seen in Figure 2.12. Interestingly after administration of ANF there was a very significant fall in sodium efflux, equivalent to a 21% mean decrease but this was accompanied by an comparatively small, insignificant, 7% change in mean perfusion pressure (Table 2.2).

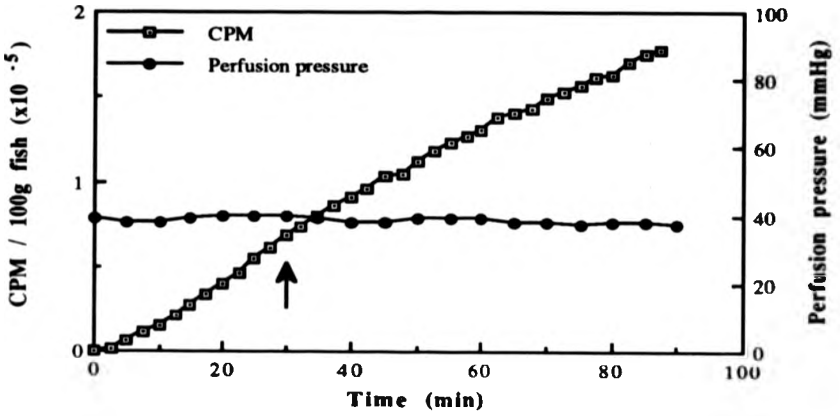

Figure 2.10. The effects of 1-oleoyl-2-acetyl glycerol ($4x10^{-6}M$) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the synthetic diacylglycerol 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

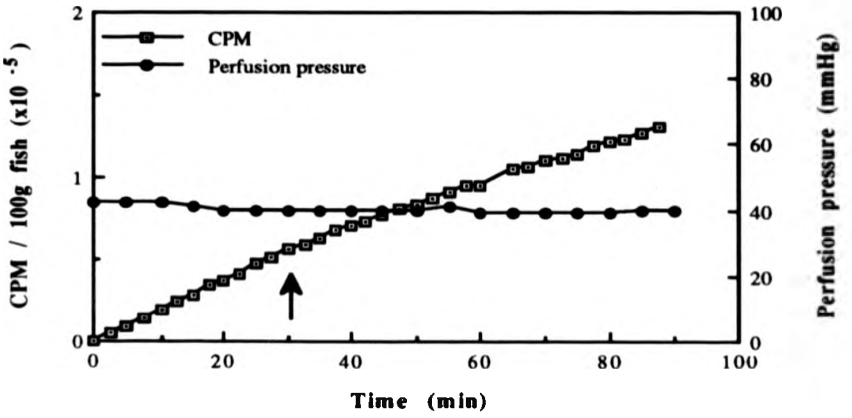

Figure 2.11. The effect of staurosporine ($3x10^{-9}M$) on sodium efflux and perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the protein kinase C inhibitor 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes and any changes were calculated from the data obtained during the following 30 minute period.

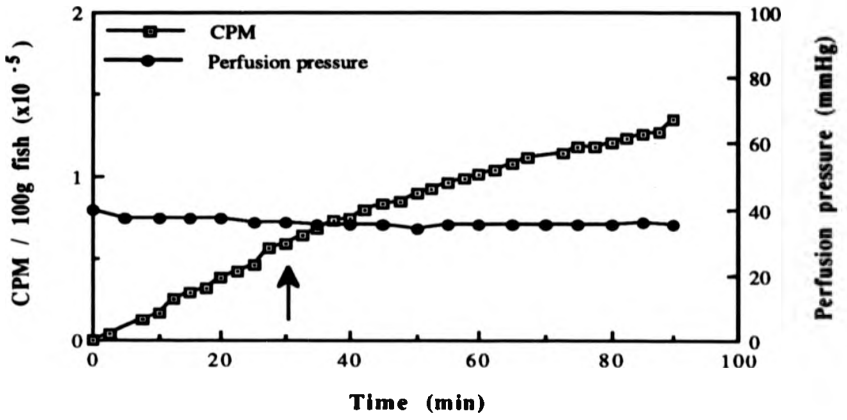

Figure 2.12. The effects of atrial natriuretic factor (10^{-7}M) on sodium efflux and afferent perfusion pressure in the gills of *Gadus morhua*. The graph shows the typical result obtained on administration of the hormone 30 minutes after the start of perfusion (indicated by the arrow). Control values were obtained during the first 30 minutes of perfusion and any changes were calculated from the data obtained during the following 30 minute period.

2.4 DISCUSSION

The morphology of the fish gill does not lend itself easily to the classic methods of study of ion transport for several reasons. The gill surface epithelium consists of at least three different cell types which are arranged in a particular fashion over the gill filament resulting in an epithelium which is not flat as, for example, is the opercular membrane. The extensive vasculature of the gill circulatory system creates additional complexities when studying the fluxes of ions and the effects of agonists or antagonists on these fluxes. In addition, the live animal can only be used for certain types of experiment (for example, see work by Greenwald *et al.* (1974), Lahlou (1975) and Girard (1976) on free swimming rainbow trout) and the use of isolated opercular membranes is only relevant in certain species of fish for example, tilapia (*Sarotherodon mossambicus*) (Foskett *et al.* 1983) and goby (*Gillichthys mirabilis*) (Marshall, 1977; Marshall & Bern, 1980).

The methods of perfusion available for the study of gill physiology have been reviewed by Perry *et al.* (1984) and he discusses the relative merits of each of the main categories (isolated branchial arches, branchial baskets, isolated heads and whole bodies). The isolated arch exhibits impaired circulation, ventilation is artificial and the whole preparation is susceptible to rapid deterioration. Isolated heads are prone to leakage and whole bodies have complicating secondary haemodynamic influences. The various techniques used to study ion exchange in the fish gill have been reviewed by Evans *et al.* (1982). It would seem that there have been relatively few studies using the whole body technique to study ion exchange in fish and no known reports of ion exchange studies in *Gadus morhua* using this method.

A whole body perfusion was used in the present study in an initial investigation into factors controlling the transport of sodium across the gills of cod. The preparation used a pithed organism which eliminated central nervous system control and also any endocrinological inputs which are under neuronal control. The perfusion method was designed to closely mimic the *in vivo* circulatory conditions of the fish and the apparatus was such that it enabled close monitoring and control of the stroke volume, stroke rate and perfusion pressure. Although few osmoregulatory studies have been performed on cod there are data available on the various circulatory parameters (Wahlquist & Nilsson, 1977; Pettersson & Nilsson, 1980; Axelsson & Nilsson, 1986). The stroke volume was set at between 0.45-0.75ml stroke^{-1} kg^{-1} and the perfusion pulse between 40-45 beats min^{-1} giving a simulated cardiac output of around 20ml min^{-1} kg^{-1} and which resulted in an afferent perfusion pressure within the range of observed ventral aortic pressures (25-50mmHg) (Wahlquist & Nilsson, 1977; Pettersson & Nilsson, 1980). The perfusion pressure remained relatively constant throughout the length of an experiment and the preparation did not exhibit the problems of incomplete gill perfusion, oedema and leakage reported by other authors in other species e.g. Part *et al.* (1982) in studies of rainbow trout. The preparation had an intact circulation with little or no leakage into the heart cavity or the surrounding medium as checked by the inclusion of the dye Evan's Blue in the perfusate. Any small leakage into the heart cavity, if it occured, was easily contained and did not affect the measurements of ion fluxes or afferent perfusion pressures.

Examination of gill filaments from control fish under a light microscope showed that no structural damage was caused by the process of perfusion. This was confirmed by scanning electron microscopy of unperfused and perfused gills from the gills of rainbow trout which showed no damage or rupture of secondary

lamellae upon perfusion (Morgan, 1992) thereby eliminating the need for inclusion of adrenaline in the perfusate as recommended by Pärt *et al.* (1982). The omission of adrenaline was beneficial to the present study since its inclusion could have complicated the results obtained from addition of other agonists.

Control experiments showed a good linear relationship between sodium efflux and time with correlation coefficients approaching unity. This provided further support for the validity of the method as did the comparison of the rates of sodium efflux obtained in this study with existing reports. The control rate of sodium efflux across the gills, as measured over the initial 30 minutes of perfusion in 40 experiments, was found to be 347±100μmol h^{-1} 100g $fish^{-1}$ which compared favourably with other reported values of sodium efflux obtained by various authors using different perfusion techniques and a variety of species (see Table 2.3). The lack of correlation between perfusion pressure and sodium efflux indicated that sodium efflux was unrelated to perfusion pressure over the range of pressures measured in 40 control preparations. Most of the data fell within the physiological ranges cited for afferent pressure and sodium efflux, thus natural fluctuations in blood pressure probably do not influence sodium efflux *in vivo*.

Further validation of the experimental technique was provided by the results obtained on addition of adrenaline and orthovanadate. Several workers have recognised the importance of circulating catecholamines in the control of circulatory parameters in teleosts (Randall, 1967; Randall & Stevens, 1967; Campbell, 1970; Rankin & Maetz, 1971; Stevens *et al.* 1972; Wood, 1974; Bolis & Rankin, 1975; Wahlquist & Nilsson, 1977; Holmgren, 1977; Wahlquist, 1980). In cod innervation of systemic blood vessels by adrenergic sympathetic fibres is well documented (Nilsson, 1972; Nilsson & Grove, 1974; Holmgren, 1978). There are

Table 2.3. Comparison of the data of sodium efflux obtained in the present study with that obtained by other authors in a number of sea-water adapted species using different experimental conditions.

Experimental conditions	**Sodium efflux** (μmol h^{-1} 100g $fish^{-1}$)	**Reference**
Whole body preparation (cod)	347±100 (40)	Present study
Whole body preparation (trout)	254± 21 (8)	Greenwald *et al.* 1974
Free swimming trout (large aquaria)	373± 73 (8)	Greenwald *et al.* 1974
Free swimming trout	300-500	Lahlou *et al.* 1975
Isolated trout head preparation (without addition of adrenaline)	298± 36 (9)	Girard, 1976
Isolated gills SW-adapted eel	138± 70 (6)	Kelly *et al.* 1981

The data represents the means and standard deviations of the number of experiments in parenthesis.

also adrenergic excitatory fibres to the cod heart (Holmgren, 1977) and adrenergic dilatory fibres to the branchial vasculature (Pettersson & Nilsson, 1979). The main effect of adrenaline in the branchial vasculature of teleosts is a dilation (Krawkow, 1913), confirmed by a number of studies on different species (see Smith, 1977 for references). It is now established in several species that the adrenaline effect has two components, an alpha-adrenergic mediated constriction partly or wholly masked by a beta-adrenergic mediated dilation (Reite, 1969; Belaud *et al.* 1971; Bergman *et al.* 1974; Wood, 1975; Dunel & Laurent, 1977; Payan & Girard, 1977; Wahlquist, 1980; Nilsson & Pettersson, 1981).

Under normal, resting nonanoxic conditions only partial perfusion of gills occurs in resting-state intact fish. Thus the control of sodium flux may be through alteration of the functional area of the gills for example, by lamellar recruitment mediated by catecholamines (Booth, 1978). Blood entering the teleost gills via the ventral aorta and afferent branchial arteries may leave either via the efferent branchial arteries to the dorsal aorta (arterio-atrial pathway) or via the branchial veins (arterio-venous pathway). Chloride cells are located at the base of the lamellae bathed by the venous system whereas the respiratory cells are located in the lamellar epithelium. Thus shunting of blood between the two pathways may be important in regulation of respiratory and ion transporting functions of the gills (Laurent & Dunel, 1980; Payan & Girard, 1984). In seawater-adapted rainbow trout there was no net sodium and chloride flux across the lamellar epithelium whereas there was a net efflux of sodium and chloride across the filamental epithelium, evidence that chloride cells are responsible for the net efflux of ions in seawater (Girard & Payan, 1980). Increased rates of sodium and water transfer across the gills have been associated with the redistribution of blood within the blood capillaries (Wood & Randall, 1973). In the present preparation perfusion of secondary lamellae seemed

to be maximal i.e. all secondary lamellae were perfused, thus this might influence the ability of the preparation to respond to circulating catecholamines. Alpha-adrenoceptor mediated nervous control of gill vasculature acts to constrict the arterio-venous pathway whereas beta-adrenoceptor mediated control acts to dilate the arterio-arterial pathway and is chiefly due to humoral catecholamines (Nilsson & Pettersson, 1981). Since adrenaline acting on beta-receptors is a known vasodilator the fall in perfusion pressure in this study, though not statistically significant, was as expected and was in accordance with that reported by other authors such as Girard (1976) for the isolated gills of the eel. The result also supports the humoral role of catecholamines in circulatory control.

In a study using a head perfusion of seawater-adapted rainbow trout Girard reported a net efflux of sodium across the gill under control conditions which was significantly reduced on administration of adrenaline (10^{-5}M) (Girard, 1976). The adrenaline caused an increase in influx and a reduction in efflux resulting in a net movement of sodium inwards across the gills. Likewise the present study revealed a reduction in sodium efflux on administration of adrenaline. However, in *Fundulus heteroclitus*, beta-adrenergic agonists stimulated salt extrusion by the opercular epithelium (Degnan & Zadunaisky, 1979). Therefore the effect may be species specific.

An inhibitor of Na^+-K^+-ATPase which co-purified with ATP prepared from equine muscle (Josephson & Cantley, 1977; Beaugé & Glynn, 1977) was identified as sodium orthovanadate (Cantley *et al.* 1977). The mechanism of the orthovanadate (VO_4^{3-}) inhibitory effect may stem from the structural similarity between orthovanadate and the terminal triphosphate group of ATP. Thus orthovanadate

would act on the Na^+-K^+-ATPase at the cytoplasmic surface. It was suggested that vanadate may act as a natural regulator of sodium pumps *in vivo* (Cantley *et al.* 1977). In this study the results obtained upon administration of orthovanadate (10^{-5}M) were in agreement with those recorded by Kelly *et al.* (1981) from the isolated gills of seawater-adapted eels, *Anguilla anguilla*. Kelly and his colleagues found that following administration of orthovanadate ($5x10^{-7}$M) the perfusion pressure rose by 40%, Na^+ and Cl^- efflux fell by 42% and 50% respectively and water efflux remained unchanged. At the higher concentration of 10^{-5}M all of these parameters were increased 2.5 fold. Microscopic examination revealed that the gills were not ruptured and it was concluded that the high perfusion pressure reflects pressure driven movement of salt and water out across the epithelium via inter- or paracellular routes between adjacent cells.

The whole body preparation used in this study was clean and easy to set up with few failures. The control sodium efflux was in the middle of the published range of values and the preparation behaved predictably with various agonists. These results confirmed the suitability and validity of the whole body preparation as a method of examining the effects of agents on the perfusion pressure and sodium efflux in the gills of *Gadus morhua* .

Mammalian atrial natriuretic factors have been used extensively to study osmoregulation in fish. Mammalian ANF causes diuresis and natriuresis in freshwater trout (Duff & Olson, 1986) and the marine aglomerular toadfish (Lee & Malvin, 1987). Mammalian ANF is also vasoactive in fish blood vessels. It vasodilated isolated vascular rings from the trout (Olson & Meisheri, 1989), the perfused gills of the toadfish (Evans *et al.* 1989) and the isolated ventral aortic rings from the dogfish shark (Solomon *et al.* 1985; Evans & Weingarten 1989).

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Injection of ANF into the coeliac artery of the toadfish reduced blood pressure (DeRenzis & Bornancin, 1984) whereas injection into the dorsal aorta of the trout led to an increase in blood pressure though this may have been a secondary effect due to vasodilation of the branchial vasculature rather than systemic vasoconstriction (Duff & Olson, 1986). The present study recorded a small decrease in afferent perfusion pressure on administration of ANF (10^{-7}M) though this change was not statistically significant. It is not clear what advantage vasodilation of the ventral aorta or branchial vasculature would confer on the teleost fish. Increased branchial perfusion would lead to a greater loss of water in marine teleosts and would therefore appear to be maladaptive unless the increase in perfusion would also stimulate branchial ionic transport.

Mammalian ANF has been shown to exert significant effects on various osmoregulatory epithelia in marine fish. Stimulation of the Na-K-Cl co-transporter was revealed in the gill epithelium (Scheide & Zadunaisky, 1988) whereas this co-transport system was inhibited by ANF in the intestine of the seawater-adapted flounder thereby reducing the extraction of water from the intestine (O'Grady *et al.* 1985). These are thought to be direct effects on the epithelia since tetrodotoxin does not inhibit the effect indicating that neural activity is not involved (Scheide & Zadunaisky, 1988; O'Grady, 1989). The role of ANF as a physiological effector in salt excretion by the shark rectal gland is well documented (Solomon *et al.* 1985a) and it is thought to exert its effect by stimulating the release of the neurotransmitter vasointestinal peptide (VIP) from neuronal sites within the gland (Silva *et al.* 1985; 1987) which in turn brings about an increase in salt excretion. Atrial natriuretic factor also enhances salt excretion from the nasal salt gland in marine birds (Langford & Holder, 1988; Schütz & Gerstberger, 1990) and stimulates chloride secretion across the opercular epithelium of *Fundulus heteroclitus* (Scheide &

Zadunaisky, 1988). How these effects are exerted is less clear at the present time.

Fish heart and brain extracts have also been used in several studies to produce vasoactive, diuretic and natriuretic responses in the trout (Duff & Olson, 1986; Olson & Meisheri, 1989) and in the toadfish (Lee & Malvin, 1987). These studies highlighted the fact that the ventricle may be an important source of ANF in the fish (Reinecke *et al.* 1987a; Evans *et al.* 1989). Electron dense granules have been seen in the cardiac tissue of hagfish (*Myxine glutinosa*) (Reinecke *et al.* 1987a), elasmobranchs (*Squalus acanthias*) (Solomon *et al.* 1985b) and teleosts (*Gila atraria*) (Westenfelder *et al.* 1988) and immunoreactivity with antibody against mammalian ANF has been shown in all three groups of fish (Chapeau *et al.* 1985; Reinecke *et al.* 1985; 1987b). Evidence of a role for a putative fish ANF in osmoregulation is provided by the fact that osmotic perturbations result in changes in the amount of circulating ANF in fish as measured by immunoprecipitation. Acclimation to reduced salinities is associated with a reduction of ANF immunoreactivity in five species of marine teleost (Galli *et al.* 1988; Evans *et al.* 1989). The use of mammalian ANF in the present study provided further evidence for the role of ANF as an osmoregulatory effector in the gills of a marine teleost and indicated that ANF may provide a primary signal in the control of sodium movement across the gill epithelium. The decrease in sodium efflux recorded here on administration of ANF contrasts with the results of other studies on different species of teleost fish. Thus the effect appears to be species specific. For example, ANF had little effect on the efflux of sodium in the rainbow trout (Eddy *et al.* 1990) but increased sodium efflux in dab (*Limanda limanda*), flounder (*Platichthyes flesus*) and plaice (*Pleuronectes platessa*) (Arnold-Reed *et al.* 1991). Whether the effect of ANF is brought about by binding of ANF to specific receptors on the surface of the salt-secreting chloride cells in the cod gill or by some other indirect

method such as the release of some other hormone or transmitter requires more detailed study of these specialised cells. Interpretation of the results obtained using the perfused gills is therefore limited.

Atrial natriuretic factor has been shown to effect the release of other hormones in several different mammalian and non-mammalian tissues and likewise many other hormones have been shown to modulate the release of ANF. In mammals ANF blocks the release of several classes of hormones, for example, aldosterone, renin, vasopressin and other pituitary hormones (reviewed by Baxter *et al.* 1988). Atrial natriuretic factor in mammalian systems inhibits prolactin secretion whereas cortisol stimulates ANF secretion. This may be of some significance since prolactin is the main osmoregulatory hormone in freshwater fish and cortisol the main hormone in seawater species and these hormones are associated with longer term adaptations to changes in salinity. In mammals ANF inhibits the release of vasopressin from the post-pituitary and this effect may be mediated at the level of vasopressin-producing cells (Samson, 1987). If the same is true in fish then this would be adaptive in marine fish since arginine vasotocin (AVT) is diuretic not antidiuretic. The putative interactions between ANF, prolactin, cortisol and AVT support the hypothesis that ANF acts primarily in salt homeostasis in fish. On the other hand ANF inhibits angiotensin II in mammals. In fish this hormone caused compulsive drinking so that ANF might act to reduce drinking. Angiotensin II may therefore play a role in seawater osmoregulation (Evans, 1990). The mineralocorticoids, alpha- and beta-adrenergic agonists and cholinergic agonists stimulate ANF secretion though their effects may be indirect via haemodynamic changes (Genest & Cantin, 1988). The glucocorticoids and thyroid hormones T_3 and T_4 act directly on the ANF gene (Gardner *et al.* 1986; Nemer *et al.* 1987).

The dominant direct stimulus for ANF secretion seems to be atrial stretch produced by volume loading or by increased salt uptake resulting in increased plasma volume (Genest & Cantin, 1988). Some evidence also exists to suggest that if the concentration of plasma sodium is increased then ANF release is stimulated from isolated rat atrial or hypothalmic fragments (Arjamaa & Vuolteenaho, 1985; Gibbs, 1987; Shibasaki *et al.* 1988). Similar mechanisms are likely to exist in fish, though at first sight the inhibition of sodium efflux by ANF appears incompatible with such a mechanism. This will be discussed again later.

Arginine vasotocin (AVT) rather than arginine vasopeptide (AVP) is the vasoactive neurohypophysial peptide in fishes (Sawyer, 1977) and it normally produces diuresis in teleost fishes (Pang, 1983). Arginine vasotocin and prolactin are important modulators of osmoregulation in freshwater where fish are volume-loaded and salt-depleted. Arginine vasotocin causes vasoconstriction of branchial vessels in *Anguilla anguilla* (Bennett & Rankin, 1986) and increased dorsal aortic pressure suggestive of vasoconstriction of systemic vessels as well (Zucker & Nishimura, 1981). These studies are consistent with the results presented in Section 2.3 which revealed a significant rise in afferent perfusion pressure on administration of AVT (10^{-7}M) indicating a vasoconstrictive effect. Presumably vasoconstriction could limit the osmotic uptake of water in the freshwater environment but it could also be effective in limiting branchial osmotic water loss in marine teleosts. Arginine vasotocin might therefore be important in either salinity.

The results of AVT administration contrast with the effects of PDB (present study) and those of orthovanadate (present study; Kelly *et al.* 1981) because despite the very high perfusion pressures experienced by the gills under the influence of AVT

there was very little effect on the rate of sodium efflux. The secondary fall in perfusion pressure seen within minutes of administration is difficult to explain because perfusion was continuous and the large volume of perfusion buffer relative to the blood volume meant that it would not have been possible for all of the hormone to have entered the body in the time span of the experiment and to have been metabolically inactivated or degraded by the fish. Some other mechanism appears to be counteracting the vasoconstriction caused by AVT.

The effects of ANF and AVT highlight the fact that changes in one of the recorded parameters can occur in the absence of changes to the other. In the case of ANF, a change in sodium efflux occurred without any significant changes in perfusion pressure, whereas in the case of AVT, the sodium efflux showed no great change but the perfusion pressure rose in a very characteristic manner. These results reinforce the earlier conclusion that Na^+ efflux and perfusion pressures can respond independently.

There have been very few reports of protein kinase C (PKC) activity in fish tissue. The first was by Kuo *et al.* (1980) in goldfish brain and this was followed by the partial characterization of PKC from trout spleen by Bell & Sargent (1987) (since the spleen is one of the richest sources of PKC in mammalian tissues) and from dogfish (*Scyliorhinus canicula*) rectal gland (reflecting the authors interest in the control of salt secretion by the rectal gland). The high specific activity of PKC in rectal gland (304±72 pmol min^{-1} mg $protein^{-1}$) was comparable with the most active mammalian tissues (Kuo *et al.* 1980). Since the rectal gland consists largely of chloride cells and is responsible for maintaining salt balance in the dogfish the high levels of PKC suggest a role for this enzyme in the control of salt secretion. Other evidence of a role for PKC as a mediator in the process of ion transport comes from

studies on intestinal Cl^- secretory cells where cholinergic regulated protein phosphorylation of an effector protein p83 from the epithelial T84 colonic cell line is mediated by PKC (Cohn, 1990), and from studies on other systems including neuronal conductance and smooth muscle contraction (reviewed by Nishizuka, 1988).

Since diacylglycerol (DAG) cannot permeate the cell membrane many analogues and mimetics of this potent physiological activator of PKC have been used to examine its effects on cellular processes and to investigate the putative role of PKC in these systems. Many synthetic analogues of DAG have been manufactured but the structural requirements for activity are quite specific, namely a *sn*-1,2-diacylglycerol with acyl side chains which are sufficiently hydrophobic to allow the molecule to partition into the membrane (Mori *et al.* 1982). The tumor-promoting phorbol esters which activate PKC directly *in vivo* and *in vitro* (Castagna *et al.* 1982; Yamanishi *et al.* 1983) mimic this *sn* 1,2-diacylglycerol structure. There is some evidence that phorbol esters intercalate into membranes and directly activate PKC in intact cell systems in a manner similar to that for the synthetic 1-oleoyl-2-acetyl-glycerol (OAG) (Castagna *et al.* 1982; Yamanishi *et al.* 1983; Kikkawa *et al.* 1984).

In this study administration of OAG and phorbol 12, 13-dibutyrate (PDB) gave interesting results. Administration of phorbol dibutyrate significantly reduced sodium efflux across the cod gill and increased the mean perfusion pressure. The correlation between sodium efflux and perfusion pressure in control studies was low indicating that afferent perfusion pressure was only controlling sodium efflux to a very small extent (8%). The effects of PDB on sodium efflux and perfusion pressure are therefore assumed to be independent of one another during the early

stages of PDB administration. As the perfusion pressure continued to rise so that it was outwith the observed control values the rate of sodium flux increased dramatically and it was no longer possible to assume that the sodium efflux remained independent of the perfusion pressure. In this situation a sustantial pressure increase may have forced tight junctions open leading to an increase in sodium efflux which might explain the third phase sometimes seen on the addition of PDB, similar to the effect seen by Kelly *et al.* (1981) in eel gills on administration of orthovanadate (10^{-5}M). The effects of OAG were less pronounced than those of PDB and this may have been due to the inability of OAG to gain access to the cell interior within the time course of the experiment.

Protein kinase C inhibitors have also been used to study the putative role of PKC. These inhibitors fall into two categories. Firstly, those that interfere with the binding of cofactors (Ca^{2+}, phospholipid, DAG) to the regulatory domain of PKC, for example, sphingosine (Hannun *et al.* 1986; Jefferson & Schulman, 1988). The second group interfere with the interaction of ATP and the protein substrate at the catalytic domain. The isoquinolines, for example, 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine (H-7) (Nakadate *et al.* 1988) and the microbial product staurosporine fall into this second class of inhibitor. Staurosporine was first identified as a weakly active antibacterial and antifungal agent (Omura *et al.* 1977) and was subsequently found to have marked cytotoxic effects at very low concentrations (10^{-11} - 10^{-9}M) (Tamaoki *et al.* 1986) and to inhibit the activity of a number of protein kinases *in vitro* (Nakano *et al.* 1987). Staurosporine inhibits PKC and cAMP-dependent protein kinase at nanomolar concentrations and is thought to interact directly with the catalytic domain of PKC since it inhibits the proteolytically generated catalytic fragment while having no effect on the binding of phorbol esters to the regulatory domain (Nakadate *et al.* 1988).

In this system where phorbol ester administration leads to a fall in sodium efflux presumably through activation of PKC then administration of staurosporine might be expected to decrease PKC activity and hence increase Na^+ efflux. However, administration of staurosporine to the whole body fish preparation in this study did not result in any significant changes in sodium efflux or perfusion pressure. As with OAG the problem here may have been one of access to the gill cells or sequestration of the inhibitor at other sites. Alternatively it may be necessary to perturb the system with an agonist before inhibition can be detected i.e. activate PKC first.

Changes in the microcirculation within the gills can occur independently of the general circulatory system. Thus it is possible that changes within the gill lamellar circulation could remain undetected by the monitoring system. Increased pressure, if due to vasoconstriction, might restrict blood flow to the gills thereby reducing the amount of blood, and thus sodium, reaching the filaments resulting in a fall in efflux across the gills. The fact that complete perfusion of all gill surfaces occured as indicated by the presence of Evan's Blue throughout the secondary lamellae suggests that such haemodynamic complications are unlikely with the present preparation and that the ability of the gills to undergo increased recruitment of lamellae is limited.

The experimental set-up proved to be a suitable and viable system with which to study the effects of agonists and hormones upon ion fluxes and the circulatory parameters of the fish gills. It also indicated which of the putative effectors might be involved in osmoregulation. The main drawback of the technique was the problem of access of some of the effectors to the chloride cells. It must be

appreciated that several barriers, for example, the vascular tissues or the cell membranes must be passed before an effector can bind to, and in some cases permeate the cell, to reach its cellular target. This is also a problem in all types of organ perfusion. Additionally, the present method does not allow one to distinguish whether the effects of PDB and ANF on sodium efflux are due to changes in the activity of the enzyme Na^+-K^+-ATPase, either directly or indirectly or whether their administration was in fact triggering the release of some other physiological hormone or hormones which were then eliciting a response.

Thus with this preparation it is possible to comment on net sodium effluxes across the gills but it is not possible to either identify or quantify the contribution of passive and active components of the system such as the active transport by Na^+-K^+-ATPase. The efflux of sodium in seawater fish is the net result of large in and out fluxes of which a major component is passive diffusional exchange (Maetz, 1971; Motais & Garcia-Romeu, 1972). It was not possible to separate the active and diffusional components of ion transport so that the results obtained did not necessarily indicate a change in the activity of Na^+-K^+-ATPase. There was therefore a need to investigate the effects of ANF at the cellular level and thus a method for the routine preparation of chloride cells was developed for this purpose (Chapter 3). The results presented in this chapter indicated which agents would be most useful for the more detailed cellular and biochemical studies aimed at understanding the control mechanisms at the molecular level.

CHAPTER 3

ISOLATION OF CHLORIDE CELLS FROM THE GILLS OF *Gadus morhua*

3.1 INTRODUCTION

3.2 METHODS

3.2.1 Isolation of chloride cells from the gills of *Gadus morhua*

(a) Collagenase digestion of gill tissue

(b) Mechanical preparation of chloride cells

3.2.2 Identification of chloride cells and measurement of cell purity and viability

3.3 RESULTS

3.3.1 Resolution of problems encountered in the isolation of chloride cells

3.3.2 Cell purity and cell viability studies

3.4 DISCUSSION

3.1 INTRODUCTION

Most of the epithelia involved in ion transport processes in fish are rich in chloride cells, which were first identified in the gills of seawater-adapted eels and named by Keys and Willmer (1932). Chloride cells are characterised by the presence of large numbers of well developed mitochondria which are in close association with the extensively infolded basal and lateral plasma membranes (reviewed by Philpott, 1980). A diagrammatic representation of a chloride cell from the opercular membrane from *Fundulus heteroclitus* which shows the main structural characteristics of such cells is shown in Figure 3.1. Chloride cells are rich in the enzyme Na^+-K^+-ATPase (Bonting, 1966), which is correlated with epithelial ion transport activity. This enzyme has been shown to be located in the baso-lateral membranes of the chloride cells using autoradiographic and cytochemical techniques (Karnaky *et al.* 1976; Hootman & Philpott, 1979), and a high level of activity of the enzyme has been shown in isolated chloride cells (Kamiya, 1972; Sargent *et al.* 1975). Ultrastructural and enzymatic studies and kinetic data have provided indirect support for the idea that branchial chloride cells are the sites of ion exchange (Maetz & Bornancin, 1975; Kirschner, 1977; Karnaky, 1980; Philpott, 1980; Avella *et al.* 1987; Ishihara & Mugiya, 1987). Studies using short-circuit techniques confirmed that the chloride cell is the active ionocyte in seawater teleost osmoregulation. A low toxicity, specific fluorescent stain for mitochondria in living cells DASPMI (Bereiter-Hahn, 1976) was used to stain chloride cells in the opercular epithelium of *Fundulus* (Zadunaisky, 1979). Further studies revealed a linear correlation between chloride cell number and the magnitude of a short circuit current (Karnaky *et al.* 1979; Marshall & Nishioka, 1980) in the same species and an increase in chloride cell density and size was correlated to the development of a short circuit current in the opercular epithelium of the tilapia (*Sarotherodon*

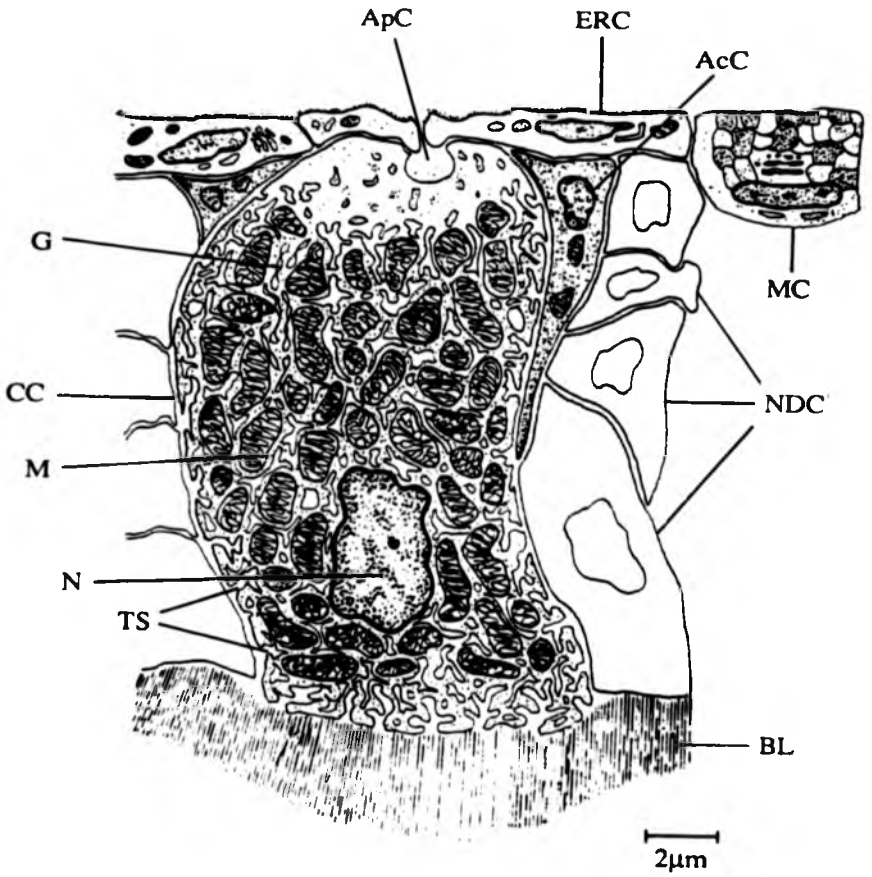


Figure 3.1. Diagrammatic representation of the ultrastructure of a chloride cell from the opercular epithelium from *Fundulus heteroclitus*. In this tissue 50-70% of the cellular population is represented by chloride cells whose cytology is identical to that described for the branchial epithelium of teleosts. CC, chloride cell; MC, mucous cell; ERC, epithelial respiratory cells; NDC, non-differentiated cells; AcC accessory cell; ApC, apical crypt; M, mitochondrion; G, golgi; TS, tubular system; N, nucleus; BL, basal lamina. Redrawn from a diagram by Degnan *et al.* 1977.

mossambicus) upon seawater acclimation (Foskett *et al.* 1981). Work by Foskett and Scheffrey (1982), using a vibrating probe technique, provided the first direct evidence that the chloride cells are indeed the site of significant electrogenic and conductive elements. Using short circuit conditions, localised current and conductive pathway studies in tilapia opercular membranes showed peak current densities only over chloride cells and current-voltage relations for individual cells showed localised sites of high conductance over the apical crypts of these cells (Foskett & Scheffrey, 1982).

Chloride cells are much more abundant and better developed in the gills of marine or seawater-adapted fish which secrete large amounts of ions to maintain homeostasis. During seawater-adaptation the levels of branchial salt excretion rise and this is accompanied by an increase in the size and/or number of chloride cells present and altered distribution in the epithelia. One of the earlier reports of this phenomenon was by Liu (1942) and this has been followed by numerous others over the past 50 or so years involving many different species of fish, e.g. Sargent *et al.* (1975), Sardet *et al.* (1979), Foskett *et al.* (1983). During seawater-adaptation there are developmental changes in the chloride cells with the tubular system becoming more extensive (Shirai & Utida, 1970; Doyle & Epstein, 1972; Karnaky *et al.* 1976; Pisam, 1981) and also mitochondria becoming larger and more numerous (Shirai & Utida, 1970; Sargent *et al.* 1975). Indeed, the main difference in gill function between freshwater and seawater-adapted gills is the activity of the chloride cells and one requirement for euryhalinity is the ability to rapidly alter the structure and function of the gill epithelium and the chloride cells (Foskett *et al.* 1983). Thus for short-term excursions between FW and SW the hypothesis of a fast acting hormonal effect to regulate chloride cell activity is feasible and for more sustained excursions between different salinities one might

envisage the initiation of differentiation processes within the gill tissue. For example, upon transfer to seawater, activation and augmentation of the immature chloride cell population was observed in the opercular membranes isolated from the tilapia (*Sarotherodon mossambicus*) up to three days and after three days there was increased differentiation of the cells which was accompanied by an enhancement and maintenance of high secretion rates (Foskett *et al.* 1981). Seawater-adaptation appears to transform the epithelium from a relatively impermeable tissue in freshwater to one in seawater that contains cells with a very high ionic permeability and transport rate.

Ultrastructural studies have indicated why this is so, with large differences in epithelial structure in the gills of FW- and SW-adapted eels. In FW eels respiratory cells and chloride cells of the gills are joined by multi-stranded (5-9 strands), tight junctions resulting in an epithelium of low permeability to ions and large molecules (Sardet *et al.* 1979; Laurent, 1984). This reduces the loss of ions via the paracellular route. In SW species the respiratory cells appear to be connected by multi-stranded junctions to one another yet junctions of only one strand exist between adjacent chloride cells resulting in so called 'leaky' junctions that are more permeable to sodium and small organic molecules (Frömter & Diamond, 1972) allowing greater ion flux via the paracellular route (Sargent *et al.* 1975; Sardet *et al.* 1979). Seawater-adaptation in euryhaline species of teleost involves a rearrangement of chloride cells within the branchial epithelium into complexes consisting of several chloride cells linked together by single strands. This compares to the FW situation where a single chloride cell is surrounded by and linked to respiratory cells by multi-stranded tight junctions.

Until recently it was believed that only one type of chloride cell existed and this cell

underwent ultrastructural modifications when fish were transferred to seawater. This picture has been complicated by the identification of two types of chloride cells in some species. The roles of these two subspecies of cells and the changes that they undego during seawater-adaptation have been discussed in a recent review which presents the current understanding of the role of mitochondria-rich cells in the gills of teleost fish (Pisam & Rambourg, 1991).

The mechanism by which chloride cells bring about the excretion of large quantities of ions across the intact gill epithelium remains to be totally elucidated. Many hypotheses have been proposed such as that by Ellis *et al.* (1977) to explain salt secretion from the avian nasal salt gland. Much experimental evidence supports the model of Kyte (1978) which was originally proposed to explain salt secretion in *Squalus* rectal gland but is equally applicable to all salt secreting epithelia and a similar model was presented by Sargent *et al.* (1980) to explain salt secretion in marine teleost gills. The model of Kyte (see Figure 3.2), suggests that NaCl enters cells passively through the basal plasma membranes and in order to maintain a constant internal sodium concentration Na^+ ions are actively pumped across the lateral membranes into the intercellular spaces by Na^+-K^+-ATPase. A very high concentration of NaCl builds up in these spaces and as a result passive diffusion out across the leaky junctions into the seawater occurs. This is also accompanied by some diffusion back to the basal side of the epithelial cells. An alternative model based on measurements of membrane potential across chloride cells in rectal gland was proposed by Epstein *et al.* (1983) (see Figure 3.3). The experimental values were interpreted as showing "a 'secondary active' co-transport of NaCl across basolateral membranes, in which chloride is transported into the cell against an electrochemical gradient, coupled to the downhill movement of Na^+. A downhill electrochemical gradient for Na^+ is maintained by activity of Na^+-K^+-ATPase."

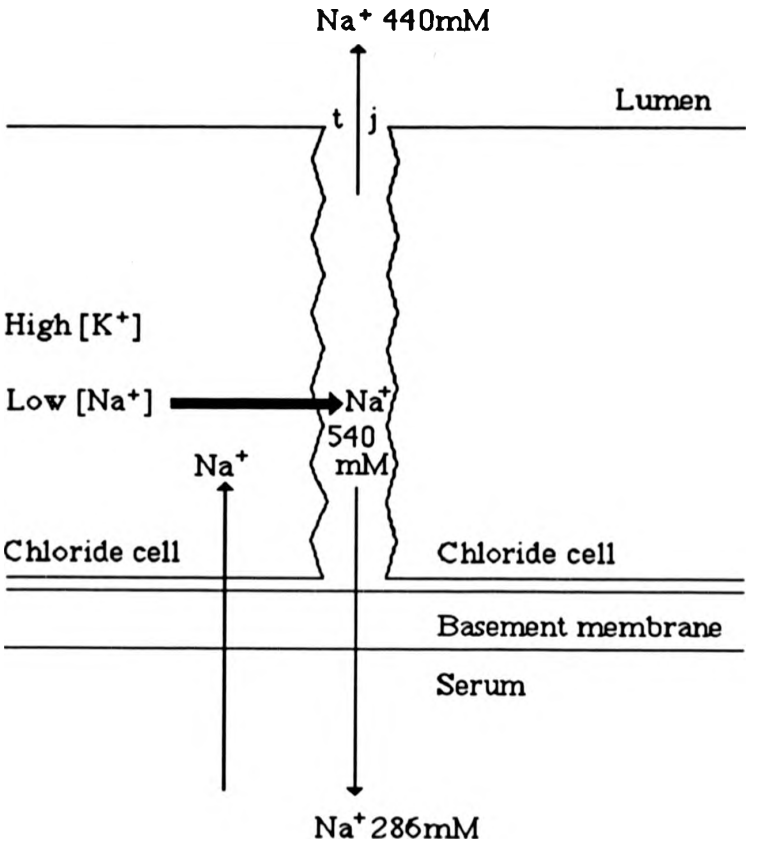


Figure 3.2. Model for the mechanism of salt pumping across the gill epithelium. The diagram represents two chloride cells connected at the luminal surface by a tight junction (tj). Sodium ions move against a concentration gradient across the baso-lateral membranes (indicated by the heavy arrow) resulting in a high intercellular sodium concentration. Diffusion of sodium then occurs down a concentration gradient (indicated by the light arrows) and results in movement of sodium out of the tight junction and some movement back across the basement membrane. The model is applicable to all salt-secreting epithelia. Diagram adapted from Kyte (1978).

	Extracellular	Intracellular	Duct Lumen
Na^+	280	47	450
K^+	5	155	10
Cl^-	270	57	460
mV	0	-83	-15

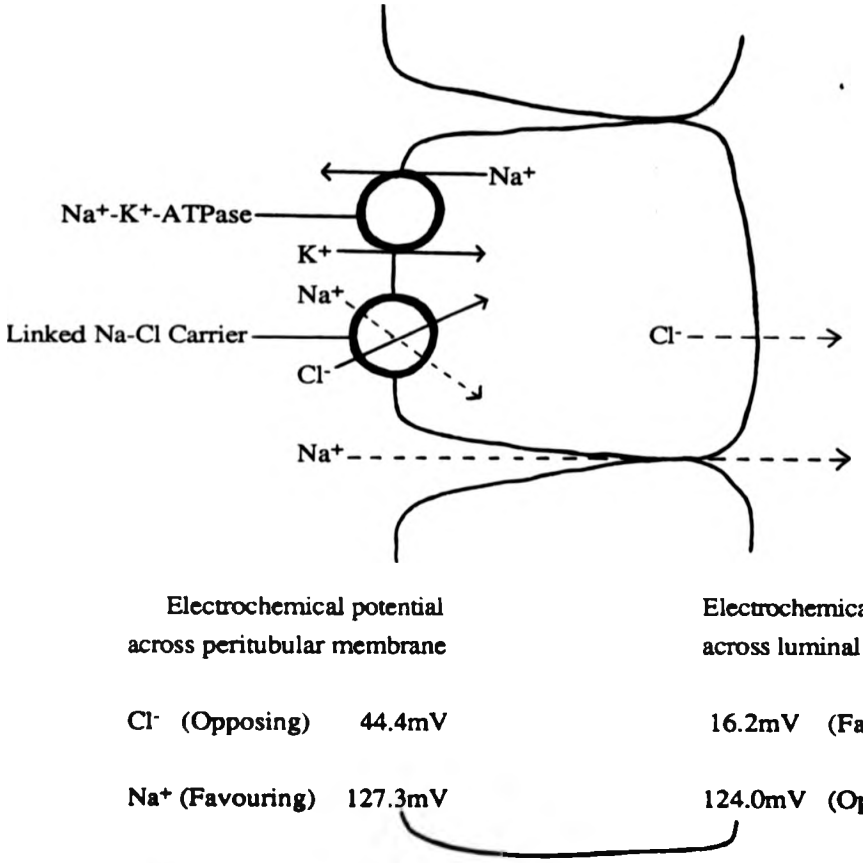


Figure 3.3. Model for the transepithelial transport of chloride. Cl^- moves into the cell against an electrochemical gradient, *via* a coupled NaCl carrier. The energy for this step is provided by the electrochemical gradient for sodium directed into the cell, which is maintained by the activity of Na^+-K^+-ATPase. Cl^- leaves the cell across the luminal border down an electrochemical gradient that favours its efflux. Na^+ moves passively into the lumen through a paracellular pathway. Diagram adapted from Epstein *et al.* 1983.

However, this model does not show Na^+-K^+-ATPase on the lateral membrane of chloride cells or show that Na^+ is pumped into the intercellular spaces. It is also unclear in this model how electrical neutrality is maintained within the chloride cell while large concentrations of sodium and chloride are moved by separate cellular routes.

In Chapter 2 a physiological approach to the study of osmoregulation was taken using a whole body perfusion technique. The isolated cell preparation provides one possible alternative for studies of ionic regulatory processes in fish gills. However, one factor which must be considered when isolating gill cells is that the epithelium is heterogenous, consisting of several cell types including respiratory cells, chloride cells and mucus cells. The advantages and disadvantages of such *in vitro* studies, discussed in a paper by Battram (1989) can be summarised. The advantages include the ease of handling of cell preparations, all cells present originate in the branchial epithelium, the cellular transport activity can be concentrated into a very small volume and it is possible to treat cells with inhibitors and other agents. In addition, there are no complicating endocrine inputs or cardiovascular effects. The one main disadvantage is that the functional juxtapositioning of the cells in the original epithelium is lost along with the ionic gradients normally present in the intercellular spaces which appear to be important in the normal function of salt excreting epithelia *in vivo* (Sargent *et al.* 1980).

As stated previously in this introduction, salt secretion has been unequivocally ascribed to the chloride cell and therefore any method which provides a pure preparation of chloride cells for investigating specific cellular functions such as ion pumping via ATPase activity is useful. Despite its drawbacks, the isolated cell preparation provides a viable alternative to other methods of investigating the

processes of ion pumping in fish gills and such studies only add to and complement the knowledge obtained from other experimental approaches.

Two main methods of preparation of isolated chloride cells were utilised in the present study. The first involved enzymatic digestion of the gill epithelium with collagenase. The second used a mechanical method of preparation. Similar methods have been described previously by several authors including Kamiya (1972), Sargent *et al.* (1975) and Hootman & Philpott (1978) but most of these separation processes produce a heterogenous collection of cells including chloride cells, respiratory cells and epithelial cells or purer preparations with low yields. The main aims of the present study were: (i) to develop these existing methods and to purify and refine the initial cell suspension in order to obtain a relatively pure preparation of chloride cells in high yield, (ii) to investigate suitable methods to check the identity, purity and viability of the cells and (iii) to use the isolated chloride cells to investigate further the putative roles of certain agonists and hormones in the process of osmoregulation in the gills of marine fish.

3.2 METHODS

3.2.1 Isolation of chloride cells from the gills of *Gadus morhua*.

(a) Collagenase digestion of gill tissue

Fish were anaesthetised in a 0.01% (w/v) SW solution of MS222. The heart was exposed and perfused with 10ml of HEPES-buffered saline (HEPES 10mM; NaCl 137mM; KCl 2.6mM; $MgCl_2.6H_2O$ 1mM; $CaCl_2.2H_2O$ 2.7mM; $NaHCO_3$ 18.5mM; Glucose 11mM; pH7.4) followed by 5ml of the same buffer containing 0.1% (w/v) collagenase to clear the gill filaments of blood, and were subsequently excised and placed into ice-cold HEPES-buffered saline. Each gill arch was blotted dry and cut at the base to produce sections of around 5-6 filaments in size and these were incubated in 50ml of buffer containing 0.1% (w/v) collagenase for 30 minutes with gentle agitation. The suspension was filtered through 190μm^2 nylon gauze and the filaments returned to a fresh 50ml of collagenase buffer for a further one hour incubation. The suspension was shaken vigorously and filtered through 190μm^2 and then 106μm^2 gauzes. Cells were pelleted by centrifugation at 700g for 10 minutes in a MSE bench centrifuge and the cell pellet weighed. Cells were resuspended to the required cell concentration and kept on ice until required.

(b) Mechanical preparation of chloride cells

Fish were anaesthetised, the heart exposed and perfused to remove blood cells as before. The gills were then excised and placed in ice-cold, HEPES-buffered saline. Individual gill arches were blotted dry and then scraped with the edge of a blunt scalpel to remove the cell epithelium from the underlying cartilage. The cells were placed in 30ml of HEPES-buffered saline in an ice bath and gently mixed using a magnetic stirrer for 15 minutes to separate the cells. The final cell suspension was

then prepared as before.

Alternatively, after the filtering stage, cells were suspended in 30ml of 21% (w/v) Ficoll-containing buffer and layered onto two step-wise Ficoll gradients in 50ml plastic centrifuge tubes with screw-on tops. The step-wise gradients consisted of 7ml each of 33%, 27% and 25% (w/v) Ficoll in cod ringer solution layered into each centrifuge tube followed by 15ml of the cell suspension in 21% (w/v) Ficoll in cod ringer with 5ml of 7% (w/v) Ficoll in cod ringer on top. The gradients were centrifuged in a swing-out rotor (MSE LR-6) at 1000g for 40-60 minutes, the bands removed and the chloride cell rich band identified using a light microscope as described in Section 3.2.2.

3.2.2 Identification of chloride cells and measurement of cell purity and viability

Mitochondrial stains

(i) Janus Green. A 10mM stock solution of the stain was prepared in HEPES-buffered saline and stored at -20°C between use. Cells were incubated in a 20-fold dilution of the stock solution and examined using a light microscope at a magnification of 40x.

(ii) [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT). A 5mg/ml stock solution was prepared and filtered through a 0.2µm Flowpore D filter (Flow Labs., Germany), and stored at -20°C. Cells were incubated in a 0.5mg/ml preparation of MTT in HEPES-buffered saline at 10°C for one hour with gentle agitation. A drop of cell suspension was then placed on a microscope slide and the

cells examined using the 40x or 100x (oil immersion) lenses of a light microscope. MTT stained viable cells only.

(iii) 2-(*p* -Dimethylaminostyryl)-1-methylpyridiniumiodide (DASPMI). Cells were incubated in a 200μM solution of DASPMI in HEPES-buffered saline at 10ºC for 30 minutes using a magnetic stirrer for gentle mixing. A drop of the cell suspension was placed on a microscope slide and the cells examined using a Zeiss Fluorescence Microscope, x40 magnification, wavelength = 450-490 units. DASPMI stained viable cells only.

Other stains

(i) Fluorescein diacetate, (FDA). A 2.5mM stock solution of FDA was prepared in acetone and was stored at -20ºC for use. Cells were suspended to a concentration of 10^6 cells/ml in HEPES-buffered saline and diluted with the FDA stock to give a final FDA concentration of 6×10^{-7}M. A drop of cell suspension was then mounted onto a microscope slide, covered with a coverslip and the edges sealed with silicone grease. Alternatively, a drop of cell suspension was mounted on a slide, covered with a coverslip and a drop of FDA solution pulled through by capillary action using a tissue. Cells were examined using a Zeiss Fluorescence Microscope, 40x magnification, wavelength 450-490 units. FDA stained only viable cells.

(ii) Trypan Blue. A stock solution of 0.5% (w/v) trypan blue in 0.85% (w/v) sodium chloride was prepared and stored at 4ºC for use. For examination using the light microsope, 1ml trypan blue stock solution was mixed with 2.5ml of cell suspension. Cells were examined within 2-3 minutes using the 40x or 100x (oil

immersion) lenses of a light microscope. Only dead cells took up the dye.

3.3 RESULTS

3.3.1 Resolution of the problems encountered in the isolation of chloride cells

Initially, the isolation of chloride cells was performed using mechanical methods similar to those described by Sargent *et al.* (1975), whereby gill filaments were scraped, cells were passed through a series of gauzes and/or syringes before being separated by high density centrifugation in Ficoll gradients. Problems encountered in the use of these preparations in the present study included the tendency for cells to aggregate together which affected their separation on the Ficoll gradients making studies of cell purity and viability difficult. Although the purity of chloride cells was enhanced to around 50% using the gradients low yields and low viability (50%) of the isolated cells meant that there was virtually no overall gain in using the Ficoll gradient separation step. Mechanical preparation without the Ficoll gradient separation step produced chloride cells that were around 35% pure and that had a viability of around 80%.

Enzymatic digestion of the gill tissue was investigated in an attempt to increase the purity and viability of cell preparations. In deciding upon the type of enzymatic digestion to employ it was important to consider the possible effects of the agent to be used upon cellular integrity and the activity of Na^+-K^+-ATPase since the enzyme is known to have a sensitive trypsin cleavage site on its extracellular surface (Bell, unpublished data). In order to minimise degradation of ATPase a digestive enzyme preparation of collagenase which was very low in trypsin activity was chosen to dissociate the gill cells. Using the method described in Section 3.2.1 it was found that higher yields of cells were obtained from collagenase digestion of gill tissues than by the mechanical methods of separation of gill cells. Both purity and viability

were increased using the collagenase separation method and cell aggregation was reduced making it easier to identify and count cells. Comparisons of the purity and viability of the different cell preparations were made and these are discussed in Section 3.3.2.

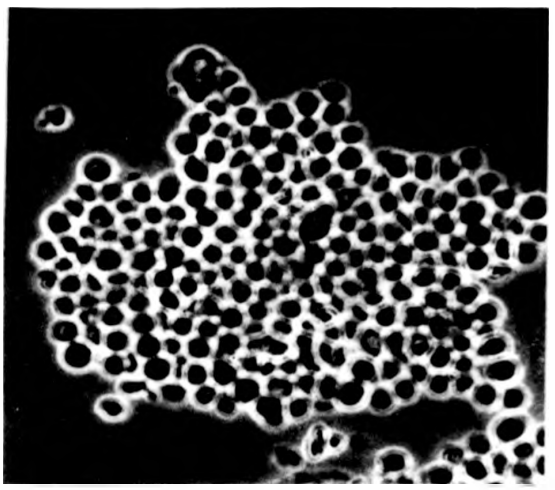
The photographs in Plate 3.1(a) & 3.1(b) show a comparison of the cell preparations obtained using collagenase digestion and mechanical separation methods. In Plate 3.1(a) the cell aggregation which routinely resulted with the mechanical dissociation of cells can be clearly seen. The large clump of cells in the centre of the picture is an aggregation of chloride cells. Much better separation of cells can be seen in Plate 3.1(b) where separation was carried out by collagenase treatment of the cells.

3.3.2 Cell purity and cell viability studies

Cell viability of the various cell preparations was examined using the trypan blue exclusion test described in Section 3.2.2. The viability of collagenase treated cells was found to be greater than 90%, much higher than that of mechanically prepared, Ficoll gradient separated chloride cells which was approximately 50%, and slightly higher than that of mechanically prepared cells without the Ficoll gradient step which was ~80% (Table 3.1).

The purity of the different types of cell preparations was examined using the various stains described in Section 3.2.2. and these results are also summarised in Table 3.1. The mechanically disrupted cell preparation contained 35.8 ± 2.8% (n=4) chloride cells and this was not significantly different from the cells obtained

(a)



(b)

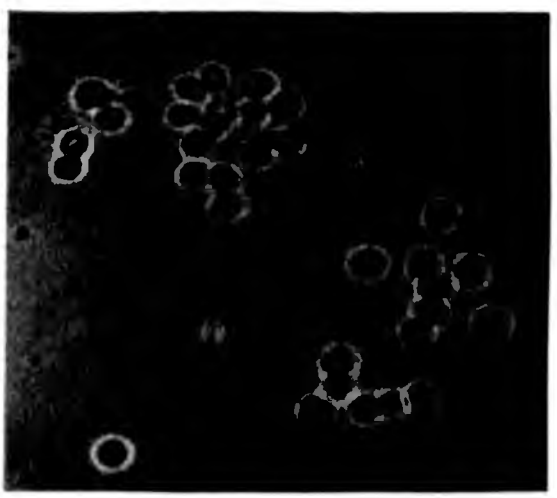


Plate 3.1. A comparison of cell suspensions from the gills of cod obtained by (a) mechanical separation and (b) collagenase digestion (refer to text for details of methods).

Table 3.1. Purity and viability of chloride cell preparations obtained by different methods.

Type of cell preparation	Purity %	Viability %	Na^+-K^+-ATPase activity (μmol Pi mg protein^{-1}hour^{-1})
Mechanical (no Ficoll)	35.8 ± 2.8 (4)	>80	44 ± 14 (4)
Mechanical (+Ficoll gradient separation)	~50	~50	n.d.
Collagenase treated (1st hour)	34.9 ± 7.3 (9)	>90	31 ± 8 (3)
Collagenase treated (2nd hour)	52.6 ± 11.7 (9)	>90	39 ± 13 (10)
Collagenase treated (2nd hour) + sedimentation of cells	74.0 ± 4.9 (5)	>90	n.d.

The various methods of cell isolation are described in Section 3.2. Cell purity was examined using MTT, Janus Green or DASPMI staining and cell viability determined using the Trypan Blue exclusion test (refer to Section 3.2). Na^+-K^+-ATPase activity was determined by the method described in Section 4.2. Values are the means and standard deviations of the number of experiments in brackets. n.d. = not determined.

from the first hour of incubation with 0.1% collagenase which contained 34.9 ± 7.3% (n=9) chloride cells. The percentage of chloride cells present after the second hour of incubation with 0.1% collagenase was greatly enhanced and the purity in this instance was 52.6 ± 11.7% (n=9). It was discovered that higher purity chloride cell preparations were obtained if the cells were allowed to settle for a few minutes in an ice bath after filtering off the filaments. The cells that had settled out of suspension were then syphoned off the bottom of the beaker. In this way, chloride cell suspensions of >70% purity were routinely obtained and subsequently it was these preparations that were used for studies on the control of osmoregulation.

As an additional check on the viability of the collagenase digestion method, the activity of Na^+-K^+-ATPase was compared in mechanically prepared and collagenase treated cells using the methods described in Section 4.2. These results are summarised in Table 3.1. The activity in mechanically prepared cells was 44 ± 14 μmol Pi mg protein^{-1} h^{-1} whereas the activity in the collagenase treated cells was 31 ± 8 and 39 ± 13 μmol Pi mg protein^{-1} h^{-1} for the first and second hours of incubation respectively. However, these differences were not significant (Student's t-test).

As part of a metabolic study it was planned to incubate chloride cells for up to five hours with radioactive isotopes. The viability of the chloride cells was crucial to the success of these experiments and this was monitored at hourly intervals for up to five hours during incubation under experimental conditions. Cell viability was checked at each time point by the trypan blue exclusion method and the results of these tests are shown in Table 3.2. There were no significant changes in chloride cell viability over the five hour incubation period.

Table 3.2. The viability of chloride cells from cod gills over a five hour incubation period.

Cell incubation time (hours)	% viability of cells
0	89.9 ± 1.0 (3)
1	88.6 ± 1.4 (3)
2	90.1 ± 0.8 (3)
3	89.7 ± 0.6 (3)
4	89.6 ± 1.8 (3)
5	87.7 ± 2.2 (3)

Cells were incubated in cod buffer under the conditions of pH and temperature employed in experiments used to radioactively label gill chloride cells with [^{32}P]-orthophosphate (refer to Section 5.2). Samples were taken at one hourly intervals. The results represent the means and standard deviations of the number of experiments in parenthesis. The Student's paired t-test indicated that there were no significant changes in cell viability throughout the experimental period.

3.4 DISCUSSION

Chloride cells from the gills have increasingly become the focus of investigation into the nature of ion exchange and regulation in teleosts in both high and low environmental salinities. The majority of these studies have aimed at elucidating the role of the chloride cells in relation to the osmoregulatory capacity of the whole fish but there is increasing awareness that the chloride cell itself presents an advantageous model system for studying key issues in cellular biology. Of particular importance are the processes of plasma membrane and mitochondrial biogenesis, the induction of the integral membrane-bound Na^+-K^+-ATPase and the cellular localisation of the enzyme. These problems have been studied by a combination of biochemical and cytochemical methods.

These studies have been more successful when applied to more homogenous tissues such as the avian salt gland and the shark rectal gland (Ernst *et al.* 1967; Karlsson *et al.* 1971; Martin & Philpott, 1974; Stewart *et al.* 1976; Simpson & Sargent, 1985) so the preparation of an enriched population of chloride cells from the heterogenous branchial epithelium is a highly desirable aim. A second reason for attempting to isolate chloride cells, as highlighted in the previous chapter, is their apparent inaccessibility *in situ* to certain types of agonists and inhibitors. Although some compounds when injected into the circulatory system of teleosts readily pass through the endothelial tissues to penetrate the basolateral membranes of the chloride cells many others can be excluded at the endothelial or basal lamina (Philpott, 1966). The development of a routine procedure for the rapid isolation of an enriched fraction of chloride cells in high yield was a necessity if this approach was to be successful. The aim of the present investigation was to find suitable methods to separate and purify viable chloride cells in high yield and to develop

methods to check the purity and viability of the cells obtained.

Isolated cells from different tissues and from different species have been utilised for ion transport studies and these have provided valuable information on the mechanism of ion transport. Some of the tissues studied include mammalian lung cells (Jones *et al.* 1982), trout liver cells (Bianchini *et al.* 1988), renal mesangial cells (Ganz *et al.* 1989) and FW-adapted trout gill epithelial cells (Battram *et al.* 1989). A number of authors have used tissue disruption and density gradient centrifugation of the resultant cell suspension as a means of partitioning heterogeneous cell populations. This approach has been particularly useful in studies on toad urinary bladder (Scott & Sapirstein, 1974; 1975), mouse gastric mucosa (Romrell *et al.* 1975; Munro *et al.* 1975), rat liver (Drochmans *et al.* 1975), mouse seminiferous epithelium (Romrell *et al.* 1976) and rabbit trachea and small intestine (Sonstegard *et al.* 1976). The teleost gill has also been investigated using similar techniques (Kamiya, 1972; Sargent *et al.* 1975) and although these studies have yielded important data on the activities of the active transport enzyme Na^+-K^+-ATPase and substantially strengthened the view that the chloride cell is the main osmoregulatory cell in the branchial epithelium, low yields of cells were obtained, sometimes subject to substantial damage according to Hootman & Philpott (1978), which would limit their use in biochemical studies of transmembrane signal transduction mechanisms. Hootman and Philpott (1978) reported a modification of the technique of Sargent *et al.* (1975) which permitted the rapid isolation of a cell fraction from the pinfish *Lagodon rhomboides* which was enriched in chloride cells (50-70%) possessing good ultrastructural integrity.

Initial work in this study used methods of tissue disruption and gravity gradient centrifugation in an attempt to isolate chloride cells from the gills of *Gadus morhua*.

However, mechanical disruption by passing the cell suspension through syringe needles tended to damage the cell membranes and separation by density gradients was often unsuccessful due to the tendency of cells to aggregate even after syringing. Alternative methods of cell preparation were therefore investigated and two different approaches were utilized. The first involved the mechanical disruption of the epithelium without subsequent density gradient centrifugation. Mechanical disruption has been used by other authors when the investigation has not required purification of the heterogeneous cell population and especially in studies of freshwater species where the number of chloride cells is relatively low (e.g. Battram *et al.* 1989). The second, and most successful, method in the present study involved disruption of the gill epithelium by enzyme digestion. Chloride cells were isolated by digestion of gill tissue with 0.1% (w/v) collagenase for two hours, followed by filtering of the cells through gauzes and finally by allowing the sedimentation of cells at 1g prior to separation from the preparation medium. This technique, as described fully in Section 3.2.1, had the additional advantage that it was relatively quick and easy to perform and thus suitable for the routine preparation of chloride cells which were to be used in further biochemical studies. Chloride cell preparations that were >70% pure and with a viability of around 90% were routinely obtained by this method which was a substantial improvement upon the purity and viability of the cells obtained using the mechanical methods of cell isolation (Table 3.1). These results compared favourably with those of Hootman and Philpott (1978) who obtained a chloride cell rich band by density gradient centrifugation composed of around 50-70% chloride cells of which ~70% were viable. The chloride cell fraction from the Hootman & Philpott study also showed an enhancement of Na^+-K^+-ATPase activity 3-4 fold over that of the heterogeneous cell population. Although Na^+-K^+-ATPase activity in collagenase treated cells in the present study appeared to be slightly lower than that in mechanically prepared

cells these differences were not significant. The higher activity in the second hour of collagenase incubation compared with the first hour of collagenase digestion was as expected since that preparation contained a greater number of chloride cells and therefore more Na^+-K^+-ATPase.

A number of dyes were investigated in an attempt to find a routine method for the identification of chloride cells from the heterogenous cell population and to determine the viability of these cells. Efforts were concentrated upon mitochondrial stains since chloride cells have abundant mitochondria and ought to stain up more readily than other cells in a heterogenous preparation of gill cells. The use of Janus green as a mitochondrial stain was reported as early as 1900 in a study on mitochondria and submitochondrial particles (Michaelis, 1900). This dye proved useful for identification of chloride cells when employed in conjunction with the light microscope though cells other than chloride cells tended to be stained to a lesser degree. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) proved to be a better mitochondrial stain, the optimum incubation period being around one hour for chloride cell identification using the light microscope. Longer periods of incubation led to staining of the other cell types present in the suspension and thus made identification more difficult. MTT was also useful in that it stained only viable cells and could therefore be used in viability studies as well as for identification of chloride cells; the tetrazolium ring is reduced in active mitochondria and the colourless substrate is modified to produce a dark coloured product by any living cell.

Fluorescent methods have the advantage of being more sensitive and specific. In a study by Bereiter-Hahn (1976) the dye 2-(*p*- dimethylaminostyryl)-1-methyl-pyridiniumiodide (DASPMI) proved to be a sufficiently specific vital stain for

mitochondria in intact cells. This dye was particularily useful as it has a low toxicity and in the present study was used in chloride cell identification, though most cells tended to fluoresce after a relatively short period. Another fluorescent dye fluorescein diacetate (FDA) is taken up by actively metabolising cells and in the process is hydrolysed to produce fluorescein which can be detected with a fluorescence microscope. FDA was useful in studies of cell viability though it did not improve cell identification since, like DASPMI, it brightly stained all actively metabolising cells in the cell preparations. Although some of these stains were useful in aiding cell identification, MTT being the most useful, it became easier to identify cells with or without their help as more experience was gained. For cell viability tests, MTT proved to be a most useful dye but more routinely the trypan blue exclusion test was used for this purpose since it was a very quick and effective test with only dead cells taking up the dye.

Tests carried out to determine the viability of chloride cell preparations over a five hour incubation period showed no significant changes over this period when checked by the paired Student's t-test ($P>0.05$) (Table 3.2) indicating the suitability of this cell preparation for future studies requiring lengthy incubations. This result is consistent with that of Battram *et al.* (1989) who found that isolated heterogeneous cell preparations from the freshwater gills of the rainbow trout had a viability of 95% or greater assessed by light microscopy and the exclusion of Trypan Blue persisted for eight hours after preparation.

In conclusion, the evidence presented in the results (Section 3.3), shows that a successful method for the preparation of relatively pure chloride cells was achieved. It was decided that the method was suitable for the routine preparation of isolated chloride cells which would be used in future biochemical studies of the

osmoregulatory processes involved in the gills of *Gadus morhua*. These studies are dealt with in Chapters 4 and 5.

CHAPTER 4

Na^+-K^+-ATPase ACTIVITY IN CHLORIDE CELLS FROM THE GILLS OF COD

4.1 INTRODUCTION

4.2 METHODS

4.2.1 Measurement of Na^+-K^+-ATPase activity

4.2.2 Protein determination

4.3 RESULTS

4.3.1 Na^+-K^+-ATPase activity in chloride cells from the gills of cod

4.3.2 The effects of various putative agonists and hormones on *in vitro* Na^+-K^+-ATPase activity

4.4 DISCUSSION

4.1 INTRODUCTION

Sodium - plus - potassium - dependent adenosine triphosphatase (Na^+-K^+-ATPase, E.C.3.6.1.3), first discovered by Skou (1957) is a membrane bound enzyme present in all animal cells. It translocates cations across the cell membrane, actively pumping Na^+ ions out of the cell and K^+ ions into the cell across the plasma membrane. It is involved in maintaining ion gradients at the cellular level which allow cells to perform their special and general functions such as the generation of action potentials in nerve and muscle, the transport of nutrients such as glucose, amino acids and phosphate and homeostatic mechanisms such as pH regulation and osmoregulation (Stein, 1967).

The enzyme is particularily abundant in the plasma membranes of cells from salt transporting epithelia and has most frequently been purified from such sources including canine kidney (Kyte, 1971; Lane *et al.* 1973), the nasal salt gland of the duck (Hopkins *et al.* 1976), the rectal gland of *Squalus acanthias* (Hokin *et al.* 1973) and the gills of seawater-adapted eels (Sargent & Thomson, 1974; Bell & Sargent, 1979). The Na^+-K^+-ATPase enzyme in all of these tissues is believed to have a common structure and mechanism of action. Various aspects of the structure and function of Na^+-K^+-ATPase have recently been reviewed by several authors (Rossier *et al.* 1987; Famborough, 1988; Jørgensen & Anderson, 1988; Kawamura & Noguchi, 1988; Sweadner, 1989; Geering, 1990; McDonough *et al.* 1990).

The enzyme belongs to the E_1E_2 type of transport ATPases which form phospho-derivatives of aspartic acid as an intermediate during catalysis but contrary to the other transport ATPases of the E_1E_2 type, the sodium pump consists of two

different subunits, alpha and beta. The minimal enzymically functional unit appears to be alpha-beta (Karlish & Kempner, 1984) though other authors favour $(alpha\text{-}beta)_2$ or $(alpha\text{-}beta)_4$ arrangements (Hah *et al.* 1985).

Until recently, lack of information on the primary structure of the subunits and their arrangement in the membrane resulted in a gap between structural and functional studies. However, with the use of molecular cloning and genetic engineering techniques, it has been possible to establish the amino acid sequence of the alpha subunit (Shull *et al.* 1985, 1986; Kawakami *et al.* 1985; Noguchi *et al.* 1986) and of both the alpha and beta subunits (Broude *et al.* 1989) from various tissue sources. These studies have also uncovered a multiplicity of isoforms of the enzyme and with the identification of various ligand binding sites, the use of antibodies to different parts of the enzyme and the use of hydrophobic probes, the position of the protein chain within and through the membrane has been elucidated. A diagrammatic representation of the orientation of the enzyme within the membrane is shown in Figure 4.1.

The catalytic alpha subunit, a protein of 112KDa, has 1016 amino acids and contains all the functional sites of the enzyme. The ATP and sodium ion binding sites and the phosphorylation site all reside on the cytoplasmic side of the alpha subunit whereas the potassium ion and ouabain binding sites are located in the regions exposed on the extracellular side of the membrane (Figure 4.1.a). The ouabain binding site is located at the extracellular junction of two transmembrane domains (Figure 4.1.b) and is linked to the phosphorylation site by a 60 residue conserved sequence that may form a major channel for energy transduction. The hydrophobic N-terminal domain may function as an ion selective barrier on the cytoplasmic side of the membrane, controlling the cation binding sites (Figure

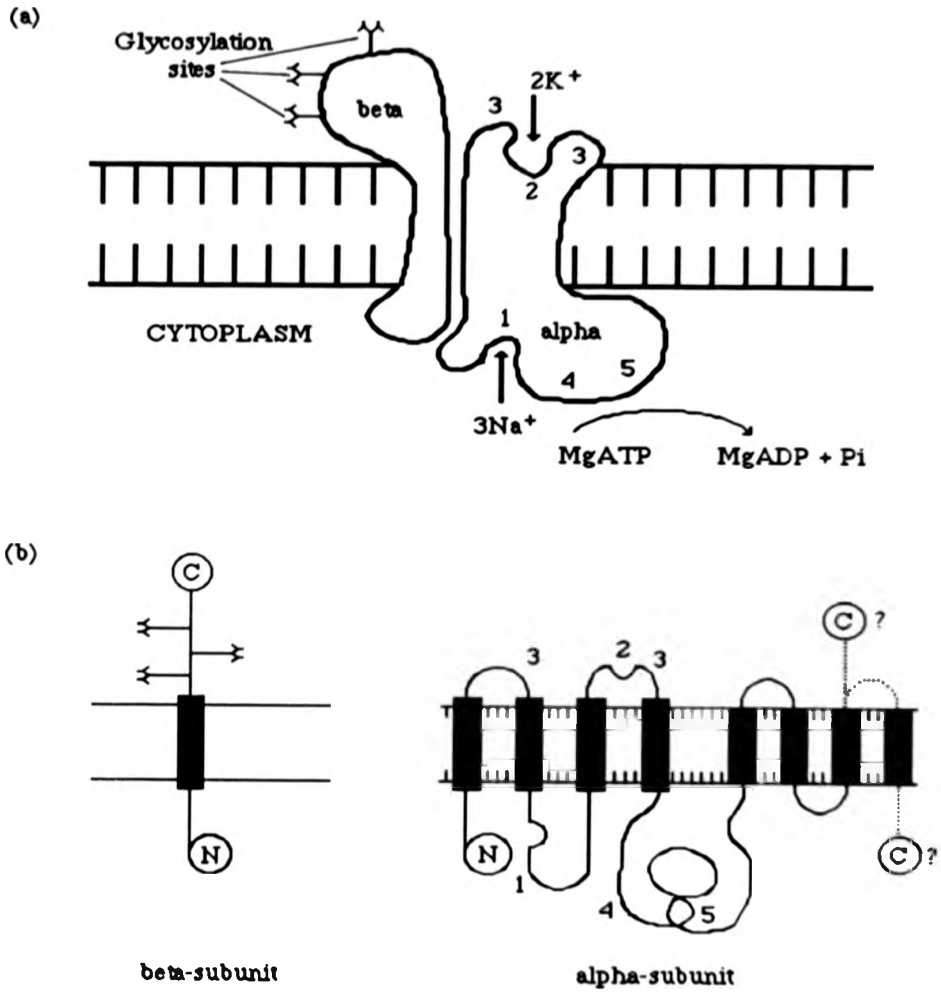


Figure 4.1. Diagrammatic representation of the arrangement of the subunits of Na^+-K^+-ATPase within the cell membrane. (a) The alpha-subunit is largely intracellular and has binding sites for sodium (1), potassium (2), cardiac glycosides (3), the phosphorylation site (4) and the ATP binding site (5). The beta-subunit is a glycoprotein which is largely extracellular. (b) The beta-subunit has only one membrane domain and the alpha-subunit either 7 or 8. Diagram adapted from Rossier *et al.* (1987).

4.1.b). Alpha-subunit structure has been reviewed by Anner (1985), Jørgensen & Andersen (1988) and Geering (1990).

The beta subunit is a glycosylated protein of around 35KDa and has only one transmembrane domain with a small portion containing the amino terminus on the cytoplasmic side (Figure 4.1.b). A large portion of the beta subunit is extracellular and contains the glycosylation sites. Beta-subunit structure has been reviewed by Kawamura & Noguchi (1988), McDonough *et al.* (1990) and Geering (1990). The exact function of the beta-subunit is not known but it is essential for pump action and appears to be involved with the proper insertion and orientation of the enzyme in the membrane (Jørgensen *et al.* 1982; Skou *et al.* 1988). Recent work by McDonough *et al.* (1990) is consistent with the hypothesis that the beta subunit regulates the number of sodium pumps transported to the plasma membrane through the assembly of alpha-beta dimers.

It appears that all ion transporting ATPases have evolved from a single ancestral protein since there is much structural homology between them all (reviewed by Geering, 1990). The alpha subunit of Na^+-K^+-ATPase shows extensive homology with the Ca^+-ATPases and the yeast plasma membrane ATPase whereas the beta-subunit is homologous with the KdpC protein, a 190 amino acid subunit of *E. coli* K^+-ATPase (Shull *et al.* 1986). A recent paper reported chimeric ion pumps made by splicing the N-terminal $^2/_3$ of the alpha-subunit of the ouabain sensitive chicken Na^+-K^+-ATPase with the C-terminal $^1/_3$ of the sarcoplasmic reticulum Ca^+-ATPase. These molecules were expressed in ouabain- insensitive mouse L cells and exhibited ouabain-sensitive ATPase activity similar to that of wild type chicken enzyme (Luckie *et al.* 1991). Since Ca^+-ATPase does not contain a beta

subunit, its ion channel must reside in the large subunit and this is also probably the case for Na^+-K^+-ATPase.

For many years there were suspicions that some types of cells had a mixture of sodium pumps with differing characteristics, especially in their responses to the inhibitory cardiac glycosides (De Pover & Godfriend, 1979). These suspicions have been confirmed using molecular biological techniques during the past ten years or so. There may be as many as five sections of DNA coding for the alpha subunit and three isoforms of this subunit have already been identified (Sweadner, 1989) while a second putative isoform of the beta subunit has also been identified (Martin-Vasallo *et al.* 1989). The physiological or regulatory relevance of the existence of different isoforms of Na^+-K^+-ATPase is as yet unknown. Do different isoforms have different functions, are they activated to the same extent by Na^+ and K^+, are they all regulated by internal Na^+ concentration and are they all equally sensitive to certain hormones? So far very little data is available to answer these questions. However, in 1985 Lytton demonstrated the presence of two different isoforms of Na^+-K^+- ATPase in rat adipocyte cells which had different Km values for $[Na]_i$ (Lytton, 1985). One isoform which had a $[Na]_i$ value of 15mM, was particularily insensitive to ouabain and was unaffected by insulin. The second isoform had a Km value for $[Na]_i$ of 50mM, was sensitive to ouabain and sensitive to insulin, the Km value being reduced upon insulin administration. Other work by Haber and Loeb (1988), demonstrated the induction of a ouabain high affinity pump isoform in the rat diaphragm upon administration of thyroid hormone. The implications of the existence of multiple isoforms of the sodium pump are far reaching and the putative role of these isoforms in the control mechanisms involved in osmoregulation remains to be determined.

The stoichiometry of Na^+-K^+-ATPase has been established by studies on reconstituted vesicles using the purified enzyme. It is now widely accepted that three Na^+ ions are exchanged for two K^+ ions per molecule of ATP hydrolysed (Hilden & Hokin, 1975; Goldin, 1977). Thus, the enzyme is intrinsically electrogenic with electrical neutrality being maintained by the outwards movement of a Cl^- ion for the movement of every three Na^+ ions out and two K^+ ions in. When Cl^- is replaced by the impermeant ion SO_4^{2-}, the stoichiometry becomes 1 Na^+ : 1 K^+ indicating that no endogenous ion is able to replace Cl^- (Dixon & Hokin, 1980). Despite numerous electrophysiological studies of salt secretory epithelia which point to these systems operating as electrogenic Cl^- pumps, there is no compelling evidence for a biochemical counterpart, i.e. a Cl^--dependent ATPase, to the physiological Cl^- pump. This holds for salt transporting epithelia and plasma membranes in general. In contrast to this, biochemical studies of salt transporting epithelia (including salt secreting epithelia), have consistently emphasised the fundamental role of Na^+-K^+-ATPase in salt movement.

Although the exact biochemical mechanism by which Na^+-K^+-ATPase translocates ions across membranes remains unknown, models similar to the one proposed by Robinson & Flashner (1979) are widely accepted (Figure 4.2). Briefly, Na^+ binds to the catalytic alpha subunit which hydrolyses ATP to become autophosphorylated on an aspartyl residue in the active site (Bastide *et al.* 1973). The phosphorylated enzyme undergoes a conformational change between the E_1 and E_2 forms of the enzyme (Post & Kume, 1973), and this promotes the translocation of Na^+ to the extracellular side of the membrane where the ion is released. A potassium ion then binds to the alpha subunit which becomes dephosphorylated and the K^+ ion is

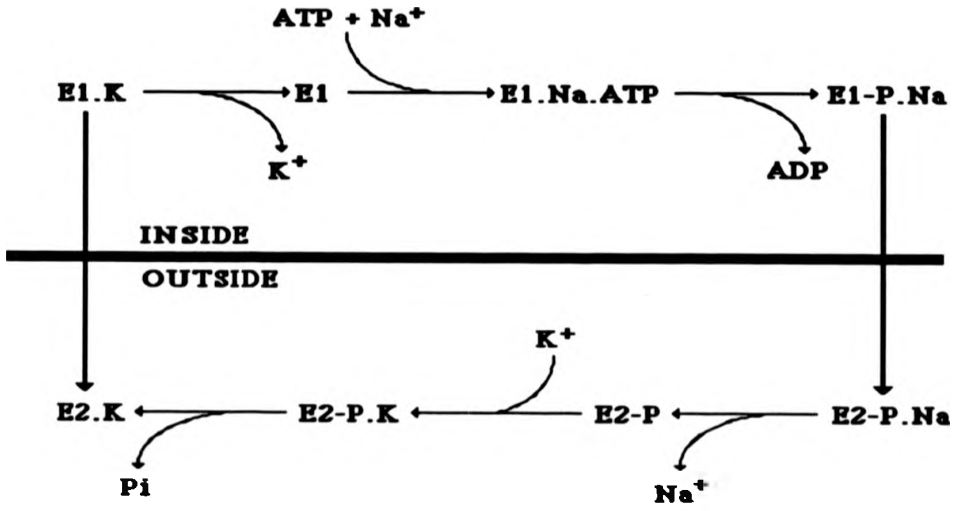


Figure 4.2. A diagrammatic representation of the model of Robinson and Flashner for the mechanism of translocation of ions across the membrane by Na^+-K^+-ATPase. E1/E2 represents a conformational change in the enzyme. The reaction sequence is explained in the text. Diagram adapted from Robinson & Flashner (1979).

translocated to the intracellular side of the membrane where it is released.

The details of the regulatory mechanisms which control Na^+-K^+-ATPase activity have also yet to be elucidated in any cell or tissue. Ultimately the regulation of any cellular process depends upon the control of enzyme activity. There are four general mechanisms by which the activity of enzymes can be regulated: (i) control by reversible binding of effectors, (ii) by covalent modification, (iii) by alteration of enzyme concentration, (iv) by alteration of substrate concentration. In the first case the enzyme is activated/inactivated as a consequence of the binding of a signal molecule which is usually a metabolite and which may, or may not, be a substrate or product of the enzyme reaction. This mechanism of control is influenced by the amount of available substrate and may provide a very rapid, instantaneous response but the extent to which activity can be changed is limited. Covalent modification of an enzyme requires a second enzyme to catalyse the covalent change and is itself subject to control. The modifier enzyme may be regulated by either intrinsic or extrinsic signals. This type of control is still comparatively fast and is normally complete within minutes. The most common example is control by kinases/phosphatases. Alteration of the concentration of enzymes in the cell result from changes in synthesis and degradation of the enzyme, by translocation of existing enzyme from one cell site to another or by changing the proportion of membrane-bound to cytosolic forms of the enzyme. Control by *de novo* synthesis of enzyme is longer term usually taking hours or days rather than minutes to complete. The intervention of extrinsic regulators is usually required.

A large body of experimental and clinical evidence (reviewed in Haber & Haupert, 1987) has led to the proposal of the existence of endogenous Na^+-K^+-ATPase inhibitor(s) which have been implicated in the control of cation pumping and the

regulation of extracellular fluid volume. Indeed for many years it has been a mystery as to why digitalis glycosides bind specifically with very high affinity to the sodium pump. It has been hypothesised that a natural agonist, the putative ouabain-like factor, must exist and attempts have been made to isolate such a factor from a range of tissues and species. A great number of factors have been nominated as having an ouabain-like action due to their ability to inhibit the sodium pump or to displace ouabain from the enzyme. Due to the complex nature of the membrane-embedded Na^+-K^+-ATPase and the number of ligands or substrates that are required for the functioning of the system the interference of such substances with the sodium pump could well occur at different sites or steps during enzyme turnover (Hansen, 1984). Several authors have concluded that the endogenous factor(s) are unesterified free fatty acids (Bidard *et al.* 1984; Tamura *et al.* 1985; Tal *et al.* 1989) and indeed, the inhibitory effect of oleic acid upon Na^+-K^+-ATPase activity has been known for many years (Skou 1964; 1965). Tamura *et al.* (1985) also observed that fatty acids interfere with anti-digoxin antibody. Kelly *et al.* (1986) on the other hand suggested that the digoxin-like immunoreactivity of plasma extracts may be due to contaminating steroids and not the fatty acids themselves. Despite the fact that both are hydrophobic, the structure of free fatty acids is very different from that of the cardiac glycosides and it is difficult to see how they could bind to the same site. It has been suggested that the double bond might be the essential common link between the two groups of substances (Schoner *et al.* 1986). The putative role of lipids and fatty acids in the control of the sodium pump and osmoregulation in general is dealt with in more detail in Chapter 5.

More recent work by Das *et al.* (1989) has led to the isolation of a Na^+-K^+-ATPase activator from kidney and brain tissue of both pig and rabbit which has a protein-like nature. As with all the other proposed endogenous substances the

mechanisms by which this protein could exert its effect upon Na^+-K^+-ATPase activity remains unknown. It has been suggested this could be by direct influence on Na^+-K^+-ATPase or by interaction with an inhibitory factor so as to remove it.

The activity of the sodium pump can be directly modulated by ligands, including monovalent cations, ATP and its analogues or cardiac glycosides and their putative physiological counterparts and also indirectly by the action of hormones and growth factors. Hormones and growth factors could regulate the activity of the sodium pump by two distinct mechanisms. Firstly, by the induction of Na^+-K^+-ATPase gene expression which represents long term adaptation to environmental changes, and secondly by post-translational changes to the pre-existing enzyme pool. This second mechanism includes the regulation of the number of pumps expressed at the cell surface by exocytosis from and endocytocis into the membrane and also the covalent modification of enzyme, for example, by phosphorylation by protein kinase C. Most of these post-translational modifications represent fast adaptation to environmental stimuli. The precise molecular mechanisms whereby hormones and factors act on the sodium pump to elicit a physiological response are still poorly understood.

The aims of the present study were to investigate the short-term control of Na^+-K^+-ATPase in chloride cells and elucidate the chain of molecular events from stimulation with a primary hormone to the change in enzyme activity, particularily with respect to diacylglycerol and protein kinase C.

4.2 METHODS

4.2.1 Measurement of Na^+-K^+-ATPase activity in chloride cells

Suspensions of chloride cells were prepared as described in Section 3.2. The cells were pelleted in a bench centrifuge at 2500rpm for 10 minutes and weighed. Ten volumes of homogenisation buffer (sucrose 0.25M; EDTA 6mM; HEPES 20mM; sodium deoxycholate 0.1%; pH6.8) were added and the cells homogenised in a teflon-in-glass homogeniser (Tri-R-Stir-R, model K43 homogeniser, 12 passes at 5000-6000rpm). Homogenates were either used immediately or stored frozen at -20°C for future use.

Each homogenate was assayed in triplicate in a control buffer lacking K^+ (NaCl 130mM; $MgCl_2$ 5mM; HEPES 20mM; ouabain 1mM; pH7.6) to measure non-Na^+-K^+-ATPase activity and in a experimental buffer (NaCl 100mM; KCl 30mM; $MgCl_2$ 5mM; HEPES 20mM; pH7.6) to measure total ATPase activity, the difference between the two giving the activity due to Na^+-K^+-dependent ATPase. 50µl of homogenate was placed in a test tube with 1ml of buffer. If necessary, agonist or hormone was added at this stage in a small volume to give the required final concentration and the volume of buffer was adjusted accordingly. The assay was started by the addition of 0.1ml of 50mM adenosine 5'-triphosphate (ATP) sodium salt, to give a final concentration of 6mM, and the reaction mixture was incubated in a shaking water-bath at 25°C for 1 hour. The method to this point was based on that described by Wheatley and Henry (1987). Reaction was stopped by addition of 1ml of ice-cold 20% trichloroacetic acid (TCA) with rapid mixing and by transferring the tubes to an ice-bath. Tubes were centrifuged at 2500rpm for 10

minutes to precipitate protein and aliquots of supernatant were taken for inorganic phosphate analysis.

Inorganic phosphate was measured using a diagnostic kit (Sigma Chemical Co.). By using reduced volumes it was possible to carry out the reaction in a 1ml cuvette. To summarise, 400µl of supernatant was added to 600µl distilled water and 200µl of acid molybdate solution (a mixture of 1-amino-2-napthol-4-sulphonic acid, sodium sulphite and sodium bisulphite, the exact composition of which was unspecified) and the contents mixed by shaking gently. 50µl of Fiske & Subbarow reducing solution (Fiske & Subbarow, 1925) was added, mixed by inversion and the cuvette allowed to stand for 10 minutes for colour development. The absorbance at 660nm was read using a Pye Unicam 1700 spectrophotometer and compared with the absorbances of standard phosphate solutions. Activities were expressed as µmol of inorganic phosphate produced per mg protein per hour (µmol mg^{-1} protein h^{-1}).

4.2.2 Protein determination

Protein was measured by the method of Lowry *et al.* (1951). A 10% (w/v) solution of sodium carbonate in 0.5M sodium hydroxide solution and a 1% (w/v) solution of sodium potassium tartrate were prepared immediately before use and kept on ice. 0.5g of copper sulphate was added to 100ml of the sodium potassium tartrate solution just before use and 10ml of this solution was then added to 100ml of the sodium carbonate in sodium hydroxide solution. A one in eleven dilution of the commercial Folin Ciocalteu reagent was prepared and kept on ice. 200µl of chloride cell homogenate was placed in a 1ml cuvette and to this 200µl of the

copper tartrate reagent was added, the solution mixed by inversion and allowed to stand for 10 minutes. 600µl of the diluted Folin Ciocalteu reagent was then added, the solution mixed again and then left to stand for 30 minutes to allow colour development. The absorbance at 650nm was measured using a spectrophotometer (Pye Unicam 1700) and the amount of protein present determined by comparison with a standard curve prepared using bovine serum albumin (BSA).

4.3 RESULTS

4.3.1 Na^+-K^+-ATPase activity in chloride cells from gills of cod

Initially Na^+-K^+-ATPase activity was measured in the relatively impure, mechanically prepared cells from gills (see Section 3.2). As different cell preparation techniques were examined the activity in these cells was also monitored and used as a check on cell viability (see Section 3.3). In collagenase prepared chloride cells which were utilised in all further enzyme studies, the Na^+-K^+-ATPase activity under normal (control) experimental conditions was around 30 μmol Pi mg protein^{-1} h^{-1}.

4.3.2 The effects of various putative agonists and hormones on *in vitro* Na^+-K^+-ATPase activity

The activity of Na^+-K^+-ATPase under control experimental conditions was found to be 30.9 ± 1.9 μmol Pi mg protein^{-1} hour^{-1} for five separate preparations. Na^+-K^+-ATPase activity was then measured in the presence of various putative agonists and hormones to examine any direct regulatory effect on the enzyme.

Phorbol 12, 13-dibutyrate showed a concentration dependent inhibition of Na^+-K^+-ATPase activity (Figure 4.3) with a concentration of 10^{-5}M giving 44% inhibition. There were no significant effects on enzyme activity upon administration of this DAG mimetic at the lower concentrations of 10^{-8}M and 10^{-7}M where enzyme activities were 34.4 ± 6.4 and 32.1 ± 7.6 μmol Pi mg protein^{-1} h^{-1},

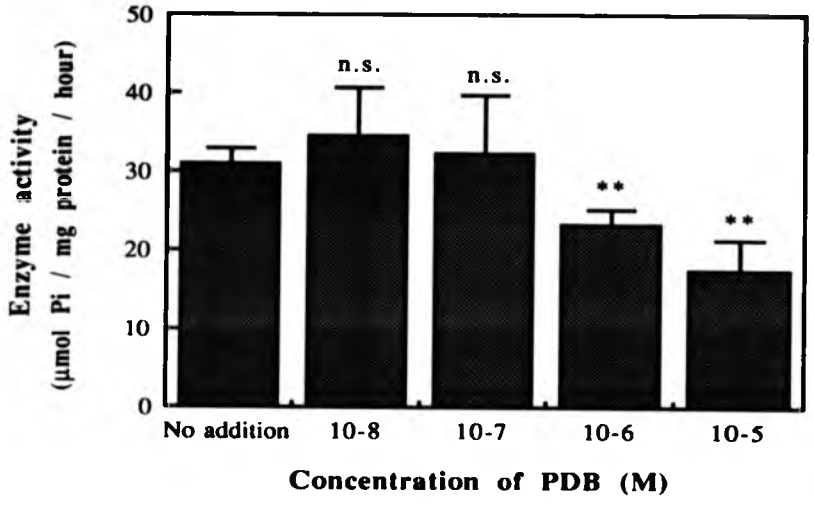


Figure 4.3. The effect of the phorbol ester phorbol 12, 13-dibutyrate (PDB) on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. ** = $p<0.001$ and n.s. = no significant difference in enzyme activity as determined by Student's t-test.

respectively. However, significant changes were observed at concentrations of 10^{-6}M and 10^{-5}M. At a concentration of 10^{-6}M the activity was reduced to 23.2 ± 2.0 μmol Pi mg protein^{-1} h^{-1} ($p<0.001$) and the activity at a concentration of 10^{-5}M was further reduced to 17.3 ± 3.9 μmol Pi mg protein^{-1} h^{-1} ($p<0.001$). The overall trend was a decrease in Na^+-K^+-ATPase activity with increasing amounts of PDB.

The DAG analogue 1-oleoyl-2-acetyl glycerol (OAG) showed a similar trend in which the activity of Na^+-K^+-ATPase decreased with increasing concentration of agonist (Figure 4.4). Significant changes in enzyme activity were measured upon administration of OAG at concentrations of 10^{-6}M ($p<0.01$) and 10^{-5}M ($p<0.001$) though these changes were less pronounced than those measured in the presence of PDB.

The effects of the protein kinase inhibitors staurosporine and H-7 were investigated but no significant effects upon Na^+-K^+-ATPase activity were recorded ($p>0.05$). The enzyme activity at three different concentrations of staurosporine ($3x10^{-9}$M, $3x10^{-8}$M and $3x10^{-7}$M) and at three different concentrations of H-7 (10^{-7}M, 10^{-6}M and 10^{-5}M) are shown in Figures 4.5 and 4.6 respectively.

Administration of the hormone atrial natriuretic factor (ANF) produced the effects upon Na^+-K^+-ATPase activity seen in Figure 4.7. Enzyme activity was significantly reduced upon administration of ANF at concentrations of 10^{-7}M and 10^{-6}M where enzyme activity was 23.7 ± 2.8 and 20.5 ± 2.0 μmol Pi mg protein^{-1} h^{-1} ($p<0.01$ and $p<0.001$ respectively). Activity was unchanged at a concentration of 10^{-8}M.

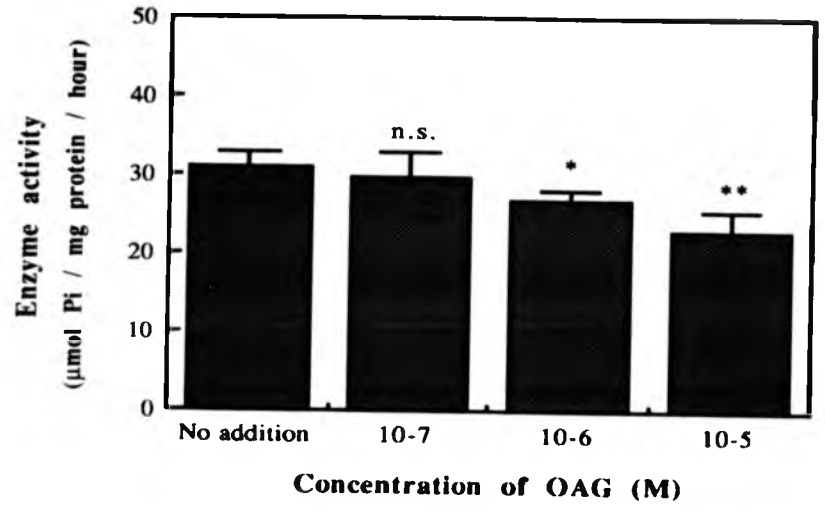


Figure 4.4. The effect of the diacylglycerol analogue 1-oleoyl-2-acetyl glycerol (OAG) on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. * = $p<0.01$, ** = $p<0.001$ and n.s. = no significant difference in enzyme activity as determined by Student's t-test.

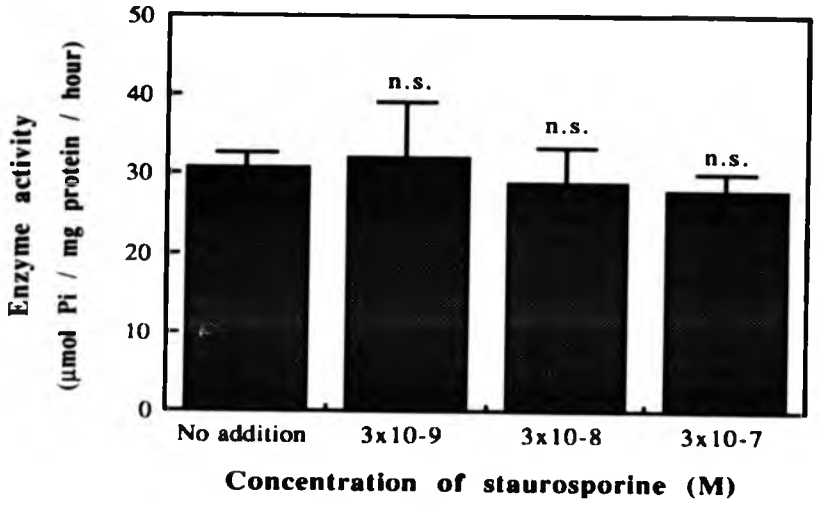


Figure 4.5. The effect of the protein kinase C inhibitor staurosporine on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. n.s. = no significant difference in enzyme activity as determined by Student's t-test.

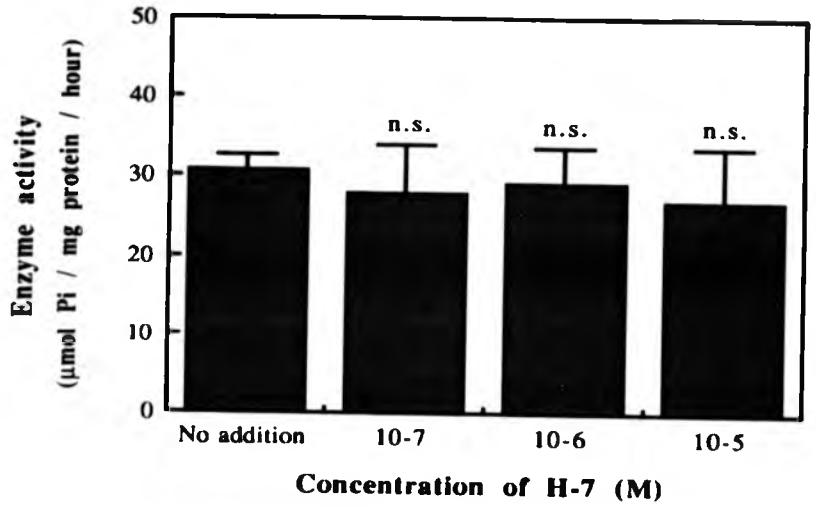


Figure 4.6. The effect of the protein kinase C inhibitor H-7 on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. n.s. = no significant difference in enzyme activity as determined by Student's t-test.

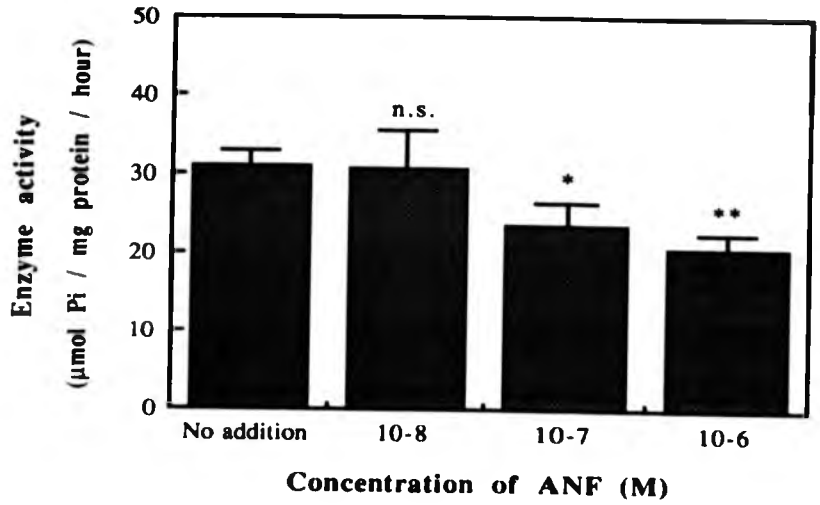


Figure 4.7. The effect of the hormone atrial natriuretic factor (ANF) on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. * = $p<0.01$, ** = $p<0.001$ and n.s. = no significant difference in enzyme activity as determined by Student's t-test.

The phorbol ester and the 1-oleoyl-2-acetyl glycerol are insoluble in water and were dissolved in the water miscible solvent dimethyl sulphoxide (DMSO). The effects of DMSO at the concentration used in the assays was therefore examined. DMSO at concentrations of $5x10^{-5}$M and 10^{-4}M produced very slight reduction in mean activity whereas higher concentrations gave mean values slightly above those of the control (Figure 4.8). At concentrations of $5x10^{-5}$M and 10^{-4}M the enzyme activity was 26.6 ± 3.8 and 28.8 ± 3.3 μmol Pi mg protein^{-1} h^{-1} respectively and at the higher concentrations of $5x10^{-4}$M and 10^{-3}M Na^+-K^+-ATPase activities of 31.4 ± 3.1 and 33.0 ± 4.5 μmol Pi mg protein^{-1} h^{-1} respectively were recorded. Generally, as the concentration of DMSO increased so too did the activity of Na^+-K^+-ATPase though none of the changes varied significantly from the control experiments ($p>0.05$).

The results of all the experiments described in this section are summarised in Table 4.1.

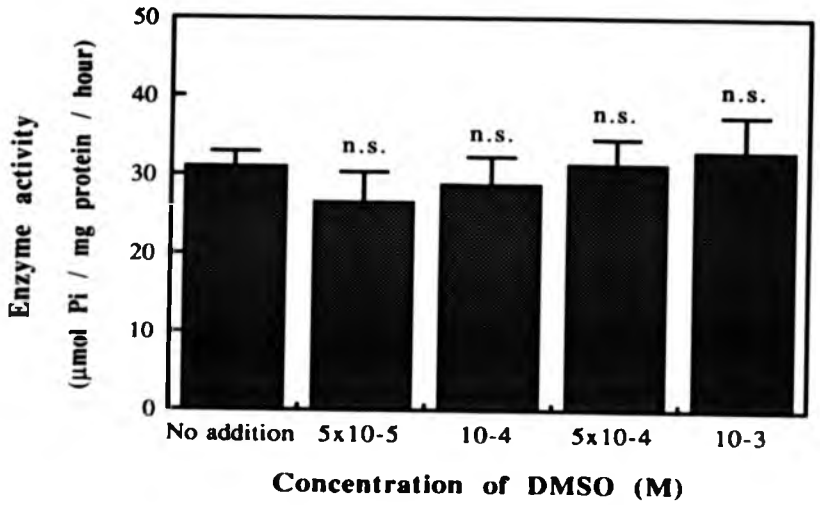


Figure 4.8. The effect of the solvent dimethylsulphoxide (DMSO) on Na^+-K^+-ATPase activity in the chloride cells from the gills of *Gadus morhua*. Enzyme activities are expressed as the means and standard deviations of five individual experiments. n.s. = no significant difference in enzyme activity as determined by Student's t-test.

Table 4.1. A comparison of the activities of Na^+-K^+-ATPase in chloride cells from the gills of *Gadus morhua* in the presence of different agents.

Substance	Concentration	Na^+-K^+-ATPase activity (μmol Pi mg protein^{-1}hour^{-1})
No additions		30.9 ± 1.9 (5)
Atrial natriuretic factor	10^{-8}M	30.6 ± 4.8 (5) n.s.
	10^{-7}M	23.7 ± 2.8 (5) $p<0.01$
	10^{-6}M	20.5 ± 2.0 (5) $p<0.001$
Phorbol 12, 13 - dibutyrate	10^{-8}M	34.4 ± 6.4 (5) n.s.
	10^{-7}M	32.1 ± 7.6 (5) n.s.
	10^{-6}M	23.2 ± 2.0 (5) $p<0.001$
	10^{-5}M	17.3 ± 3.9 (5) $p<0.001$
1-oleoyl-2-acetyl glycerol	10^{-7}M	29.8 ± 3.0 (5) n.s.
	10^{-6}M	26.8 ± 1.3 (5) $p<0.01$
	10^{-5}M	23.0 ± 2.6 (5) $p<0.001$
H-7	10^{-7}M	27.9 ± 6.0 (5) n.s.
	10^{-6}M	29.2 ± 4.6 (5) n.s.
	10^{-5}M	26.9 ± 6.9 (5) n.s.
Staurosporine	3×10^{-9}M	31.9 ± 7.3 (5) n.s.
	3×10^{-8}M	28.8 ± 4.5 (5) n.s.
	3×10^{-7}M	27.9 ± 2.2 (5) n.s.
Dimethylsulphoxide	5×10^{-5}M	26.6 ± 3.8 (5) n.s.
	10^{-4}M	28.8 ± 3.3 (5) n.s.
	5×10^{-4}M	31.4 ± 3.1 (5) n.s.
	10^{-3}M	33.0 ± 4.5 (5) n.s.

Data are expressed as the means and standard deviations of the number of experiments in parenthesis. The Student's t-test was used to determine whether the addition of agent resulted in any significant change in enzyme activity. n.s. = not significantly different from control data.

4.4 DISCUSSION

The regulation of Na^+-K^+-ATPase has been extensively studied and the putative roles of many effectors and hormones have been investigated in a large number of different tissues and organisms (see review by Rossier *et al.* 1987). In spite of this research effort the control of Na^+-K^+-ATPase activity in many systems remains unclear. The activity of the sodium pump can be modulated either directly by the binding of ligands, including monovalent cations, ATP and its analogues or indirectly by the actions of hormones and other effectors. The action of hormones can be subdivided into those that affect transcription and gene expression to generate new enzyme molecules and those that act to modify the activity of existing Na^+-K^+-ATPase molecules.

It has generally been believed that the dynamic regulation of the sodium pump is achieved by alterations in the concentration of intracellular sodium ions (Van Dyke & Scharschmidt, 1983; Brodie & Sampson, 1989). The K_m or binding constant for the binding of Na^+ to Na^+-K^+-ATPase is 20-40mM and the intracellular sodium concentration is generally below this level (Cohen & Lechene, 1989). The internal sodium site of the sodium pump is therefore not normally saturated and the sodium pump works below its maximal capacity (V_{max}) (Skou, 1965; Jorgensen, 1986). Any increase in intracellular sodium concentration therefore immediately increases enzyme activity up to the maximum capacity of the system. Such effects have been referred to as the ion concentration dependent stimulation of Na^+-K^+-ATPase and are regarded by many authors to be the short-term regulatory mechanism of the enzyme (Pressley, 1988; Lamb, 1990) which ultimately triggers long-term regulation thereby increasing the number of functional pumps in the membrane.

Lamb (1990) suggested that there was no good evidence that the maximal rate of pumping could be changed by altering the shape or structure of the enzyme, for example by phosphorylation, though there have been an increasing number of reports providing evidence which might contradict this view (discussed later in this section). Upregulation can occur by the biosynthesis of new enzyme, by increasing the rate of insertion of enzyme into the membrane or by decreasing the rate of removal and rate of turnover of the enzyme. Cook *et al.* (1982) showed that Na^+ pumps were continuously being inserted and removed from the cell membrane. Both increased insertion and decreased turnover have been shown to occur in various tissues (Will *et al.* 1977; Pollack *et al.* 1981; Graves & Wheeler, 1982; Karin & Cook, 1986; Pressley *et al.* 1986; Tamkun & Fambrough, 1986; Wolitzky & Fambrough, 1986; Ismail-Beigi *et al.* 1988).

Another potential mechanism of upregulation exists and involves the recruitment of existing enzyme from some previously inactive pool. These recruitment processes can be divided into two groups: (i) those that involve conversion of a non-functional pump or its constituents into an active form by allosteric or covalent means and (ii) those that involve transfer of potentially functional pumps from a protected 'masked' site to the plasma membrane. Evidence for protected sites may be demonstrated by an increase in Na^+-K^+-ATPase activity in cell lysates following treatment with detergents (Jørgensen & Skou, 1971; Pollack *et al.* 1981; Ismail-Beigi *et al.* 1988). However other work suggests that recruitment from an inactive pool does not account for upregulation of the Na^+-K^+-ATPase pump in some systems (Wolitzky & Fambrough, 1986). There are several reviews on the topic of up and down regulation of the sodium pump including those by Karin & Cook (1983), Algerably *et al.* (1985), Pressley (1988) and Lamb (1988; 1990).

The steroids aldosterone, corticosterone, cortisol and the thyroid hormones have been implicated in the control of the transcription of the Na^+-K^+-ATPase gene and to a lesser extent in the processing and stability of the corresponding mRNA, and also in the translation process, membrane assembly and cell surface expression of newly synthesised enzyme molecules (Smith & Edelman, 1979; Bastl *et al.* 1980; Garty 1986; Jørgensen, 1986; Morel & Doucet, 1986; Barlet & Doucet, 1987; Clauss *et al.* 1987). The action of these hormones is mediated through DNA-binding receptors. In fish the enzymes cortisol and prolactin have emerged as mediators of longer term osmoregulatory changes in sodium pump activity and are associated with changes in the number and proliferation of chloride cells. Such processes extend over hours or days representing chronic adaptation to environmental changes in salinity.

The short-term hormonal control of Na^+-K^+-ATPase is just beginning to be understood. Hormones including insulin, glucagon, epidermal growth factor, vasopressin, catecholamines and possibly progesterone may act on pre-existing pumps by modulating their activity. For example, in rat proximal collecting tubule cells dopamine inhibits Na^+-K^+-ATPase (Aperia *et al.* 1987; Bertorello *et al.* 1988) whereas in brain tissue noradrenaline stimulates the enzyme (Swann, 1983). The action of such hormones is mediated by plasma membrane receptors and second messengers. These hormones may act by triggering retrieval and membrane insertion of pre-existing pumps (Richter *et al.* 1984; Cook *et al.* 1985) or as suggested by another study on transformed cells, via PKC-dependent phosphorylation of the alpha subunit thereby modulating activity of the sodium pump (Ling & Cantley, 1984). Both of these putative mechanisms are likely to be fast-acting, occuring within minutes of receptor stimulation.

By investigating the effect of protein kinase C activators and inhibitors on the activity of Na^+-K^+-ATPase it was hoped that it would possible to reveal the putative role of PKC in the short-term control of gill osmoregulation. Several reports suggest that PKC activation can regulate the activity of Na^+-K^+-ATPase. In isolated rat hepatocytes phorbol esters stimulated Na^+-K^+-ATPase-mediated transport activity (Lynch *et al.* 1986) and similar effects were reported in pancreatic acinar cells by Hootman *et al.* (1987) and in rabbit aorta by Gupta *et al.* (1991). In contrast with these studies DAG inhibited Na^+-K^+-ATPase in the rat brain (Goldberg *et al.* 1985) and the PKC activators, OAG and phorbol ester inhibited Na^+-K^+-ATPase activity in renal proximal tubule cells (Bertorello & Aperia, 1989) so it would seem that the response depends on the tissue involved. In this study the phorbol ester PDB inhibited Na^+-K^+-ATPase activity in a concentration dependent manner with inhibition of the enzyme becoming significantly different from the control at a concentration of 10^{-6}M. The diacylglycerol analogue OAG produced a similar but smaller effect and was again concentration dependent becoming significantly different from the control at 10^{-6}M. These results are consistent with those of Bertorello & Aperia (1989), and suggest that Na^+-K^+-ATPase may be an effector protein for PKC in the chloride cells of the cod gill. No significant changes in enzyme activity were detected upon administration of the PKC inhibitors staurosporine or H-7 but this may have been due to the fact that perturbation of the system is necessary before the effects of these effectors can be seen. Pre-incubation in the presence of phorbol ester may be all that is needed to elicit a response with these PKC inhibitors.

Some caution should be exercised when interpreting the effects of phorbol esters

since Lynch *et al.* (1986) found that, in addition to directly stimulating PKC, phorbol ester can increase the production of DAG without stimulating Ins1,4,5P_3 formation. Thus the observed stimulation of the sodium pump by phorbol ester in that study could have been due to a general alteration in DAG and other membrane lipids rather than a direct stimulation of PKC. In other systems phorbol esters have been shown to increase the activity of the Na^+-H^+-antiporter, an effect that leads to increased Na^+ entry into the cell resulting in stimulation of the sodium pump (Dicker & Rozengurt, 1981; Grinstein *et al.* 1985). However, this effect is transient, lasting only a few seconds in kidney proximal tubule (Mellas & Hammerman, 1986). The mechanism by which activation of PKC might inhibit Na^+-K^+-ATPase in the present study was not determined but could be by direct phosphorylation or by the activation of some other intracellular pathway.

Since the publication of the paper by Lamb in 1990 in which he states that there is no good evidence to support the phosphorylation of the sodium pump as a mechanism of control at least two reports have been published to the contrary. Lowndes *et al.* (1990) showed that Na^+-K^+-ATPase from dog kidney and duck salt-gland could be phosphorylated *in vitro* by PKC indicating that Na^+-K^+-ATPase may serve as a substrate for PKC in intact cells. In addition Bertorello and his co-workers have recently shown that phosphorylation of purified Na^+-K^+-ATPase from shark rectal gland is associated with an inhibition of sodium pump activity of about 40-50% (Bertorello *et al.* 1991). Protein kinase C also inhibited the activity of Na^+-K^+-ATPase from rat renal cortex in the same study.

Having established a putative role for the cardiac hormone ANF in the transport of sodium across the gills of cod in Chapter 2 by showing that administration of the

hormone at a concentration of 10^{-7}M significantly reduced Na^+ efflux, the direct effect upon the sodium pump itself was investigated. The results presented in this chapter indicate that Na^+-K^+-ATPase activity was affected *in vitro* in a concentration dependent manner with higher concentrations of hormone decreasing activity. Since the Na^+-K^+-ATPase enzyme in this preparation is still associated with plasma membranes it is probable that putative ANF receptors are also present and still intact though the existence of ANF receptors on chloride cells remains unreported in the literature. It seems that hormone binding to these receptors is able to initiate a series of steps leading to a change in the activity of the sodium pump. The author is unaware of any other study in which ANF has been shown to have this effect upon the activity of Na^+-K^+-ATPase *in vitro*. Several other investigations have indicated that ANF does not alter sodium pump activity (Pollack *et al.* 1983; Thibault *et al.* 1983; Pamnani *et al.* 1984; Zeidel *et al.* 1986), whilst an indirect relationship between the hormone and Na^+-K^+-ATPase has been implicated in rabbit aorta since the vasodilatory effect of ANF can be inhibited by ouabain (Sybertz & Desiderio, 1985).

In this chapter two different aspects of the control of the sodium pump have emerged. Firstly, the role of the cardiac hormone ANF as a putative physiological effector of the enzyme and secondly, the possible role for PKC as a subcellular modulator of enzyme activity. Atrial natriuretic factor has been shown to bring about a cellular response via the production of the second messenger cGMP in several systems (reviewed by Baxter *et al.* 1988; Hamet & Trembley, 1988; Schiffrin, 1988). However, other second messengers such as cAMP and calcium have also been implicated in some systems though their role is less well understood (reviewed by Anand-Srivastava, 1988; Baxter *et al.* 1988). Whether receptor stimulation of chloride cells by ANF initiates second messengers which activate

PKC remains open to speculation. In order to investigate the possible link between ANF and PKC activation a series of further experiments were planned and these subcellular biochemical studies form the subject matter of Chapter 5.

CHAPTER 5

OSMOREGULATORY CONTROL MECHANISMS IN THE CHLORIDE CELLS FROM THE GILLS OF *Gadus morhua*

5.1 INTRODUCTION

5.1.1 Phosphatidylinositol metabolism and the formation of inositol phosphates and diacylglycerol

5.1.2 Phosphatidylcholine metabolism and the formation of diacylglycerol

5.1.3 Protein kinase C

5.1.4 Other lipid-derived cellular messengers

5.1.5 Objectives of the present study

5.2 METHODS

5.2.1 Incorporation of radioactively-labelled precursors into chloride cells

5.2.2 Preparation of aqueous and organic soluble components of gill tissue

(i) Total lipid

(ii) Phospholipids (including the polyphosphoinositides)

(iii) Inositol phosphates

(iv) Choline metabolites (water-soluble)

5.2.3 Separation of the lipid fraction from chloride cells

5.2.4 Determination of chloride cell lipid composition

(i) by densitometry

(ii) by mass determination

(iii) by phosphate determination

5.2.5 Measurement of the incorporation of [^{32}P]-orthophosphate into

phospholipids and inositol phosphates

5.2.6 Separation of water-soluble choline metabolites from chloride cells and measurement of radioincorporation into water-soluble choline metabolites

5.2.7 Analysis of the fatty acid composition of lipids from chloride cells

5.2.8 Measurement of agonist stimulated DAG production

5.3 RESULTS

5.3.1 Determination of phospholipid composition of cells

5.3.2 Composition of phospholipid from chloride cells

5.3.3 Fatty acid composition of phospholipid classes from chloride cells

5.3.4 Studies on incorporation of radioisotopes into chloride cells

5.3.5 The effects of atrial natriuretic factor upon chloride cell metabolites

(i) Effects on phospholipids

(ii) Effects on water-soluble choline metabolites

(iii) Effects on fatty acid composition of diacylglycerol

5.4 DISCUSSION

5.1 INTRODUCTION

5.1.1 Phosphatidylinositol metabolism and the formation of inositol phosphates and diacylglycerol

The first experiments which ultimately led to the present understanding of phosphatidylinositol signalling were carried out by Hokin & Hokin in the 1950's. In 1953 they reported acetlycholine-stimulated phospholipid turnover in pancreas and brain slices (Hokin & Hokin, 1953) and their work from this period has been recently reviewed (Hokin & Hokin, 1989). In the mid 1970's it was discovered that an inositol lipid-specific phospholipase C catalysed receptor-stimulated inositol lipid hydrolysis (Durell, 1969; Lapetina & Michell, 1973). Several other discoveries aided progress in this field of research; (i) the hydrolysis of inositol lipid is closely coupled to receptor occupation (Michell, 1975); (ii) inositol lipid hydrolysis appeared to be coupled to mobilisation of intracellular calcium (Michell, 1975); (iii) the lipid hydrolysed upon stimulation was PtdIns4,5P_2 (Michell *et al.* 1981). Subsequently in 1983 Ins1,4,5P_3 was identified as the calcium-mobilising second messenger (Streb *et al.* 1983). At around the same time protein kinase C was discovered and its control by DAG and phorbol ester tumour promoters was being investigated (Castagna *et al.* 1982; Takai *et al.* 1979). Hence from the initial discovery of receptor-stimulated phosphatidylinositol (PtdIns) metabolism by Hokin and Hokin in 1953 it was a further 30 years before the formulation of the general hypothesis that many receptors caused the activation of phosphoinositidase C-catalysed PtdIns4,5P_2 hydrolysis directly yielding two cellular messengers, 1,2-diacylglycerol and Ins1,4,5P_3 and indirectly causing the increase in the cytosolic concentration of a third, the calcium ion (Berridge & Irvine, 1984; Marx, 1984;

Nishizuka, 1984). This picture of a signal transduction mechanism was more complex than the previously characterised and essentially linear sequence leading from receptors via adenylate cyclase to the protein substrates of cAMP-dependent protein kinase but none the less the hypothesis was relatively straightforward. However, work since 1984 has shown that the roles of inositol lipids in cellular control processes is in fact very much more complex.

It is now recognised that many cell types contain and interconvert a large number of inositol polyphosphates, some of which are present in high intracellular concentrations (Michell, 1989). In addition to Ins1,4,5P_3 a variety of cyclic and non-cyclic inositol monophosphates, diphosphates, triphosphates, tetraphosphates etc. have been isolated (Majerus *et al.* 1988). Modern analytical techniques have identified almost every possible inositol phosphate in cellular extracts (reviewed by Berridge, 1993). Enzymatic pathways from PtdIns4,5P_2 via Ins1,4,5P_3 have been discovered that lead via other enzymatic pathways to Ins1,4P_2, Ins1,3,4,5P_4, Ins1,3,4P_3, Ins1,3,5,6P_4, Ins1,3P_2 and Ins3,4P_2. The complete pathways to some other inositol metabolites are still unknown, for example, Ins3,4,5,6P_4, Ins1,3,4,5,6P_5 and Ins1,2,3,4,5,6P_6. The metabolite Ins1,3,4,5P_4 has been shown to antagonise Ins1,4,5,P_3 function by stimulating the sequestration of cytosolic Ca^{2+} into intracellular stores (Hill *et al.* 1988). The inositol phosphates have recently been reviewed by Irvine *et al.* (1988), Shears (1989a; 1989b), Berridge & Irvine (1989), Downes & MacPhee (1990) and Berridge (1993) but the possible roles of many of these compounds still remain to be determined. The polyphosphoinositides appear to include PtdIns3,4,5P_3, PtdIns3P and PtdIns3,4P_2 in addition to the more widely recognised PtdIns4P and PtdIns4,5P_2

(Traynor-Kaplan *et al.* 1988; Whitman *et al.* 1988; Augur *et al.* 1989). The inositol lipids also have an undefined role in the control of cellular behaviour by cell surface receptors and oncogenic encoded proteins that possess intrinsic protein kinase activity.

Another cellular signalling system uses a different form of PtdIns as a precursor. Phosphatidylinositol glycans can act as lipophilic anchors for many of the diverse proteins that are exposed on the surface of eukaryotic cells, see Homans *et al.* (1988) for structures. Mammalian cells contain very small quantities of PtdIns glycans of undetermined structure that are hydrolysed during insulin stimulation. The water-soluble inositol phosphate glycans that are liberated, (presumably within the cells), have some of the properties of the long-sought intracellular messengers of insulin action (Low & Saltiel, 1988; Low, 1989). The metabolic relationship between the intracellular events of PtdIns glycan metabolism in response to insulin and the origin and fate of PtdIns glycan anchored cell surface proteins is a topic of intense study.

Diacylglycerol, the other product of receptor-mediated PtdIns4,5P_2 hydrolysis, is the most potent physiological activator of protein kinase C. Naturally occuring 1,2-*sn* -DAG but not the 2,3-*sn* -enantiomer nor the 1,3-*sn* -diastereomer is capable of activating PKC (Boni & Rando, 1985). The carboxyl moieties of the esters and the 3-hydroxyl moiety of DAG are required for maximal activation (Ganong *et al.* 1986). The hydrophobic domain of DAG is critical in ensuring the proper orientation of membrane insertion so that the carbonyl and hydroxyl groups are present at the interface. One or more of these groups may interact specifically with protein kinase C and at least one may ligate directly to Ca^{2+}. The multiple-point attachment of DAG to the PKC/Ca^{2+}/PtdSer complex is prerequisite for activation

(Ganong *et al.* 1986). Diacylglycerol stimulates various PKC isozymes with different potency in asays containing different phospholipids, PKC I being more sensitive to DAG or phorbol ester than PKC II and PKC III. In addition to PKC, diacylglycerol also stimulates or inhibits several enzymes (Farooqui *et al.* 1988) including Na^+-K^+-ATPase at *in vivo* concentrations (Goldberg *et al.* 1985).

Guanine nucleotide binding proteins (G-proteins) are key components of receptor-mediated PtdIns metabolism as they are in many other cellular signalling processes. They allow communication between receptors, coupling or transducing the extracellular signal to the intracellular effector systems. These can be either ion channels or enzymes (e.g. adenylate cyclase, phospholipase C, cGMP phosphodiesterase) which in turn alter the intracellular concentration of second messengers. The role of G-proteins in signal transduction has been reviewed by Gilman (1987) and Milligan (1989) and their regulation of phospholipase C has been discussed in a recent review by Sternweis & Smrcka (1992).

5.1.2 Phosphatidylcholine metabolism and the formation of diacylglycerol

The potential of other pathways of phospholipid turnover in hormonal signal transduction have only recently been appreciated. There is now accruing evidence of agonist-induced phospholipid cycles where the catabolism of phosphatidylcholine (PtdCho) serves as a source of diacylglycerol. Like the inositol phospholipids, phosphatidylcholine is found almost exclusively in eukaryotic cell membranes. The DAG from PtdCho metabolism can be generated directly in response to certain hormones via a phosphocholine-specific phospholipase C reaction which is analagous to the stimulation of PtdIns-specific phospholipase C.

Enhanced production of DAG from PtdCho has been found for various cell types treated with P_2 purinergic agents and vasopressin (Irvine & Exton, 1987), platelet derived growth factor (Besterman *et al.* 1986) and bombesin (Muir & Murray, 1987). Most of these substances also stimulate PtdIns4,5P_2 hydrolysis but other substances such as interleukins do not (Roscoff *et al.* 1988). In most of the studies it was concluded that the DAG was generated directly from PtdCho via phospholipase C-catalysed degradation because phosphocholine appeared as a by-product.

Diacylglycerol can also be produced indirectly by the activity of phospholipase D to yield phosphatidic acid which is then cleaved to DAG by the action of phosphatidic acid phosphohydrolase (PAP). Work on a rat embryo cell line (REF52) and rat hepatocytes has implicated phospholipase D in vasopressin-induced phosphatidylcholine metabolism. REF52 cells displayed an increase in phosphatidic acid and choline catabolism (Cabot *et al.* 1988a; 1988b) whereas rat hepatocytes displayed phosphatidic acid production before DAG accumulation (Bocckino *et al.* 1987).

Lyso-PtdCho, produced by the action of phosphlipase A_2, may be re-esterified to PtdCho or catabolised to glycerophosphocholine (GPC), which can be further degraded to glycerol-3-phosphate (G3P), which in turn can be converted back to diacylglycerol via phosphatidic acid synthesis. Diacylglycerol can react with CDP-choline to complete a cycle of PtdCho turnover. Figure 5.1 summarises the present knowledge of phosphatidylcholine metabolism and its products.

In rat hepatocytes a G-protein appears to mediate the P_2-purinergic induced

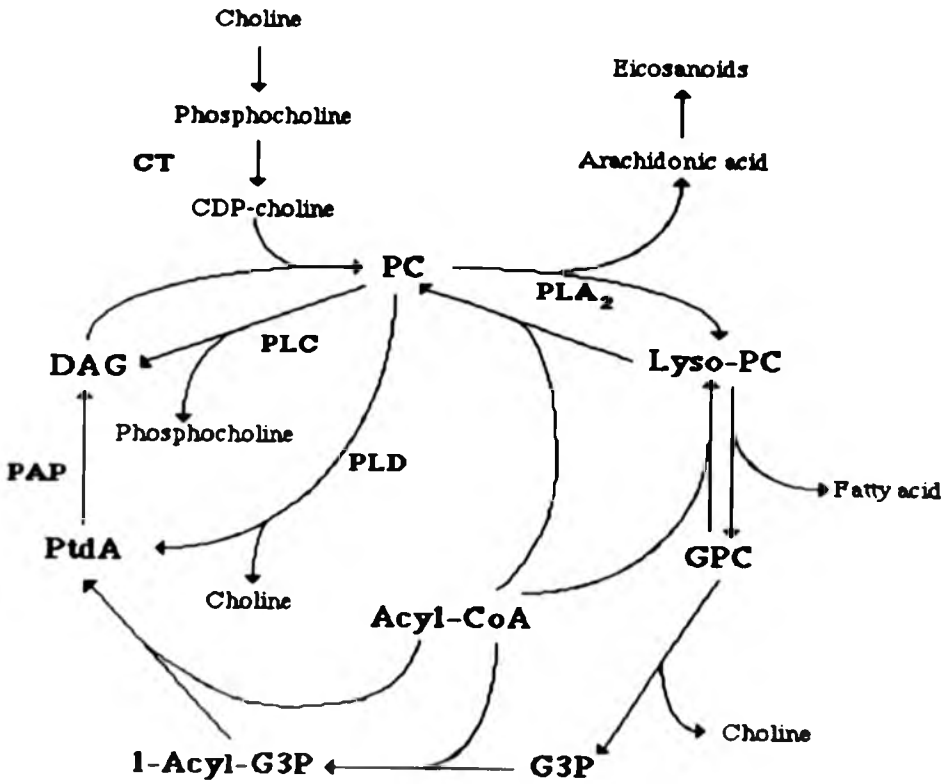


Figure 5.1. Principal features of phosphatidylcholine (PC) metabolism and the production of the second messenger diacylglycerol (DAG). See text for details. PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2; PtdA, phosphatidic acid; PAP, PtdA phosphohydrolase; GPC, glycerophosphocholine; G3P, glycerol 3-phosphate; Acyl-CoA, acyl coenzyme A; CDP, cytidine diphosphate; CT, cytidine triphosphate:phosphocholine cytidyltransferase. Diagram adapted from Pelech & Vance (1989).

stimulation of PtdCho-specific phospholipase C and is analagous to the novel G-protein (Gp) implicated in the coupling of hormone receptor activation to the breakdown of inositol phospholipids to phospholipase C (Cockcroft, 1987). GTP and related guanine- containing analogues have been shown to stimulate PtdCho catabolism in rat hepatocyte membranes whereas other nucleotide triphosphates had little or no effect (Bocckino *et al.* 1987; Irving & Exton, 1987; Exton, 1988).

In contrast to the phospholipase C-catalysed breakdown of PtdInsP4,5_2 the hydrolysis of PtdCho produces DAG without raising cytoplasmic Ca^{2+} levels. The phosphocholine generated from PtdCho is unlikely to act as a second messenger because of the large existing pool of phosphocholine already present in the cell (Pelech & Vance, 1984; 1989). DAG produced from PtdCho catabolism could, however, propagate a hormonal signal via the activation of protein kinases in a Ca^{2+} independent fashion. Misra & Sahyoun (1987) showed that DAG and tetradecanoyl-phorbol acetate (TPA), a DAG mimetic, stimulated protein kinase C independently of Ca^{2+}. Other workers have shown DAG-enhanced phosphorylation of histone H1 in the absence of calcium ions by a PKC-related kinase (Ohno *et al.* 1988) and by a protease-activated kinase II (Gonzatti-Haces & Traugh, 1986). *In vitro* studies have shown that protein kinase C can be activated in the absence of, or in the presence of low concentrations of, Ca^{2+} by free fatty acids released via the action of phospholipase A_2 on phospholipids (Murakami *et al.* 1986).

Diacylglycerol may also act as an activator of phosphatidlycholine synthesis as well as a substrate of that process. The mechanism for PtdCho synthesis is dependent upon the rate limiting enzyme CTP:phosphocholine cytidyltransferase (CT) (Vance

& Pelech, 1984; Pelech & Vance, 1984; 1989). This enzyme appears to be regulated by the availability of CTP and phosphocholine substrate molecules and by the translocation of the enzyme between the cytoplasm, where it exists as an inactive reservoir, and the endoplasmic reticulum where it is activated by phospholipids (Pelech & Vance, 1984). The translocation of CT has been shown to occur by at least two mechanisms. The first is mediated by cAMP-dependent protein kinase which releases the enzyme from the endoplasmic reticulum (ER), into the cytoplasm. This mechanism involves the direct phosphorylation of a CT serine residue (Vance & Pelech, 1984), and the process has been shown to be reversed by an unidentified protein phosphatase. The second mechanism involves translocation to the ER and activation of CT by fatty acids (Vance & Pelech, 1984; Pelech & Valance, 1984; Cornell & Vance, 1987). Some evidence also exists for the translocation of CT to the ER by DAG. Phospholipase C treatment of cultured cells leads to an increase in DAG and CT translocation to the ER and this is reversed when the phospholipase is removed (Wright *et al.* 1985; Terce *et al.* 1988).

Attention has been drawn to the remarkable parallels between protein kinase C and CTP:phosphocholine cytidyltransferase (Pelech & Vance, 1989). Both enzymes are similarily regulated via changes in their bimodal distribution in cells. Both can be activated by acidic phospholipids and fatty acids and DAG promotes translocation of both to membranes from the cytosol, protein kinase C being activated on the plasma membrane (Kikkawa *et al.* 1982; Nishizuka, 1984)), and CT being activated on the ER (Pelech & Vance, 1984; Vance & Pelech, 1984).

A phosphatidylcholine cycle features a number of metabolic advantages over signal transduction via the phosphatidylinositol cycle. The breakdown of PtdCho may be

more sustained since the pool of PtdCho is 5-10 fold that of PtdIns. Furthermore, the replenishment of PtdCho from DAG only involves one enzymatic step compared to five for PtdIns4,5P_2 synthesis from DAG and only one molecule of CTP is needed for phosphocholine conversion to PtdCho whereas one molecule of CTP and three molecules of ATP are needed for synthesis of PtdIns4,5P_2 from DAG. Phosphatidylcholine metabolism is therefore not as expensive energetically and as a signalling system it releases one rather than two second messengers which may also be useful in some situations.

The fatty acid composition of the second messenger diacylglycerol produced in any signal transduction mechanism ought to reflect the distinctive fatty acid composition of the parent phospholipid class (unless there are separate pools). Thus, fatty acid analysis of any diacylglycerols produced following agonist stimulation should indicate which phospholipid substrate and therefore which second messenger pathways are involved in the control of gill ion fluxes. The main phospholipids in fish are generally rich in (n-3) polyunsaturated fatty acids and have high (n-3)/(n-6) ratios. (n-3) Polyunsaturates are essential dietary components in seawater fish but (n-6) polyunsaturates have been thought to be non-essential and *vice-versa* for freshwater fish. However, both views are over simplifications (Bell *et al.* 1986) and (n-3) and (n-6) PUFA are now believed to be essential dietary components in both freshwater and marine fish though the absolute requirements are probably different. In view of the importance of the 2-series prostaglandins, and therefore arachidonic acid, it is probable that (n-6) PUFA are essential in freshwater and seawater fish.

5.1.3 Protein kinase C

The phosphorylation of proteins is an important mechanism by which many cellular processes are regulated (reviewed by Nestler *et al.* 1984). Many protein kinases have been described and among these much interest has centered on protein kinase C (PKC), a serine/threonine-specific kinase, first identified by Inoue *et al.* (1977) in rat brain, and which is now thought to play a pivotal role in many cellular control processes. Protein kinase C has a broad protein substrate specificity which is different from cyclic nucleotide-dependent and calmodulin-dependent protein kinases (Takai *et al.* 1979; Wise *et al.* 1982). The enzyme not only phosphorylates a number of proteins both *in vivo* and *in vitro* (Woodgett *et al.* 1987) but also, like many kinases, undergoes auto -phosphorylation, in which multiple sites are phosphorylated on serine and threonine residues (Huang *et al.* 1986; Parker *et al.* 1986; Woodgett *et al.* 1987). Protein kinase C was originally characterised as being dependent on diacylglycerol, Ca^{2+} and phospholipid for its activity (Nishizuka, 1984). However, several new members of the PKC family have been recently identified whose activities are dependent on DAG and phospholipid but are independent of Ca^{2+} (reviewed by Nishizuka, 1988). Protein kinase C is also stimulated by the tumour promoting phorbol esters through their ability to mimic the effects of diacylglycerol and PKC has been shown to be the major receptor for phorbol ester in the cell (Ashendel, 1985). More recently there have been significant developments in studies on the activation of PKC by other lipids such as free fatty acids, gangliosides and sulphatides (Farooqui *et al.* 1988).

In the resting cell PKC is found mainly in the cytosol, presumably in the inactive state. Agonist stimulation activates a specific phospholipase C which cleaves

phosphoglycerides resulting in an increase of diacylglycerol in the plasma membrane. This causes a translocation of PKC to the plasma membrane. Diacylglycerol activates PKC by increasing its affinity for Ca^{2+} and phosphatidylserine (Takai *et al.* 1979; Kikkawa *et al.* 1982) so that in the presence of DAG protein kinase C is activated at physiological concentrations of calcium (10^{-7}M). Thus the increase in DAG in the plasma membrane can markedly stimulate PKC without the mobilization of calcium from intracellular stores.

At least seven subspecies of PKC have been identified to date and there have been several recent comprehensive reviews on the PKC family (Kikkawa *et al.* 1989; Parker *et al.* 1989; Shearman *et al.* 1989). The PKC subspecies are monomeric proteins with molecular weights between 70,000 and 90,000. The enzyme has a hydrophobic regulatory domain which interacts with Ca^{2+} and phospholipids and a hydrophilic catalytically active domain each containing both highly conserved regions and variable regions. These kinases are not isoenzymes (i.e. different gene products acting to catalyse the same reaction), but rather the PKCs are linked together as a family through their regulatory properties. Biochemical and immunocytochemical studies with subspecies-specific antibodies suggest that the PKC subspecies may be differently located in particular cell types and at limited intracellular locations. Many cell types express more than one subspecies in variable ratios, and their intracellular distribution may depend on the state of activation of the cells. In response to extracellular signals these subspecies are frequently down regulated at different rates. Although there is little evidence to implicate PKC subspecies with specific functions, each PKC family member may have a distinct role in the processing and modulation of a variety of physiological and pathological cellular responses.

5.1.4 Other lipid-derived cellular messengers

Arachidonic acid metabolism and the eicosanoids

Upon cell stimulation, arachidonic acid is released from membrane phospholipids via cleavage by phospholipase A_2 from the 2-position of the phospholipid. For example, in rat pineal glands stimulated with norepinephrine or calcium ionophore A23187, hydrolysis of PtdCho by phospholipase A_2 produces lyso-PtdCho and arachidonic acid (Ho & Klein, 1987). Lipoxygenases act upon arachidonic acid to produce leukotrienes and hydroxy-eicosatetraenoic acids whereas cycloxygenase action produces the prostaglandins, prostacyclins and thromboxanes. Thus arachidonic acid is the precursor of many very important bioactive compounds which are involved in the control of regulation of many processes such as immunity, inflammation, water reabsorption, vasocontraction, vasodilation, platelet aggregation and activation of adenylate cyclase (Merrill, 1989). Prostaglandins PGE_1 and PGF_{2a} influence Na^+ and Cl^- fluxes in gills of seawater *Mugil capito* (Pic, 1975).

In terrestrial animals arachidonic acid is the predominant C20 PUFA and is found in all phosphoglycerides. Therefore in theory arachidonic acid could be derived from PtdCho, PtdEth (diacyl or alkenylacyl), PtdSer or PtdIns. However, in marine fish arachidonic acid is found predominantly in PtdIns as the 18:0/20:4 molecular species, as in terrestrial animals, though there is marked tissue specificity in cod (Bell & Dick, 1990). Several fatty acids are potential substrates for prostaglandin synthesis in fish tissue including arachidonic acid (reviewed by Mustafa & Srivastava, 1989). For example, in trout gill PGE_1 is synthesised from

eicosatrienoic acid, 20:3(n-6), (Christ & van Dorp, 1972) and PGE_2 from arachidonic acid, 20:4(n-6), (Mustafa & Srivastava, 1989). The other abundant C20 PUFA, 20:5(n-3), can undergo similar reactions to those of arachidonic acid via cycloxygenase action (Sprecher *et al.* 1982). These PUFA can therefore act as substrates for prostaglandin formation and also inhibit conversion of other fatty acids (Hwang & Carroll, 1980). Since the final products have different efficacies the final balance of prostaglandin activity reflects the PUFA composition in the cellular phospholipids.

The regulation of phospholipase A_2 is not well understood (Crooke *et al.* 1988) but there is evidence that G-proteins are involved and that interaction between different signal transduction pathways impinging upon protein kinase C may bring about the release of arachidonic acid (Burch, 1988).

Platelet activating factor (PAF)

Platelet activating factor (PAF, 1-*O* -alkyl-2-acetyl-*sn* -glycero-3-phosphocholine) is a biologically active phospholipid synthesized by a number of inflammatory cells (Hanahan, 1986). Several tissues are also known to synthesize PAF or respond to it and it has been detected in many of the body fluids of several mammalian species (reviewed by Snyder, 1987). While the physiological role of PAF is poorly understood, it is involved in the activation and/or aggregation of mammalian platelets and leukocytes (Camussi *et al.* 1981; O'Flaherty *et al.* 1981; Yasaki *et al.* 1982) and may be a mediator of hypotensive activities in mammals (Muirhead, 1980). PAF is also known to cause an increase in vascular permeability, vasoconstriction and contraction of smooth muscle (Humphrey *et al.* 1982; Findley *et al.* 1981). A recent report demonstrated the synthesis of PAF in gill, kidney,

liver and spleen from the rainbow trout, the first report of PAF synthesis in a teleost (Turner & Lumb, 1989). The role of PAF as a lipid mediator in fish tissues remains to be determined.

Phospholipase A_2 action produces lysophospholipids which can affect protein kinase C activity and if the substrate of PLA_2 is 1-alkyl-2-acyl phosphatidylcholine then platelet activating factor (PAF) can be formed by reacylation with acetyl-coenzyme A.

Sphingosine

Sphingolipids are a large class of compounds which includes sphingomyelin, gangliosides and cerebrosides. They are major components of all animal tissues found mainly in the plasma membrane. Because the sphingolipids were first identified in the brain their names imply that they are specific to nervous tissues but they are important in all cell types. As well as being involved in the maintenance of membrane and lipoprotein structure, sphingolipids have also been implicated in the process of cell to cell communication and can act by modulation of cell surface receptors (Bremner *et al.* 1988), perhaps after structural modification of the parent sphingolipid (Hanai *et al.* 1988). In certain systems gangliosides can stimulate protein phosphorylation which may be a mechanism by which cell-surface receptors mediate an intracellular response (Chan, 1987). Another hypothesis for the way in which sphingolipids act in signal transduction suggests that they are hydrolysed to free sphingosine which is a potent inhibitor of protein kinase C *in vitro* and of cellular events which depend on this enzyme (Hannun *et al.* 1987; Hannun & Bell, 1987). Hannun & Bell (1987) have also shown that lysosphingolipids may be important in some cases and work by Jefferson & Schulman (1988) indicates that

sphingosine can inhibit calmodulin-dependent protein kinases. Studies of the epidermal growth factor receptor indicate that sphingosine may serve as a pleotrophic modulator of cellular function by activating one class of kinase whilst inhibiting protein kinase C (Northwood & Davis, 1988). Ongoing research is investigating whether or not cells naturally use free sphingosine as a second messenger. The potent effects of exogenous sphingosine suggests that endogenous sphingosine could be a biomodulator (Merrill & Stevens, 1989).

5.1.5 Objectives of the present study

The preceeding introduction is not an exhaustive review of what is now a dauntingly large field of very active research. Rather, it sought to show the many hypothetical receptor and second messenger systems which could be used to control osmoregulation and the sodium pump at the molecular level, with particular emphasis on diacylglycerol and protein kinase C. The following series of experiments were designed to identify possible second messenger pathways based on DAG production from either inositol lipids or PtdCho. These involved:

(i) Identification of the main phospholipid classes of chloride cells by HPTLC and the analysis of the fatty acid compositions of these lipids by gas liquid chromatography. The phospholipid and fatty acid compositions of cells may reflect their specialised functions.

(ii) Radiolabelling of chloride cells with [^{32}P]-orthophosphate and/or [methyl-^{3}H]-choline and the separation of the aqueous and lipid fractions by chromatography in order to identify patterns of labelling of the phospholipids, inositol phosphates and

choline metabolites.

(iii) Similar radiolabelling studies in ANF-stimulated cells to monitor any changes in metabolite turnover.

(iv) Determination of the fatty acid composition of DAG in resting and ANF-stimulated cells.

5.2 METHODS

5.2.1 Incorporation of radioactively-labelled precursors into chloride cells

Chloride cells were prepared by the method described in section 2.2.3. One gram wet weight of chloride cells was resuspended in 12ml phosphate-free, HEPES-buffered saline for incubation. For labelling the phosphate group of phospholipids and inositol phosphates, 500μCi of carrier-free [^{32}P]-orthophosphate was added to the cell suspension. For labelling of choline metabolites, 50μCi of [methyl-^{3}H]-choline chloride was added to the incubation mixture. A sample of chloride cells (2ml) was taken at the start of the experiment and at subsequent hourly intervals up to a maximum of 5 hours. Cell were incubated in a shaking waterbath at 15°C. Each sample was immediately washed with chilled phosphate buffer if only [^{32}P]-orthophosphate was used in the incubation or with phosphate and choline buffer if both isotopes were used. Cells were centrifuged at 700*g* for 10 minutes in a bench centrifuge and then washed a further two times in the same way before the lipids were extracted.

If the effect of hormone was being investigated the cells were resuspended in 4ml of HEPES buffer after washing to remove radioisotopes and a control sample removed for extraction. Agonist was added to the required concentration and the cells incubated for up to 1 hour. In the investigation into the effects of agonists on incorporation into the inositol phosphates 1ml samples were removed at 5min, 20min and 1 hour and for the investigation into the effects of incorporation into the choline metabolites and phospholipids, 1ml samples were removed at 30s, 5min and 1hour.

5.2.2 Preparation of aqueous and organic soluble components of gill tissue

(i) Total lipid.

Chloride cells were pelleted and, if radioactively labelled, washed as described in section 5.2.1. Extraction was carried out using a modified version of the method of Folch *et al.* (1957). Ten volumes of chloroform/methanol (2:1 v/v) were added to cells and homogenised in a glass-in-teflon homogeniser at 5000-6000rpm for 12 strokes. A quarter volume of 0.88% (w/v) potassium chloride was added and the contents shaken vigorously. Mixtures were centrifuged at 2500rpm for 5 minutes and the upper aqueous layer either discarded or dried down using the rotary evaporator as required. The organic layer was passed through phase separation paper, dried down on the nitrogen evaporator, desiccated under vacuum for at least one hour and weighed. Samples were redissolved in chloroform / methanol (2:1 v/v), containing 0.01% (w/v) butylated hydroxytoluene (BHT) to a concentration of 10mg ml^{-1} and stored under nitrogen at -20°C.

(ii) Phospholipids (including the polyphosphoinositides).

Following incorporation of [^{32}P]-orthophosphate and washing of cells as described in section 5.2.1, chloride cells were resuspended in HEPES buffer and extracted in 10 volumes of chloroform / methanol (2:1 v/v), and 1M hydrochloric acid to a final concentration of 70mM (Billah & Lapentina, 1982; Berridge, 1983). The mixture was homogenised immediately in a glass-in-teflon homogeniser at 5000-6000rpm for 12 strokes and then centrifuged at 700*g* for 5 minutes to separate the phases. The organic layer was removed, washed with 0.3mM HCl to a final concentration of 70mM and centrifuged as before. The final organic layer was passed through phase separation paper and lipid samples were dried down under nitrogen.

Samples were desiccated overnight, under vacuum, and then resuspended in chloroform/methanol (2:1 v/v), containing 0.01% (w/v) BHT to a concentration of 10mg ml^{-1} for storage under nitrogen at -20°C.

(iii) Inositol phosphates

Following incorporation of [^{32}P]-orthophosphate and washing of cells as described in Section 5.2.1, approximately 0.1-0.2g chloride cells were resuspended in 1ml HEPES buffer for extraction. Fifty µl of phytate hydrolysate was added at this point as a standard, prepared by a method based on that described by Wreggett *et al.* (1987). One gram of sodium phytate was dissolved in 10ml of 0.1M sodium acetate/acetic acid buffer, pH 4.0 and placed on a hot block at 100°C for 8 hours. The hydrolysate was desalted by passage through 4ml of Amberlite resin (1R-120, Analar, BDH) in a Pasteur pipette. The column was in the H^+ form, prepared by washing the column with 1M hydrochloric acid and then with acetate buffer. The hydrolysate was dried down using a rotary evaporator, redissolved in distilled water to a concentration of 50mg ml^{-1} and stored at 4°C.

3.76ml of chloroform/methanol/conc HCl (100:200:2 v/v/v) were added to the chloride cells followed by homogenisation at 5000-6000rpm for 12 strokes. 1.2ml chloroform and 1.2ml distilled water were then added, the mixture shaken and centrifugation carried out at 2500rpm to separate phases. The lower organic layer was passed through phase separation paper, dried down under nitrogen, desiccated, weighed and resuspended in chloroform/methanol (2:1, v/v), containing 0.01% (w/v) BHT and stored for use under nitrogen at -20°C. The upper aqueous layer was removed to a test tube and dried under reduced pressure on the rotary evaporator. The inositol phosphates were resuspended in 1ml 0.1M acetic

acid/sodium acetate buffer, pH 4.0, passed down a prepared Amberlite column, dried by rotory evaporation, weighed and redissolved in distilled water at a concentration of approximetely 50mg ml^{-1}.

(iv) Choline metabolites (water-soluble)

Radioactively-labelled cells were washed as before (Section 5.2.1), resuspended in 1ml HEPES-buffered saline and extracted as for total lipid [Section 5.2.2(i)]. The aqueous phase was retained, dried down by rotary evaporation (35°C) and redissolved in 50% ethanol to a final concentration of 100mg/ml.

5.2.3 Separation of the lipid fraction from chloride cells

The lipid classes from chloride cells were prepared by high performance thin layer chromatography (HPTLC). All HPTLC plates were pre-run in hexane/diethyl ether (1:1, v/v) to wash the plates and after air-drying placed in a desiccator under vacuum for 30min/1hr before use. Plates were pre-activated at 110°C for 30 minutes before use. Lipid samples were loaded on an origin 1cm from the bottom of the plate, either as spots (10µg lipid), if the samples were for quantitation by densitometry, or as streaks (10µg lipid mm^{-1}), if the plates were to be used for autoradiography or the lipid eluted. All plates were air-dried then desiccated under vacuum after each development in solvent. Samples of total lipid were separated using a double development system, the plates being run to a height of 6cm in methyl acetate/propan-2-ol/chloroform/methanol/ potassium chloride 0.25% (w/v aq.) (25:25:25:10:9, v/v/v/v/v) to resolve polar lipids, followed by development in hexane/diethyl ether/acetic acid (85:15:1.5, v/v/v) to the top of the plate to resolve neutral lipids (Olsen & Henderson, 1989). When only phospholipids were being

examined then only the first solvent of this system was used (Vitiello & Zanetta, 1978).

Phospholipids were occasionally separated using a two dimensional system where HPTLC plates were run in one direction using the solvent by Vitiello and Zanetta (1978) and then in the second direction using chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) (Parsons & Patton, 1967). If the polyphosphoinositides were to be investigated chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v/v/v/v) (Morse *et al.* 1987) was used.

Lipids were visualised for densitometry by spraying plates with a solution of 3% (w/v) copper acetate in 8% (v/v) phosphoric acid and charring at 160°C for 20 minutes (see Plate 5.1), and in preparative work by spraying plates with 0.1% (w/v) 2,7-dichlorofluorescein (DCF) in 97% (v/v aq.) methanol containing 0.01% BHT and visualised under U.V. light.

5.2.4 Determination of chloride cell lipid composition

(i) by densitometry

Chloride cell total lipid and phospholipid compositions were determined, in the main, by densitometry of charred HPTLC plates using a Shimadzu CS-9000 dual-wavelength flying-spot densitometer.

(ii) by mass determination

Phospholipids were also analysed by determination of lipid mass following separation of lipid classes by HPTLC. Approximately 2mg of lipid on a 10cm

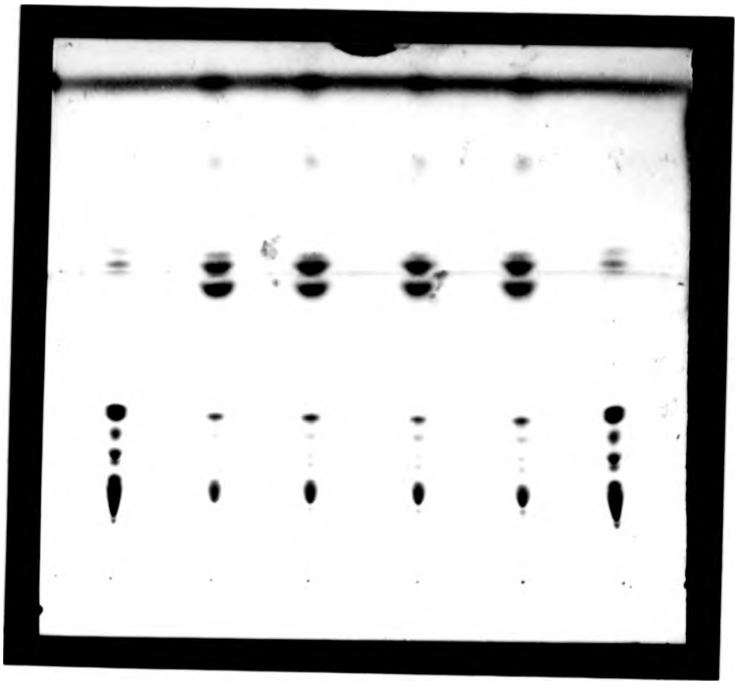


Plate 5.1. Visualisation of the separation of lipid from chloride cells from the gills of *Gadus morhua*. Lipid was separated by the double development h.p.t.l.c. method described by Olsen & Henderson (1989) and visualised for densitometry by charring. See Section 5.2 for further details of methods. Lanes 1 and 6, herring liver phospholipid standard 10μg; Lanes 2, 3, 4, and 5, cod gill total lipid 10μg.

origin of a 20 x 20cm thin layer chromatography plate was chromatographed and the position of individual phospholipids determined by spraying the edge of the plate with DCF. Individual phospholipid zones were scraped into test-tubes and eluted from the silica by washing with 2.5ml chloroform/methanol/water (5:5:1, v/v/v) and filtering through Whatman 1PS phase separation paper. Phospholipids were dried down under nitrogen and quantified by weighing.

(iii) by phosphate determination

Phospholipids were prepared as in (ii) above and the individual zones were assayed for phospholipid phosphorus using a test-combination kit by Boehringer Mannheim, GmbH, Germany.

5.2.5 Measurement of the incorporation of [^{32}P]-orthophosphate into phospholipids and inositol phosphates

After extraction, 5µl samples of total lipid were placed in scintillation vials with 5ml of Ecoscint A (National Diagnostics, USA) and radioactivity counted in the scintillation counter (Canberra Packard Tricarb 2000CB). Phospholipids were separated by h.p.t.l.c. and radioactive bands detected by autoradiography (HPTLC plates were exposed to Konika A2 X-ray film overnight, film was developed in a 1:18 dilution of Kodak LX24 developer for three minutes, rinsed for several minutes in running water, fixed in a 1:3 dilution of Kodak FX-40 liquid fixer for 5 minutes and finally rinsed in water). Alternatively, lipids were detected by use of standards stained with DCF and the individual lipid zones scraped into scintillation vials and counted in 5ml of scintillation fluid as before.

[^{32}P]-Orthophosphate labelled inositol phosphates were separated by paper chromatography based on the method of Dean & Beaven (1989). Samples were loaded as spots on a 27cm x 50cm strip of Whatman No1 chromatography paper and developed in the solvent propan-1-ol/concentrated ammonia/water (5:4:1, v/v/v), for approximately 20 hours until the solvent front was 40cm from the origin. The paper was dried and then each sample lane was cut into 0.5 or 1cm strips which were placed in scintillation vials and assayed for radioactivity in 5ml of scintillation fluid. Lanes alongside containing standard samples were sprayed with 60% (w/v) perchloric acid/1M HCl/5% (w/v) ammonium molybdate/water (5:10:20:65, v/v/v/v), the paper dried and exposed to pulses of U.V. light for approximately 30 seconds to detect standard inositol phosphates which were seen as blue spots. The migration of glucose monophosphate and glucose diphosphate was also investigated since these substances served as standards for Ins1P and Ins2P. These standards were desalted before loading using an Amberlite column as described in Section 5.2.2.

5.2.6 Separation of water-soluble choline metabolites from chloride cells and measurement of radioincorporation into water-soluble choline metabolites

The aqueous extracts from samples labelled with [methyl-^{3}H]-choline and [^{32}P]-orthophosphate were separated by HPTLC. Plates were pre-run and pre-activated as described in Section 5.2.3 before use. Samples containing approximately 2000 - 4000 cpm were streaked onto 1cm origins and 50µg of choline and phosphocholine standards were loaded on spots on either side of the samples. [^{32}P]-Orthophosphate labelled sample was also loaded onto a spot as a standard. Plates were developed in the solvent 0.5% (w/v) sodium

chloride/methanol/ammonia (100:100:2, v/v/v) (Yavin, 1976) to 1cm from the top of the plate and the plate air-dried and finally desiccated *in vacuo*. Radioactive bands were identified by autoradiography. Individual sample lanes on the plates were marked out into 5mm sections, these portions scraped into scintillation vials and 5ml of scintillation fluid added. This method was used to analyse incorporation of [methyl-^{3}H]-choline into choline, phosphocholine, acetylcholine, and CDP-choline. Lipid extracts were also analysed by HPTLC using the method of Vitiello and Zanetta (1978) described in section 5.2.3. This method of separation enabled the determination of the extent of incorporation of radioactive choline into phosphatidylcholine as well as incorporation of [^{32}P]-orthophosphate into phospholipids.

5.2.7 Analysis of the fatty acid composition of lipids from chloride cells

One mg of total lipid was loaded onto a 6cm origin on a 10 x 10cm HPTLC plate with a total lipid standard spotted on either side. The plate was developed using the double development system described in Section 5.2.3, air-dried, desiccated and then sprayed with 0.1% (w/v) DCF in 97% (v/v) aq. methanol containing 0.01% BHT and viewed with a U.V. lamp to locate lipid bands. Individual phospholipid bands were scraped into test-tubes, 2ml of 1% (v/v) sulphuric acid in methanol added and the contents mixed. 19:0 free fatty acid was added to phospholipids at this stage as an internal standard (approximately one tenth of the total amount of phospholipid present). The test-tubes were flushed with nitrogen, lightly stoppered with a piece of tissue placed between the stopper and the tube, and placed on a dry-block heating mantle at 50°C for 16 hours. Tubes were then cooled on ice, 5ml of water and 5ml of hexane/diethyl ether (1:1, v/v) containing 0.01% (w/v) BHT

added and the tubes shaken vigorously. The tubes were then centrifuged at 700*g* for 5 minutes in a bench centrifuge and the upper organic layer removed to a clean tube. The aqueous layer was re-extracted with another 5ml of hexane/diethyl ether (1:1, v/v). The organic layers were pooled and extracted with 3ml of 2% (w/v) potassium hydrogen carbonate to remove dichlorofluorescein. Tubes were centrifuged as before and the upper phase removed to a separate tube, the solvent evaporated off under nitrogen and the fatty acid methyl esters desiccated *in vacuo* for at least 30 minutes. The extract was finally redissolved in 1ml of hexane and transferred to 2ml glass bottles for storage at -20°C under nitrogen. Before analysis the hexane was removed by evaporation under nitrogen and the extract redissolved in hexane to a concentration of approximately 5mg ml^{-1}. Aliquots of this solution (1µl) were injected onto the Carlo-Erba gas liquid chromatograph equipped with a Free Fatty Acid Phase (FFAP) capillary column 50m x 0.32mm i.d. (Scientific Glass Engineering, U.K.), on-column injection and using H_2 as carrier gas with a thermal gradient from 50-225°C. Fatty acids were identified by reference to GLC traces of a standard sample of FAME prepared from a marine oil (marinol), and the inclusion of a known amount of 19:0 internal standard enabled the phospholipid classes to be quantitated.

5.2.8 Measurement of agonist stimulated DAG production

One mg samples of lipid from chloride cells that had been incubated in the absence of, or in the presence of ANF (10^{-7}M) were separated by double development HPTLC (Olsen & Henderson, 1989) and the lipid band corresponding to DAG was visualised by spraying plates with DCF and viewing under U.V. light (Section

5.2.3 for details). Analysis of the fatty acid composition of the DAG was then carried out as described for the phospholipids in Section 5.2.7 except that 1ml toluene was added to the silica to help solubilise the lipid and 10μg di-20:0 diacylglycerol was added as internal standard.

5.3 RESULTS

5.3.1 Determination of phospholipid composition of cells

Initial studies involved an investigation to determine a reliable and reproducible method of measuring the phospholipid composition of chloride cells. Before the chloride cell separation technique was fully worked out studies were carried out on mechanically prepared heterogeneous cell samples. The phospholipid composition of these cells was determined in one of three ways. The first method separated total gill phospholipid in a one dimensional TLC system (Parsons & Patton, 1967), scraped the bands from the plate, eluted the individual phospholipid from the silica and weighed the lipid. This method gave relatively inconsistent results due to the difficulty in removing all of the silica from the sample. This was especially so for the less abundant phospholipids where a small amount of silica had a large effect on the result. This method was of no use when attempting to calculate the amount of polyphosphoinositides present in cod gill.

The second method involved HPTLC separation of phospholipid by the method described by Morse *et al.* (1987), elution of phospholipid from the silica and quantitation of phospholipid by phosphorus determination using a kit from Boehringer Mannheim. This proved to be useful for the measurement of the polyphosphoinositides when used in conjunction with standards to locate bands.

A third, more routinely used method was that which separated the phospholipids by HPTLC in a one-dimensional system (Vitiello & Zanetta, 1978). Quantitation of the individual phospholipids was made by charring the plates and scanning with a densitometer. This method gave good separation of all the major phospholipids

(Plate 5.2) and gave consistent results. There was relatively good agreement between the methods (Table 5.1). However, the method utilising densitometry was much faster and appeared to give more consistent results and was therefore the method of choice for routine phospholipid analysis.

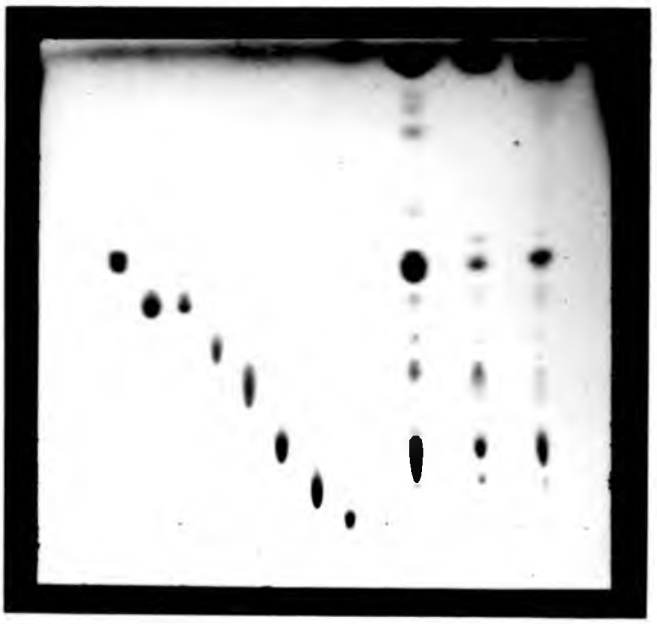
5.3.2 Composition of phospholipids from chloride cells

Using the methods described in Section 5.2 the composition of phospholipid from pure fractions of chloride cells from the gills of cod was determined (Table 5.2). The bulk of the phospholipid was phosphatidylcholine and phosphatidyl-ethanolamine which formed 41% and 27% respectively. Phosphatidylserine comprised 10% of the total and sphingomyelin, phosphatidylinositol and cardiolipin/phosphatidic acid formed the remainder in almost equal quantities.

5.3.3 Fatty acid composition of phospholipid classes from chloride cells

The fatty acid compositions of the phospholipids sphingomyelin (SM), phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), phosphatidylethanolamine (PtdEth), phosphatidylserine (PtdSer) and cardiolipin (CL)/phosphatidic acid (PtdA) from the chloride cells from the gills of the cod were analysed (Table 5.3).

The major fatty acids in PtdCho were 16:0, 18:1, 20:5(n-3) and 22:6(n-3) with saturates, total monounsaturates and polyunsaturates present in roughly equal amounts (32.8%, 32.1% and 35.1% respectively). The majority of the



1 2 3 4 5 6 7 8 9 10 11

Plate 5.2. Separation of standard phospholipids and samples of lipid from cod tissue by the HPTLC method described by Vitiello & Zanetta (1978). Phospholipids were visualised by charring. See Section 5.2 for further details of methods. Lane 1, PtdEth standard 10μg; Lane 2, CL standard 10μg; Lane 3, PtdA standard 10μg; Lane 4, PtdIns standard 10μg; Lane 5, PtdSer standard 10μg; Lane 6, PtdCho standard 10μg; Lane 7, SM standard 10μg; Lane 8, Ptd4,P_2 standard 5μg; Lane 9, cod brain lipid 10μg; Lane 10, cod liver lipid 10μg; Lane 11, cod gill lipid 10μg.

Table 5.1. A comparison of the phospholipid composition of heterologous cell preparations from the gills of *Gadus morhua* using different methods of determination.

Phospholipid	Method of quantitation			
	(i)	(ii)	(iii)	(iv)
PtdCho	41.9	40.0	41.5 ± 0.4 (3)	n.d.
PtdEth	30.0	24.4	24.5 ± 0.1 (3)	n.d.
PtdIns	5.4	14.6	5.3 ± 0.2 (3)	n.d.
PtdSer	9.7	10.5	12.4 ± 0.3 (3)	n.d.
Cardiolipin / PtdA	13.1	10.2	11.1 ± 0.1 (3)	n.d.
Sphingomyelin	n.d.	n.d.	4.5 ± 0.3 (3)	n.d.
PtdIns4P	n.d.	n.d.	n.d.	1.3 ± 0.3 (3)
PtdIns4,5P_2	n.d.	n.d.	n.d.	0.8 ± 0.2 (3)

(i) Thin layer chromatographic separation using the solvent system of Vitiello & Zanetta (1978), elution of phospholipid bands from silica and quantitation by weighing. (ii) Separation and elution as in (i) and quantitation by lipid phosphorus determination (Boehringer Mannheim kit). (iii) Separation as in (i) and quantitation by charring and densitometry. (iv) Thin layer chromatographic separation using the solvent system of Morse *et al.* (1987), elution of phospholipid bands from silica and quantitation by lipid phosphorus determination. Further details of the methods can be found in Section 5.2. Data are expressed as the percentage of the total phospholipid recovered and where appropriate are expressed as the means and standard deviations of the number of experiments in parenthesis. n.d. = not determined.

Table 5.2. Phospholipid composition of chloride cells from the gills of *Gadus morhua*.

Phospholipid	Composition (wt %)
Sphingomyelin	7.6 ± 0.2 (3)
Phosphatidylcholine	41.1 ± 0.8 (3)
Phosphatidylserine	10.3 ± 0.8 (3)
Phosphatidylinositol	7.0 ± 0.5 (3)
Cardiolipin / Phosphatidic Acid	7.1 ± 0.5 (3)
Phosphatidylethanolamine	26.9 ± 0.8 (3)

Phospholipids were separated by thin layer chromatography using the solvent system of Vitiello & Zanetta (1978) and quantitation carried out by charring and densitometry (refer to Section 5.2 for further details). Data are expressed as a percentage of the total phospholipid recovered. Values are the means and standard deviations of the number of experiments in parenthesis.

Table 5.3. Analyses of fatty acid methyl esters and dimethyl acetals prepared from phospholipids isolated from chloride cells from the gills of *Gadus morhua*.

	SM	PtdCho	PtdSer	PtdIns	Cardiolipin /PtdA	PtdEth
FAME						
14:0	5.3 2.9	1.3 0.5	0.5 0.2	0.7 0.3	0.8 0.2	tr
16:0	30.7 1.5	26.2 0.8	8.6 2.2	8.6 1.3	12.9 0.9	7.9 0.3
16:1*	3.5 0.9	5.0 0.4	2.1 0.7	2.0 0.3	6.1 2.8	3.2 2.6
17:0	1.8 0.5	1.9 0.2	5.1 1.3	1.0 0.8	2.4 0.8	3.4 0.6
18:0	15.2 2.2	3.2 0.6	26.8 2.2	33.8 3.4	13.6 6.0	11.6 1.9
18:1(n-9)	9.7 1.4	18.8 0.4	9.0 1.0	7.1 0.8	12.8 2.8	6.5 0.2
18:1(n-7)	2.7 0.4	3.5 0.1	4.2 0.3	3.5 0.7	7.5 2.3	5.1 1.8
18:2(n-6)	1.8 0.9	tr	1.0 0.2	0.8 0.2	1.2 0.4	tr
20:0	3.8 0.2	tr	1.3 0.2	-	-	tr
20:1*	6.9 2.9	4.1 0.6	10.3 3.0	5.6 1.8	3.7 0.3	4.3 1.6
20:4(n-6)	-	2.5 0.2	2.1 0.3	11.5 1.5	2.7 0.3	3.5 0.4
20:5(n-3)	1.5 0.4	12.3 0.9	4.0 0.5	11.5 1.7	6.2 2.2	10.3 1.1
22:1*	2.1 0.3	-	-	-	-	-
22:5(n-3)	-	0.9 0.1	0.8 0.1	0.8 0.0	0.9 0.1	1.2 0.1
22:6(n-3)	3.3 1.8	18.9 1.1	24.0 3.8	12.7 2.4	26.3 4.6	32.4 2.0
24:1*	12.4 2.0	0.6 0.3	-	-	-	-
DMA						
16:0						0.9 0.3
18:0						3.1 0.7
18:1(n-9)						4.1 1.5
18:1(n-7)						1.2 0.1
ΣSFA	56.1	32.8	40.3	44.3	29.7	27.2
ΣMUFA	37.1	32.1	25.8	18.1	32.4	24.3
Σ (n-6) PUFA	1.8	2.8	3.1	12.3	3.9	3.9
Σ (n-3) PUFA	4.9	32.3	28.5	24.7	33.4	44.3
Σ PUFA	6.7	35.1	31.6	37.0	37.3	48.2
(n-3)/(n-6)	2.7	11.5	9.2	2.0	8.6	11.4

Data are expressed as weight percentage and are the means and standard deviations of three individual experiments each based on the chloride cells from all the gill arches of three fish. The monounsaturates denoted * are the sum of isomers but are predominently the (n-9) isomer except for 16:1 which is predominently the (n-7) isomer. - signifies not detected and tr signifies present in at least two experiments at a value of less than 0.5%. FAME = fatty acid methyl esters, DMA = dimethyl acetals, ΣSFA = total saturated fatty acids, ΣMUFA = total monounsaturated fatty acids, ΣPUFA = total polyunsaturated fatty acids.

polyunsaturates were of the (n-3) series resulting in an (n-3)/(n-6) ratio of 11.5.

Phosphatidylethanolamine was characterized by having the highest amount of polyunsaturates (48.2%), again the majority of this being of the (n-3) series (44.3%), with the (n-6) series contributing only 3.9%. This resulted in PtdEth having a similar (n-3)/(n-6) ratio to that of PtdCho (11.4). The saturated and monounsaturated fatty acids each comprised about a quarter of the sample (27.2% and 24.3% respectively). Phosphatidylethanolamine differed from all the other phospholipids analysed in that 9.6% of the acid-catalysed transmethylation products were in the form of dimethylacetals indicating that approximately one fifth of the total PtdEth was present as PtdEth plasmalogen (1-*O* -alkenyl-2-acyl glycerophosphoethanolamine). This could however be an underestimate since this is an unreliable method of determining plasmalogen (Christie, 1982).

In PtdSer 18:0 and 22:6(n-3) each comprised about a quarter of the total fatty acid with 16:0, 18:1 and 20:1 also abundant. Saturated fatty acids totalled 40.3% whereas monounsaturated fatty acids and polyunsaturated fatty acids totalled 25.8% and 31.6% respectively. The polyunsaturates again formed a relatively high (n-3)/(n-6) ratio of 9.2.

In sphingomyelin saturated fatty acids constituted more than half (56.1%), and the monounsaturated fatty acids 37.1% of the total fatty acids. The amount of polyunsaturated fatty acids in SM was the lowest of all the phospholipids examined at 6.7%. Of this 4.9% was of the (n-3) series (much lower than in any of the other phospholipids), and 1.8% was of the (n-6) series. The resulting (n-3)/(n-6) ratio of 2.7 was more akin to that of phosphatidylinositol than to the other phospholipids.

The band containing cardiolipin and phosphatidic acid had roughly equal amounts of saturates, monounsaturates and polyunsaturates (29.7%, 32.4% and 37.3%) and again (n-3) polyunsaturates predominated resulting in a relatively high (n-3)/(n-6) ratio of 8.6.

Phosphatidylinositol showed characteristically large amounts of 18:0 and 20:4(n-6), the latter about five times more abundant than in any other phospholipid class. Arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were almost equally abundant resulting in a much lower (n-3)/(n-6) ratio of 2.0. Phosphatidylinositol had a similar pattern of fatty acids to PtdSer with the saturated fatty acids forming 44.3%, the monounsaturates 18.1% and the polyunsaturates 37.0% of the total.

5.3.4 Studies on incorporation of radioisotopes into chloride cells

Initially a series of time course experiments was carried out to determine the time taken to reach equilibrium for incorporation of [^{32}P]-orthophosphate into the phospholipids of chloride cells. Chloride cells were incubated in the presence of [^{32}P]-orthophosphate and samples removed at hourly intervals up to 5 hours (Figure 5.2). The amount of radioactivity increased over the first three hours of incubation then slowed and plateaued after four to five hours. The incorporation into the major individual phospholipid classes over the same period is shown in Figure 5.3. This shows a similar trend to that seen for the total lipid sample. Radioincorporation increased throughout the first two to three hours and reached equilibrium in most of the phospholipids by around 3-4 hours. A minimum incubation time of four hours with [^{32}P]-orthophosphate was thus thought to be

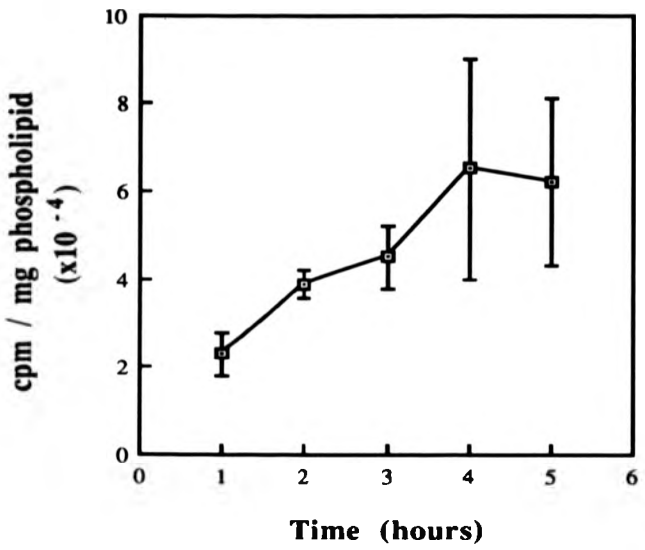


Figure 5.2. Time course of incorporation of [^{32}P]-orthophosphate into the lipid fraction from chloride cells from the gills of *Gadus morhua*. Chloride cells (1g) were incubated in phosphate-free HEPES buffer (10ml) and 500µCi of carrier-free [^{32}P]-orthophosphate at 10°C for up to 5 hours. Reactions were terminated and phospholipid extracted as described in Section 5.2. Data represent the means and standard deviations of three individual experiments.

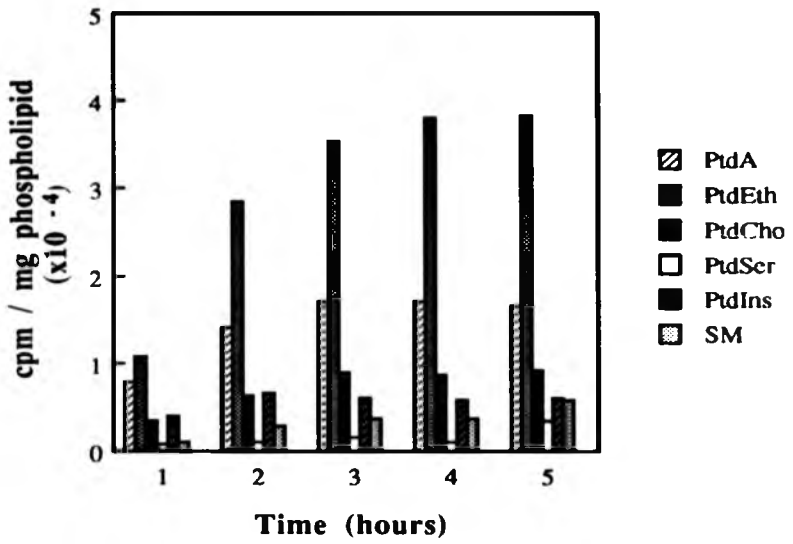


Figure 5.3. Time course of incorporation of [^{32}P]-orthophosphate into individual phospholipids from chloride cells from the gills of *Gadus morhua*. Chloride cells (1g) were incubated in phosphate-free HEPES buffer (10ml) and 500μCi of carrier-free [^{32}P]-orthophosphate at 10°C for up to 5 hours. Reactions were terminated and phospholipid extracted and purified as described in Section 5.2.

necessary in order to label the gill phospholipids to equilibrium.

The pattern of [^{32}P]-orthophosphate incorporation into phospholipids labelled to equilibrium and separated by HPTLC can be seen in Plate 5.3. The majority of the radioactivity appears to correspond to the PtdEth band with lesser amounts in the bands corresponding to PtdCho, PtdIns and PtdA.

The preliminary radioincorporation and autoradiographic studies provided no evidence for the rapid labelling, and therefore turnover, of the polyphosphoinositides. However with the use of standards to locate PtdIns4P and PtdIns4,5P_2 and separation of the phospholipids in the solvent described by Morse *et al.* (1987) it was possible to assay the appropriate band for radioactivity. The results of incorporation of [^{32}P]-orthophosphate over a 5 hour incubation period are presented in Figure 5.4. This shows a relatively high level of incorporation compared with the more abundant phospholipids and the specific activity of incorporation into PtdIns4,5P_2 is comparable with that in PtdEth after 5 hours.

The high level of radioactivity in the phospholipid band corresponding to PtdEth was unexpected. Additionally the radioactivity often corresponded with the region at the trailing edge of the PtdEth band. In an attempt to determine whether the radioactivity was indeed associated with PtdEth or whether it was associated with some other lipid co-migrating with PtdEth the band was scraped from the TLC plate and treated with phospholipase C, re-extracted and the lipid and aqueous fractions assayed for radioactivity. It was found that all of the radioactivity was lost to the aqueous fraction indicating that the 'unknown'/PtdEth radioactive band was in fact a phosphoglyceride. Additionally when subjected to acid digestion the 'unknown'

Plate 5.3. Autoradiogragh showing the separation of radioactively labelled phospholipids from the chloride cell lipid fraction. Phospholipid was separated by the HPTLC method described by Vitiello & Zanetta (1978) and the dried HPTLC plate exposed to X-ray film overnight. See Section 5.2 for details of methods.

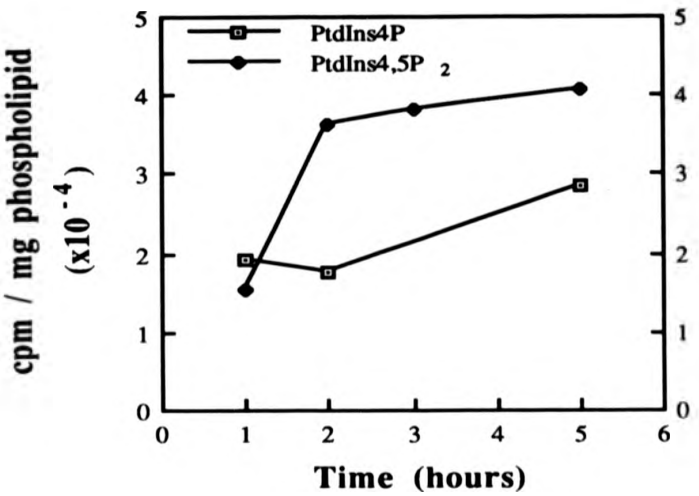


Figure 5.4. Time course of incorporation of [^{32}P]-orthophosphate into the polyphosphoinositides from chloride cells from the gills of *Gadus morhua*. Chloride cells (1g) were incubated in phosphate-free HEPES buffer (10ml) and 500μCi of carrier-free [^{32}P]-orthophosphate at 10°C for up to 5 hours. Reactions were terminated and phospholipid extracted and purified as described in Section 5.2.

phosphoglyceride had an unchanged Rf value upon chromatography.

Because an unusual pattern was detected in the labelling of chloride cells a comparison of incorporation into the brain and liver tissues of cod with gill tissue was carried out. Labelling of bands corresponding to PtdIns4P and PtdIns4,5P_2 was detected in both liver and brain samples (data not presented). Thus the unusual pattern observed in gills was unique to that tissue.

A series of experiments were carried out to investigate the incorporation of [^{32}P]-orthophosphate into the water-soluble inositol phosphates. Separation of inositol phosphates by paper chromatography proved to be troublesome since there appeared to be a large amount of background radioactivity making it impossible to detect individual inositol phosphates especially near to the origin. Additionally individual inositol phosphates were often not well separated and therefore difficult to analyse. These problems could not be resolved so that further experiments using this method were thought to be unjustified especially since other evidence suggested that the PtdIns cycle was not particularily active in chloride cells.

A study of the incorporation of [methyl-^{3}H]-choline into individual choline metabolites was performed and the typical pattern of separation by HPTLC of the labelled water-soluble compounds after a 4 hour incubation is presented in Figure 5.5. Almost half of the total radioactivity recovered was found in the band corresponding to choline/acetylcholine, 32% in the phosphocholine band, 6% in the glycerophosphocholine band and 2% in the band corresponding to CDP-choline.

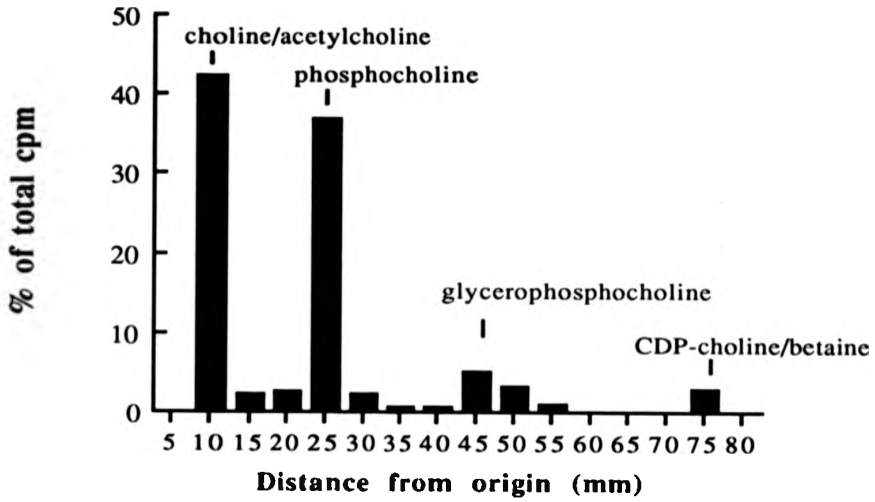


Figure 5.5. Separation of water-soluble choline metabolites from the chloride cells from the gills of *Gadus morhua*. Chloride cells (1g) were incubated in HEPES buffer (10ml) and 50μCi of [methyl-^{3}H]-choline at 10°C for 4 hours. Reactions were terminated and the aqueous phase extracted as described in Section 5.2. Choline metabolites were separated by thin layer chromatography using the solvent system of Yavin (1976). The graph shows the amount of radioactivity detected along the length of the TLC plate and the expected positions of the various metabolites.

5.3.5 The effects of atrial natriuretic factor upon chloride cell metabolites

(i) Effects on phospholipids

The effect of atrial natriuretic factor (10^{-7}M) on the phospholipid composition of chloride cells over a 1 hour period was investigated (Table 5.4). No significant differences were detected on incubation with the hormone. Additionally, a series of experiments were carried out to determine the effects of ANF on the incorporation of [^{32}P]-orthophosphate into the major phospholipids. After preincubation to equilibrium and treatment of chloride cells with ANF (10^{-7}M) analysis of incorporation into the phospholipids PtdEth, PtdIns, PtdCho and PtdA was performed and expressed as cpm per mg lipid. The results of sampling these phospholipids at 30 seconds, 5 minutes and 1 hour after ANF administration are summarised in Figures 5.6-5.9 respectively. There were no significant changes in the amounts of radioactivity in these phospholipids between control and experimental samples. Likewise the incorporation of [^{32}P]-orthophosphate into the polyphosphoinositides was also examined after treatment of chloride cells with ANF but again there were no significant changes upon hormone administration (Figure 5.10). The results of these and additional [^{32}P]-orthophosphate incorporation experiments are summarised in Table 5.5 where the data are expressed as % change of initial radioactivity.

The effects of atrial natriuretic factor on the incorporation of [methyl-^{3}H]-choline into the phospholipids revealed that most of the radioactivity (95%) was to be found in PtdCho as would be expected but there was very little incorporation into sphingomyelin (<2%). These values did not change on administration of ANF to chloride cells (Table 5.6).

Table 5.4. The effect of atrial natriuretic factor (10^{-7}M) on the phospholipid composition of chloride cells from the gills of *Gadus morhua*.

Phospholipid	% Composition			
	Time after hormone administration			
	Control	30sec	5min	1hour
Sphingomyelin	7.3 ± 1.7	7.2 ± 1.8	8.2 ± 2.1	8.1 ± 0.6
Phosphatidylcholine	46.3 ± 4.6	46.1 ± 3.1	45.6 ± 3.9	46.4 ± 6.6
Phosphatidylserine	7.1 ± 1.7	6.9 ± 0.5	7.5 ± 0.1	8.1 ± 0.5
Phosphatidylinositol	5.0 ± 0.8	4.6 ± 1.7	4.3 ± 0.9	5.6 ± 0.9
Cardiolipin / Phosphatidic Acid	9.2 ± 3.6	9.5 ± 3.8	9.6 ± 4.9	8.3 ± 2.3
Phosphatidyl-ethanolamine	25.1 ± 4.4	25.7 ± 2.6	24.7 ± 3.2	23.6 ± 4.8

Chloride cells (1g) were resuspended in 4ml of HEPES buffer and split into 4 x 1ml aliquots. ANF was added to three of these to a final concentration of 10^{-7}M and the flasks containing hormone were incubated at 10°C. Control and experimental reactions were terminated and phospholipid extracted and purified as described in Section 5.2. Data are expressed as the percentage of total phospholipid and are the means and standard deviations of three separate experiments. There were no differences in phospholipid composition between control and hormone treated samples as determined by Student's paired t-test.

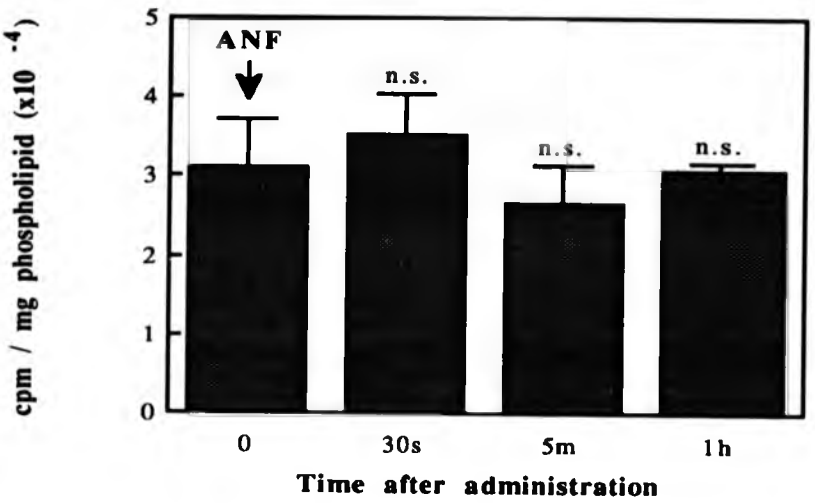


Figure 5.6. The effect of atrial natriuretic factor (ANF) on phosphatidylethanolamine metabolism in the chloride cells from the gills of *Gadus morhua*. Chloride cells were preincubated in HEPES buffer (10ml) containing 500μCi of carrier-free [^{32}P]-orthophosphate at 10°C for 4 hours. Radioactive labelling was monitored at 30 seconds, 5 minutes and 1 hour after addition of the hormone. Reactions were terminated and phospholipids extracted and purified as described in Section 5.2. Data represent the means and standard deviations of three individual experiments. None of the observations were significantly different from the control value as determined by Student's t-test.

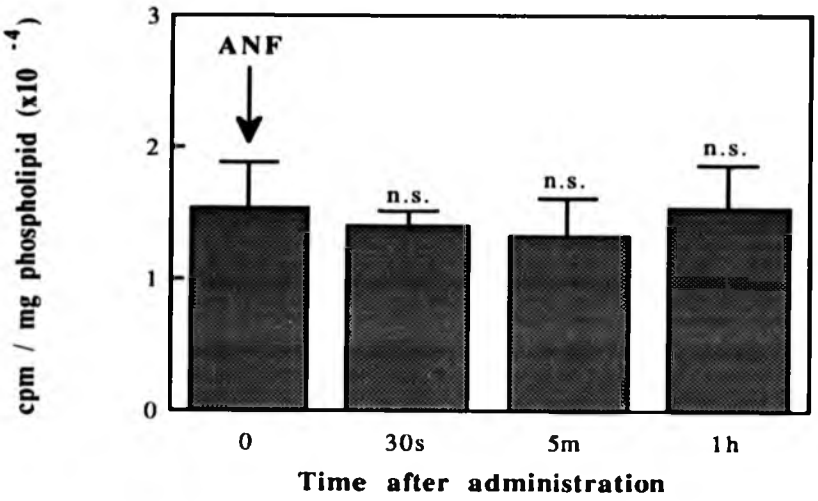


Figure 5.7. The effect of atrial natriuretic factor (ANF) on phosphatidylinositol metabolism in the chloride cells from the gills of *Gadus morhua*. Methods as described in Figure 5.6. Data represent the means and standard deviations of three individual experiments. None of the observations were significantly different from the control value as determined by Student's t-test.

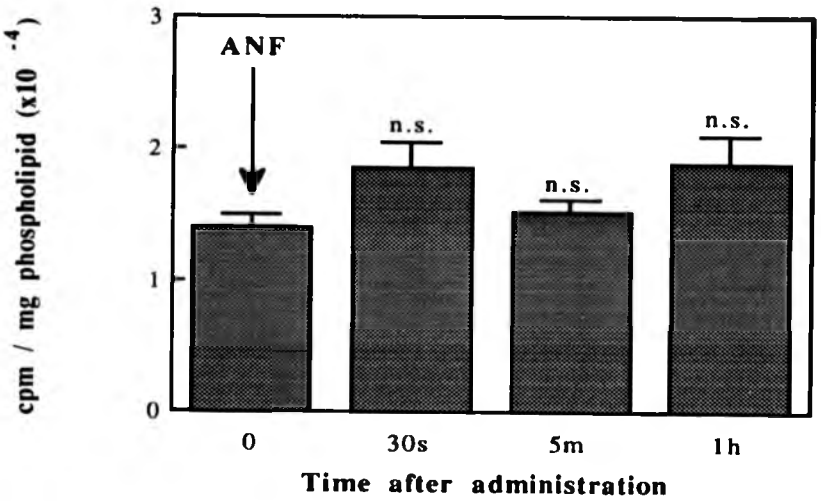


Figure 5.8. The effect of atrial natriuretic factor (ANF) on phosphatidylcholine metabolism in the chloride cells from the gills of *Gadus morhua*. Methods as described in Figure 5.6. Data represent the means and standard deviations of three individual experiments. None of the observations were significantly different from the control value as determined by Student's t-test.

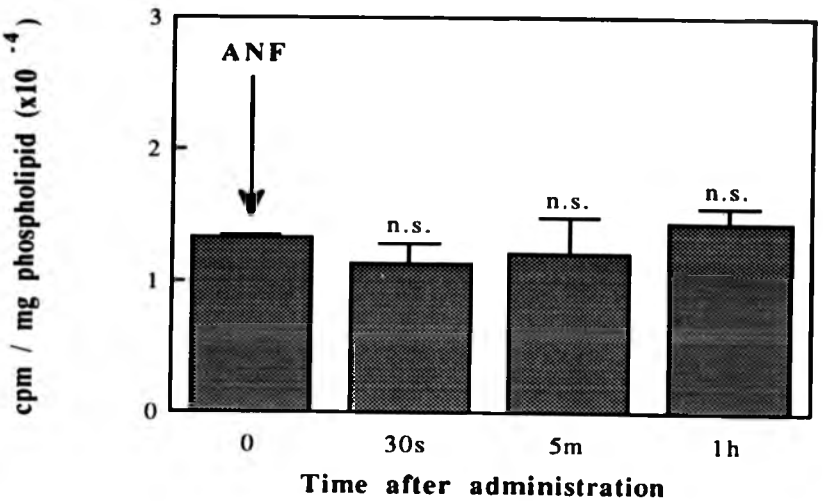


Figure 5.9. The effect of atrial natriuretic factor (ANF) on phosphatidic acid metabolism in the chloride cells from the gills of *Gadus morhua*. Methods as described in Figure 5.6. Data represent the means and standard deviations of three individual experiments. None of the observations were significantly different from the control value as determined by Student's t-test.

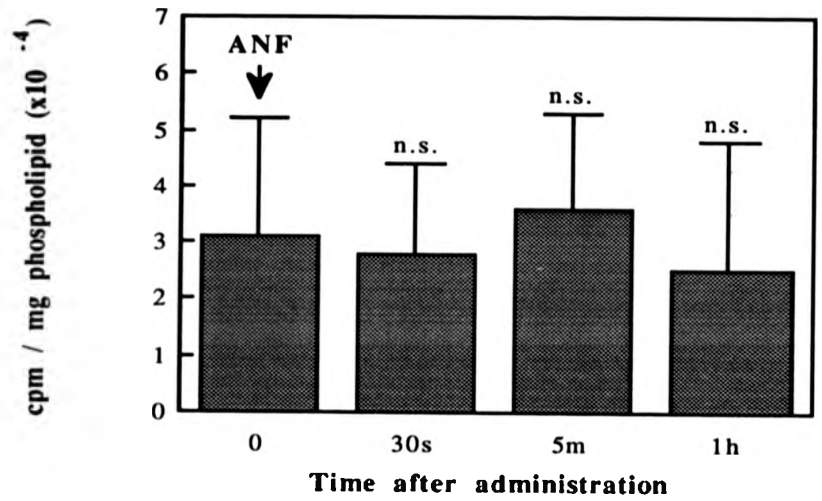


Figure 5.10. The effect of atrial natriuretic factor (ANF) on polyphosphoinositide (PtdIns4P and PtdIns4,5P_2) metabolism in the chloride cells from the gills of *Gadus morhua*. Methods as described in Figure 5.6. Data represent the means and standard deviations of three individual experiments. None of the observations were significantly different from the control value as determined by Student's paired t-test.

Table 5.5. The effect of atrial natriuretic factor (10^{-7}M) on incorporation of [^{32}P]-orthophosphate into individual phospholipid classes from the gills of *Gadus morhua*.

Phospholipid	% of total radioincorporation			
	Time after administration			
	Control	30sec	5min	1hour
Sphingomyelin	4.9 ± 3.0	5.0 ± 2.5	6.5 ± 3.7	3.8 ± 2.4
Phosphatidylcholine	13.6 ± 1.7	16.6 ± 3.6	15.5 ± 2.5	16.9 ± 1.5
Phosphatidylserine	1.8 ± 1.8	2.0 ± 1.5	1.6 ± 0.9	2.5 ± 1.9
Phosphatidylinositol	24.9 ± 3.7	21.7 ± 4.5	22.8 ± 3.9	23.2 ± 2.2
Cardiolipin / Phosphatidic acid	22.1 ± 4.6	20.1 ± 2.6	20.2 ± 2.0	21.0 ± 1.7
Phosphatidyl-ethanolamine	34.7 ± 2.9	34.7 ± 6.0	33.4 ± 2.9	32.6 ± 5.8
Polyphospho-inositides	13.2 ± 7.6	12.5 ± 6.1	16.9 ± 5.6	14.4 ± 9.3

Chloride cells (1g) were incubated in phosphate-free HEPES buffer (10ml) containing 500μCi [^{32}P]-orthophosphate for 4 hours at 10°C. Cells were washed in phosphate-containing HEPES buffer, resuspended in 4 x 1ml aliquots. Atrial natriuretic factor (10^{-7}M) was added to three of these and incubated at 10°C. Control and experimental reactions were terminated and phospholipids extracted, purified and assayed for radioactivity as described in Section 5.2. The polyphosphoinositide data was obtained from a series of separate experiments. Data are expressed as percentage of total radioactivity recovered and are the means and standard deviations of three individual experiments.

Table 5.6. The effect of atrial natriuretic factor (10^{-7}M) on incorporation of [methyl-^{3}H]-choline into individual phospholipid classes.

Phospholipid	% of total radioincorporation			
	Time after administration			
	Control	30sec	5min	1hour
Sphingomyelin	1.3 ± 0.9	1.3 ± 0.7	1.3 ± 0.9	2.2 ± 0.4
Phosphatidylcholine	90.9 ± 1.9	90.5 ± 0.5	90.9 ± 1.0	89.7 ± 2.1
Phosphatidylserine	1.9 ± 0.2	2.6 ± 0.8	2.5 ± 0.4	3.0 ± 0.5
Phosphatidylinositol	1.3 ± 0.1	0.9 ± 0.3	2.2 ± 1.6	1.0 ± 0.3
Cardiolipin / Phosphatidic acid	1.7 ± 1.1	1.9 ± 0.7	1.8 ± 0.6	2.0 ± 1.1
Phosphatidyl-ethanolamine	3.1 ± 1.3	2.8 ± 1.0	3.0 ± 0.7	2.8 ± 1.7

Chloride cells (1g) were incubated in choline-free HEPES buffer (10ml) containing 50μCi [methyl-^{3}H]-choline for 4 hours at 10°C. Cells were washed in choline -containing HEPES buffer, resuspended in 4 x 1ml aliquots. Atrial natriuretic factor (10^{-7}M) was added to three of these and incubated at 10°C. Control and experimental reactions were terminated and phospholipids extracted, purified and assayed for radioactivity as described in Section 5.2. Data are expressed as percentage of total radioactivity recovered and are the means and standard deviations of three individual experiments.

(ii) Effects on water-soluble choline metabolites

The effects of ANF on the incorporation of [methyl-^{3}H]-choline into the water-soluble choline metabolites was investigated. The metabolites were separated by HPTLC as described in the methods section (Yavin, 1976) and bands corresponding to choline/acetylcholine, phosphocholine, glycerophosphocholine and CDP-choline were assayed for radioactivity after administration of ANF. The results obtained are presented in Table 5.7 and are expressed as the % of the radioactivity found in control samples. The amount of radioactivity in the band corresponding to choline/acetylcholine was significantly reduced to 80% of the control value 1 hour after administration of ANF ($p<0.05$) (Figure 5.11). Incorporation into the band corresponding to phosphocholine fell slightly 30 seconds after hormone treatment, remained at around this level at 5 minutes and returned to basal levels 1 hour after administration. None of these changes were significant. There was a slight decrease in the amount of incorporation in the glycerophosphocholine band at 30 seconds but by 5 minutes there was an increase in the amount of radioincorporation corresponding to a rise of 35%. After 1 hour the increase was significantly different from that of the initial level ($p<0.05$). The most pronounced change in incorporation of [methyl-^{3}H]-choline was observed in the band corresponding to CDP-choline. A 59% increase in incorporation into CDP-choline was recorded 30 seconds after hormone administration though this was not significantly different from control values. Incorporation rose further by 62% and 130% after 5 minutes and 1 hour respectively both results being significantly different from the control. These results suggest rapid turnover of CDP-choline and may signify a role for phospholipase A_2 in agonist stimulated choline metabolism.

Table 5.7. The effect of atrial natriuretic factor (10^{-7}M) on the incorporation of [methyl-^{3}H]-choline into choline metabolites from the chloride cells from the gills of *Gadus morhua*.

Metabolite	% of total [methyl-^{3}H]-choline incorporation	% of initial value		
		Time after administration		
		30sec	5min	1hour
Choline	47.8 ± 15.3	115 ± 14	107 ± 6	81 ± 6*
Phosphocholine	31.2 ± 8.7	76 ± 27	81 ± 18	108 ± 2
Glycerophospho-choline	6.3 ± 3.4	84 ± 27	135 ± 43	138 ± 27*
CDP-choline	1.6 ± 0.9	159 ± 56	162 ± 6**	230 ± 6***

Chloride cells (1g) were incubated in choline-free HEPES buffer (10ml) containing 50μCi [methyl-^{3}H]-choline for 4 hours at 10°C. Cells were washed in choline-containing HEPES buffer and resuspended in 4 x 1ml aliquots. Atrial natriuretic factor (10^{-7}M) was added to three of these and the samples incubated at 10°C. Control and experimental reactions were terminated and choline metabolites extracted, purified and assayed for radioactivity as described in Section 5.2. Data are expressed as percentage of total radioactivity recovered and are the means and standard deviations of three individual experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

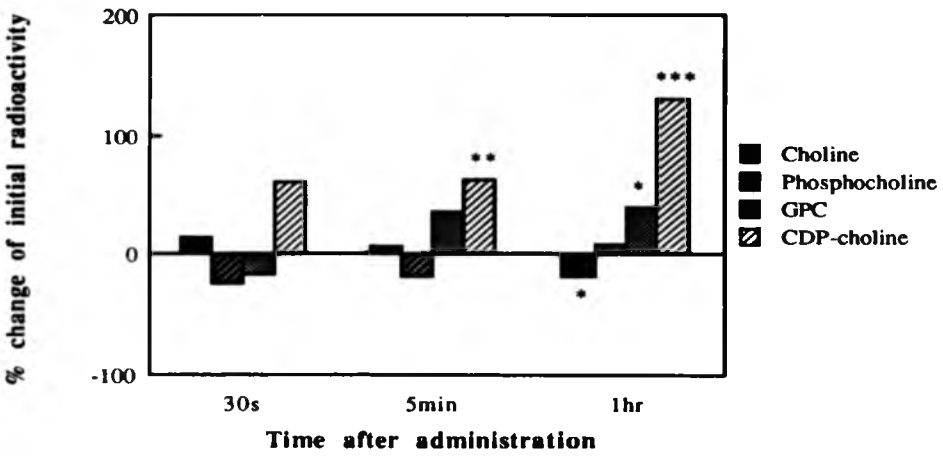


Figure 5.11. The effect of atrial natriuretic factor (ANF) on choline metabolism in the chloride cells from the gills of *Gadus morhua*. Chloride cells were preincubated in HEPES buffer (10ml) containing 50μCi of [methyl-^{3}H]-choline at 10°C for 4 hours. Radioactive labelling was monitored at 30 seconds, 5 minutes and 1 hour after addition of the hormone. Reactions were terminated and the aqueous choline metabolites extracted and purified as described in Section 5.2. Data represent the means of three individual experiments (standard deviations not shown). Student's paired t-test was used to determine the significance of the difference between control and experimental systems. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

(iii) Effects on fatty acid composition of diacylglycerol

In order to investigate whether ANF could initiate the phospholipase C-mediated release of DAG from either PtdCho and/or PtdIns the fatty acid composition of DAG was analysed before and after treatment of chloride cells with ANF. The results of this experiment reveal that no significant changes occured in the amounts of the predominant fatty acids after administration of hormone (Table 5.8).

Table 5.8. The effect of atrial natriuretic factor (10^{-7}M) on the fatty acid composition of diacylglycerol isolated from chloride cells from the gills of *Gadus morhua*.

FAME	Time after administration							
	Control		30 sec		5 min		1 hour	
14:0	4.0	1.6	4.0	2.2	3.9	1.9	5.6	4.1
16:0	19.5	3.1	19.6	5.1	20.2	4.0	20.5	3.9
16:1*	1.5	0.1	2.6	1.0	2.7	1.5	2.9	1.2
18:0	19.8	4.4	25.2	9.6	25.0	14.3	22.9	14.6
18:1*	19.8	8.2	20.3	4.7	16.0	4.5	20.5	3.7
18:2	2.3	1.3	3.4	1.9	3.1	1.7	2.0	0.5
20:1*	6.4	1.6	6.1	2.4	5.4	3.1	4.6	2.9
20:4(n-6)	3.0	2.0	2.6	1.7	2.6	1.4	2.3	1.3
20:5(n-3)	7.1	4.4	6.6	4.2	7.7	2.2	7.0	5.0
22:6(n-3)	16.7	8.9	14.4	10.7	13.5	9.7	11.8	8.7

Chloride cells (1g) were resuspended in 4 x 1ml aliquots of HEPES buffer and hormone was added to three of these to a final concentration of 10^{-7}M. Cells were incubated at 10°C and samples removed at appropriate time intervals. Control and experimental reactions were terminated, lipid extracted and purified and fatty acid methyl esters (FAME) prepared as described in Section 5.2. Data are expressed as weight percent and are the means and standard deviations of three individual experiments. The monosaturates denoted * are the sum of the isomers but are predominently the (n-9) isomer except for 16:1 which is predominently the (n-7) isomer. There were no significant differences between the control and experimental data as determined by Anova one-way analysis of variance.

5.4 DISCUSSION

Having established possible roles for the hormone ANF and the second messenger DAG (Chapters 2 and 4 of this thesis) this set of experiments aimed to investigate whether the action of ANF as a primary message was linked to the generation of the second messenger DAG and which, if any, of the phospholipids were involved in the transduction of the primary signal.

Initial work focussed on the separation and quantitation of phospholipids from chloride cells, a necessary prerequisite to further studies. A comparison of different methods was conducted and it was concluded that for routine analysis of the main phospholipid classes HPTLC using the solvent described by Vitiello & Zanetta (1978) gave the most satisfactory resolution. Phospholipids were quantitated by charring followed by densitometry since gravimetric quantification gave inconsistent results and was useful only for relatively large amounts of lipid. Since the polyphosphoinositides are present in very small amounts they could not be visualised by charring and therefore could not be quantitated by densitometry. Phosphorus determination was used to quantify the polyphosphoinositides.

With respect to lipid composition the epithelium of the teleost gill is similar to that of most other tissues (Mommsen, 1984). Phosphatidylcholine, phosphatidyl-inositol and phosphatidylethanolamine together account for over 80% of total phospholipid (Thomas & Patton, 1972; Phleger, 1978) but a greater percentage of phosphatidylserine and sphingomyelin occurs in the osmoregulatory organs (gill, gut and kidney) of *Anguilla anguilla*. The phospholipid class composition and the fatty acid composition of the individual phospholipid classes of chloride cells from cod gills has not been determined before. The phospholipid class composition in

the present study was found to be very similar to the composition of whole gill cell populations. Lie & Lambertsen (1991) reported the phospholipid composition of cod gill tissue to be PtdCho 61%, PtdEth 21%, PtdIns 7% and PtdSer 11% which compares well with the findings of the present study. In addition the class composition of the phosphatidylinositol lipids compares well with the study by Simpson & Sargent which found that PtdIns, PtdIns4P and PtdIns4,5P_2 comprised 9.1%, 1.0% and 0.9% of total cellular phospholipid in dogfish rectal gland (Simpson & Sargent, 1985).

Just as the composition of phospholipids can often provide valuable information about the possible function of the tissue from which they were extracted so too can the fatty acid composition of these lipids. The main phospholipid classes showed the characteristic fatty acid compositions now well established for fish tissues. In PtdCho 16:0, 18:1, 20:5(n-3) and 22:6(n-3) were the main fatty acids, while PtdEth was also dominated by these four fatty acids in slightly different proportions. Phosphatidylserine was characterised by large amounts of 18:0 and 22:6(n-3) while PtdIns also contained a high proportion of 18:0 and the highest proportion of 20:4(n-6) of any phospholipid class. Sphingomyelin was dominated by saturated and monosaturated fatty acid, including 24:1 which was barely present in any other phospholipid class. These results are in good agreement with earlier studies which determined the fatty acid composition of phospholipids from the microsomal fraction from an unpurified cell preparation for cod gills (Bell *et al.* 1983), the results of Lie & Lambertson (1991) for whole gill tissue and with more recent molecular species compositional data obtained from several tissues from cod (Bell & Dick, 1990; 1991). This is as expected since chloride cells, although not the predominant cell type in the gills, do have extensively infolded basal and lateral plasma membranes which probably contribute the major proportion of the total

membrane present in a mixed cell population and most of the microsomes in *in vitro* studies.

It has been known for some years that even in species of fish where (n-3) PUFA are predominant in the structural phospholipids PtdCho, PtdEth and PtdSer, in PtdIns arachidonic acid is often the most abundant PUFA (Bell *et al.* 1983). More recent studies have shown that 18:0/20:4(n-6) is the main molecular species of PtdIns in most fish tissues as it is in terrestrial animals (Bell, 1989; Bell & Tocher, 1989; Bell & Dick, 1990). As for mammalian PtdIns, the high level of arachidonic acid in PtdIns from fish points to a pivitol metabolic role for this minor phospholipid as the source of arachidonic acid for eicosanoid synthesis in marine species. However, 20:5(n-3) was equally abundant in PtdIns from chloride cells suggesting that there could be production of the 3-series prostaglandins in the chloride cells of the gills of cod. The 18:0/20:5(n-3) molecular species of PtdIns was recently found to be predominant in the brains of trout and cod (Bell & Tocher, 1989; Bell & Dick, 1990).

The phosphatidylinositol lipid composition in the present study compares well with that obtained from the spotted dogfish (*Scyliorhinus canicula*) rectal gland (Simpson & Sargent, 1985). The shark rectal gland is rich in phosphatidylinositol lipids and studies with [^{32}P]-orthophosphate have shown active turnover of PtdIns in non-stimulated rectal gland slices which was reduced upon cAMP administration (reviewed by Sargent *et al.* 1987). The same paper reviews the role of cAMP in salt excretion in the rectal gland. When cAMP levels are increased there is reduced turnover of PtdIns and an increased excretion of salt by the gland. In contrast to this administration of adrenaline (10^{-5}M) results in increased PtdIns turnover and reduced salt secretion in the gills of seawater eels (Girard *et al.* 1977) and

acetylcholine increases PtdIns turnover and salt excretion in the avian nasal salt gland (Hokin & Hokin, 1959; 1960). Although cAMP plays a central role in controlling salt secretion in rectal gland exactly how it exerts its effect on Na^+-K^+-ATPase is unknown though a cAMP-dependent protein kinase is involved (Yeh *et al.* 1983). A role for cAMP in the control of sodium transport in the gills from a marine fish has yet to be determined.

Radioactive incorporation studies with [^{32}P]-orthophosphate were initially carried out in chloride cells to see if PtdIns turnover was occuring in the resting, unstimulated state in the cod gill. Examination of autoradiographs revealed an unusual pattern of radioactive labelling with the highest level of incorporation of [^{32}P]-orthophosphate in a band corresponding to or running just below the main bulk of PtdEth (Plate 5.3). Although the other major phospholipids were also labelled to a lesser extent no radioactivity was seen in autoradiographs in the areas corresponding to PtdIns4P or PtdIns4,5P_2. Assay of the silica bands for radioactivity confirmed the high level of incorporation into PtdEth with lesser amounts in the other main phospholipids. Analysis of bands corresponding to standards of PtdIns4P and PtdIns4,5P_2 revealed that these phospholipids did incorporate relatively high specific activities of radioactivity though due to the very small quantity of polyphosphoinositides present this radioactivity could not be detected by autoradiography. The specific activities of the phosphoinositides in the present study were two orders of magnitude lower than those described for the rectal gland of *Scyliorhinus canicula* (Simpson, 1984). Difficulty in labelling the phosphatidylinositides in cod gills was also experienced by Bell (personal communication) when determining the fatty acid composition of PtdIns, PtdIns4P and PtdIns4,5P_2 from cod gills where radiolabelled carrier lipid prepared from eels

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was used in order to detect these products (Bell *et al.* 1985). Due to the unexpected labelling pattern of gill phospholipids observed here cod liver and brain tissue were also labelled with [^{32}P]-orthophosphate. These tissues showed the expected labelling pattern with high incorporation into phosphatidylinositol metabolites and much less incorporation in other phosphoglycerides, including PtdEth. Interestingly work by Zwingelstein and his co-workers revealed that in the gut and the gill of the eel PtdEth showed a greater turnover rate than PtdCho (Zwingelstein *et al.* 1975; 1980; Zwingelstein, 1979) and a recent study by Stark *et al.* (1993) showed that PtdEth was the most highly labelled major phospholipid in *Drosophila* heads when incubated with [^{32}P]-orthophosphate.

The unusual pattern of labelling of phospholipids with [^{32}P]-orthophosphate in gill chloride cells, and especially the nature of the radioactive band corresponding to PtdEth, warranted more attention. The lipid behaved as PtdEth in two chromatography systems, was hydrolysed by phospholipase C but not by dilute acid. This behaviour is compatible with diacyl- or 1-*O* -alkyl-2-acyl-glycero-phosphoethanolamine (GPE) but not with 1-*O* -alkenyl-2-acyl GPE (PtdEth plasmalogen). Ethanolamine glycerophospholipids have not previously been implicated in cellular signalling systems and this seems to be the case here since there was no change in [^{32}P]-orthophosphate labelling on administration of ANF. The high incorporation of [^{32}P]-orthophosphate into PtdEth therefore remains something of a mystery.

Administration of the hormone ANF to chloride cells labelled to equilibrium with [^{32}P]-orthophosphate did not reveal any changes in the pattern of labelling in the lipid products of PtdIns metabolism (PtdIns, PtdA, PtdIns4P and PtdIns4,5P_2)

though the change in the amount of radioincorporation in PtdCho upon ANF administration, though small, warranted further investigation. The low incorporation of [^{32}P]-orthophosphate into chloride cells from cod compared with dogfish rectal gland or eel gills suggests a fundamental difference between PtdIns metabolism in stenohaline fish and sharks and euryhaline fish.

As a complimentary approach to the investigation of PtdIns metabolism an examination of the water-soluble inositol phosphates produced during PtdIns turnover was carried out using paper chromatography. In metabolic studies it is always technically easier to look for an increase in a product from a low level or zero (e.g. inositol phosphates) rather than for a small decrease in a substrate (e.g. phosphoinositides). Complicated protocols have been described for the resolution and identification of inositol phosphate isomers but for this study it was felt that a simple method to detect total inositol phosphate classes would be sufficient and paper chromatography was tried. Though the technique gave inconsistent and irreproducible results there was no evidence of production of inositol phosphate. This confirmed the results of the phosphoinositide labelling experiments.

Studies with [methyl-^{3}H]-choline labelled cells to investigate the effects of ANF on the production of water-soluble PtdCho metabolites in chloride cells revealed a substantial rise in radioactively labelled CDP-choline 30 seconds after administration and a continued increase up to 1 hour which was also accompanied by an increase of radioincorporation into glycerphosphocholine. The production of CDP-choline via phosphocholine is dependent on CT activity and it would appear that ANF might be involved in the control of CT activity. The mechanism by which this could occur is unknown though CT has been likened to PKC (refer to Chapter 5.1 for more details) and control might be by translocation of the enzyme between

cytoplasmic and membrane-bound forms and/or by a phosphorylation/dephosphorylation reaction (Pelech & Vance, 1989). The initial slight fall in incorporation in phosphocholine and the subsequent fall in incorporation in choline supports CT-mediated generation of CDP-choline. Phospholipase A_2 activity on PtdCho produces arachidonic acid and lyso-PtdCho which can be further catabolised to glycerophosphocholine. Thus the increased turnover of glycerophosphocholine after 1 hour might indicate a putative role for phospholipase A_2 in the response to ANF receptor stimulation.

Arachidonic acid and other unsaturated fatty acids can be derived from inositol lipids through consecutive reactions involving phospholipase C and diacylglycerol lipase or via PtdA by its specific lipase and may be important '3rd messengers' in PtdIns turnover (McPhail *et al.* 1984). However in most mammalian tissues arachidonic acid is released by phospholipase A_2 action on PtdCho and PtdEth and it is possible that after receptor stimulation both PtdCho and PtdEth phospholipases act in concert (Nishizuka, 1984). The differential effects of arachidonic acid on isolated PKC isozymes suggest that this fatty acid may be an isozyme selective PKC activator (Shearman *et al.* 1989) but although it is clear that arachidonic acid activates isolated PKC it remains to be determined whether it does so in intact cells. However, this effect is not specific to arachidonic acid since other free fatty acids can also activate PKC *in vitro* (Merrill, 1989). A recent study suggested that stimulation of phospholipase A_2 and arachidonic acid release occurring during dopamine inhibition of Na^+-K^+-ATPase activity in the rat cortical collecting duct involved receptor-mediated cAMP-dependent protein kinase (PKA) (Satoh *et al.* 1992). This pattern of action is shared by other agonists that increase cellular cAMP and thus stimulate PKA activity. 12(R)-Hydroxyeicosatetraenoic acid, a

major arachidonate metabolite, inhibits Na^+-K^+-ATPase from bovine corneal epithelium, rat kidney and rat heart ventricle (Masferrer *et al.* 1990). In addition many other lipid modulators such as sphingosine, lysophosphatidylcholine and unsaturated fatty acids like oleic acid also inhibit Na^+-K^+-ATPase from various species and tissues *in vitro* (Oishi *et al.* 1988; 1990; Swarts *et al.* 1990). Although the identity of the endogenous 'ouabain-like' inhibitory factor remains to be elucidated evidence suggests that lyso-PtdCho and unesterified fatty acids may account for some of this endogenous activity (Tamura *et al.* 1985; Kelly *et al.* 1986; Tamura *et al.* 1987).

The fatty acid composition of DAG has been used by other workers to determine the source of DAG produced as a second messenger. For example, Moscat *et al.* (1989) used this method of analysis to determine the source of DAG in PDGF-stimulated mammalian NIH/3T3 fibroblasts and those transformed by the *ras* oncogene. Phosphatidylcholine from these cells is rich in oleic (18:1) and linoleic acid (18:2) so that DAG rich in these fatty acids might implicate phospholipase C-mediated degradation of PtdCho as their source. In cod this approach should be even easier since PtdIns is rich in stearic, 18:0 and arachidonic acid, 20:4(n-6) whereas the other phosphoglycerides have low levels of arachidonic acid and large amounts of 20:5(n-3) and 22:6(n-3). Thus DAG abundant in 18:0 and 20:4(n-6) fatty acids would implicate PtdIns as the source of DAG and DAG containing 16:0, 18:1, 20:5(n-3) and 22:6(n-3) would possibly implicate PtdCho or PtdEth as the precursor lipid. Thus the present study aimed to use this approach to determine whether or not the phospholipase C-mediated degradation of either PtdIns or PtdCho provided the link between ANF as the primary physiological hormone and DAG as a second messenger in osmoregulation in the gill. If, as the evidence in Chapter 4 suggested the chloride cells contain ANF receptors then

hormone binding could initiate phospholipase C-mediated breakdown of membrane PtdCho and/or PtdIns. Phosphatidylinositol metabolism would result in the production of Ins1,4,5P_3 and DAG from PtdIns4,5P_2 or Ins1P and DAG from PtdIns (reviewed by Berridge, 1993) whereas PtdCho metabolism would result in production of phosphocholine and DAG (reviewed by Pelech & Vance, 1989; Exton, 1990). Phospholipase C-mediated effects on PtdIns are usually fast acting and transient and are detected within a few seconds or minutes of stimulation (Wright *et al.* 1988; Augert *et al.* 1989; Pessin & Raben, 1989; Exton, 1990). On the other hand phospholipase C-mediated effects on PtdCho metabolism tend to be slower and peak at around one hour after agonist administration (Bocckino *et al.* 1985; Cabot *et al.* 1988b; Wright *et al.* 1988). Analysis of DAG composition in the present study ought to have revealed any time related differences in composition after administration of ANF. The experimental protocol was based on the assumption that 1% of PtdCho and 5% of PtdIns would be hydrolysed yielding approximately 5µg and 2.5µg of DAG respectively which should have been detectable. Di 20:0 diacylglycerol included as an internal standard in cell extracts allowed quantitation of any DAG produced. Unfortunately there was no evidence of DAG production above the basal level or that the fatty acid composition of DAG changed in any way after treatment of chloride cells with the hormone. This result suggests that neither phospholipase C-mediated degradation of PtdIns or PtdCho link the receptor mediated action of ANF to the release of DAG as a second messenger in this system. However, a recent study by Pettitt & Wakelam (1993) in Swiss 3T3 mouse fibroblasts suggested that there can be a substantial receptor-stimulated turnover of DAG molecular species in the absence of any measurable change in mass of DAG. It is therefore possible that the approach employed in this study was not sophisticated enough to pick up any changes in DAG composition.

In mammalian systems the common intracellular second messenger in tissues sensitive to ANF is cGMP (reviewed by Genest & Cantin, 1988) with cAMP production being inhibited (Anand-Srivastava *et al.* 1984; Leitman & Murad, 1988). However the ANF inhibition of aldosterone secretion may not be mediated by cGMP but by Ca^{2+} (Matsuoka *et al.* 1987; Leitman & Murad, 1988). Although the role of cGMP is well established the mechanism by which this nucleotide functions remains unknown though Fiscus *et al.* (1985) demonstrated that the activity of a cGMP-dependent protein kinase is stimulated by ANF. In the retina cGMP can work directly without any involvement of phosphorylation via its physical interaction with sodium channels thereby reducing sodium influx (Fesenko *et al.* 1985). A direct inhibitory effect of ANF has also been demonstrated on Na^+/H^+ transport in the growing phase of the kidney epithelial cell line LLC-PK (Inui et al. 1985).

Although the results presented here do not exclude a link between ANF and DAG by some mechanism other than receptor-mediated phospholipase C action on PtdIns or PtdCho either directly or indirectly it seems likely that the effects brought about by ANF and phorbol ester on sodium efflux (Chapter 2) and Na^+-K^+-ATPase activity (Chapter 4) act independently of one another.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The present study aimed to investigate the putative role of diacylglycerol (DAG), one of the products of receptor-mediated hydrolysis of the phosphatidylinositol lipids in the control of Na^+-K^+-ATPase-mediated active sodium efflux across the gills of a marine teleost *Gadus morhua*. A succession of experiments was designed which aimed to progressively reveal the details of osmoregulatory control in this species. A requisite of this approach was to identify a primary agonist responsible for initiating the signalling cascade which could then be used in isolated cell studies to dissect the details of the signal transduction process. Initial studies utilised the whole body perfusion technique in a physiological approach to identify any hormones and/or any other agonists which might fulfil this primary role in modulating sodium transport. Subsequent studies involved more detailed biochemical analysis on isolated chloride cells. The conclusions of the present study are summarised in Figure 6.1.

The cardiac hormone atrial natriuretic factor (ANF) was found to significantly reduce the sodium efflux across the gills of *Gadus morhua*. The role of ANF as a vasodilator and of its natriuretic response in mammalian systems is well documented (reviewed by Baxter *et al.* 1988; Genest & Cantin, 1988; Goetz, 1988; Kramer, 1988; Sampson, 1992) and the role that it plays in fishes is at present being rigorously investigated (reviewed by Evans, 1990). The present study also revealed that ANF significantly inhibited Na^+-K^+-ATPase activity *in*

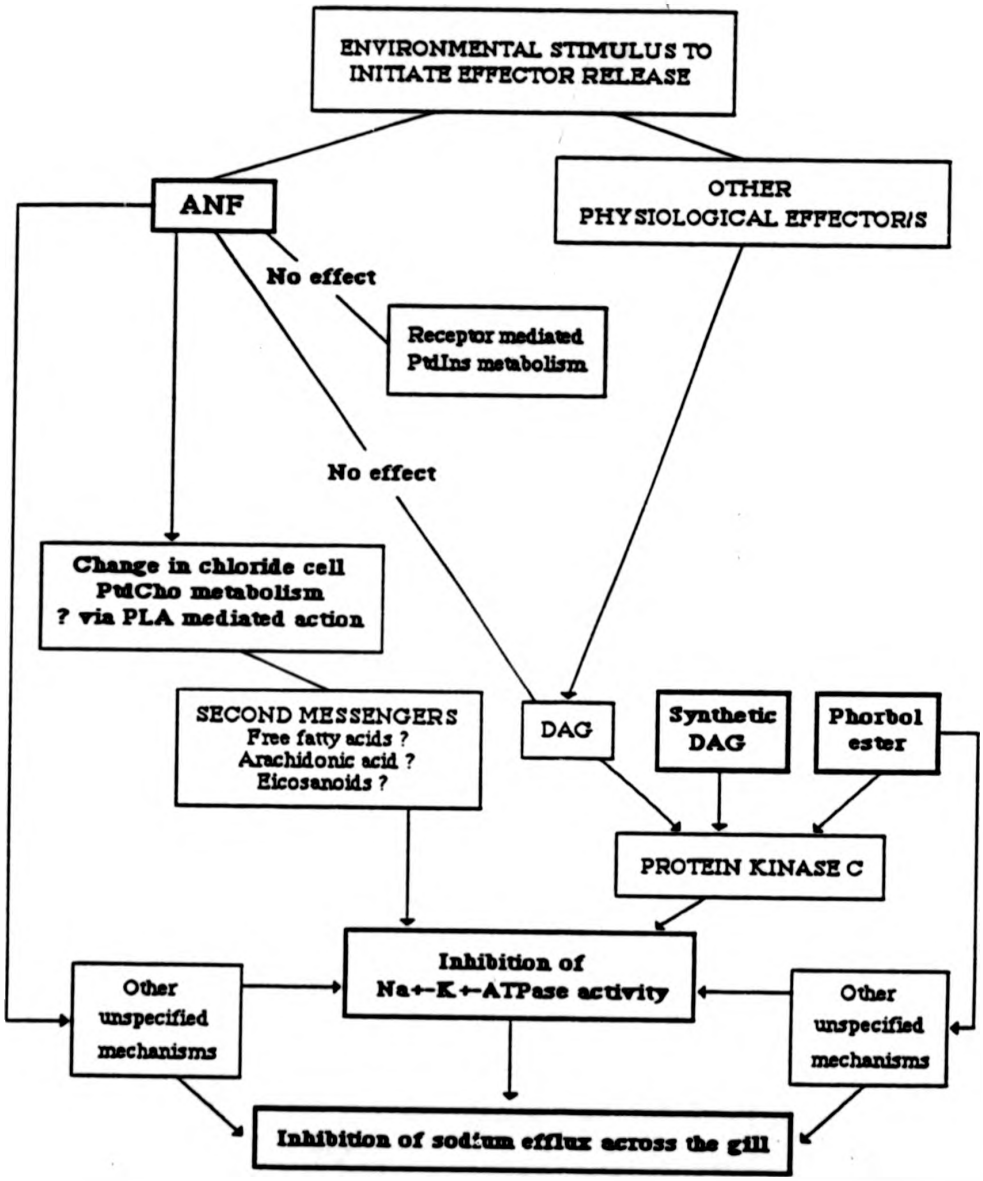

Figure 6.1. Summary of the roles of atrial natriuretic factor (ANF) and diacylglycerol (DAG) mimetics (phorbol ester and synthetic DAG) in the control of Na^+-K^+-ATPase mediated branchial ion exchange in *Gadus morhua*. Steps and pathways that have been established in this study are highlighted in bold type and those yet to be confirmed are in plain type.

vitro thereby establishing a physiological role for ANF in the control of active sodium transport by Na^+-K^+-ATPase across the gills of cod. This finding suggested that chloride cells most probably contained ANF-specific cell surface receptors and that ANF appeared to initiate an unspecified receptor-mediated process which ultimately brought about a change in the activity of Na^+-K^+-ATPase via the production of second messengers.

The present study revealed that the phorbol ester, phorbol 12, 13-dibutyrate, and the synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol, significantly reduced sodium efflux across the gills of cod whilst enzymatic studies revealed an inhibitory effect of the same agents on the activity of Na^+-K^+-ATPase. It is widely accepted that the primary role of DAG *in vivo* is to activate protein kinase C which then phosphorylates a wide range of cellular proteins (Nishizuka, 1986; 1989). Since its discovery in 1977 PKC has been established as a central component for the activation of a large array of biological processes including metabolism, secretion, contraction, proliferation and differentiation (Nishizuka, 1986). The PKC field has recently become much more complicated by the identification of a number of isozymes which might be tissue specific and which may not have the same properties with respect to their requirements for DAG, PtdSer and Ca^{2+}. Diacylglycerol and phorbol esters bind to PKC thereby increasing its affinity for calcium so as to permit its full activation at ambient concentrations of intracellular calcium (Nishizuka, 1984). In intact cells PKC is predominently cytosolic and undergoes stimulus-induced translocation to the plasma membrane where it binds PtdSer which is essential for activation (Nishizuka, 1988).

In elasmobranchs salt excretion occurs in the rectal gland and in the spotted

dogfish this organ was found to have high PKC activity (Bell & Sargent, 1987). It was therefore deduced that other cells involved in salt excretion such as the chloride cells of the marine gill might share this characteristic and that DAG could therefore be involved in the cellular control of salt secretion. It is usually assumed, based on histological evidence, that chloride cells from all tissues are similar but it is possible that the progenitor cells from which chloride cells evolved in fish gills, elasmobranch rectal gland and avian salt glands are fundamentally different with respect to cellular control mechanisms. Extrapolating between the three basic groups of chloride cells may not therefore be valid.

Initial indications from the investigation using the perfusion technique were that DAG and hence PKC did have a role to play in osmoregulation in the gills of a marine fish and this was again supported by the concentration-dependent inhibitory effect of PDB and OAG on Na^+-K^+-ATPase activity. In rat proximal tubule cells dopamine has been demonstrated to be a physiological inhibitor of Na^+-K^+-ATPase (Bertorello *et al.* 1988) and this inhibition is mediated by PKC (Bertorello & Aperia, 1989). On the other hand PKC activation has been reported to stimulate Na^+-K^+-ATPase activity in other cell types (Lynch *et al.* 1986). Work by Lowndes *et al.* (1990) indicated that Na^+-K^+-ATPase may serve as a substrate for PKC in intact cells from dog kidney, duck salt gland and duck salt gland microsomes further supporting a role for PKC in osmoregulation. The results of the present study suggest that phorbol ester inhibits Na^+-K^+-ATPase via the activation of PKC but this is inconsistent with the hypothesis proposed by Robinson and Flashner (1979) whereby direct phosphorylation of Na^+-K^+-ATPase, for example by PKC, leads to a

conformational change in the enzyme which promotes translocation of sodium across the membrane i.e. an increase in enzyme activity. However, a more recent report has demonstrated that phosphorylation of the catalytic subunit of Na^+-K^+-ATPase from shark rectal gland by PKC or cAMP-dependent protein kinase inhibits the activity of the enzyme (Bertorello *et al.* 1991). The present results might suggest a similar control mechanism in the chloride cells of the cod gill where DAG activates PKC which in turn inhibits the activity of Na^+-K^+-ATPase. The possibility remains however that PKC exerts its effect on Na^+-K^+-ATPase by some other indirect mechanism or that the phorbol ester is exerting its effect by means other than through PKC. The alteration in intracellular sodium concentration is an important physiological regulator of Na^+-K^+-ATPase activity since normally the intracellular concentration of sodium is too low to saturate the enzyme (Skou, 1965; Jørgensen, 1986). Phorbol esters have been shown to increase the activity of the Na^+-H^+-antiporter which results in an increase in intracellular sodium levels (Dicker & Rozengurt, 1981; Grinstein *et al.* 1985).

Studies on a number of systems have shown that DAG required for PKC activation can arise from receptor-mediated hydrolysis of PtdCho or even PtdEth (Grove & Schimmel, 1982; Besterman *et al.* 1986; Daniel *et al.* 1986). The initiating event of DAG accumulation from PtdCho is not clear and may differ for different cell types though evidence has been obtained to support activation of phospholipase C leading to the direct formation of DAG (Besterman *et al.* 1986; Irvine & Exton, 1987; Muir & Murray, 1987). Diacylglycerol can be produced not only from phospholipase C mediated hydrolysis of phospholipids but also via phospholipase D action producing PtdA which is subsequently cleaved to diacylglycerol by PtdA

phosphohydrolase. Alternatively phospholipase A_2 can catalyse the hydrolysis of phospholipids to produce lyso-phospholipid and free fatty acid. Lyso-phospholipid can be catabolised to glycerol-3-phosphate and then converted to DAG via PtdA synthesis. Phosphatidic acid itself could be active as a signalling molecule.

An added complication of receptor-mediated PtdCho and PtdEth metabolism is that in many cells these phospholipids consist of a mixture of 1,2 diacyl, 1-alkyl-2-acyl and 1-alkenyl-2-acyl subspecies. The biochemical properties of the different subspecies varies widely in different tissues and cultured cells, and in some cells ether-linked phospholipids are metabolically the most active. Phorbol ester has been shown to stimulate PKC-dependent phospholipase D-mediated formation of ester- and ether-linked PtdA in cultured mammalian HeLa cells (Hii *et al.* 1990). The results of these studies by Hii and his co-workers suggest that alkyl and alkenyl sub-classes of PtdCho and PtdEth may be hydrolysed following activation of PKC by phorbol esters or by agonists with the resultant production of ether-linked diacylglycerols. Thus mixtures of diacylglycerols, alkylacylglycerols and alkenylacylglycerols can be produced (Martin 1988; Rider *et al.* 1988). Several studies have indicated that 1-*O* -alkyl-2-acyl-*sn* -glycerol does not activate but inhibits PKC (Ganong *et al.* 1986; Daniel *et al.* 1988) but a more recent study using purified PKC from rat brain concludes that this species can activate PKC but only at relatively high concentrations of calcium compared with diacylglycerol (Ford *et al.* 1989). The differential responsiveness of PKC to DAG molecular species underscores the possible significance of multiple PKC isoforms. These isoforms, of which there are at least seven, have different tissue distributions and varying sensitivities to various activating factors including DAG and phorbol esters

(Nishizuka, 1989). Diacylglycerol molecular species formed during cell stimulation will reflect the cell type and the type of agonist involved. It is possible that *in vivo* different DAG molecular species will affect different PKC isoforms thereby producing distinct patterns of PKC activation and inactivation with respect to subcellular translocation, substrate recognition and duration. The present study determined the amount and the fatty acid composition of DAG in ANF-stimulated cells but no changes were found in either parameter on administration of the hormone. This result suggests that ANF did not exert its effect via DAG production and that DAG formation must involve some other physiological mediator in this system.

Chloride cells stimulated with ANF were found to display altered PtdCho metabolism. The changes involved an increase in radiolabelled CDP-choline and glycerophosphocholine (GPC) and a small decrease in labelled choline. The fall in radiolabelled choline and rise in radiolabelled CDP-choline are indicative of increased activity of cytidyltransferase (CT) the major controlling enzyme in the PtdCho metabolic cycle and the increase in the labelling of GPC implies increased PtdCho metabolism via phospholipase A_2 activity. There was no evidence to support a phospholipase C or a phospholipase D mediated action of ANF. The similarity between the control of cytidyltransferase and protein kinase C has already been pointed out in this thesis (Section 5.1.2) and the implications of this are important in understanding the cellular control mechanisms involved here.

The unusual pattern of labelling of phospholipids from gill chloride cells was of particular interest. The high incorporation of [^{32}P]-orthophosphate into PtdEth remains a mystery and may indicate a special role for PtdEth in the structural

phosphohydrolase. Alternatively phospholipase A_2 can catalyse the hydrolysis of phospholipids to produce lyso-phospholipid and free fatty acid. Lyso-phospholipid can be catabolised to glycerol-3-phosphate and then converted to DAG via PtdA synthesis. Phosphatidic acid itself could be active as a signalling molecule.

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The unusual pattern of labelling of phospholipids from gill chloride cells was of particular interest. The high incorporation of [^{32}P]-orthophosphate into PtdEth remains a mystery and may indicate a special role for PtdEth in the structural

make-up of the membrane. Whether PtdEth has a role in the transmission of information across the cell membrane remains to be determined as do the physiological effectors of such a system. The present study did not find an active PtdIns cycle in "resting state" chloride cells as reported by Simpson & Sargent (1985) for the shark rectal gland nor did treatment of these cells with ANF induce any changes in the phospholipid class composition or the pattern of radioactive labelling. Inositol phosphate production was not increased following stimulation with ANF (10^{-7}M). It was therefore concluded that PtdIns turnover is very low in resting chloride cells from cod gills and is not stimulated on administration of ANF. The fact that chloride cell membranes from cod gills contain amounts of polyphosphoinositides similar to those found in other tissues does suggest that Ins1,4,5P_3 and Ca^{2+} play a role in cellular signalling in these cells but if they are involved in the control of salt secretion then another primary agonist initiates the process.

The potential for cross-talk between different receptor systems is becoming increasingly apparent and adds a further level of complexity to attempts to understand cellular control processes. The possibility of different agonists countering each other, acting additively or synergistically are almost endless. Future work in this and other systems will have to recognise these possibilities.

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