EPINEPHRINE AND GLUCAGON EFFECTS ON THE SECOND MESSENGERS cAMP AND IP_3 IN FISH HEPATOCYTES

by

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ABSTRACT

The transduction systems that link hormone stimulation to cellular metabolic processes have been extensively studied in several mammalian cell types of which hepatocytes are preferentially used. Comparative studies in the more primitive vertebrates (e.g. fish, amphibians and reptiles) support the hypothesis that these animals lack the complexity and the degree of specificity for these processes demonstrated in mammals.

It is already known that epinephrine (EPI) and glucagon (GLU) enhance hepatic glucose output in all vertebrates. Evidence in fish, amphibians and reptiles suggest that only the cyclic AMP (cAMP)-mediated transduction mechanism exists in liver, as the inositol trisphosphate (IP_3) system has not been reported to occur. The recent discovery of inositol phosphates and IP_3-binding in fish non-hepatic tissues and Ca^{2+}-transients in some fish hepatocytes has raised the question as to whether the IP_3 pathway may contribute in the modulation of hepatic glucose metabolism in conjunction with the cAMP system in response to EPI and GLU. An EPI-mediated effect through both transduction mechanisms implies the presence of α- or β- or both adrenoceptors as in mammals.

This thesis demonstrates the production of IP_3 and confirms the production of cAMP in eel and bullhead hepatocytes following exposure to either EPI or GLU. These findings indicate the presence of both transduction systems at least in these two fish species. As in mammals, both hormones function by stimulating the production of different amounts of second messengers. In fish hepatocytes the production of these intracellular mediators was significantly less with GLU than with EPI. The activity of the enzyme adenylyl cyclase (ACase) was assessed to correlate the enzyme activities in crude membrane preparations with the production of cAMP in intact cells following EPI and GLU stimulation. Moreover the use of the adrenergic α- and β-antagonists phentolamine and propranolol provided evidence for an effect of EPI on both α_1- and β_2-adrenoceptors.

The presence of the α_1-subtype was confirmed in black bullhead and assessed for the first time in American eel liver membranes with the specific-binding of radioactive prazosin (^{3}H-PRZ), a classical α_1-antagonist, and the displacement of ^{3}H-PRZ with other non-radioactive, adrenergic

antagonists. The order of potency displayed by these ligands was typical for α_1-adrenoceptors of mammals. Similar affinity for PRZ was found in the two species, whereas eel showed less receptor density than bullhead membranes. However, the binding capacity was found to be within the range of the mammalian receptors.

Moreover the existence of IP_3-binding sites, that may account for the involvement of a Ca^{2+} messenger system in response to EPI and GLU action was assessed for the first time in fish hepatocytes. Eel and bullhead microsomal preparations showed different affinities and binding capacities for IP_3. These results give support to the hypothesis that both hormones may induce intracellular Ca^{2+} oscillations.

Overall these findings confirm that EPI and GLU stimulate fish liver metabolism through both the signalling molecules cAMP and IP_3. The presence of IP_3-binding sites and of the α_1-adrenergic receptors places fish liver cells at the same level of complexity of some mammalian hepatic systems. Species differences may account for the different contribution of each pathway in the regulation of hepatic metabolism.

SOMMAIRE

Les systèmes de transduction reliant la stimulation hormonale aux procédés métaboliques cellulaires ont été rigoureusement étudiés dans plusieurs types de cellules mammifères, parmi lesquelles les hépatocytes sont utilisées de préférence. Des études comparatives dans les vertébrés plus primitifs (poissons, amphibiens et reptiles) sont en accord avec l'hypothèse que ces animaux manquent la complexité et le degré de spécificité pour ces procédés démontrés chez les mammifères.

C'est déjà connu que l'épinéphrine (EPI) et le glucagon (GLU) améliorent la production de glucose hépatique dans tous les vertébrés. Les données expérimentales dans les poissons, les amphibiens et les reptiles semblent démontrer que, dans le foie, seul le mécanisme de transduction ayant le AMP cyclique (cAMP) comme médiateur existe, puisque le système avec le triphosphate d'inositol (IP3) n'avais jamais été observé. La découverte récente de la fixation des phosphates d'inositol et de IP3 dans les tissus non-hépatique des poissons ainsi que des espèces transitoires de Ca2+ dans certaines hépatocytes dans les poissons laisse supposer que le mécanisme IP3 puisse contibuer à la modulation du métabolisme du glucose hépatique en conjonction avec le système cAMP en réponse de l'EPI et du GLU. Un effet assisté par l'EPI pour les deux mécanismes de transduction implique la présence d'adrénocepteurs α- ou β les deux dans les mammifères.

Cette thèse démontre la production de IP3 et confirme la production de cAMP dans les hépatocytes de l'anguille et du "bullhead" après une exposition à l'EPI et au GLU. Ces observations sont en accord avec l'existence des deux systèmes de transductions, au moins pour les deux espèces de poissons étudiées. Comme pour les mammifères, chacunes de ces deux hormones agissent en produisant des quantités différentes de seconds messagers. Dans les hépatocytes de poissons, la productions de ces médiateurs intracellulaires est nettement moindre avec le GLU qu'avec l'EPI. L'activité de l'enzyme cyclase adenylyl (ACase) a été estimée afin de trouver une corrélation entre l'activité des enzymes dans des préparations de membranes brutes et la production de cAMP dans les cellules intactes après stimulation avec

EPI et GLU. De plus, l'usage des antagonistes adrénergiques α- et β- phentolamine et propranolol a mis en évidence l'effet de l'EPI à travers les deux adrénocepteurs α1- et β2-.

La présence du soustype α1 a été confirmée dans le "black bullhead" et estimée pour la première fois dans les membranes du foie de l'anguille américaine avec la fixation spécifique du prazosin radioactif (3H-PRZ), un antagoniste α1 classique, et le déplacement de 3H-PRZ avec d'autres antagonistes adrénergiques non-radioactifs. L'ordre de puissance montré par ces ligands a été typique des adrénocepteurs α1 des mammifères. Une affinité semblable pour PRZ a été trouvée dans les deux expèces, quoique l'anguille a démontrée moins de densité de récepteurs que les membranes du "bullhead". Néan moins, la puissance de fixation se trouvait dans la zone des récepteurs mammifères.

De plus, le fait que la présence de sites de fixation pour IP3 peut rendre compte du rôle d'un système messager du Ca2+ en réponse à l'action de l'EPI et du GLU a été estimé pour la première fois dans les hépatocytes des poissons. Les préparations microsomales des anguilles et des "bullheads" ont montré de différentes affinités et puissances pour IP3. Ces résultats supportent l'hypothèse que les deux hormones pourraient induire des oscillations intracellulaires du Ca2+.

Dans l'ensemble, ces retrouvailles confirment que l'EPI et le GLU stimulent le métabolisme du foie des poissons à travers des deux molécules signalantes cAMP et IP3. La présence de sites de fixation pour le IP3 ainsi que des récepteurs adrénergique α1 situe les cellules du foie des poissons à un niveau de complexité semblable à quelques systèmes hépatiques mammifères. Des différences entre les espèces pourrait rendre compte de la regulation du metabolisme hépatique

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CHAPTER 1:
INTRODUCTION

It is well known that the main metabolic responses induced by epinephrine and glucagon on vertebrate liver cells are the stimulation of glycogenolysis (glycogen breakdown) and gluconeogenesis (glucose synthesis from C-3 precursors) (Exton, 1985; 1988; Moon, 1988). These cellular events have the primary function to raise blood glucose levels. Within the hepatocyte, glucose is produced from lactic acid derived from muscle contraction, from amino acids from the blood or skeletal muscle and from glycerol liberated by fat breakdown (lipolysis). Under conditions of glucose mobilization, hormonal stimulation of hepatic glycogen phosphorylase leads to the cleavage of the glycogen polymer into monomeric units of glucose-1-phosphate. Glucose is produced from glucose-1-phosphate after conversion into glucose-6-phosphate, and then released into the circulating blood (Hadley, 1984). Even in teleost fishes, the hyperglycemic effect of the two hormones is mainly based on the stimulation of hepatic glycogen breakdown, glucose synthesis and glucose output. Experiments *in vivo* and *in vitro* have demonstrated these piscine physiological responses (Thorpe and Ince, 1974; Birnbaum *et al.*, 1976; Morata *et al.*, 1982; Ottolenghi *et al.*, 1984a; 1985; Brighenti *et al.*, 1987a; Janssens and Lowrey, 1987; Wright *et al.*, 1989; Mommsen *et al.*, 1988; Mommsen and Moon, 1989; 1990). The effect of epinephrine and glucagon on intracellular mediators that modulate these metabolic responses is the main objective of this thesis. A description of the actions of these hormones on their target tissues is necessary to understand the dynamic mechanism of the transduction systems that activate metabolic pathways.

A. Epinephrine and its Mechanisms of Action on Target Cells

The catecholamines epinephrine (EPI) and norepinephrine (NEPI) are secreted from the chromaffin cells that form the medulla of the adrenal glands found at the anterior poles of mammalian kidneys (Coupland, 1965; Hadley, 1984). The cortical cells of these glands are steroidogenic tissue. In birds and reptiles the adrenal glands are still anatomically detected anterior to the kidneys, but internally the chromaffin tissue is increasingly intermingled with the

cells of the cortex. In amphibians, the adrenal glands or "bodies" are elongated structures on the ventral surface of the kidneys or partially embedded in this surface. In this vertebrate class the chromaffin and cortical cells can still be detected close to each other (Coupland, 1965; Hadley, 1984). Extra-chromaffin cells can be observed in anurans and urodeles associated with symphathetic ganglia in the abdominal and para-vertebral regions.

In fishes adrenal glands cannot be detected as separate organs. The organization of the secretory cells in teleosts shows species differences. Generally the chromaffin and steroidogenic cells are in variable association and close to sympathetic ganglia, but always in a close anatomical association with the kidneys. The chromaffin tissue is dispersed in the walls of the anterior region of the posterior cardinal vein and embedded in the anterior portion of the kidneys. Here the chromaffin cells can be found as single cells, in clusters, in cord-like agglomerates or in unicellular layers (Coupland, 1965). In Atlantic cod, *Gadus morhua*, the chromaffin tissue, other than being found in the walls of the posterior cardinal vein, is arranged in the anterior portion of the kidney that forms a structure called the "head kidney" that resembles the adrenals of more evolved vertebrates (Nilsson *et al.*, 1983).

The chromaffin cells have the same neural embryological origin as the postganglionic fibres of the sympathetic nervous system (SNS) that secrete norepinephrine as an excitatory transmitter in all organs innervated by it. The quantity of humoral catecholamines secreted by the chromaffin tissue into the blood stream is greater than the neurally-released catecholamines in classes of vertebrates where the SNS is less developed than in mammals (Sulakhe *et al.*, 1988). Measurements of plasma catecholamine concentrations under resting conditions, without imposing any stress or treatment, present some difficulty, as catecholamines are secreted in response to stress stimuli. However EPI and NEPI concentrations in fish under physiological conditions are estimated to be between 1 and 10 nM (Thomas and Perry, 1992), while mammalian blood titres are 10 times less (Gorbman *et al.*, 1983). The lower mammalian level is consistent with a more developed neural adrenergic control. Depending upon the nature and the severity of the particular stress, the values of fish blood catecholamines may increase up to 100

times basal levels (Perry *et al.*, 1991; Thomas and Perry, 1992). The catecholamine mobilization and the responsiveness of target tissues show species-dependency, are subjected to seasonal cycles and interactions with other hormones, and are influenced by the physiological status of the animal (Gorbman *et al.*, 1983; Randall and Perry, 1992; Thomas and Perry, 1992). American eels (*Anguilla rostrata*) show especially low levels of plasma catecholamines (Reid and Perry, 1994). In teleosts EPI secretion appears to be predominant over NEPI secretion (Randall and Perry, 1992).

Even though the information on the biosynthetic pathway of catecholamines in fish is based on studies of few species (Nilsson, 1983; Randall and Perry, 1992), the process starts from the amino acids phenylalanine and tyrosine as in other vertebrate groups. A sequence of enzymatic reactions that take place in the cytoplasm of the chromaffin cells and adrenergic neurons converts the two amino acids to dopamine (DA), another catecholamine, that is the direct precursor of NEPI. DA is included into storage vesicles for release purposes or is converted inside these vesicles into NEPI. The last step of this biosynthetic pathway is the direct formation of EPI by methylation of NEPI in the cytoplasm (Randall and Perry, 1992). Stored in membrane bound vesicles, EPI and NEPI are released into the circulating blood in response to numerous "physical and environmental disturbances" such as anemia, external hypoxia, hypercapnia, exhaustive exercise and metabolic acidosis. The ratio of circulating EPI/NEPI during stress is species dependent (Perry and Reid, 1992; Randall and Perry, 1992). The magnitude of the stress required to elicit catecholamines is species- and environmental-dependent (Kinkead and Perry, 1991; Perry and Reid, 1992; Randall and Perry, 1992; Thomas and Perry, 1992), but generally blood catecholamine content does not change significantly from basal levels during mild to moderate stress (Perry *et al.*, 1992). For instance catecholamines do not seem to regulate ventilation in fish as they are released only when critical thresholds of water O_2 and CO_2 content are reached, whereas hyperventilation is triggered at very slight changes in "blood/water chemistry". When blood catecholamine levels do rise following severe stress conditions, they initiate a series of physiological and biochemical events

that represent a "final survival strategy" to compensate for the effects of the extreme stress, such as sustained breathing, increased gill diffusion capacity, dorsal aortic blood pressure, blood oxygen capacity, activating the Na^+/H^+ antiport exchanger of the erythrocyte membrane and increasing the hematocrit through vasoconstriction of the spleen (Kinkead *et al.*, 1991; Randall and Perry, 1992; Thomas and Perry, 1992; Perry and Reid, 1993; Perry and Thomas, 1993). All these responses have the common target to enhance oxygen transport and regulate blood acid-base status. Other catecholamine-mediated effects such as hyperglycemia, lipolysis, inhibition of insulin release and enhancement of glucagon release are adaptive responses to increased metabolic demands. The teleost red blood cell (rbc) and hepatocyte have been chosen as "model systems" to study the mechanism of catecholamine action. The adaptive response induced by catecholamines in the rbc results in intracellular alkalinization and increase in oxygen transport (Randall and Perry, 1992; Thomas and Perry, 1992), whereas, in hepatocytes these agonists enhance the mobilization of glucose (Ottolenghi *et al.*, 1984a; 1985; Brighenti *et al.*, 1987a; Janssens and Lowrey, 1987; Mommsen *et al.*, 1988).

The two catecholamines are quickly removed from the blood stream by tissue storage, by the action on target tissues, through receptor-binding and mainly by degradation in gills, liver and kidney. The gill is the main organ of blood borne catecholamine degradation and chloride cells may be directly involved in this catabolic process (Randall and Perry, 1992).

Even though circulating catecholamines may increase by 100-fold under extreme disturbances, tissues must respond to relatively low hormone concentrations within physiological limits. The hydrophilic character of these molecules hinders their passage through the membrane lipid bilayer of the target cells, requiring that the hormone interacts with surface receptors embedded within the cell membrane (Alberts *et al.*, 1989). These receptors, called adrenergic receptors or adrenoceptors, have specific affinities for natural and synthetic catecholamines generally termed agonists, and selective antagonists or compounds which block agonist binding to the receptor.

B. Adrenoceptors Studies in Mammals and Lower Vertebrates

Using adrenergic agonists and antagonists, Ahlquist (1948) classified adrenoceptors into two types, α and β, based upon pharmacological responsiveness of several tissues in mammals. The further subclassification of the β-type into β_1 and β_2 (Lands *et al.*, 1967a; Furchgott, 1967), and of the α-type into α_1 and α_2 (Langer, 1974) was made in the decades that followed on the basis of the order of potency of new specific adrenergic ligands in mammalian organ bath experiments. The same approach supported by radioligand binding and complementary DNA cloning techniques has provided a further subclassification into multiple subtypes of α_1- and α_2-adrenoceptors and a third kind of β (β_3, typical of the adipose tissue) (Summers and McMartin, 1993). The classification of the α-subtypes was made according to the pharmacological responses rather than considering the localization and the function of the receptors (Johansson, 1983). However, α_2-adrenoceptors are generally located pre- and post-synaptically with an inhibitory effect, while the α_1-adrenoceptors seem to be solely postsynaptic, with a stimulating action on the cell (Exton, 1985). There has been a tendency to believe that in mammalian liver the neural catecholamine-dependent control functions through the α_1-adrenergic receptor, whereas the circulating catecholamines act through the β-type (Sulakhe *et al.*, 1988). The β-adrenoceptors are always coupled to a stimulatory effect on the target cells.

The rank order of potency of specific agonists/antagonists that characterize each subtype of adrenoceptor in mammalian cells is now used to identify adrenoceptors in non-mammalian vertebrates tissues. The classical sensitivity to agonists of a mammalian β_2-adrenoceptor is isoproterenol (ISO) > epinephrine (EPI) > norepinephrine (NEPI) > phenylephrine (PE). The rank of potency displayed by a mammalian α_1-adrenoceptor is instead epinephrine (EPI) > norepinephrine (NEPI) > phenylephrine (PE) > isoproterenol (ISO) (Exton 1980, 1985). The pharmacological approach that employs specific antagonists to prevent normal adrenergic functioning has shown uniformity in the induced responses in all vertebrates studied to date, including fish (Birnbaum *et al.*, 1976; Janssens and Grigg, 1984; Brighenti *et al.*, 1987a,b;

Wright *et al.*, 1989; Michelsen and Sheridan, 1990; Moon and Mommsen, 1990; Janssens and Grigg, 1992). Radioligand binding techniques, based on the different affinities of the receptor for highly selective radiolabelled agonists and antagonists, are employed to give evidence of a conservative trend in the adrenoceptor types and subtypes also in lower vertebrate classes (Johansson, 1984; Janssens and Lowrey, 1987; Sulakhe *et al.*, 1988; Janssens and Grigg, 1988, 1992; Fabbri *et al.*, 1992; Reid *et al.*, 1992; Gutiérrez-Venegas and García-Sáinz, 1993; Moon *et al.*, 1993.)

The selective radioactive antagonists prazosin (PRZ) and yohimbine (YOH) are usually chosen to define α_1- and α_2-adrenoceptors, respectively (Exton, 1985; Summers and McMartin, 1993; Fabbri *et al.*, 1994), while labelled cyanopindolol, because of its high affinity (Summers and McMartin, 1993) and the non selective radioactive alprenolol can be used to identify the β-type (Fabbri *et al.*, 1992).

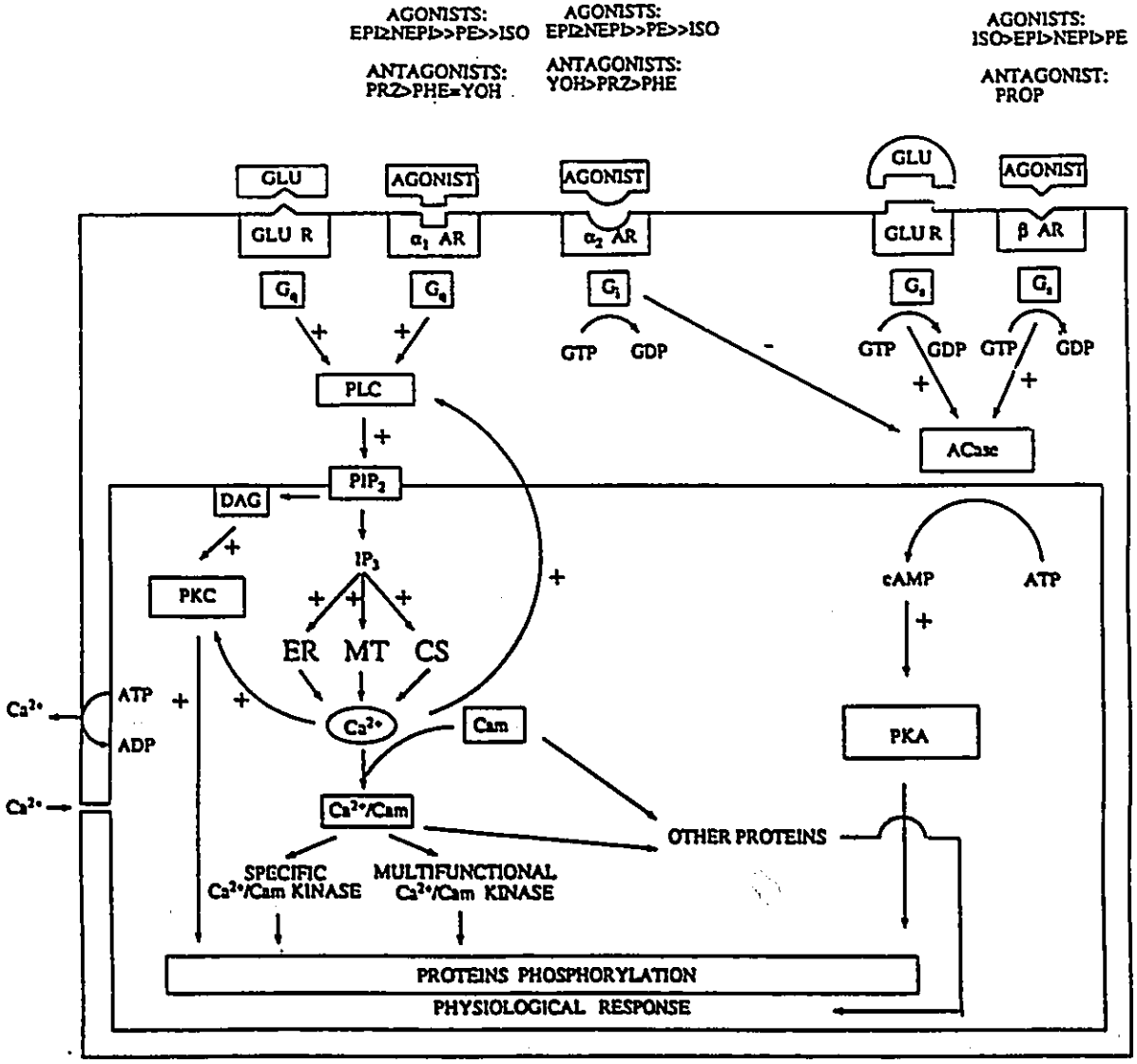
The advent of molecular biology techniques has provided structural information on many "subtype designations" of adrenoceptors and on other constituents of the transduction pathways interposed between hormone-receptor binding and the metabolic response (Liggett and Raymond, 1993). The functional significance of different subtypes probably permits a more sensitive control on the cell response. The α_1- and α_2-adrenoceptors are now considered two different receptors and are not related to each other more than the β-adrenoceptor would be to the α (Bylund, 1992). All three types, however, are transmembrane proteins with seven hydrophobic spanning regions, an extracellular amino terminus, containing glycosylation sites and an intracellular carboxy terminus tail, that with other amino acids of the third intracellular loop are possible locations of phosphorylation reactions that trigger the desensitization process of the receptor. The third cytoplasmic intracellular hydrophilic loop seems also to be involved in the interaction between the receptor and a GTP-dependent regulatory protein called the G protein (Ligget and Raimond, 1993; Summers and McMartin, 1993). Desensitization is a complex phenomenon that induces a decrease in receptor expression in order to maintain metabolic responses within physiological limits. The rapid uncoupling of the receptor from its G

protein represents the first step in this process and is due to phosphorylation of the receptor by various kinases. The phosphorylation induced by a single agonist (due to activation of a kinase) may reduce the sensitivity of the receptor to that specific ligand or the attenuation of its response to other agonists (Liggett and Raymond, 1993). The transmembrane spanning regions are the site for the agonist/antagonist binding. The length of the protein that forms the adrenoceptor is from 402 to 560 residues (Liggett and Raymond, 1993; Summers and McMartin, 1993).

All adrenoceptors studied to date are linked to a G protein (Birnbaumer, 1990; Ligget and Raymond, 1993; Summers and McMartin, 1993). Receptor number and the level of G proteins can regulate cell responsiveness (García-Sáinz *et al.*, 1989). The activation of the adrenoceptor by the binding of agonists/antagonists induces a transient allosteric change in its protein structure that triggers a complex cascade mechanism ultimately leading to the modulation of intracellular messengers and the extent of the metabolic response. The activation of the receptors induces an exchange of a GDP molecule for a GTP at the level of the G protein. The activated G protein complex will in turn regulate the activity of the enzyme responsible for the synthesis of the relevant second messenger (Exton, 1985, 1988; Birnbaumer, 1990). Several distinct G proteins have been isolated and cloned. They have been divided into three major classes on the basis of their respective effector systems to which they are linked (Fig. 1-1). The G_s protein couples β-adrenoceptors with the stimulation of the enzyme adenylyl cyclase (ACase) which results in the synthesis of the second messenger cyclic AMP (cAMP). A "tentatively named" G_q protein (Berridge, 1987; Guillon *et al.*, 1992) couples α_1-adrenoceptors with the enzyme phosphoinositide-specific phospholipase C (PLC) responsible of the cleavage of the membrane bound phosphatidylinositol bisphosphate (PIP_2) into the two second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Guillon *et al.*, 1992). IP_3 induces an increase in intracellular Ca^{2+} levels $[Ca^{2+}]_i$ (Exton, 1979b; 1985; 1988). α_2-Adrenoceptors are linked to a G_i protein that inhibits the enzyme ACase (Fig. 1-1) (Exton, 1985; García-Sáinz *et al.*, 1989; Duzic *et al.*, 1992; Summers and McMartin, 1993).

Each G protein contains three subunits, the α, β, and γ (Birnbaumer, 1990; Duzic *et al.*,

Fig. 1-1: Transduction mechanisms related to the α- and β-adrenergic ligands and glucagon stimulation of a liver cell. β-adrenergic agonists and glucagon stimulate cAMP synthesis through their own receptors. IP_3 production depends upon binding of α-adrenergic agonists and glucagon to α_1-adrenoceptors and glucagon receptors, respectively. The synthesis of IP_3 induces an increase of $[Ca^{2+}]_i$. The binding of α-adrenergic agonists to α_2-receptors induces an inhibitory effect on cAMP synthesis through the action of an inhibitory G protein. The names of the G proteins are generic terms. Abbreviations: GLU, glucagon; R, receptor; AR, adrenoceptor; G_s and G_q, stimulatory G proteins; G_i, inhibitory G protein; GDP, guanosine diphosphate; GTP, guanosine triphosphate; ACase, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PLC, phospholipase C; PIP_2, phosphoinositol 4,5-bisphosphate; IP_3, phosphoinositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; ER, endoplasmic reticulum; MT, mitochondria; CS, calciosomes; Cam, calmodulin; Ca^{2+}/Cam, Ca^{2+} bond to calmodulin. Adrenergic agonists and antagonists: EPI, epinephrine; NEPI, norepinephrine; PE, phenylephrine; ISO, isoproterenol; PRZ, prazosin; YOH, yohimbine; PHE, phentolamine.


AGONISTS:
EPI≥NEPI>>PE>>ISO
ANTAGONISTS:
PRZ>PHE=YOH
AGONISTS:
EPI≥NEPI>>PE>>ISO
ANTAGONISTS:
YOH>PRZ>PHE
AGONISTS:
ISO>EPI>NEPI>PE
ANTAGONIST:
PROP
GLU
AGONIST
AGONIST
GLU
AGONIST
GLU R
α1 AR
α2 AR
GLU R
β AR
Gq
Gq
Gi
Gs
Gs
GTP
GDP
GTP
GDP
GTP
GDP
PLC
ACase
PIP2
DAG
IP3
PKC
ER
MT
CS
cAMP
ATP
Ca2+
ATP
ADP
Ca2+
Ca2+
Cam
PKA
Ca2+/Cam
OTHER PROTEINS
SPECIFIC
Ca2+/Cam KINASE
MULTIFUNCTIONAL
Ca2+/Cam KINASE
PROTEINS PHOSPHORYLATION
PHYSIOLOGICAL RESPONSE

1992; Guillon *et al.*, 1992). The α subunit differs markedly among G proteins. The β and γ subunits have instead a consistent structure among the different G proteins and are tightly associated together (Federman *et al.*, 1992; Summers and McMartin, 1993). The α subunit readily uncouples from the β/γ complex, binds the receptor, induces release of GDP, interacts with the effector protein (ACase, PLC), and has intrinsic GTPase activity (Fig. 1-1). The hydrolysis of GTP to GDP results in the reassociation of the α subunit with the β/γ complex (Summers and McMartin, 1993). A limiting factor for the activation of the G proteins is the guanine nucleotide concentration (Houslay, 1986). The G proteins and the effector proteins are embedded in the cell membrane. Due to the "fluidity" of the lipid bilayer (Alberts *et al.*, 1989), hormone receptors just as other proteins can move laterally within the membrane. These findings have lead to the suggestion that the activation of the enzyme ACase is due to collisions between the molecular components of this transduction mechanism and that changes in bilayer "fluidity" directly influence the activity of this system (Houslay, 1986).

The best characterized member of the "superfamily of G-coupled receptors" (Bylund, 1992) is the β-adrenoceptor (Summers and McMartin, 1993). The β_1- and β_2-subtypes have been pharmacologically characterized and there is similarity in their sequence between mammalian species. Both subtypes are coupled to ACase (Summers and McMartin, 1993). The distribution of these two subtypes is tissue- (Ota *et al.*, 1993) and species- (Summers and McMartin, 1993) dependent. However the β-type is present in livers of all vertebrates, including fish (Janssens and Lowrey, 1987; Sulakhe *et al.*, 1988; Janssens and Grigg, 1988; 1992; Fabbri *et al.*, 1992). The functional domains of the β-adrenoceptor, such as specific regions for ligand binding, for coupling to the G protein and for modulation of its expression (desensitization phenomenon) through the action of several protein kinases, are well characterized especially for the β_2-subtype (Summers and Martin, 1993). Liver β-receptors are mainly of the β_2-subtype (Morgan *et al.*, 1983).

α_1-Adrenoceptors induce changes in cellular activities by increasing hepatic intracellular free Ca^{2+} concentrations $[Ca^{2+}]_i$ (Fig. 1-1) (Joseph *et al.*, 1984). This mechanism may take part

in the regulation of cellular Ca^{2+} flux. α_1-Adrenoceptors are selectively stimulated by methoxamine or phenylephrine (PE) and selectively blocked by prazosin (PRZ). Three types of α_1-adrenoceptors have been identified by pharmacological and molecular cloning (Minneman, 1988; Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomansey *et al.*, 1991). The α_{1A} and α_{1B} have been defined pharmacologically on the basis of reversible and irreversible antagonists (Bylund, 1992). Activation of any of these α_1-adrenoceptor subtypes increases $[Ca^{2+}]_i$ levels, but there are differences in their underlying mechanisms (García-Sáinz *et al.*, 1992a). The α_{1A} is related principally to an extracellular Ca^{2+} influx through the activation of Ca^{2+} channels rather then to the synthesis of IP_3, whereas the α_{1B} is directly linked to the phosphoinositide pathway with the consequent Ca^{2+} mobilization from intracellular stores (Han and Minneman, 1987; Han *et al.*, 1990; Michel *et al.*, 1990; Bylund, 1992; Summers and McMartin, 1993). The α_{1C} is a novel adrenoceptor that seems to be restricted to a few tissues (García-Sàinz, 1992). Only the α_{1B} is reported to be present in rat livers, while in guinea pig hepatocytes the subtype α_{1A} seems to represent the α_1-type (García-Sáinz *et al.*, 1992a, b; Summers and McMartin, 1993). The stimulation through α_1-adrenoceptors promotes glycogenolysis through glycogen phosphorylase activation in mammalian hepatocytes (Joseph *et al.*, 1984; García-Sáinz *et al.*, 1992a). The α_1-adrenoceptors are characterized by a longer amino acid chain and a longer third cytoplasmic loop than the β-adrenoceptors, which may be critical attributes for binding to the G_q protein (Minneman, 1988; Liggett and Raymond, 1993; Summers and McMartin, 1993).

Molecular cloning, DNA sequencing and radioligand binding data have provided evidence of four α_2-subtypes (Summers and McMartin, 1993). α_2-Adrenoceptors can modulate a variety of transduction systems including the ACase- and phospholipase-mediated pathways and various ion transport mechanisms (Duzic *et al.*, 1992). The coupling to various transduction pathways is tissue-dependent and linked to the specific function of the cell. α_2-Adrenoceptors inhibit ACase through a G_i protein (Fig. 1-1) or directly modify the activity of ion channels including Ca^{2+} channels, the Na^+/H^+ exchanger and K^+ channels (Bylund, 1988). The α_2-type has high affinity for the agonist clonidine and the antagonists rauwolscine and yohimbine

(YOH) and low affinity for the α_1-antagonist PRZ. The liver possesses α_2-adrenoceptors, however, their number is smaller than the α_1-subtype and it is not yet clear whether they play a significant role in the regulation of liver cAMP levels (Exton, 1988).

The characterization of adrenoceptors and description of their structure come almost exclusively from studies carried out on mammalian tissues. In particular the metabolically important adrenoceptors in mammalian liver are the α_1- and β_2-subtypes. The transduction mechanisms that link these two receptors to the metabolic response in liver cells will be discussed presenting the results of studies carried out mainly on mammalian hepatic tissue.

1) β_2-Adrenoceptor transduction pathway

Comparative studies employing radioligand binding techniques showed the presence of β-adrenoceptors in liver membranes of selected amphibians, reptiles, birds and mammals (Sulakhe *et al.*, 1988). These receptors were indirectly identified by competitive inhibition of glycogenolysis with the use of adrenergic antagonists in goldfish (Birnbaum *et al.*, 1976), and detected in other teleosts, including carp (Janssens and Lowrey, 1987), black bullhead (Fabbri *et al.*, 1992) and rainbow trout (Reid *et al.*, 1992).

These studies showed a marked species variation and a phylogenetic trend which suggested that the β-adrenoceptor was the primitive liver adrenoceptor type. β-Adrenoceptors seem to dominate in lower vertebrates, where the SNS is less evolved. In these classes there is less neural catecholamine-dependent control and consequently less need for α-adrenoceptors (Sulakhe *et al.*, 1988). Non-mammalian vertebrates show the β-adrenoceptor transduction pathway ubiquitously distributed in their tissues (Sulakhe *et al.*, 1988; Fabbri and Moon, 1994). The β-type seems to be the sole adrenergic receptor expressed in amphibians and reptiles (Janssens and Grigg, 1988; 1992). In mammals there are significant species exceptions like guinea pig, beef (Sulakhe *et al.*, 1988) and humans (Exton, 1985), where the β component prevails over the α_1.

Activation of adenylyl cyclase (ACase) through G_s proteins (Fig. 1-1) increases

intracellular levels of cAMP (Exton, 1979a; 1985; Fabbri *et al.*, 1992). The typical β_2-adrenergic response is glycogen breakdown and gluconeogenesis in liver (Exton, 1979a; 1985), both mechanisms that increase glucose availability. The plasma membrane-bound ACase activated by a G_s protein, catalyses the conversion of ATP to cAMP (Alberts *et al.*, 1989) in the presence of Mg^{2+} and traces of Ca^{2+} (Ottolenghi *et al.*, 1988). ACase is a glycoprotein of 1,134 amino acids. It is inserted into the cell membrane by two hydrophobic regions, each represented by six transmembrane spanning regions, separated by large cytoplasmic domains. Several forms of this enzyme are suggested by molecular biology studies (Summers and McMartin, 1993). Once the second messenger cAMP is synthesized it either binds and activates the cAMP-dependent protein kinase (Fig. 1-1) (Exton, 1988) or is rapidly degraded to adenosine 5'-monophosphate (5'-AMP) by a family of phosphodiesterase isoenzymes (PDEs). Hormones such as insulin and glucagon regulate the level of cAMP by controlling the rate of cyclic nucleotide degradation through a short- or long-term activation of the different forms of PDEs (Gettys *et al.*, 1988; Houslay, 1990; Conti *et al.*, 1991; Houslay *et al.*, 1992). Increases in the concentration of cAMP that can achieve levels of up to 400-fold over basal are temporary, as even when the agonist is continuously present, intracellular cAMP levels generally plateau or return towards basal levels within a few minutes. The enzymatic degradation of cAMP by PDEs and the desensitization of the β-adrenoceptor through several kinases (at least two, the cAMP-dependent protein kinase A and the protein kinase C, which are activated under different conditions; Lefkowitz and Caron, 1986), reduce the rate of cAMP synthesis maintaining the cell response within physiological limits. Apparently β-adrenoceptor sequestration is a mechanism that takes place in cases of long-term agonist exposure, while for rapid desensitization only the phosphorylation of the receptor is required (Hausdorff *et al.*, 1990; Ligget and Raymond, 1993).

The cAMP-dependent protein kinase (PKA) activated by cAMP is one of the major enzymes mediating the phosphorylation of target proteins (Fig. 1-1) leading to metabolic and physiological responses. Many key enzymes of particular metabolic pathways are substrates for its ATP-dependent phosphorylation (Exton, 1979a). PKA may be found bound to the membrane

or in the cytoplasm (Summers and McMartin, 1993). EPI stimulation through β-adrenoceptors leads primarily to the activation of PKA through the binding of cAMP to the regulatory subunits of PKA. This results in the dissociation of the catalytic subunits that will activate phosphorylase kinase which in turn will convert glycogen phosphorylase into its active form. The activation of glycogen phosphorylase, the key enzyme of glycogen breakdown, leads to increased glycogenolytic flux and glucose output (Exton, 1979a). The phosphorylation of the key glycolytic enzyme pyruvate kinase by PKA induces its inactivation, and this appears to be the major component of the stimulatory action of EPI on gluconeogenesis (Exton, 1979a). Another substrate for PKA and several other protein kinases is the enzyme glycogen synthase (Imazu *et al.*, 1984). The enzyme that catalyzes the "rate-limiting reaction of glycogen synthesis" is inactivated by protein kinase phosphorylation and the direct consequence is the inhibition of glycogen formation (Exton, 1979a). This transduction system appears to be conserved in all vertebrates (Exton, 1979a: mammals; Janssens *et al.*, 1983: axolot; Janssens and Grigg, 1984, 1987: toad; Ottolenghi *et al.*, 1984a; 1985; Brighenti *et al.*, 1987a: bullhead; Janssens and Lowrey, 1987: carp; Mommsen *et al.*, 1988; Wright *et al.*, 1989: trout).

2) α_1-Adrenoceptor transduction pathway

Studies carried out with radioligand binding techniques on hepatic tissues in different classes of vertebrates (Janssens and Lowrey 1987: teleost; Sulakhe *et al.*, 1988: species of all classes except fish; Janssens and Grigg, 1988: amphibians; 1992: reptiles) have shown that the α_1-type is apparently absent in the more primitive vertebrates. In fact, only in birds and mammals did Sulakhe *et al.* (1988) find α_1-adrenoceptors. Only recently has the α_1-subtype been characterized in one teleost species, the bullhead *Ictalurus melas* (Fabbri *et al.*, 1994). In mammals the α_1-adrenoceptor dominates over the β_2 in rat and other species hepatic tissue (Sherline *et al.*, 1972; Sulakhe *et al.*, 1988), although the β_2-adrenoceptor continues to be expressed at different levels, depending upon the species. In birds the hepatic α_1- and β-adrenoceptors are "co-expressed, both at high levels", but the α_1-subtype predominates over

the β-subtypes (Sulakhe *et al.*, 1988). Several β-adrenergic ligands used in binding experiments to liver plasma membranes of reptiles, amphibians and teleosts displace the most common radioactive ligand ([^{125}I] iodocyanopindolol) used for the characterization of β-adrenoceptors with the same order of potency as in mammalian studies, whereas α-adrenergic antagonists do not bind specifically to the membranes suggesting that α_1-adrenoceptors are not present in these classes. A recent confirmation of the absence of α_1-adrenoceptors in the axolotl was reported by Kleineke and Janssens (1993). They demonstrated that the increase in cytosolic Ca^{2+} in isolated axolotl hepatocytes was not by IP_3-induced extrusion of the cation from internal stores but by a cAMP-dependent regulation of Ca^{2+} channels in the plasma membrane which allowed Ca^{2+} influx.

Rat hepatocytes are the model system used for the study of the mechanism of the α_1-adrenoceptor-mediated responses (Exton, 1988). The complex mechanism of IP_3 mobilization is triggered by the coupling of the agonist/receptor complex to a stiumlatory G_q protein that in turn stimulates a Ca^{2+}-dependent phospholipase termed phospholipase C (PLC), specific for the hydrolysis of phosphatidylinositol-1,4-bisphosphate (PIP_2) resulting in the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Fig. 1-1) (Joseph *et al.*, 1984; Exton, 1985; 1988; Alberts *et al.*, 1989; Berridge *et al.*, 1987; Guillon *et al.*, 1992; Berridge, 1993; Fabbri and Moon, 1994). The enzyme PLC is a component of an enzyme family that apparently does not display areas of hydrophobicity required for membrane insertion (Guillon *et al.*, 1992; Exton, 1992; Summers and McMartin, 1992). Both cleavage products of PIP_2 are considered second messengers (Joseph *et al.*, 1984; Exton, 1985). DAG remains bound within the cell membrane with its hydrophobic fatty acid chains (Fig. 1-1) and has two potential signalling roles. It can be further cleaved and its 20-carbon fatty acid derivatives can be used in the synthesis of local mediators called eicosanoids or, it can activate the Ca^{2+}-phospholipid-dependent protein kinase C (PKC) (Fig. 1-1) that phosphorylates a large number of proteins (Exton, 1988). The second cleavage product generated from PIP_2 is IP_3, a water soluble molecule that is released into the cytosol and has an important role in the complex

mechanism of cellular Ca^{2+} regulation (Berridge, 1989; 1993). Unless it is continuously generated, its action is short-lived because it is rapidly metabolized. Studies using α_1-adrenergic agonists and other Ca^{2+}-mobilizing agents have shown that in many tissues, including liver, the increase of IP_3 is detected in seconds (Exton, 1988; 1992). Small increases in IP_3 induce large physiological responses due to a transient increase of $[Ca^{2+}]_i$ that amplifies the initial signal. The IP_3-mediated Ca^{2+} mobilization from intracellular stores or extracellular fluids is possible due to the presence of IP_3-sensitive Ca^{2+} channels in cell membranes (Joseph *et al.*, 1984; Meldolesi *et al.*, 1990; Kraus-Friedmann, 1990). The IP_3 released into the cytoplasm binds to specific sites associated with these Ca^{2+} channels inducing their activation or opening (Mauger and Claret, 1988; Berridge, 1993). Given the very low $[Ca^{2+}]_i$, Ca^{2+} moves along its concentration gradient into the cytoplasm (Berridge, 1993).

The C-terminal region of the proteic molecule that forms the IP_3 receptor has typical membrane-spanning domains that anchor the protein in the membrane and that form the "IP_3-sensitive Ca^{2+} channel". The large N-terminal domain lies free in the cytoplasm and contains the IP_3-binding site (Berridge, 1993). The IP_3 receptor is associated with membranes of intracellular organelles and the plasma membrane through anchorage with the cytoskeleton, as suggested by recent studies on rat liver plasma membranes (Feng and Krauss-Friedmann, 1993). The specific IP_3 receptor has been characterized in many different mammalian tissues (Spät *et al.*, 1986; Parys *et al.*, 1992). The binding of IP_3 molecules on their receptor-channel (references 10, 13, 35 from Feng and Kraus-Friedman, 1993) induces the opening of these channels and the mobilization of Ca^{2+} into the cytoplasm (Berridge, 1993). Ca^{2+} ions are transiently mobilized either from intracellular compartments like mitochondria, the endoplasmic reticulum, specialized Ca^{2+} storage compartments named calciosomes or from extracellular sources (Exton, 1979; 1985; 1988; Volpe *et al.*, 1988; Putney *et al.*, 1989; Meldolesi *et al.*, 1990; Zhang *et al.*, 1992a). A system that sequesters Ca^{2+} from the cytoplasm to maintain the cellular response within the physiological limits is provided by Ca^{2+}-ATPase pumps in cell membranes (Sulakhe *et al.*, 1990) and the Ca^{2+}-binding proteins, calmodulin and a calsequestrin-like molecule, such

as the one found in skeletal muscle (Volpe *et al.*, 1988; Meldolesi *et al.*, 1990), present inside the storage organelles. The former Ca^{2+}-binding protein is characterized by high affinity but low binding capacity for Ca^{2+} (Alberts *et al.*, 1989; Meldolesi *et al.*, 1990), while the latter is characterized by moderate affinity but high capacity for the ion (Volpe *et al.*, 1988). A way to regulate the hormonally induced Ca^{2+} accumulation in rat hepatocytes is a cAMP-dependent control on Ca^{2+} mitochondrial uptake (Morgan *et al.*, 1983).

Among the pathways that increase the availability of glucose from liver cells, Ca^{2+} promotes the activation of various key enzymes. Adenylyl cyclase requires traces of the ion for its normal activity. Ca^{2+} exceeding basal levels following hormone stimulation cooperate with phosphatidylserine in the activation of the membrane bound PKC (Exton, 1988). In liver PKC in turn activates glycogen phosphorylase and inactivates glycogen synthase (Exton, 1981; Blackmore *et al.*, 1986) enhancing the cAMP-mediated glucose output. Ca^{2+} forms a complex with the ubiquitous Ca^{2+}-dependent regulatory protein calmodulin (Exton, 1985) or is directly involved as an enzymatic cofactor in the activation of other enzymes (Exton, 1988). The importance of Ca^{2+} as an intracellular mediator is also suggested by the Ca^{2+}-calmodulin complex (Ca^{2+}/Cam) that becomes part of the molecular structure of enzymes like phosphorylase kinase (Exton, 1985; 1988), a key enzyme in the glycogen breakdown pathway, and consequently Ca^{2+} takes part in the activation of this enzyme. The enzyme is stimulated allosterically by increases in Ca^{2+} within the cytosolic range (Chrisman *et al.*, 1982; Charest *et al.*, 1983). The major role of phosphorylase kinase is to activate glycogen phosphorylase (Exton, 1985), the enzyme that acts directly on glycogen and catalyzes the synthesis of glucose-1-phosphate. The complex activates other multifunctional Ca^{2+}/Cam-dependent protein kinases that modulate the activity of a variety of enzymes. A Ca^{2+}/Cam-dependent protein kinase seems to inactivate the enzyme glycogen synthase (Exton, 1988). In rat liver, apparently phosphorylase kinase is the predominant enzyme which phosphorylates and inactivates glycogen synthase, probably in a Ca^{2+}-dependent manner (Imazu *et al.*, 1984). The Ca^{2+}/Cam complex interacts also with other cellular proteins altering their activity and inducing various

physiological responses (Exton, 1985). Ca^{2+} is also required for the inactivation of pyruvate kinase, the regulation of PLC activities (Guillon *et al.*, 1992) and the stimulation of mitochondrial pyruvate carboxylation (Exton, 1985; 1988).

The cAMP-mediated mechanism and the IP_3-Ca^{2+}-induced release show a synergy in modulating the activity of key enzymes that regulate glycogenolytic and gluconeogenic processes in liver cells. In some cases, one pathway may regulate the sensitivity of the response obtained from the other pathway acting on non-enzymatic targets (Imazu *et al.*, 1994). Guinea pig hepatocytes show, for example, a potentiation of the sensitivity of the IP_3-binding sites by the action of a cAMP-dependent protein kinase (Burgess *et al.*, 1991). The amplification of the external initial signal through the production of the second messengers and the "cross-talk" between the two pathways provide adequate temporary metabolic responses that result in an increased hepatic output. The α_1- and β-mediated pathways interact in liver cells to give a common metabolic response. An example of this interdependency is the activation of the Ca^{2+}/Cam-dependent phosphorylase kinase through the cAMP-dependent protein kinase A.

3) Adrenoceptor transduction in fishes

Studies of catecholamine effects on hepatocyte metabolism have been carried out mainly on mammals, but there is conservation of action in all vertebrate hepatic tissue. Glycogenolysis is in fact stimulated by catecholamines and other hormones in livers of birds (Gutiérrez-Venegas and García-Sáinz, 1993), reptiles (Janssens and Grigg, 1992), amphibians (Janssens *et al.*, 1983; Janssens and Grigg, 1984; 1987) and teleosts (Birnbaum *et al.*, 1976; Janssens and Lowrey, 1987; Wright *et al.*, 1989; Brighenti *et al.*, 1987; Mommsen *et al.*, 1988; Michelsen and Sheridan, 1990).

The hepatic tissue of fish has also become an important "model system" to study the mechanisms of adrenergic glucose production (Moon *et al.*, 1985). Studies on catecholamine-induced metabolic responses in bullhead hepatocytes showed that the catecholamine effect, as in more evolved vertebrates, results in the continuous decrease in

glycogen levels through increased glycogen phosphorylase activities and that the hormone-induced hyperglycemia is mainly due to glycogenolysis (Ottolenghi *et al.*, 1984a; 1985). Fish hepatic tissue has only recently been the object of studies to confirm the presence of β-adrenoceptors and to provide evidence for the first time of the presence of α_1-adrenoceptors. The following studies show the development of our understanding of both receptor types in fish liver.

The response of the bullhead liver following exposure to various catecholamines demonstrated consistency with the presence of β-adrenoceptors, as propranolol (PROP), the classical β-antagonist but not phentolamine (PHE), the classical α-antagonist (Exton, 1985; 1988), blocked cAMP increases, the activation of the enzyme glycogen phosphorylase, decreases in glycogen content in cells, with the final result of decreased glucose output into the medium (Brighenti *et al.*, 1987a, b). The classical rank of potency for the β_2-adrenoceptor (ISO > EPI > NEPI > PE) seems not to be followed precisely in fish since EPI had a greater effect than ISO on cAMP levels, glycogen phosphorylase activation and glucose output. Some complexity arises from the findings that PE, a synthetic α-agonist, induced similar phosphorylase activation, glycogen depletion and glucose output, but lower cAMP synthesis than the β-mediated EPI and NEPI effects and that these responses were again blocked by PROP but not PHE. These experimental results suggested that in fish, PE may behave as a β-agonist or assuming that PE acts as an α-antagonist, PROP may also bind to α-receptors. In light of these results the phosphorylase activation, the consequent glycogen breakdown, and glucose release may be due to the activation of both types of receptors. The presence of an α-subtype was indirectly suggested by these studies of Brighenti *et al.* (1987a, b).

Further evidence for the presence of β_2-adrenoceptors was provided by studies on intact rainbow trout (*Oncorhynchus mykiss*) injected with EPI and NEPI and exposed to a moderate hypoxic stress (reduced P_wO_2) (Wright *et al.*, 1989). The observed sustained levels of blood glucose were suggestive of an increased glycogenolytic response through the activation of glycogen phosphorylase and inhibition of glycolysis and/or enhancement of gluconeogenesis

through a decrease in the activity of pyruvate kinase. Pre-treatment with PROP eliminated these effects and provided strong evidence for a β-mediated action of EPI and NEPI in trout (Wright *et al.*, 1989).

Experiments carried out on EPI treated trout hepatocytes exposed to hypercapnic conditions (increased medium PCO_2 and decreased medium pH), confirmed glycogenolysis as a major source of glucose production (Mommsen *et al.*, 1988). This report also showed that the activation of glycogen phosphorylase and the inactivation of pyruvate kinase lead to the degradation of glycogen and an increase in the flux through the gluconeogenic pathway from lactate. Pre-treatment with PROP reduced the EPI effect and supported the presence of the β-subtype. Exposure to PE also resulted in an activation of glycogenolysis and gluconeogenesis and further suggested the possible involvement of an α_1-receptor (Mommsen *et al.*, 1988). Environmental disturbances like hypercapnia and hypoxia increase the need to produce glucose for tissues suffering from a lack of oxygen. Liver glucose metabolism switches from the oxidative to the glycolytic pathway to compensate for this enhanced glucose demand. Injection of catecholamines stimulates glycogenlysis to maintain glucose availability (Perry *et al.*, 1988; Wright *et al.*, 1989). These findings support the role of catecholamines to counteract threatening situations.

A study by Moon and Mommsen (1990) using three teleost species, the American eel, the brown bullhead and the rainbow trout showed that PE influences glycogenolysis and gluconeogenesis in a significant species-dependent fashion. The PE-induced effects were abolished by PROP and partially by PRZ and YOH, α_1- and α_2- antagonists, respectively, supporting either a mixed α/β transduction system or a different effectiveness of the classical α- and β-agonists and antagonists in fish liver. The use of selective β_1-antagonists supported the presence of the β_2-adrenoceptors.

The existence of β-adrenoceptors in teleost hepatocytes has been reported using radioligand binding techniques only in carp (Janssens and Lowrey, 1987), rainbow trout with chronically elevated plasma cortisol levels (Reid *et al.*, 1992), and black bullheads (Fabbri *et al.*,

1992). In trout the β-adrenoceptor appears to be of the β_2-subtype based on the order of potency demonstrated by adrenergic agonists in displacing the radioactive synthetic β-antagonist (±)-4-(3-t-butylamino-2-hydroxypropoxy)- [5,7-^{3}H]benzimidazol-2-one (CGP), that for its hydrophilic character is selectively retained outside the cell and detects β-receptors more specifically than other commonly used lipophilic antagonists (Reid *et al.*, 1992). All three cases support the coupling of the β-receptor with cAMP synthesis. Measurements indicated that each species differed in the amount of cAMP produced at a given agonist concentration.

Comparative studies on hormone-stimulated hepatic glycogenolysis in goldfish (Birnbaum *et al.*, 1976), carp (Janssens and Lowrey, 1987), in the urodele and anuran amphibians *Ambyostoma mexicanum* (Janssens *et al.*, 1983; 1986; Janssens and Grigg, 1988) and *Xenopus laevis* (Janssens and Grigg, 1984; 1988), and in turtles (Janssens and Grigg, 1992), supported the existence of the β-mediated pathway, but not the α-adrenoceptor system. Competitive binding studies carried out with liver membranes from the previous mentioned species (except goldfish) characterized β-adrenoceptors, while no study found any specific binding to the α-subtype or any displacement by classic α-antagonists. The same studies in carp, toad, axolotl and turtles showed no evidence that changes in cell Ca^{2+} played any role in the hormone-mediated changes in hepatic glycogenolysis. These studies create a paradox with the PE effect in eel, bullhead and trout hepatocytes which suggested the presence of an α-type population of hepatic adrenoceptors.

Ca^{2+} oscillations measured in single *Xenopus* oocytes stimulated by different doses of IP_3 and its derivatives gave indirect evidence that the effect of cell surface agonists may cause an increase of IP_3 (DeLisle *et al.*, 1990). ATP-dependent Ca^{2+} transport activities measured in liver plasma membranes of all classes of vertebrates except fish (Sulakhe *et al.*, 1990), supported the involvement of Ca^{2+}-mediated regulation of metabolic pathways also in non-mammalian vertebrates.

Recently the role of the linked G protein-PLC system that involves IP_3 and Ca^{2+} as intracellular mediators has been shown in non-hepatic tissues of a variety of lower vertebrates

including fish (Ecat and Valentich, 1991; Kwon and Lee, 1991; Lacy *et al.*, 1992; Kalinoski *et al.*, 1992). Moreover the presence of α-adrenoceptors in non-hepatic tissues of non-mammalian vertebrates has been reported (Johansson, 1984). α-Adrenoceptors have been reported in trout vasculature by the hypotensive effects of the α-antagonists PHE and PRZ (Xu and Olson, 1993).

Spectrofluorimetric measurements of changes in $[Ca^{2+}]_i$ in catecholamine-induced single fish hepatocytes (Zhang *et al.*, 1992a) gave indirect support for the presence of α-adrenoceptors. EPI stimulation strongly modulated $[Ca^{2+}]_i$ in eel and bullhead hepatocytes, while it had little effect on trout. Apart from species variations in the basal cell Ca^{2+} level, the amplitude of the response and the source of Ca^{2+}, the connection between EPI and increases in $[Ca^{2+}]_i$ were clear. The % increase above control $[Ca^{2+}]_i$ was similar in eel and bullhead. Continuous EPI exposure induced Ca^{2+} oscillations only in eel and were quantitatively similar to those observed in rat hepatocytes exposed to α-agonists. In bullhead, EPI induced a biphasic response with an initial transient increase in $[Ca^{2+}]_i$ followed by a sustained component. This pattern of response suggested Ca^{2+} release from intracellular stores for eel, while in bullhead the initial transient probably came from intracellular Ca^{2+} stores, while the prolonged phase was postulated to be derived from extracellular sources through increased Ca^{2+} influx across the hepatocyte membrane.

The classic α- and β-agonists and antagonists (Ahlquist, 1948; Exton, 1985; 1988) employed in the studies of isolated hepatocytes from eel and bullhead by Zhang *et al.* (1992b) linked for the first time changes in $[Ca^{2+}]_i$ to an α-adrenoceptor system in liver membranes of these fish. The α-agonist mediated increase in $[Ca^{2+}]_i$ was prevented by the α-antagonist PHE but not by the β-antagonist PROP, even when used at concentrations 100 times higher than the agonist. The β-agonist ISO was ineffective in all of these studies.

Pharmacological evidence for the presence of α_1-adrenorecsptors in fish hepatocytes comes from successive spectrofluorimetric analysis of changes in $[Ca^{2+}]_i$ on bullhead liver cells exposed to different catecholamines (Moon *et al.*, 1993). The response to the different adrenergic ligands described a rank of potency that was the same as that described for

mammalian α_1-adrenoceptors (Exton, 1985). The lack of effectiveness of the specific α_2- and β-antagonists clonidine and PROP, but not of the generic α-antagonist PHE, gave further support for an α_1-mediated transduction pathway in these fish liver cells. The use of the classic α_1-antagonist PRZ (Exton, 1985) had to be avoided because of its high fluorescence background that would interfere with the fluorescence measurements.

The presence of α_1-adrenoceptors was confirmed in black bullhead purified liver membranes (Fabbri *et al.*, 1994) using tritiated prazosin (^{3}H-PRZ) for its high selectivity and affinity for the α_1-receptor type in mammals (Exton, 1985). Competition experiments showed that the bound ^{3}H-PRZ was displaced by the adrenergic ligands that showed the rank of potency typical for mammalian α_1-antagonists (PRZ > YOH > PHE >> PROP). The adrenergic agonists used to displace the radioactive PRZ showed definitely more affinity for an α_1-adrenoceptor than for an α_2- and even less for a β-adrenoceptor.

These studies clearly show that both α_1- and β_2-adrenoceptors exist on the hepatic membranes of at least some fish species.

C. Glucagon and its Effect on Liver Cells

Glucose homeostasis is regulated by a number of hormones, but glucagon (GLU) and insulin released from the alpha (α) and beta (β) cells of the endocrine pancreas, respectively, play key roles. In the more advanced vertebrates, the endocrine pancreas also has an exocrine function. In teleosts the endocrine cells form several compact tissue masses called Brockman bodies, completely separated from the exocrine component and scattered in the body cavity. These bodies can be found within the mesenteric membranes and also in the wall of the intestine, in close proximity to the liver. The ratio of α and β cells varies in the different classes of vertebrates suggesting that in each class the relative importance of hyperglycemia and hypoglycemia may differ. The α and β cells are always in intimate contact with blood capillaries. Since the endocrine cells originate from the gut, endocrine pancreatic components can be observed in the gastrointestinal tract, secreting glucagon-like substances (Hadley, 1984).

Insulin has a dominant role in the control of carbohydrate metabolism because it is the only hormone that directly lowers blood glucose levels. Some hormones have hyperglycemic effects, but GLU is the hormone that plays a primary counter-regulatory role to the action of insulin (Hadley, 1984). The physiological status of the organism and environmental stress conditions influence this very sensitive regulation system. Catecholamines compensate and become critical when GLU secretion is not sufficient (Boyle *et al.*, 1989). In most vertebrates GLU release is induced by a diminished level of blood glucose and stimulates mainly the hepatic tissue to release glucose into the blood stream to restore the physiological circulating levels. In contrast, when blood glucose is elevated the release of insulin induces glucose uptake into the liver and other cells (Hadley, 1984). Adipose tissue and muscle are two other target tissues for insulin and GLU to control glucose flux in and out of the circulating blood. Many physiological processes constantly affect blood glycemia, thus constant monitoring is necessary to maintain a normal internal environment. It is this balance between insulin and GLU which acts to maintain appropriate blood glycemic levels.

Insulin and GLU are peptide hormones. Insulin consists of two polypeptide chains, A and B, of 21 and 30 amino acids, respectively. The 2 chains are linked together by a pair of disulfide bounds. GLU is instead a single 29 amino acid peptide. The high degree of conservation of the primary structure of these peptides in all vertebrate classes strongly suggests that the integrity of the molecules is essential for their biological activity (Hadley, 1984). Both hormones mediate responses of short duration and their water-soluble molecular characteristics require that they interact with distinct cell-surface receptors.

This thesis examines only GLU, so the action of this hormone will be described.

1) Role of glucagon in mammalian liver cells

Studies on mammalian liver GLU receptors suggest that this receptor is a lipoprotein (Rodbell *et al.*, 1971). The use of anti-GLU receptor antibodies has identified different antigenic determinants located within distinct receptor domains and has lead to the description of the

receptor as a seven-pass transmembrane protein with the GLU-binding site on the third extracellular loop (Iwanij and Vincent, 1990). The GLU sequence is identical in most mammals (Hadley, 1984) suggesting that the receptor will also be similar. A GLU receptor has been cloned and sequenced (Jelinek *et al.*, 1993).

Glucagon stimulates glucose release in the short-term through an increase in glycogenolysis, whereas it acts through gluconeogenesis (from amino acids and glycerol) for longer term glycemic changes (Hadley, 1984). The hormone also stimulates lipolysis and amino acid uptake (Mine *et al.*, 1983). GLU does regulate some forms of phosphodiesterase ("membrane bound" form, Heyworth *et al.*, 1983; "dense vesicle" form, Houslay, 1986), the enzyme that degrades cAMP, which does influence GLU actions (Gettys *et al.*, 1988).

Like EPI, GLU stimulates ACase activity (Rodbell *et al.*, 1971; Exton, 1979a; Wright and Rodbell, 1979) through a distinct stimulatory G protein (Houslay, 1986; Padrell *et al.*, 1987). The levels of G protein and glucagon receptors differ among mammals (Padrell *et al.*, 1987). Intracellular cAMP level increases and triggers phosphorylation of a number of proteins through activation of a cAMP-dependent PKA that leads to glucose release and inactivation of glycogen synthesis (Hadley, 1984). Some mammalian cells (Mine *et al.*, 1983; Kurstjens *et al.*, 1990) demonstrate a transient rise in $[Ca^{2+}]_i$ to applied GLU. The presence of two sub-populations of GLU receptors linked through these two signalling pathways in mammalian hepatocytes supports the potential of contributing in different ways to the metabolic response (Wakelman *et al.*, 1986; Grady *et al.*, 1987). Apparently an inhibitory G protein is not involved in the desensitization of ACase that may lead to a consequent stimulation of inositol phospholipid metabolism (Murphy *et al.*, 1989). The mobilization of Ca^{2+} induced by GLU may also be due to a cAMP-dependent activation of PLC, leading to stimulation of IP_3 formation (Blackmore and Exton, 1986). There is also the possibility that the two classes of receptors interconvert between affinity states leading to these two signalling pathways (Horwitz *et al.*, 1985). However the formation of IP_3 in GLU-mediated intracellular effects in mammalian hepatocytes is still controversial as experiments using rats and guinea pigs suggested a

cAMP-dependent increase in $[Ca^{2+}]_i$ followed glucagon exposure, possibly through a cAMP-dependent phospohrylation of the IP_3 receptor/channels (Staddon and Handsford, 1989; Burgess *et al.*, 1991).

Prolonged exposure of rat hepatocytes to GLU induced a decrease in the concentration of glucagon receptors, called desensitization (Santos and Blazquez, 1982). Electron microscopy has shown GLU-receptor complex internalization and association with lysosome-like structures in rat hepatocytes (Authier *et al.*, 1992). The translocation from the cell surface is rapid and reversible.

2) Role of glucagon on teleost liver cells

Recently GLU receptors have been detected on hepatocytes of two teleost fishes, the American eel and the brown bullhead, but not in the rainbow trout (Navarro and Moon, 1994). The description of these receptors in fish agrees with those in mammals; the analysis of the data suggest the presence of two classes of receptors with different affinities for GLU and different membrane densities. Pre-incubation with GLU reduced the number of high-affinity binding sites suggesting receptor desensitization, probably by receptor internalization, as for mammalian hepatocytes.

GLU hyperglycemic effects in fish are due to the activation of glycogenolysis and gluconeogenesis through the stimulation of ACase and the consequent increase of intracellular cAMP concentrations (Birnbaum *et al.*, 1976; Janssens and Lowrey, 1987; Moon *et al.*, 1989; Mommsen and Moon, 1989; 1990). GLU is also found in fish to be less effective than EPI at low concentrations in activating cAMP synthesis (Mommsen and Moon, 1989). Experiments on eel, bullhead and trout hepatocytes showed that the hormone-induced increases in cAMP and the magnitude of the metabolic responses (glycogenolysis and gluconeogenesis) showed marked intra- and interspecific differences (Mommsen and Moon, 1989; 1990). In goldfish the glycogenolytic effect of GLU is much stronger than that induced by EPI (Birnbaum *et al.*, 1976). The lipolytic action of GLU through the activation of triglyceride lipase, is pronounced in

several species. The triglyceride-derived glycerol may represent a major source of C-3 glucose precursors. Amino acid uptake is also considerably stimulated by GLU (Thorpe *et al.*, 1974; Mommsen and Moon, 1989; Janssens and Grigg, 1992).

There is no evidence that the metabolic responses induced by GLU in these less advanced classes of vertebrates involve the inositol phosphates as possible intracellular mediators. In reptile hepatic tissue GLU stimulated a greater increase in cAMP as well as greater metabolic responses than EPI, but there was no evidence of any transient increase of $[Ca^{2+}]_i$ in the transduction pathway for either hormone (Janssens and Grigg, 1992). In studies on axolotl livers it was clear that GLU stimulated cAMP which provoked an increase in $[Ca^{2+}]_i$ but without concurrent changes in IP_3 (Kleineke and Janssens, 1993). To date there are no studies in fish regarding the possible impact of IP_3 in the GLU transduction mechanism. The possible role of IP_3 as a second messenger related to $[Ca^{2+}]_i$ in fish arises indirectly from studies in non-hepatic tissues of teleosts and elasmobranchs where IP_3 binding and inositol phosphate metabolism have been detected, respectively (Kalinoski *et al.*, 1992, bullhead; Ecay and Valentich, 1991, shark). Moreover the adrenergic-induced changes in $[Ca^{2+}]_i$ by Zhang *et al.* (1992a) may lead one to propose that the GLU transduction pathway in fish may also involve Ca^{2+} mobilization.

D. Questions and Hypotheses

The information collected from studies on hormone receptors, transduction mechanisms and cellular responses of hepatic tissue shows a conservative evolutionary trend. EPI and GLU through their respective receptors induce the same metabolic effect in the liver cells of all classes of vertebrates. However, our understanding of the cellular transduction pathways for EPI and GLU in fish livers is limited, since until recently research has been restricted to descriptive studies of the metabolic actions of the hormones.

Given the similarities of the metabolic responses induced by EPI and GLU in all vertebrate classes, it can be hypothesized that the transduction mechanisms may retain the same

characteristics in all vertebrate classes including fish. On the other hand, as vertebrates closer to fish display the absence of some components of the system, including α-adrenoceptors and IP_3 synthesis, the necessity to shed some light on the degree of complexity of the transduction mechanisms in fish is evident.

Several questions arise at this point. Is catecholamine action in fish mediated by both α- and β-adrenoceptors or is the α-type absent as is thought in amphibians and reptiles?

The recent studies concerning the stimulatory effect of EPI on fish cytosolic Ca^{2+} support the presence of α_1-adrenoceptors and these studies have recently been substantiated with the detection of this receptor subtype in black bullhead hepatic membranes (Fabbri *et al.*, 1994). But is this kind of responsiveness an isolated case or do other fish species also have these receptors? The presence of this receptor subtype must be shown in other species before concluding that α_1-adrenergic receptors are included in the population of receptors on fish liver membranes.

The presence of α_1-adrenoceptors leads one to hypothesize the possibility of an IP_3-mediated change in $[Ca^{2+}]_i$. If IP_3 can be detected after hormone exposure in different species, it is possible to include the inositol-phosphate mechanism as part of the transduction pathway used by fishes to modulate metabolic responses. Moreover the assessment of IP_3-binding sites on internal liver membranes would not only support the hypothesis of IP_3-dependent intracellular Ca^{2+} regulation, but further support the presence of α_1-adrenoceptors in the species that show IP_3 synthesis.

Thus, as in mammals, more than one second messenger may be involved in regulating the stimulation by a single hormone in fish liver. Another important point is to examine the differences in second messenger synthesis to quantitate the involvement of different transduction systems in the stimulation of the cells with hormones. According to previous results, GLU induced a far greater amount of cAMP in eel than in bullhead hepatocytes (Mommsen and Moon, 1990), but in terms of total glucose released, the two species responded in a similar way. To explain this metabolic behaviour it is possible to hypothesize that bullhead

hepatocytes compensate for the lower cAMP synthesis by the synthesis of IP_3, while the eel cells respond preferentially through the cAMP-mediated pathway.

And finally, a comparison of second messengers synthesis between EPI and GLU may answer the question as to whether the two hormones have different potentials in evoking a cellular response.

E. Purpose and intention of the Study

In this thesis livers from American eel (*Anguilla rostrata*) and black bullhead (*Ictalurus melas*) will be used to test the hypothesis of different cAMP and IP_3 levels after hormone stimulation, the presence of IP_3-binding sites and the existence of α_1-adrenoceptors. The data gathered from the two species will allow to compare the relative capabilities of these species to use the two transduction pathways. In order to accomplish this, the synthesis of the second messengers cAMP and IP_3 will be measured in isolated hepatocytes exposed to both hormones. The addition of α- and β-antagonists with EPI will provide support for the involvement of α- and β-adrenoceptors in the synthesis of the second messengers.

The presence of IP_3-binding sites will be tested on microsomal preparations that are enriched in internal membrane fractions. The binding sites will be identified with the use of tritiated IP_3 (^{3}H-IP_3) in time course experiments to study the time necessary for the binding to occur. The characterization of the IP_3-binding sites will be carried out with the displacement of ^{3}H-IP_3 with non-radioactive IP_3 and quantitated by Scatchard analysis (dissociation constant, K_D, and binding capacity, B_{max}).

The presence of α_1-adrenoceptors will be assayed in purified liver membranes of both species, even though these receptors have been recently reported in black bullhead membranes. Only a few experiments will be undertaken with this species, as the main characteristics of the binding sites have already been presented (Fabbri *et al.*, 1994). Saturation of the binding sites to obtain specific binding will be performed with increasing concentrations of tritiated prazosin (^{3}H-PRZ). The characterization of the receptors will be carried out by displacing ^{3}H-PRZ with

increasing concentrations of non-radioactive antagonists with different degrees of specificity for the α_1-adrenoceptors. The K_D and B_{max} values will be assessed by Scatchard analysis.

The activity of the enzyme ACase will be measured in crude membrane preparations. The aim of this last part of the study is to verify whether the enzyme has different activities in the two species in order to explain the different levels of cAMP synthesis following EPI and GLU stimulation.

These studies will provide a better understanding of EPI and GLU transduction in fish liver cells, but more importantly, may identify areas for further study of species differences in these transduction systems.

CHAPTER 2:
MATERIALS AND METHODS

A. Animals and Their Maintenance

Sexually immature American eels, *Anguilla rostrata* LeSueur were obtained from the St. Lawrence River at the Saunders Hydroelectric Dam (Ontario Hydro) during their upstream migration in the summer of 1993. Eels were transported to Ottawa on ice. Sexually mature black bullheads, *Ictalurus melas* were supplied by Dr. Joe Buttner, State University of NY, Brockport. These fish were raised from eggs in their pond culture facility. Bullheads were transported to Ottawa in constantly aerated water; transit time was approximately 5 h.

All fish were kept in tanks of flowing, well aerated, dechloraminated City of Ottawa tap water at 10°C for eel and 15°C for bullheads. Photoperiod was constant at 12L:12D. Eels were maintained without feeding, while bullheads were fed daily with commercial catfish pellets (Purina Mills Inc; St Louis, MO, USA).

Fish were acclimated to laboratory conditions for approximately 3 weeks prior to use. All experiments were conducted within 6 months of the fish arriving in the aquatic facility.

B. Hepatocyte Preparation

Viable hepatocytes were routinely obtained in good yield following the procedures described by Moon *et al.* (1985) and Moon and Mommsen (1990). Slight modifications to the general protocol were made to optimize hepatocyte yield for the two species. The composition of solutions used in the preparation is listed on Table 2-1.

Fish (approximately 150-200 g for eels and 200-300 g for bullheads) were anesthetized in 2-phenoxyethanol (approximately 20 ml/l tap water). Once immobilized, a mid-ventral incision was made to expose the liver. The hepatic portal vein was cannulated using Intramedic polyethylene tubing. Medium A was pumped through the liver using a Haake Buchler peristaltic pump at a flow rate adjusted to approximately 2 ml/min for approximately 5 min to clear the organ of circulating blood. Perfusions were performed *in vitro* on a watch glass for the eel and *in*

Table 2-1. Composition of perfusion media for hepatocyte isolation.

MEDIUM	COMPOSITION	NOTES
A	(Hanks Salts) 0.8 mM $MgSO_4$ 0.33 mM NaH_2PO_4 0.44 mM KH_2PO_4 136.7 mM NaCl 5.4 mM KCl 5.0 mM HEPES 5.0 mM HEPES-Na	5.0 mM $NaHCO_3$ was added, pH was adjusted to 7.63 at RT. For eel hepatocytes the medium A used for rinsing the liver was enriched with 1mM EGTA.
B	Medium A + collagenase: 0.25 mg/ml for eel 0.15 mg/ml for bullhead	pH was adjusted to 7.63 at RT.
C	Medium A + 1.5 mM $CaCl_2$ + 1.5% BSA for eel or 2.0% BSA for bullhead	pH was adjusted to 7.63 at RT.

RT, room temperature (ca. 18°C).
BSA, bovine serum albumin.

situ for the bullhead. Gentle massaging of the liver enhanced the clearing process.

The perfusion solution was then switched to medium B and the perfusion was continued for an additional 45 or 30 min for eel and bullhead, respectively, to obtain satisfactorily digested tissue and cell separation. Repeated massaging facilitated the collagenase to circulate through the entire tissue mass. At the first visible signs of disintegration (when the liver was expanded and soft), the perfusion was stopped and the liver was freed from the gall bladder and all other adhering tissues. The digested liver was placed on a Petri dish and finely minced with a razor blade in ice-cold medium A. The suspension was then filtered through two layers of nylon netting (253 and 73 µm, respectively) to remove liver pieces; this involved gently massaging the minced tissue through the coarser screen to increase the final yield.

Hepatocytes were rinsed and collected after a series of four centrifugations at 90 xg (1,000 rpm) for 2 min at 4°C in a Sorval RC28S centrifuge (SS34 rotor; Du Pont Canada). The washing procedures were performed twice with medium A followed by twice with medium C. The final resuspension of the cells was in medium C. The hepatocyte concentration was taken to approximately 50 mg cells per ml suspension medium. Cell concentrations were determined by weighing in duplicate cells from a given volume of suspension after centrifugation at 13,000 xg for 1 min in a Fisher 235B microcentrifuge, elimination of the supernatant and wiping the microfuge tube walls with lint-free absorbent tissue.

C. Hepatocyte Incubation

Time course and dose-response experiments were carried out at room temperature (18°C) on hepatocytes to study the time dependency and the effect of different concentrations of agonists used to stimulate cAMP and IP_3 production.

Cells remained on ice for the time needed to complete the experimental set-up. Just prior to initiating an incubation, the cell suspension was brought to room temperature for 5 min. The agonists used were epinephrine (EPI) and glucagon (GLU). Phentolamine (PHE) and propranolol (PROP), generalized α- and β-adrenoreceptor antagonists, respectively (Exton,

1985), were always added 10 min before agonists.

Hormones and α- and β-antagonists when used, were diluted at the last moment to avoid photodegradation. EPI was made as 1 mM stock and diluted to the required concentrations in medium A (Table 2-1). Antagonists were similarly dissolved. Glucagon was dissolved in 1 ml of 2.5 mM HCl to obtain a stock concentration of 0.15 mM; further dilutions were made in medium A.

In both time course and dose-response experiments, incubations were carried out in a total volume of 200 µl in plastic microcentrifuge tubes. 180 µl of cells were added to 20 µl of diluted hormone; medium A replaced the agonists in the control samples. When the α- and β-antagonists were used, 10 µl of the antagonist was added to the cells and left to incubate for 10 min; 10 µl of EPI was added to stimulate the cells.

Incubations were terminated at the specified time by adding 25 µl of "cocktail for standard curve" (Table 2-2), vortexing and leaving the samples on ice for 20 min. Samples were then centrifuged for 5 min (13,000 xg), then 180 µl of the supernatant recovered and neutralized with one half volume of 1M $KHCO_3$. The neutralized samples could be stored at 4°C for one day or at -20°C for a longer time period before assaying. Just prior to assaying cAMP and IP_3, the samples were again centrifuged for 5 min at 13,000 xg. From the supernatant, 25 µl was used to determine cAMP levels, and 20 µl and 10 µl were used for IP_3 in eel and bullhead, respectively.

D. cAMP Assay

The determination of cAMP levels followed the procedures described by Brown *et al.* (1972) and used previously in fish studies (Fabbri *et al.*, 1991).

cAMP was assayed using the solutions noted in Table 2-2. The assay is based on the competitive binding between cAMP (produced by the intact cells or by the membranes prepared to test the activity of the enzyme adenylyl cyclase, ACase) and a fixed quantity of ^{3}H-cAMP for a cytoplasmic protein, the enzyme protein kinase A, which has a high specificity and affinity for

Table 2-2. Solutions for cAMP assay

SOLUTION	COMPOSITION		NOTES
ASSAY BUFFER	50 mM Tris-HCl 10 mM theophylline 6 mM β-mercaptoethanol		pH was adjusted to 7.4 at RT with HCl. Theopylline was used to preserve cAMP from degradation.
COCKTAIL FOR STANDARD CURVE	10 mM LiCl 4 mM EDTA 20% PCA (solvent)	A	1 ml H_2O was added to 125 µl of A and 550 µl of B. H_2O substituted for the cocktail when testing the dilution of the new binding protein. Used the same volumes in the blank and standard curve to have the same acidity and ionic strength of the sample.
	1 M $KHCO_3$	B	
cAMP STANDARD	0.2 mM cAMP, 1:1000 (10 pmoles/50 µl)		The other dilutions were 7, 4, 2, 1 pmoles/50 µl.
^{3}H-cAMP STOCK SOLUTION	1:100 in 95% EtOH-H_2O, 1:1		The stock solution was diluted to give approximately 20,000 cpm/25 µl.
BINDING PROTEIN	Diluted in assay buffer		The dilution was determined by running standard curves of different dilutions. 100 µl used in the assay.
CHARCOAL	2% BSA 10% Charcoal		In assay buffer.

RT, room temperature.
PCA, perchloric acid.

cAMP (Brown *et al.*, 1972). The amount of the radioactive protein-cAMP complex generated in the assay is inversely proportional to the amount of cAMP produced by the hepatocytes or by the crude membranes. Consequently, measurement of the radioactivity allows one to calculate the non-radioactive cAMP present in the sample from a standard curve.

The binding protein was extracted from bovine adrenal cortex, as this tissue is rich in protein kinase A. The method of preparation is described by Brown *et al.* (1971, 1972). Bovine adrenal glands were obtained from a local slaughter house (C.H. Thomas, Ltd, Ottawa). Sufficient adrenal glands were used to obtain 50-80 g of cortex which were separated from the surrounding fat and demedullated. The tissue was then minced with scissors on ice and homogenized in a ice cold blender for 6 min in 2 vol (w/v) of ice cold 0.32 M sucrose solution. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 9,200 xg (10,000 rpm; Sorval RC28S) for 10 min at 4°C. The fat deposited on the surface of the supernatant was removed and the supernatant was centrifuged at 4°C in a Beckman L8-70 M using a TI 50 rotor at 100,000 xg for 1 h. After removal of the fat, the supernatant was collected in a beaker; 0.5 ml aliquots were prepared and stored at -20°C for at least 15 days before use.

To choose the right binding protein concentration to be used in the assay, 200 µl of the preparation was diluted 1:10 and 1:15 in cAMP assay buffer (Table 2-2). The binding strength of the two dilutions was tested by running cAMP standard curves as described below. The best dilution was found by plotting the concentration in pmol of cAMP used in the standard curve against the corresponding Co/Cx ratios (see below) in cpm. The line drawn through the points for each dilution that was the closest to a 45° angle represented the most appropriate dilution.

The binding protein was thawed, diluted in assay buffer the day preceding its use and re-frozen. The day of the assay, the protein was thawed and kept on ice. The assay was carried out in duplicate in 1.5 ml plastic microcentrifuge tubes on ice. The total volume of the assay was 350 µl. The neutralized samples were centrifuged for 5 min at 13,000 xg in a microcentrifuge and 25 µl of the supernatant used in the assay. A blank was prepared with 25 µl of "cocktail for the standard curve", 125 µl of assay buffer and 25 µl of the diluted ^{3}H-cAMP stock solution

(containing approximately 20,000 cpm). Binding protein was replaced by distilled water to reach the total assay volume. This blank represents the background radioactivity.

The cAMP standard curve consisted of samples containing 50 µl of 0 (water; total binding or T), 1, 2, 4, 7 and 10 pmoles cAMP. The standard cAMP concentrations were prepared by diluting the 0.2 mM cAMP stock solution (10 nmoles per 50 µl) 1:1,000 to obtain 10 pmoles per 50 µl. All the dilutions were prepared from this in a volume to provide the amount for duplicate assays. To each standard was added the "cocktail for the standard curve" (Table 2-2), the assay buffer and ^{3}H-cAMP in the same amount as in the blank. The binding protein (100 µl) was added to all tubes and the final volume of 350 µl was adjusted with water. The neutralized samples were run in parallel with the same volume additions. All procedures were undertaken in a ice-water slush bath. Once all components were added to the tubes, the tubes were vortexed and incubated a 10°C for 90 min. The tubes were shaken periodically. At the end of the incubation period, 100 µl of charcoal-BSA suspension was added. The tubes were vigorously vortexed followed by centrifugation at 13,000 xg at 10°C for 5 min. 200 µl of the supernatant were carefully withdrawn and placed in scintillation vials and 4.5 ml of ACS II (Amersham, Canada Ltd, Oakville, ON) added. The radioactivity was counted using a 1215 Rack Beta Liquid Scintillation Counter (LKB WALLAC) after the vials sat in the dark for 12 h.

The production of cAMP in the unknown samples was calculated by comparison with the radioactivity measured by the standard curve. The cpms of the duplicates were averaged and the blank value subtracted from these averaged values. The ratio between the total counts (0 pmoles cAMP; Co) and the correspondent counts of each sample (Cx), was calculated. The Co/Cx values of the standard were linearized by using regression analysis, and the pmoles cAMP for each of the unknown sample was calculated from this curve. The results were corrected according to the dilution factors used in the protocol and then referred to g of cell wet wt.

E. IP_3 Assay

The IP_3 assay was that previously described by Challiss *et al.* (1990), and again required

the preparation of an IP_3 binding protein from bovine adrenal cortex. The basis of this determination is similar to that described for cAMP (above, D). The solutions used for this assay are noted on Table 2-3.

The preparation of the IP_3 binding protein followed the methods described by Challiss *et al.* (1990) and Palmer *et al.* (1988). The cortex obtained from bovine adrenal glands was minced with scissors, weighed and homogenized in an ice cold blender for 6 min in 8 vol (w/v) of homogenization buffer, pH 8.0, containing 2 mM $NaHCO_3$ and 1 mM dithiothreitol (DTT). The homogenate was filtered through one layer of cheesecloth and centrifuged at 5,000 xg (7,500 rpm; RC28S) for 15 min at 4°C. The supernatant was collected and re-centrifuged at 38,000 xg for 20 min at 4°C. The resulting pellet was re-homogenized with one stroke of an ice cold Dounce homogenizer in 4 vol (w/v) of homogenization buffer and centrifuged again at 38,000 xg. The pellet was resuspended in a total volume of 50 ml of cold homogenization buffer. After gently mixing the resuspension, 0.5 ml aliquots were pipetted into 1.5 ml plastic centrifuge tubes and stored at -20°C.

To assay IP_3, the binding protein was thawed and diluted to 100 µg protein per 100 µl with assay buffer. Protein was determined using the method of Lowry *et al.* (1951); 1 N NaOH was used to dilute the IP_3 binding protein 1:5 or up to 1:40 for this determination. BSA was used as a standard and absorbance was measured at 500 nm with a Spectronic 1001 Plus (Milton Roy). The IP_3 assay was run on ice, in 1.5 ml plastic microcentrifuge tubes in duplicates. The total assay volume was 500 µl. A standard curve (see Table 2-3) consisting of 50 µl of increasing concentrations of unlabelled IP_3 was prepared from a 1:10 dilution of a 100 µM stock IP_3 (5,000 pmoles per 50 µl) in a manner similar to that described for the cAMP assay (above, D). In addition, a total (T; 0 added non-radioactive IP_3) and a non-specific (NS; 500 pmoles per 50 µl, 10 µM) were run for each assay. The neutralized samples to be assayed for IP_3 content were centrifuged for 5 min at 13,000 xg. Ten and 20 µl of supernatant were assayed for bullhead and eel, respectively. The sample and standard were incubated in parallel for 30 min on ice. Bound from free ^3H-IP_3 was separated by a 9 min centrifugation at 13,000 xg at 10°C. The

Table 2-3. Solutions for IP_3 assay

SOLUTION	COMPOSITION		NOTES
ASSAY BUFFER	100 mM Tris-HCl 4 mM EDTA 0.04% BSA		pH was adjusted to 9.0. Used 180 µl and 200 µl for bullhead and eel, respectively. 300 µl were used in assaying IP_3 binding sites.
COCKTAIL FOR STANDARD CURVE	10 mM LiCl 4 mM EDTA 20% PCA	A	1 ml H_2O was added to 125 µl of A and 550 µl of B. H_2O substituted for cocktail when testing the dilution of the new binding protein.
	1 M $KHCO_3$	B	
IP_3 STANDARD	100 µM IP_3 (500 pmoles/50 µl)		The NS and the 25 pmoles per 50 µl were made from the stock, diluting 1:10 and 1:100, respectively. Other dilutions were: 12.5, 6.4, 3.2, 1.6, 0.8, 0.4 pmoles/50 µl.
^3H-IP_3 STOCK SOLUTION	95% EtOH-H_2O, 1:1		The dilution factor was calculated depending on the dpm of 50 µl of the stock, since the assay was run with 7,000 to 9,000 dpm per tube.
BINDING PROTEIN	Diluted in assay buffer (200 µl/sample)		The dilution factor was calculated on the basis of the protein content, needing 100 µg protein/100 µl. The dilution was made in assay buffer the same day of the assay, and kept on ice.

NS, non-specific.

supernatant was carefully aspirated and the pellet was resuspended in 300 µl water and quantitatively transferred to scintillation vials. ACS II scintillant (4.5 ml) was added and radioactivity was determined after the vials remained in the dark for 12 h (above, L). The results in nmoles IP_3 per g of cell wet wt were calculated as for the cAMP determination (above, D) by subtracting the NS from T and by referring the Co/Cx ratios of each sample to those of the standard curve.

F. IP_3 Binding Assay

Intracellular IP_3 binding sites were characterized in microsomes prepared from eel and bullhead livers according to Challis *et al.* (1990).

Fish were anesthetized as previously described. Livers were quickly removed, cleaned and weighed. Livers were minced with scissors in ice cold homogenization buffer composed of 2 mM $NaHCO_3$ and 1 mM DTT, pH 8.0. After decanting the rinsing fluid and the blood, each liver was homogenized on ice in cold buffer with a 10 sec burst of a Polytron (KINEMATICA, Brinkmann Instruments, Rexdale, ON) homogenizer, followed by 3 passes with an ice cold tight-fitting hand homogenizer (teflon-glass, Wheaton 30 ml). The suspension was made to a total 10-15 vol (w/v) with homogenization buffer.

The homogenate was centrifuged at 5,000 xg (7,500 rpm; Sorval RC28S) for 15 min at 4°C. The fat deposited at the top of the supernatant was aspirated, and then the supernatant was re-centrifuged at 34,500 xg (19,250 rpm; RC28S) for 20 min at 4°C. The pellet was resuspended using an ice cold Dounce hand homogenizer in a few ml of cold homogenization buffer. A further centrifugation at 34,500 xg for 20 min at 4°C recovered the microsomal pellet that was resuspended in 1 vol (w/v) of IP_3 assay buffer without BSA (Table 2-3). The protein content of the preparation was estimated as before using 0.1 N NaOH to dilute the pellet prior to the protein evaluation.

The membrane-enrichment of the microsomal preparation was estimated by assaying membrane bound 5'-nucleotidase (5'NTase; EC 3.1.3.5) activity as this enzyme is routinely

used as an indicator of intracellular and plasma membranes (Solyom and Trams, 1972). The enzyme was assayed according to Solyom and Trams (1972), using the production of inorganic phosphate as measured according to Bonting (1970). The solutions for the 5'NTase assay are listed on Table 2-4. Ten µl of the microsomal fraction were incubated with 200 µl of substrate solution for 15 min at 28°C. The reaction was stopped by adding 200 µl 25% TCA, the tubes vortexed and after 20 min on ice or optional storage at -20°C, the samples were centrifuged at 13,000 xg for 5 min. Inorganic phosphate was measured in 300 µl of this supernatant against a blank and a standard phosphate concentration measured concurrently. 420 µl of 0.6 N PCA and 600 µl of color reagent were added, the tubes were vortexed and incubated for 15 min at RT (18°C). The absorbance was measured at 700 nm and compared with the standard. Results are expressed as µg Pi/g protein/min.

IP_3 binding sites were characterized in the hepatic microsomal preparation using the same principle described for the IP_3 assay (above, E). Measurements of the radioactivity of the protein-^{3}H-IP_3 complex provided evidence for binding. Time course experiments were run to test the association of the ^{3}H-IP_3 with the microsomal fraction and to determine the time of maximum binding. Each sample was run in duplicate at 1, 2, 5, 10, 15, 30 and 60 min. At each time, a T (total; 0 IP_3) and a NS (non-specific; 1 µM IP_3) were run to evaluate IP_3-specific binding (T - NS = S). Incubations contained 100 µl of microsomes (800 µg of protein), 50 µl ^{3}H-IP_3 (corresponding to approximately 14,000 dpm and 0.2 nM IP_3), 50 µl non-radioactive IP_3 where required, and 300 µl IP_3 assay buffer (Table 2-3). Incubations were run on ice and terminated at the appropriate time by centrifugation (13,000 xg for 9 min at 10°C); the supernatant was removed by aspiration. The pellet was resuspended in 200 µl of water and quantitatively transferred to scintillation vials and counted as described for the IP_3 assay (above, D).

The specific binding at each time period was obtained by subtracting the NS from the corresponding T counts. The dpm of IP_3-specific binding was transformed into fmoles/mg protein by the correlation of the dpm with the specific activity of ^{3}H-IP_3 (54 Ci/mmol) and mg

Table 2-4. Solutions for 5'-nucleotidase assay.

SOLUTION	COMPOSITION	NOTES
SUBSTRATE SOLUTION	75 mM Trizma base 75 mM Tris-HCl 5 mM $MgCl_2$ 10 mM KCl 5 mM 5'AMP	For 100 ml solution, 15 ml Trizma base were combined with approximately 85 ml Tris -HCl, 29 µl of a 17.24 M $MgCl_2$ solution and 75 mg KCl were added to the buffer. pH was adjusted to 9.0 at RT. 5mM 5'AMP was added to the buffer just prior to use.
TCA SOLUTION	25% in water	
PCA SOLUTION	6% in water	Diluted from 70% PCA.
COLOR REAGENT	16 mM NH_4molybdate.$4H_2O$ 3.16 ml H_2SO_4 conc/100 ml 0.14 M $FeSO_4.7H_2O$	$FeSO_4$ was dissolved immediately before use.
STANDARD PHOSPHATE	1.6 mM $PO_4^=$	22 mg NaH_2PO_4 were dissolved in order to obtain 5 µg $PO_4^=$/100 µl of solution.

RT, room temperature.
TCA, trichloroacetic acid.

protein used (800 µg). Results were expressed in % of maximal association considering 100% the maximum or total radioactivity bound at 30 min.

IP_3 binding was further characterized by displacement experiments. The experiments involved the competition between the radioactive tracer (^{3}H-IP_3) and the non-radioactive ligand for IP_3 binding sites. Each assay was run in duplicate. Microsomal protein was incubated for 30 min on ice in the presence of 10,000-15,000 dpm of ^{3}H-IP_3 with increasing concentrations of the non-radioactive IP_3. T and NS binding were run for the assay to obtain the concentration of the sites relative to the specific IP_3 bound. Increasing dilutions of the stock IP_3 (100 µM) ranging from 0.01 nM to 1 µM were tested in competition with the ^{3}H-IP_3 for the IP_3 binding sites. The total volume of the assay was 500 µl and the volume of the ^{3}H-IP_3 and of the dilutions of the standard IP_3 tested was 50 µl. IP_3 buffer (300 µl; Table 3-3) and 100 µl of microsomes were added to all samples. Incubations were terminated and IP_3 binding analyzed as described in the association experiments. The displacement of ^{3}H-IP_3 was presented as % of total binding by referring to the maximum specific bound in the total (T) as 100%.

G. α-Adrenoceptor Binding Assay

The presence of α_1-adrenoceptors in livers of eel and bullhead was detected on purified membrane fractions prepared and assayed following the methods described by Ray (1970) for rat liver and modified by Fabbri *et al.* (1991, 1994) for fish liver. The solutions used are presented on Table 2-5.

Fish were anesthetized as previously described. The liver was removed, cleaned and weighed. The tissue was minced with scissors in a beaker containing ice cold homogenization buffer (Table 2-5) and then homogenized on ice with a Dounce homogenizer (8 strokes) followed by a tight-fitting hand homogenizer (2 strokes). The homogenate was brought to 25 vol (w/v) with ice cold homogenization buffer, stirred on ice for 2 to 5 min and filtered through a coarse nylon mesh (253 µm). After centrifugation at 3,000 xg (5,750 rpm; Sorval RC28S) for 10 min at 4°C, the pellet was resuspended in 10 vol (w/v) of ice cold homogenization buffer,

Table 2-5. Composition of the solutions for purified plasma membrane preparations.

SOLUTION	COMPOSITION	NOTES
HOMOGENIZATION BUFFER	1 mM $NaHCO_3$ 5 mM $CaCl_2$ 1 mM leupeptin	pH was adjusted to 7.75. Leupeptin was added daily to the amount of buffer used.
SUCROSE SOLUTIONS	8% 37%(3.7ml for gradient) 41%(4.4ml for gradient) 45%(1.9ml for gradient) 80%	Sucrose was dissolved in homogenization buffer without leupeptin. Gradients were prepared at 10°C.
INCUBATION BUFFER	1.0 mM $NaHCO_3$ 100.0 mM HEPES 120.0 mM NaCl 1.2 mM $MgSO_4$ 15.0 mM CH_3COONa 1.0 mM EDTA 2.5 mM KCl 10.0 mM glucose	Glucose was added to the amount of buffer used for the preparation. pH was 5.5 to store the buffer without glucose at 4°C. After glucose addition, the pH was adjusted to 7.75. Unless otherwise specified, the buffer contained glucose.

filtered through a fine nylon mesh (73 µm) and re-centrifuged as before. This pellet was resuspended in 2 vol (w/v) of the homogenization buffer, filtered through a fine nylon mesh and mixed with 80% (w/v) sucrose to reach a final sucrose concentration of 31%. Two ml of this resuspension was layered over a discontinuous sucrose gradient composed of 45%, 41% and 37% sucrose solutions and centrifuged at 4°C in a Beckman L8-70M (SW 41 rotor) at 100,000 xg (30,500 rpm) for 2 h. The purified plasma membranes were recovered from the 37% sucrose fraction, diluted with cold homogenization buffer to a final 8% sucrose concentration and centrifuged at 25,000 xg (16,500 rpm; Sorval RC28S) for 15 min at 4°C.

The pellet was resuspended in 8% sucrose and re-centrifuged under the same conditions. The final pellet was resuspended in 0.5-1.0 ml of incubation buffer (Table 2-5). Protein content and 5'NTase activities were assayed as previously described. The protein content was taken to 350 µg and to 150 µg per 50 µl for eel and bullhead, respectively.

Saturation and displacement experiments were used to identify and to characterize the α-adrenoceptors on these membranes. Increasing concentrations of ^{3}H-prazosin (PRZ; Du Pont), a specific α_1-adrenoreceptor antagonist ($\alpha_1 > \alpha_2$; Exton, 1985), were used to saturate the binding sites existing on the purified plasma membranes. An aliquot of ^{3}H-PRZ was diluted 1:100 in an ethanol-water 1:1 mixture to give a 225 nM stock solution; 10 µl of this stock contained approximately 450,000 cpm. The total volume of the assay was 150 µl, so that 10 µl of the stock was 15 nM in the assay and this stock was used to prepare 10, 5, 2, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 nM dilutions. All dilutions were made in incubation buffer without glucose (Table 2-5). A stock non-radioactive PRZ solution was prepared in DMSO (dimethylsulfoxide) and diluted in incubation buffer to 10 µM. At each ^{3}H-PRZ concentration, a total (T; only ^{3}H-PRZ) and a non-specific (NS; ^{3}H-PRZ + 10 µM PRZ) were run in duplicate. In addition, 50 µl of membranes (containing 350 or 150 µg protein for eel and bullhead, respectively) and incubation buffer (Table 2-5) were added to a final volume of 150 µl. The samples were vortexed and incubated for 15 min at RT. The incubation was terminated by sudden cooling (0°C), and 130 µl of the incubate was carefully layered over 300 µl of 3% ice cold RIA-grade BSA in incubation

buffer in a cold room (10°C). The samples were centrifuged at 13,000 xg for 3 min at 10°C, the supernatant removed by aspiration and the pellet resuspended in 100 µl of 0.2% ice cold RIA-grade BSA and re-centrifuged as before. The pellet obtained was resuspended in 100 µl of 50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.4, quantitatively transferred to scintillation vials and counted 12 h later as previously described (above, D). The dpm of specific binding for each concentration of ^{3}H-PRZ was calculated by subtracting the NS dpm from the correspondent T. The dpm of T, NS and specifc binding of each dilution of ^{3}H-PRZ was expressed in fmoles by referring the total radioactivity present in the sample to the specific activity of the radioisotope. Results were plotted as fmoles/mg protein against ^{3}H-PRZ concentrations, obtaining a saturation curve (see Results).

Displacement experiments were used to characterize this adrenoceptor binding as α_1-type. Increasing concentrations of the non-radioactive antagonists PRZ, phentolamine (PHE), and propranolol (PROP) (from 0.01nM to 10 µM for PRZ and PHE; from 0.1 µM to 0.1 mM for PROP) were used to displace ^{3}H-PRZ. The assay was not run in duplicate, with the exception of the total (T; only ^{3}H-PRZ), unless enough sample was available. A T for the whole assay was prepared with 10 µl of ^{3}H-PRZ at 1 nM or 30,000 dpm. The NS sample was represented by the highest non-radioactive PRZ concentration for all the displacing agents tested. For each displacing agent tested samples containing 10 µl of ^{3}H-PRZ and 50 µl of a particular dilution of non-radioactive agonist were run. PHE and PROP were prepared in incubation buffer (Table 2-5) at the above mentioned concentrations. The assay was run and the data were calculated as noted for the saturation study. Final results were expressed as % of maximum binding referring the specific binding of each displacing agent dilution to the specific binding of ^{3}H-PRZ (T, no addition) as 100%.

H. Adenylyl Cyclase Assay

Adenylyl cyclase (ACase; EC 4.6.1.1) was assayed in crude liver membranes prepared according to Rugg *et al.* (1978) for rat lung membranes and previously used by Ottolenghi *et al.*

(1988) for bullhead liver.

As previously, the liver was removed and minced with scissors in cold homogenization buffer consisting of 5 mM Tris-HCl, pH 7.4, 1 mM $MgSO_4$ and 0.25 M sucrose. The tissue was rinsed and homogenized on ice in cold buffer with two 10 sec bursts of the Polytron. This homogenate was subjected to 2 strokes of an ice-cold tight-fitting hand homogenizer. The homogenate was filtered through a double layer of cheesecloth and more cold homogenization buffer was added to a total of 10 vol (w/v). The homogenate was centrifuged at 480 xg (2,250 rpm; Sorval RC28S) for 10 min at 4°C. The supernatant was collected and centrifuged at 30,000 xg (18,000 rpm) for 10 min at 4°C. The pellet was resuspended in homogenization buffer without sucrose and re-centrifuged under the same conditions. The final pellet was resuspended in 2 vol (w/v) of assay buffer consisting of 50 mM Tris-HCl, pH 7.4 and 4 mM EDTA. The protein content of the crude membrane was assayed and diluted to 250 and 150 µg per 100 µl for eel and bullhead membranes, in order to obtain comparable activities.

ACase activity in this membrane fraction was carried out as described by Ottolenghi *et al.* (1988). Membranes were incubated for 10 min at RT with increasing concentrations (from 0.01 nM to 100 µM) of EPI or GLU. Incubations were undertaken in 1.5 ml plastic microcentrifuge tubes with a total volume of 400 µl. This consisted of 100 µl of ACase assay mix (final concentration 1mM ATP, 3.75 mM $MgCl_2$, 0.1 mM EGTA in 100 mM Tris-HCl, pH 8.0), 50 µl GTP (0.5 mM final), 50 µl theophylline (3 mM final), 100 µl membranes and 100 µl assay buffer or agonist. The tubes were gently shaken during the incubation to continuously mix the components. The reaction was stopped by immersing the tubes in boiling water for 2 min. After cooling to RT, the samples were stored at -20°C. ACase activity was estimated by determining cAMP levels as previously described (above, D). Modifications to the cAMP assay were as follows. The "cocktail for the standard curve" (Table 2-2) was replaced by the same amount of the assay buffer used to resuspend the final pellet. The volume of the supernatant assayed was 50 µl. Results are expressed as pmoles cAMP/mg of protein /10 min.

I. Chemicals

Epinephrine, phentolamine, propranolol, synthetic cAMP and IP_3 and all of the components of the solutions prepared for the different procedures were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), BDH (Toronto, ON), ICN Biochemical Inc. (Mississagua, ON) or Amersham Canada Ltd (Oakville, ON). Crystalline glucagon was generously provided by Eli Lilly and Co. (Indianapolis, IN, USA). ^{3}H-cAMP and ^{3}H-IP_3 were purchased from Amersham Canada Ltd. and ^{3}H-PRZ was purchased from Du Pont Canada Inc. (Mississagua, ON). All other chemicals were obtained from local suppliers and were of the highest available purity.

All hormones and α- and β-adrenergic antagonists were diluted in physiological saline (medium A, Table 2-1) except prazosin which was diluted in DMSO. The agonists and antagonists solutions were prepared at the last moment in wrapped containers to reduce photodegradation of these compounds.

L. Statistics

Lines of graphs were drawn between points by eye. Linear regression analysis was used to calculate the cAMP and IP_3 concentrations in cell and membrane samples. When there were 3 or more independent experiments, data are expressed as the mean ± SEM. Values representing data with large SEM intervals were compared with controls to determine significant difference by one-way ANOVA (analysis of variance) (Zar, 1974) using STATISTIX software package (Analytical Software, St. Paul MN USA). Where necessary to find difference amongst groups, LSD (T) or Student's t-test were performed; a $p < 0.05$ was considered significant and a $p < 0.01$ as highly significant.

The "EBDA" and "LIGAND" (Munson and Rodbard, 1980) programs (Biosoft-Elsevier) were used to perform Scatchard analysis of the IP_3 and α_1-binding sites.

CHAPTER 3:
RESULTS

A. cAMP and IP_3 Studies

Eel (*Anguilla rostrata*) and bullhead (*Ictalurus melas*) hepatocytes were stimulated with epinephrine (EPI) with and without antagonists and glucagon (GLU) to study cAMP and IP_3 production. The cells were used at a concentration of 50 mg/ml suspension. Time course and dose-response experiments were performed to examine the changes in levels of the second messengers after exposure to the two agonists and to phentolamine (PHE) and propranolol (PROP), α- and β-adrenergic antagonists, respectively.

1) Time dependency of cAMP stimulated synthesis

Fig. 3-1 shows time course curves of control and EPI-stimulated cAMP production in bullhead hepatocytes. Incubations with 10 μM EPI at different time periods, up to 30 min, induced an increase in cAMP concentrations which peaked at 5 min and 985% above control. At this time, cAMP concentration in the control cells was 0.81 ± 0.2 nmoles/g (n = 7) compared with 8.0 ± 1.6 nmoles/g (n = 6). This transient rise slowly fell thereafter back towards control levels, but remained elevated for at least 30 min.

Subsequent time course experiments with time of exposure restricted to 5 min confirmed these results. Hepatocytes of both species were stimulated by 10 μM EPI with eels showing a 51-fold increase (0.70 ± 0.1 to 35.8 ± 1.18 nmoles/g, n = 4; Fig. 3-2A) and bullheads a 14-fold increase (1.05 ± 0.05 to 14.8 ± 0.49 nmoles/g, n = 4; Fig. 3-2B) in cAMP content at 5 min. Pre-incubation of these same cells with 100 μM PHE or PROP provided evidence that the α-antagonist did not oppose EPI-stimulation while the β-antagonist PROP totally abolished the EPI effect on cAMP production (Fig. 3-2). Eel and bullhead control values were significantly different at 0 min ($p<0.05$) and at 5 min ($p<0.05$), but not at 1 min.

GLU addition at 0.1 μM resulted in a lower transient elevation in cAMP compared to EPI, but at 1 and 5 min cAMP levels were significantly different from control (1 min, eel

Fig. 3-1: Effect of epinephrine (EPI) on intracellular concentrations of cAMP in isolated bullhead hepatocytes. Hepatocytes (50mg/ml) were incubated in the absence (close circle, n = 7) or presence (open triangle n = 6) of 10 µM EPI for the time period indicated. Incubations were terminated by adding 25 µl of "cocktail for standard curve" (Table 2-2), and cAMP assayed as in Materials and Methods. Values are the mean ± SEM of (n) experiments.

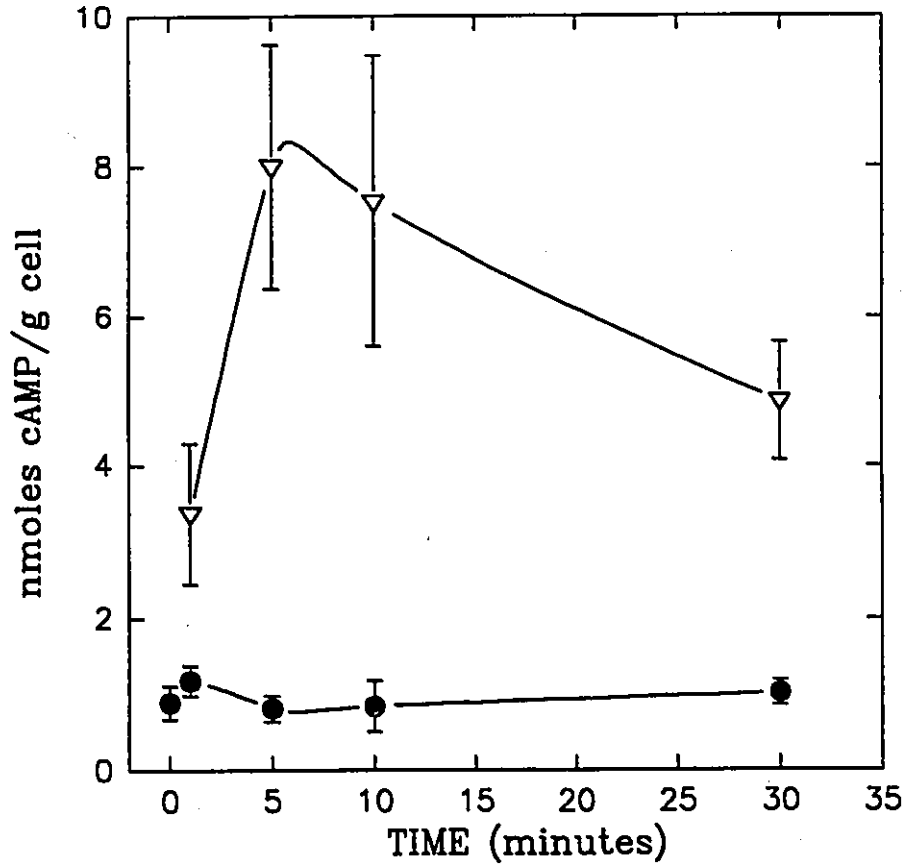
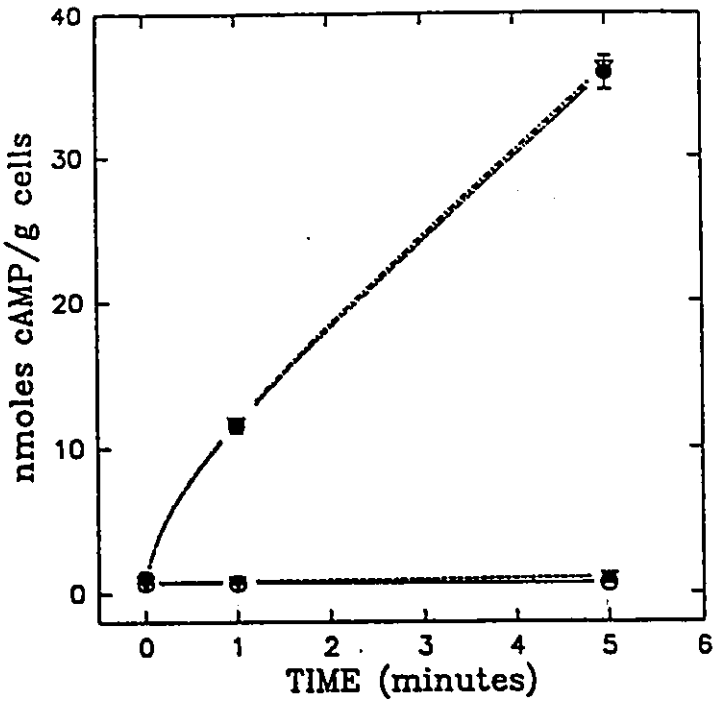
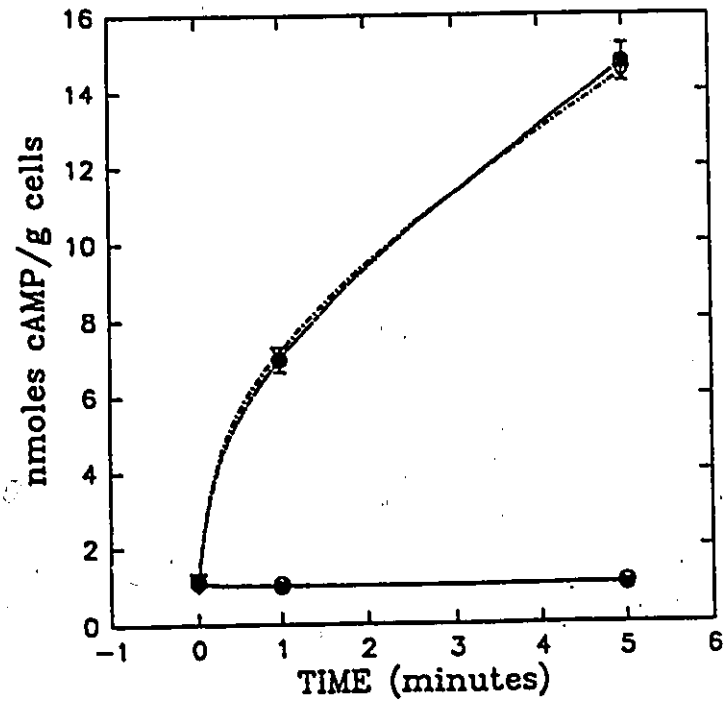

nmoles cAMP/g cell
TIME (minutes)
0
2
4
6
8
10
0
5
10
15
20
25
30
35

Fig. 3-2A, B: Time course for the epinephrine (EPI) effect tested with and without α- and β-antagonists on cAMP levels in isolated eel (A) and bullhead (B) hepatocytes. Hepatocyte incubations were as in Fig. 3-1 without (open circle) or with (close circle) 10 μM EPI plus phentolamine (open triangle, PHE 100 μM) or propranolol (close triangle, PROP 100 μM) for 5 min. Where antagonists were used, cells were preincubated for 10 min prior to adding EPI. Values are given for total cellular cAMP in nmoles cAMP/g cells ± SEM of n = 4 independent determinations. Student t-test showed control values of the two fish significantly different at 0 min and at 5 min ($p<0.05$), but not at 1 min.

A


nmoles cAMP/g cells
40
30
20
10
0
0
1
2
3
4
5
6
TIME (minutes)

B


nmoles cAMP/g cells
16
14
12
10
8
6
4
2
0
−1
0
1
2
3
4
5
6
TIME (minutes)

$p<0.001$, bullhead $p<0.05$; 5 min, eel $p<0.05$, bullhead $p<0.001$; Fig. 3-3A, B). The response at 5 min after addition for eel was 19-fold (0.70 ± 0.1 to 13.4 ± 1.39 nmoles/g, $n = 3$) compared with 2.3-fold for bullhead (1.05 ± 0.05 to 2.47 ± 0.16 nmoles/g, $n = 3$) hepatocytes. This cAMP increase from control was highly significant ($p<0.001$) in both species. Exposure of the same cells to GLU at a concentration 10,000-fold lower (0.01 nM) (Fig. 3-3A, B) induced a significant increase in cAMP levels (2.37 ± 0.42 and 1.32 ± 0.05 nmoles/g in eel and bullhead hepatocytes, respectively) compared with control values ($n = 3$) at 5 min ($p<0.05$), whereas at 1 min it was significantly different only for eel ($p<0.001$).

These results show that the two hormones, EPI and GLU, induced a greater cAMP response in eel than in bullhead hepatocytes, and a greater response to EPI than GLU at the concentrations tested.

2) Dose-dependent effect of agonists on cAMP levels

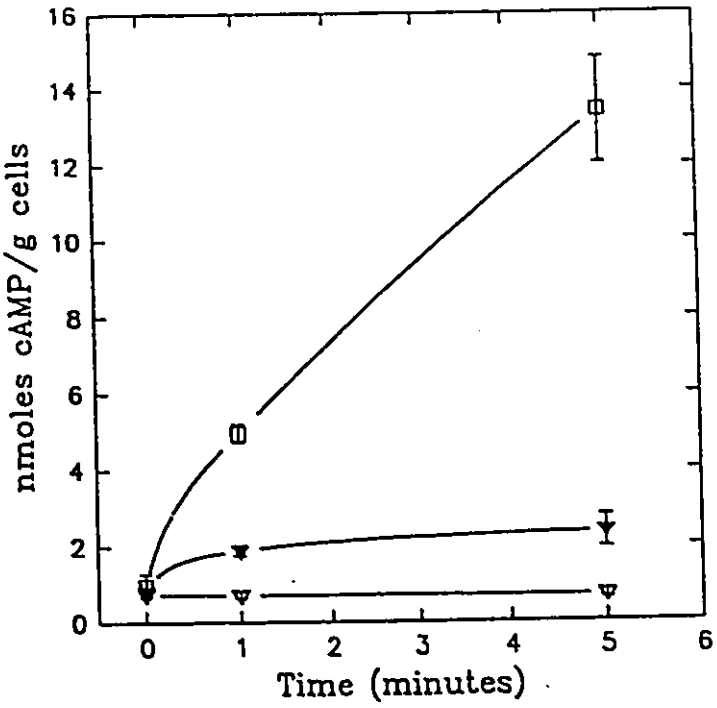
The response to different concentrations of the two hormones were tested on cells of both species after a 5 min incubation. EPI, ranging from 0.1 nM to 0.1 mM, produced a maximal cAMP stimulation at approximately 10 µM in both eel and bullhead (Fig. 3-4) hepatocytes. The amount of cAMP produced at 10 µM EPI was significantly higher in eel (39.6 ± 2.19 nmoles/g, $n = 7$) compared with bullhead (15.2 ± 0.37 nmoles/g, $n = 7$) cells ($p<0.001$). The K_a (50% of activation of the cAMP response under EPI stimulation) were calculated to be $5.7 \pm 1.6 \times 10^{-7}$ M and $1 \pm 0.25 \times 10^{-7}$ M for eel and bullhead, respectively and were not significantly different.

The effect of GLU from 0.001 nM to 10 µM was maximal at 1 µM in both species (Fig. 3-5). The absolute cAMP production was approximately 3.6-fold higher in eel than in bullhead (16.3 ± 0.94 vs 4.54 ± 0.05 nmoles/g, $n = 3$) hepatocytes. The K_a-values with GLU were $1.8 \pm 0.55 \times 10^{-8}$ M and $9.1 \pm 5.9 \times 10^{-8}$ M and were not significantly different.

These data show that there is a dose-dependent effect of EPI and GLU on the cAMP content of hepatocytes of the two species, and although the sensitivities of the hepatocytes are similar, the maximal responsiveness to EPI exceeds that to GLU in both species and confirms

Fig. 3-3A, B: Glucagon (GLU) effect on cAMP concentrations in isolated eel (A) and bullhead (B) hepatocytes. Hepatocyte incubations were as in Fig. 3-2 except GLU replaced EPI. Open square, GLU 0.1 μM; close triangle, GLU 0.01 nM; open triangle, control. All values are means ± SEM of n = 3 independent experiments. All increases were significantly different from control ($p<0.05$ and $p<0.001$) except for the GLU-induced response at 1 min at 0.01 nM GLU in bullhead.

A


nmoles cAMP/g cells
Time (minutes)
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6

B

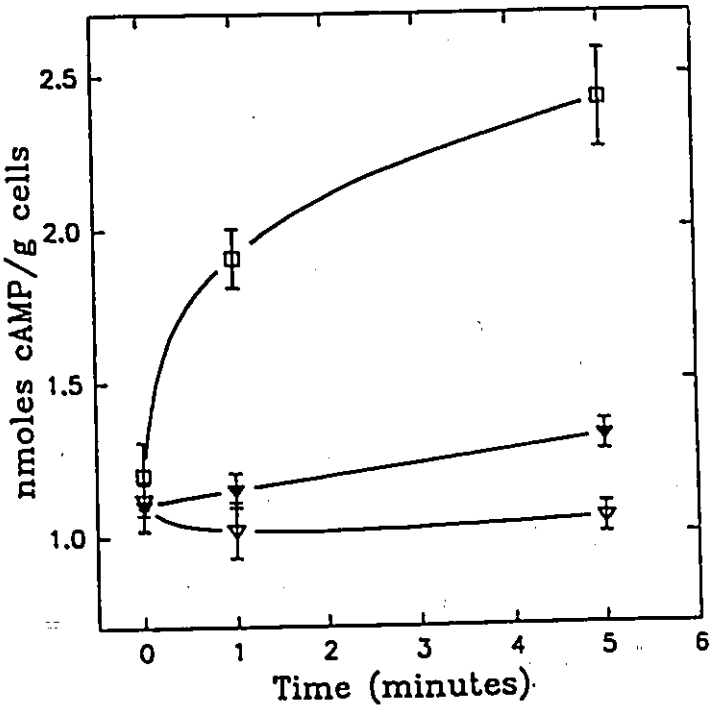

nmoles cAMP/g cells
Time (minutes)
1.0
1.5
2.0
2.5
0
1
2
3
4
5
6

Fig. 3-4: Effect of increasing concentrations on the cAMP response to epinephrine (EPI) in eel (open circle) and bullhead (close circle) hepatocytes. cAMP levels were measured after a 5 min incubation of the hepatocyte suspensions with increasing EPI concentrations. Peaks at 10^{-5} M EPI were significantly different ($p<0.001$), when compared with control. Values are means ± SEM of n = 7 independent experiments. K_a values (eel, 5.7 (± 1.6) x 10^{-7} M; bullhead, 1.0 (± 0.25) x 10^{-7} M) were calculated with the Hill plot and were not significantly different.

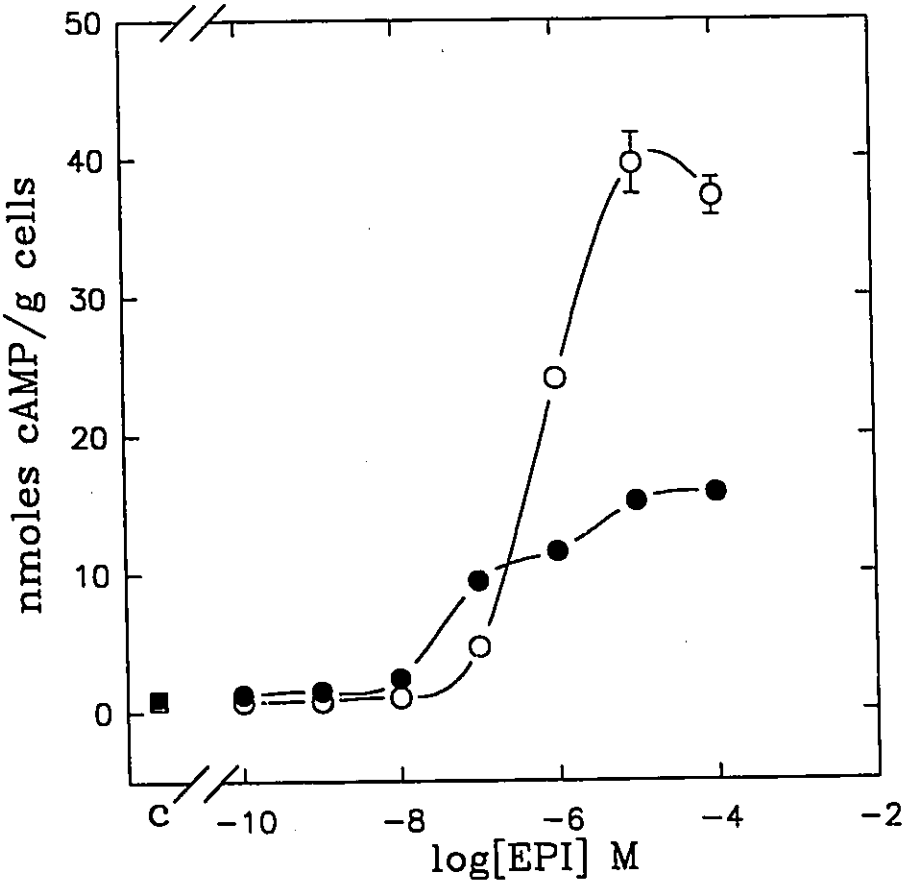
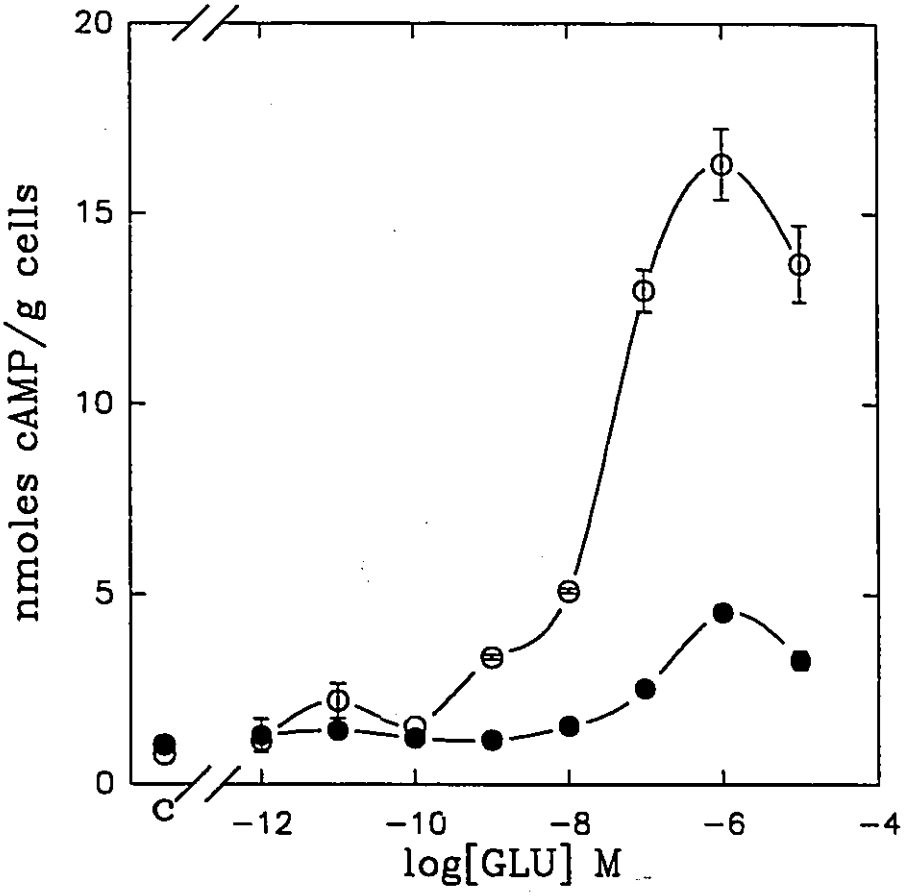

50
40
30
20
10
0
nmoles cAMP/g cells
C
−10
−8
−6
−4
−2
log[EPI] M

Fig. 3-5: Dose-effect of glucagon (GLU) on cAMP levels in isolated eel (open circle) and bullhead (close circle) hepatocytes. Hepatocytes were incubated for 5 min and cAMP assessed. Values are means ± SEM of n = 3 independent experiments. K_a values (eel, 1.8 (± 0.6) x 10^{-8} M; bullhead, 9.1 (± 5.9) x 10^{-8} M) were calculated by Hill plot and were not significantly different.


20
15
10
5
0
nmoles cAMP/g cells
C
−12
−10
−8
−6
−4
log[GLU] M

previous result that cAMP increase is higher in eel than in bullhead.

3) Time dependency of IP_3 synthesis

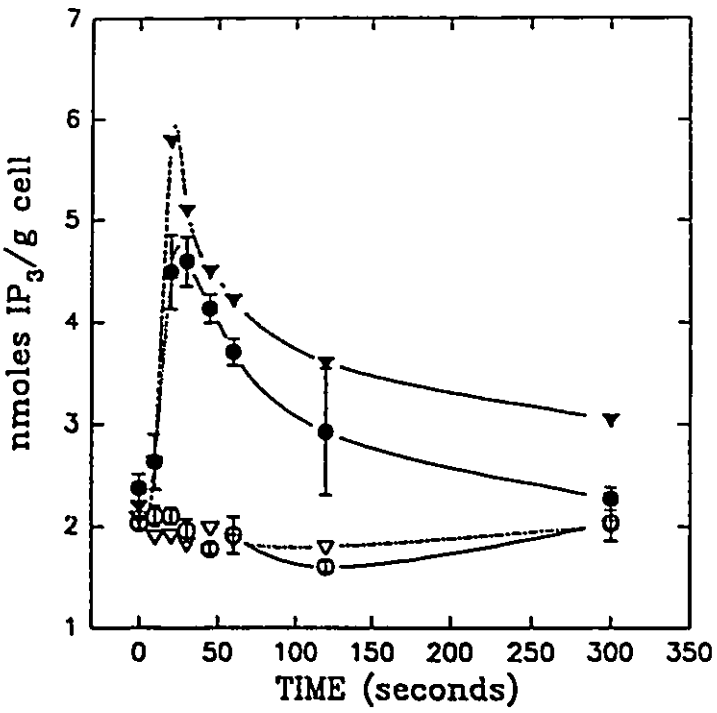
The stimulation of eel and bullhead hepatocytes with 10 μM EPI with or without 100 μM PHE or PROP showed a significant increase in IP_3 levels between 20 and 45 sec with a peak at approximately 30 sec (Fig. 3-6A, B) ($p<0.001$). This time course is consistent with previous studies in the literature using rat tracheal smooth muscle slices stimulated with 1 mM carbachol (Challiss *et al.*, 1990). At 30 sec of EPI stimulation, the peak levels of IP_3 were 4.59 ± 0.24 and 9.39 ± 0.33 nmoles/g ($n = 4$) in eel and bullhead, respectively. In the presence of EPI + PROP (β-antagonist), the peak IP_3 concentration increased slightly and may be shifted slightly. In eel hepatocytes the maximal level rose to 5.8 nmoles/g at 20 sec ($n = 2$), while to 10.0 nmoles/g in blullhead hepatocytes at 45 sec ($n = 2$). The pre-incubation with the α- and β-antagonists showed that PHE (α-antagonist) completely blocked the EPI-stimulated increase in IP_3, while PROP (β-antagonist) did not block but slightly increased IP_3 levels (Fig. 3-6A, B). The EPI peaks compared with the peaks obtained with pre-incubation with PROP were not significantly different in eel, while they were significantly different in bullhead ($p<0.05$).

The control levels of IP_3 were significantly different between the two species (2.03 ± 0.05 and 5.53 ± 0.12 nmoles/g, for eel and bullhead, respectively; Fig. 3-6A, B) ($p<0.001$ at any of the noted time). The comparison between the peak IP_3 production and the correspondent control value, indicated that the absolute concentration of IP_3 produced at 10 μM EPI was similar in both species (approximately 4 nmoles/g cells).

GLU at 0.1 μM generated a peak IP_3 stimulation at 30 sec in eel of 4.76 ± 0.43 nmoles/g ($n = 3$) and at 45 sec in bullhead of 10.2 ± 1.09 nmoles/g ($n = 3$) (Fig. 3-7A, B). The hormone stimulated eel hepatocytes less than bullhead hepatocytes when these maxima are compared with the corresponding control levels of 1.96 ± 0.11 and 5.93 ± 0.08 nmoles/g, respectively. The peaks were significantly different from control ($p<0.001$, eel; $p<0.05$, bullhead). Cells exposed at 0.01 nM GLU were poorly stimulated (Fig. 3-7A, B) although eel cells did show a significant

Fig. 3-6A, B: Time dependency of the epinephrine (EPI) effect on IP_3 synthesis tested with or without the presence of the α- and β-antagonists phentolamine (PHE) and propranolol (PROP) in isolated eel (A) and bullhead (B) hepatocytes. Hepatocytes (50 mg/ml) were incubated without (open circle) or with (close circle) 10 µM EPI (n = 4) plus 100 µM PHE (open triangle) or PROP (close triangle) (n = 2) and terminated at the specified time by adding "cocktail for standard curve" (Table 2-3). IP_3 content was assayed by competitive binding (see Materials and Methods). Values represent mean values ± SEM of (n) experiments. EPI peaks compared with those obtained with pre-incubation with PROP were not significantly different in eel, but significantly different in bullhead ($p<0.05$). However all peaks were significantly different from control ($p<0.001$), as were the control levels of the two fish compared together.

A


nmoles IP3/g cell
7
6
5
4
3
2
1
0
50
100
150
200
250
300
350
TIME (seconds)

B

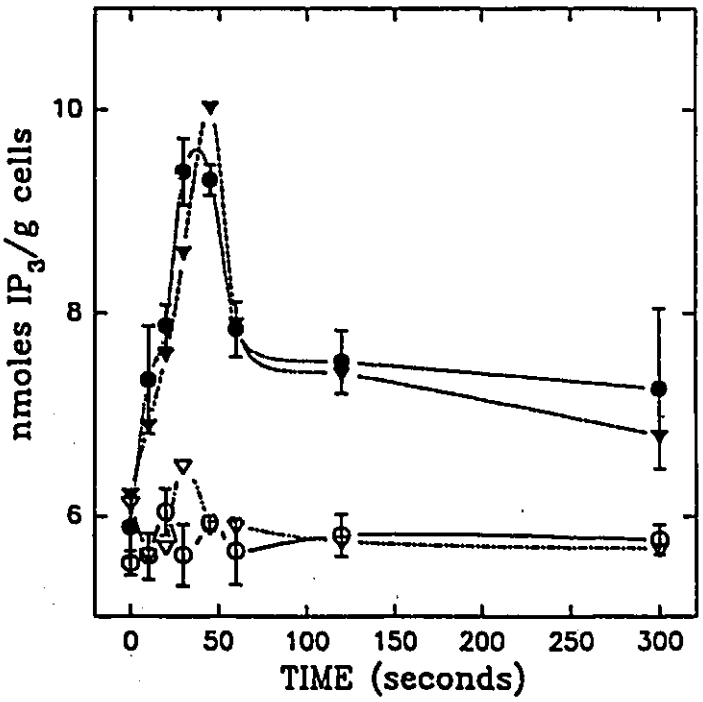
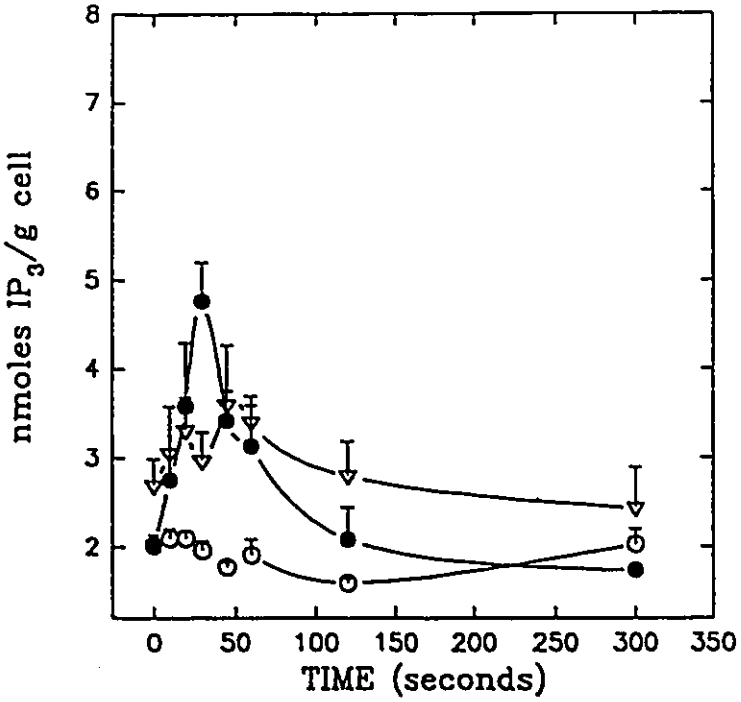
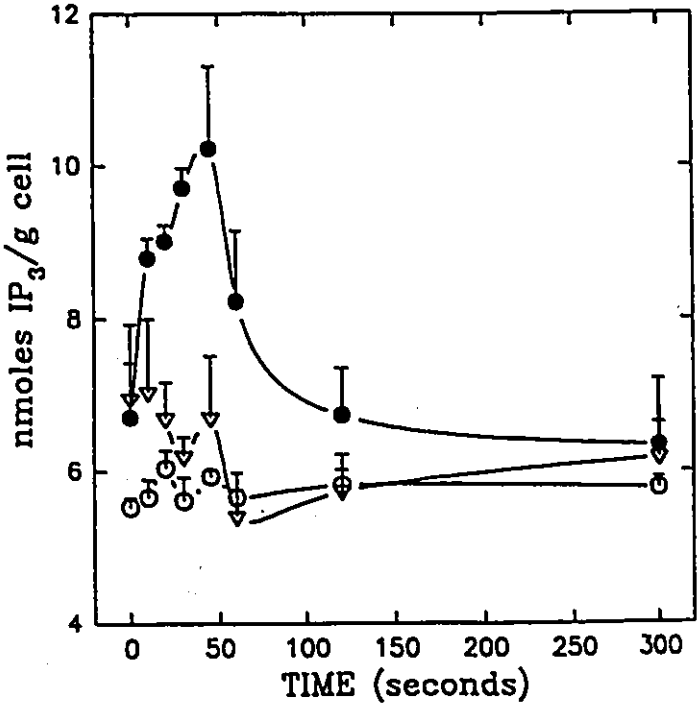

nmoles IP3/g cells
10
8
6
0
50
100
150
200
250
300
TIME (seconds)

Fig. 3-7A, B: Time course for the glucagon (GLU) effect on IP_3 levels in isolated eel (A) and bullhead (B) hepatocytes. Experiments were conducted as on Fig. 3-6, except GLU replaced EPI. Open circle, control; close circle, 0.1 µM GLU; open triangle, 0.01 nM GLU. Values represent means ± SEM of n = 3 experiments. Student t-test was performed and the peaks were considered significantly different (0.1 µM: eel, $p<0.001$; bullhead, $p< 0.05$; 0.01 nM: eel, $p<0.05$) from the control except for 0.01nM GLU in bullhead.

A


nmoles IP3/g cell
8
7
6
5
4
3
2
0
50
100
150
200
250
300
350
TIME (seconds)

B


nmoles IP3/g cell
12
10
8
6
4
0
50
100
150
200
250
300
TIME (seconds)

increase compared with the control level ($p<0.05$). The peak IP_3 values were obtained at 45 sec and were 3.59 ± 0.67 and 6.70 ± 0.82 nmoles/g for eel and bullhead, respectively ($n = 3$).

The two hormones, EPI and GLU, at concentrations of 10 and 0.1 µM, respectively, caused approximately the same fold-increase in IP_3 (2-fold), even though the response in terms of nmoles/g was different. This results from the significantly higher basal IP_3 level in bullhead compared with eel hepatocytes.

4) Dose-dependent effect of agonists on IP_3 synthesis

All dose response experiments were run with a 30 sec incubation period. The curves (Fig. 3-8) show that 10 µM EPI gave a maximal IP_3 response reaching 5.48 ± 0.66 and 11.1 ± 0.78 nmoles/g in eel and bullhead, respectively ($n = 4$). Eel hepatocytes showed significantly lower ($p<0.001$) control values (2.24 ± 0.16, $n = 15$ vs 6.17 ± 0.17 nmoles IP_3/g, $n = 13$) than bullhead, but the fold-changed at maximum were approximately equal for both species. An LSD (T) pairwise comparison following a one-way ANOVA showed that the responses were all significantly different from control values in both fish, except for the value obtained for bullhead at 100 µM EPI. It is important to note that cAMP concentration did not increase until 10 nM EPI (Fig. 3-4).

Glucagon addition resulted in two peaks of IP_3 production, at 0.01 nM and 0.1 µM in both eel and bullhead hepatocytes ($n = 4$). In eel cells, the two maxima were, respectively, 4.82 ± 0.32 and 5.14 ± 0.27 nmoles/g, while in bullhead, the peaks were 10.3 ± 1.03 and 10.6 ± 0.72 nmoles/g (Fig. 3-9). A LSD (T) pairwise comparison following a one-way ANOVA showed that in eel all values obtained in the dose-response are significantly different from control. In bullhead only the responses induced by GLU concentrations in the range from 0.1 µM to 0.01 nM are significantly different from control.

B. IP_3 Binding Studies

The specific activities of 5'-nucleotidase (5'NTase) were determined in eel and bullhead

Fig. 3-8: Epinephrine (EPI) dose-effect on IP_3 levels in isolated eel (open circle) and bullhead (close circle) hepatocytes. IP_3 synthesis was measured as on Fig. 3-7 after 30 sec of exposure of the hepatocyte suspensions to the different EPI concentrations. Values represent means ± SEM of n = 4 independent experiments. Control values are means ± SEM (error bars within symbols) of n = 15 (eel) or 13 (bullhead) and are significantly different between the two species ($p<0.001$). The levels obtained in the dose-response are significantly different from control in both fish, except the bullhead response at 10^{-4} M EPI, by a LSD (T) pairwise comparison following a one-way ANOVA.

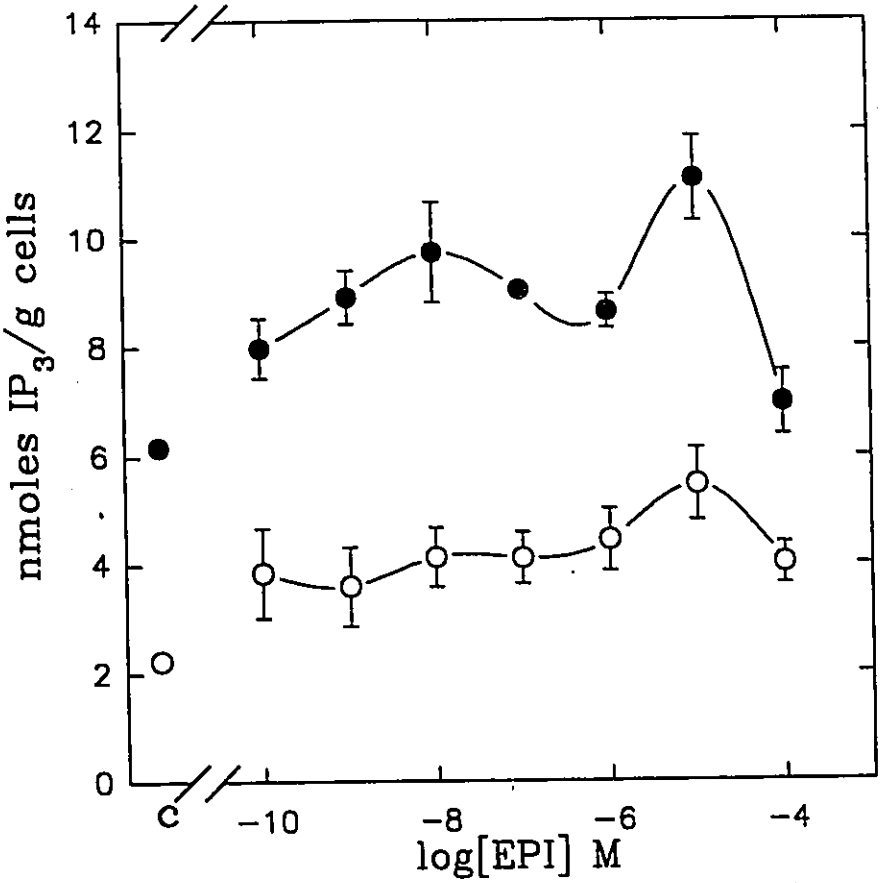
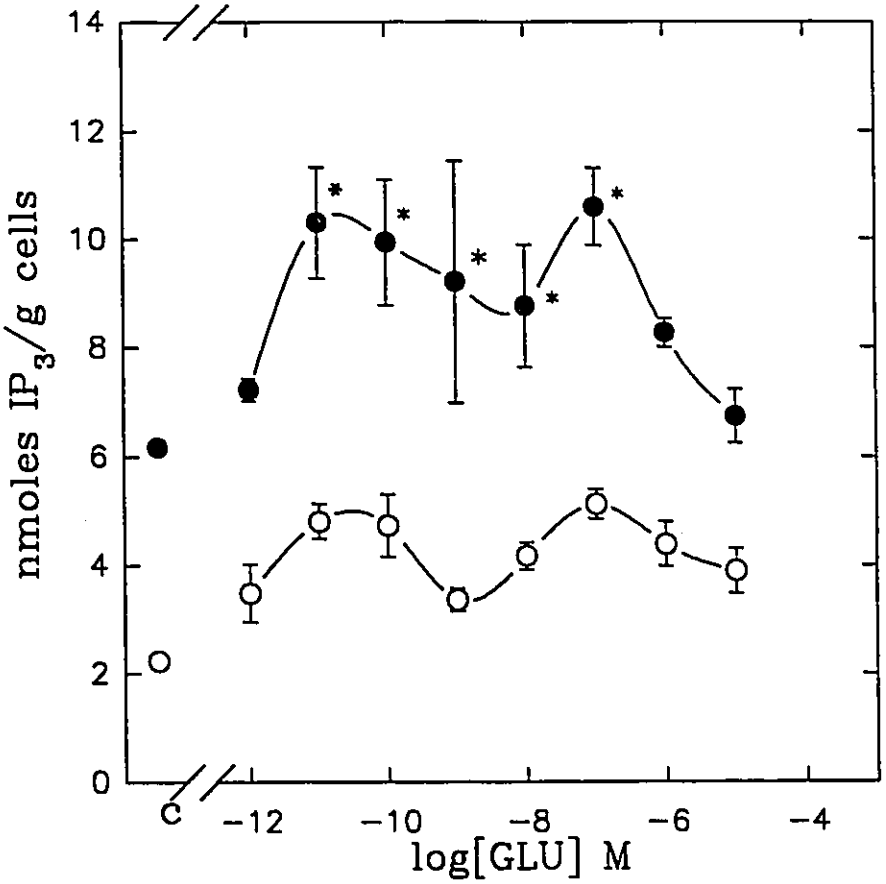

14
12
10
8
6
4
2
0
nmoles IP3/g cells
C
−10
−8
−6
−4
log[EPI] M

Fig. 3-9: Glucagon (GLU) dose-effect on IP_3 synthesis in eel (open circle) and bullhead (close circle) liver cells. Hepatocytes were incubated for 30 sec in increasing concentrations of GLU and IP_3 assayed as in Fig. 3-7. Values represent means ± SEM of n = 4 independent experiments. Control values are means ± SEM (error bars within symbols) of n = 15 (eel) or 5 (bullhead). * are significantly different ($p<0.05$) from control by a LSD (T) pairwise comparison following a one-way ANOVA.


14
12
10
8
6
4
2
0
nmoles IP3/g cells
C
−12
−10
−8
−6
−4
log[GLU] M
*
*
*
*
*

liver homogenates and in the microsomal fraction as a marker for the presence of membranes in the preparation. Table 3-1 shows that the final pellet was approximately 3 times enriched in this enzyme activity.

IP_3-binding sites were characterized in this microsomal fraction of both species using association and competition experiments. ^3H-IP_3 binding was measured on 100 µl of microsomal fraction containing 800 µg of protein and approximately 14,000 dpm of ^3H-IP_3 at a final IP_3 concentration of 0.2 nM. Non-specific (NS) binding represented the amount of ^3H-IP_3-bound in the presence of 1 µM non-radioactive IP_3.

1) Association experiments

The rate of association of ^3H-IP_3 with the microsomal membrane fraction of both species is shown in Fig. 3-10. The amount of radioactivity bound increased rapidly over the first 10 min, reached an optimum at 30 min, and slowly declined over the next 30 min. At optimum, specific binding was represented by 5.3 and 9.88 fmol of tracer bound/mg and by 4.17 and 7.86% of the total radioactivity in the incubate in eel and bullhead preparations, respectively.

2) Characterization of IP_3-binding sites

Displacement experiments were carried out incubating 100 µl of membranes from both species for 30 min with the same amount of ^3H-IP_3 previously described (Fig. 3-10) and increasing concentrations of non-radioactive IP_3, from 0.01 nM to 1 µM. Non-specific binding (NS) was determined in the presence of 1 µM IP_3; total binding (T; only ^3H-IP_3, no non-radioactive IP_3) was run to calculate the specific binding in fmoles/mg of protein that represented 100% of the ^3H-IP_3 bound. Increasing concentrations of non-radioactive IP_3 induced a competition for the binding sites between the non-radioactive and the radioactive IP_3, until all ^3H-IP_3 was displaced by the non-radioactive ligand (0 ^3H-IP_3 bound). Total displacement was achieved at concentrations slightly lower than 0.01 µM in eel and 0.1 µM in bullhead (Fig. 3-11; n = 3). The non-specific binding of ^3H-IP_3 rappresented 4.1 and 3.4% of the total binding for eel

Table 3-1. Assessment of enrichment of microsomal membrane fractions prepared from eel and bullhead liver.

SPECIES	HOMOGENATE	MICROSOMAL FRACTION
EEL	15.4 ± 1.1	44.6 ± 7.7 (291%)
BULLHEAD	20.5 ± 2.6	54.5 ± 7.0 (268%)

The enrichment of 5'NTase activities in membranes (in %) was measured by comparing the specific activities in the membrane with those in the crude homogenate. Results are expressed in nmoles/mg of protein/min and presented as mean ± SEM of 4 experiments.

Fig. 3-10: ^{3}H-IP_3 binding to eel (close circle) and bullhead (open circle) liver microsomal fraction. Microsomal protein (800 µg) was incubated in ^{3}H-IP_3 (14,000 dpm, 0.2 nM IP_3) and terminated at the specified time by centrifugation at 13,000 xg for 9 min at 10 °C. The pellet was resuspended and radioactivity was estimated. For each time a total (T) and a non-specific (NS; 1 µM IP_3) sample were run to obtain the specific binding (T - NS = specific binding). Specific binding is expressed as a % of the maximum binding obtained at 30 min. The % of total radioactivity at optimal were 4.17 and 7.86 for eel and bullhead, respectively. Results are the means of 3 experiments, with variations not exceeding 5% (eel) or 11% (bullhead).

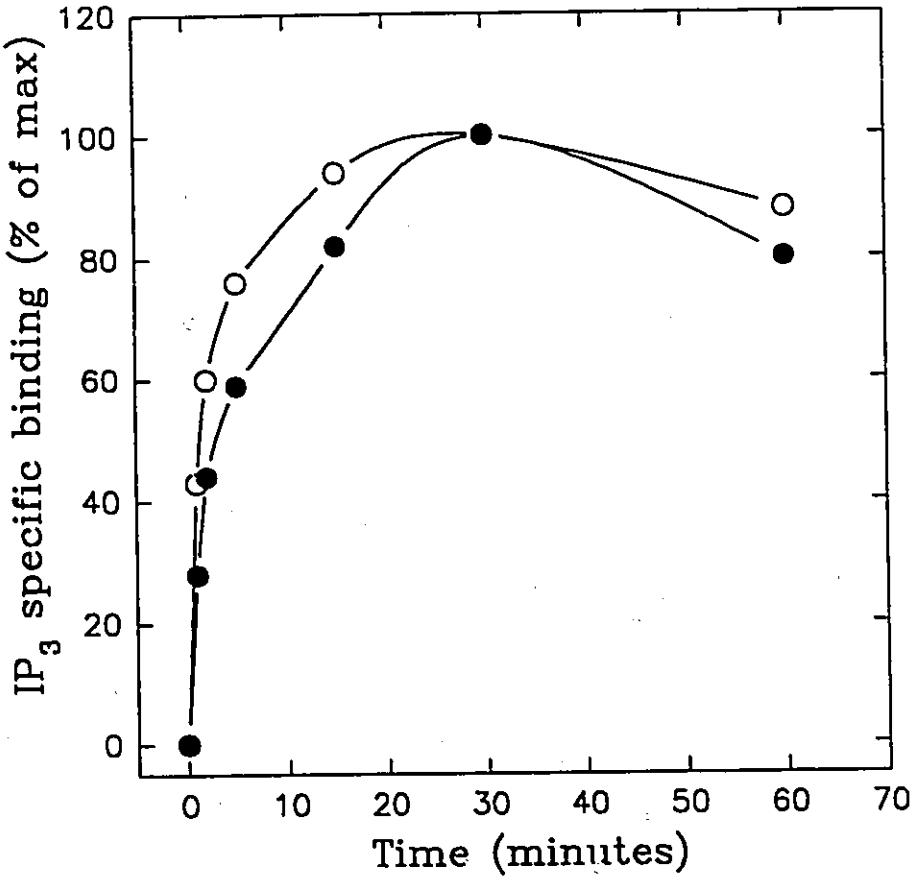
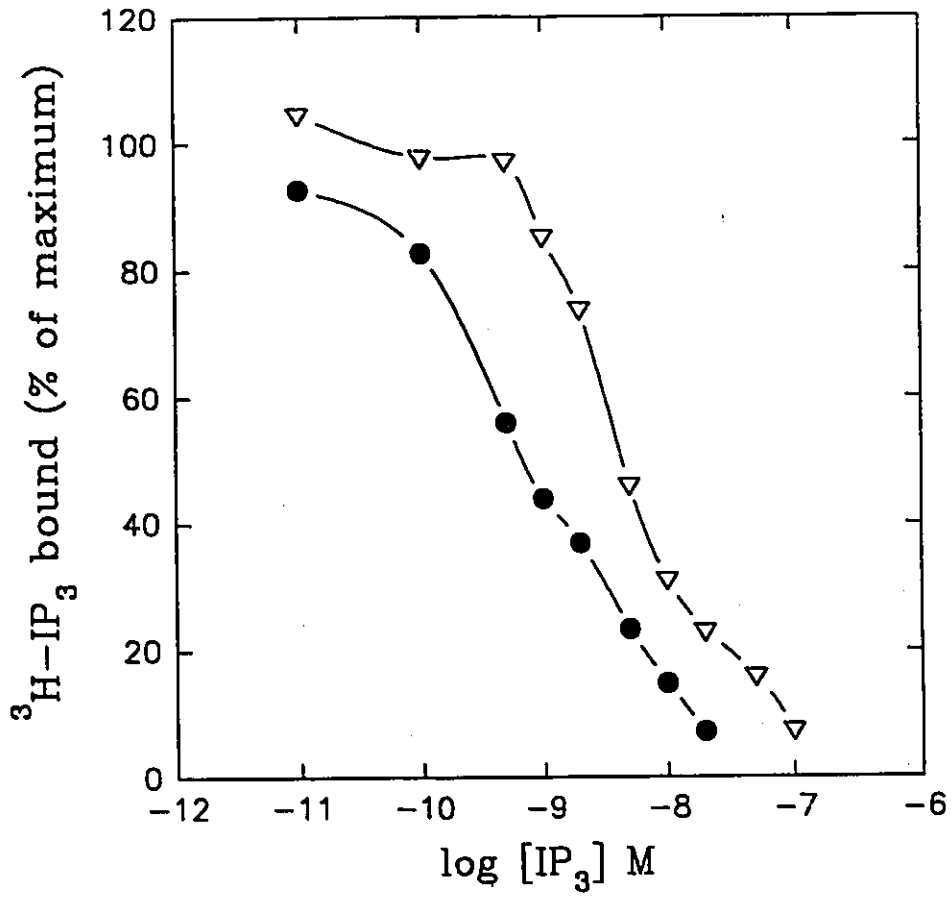

120
100
80
60
40
20
0
IP_3 specific binding (% of max)
0
10
20
30
40
50
60
70
Time (minutes)

Fig. 3-11: Displacement of ^{3}H-IP_3 from microsomal binding sites by non-radioactive IP_3 in eel (close circle) and bullhead (open triangle). Microsomal protein (800 µg) was incubated at increasing IP_3 concentrations for 30 min on ice and terminated as in Fig. 3-10. Results are the average of 3 experiments for each species; variation between values is less than 19 and 12 % of the absolute values for eel and bullhead, respectively, at the highest IP_3 concentrations.


120
100
80
60
40
20
0
−12
−11
−10
−9
−8
−7
−6
^{3}H−IP$_3$ bound (% of maximum)
log [IP$_3$] M

and bullhead, respectively, in the range of tracer routinely used.

Analysis of this IP_3-binding to the microsomal fraction was undertaken using Scatchard plots, and are reported in Fig. 3-12 (n = 3). This analysis suggests a two-site model to describe the existence of two classes of IP_3-binding sites of different characteristics on the membranes isolated from the two species. One class of binding sites is characterized by a high affinity for the displacing agent, described by a low K_D (nM concentration of competitive ligand needed to displace 50% of the radioactive ligand) and these sites are less represented on the membranes; the B_{max}(maximal binding capacity in fmoles/mg of protein) that represents the concentration of these binding sites is relatively low. The second class of binding sites is a low affinity (high K_D) and high capacity (high B_{max}) group compared with the other class. Table 3-2 demonstrates a significant difference between the two species with respect to IP_3-binding. The Student's t-test showed significant difference between the K_D and the B_{max} of the two groups of binding sites of the two fish ($p<0.001$ for low K_D and $p<0.05$ in all other cases of comparison). For both binding site classes, the eel membrane showed lower K_D and B_{max} values compared with the bullhead.

C. α-Adrenergic Receptors Characterization

The membranes recovered in the 37% sucrose fraction following the ultracentrifugation preparation described by Fabbri *et al.* (1992) for bullhead liver are known to display the highest enrichment of 5'NTase of all fractions present in the discontinuous sucrose gradient. Therefore this fraction was used to study the presence of α-adrenoceptors just as Fabbri *et al.* (1992) did for β- and α-adrenoceptors (1994).

1) Receptor affinity for the α_1-antagonist prazosin

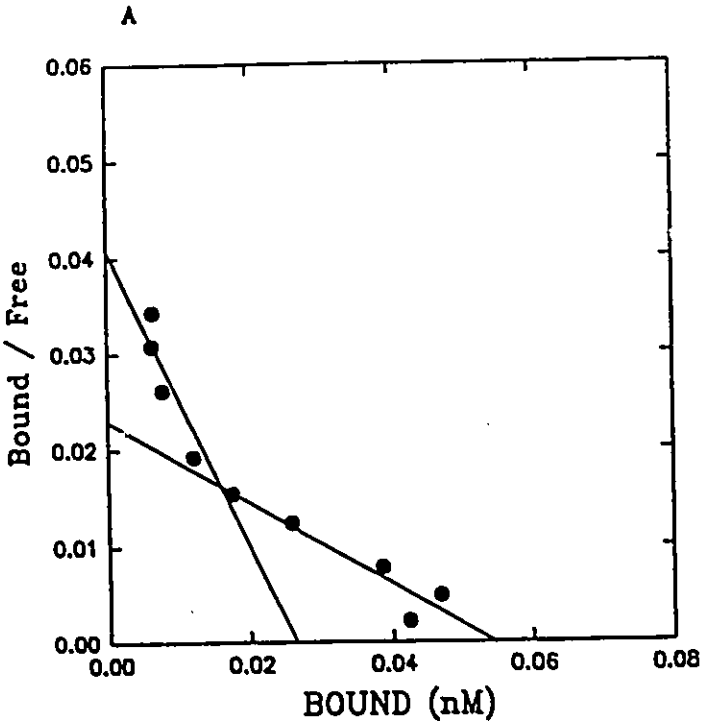
The binding of ^{3}H-prazosin (^{3}H-PRZ), a specific α_1-antagonist, was used to provide evidence for the presence of an α_1-adrenoceptor population on liver membranes of the two species. An incubation time of 15 min was selected based upon trials and a recent study by Fabbri *et al.* (1994) which examined ^{3}H-PRZ binding to bullhead hepatic membranes.

Table 3-2. IP_3 binding characteristics for eel and bullhead microsomal membranes.

CHARACTERISTICS	EEL	BULLHEAD
K_D (nM; high affinity)	0.74 ± 0.07	3.63 ± 0.3
B_{max} (fmoles/mg protein)	18.7 ± 1.4	164.5 ± 10.1
K_D (nM: low affinity)	2.21 ± 0.28	19.8 ± 1.5
B_{max} (fmoles/mg protein)	34.7 ± 1.8	384 ± 49

Results are the means ± SEM of 3 experiments carried out with each species.

Fig. 3-12A, B: Scatchard analysis of the IP_3-binding data in eel (A) and bullhead (B). Data are transformed from those in Fig. 3-11 using the program Ligand.


A
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Bound / Free
0.00
0.02
0.04
0.06
0.08
BOUND (nM)

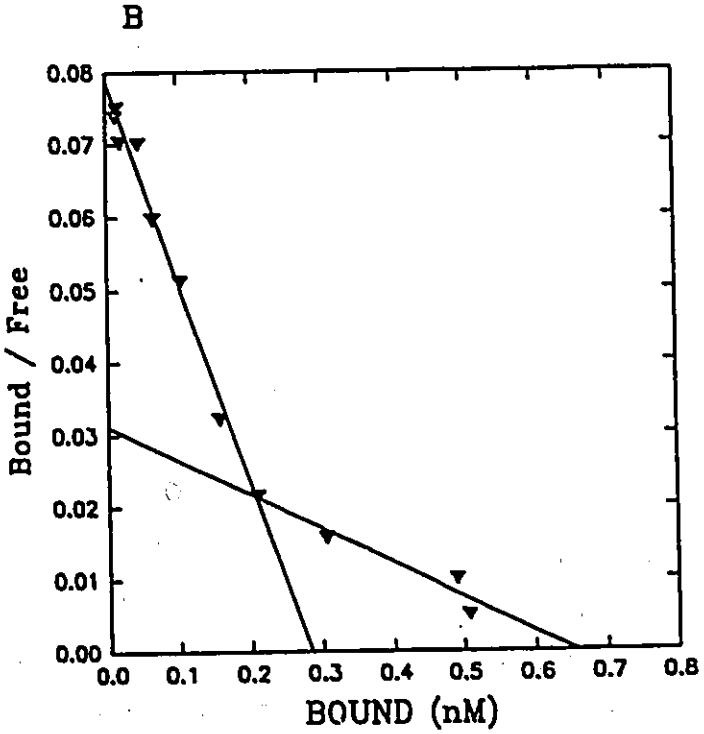

B
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Bound / Free
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
BOUND (nM)

Fig. 3-13A, B show that the specific binding, calculated by the difference between T- and NS-binding, reached a plateau at 10 nM ^{3}H-PRZ in both species. NS-binding increased with concentration of the radioligand, and the plateau observed indicates that the α_1-adrenoceptors did saturate in both species. The maximal variation of the specific binding over total radioactivity for bullhead in 2 experiments was less than 26%. The affinity and maximal binding capacity of the α_1-binding sites were assessed by Scatchard analysis. A single class of α_1-binding sites were indicated with K_D values for eel and bullhead that were similar (2.92 ± 0.24 nM for eel vs 2.75 ± 0.29 nM for bullhead). The eel membranes showed fewer binding sites than the bullhead (B_{max}= 108 ± 20.4 vs 197 ± 66 fmoles/mg protein for eel and bullhead, respectively). A recent study by Fabbri *et al.* (1994) characterized α_1-adrenoceptors in the hepatic membranes of this same bullhead species, and their result showed a slightly lower K_D (1.6 nM) but a similar receptor density (182 fmoles/mg protein) as noted in my study.

2) Characterization of α_1-adrenoceptors

The ability of the non-radioactive adrenergic ligands prazosin (PRZ), phentolamine (PHE) and propranolol (PROP) to displace specific ^{3}H-PRZ-binding was used to characterize the α_1-adrenoceptors of the two species. Increasing concentrations of non-radioactive PRZ, PHE and PROP displaced ^{3}H-PRZ in a dose-dependent fashion (Fig. 3-14A, B). The potency of each ligand was the same in the two species as demonstrated by the concentration needed to displace 50% of the bound ^{3}H-PRZ (Table 3-3).

PRZ displaced the radioactive analogue at a lower concentration than the other two adrenergic antagonists; at a concentration equivalent to that of the affinity of PRZ for the α_1-adrenoceptors, PHE competed with ^{3}H-PRZ less well than the non-radioactive PRZ, with values about 100 to 200-times higher than that for PRZ. The β-adrenergic antagonist PROP competed poorly, with only a 20% displacement at 100 μM (approximately 23.5% and 21% in eel and bullhead, respectively). The order of potency of the tested antagonists was PRZ > PHENT >> PROP. The number of bullhead experiments were reduced as α_1-adrenoceptors have

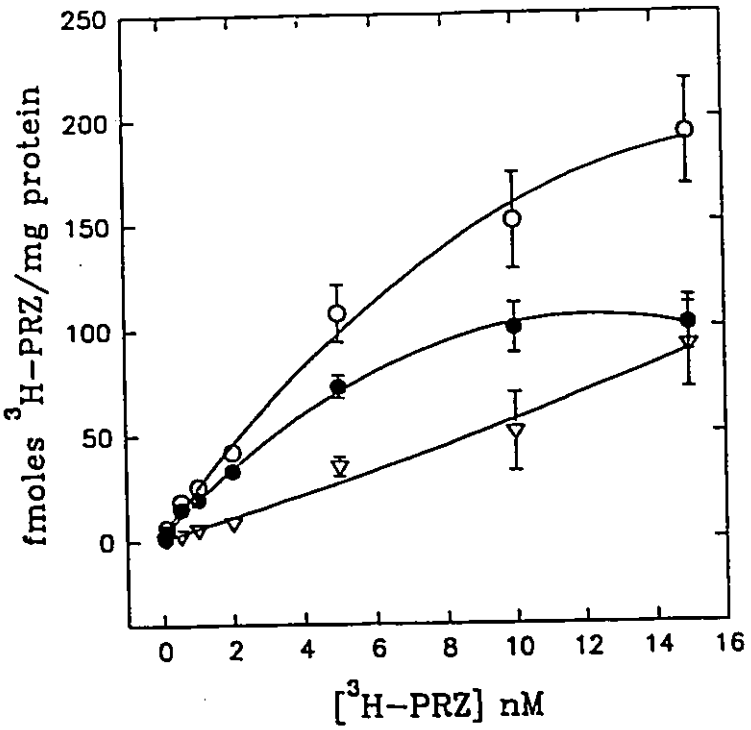
Table 3-3. Effects of adrenergic antagonists on the displacement of ^{3}H-PRZ binding to eel and bullhead liver membranes.

SPECIES	LIGAND	CONCENTRATION*
EEL	PRZ (3)	$2.51 \pm 0.27 \times 10^{-9}$
	PHE (3)	$1.06 \pm 0.02 \times 10^{-7}$
	PROP (2)	———
BULLHEAD	PRZ (2)	2.56×10^{-9}
	PHE (1)	0.92×10^{-7}
	PROP (2)	———

Numbers in parentheses represent number of experiments done.
* represents ligand concentration required to displace 50% of the bound ^{3}H-PRZ (IC_{50}). This value was calculated using linear regression. No values for PROP were presented as this antagonist did not reach 50% displacement even at 100 µM (see Fig. 3-14).

Fig. 3-13A, B: Saturation of α-adrenoceptors from purified eel (A) and bullhead (B) liver membranes with tritiated prazosin (^{3}H-PRZ). ^{3}H-PRZ was added at various concentrations in the absence (Total, open circle) and presence of 10 μM PRZ (NS-binding, open triangle). Specific binding (close circle) was obtained by subtracting the NS from the T. Membrane protein content was 350 or 150 μg for eel and bullhead, respectively, and all incubations were for 15 min at RT. Incubations were terminated by cooling the samples on ice. Bound from free was separated by centrifugation at 13,000 xg for 3 min at 10°C through a medium of 3% ice-cold BSA. The data for eel are means ± SEM of n = 3, while for bullhead values are averages of 2 experiments. The variation of the specific binding over the total radioactivity was less than 26% for bullhead.

A


250
200
150
100
50
0
fmoles ^{3}H-PRZ/mg protein
0
2
4
6
8
10
12
14
16
[^{3}H-PRZ] nM

B

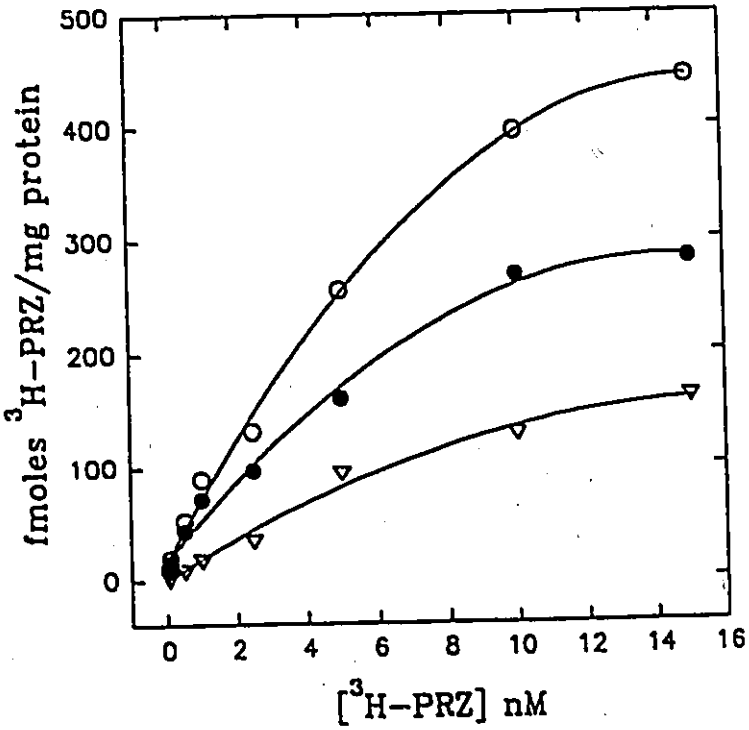
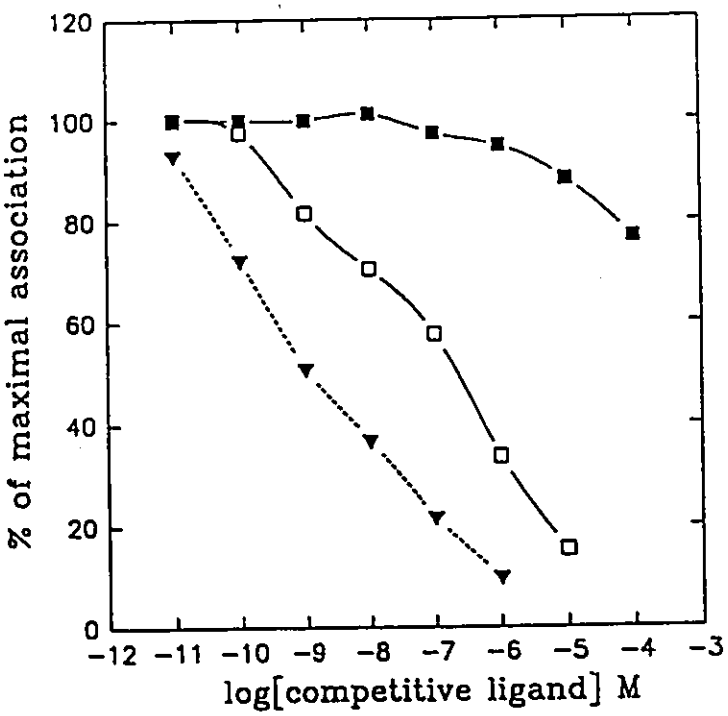
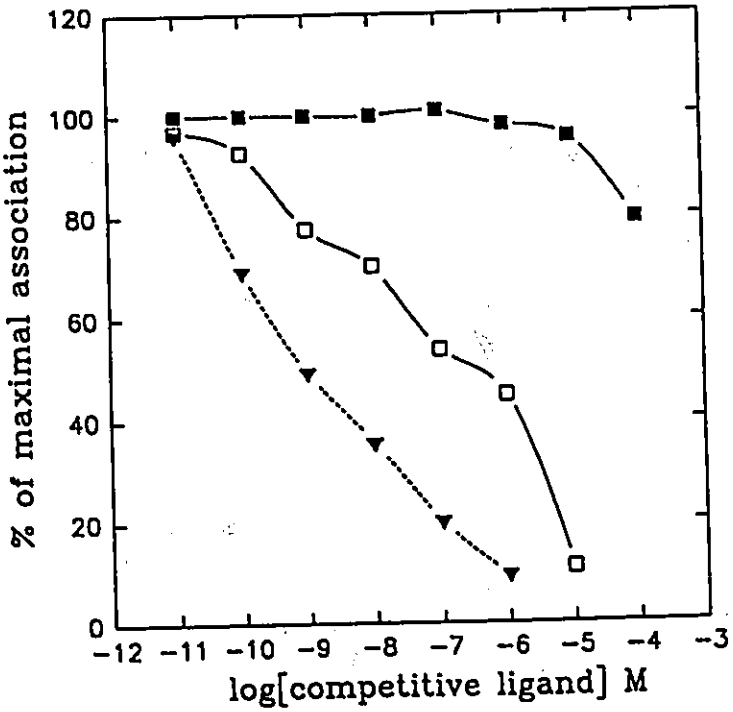

500
400
300
200
100
0
fmoles ^{3}H-PRZ/mg protein
0
2
4
6
8
10
12
14
16
[^{3}H-PRZ] nM

Fig. 3-14A, B: Identification of α_1-adrenoceptors in eel (A) and bullhead (B) purified liver membranes. Incubations were as in Fig. 3-13, except that 1nM ^{3}H-PRZ (approximately 30,000 dpm) was used and increasing concentrations of the non-radioactive ligands prazosin (PRZ, close triangle), phentolamine (PHE, open square) and propranolol (PROP, close square) were tested. IC_{50}, the concentration necessary to inhibit 50% of ^{3}H-PRZ binding, was calculated for each displacing agent from the experimental values, using linear regression without any computerized elaboration. Values for eel represent means of 3 independent experiments; for the bullhead, data for PRZ and PROP were the average of 2 experiments, while for PHE only one experiment. An indication of variation between experiments is noted on Table 3-3.

A


120
100
80
60
40
20
0
% of maximal association
−12 −11 −10 −9 −8 −7 −6 −5 −4 −3
log[competitive ligand] M

B


120
100
80
60
40
20
0
% of maximal association
−12 −11 −10 −9 −8 −7 −6 −5 −4 −3
log[competitive ligand] M

already been characterized in this species (Fabbri *et al.*, 1994).

D. Adenylyl Cyclase Activities

The determination of the activity of the enzyme responsible for the synthesis of the second messenger cAMP, ACase, was included in this study as a possible explanation for the relatively high cAMP levels in hepatocytes of eels compared with bullheads. The enzyme activity was tested on crude membrane preparations from liver cells of both species. Activities were determined as noted in the Materials and Methods after the cells were exposed to increasing concentrations of EPI and GLU to stimulate ACase activity. The incubation conditions were the same used in previous experiments testing the enzyme activity in bullhead liver membranes by Ottolenghi *et al.* (1988) and Fabbri *et al.* (1992). Activities were assessed under optimal conditions of pH and substrate concentrations, and after 10 min of hormone exposure.

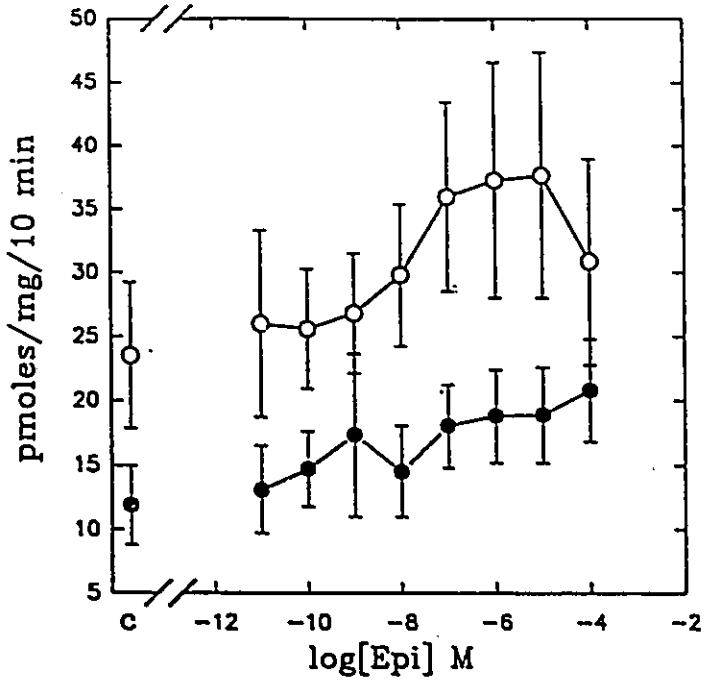
The EPI dose-response curve for the two species is presented in Fig. 3-15A. In both species the maximal response was obtained at approximately 10 µM EPI concentration. Bullhead crude hepatic membranes showed higher control ACase activities compared with the eel preparation (23.5 ± 5.7 vs 11.9 ± 3.1 pmoles cAMP/mg/10 min), but they were not significantly different. A one-way ANOVA showed that values of eel and bullhead dose responses were also not significantly different from their control levels. At 1 µM EPI, the % activation by EPI was actually higher in the eel than in the bullhead (Fig. 3-15B). The extent of activation of ACase in the bullhead preparation was below that previously reported by Ottolenghi *et al.* (1988).

Eel and bullhead liver ACase was less sensitive to GLU stimulation than to EPI (Fig. 3-16A). Activities with GLU were shown to be not significantly different from control values. The stimulation of bullhead ACase with mammalian GLU reported in Fig. 3-16A was well below values presented by Ottolenghi *et al.* (1988). Fig. 3-16B shows that the % ACase activation by GLU was higher in the eel than in the bullhead membranes. In both species GLU

Fig. 3-15A: Epinephrine (EPI) dose-effect on adenylyl cyclase (ACase) activities in eel (close circle) and bullhead (open circle) crude membranes. Crude membrane protein (250 and 150 µg for eel and bullhead, respectively) was incubated at RT for 10 min in an ACase assay mix (see Materials and Methods) and increasing concentrations of EPI. Reactions were terminated by boiling for 2 min, and cAMP assayed. In the absence of EPI, activities were 11.9 ± 3.1 and 23.5 ± 5.7 pmoles/mg protein/10 min in eel and bullhead, respectively. Enzyme activity is presented as means ± SEM of 7 and 9 experiments, in eel and bullhead, respectively. Eel and bullhead activities were compared with their control and found not significantly different by a one-way ANOVA. A Student's t-test showed no significant difference between eel and bullhead control values.

Fig. 3-15B: % change from control ACase activities of eel (close circle) and bullhead (open circle) crude liver membranes following addition of epinephrine (EPI). Values replotted from Fig. 3-15A.

A


pmoles/mg/10 min
50
45
40
35
30
25
20
15
10
5
C
−12
−10
−8
−6
−4
−2
log[Epi] M

B

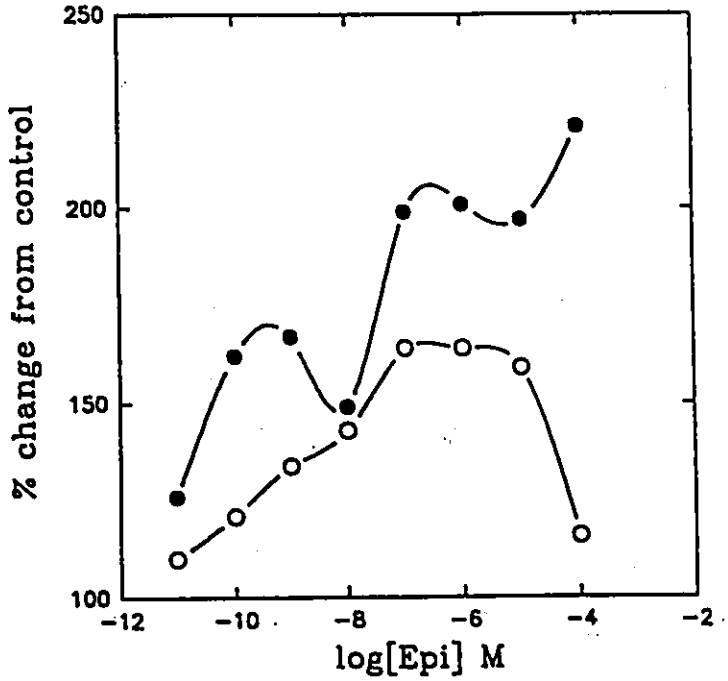
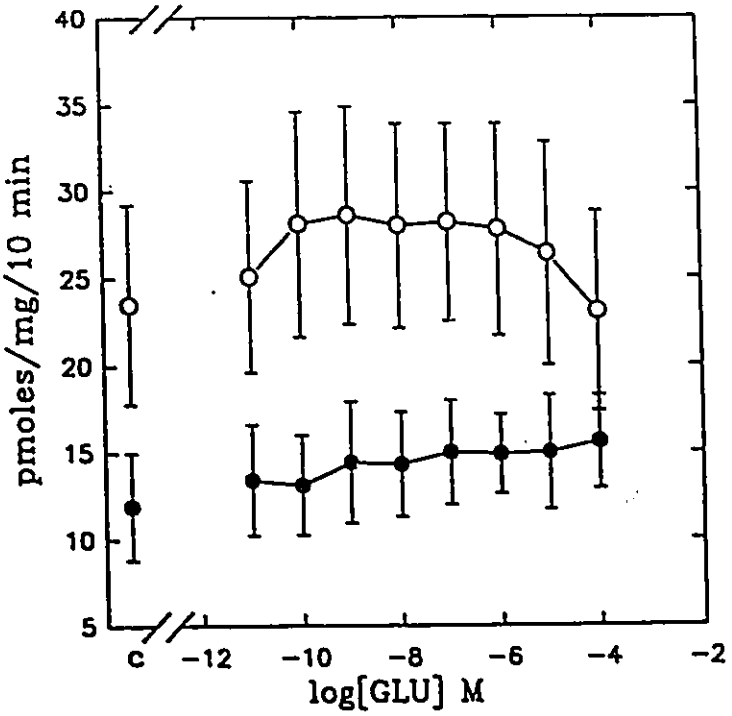

% change from control
250
200
150
100
−12
−10
−8
−6
−4
−2
log[Epi] M

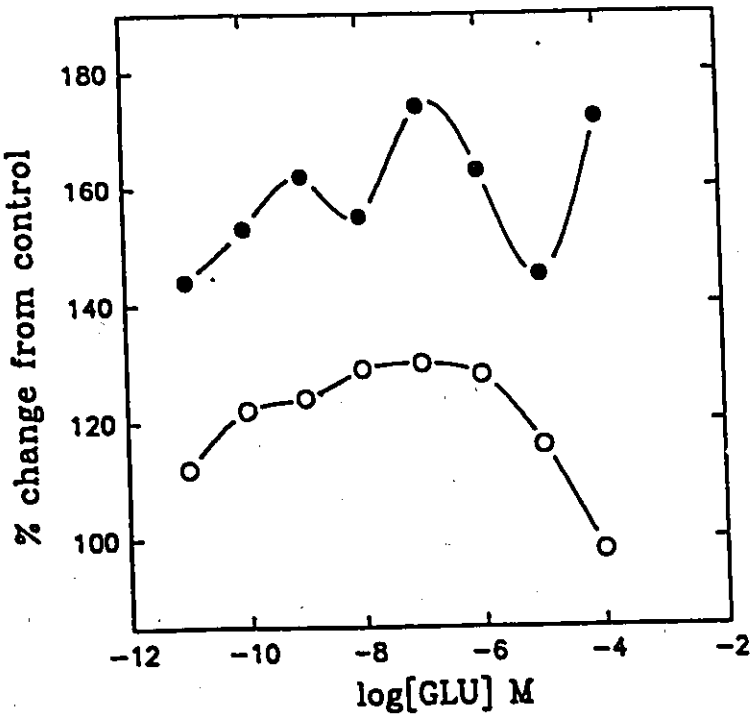
Fig. 3-16A: Effect of increasing glucagon (GLU) concentrations on ACase activities of eel (close circle) and bullhead (open circle) crude membranes. Experiments undertaken as in Fig. 3-15A, except GLU replaced EPI. Control values as noted in Fig.3-15A; n = 7, bullhead, and n = 9 for eel. Eel and bullhead control levels were not significantly different, (t-test, $p>0.05$). Eel and bullhead activities were not found significantly different from their control levels by a one-way ANOVA.

Fig. 3-16B: % change from control ACase activites of eel (close circle) and bullhead (open circle) crude membranes following glucagon (GLU) addition. Calculated from values obtained on Fig. 3-16A.

A


40
35
30
25
20
15
10
5
pmoles/mg/10 min
C
−12
−10
−8
−6
−4
−2
log[GLU] M

B


180
160
140
120
100
% change from control
−12
−10
−8
−6
−4
−2
log[GLU] M

induced a peak at the concentration of 0.1 μM.

A species comparison of the activities induced by the two hormones shows that EPI always had a greater effect than GLU (Fig. 3-15A and 3-16A), and that the activation above control was always greater in the eel than in the bullhead membranes (Fig. 3-15B and 3-16B).

CHAPTER 4:
DISCUSSION

Previous studies (see Introduction) have shown that hormone-induced increases in cAMP are linked to metabolic changes in fish liver cells; there is significant evidence that both epinephrine (EPI) and glucagon (GLU) induce increases in cAMP. This thesis has further evaluated the effect of EPI and GLU on the hepatic cAMP transduction system of American eels and black bullheads. More critically, however, this thesis has examined the role of the other transduction system which is linked through changes in IP_3 and ultimately changes in the concentration of intracellular Ca^{2+} $[Ca^{2+}]_i$. This process has not been previously examined in fish hepatic tissue and represents a potentially important hormone transduction pathway.

This discussion will place my Results in the context of other studies reported for the β_2- and α_1-mediated adrenergic system and the GLU transduction pathway.

A. β_2-Mediated Adrenergic Effects

EPI has been reported to stimulate hepatic glycogen breakdown and glucose output in a number of fish species using different experimental systems (Birnbaum *et al.*, 1976; Ottolenghi *et al.*, 1983, 1984; Brighenti *et al.*, 1987a, b; Mommsen *et al.*, 1987; Wright *et al.*, 1989; Michelsen and Sheridan, 1990). The same authors have shown that this effect is mediated through a β_2-adrenoceptor system as cAMP production increases and the effects are mimicked and blocked by classic β-agonists and antagonists (Ahlquist, 1948; Exton 1985, 1988). The effects of phenylephrine (PE), however, have been shown not to fit this classic scheme (Brighenti et al., 1987a, b; Moon and Mommsen, 1990).

Experiments carried out in my study to test cAMP stimulation following EPI exposure used the same concentrations of agonist and antagonists reported by Brighenti *et al.* (1987b). At 10 μM EPI, cAMP levels rose in bullhead hepatocytes, reaching a peak at about 5 min of incubation, followed by a slow decrease thereafter (Fig. 3-1). Brighenti *et al.* (1987b) found a peak in cAMP production at 30 min followed by a slow decline to 60 min. This difference with

my data probably reflects the presence of theophylline, a phosphodiesterase inhibitor, in their cell incubation system. The decline in cAMP accumulation towards basal levels after the peak observed in my study was more rapid and evident, due to the reported phosphodiesterase activation by catecholamines (Solomon, 1975; Brighenti *et al.*, 1987b), as no inhibitor was employed in my experiments. The incremental increase in cAMP above control values was at least 10-fold (Fig. 3-1, 3-2B) compared with 15-fold in Brighenti *et al.* (1987b). It is also significant that the cAMP production in bullheads followed the same pattern observed in this species by Moon *et al.* (1993), although the EPI concentration used (0.1 µM) was 2 orders of magnitude lower than that used in the present study and the peak production was reached after approximately 10 min. The cAMP basal levels observed in the two species (approximately 0.7 and 1 nmoles/g wet wt in eel and bullhead, respectively) are in the range of values reported for rat hepatocytes (0.2 to 1.4 nmoles/g wet wt: Grojec *et al.*, 1990; Yamanouchi *et al.*, 1992; Díaz *et al.*, 1991; García-Sáinz, 1990).

All subsequent experiments exposed eel and bullhead hepatocytes to EPI for 5 min (Fig. 3-2 A, B), as suggested from my previous experiments (Fig. 3-1), and in experiments using amphibians (Janssens and Grigg, 1984: *Xenopus laevis*) and teleosts (Janssens and Lowrey, 1987: carp; Mommsen and Moon, 1990: eel, bullhead). At 10 µM the EPI-induced cAMP production reached a much greater extent in eel than in bullhead cells (51- vs 14-fold increase, respectively), reflecting the same trend reported by Mommsen and Moon (1990), using these species and GLU. The EPI-induced cAMP peak levels of my experiments are an order of magnitude greater than those reported in toad, carp and reptile livers by Janssens and colleagues (Janssens and Grigg, 1984: toad; Janssens and Lowrey, 1987: carp; Janssens and Grigg, 1992: turtles) probably as a result of a 10-fold higher EPI concentration in my studies than that used in their studies and of their use of liver pieces or organ culture where EPI stimulation may be less effective than on isolated hepatocytes.

A β_2-mediated effect of EPI on cAMP production was supported by pre-incubation of the cells with the α- and β-antagonists phentolamine (PHE) and propranolol (PROP) (Fig. 3-2 A,

B). PHE was ineffective in blocking the EPI-induced cAMP increase, while in contrast PROP brought cAMP levels back to the control levels in both species. The data are strong evidence for the involvement of a β_2-mediated response, as previously reported by Birnbaum *et al.* (1976) for goldfish hepatocytes, Brighenti *et al.* (1987b) for bullhead hepatocytes and Janssens and Lowrey (1987) for carp liver culture.

cAMP production increased in a dose-dependent manner as EPI concentrations increased (Fig. 3-4). cAMP production rapidly increased in both species above 0.01 µM EPI, peaking at approximately 10 µM EPI, confirming that this concentration was the best choice for the time course experiments. The K_a values for cAMP production of approximately 10^{-7} M are well above the K_D of 2 nM reported for the binding of dihydroalprenolol to bullhead liver membranes (Fabbri *et al.*, 1992). At EPI concentrations of 0.1 µM, bullhead hepatocytes were more responsive than eel cells, but at higher EPI concentrations, eel hepatocytes significantly increased cAMP levels compared with the bullhead, producing up to 2.6 times the amount of cAMP of bullhead cells. The dose effect obtained by Brighenti *et al.* (1987b) in bullhead hepatocytes was similar to that observed here, although no maximum was achieved probably due to the presence of theophylline. Goldfish hepatocytes (Birnbaum *et al.*, 1976) incubated for 10 min responded to changes in EPI by a 3-fold increase in cAMP between 0.01 to 10 µM EPI. The results obtained for goldfish at 10 µM EPI (expressed in pmoles/10^6 cells; converted to g of wet wt using the assumption that 1 g wet wt of fish hepatocytes corresponds to 12×10^8 cells; Moon, unpublished data) indicate that this response is 4.1- and 1.6-fold lower than that obtained in eel and bullhead, respectively. This thesis did not specifically examine the bases for these differences in cAMP production between eel and bullhead hepatocytes or other fish species. However, adenylyl cyclase (ACase) activities were examined (Fig. 3-15), and one could hypothesize that these differences may relate to differences in activities of this enzyme or the phosphodiesterases which breaks down cAMP.

ACase has been extensively studied in mammalian cells, including the cloning of different isoforms of the enzyme (Tang *et al.*, 1991; Taussig *et al.*, 1993), activities of the enzyme in a

variety of mammalian cells and the effects of hormone stimulation, guanyl nucleotides and ions in the activation or inhibition of the enzyme (Birnbaumer *et al.*, 1969; Pohl *et al.*, 1971; Rodbell *et al.*, 1971; Caldwell *et al.*, 1992). In contrast, little is known about whether there is a conservative trend in the molecular organization of this enzyme in lower vertebrates. There is adequate indirect evidence that this enzyme is a component of the cAMP transduction pathway in goldfish (Birnbaum *et al.*, 1976), carp (Janssens and Lowrey, 1987), bullhead (Brighenti *et al.*, 1987) and trout (Michelsen and Sheridan, 1990) livers and a few amphibians (Herman and Brown, 1983; Janssens *et al.*, 1983: axlolotl; Janssens and Grigg, 1984, 1987: toad) and reptiles (Janssens and Grigg, 1992: turtles).

A study by Ottolenghi *et al.* (1988) has characterized this enzyme in bullhead hepatic tissue and recently Fabbri *et al.* (1991) examined the effects of adenosine analogues (often employed as ACase inhibitors in mammalian cells) on ACase activities from the same bullhead membranes in order to demonstrate a certain uniformity in the enzyme characteristics between the mammalian and the fish systems. A similar characterization has been undertaken in trout gills membranes by Guibbolini and Lahlou (1987).

ACase activities, as assessed by cAMP production, increased in crude membranes isolated from eel and bullhead liver homogenates as EPI concentrations increased. The two species did not show significantly different control activities (Fig. 3-15A) and responded poorly to EPI, both showing maximal activation at approximately 10 μM EPI. The higher control values of bullhead may be explained by a higher density of β_2-adrenoceptors and/or more ACase molecules in these membranes compared with eels. The use of forskolin, an ACase activator (Exton, 1985, 1988), could possibly separate these two possible explanations. Ottolenghi *et al.* (1988) compared the effects of natural (EPI and norepinephrine, (NEPI) and synthetic (isoproterenol, ISO and phenylephrine, PE) catecholamines and GLU on ACase activities in bullhead hepatic membranes with those of rat. Catecholamines were stronger inducers of ACase than GLU, and the β-antagonist PROP inhibited this catecholamine-stimulated ACase activity in bullhead. In rat GLU had a greater effect on ACase than did catecholamines, consistent with the prevalence

of the α_1- over the β_2-adrenoceptor subtype in rat liver (Sulakhe *et al.*, 1988). Frog liver ACase showed an intermediate situation between rat and bullhead; enzyme activity was higher than in bullhead but below the activation levels of rat, and frog ACase showed approximately the same sensitivities to EPI and GLU when tested at equal concentrations (Herman and Brown, 1983). This result is consistent with the lack of α_1-adrenoceptors in frog (Sulakhe *et al.*, 1988). Bullhead liver ACase (Ottolenghi *et al.*, 1988) had a lower K_a for ATP than the ACase systems of rat (Pohl *et al.*, 1971), frog (Herman and Brown, 1983) and trout gill epithelium (Guibbolini and Lahlou, 1987), while Ca^{2+}, Mg^{2+} and F^- requirements were similar to those in rat and frog. The temperature dependency was much more similar to that of rat than to that of frog, while the ACase sensitivity to pH was similar in all the systems.

Maximal activities of bullhead ACase achieved in my study are slightly lower than those obtained by Ottolenghi *et al.* (1988), but were of the same order of magnitude; these small differences may relate to small changes in experimental conditions, especially temperature. ACase activities in partially purified hepatic plasma membranes of rats exposed to 60 µM EPI was approximately 12- and 7-fold higher than the eel and bullhead activity at the same EPI concentration (Pohl *et al.*, 1971). The reason for these higher ACase activities in rat is not related to the density in β-adrenoceptors as the binding capacities reported for rat (Sulakhe *et al.*, 1988) is similar to that reported for the high affinity β-adrenoceptors characterized in bullhead (Fabbri *et al.*, 1992).The data gathered for eel crude membranes are the first ACase activities to be reported for this species. Comparable activities to those in bullhead required more eel membranes (150 µg vs 250 µg/100 µl). The % change from control ACase activities with EPI (Fig. 3-15B), however, demonstrated that eel ACase is more responsive than the bullhead preparation; this is in agreement with the differences obtained in cAMP production from isolated hepatocytes of these two species stimulated with EPI (Fig. 3-2A, B). An enhanced phosphodiesterase activity may be one possible explanation for the lower cAMP levels in bullhead hepatocytes. This hypothesis is consistent with higher ACase activities in bullhead than in eel membranes incubated with theophylline; phosphodiesterase activities would reduce cAMP

levels during hepatocyte incubation.

A study on ACase activities reported in trout gill epithelium (Guibbolini and Lahlou, 1987) using similar experimental conditions to those in my study, showed basal and stimulated cAMP levels 10 times greater than those found in fish liver membranes. These authors as well as Ottolenghi *et al.* (1988; bullhead hepatocytes) reported that ACase was sensitive to GTP, Mg^{2+}, Ca^{2+} and F^- in a manner qualitatively identical to that reported in mammalian cells (Birnbaumer, 1990). The stimulating effect of fluoride ions (NaF, 10 mM final concentration) was also tested on ACase activity in a few of my experiments and the results support these findings. In eel (n = 1) and bullhead (n = 2) membranes, F^- induced an increase of ACase activity of approximately 900 and 260%, respectively, when compared to the same membranes treated with an equal amount of sodium chloride as a control. The precise mechanism of F^- activation is not understood (Ottolenghi *et al.*, 1988), although it is generally thought to fully activate ACase. All of these studies suggest that ACase of fish resembles the mammalian enzyme system (Alexander *et al.*, 1975).

These studies on cAMP and ACase in eel and bullhead livers support previous studies which show that EPI operates through a cAMP signal transduction pathway in many different vertebrate systems.

B. α_1-Mediated Adrenergic Effects

As noted in the Introduction, the existence of α_1-adrenoceptors in lower vertebrates, including fish (Birnbaum *et al.*, 1976: goldfish; Janssens *et al.*, 1983, 1986: toad and axolotl; Janssens and Grigg, 1984, 1987: toad; Janssens and Lowrey, 1987: carp; Sulakhe, 1988: all vertebrate classes, except fish; Janssens and Grigg, 1992: turtles) is debatable. The intriguing results reported by Brighenti *et al.* (1987a, b) and Moon and Mommsen (1990) for phenylephrine (PE) (a generic α-antagonist; Exton, 1985) could not exclude the existence of α-adrenoceptors in fish liver membranes. The mammalian transduction mechanism mediated by the IP_3 synthesis and consequent increase in $[Ca^{2+}]_i$ levels is well documented in rat liver

(Exton, 1979b, 1985, 1988), where α_1-adrenoceptors prevail over the β_2-type (Sulakhe *et al.*, 1988). This same pathway was indirectly supported at least in eel and bullhead liver cells by studies on the transient increase in $[Ca^{2+}]_i$ induced by EPI and the blockade of this effect by the α-antagonist PHE but not by the β-antagonist PROP (Zhang *et al.*, 1992b). A clear involvement of α_1-adrenoceptors in the change in $[Ca^{2+}]_i$ was provided by Moon *et al.* (1993) using bullhead hepatocytes and several α-agonists that showed the same order of potency reported for α_1-adrenoceptors of mammalian hepatocytes (Exton, 1985). Fabbri *et al.* (1994) have only recently provided direct evidence for α_1-adrenoceptors with the report of specific binding of ^{3}H-prazosin (PRZ) to bullhead hepatic membranes. Other fish tissues demonstrate α_1-mediated effects (shark rectal glands: Ecay and Valentich, 1991; catfish olfactory cilia: Kalinoski *et al.*, 1992; trout vasculature: Xu and Olson, 1993; goldfish follicles: Goetz and Bradley, 1994).

The binding of the specific α_1-antagonist PRZ is the tool commonly used to specifically define the α_1-adrenoceptor in mammals (Exton, 1985). The high selectivity and affinity of this specific ligand allows the use of the tritiated analogue, ^{3}H-PRZ, to identify the α_1-subtype. In fact, it has been successfully employed in the recent assessment of these receptors in bullhead purified liver membranes (Fabbri *et al.*, 1994).

My studies demonstrate the specific binding of increasing concentrations of ^{3}H-PRZ to purified liver membranes of eel and bullhead (Fig. 3-13 A, B). The saturation of the process indicated the presence of a limited number of receptors on the liver membranes. The specific binding at 10 and 15 nM ^{3}H-PRZ was approximately 3 times lower in eel than in bullhead. This implies that these binding sites are less represented on eel than on bullhead membranes. Scatchard analysis supported a single-site model with a similar K_D (2.92 ± 0.24 and 2.75 ± 0.29 nM in eel and bullhead, respectively) but a lower B_{max} value in eel than in bullhead (108 ± 20.4 vs 197 ± 66 fmoles/mg). The different receptor densities supports a species dependency in binding capacities. The data gathered from my experiments on bullhead membranes are in agreement with those recently obtained by Fabbri *et al.* (1994) on the same species (single class; K_D = 1.6 nM; B_{max} = 182.1 fmoles/mg protein). Gutiérrez-Venegas and García-Sáinz (1993)

also found a single class of PRZ binding sites on chicken hepatocytes characterized by a higher affinity for ^{3}H-PRZ (K_D = 0.30 nM) and even higher density (B_{max} = 870 ± 102 fmoles/mg protein) which is similar to that reported by Sulakhe *et al.* (1988) for this species. These chicken hepatic membranes saturated at 180 pM ^{3}H-PRZ, or a 100-fold lower concentration than that observed in my study. An ontogenic study recorded a dramatic rise of rat hepatic α_1-adrenoceptors from 40 to levels of 110-140 fmoles/mg in adult rat (McMillan *et al.*, 1983), but these levels are well below the 600 and 760 fmoles/mg protein reported by Sulakhe *et al.* (1988) and by García-Sáinz *et al.* (1992b), respectively for adult rat. It should also be remembered that the α_1-subtype represents 75% of all adrenoceptors in the adult rat (Hoffman *et al.*, 1980; Sulakhe *et al.*, 1988). Certainly the specific α_1-adrenoceptor binding found in eel and bullhead liver is within the range of values reported for various mammals by Sulakhe *et al.* (1988) and García-Sáinz *et al.* (1992b).

It is interesting to compare the kinetic constants reported in my experiments for the α_1-adrenoceptors with those previously reported by Fabbri *et al.* (1992) for bullhead β_2-adrenoceptors. Using the same preparation and species, but obviously different individual fish, Fabbri *et al.* (1992) reported two classes of hepatic β_2-adrenoceptors with K_Ds of 2 and 62 nM and B_{max}s of 47 and 452 fmoles/mg protein, corresponding to high and low affinity β-adrenoceptors. Comparing only the high affinity β_2-adrenoceptors with the α_1-subtype assessed in my study, K_D values are equivalent, but B_{max} values for the α_1-sites are 4 times higher than the β_2-sites; as in rats, in bullhead the α_1-subtype appears to account for at least 75% of the adrenoceptor population. At least this comparison would suggest that the α_1-adrenoceptor effect in bullhead would be as great as that of the β_2-effect; obviously, referring to IP_3 production that is examined in the following pages, this is not the case.

Displacement experiments based on the competition of different non-radioactive ligands for the specific ^{3}H-PRZ-binding sites were used to characterize the α_1-adrenoceptors in both species (Fig. 3-14 A,B). The ligands used were the non-radioactive analogue PRZ, the generic α-antagonist PHE and the β-antagonist PROP, that show a decreasing affinity for the α_1-subtype

in mammals (Exton, 1985). PRZ was the most effective as assessed by both the low IC_{50} value (Table 3-3) and the fact that PRZ displaced 100% the radioactive analogue at 1 μM (Fig. 3-14A, B); PHE was less effective. PROP was ineffective, displacing only 20% of the ^{3}H-PRZ at a concentration of 100 μM. The potency exhibited by the displacing agents followed the classical order for α_1-adrenoceptors of mammalian cells (Exton, 1985), and for this reason this study provides evidence for the existence of these receptors at least in the livers of these two fish species. The results obtained for eel α_1-adrenoceptors are the first reported for this species in the literature.

Heterogeneity of α_1-adrenoceptors was discussed in the Introduction. Based upon the displacement of [^{3}H] bunazosin by 5-methyl urapidil and PRZ, García-Sáinz *et al.* (1992b) identified α_{1A}-adrenoceptors to guinea pig, α_{1B}- to rat and α_{1C}- to rabbit hepatic tissues. Each α_1-subtype has a specific order of antagonist displacement. PRZ showed an IC_{50}-value for these mammalian receptors of 1.7, 0.3, and 0.55 nM, respectively, below that reported for either eel or bullhead (Table 3-3). It is not possible to identify which α_1-adrenoceptor subtype is present on the fish hepatic membranes given the absence of appropriate pharmacological studies, but the α_{1B}- and α_{1C}-subtypes seem to be directly linked to IP_3 formation (Han *et al.*, 1987; Michel *et al.*, 1990; Summers and McMartin, 1993), which was found to occur in my study (see below). Moreover studies on rat livers showed the highest selectivity of PRZ for the α_{1B}-subtype (Torres-Márquez *et al.*, 1992; García-Sáinz *et al.*, 1993), which appears to be the most common α_1-subtype in rat hepatocytes.

Even though the literature on amphibians, reptiles and some teleost (Janssens *et al.*, 1986; Janssens and Grigg, 1987, 1992; Janssens and Lowrey, 1987) suggests that the α-mediated pathway observed in livers of birds and mammals (Exton, 1985, 1988; Sulakhe *et al.*, 1988; Putney *et al.*, 1989; Gutiérrez-Venegas and García-Sáinz, 1993) may have evolved after these classes separated from the common vertebrate ancestors, the presence of α_1-adrenoceptors in eel and bullhead liver membranes indicates a conservative trend within the classes of vertebrates. The existence of α_1-adrenoceptors in eel and bullhead livers leads one to ask whether these

receptors are linked to Ca^{2+} by IP_3, a second messenger which has been found even in insect salivary glands (Berridge *et al.*, 1983).

Exposure of eel and bullhead hepatocytes to 10 µM EPI induced an immediate, transient, increase in IP_3 production, achieving a maximum between 20 and 45 sec (Fig. 3-6A, B). This time course is consistent with the large decrease in PIP_2 levels observed within a few seconds in isolated rat hepatocytes exposed to EPI (Augert *et al.*, 1989). A rapid metabolism (Exton, 1988b) by a complex pathway that dephosphorylates IP_3 to phosphatidylinositol (Berridge and Irvine, 1984; Putney *et al.*, 1989) may account for the rapid decrease of IP_3 levels observed in eel and bullhead. PHE (100 µM) completely blocked this EPI-inducted IP_3 increase, whereas PROP had a stimulating effect, increasing IP_3 production to values slightly higher than those reached with EPI alone. This probably reflects PROP blocking β_2-adrenoceptors, freeing the EPI to bind only the α_1-adrenoceptor. Comparing the difference between the control (2.1 ± 0.08 and 5.93 ± 0.08 nmoles/g at 20 sec for eel and at 45 sec for bullhead, respectively) and the peak IP_3 values obtained at the same time from the combined effects of EPI and PROP, the absolute IP_3 production was similar in the two species (approximately 4 nmoles/g). Bullhead hepatocytes always showed higher control IP_3 levels than the eel cells. This higher basal IP_3 level may be partially accounted for by the higher α_1-adrenoceptor density found on bullhead compared with eel liver membranes (197 and 108 fmoles/mg protein, respectively). Other factors, however, must be involved in setting this level. This higher basal IP_3 level of bullheads may partially explain the higher basal $[Ca^{2+}]_i$ reported by Zhang et al. (1992a) in these cells relative to eels.

The synthesis of inositol phosphates by the incorporation of $[^3H]$- and $[^{32}P]$-precursors following ligand addition, or the conversion from one phosphorylated form to another, is extensively reported in the literature. However, estimates of cellular IP_3 content are less available. Meek (1986) reported values of 3-40 nmoles IP_3/g in various mammalian tissues, while Püschel *et al.* (1993) and Palmer *et al.* (1989) report basal IP_3 concentrations of 0.76 and 0.4 nmoles/g, respectively in rat hepatocytes. The latter concentration increased by 10-fold with 230 nM vasopressin within 15 sec (Palmer *et al.*, 1989). My study demonstrates that eel and

bullhead hepatocytes (basal levels of 2 and 5.5 nmoles/g in eel and bullhead, respectively) contain IP_3 at concentrations in the range of those found in rats and that both EPI and GLU increase these concentrations. The IP_3 time-dependency in both fish is similar to that observed in mammals.

EPI dose curves (Fig. 3-8) showed that peak IP_3 production occurred at 10 μM EPI in both species. The responsiveness was similar in both fish, given that the control values showed the same proportionality with the stimulated values. No similar dose-response curves could be found in the literature, but they are characterized by a heightened plateau response so that even at 0.1 nM EPI, IP_3 content was above control or basal. Unlike the cAMP response which is not induced below 10 nM EPI, the IP_3 response is already maximal. This implies that those biochemical processes which respond to EPI at concentrations below 10 nM may be modulated by the IP_3 rather than the cAMP pathway. As discussed below, this idea follows from a model proposed by Wakelam *et al.* (1986) for GLU.

The α-mediated modulation of Ca^{2+} homeostasis in fish hepatocytes reported by Zhang *et al.* (1992a) and Moon *et al.* (1993), the detection of α_1-adrenoceptors (Fabbri *et al.*, 1994; this thesis) and production of IP_3 (this thesis) with EPI stimulation in eel and bullhead liver cells leads one to propose the presence of IP_3-binding sites in order to complete the sequence of events that leads to the Ca^{2+}-mediated transduction mechanism. The IP_3-binding site may play a pivotal role in the stimulation of the transient fluctuations of $[Ca^{2+}]_i$.

IP_3 binding studies were carried out in both species on microsomal fractions prepared from liver homogenates. This preparation is composed primarily of cellular membranes as indicated by the enrichment of the specific activity of the enzyme 5'NTase (Table 3-1). A family of 5'NTase isozymes is intimately associated with cell membranes (Solyom and Trams, 1972; Feng and Kraus-Friedmann, 1993), and the enrichment found in my study was similar to that noted by Fabbri *et al.* (1992) in bullhead and Pohl *et al.* (1971) in rat liver using similar preparation techniques.

^3H-IP_3 was found to associate with this microsomal preparation from both species (Fig.

3-10). Non-specific binding was always less than 10% of the total radioactivity, well below such values reported in bullhead olfactory cilia (Kalinoski *et al.*, 1992) which were 50% at a non-radioactive ligand concentration 50 times higher than that used in eel and bullhead liver microsomes. Association of ^{32}P-IP$_3$ to specific receptors in permeabilized guinea pig hepatocytes and rabbit neutrophils (Spät *et al.*, 1986) showed a non-specific binding that was 15-20% of the total, in the presence of a 10-fold higher concentration of non-radioactive IP$_3$. These findings indicate that the concentration of the non-radioactive ligand chosen in my experiments was appropriate, giving a low non-specific binding. Under my experimental conditions, ^{3}H-IP$_3$ binding increased rapidly in a time-dependent fashion, achieved a maximum at 30 min in both species, and then slowly declined. The 50% association of ^{3}H-IP$_3$ occurred between 2 and 5 min in eel and after 1 min in bullhead. These kinetics are much slower than those in rat hepatic membranes where maximal binding was achieved within 30 to 60 sec (Spät *et al.*, 1986). Specific ^{3}H-IP$_3$ binding showed differences between the two fish species; the % maximal association in eel was 18 to 35% lower than in bullhead (Fig. 3-10). Some characteristics of the binding sites, such as density or affinity, as shown by Scatchard analysis (Table 3-2), may explain the different association times of ^{3}H-IP$_3$ in eel and bullhead.

To characterize the IP$_3$-binding sites in both species, the displacement of ^{3}H-IP$_3$ was carried out with increasing concentrations of the specific non-radioactive ligand IP$_3$ (Fig. 3-11). Increasing concentrations of non-radioactive IP$_3$ competed for the binding sites with the radioactive analogue, which was displaced up to 100% at approximately 0.01 and 0.1 µM IP$_3$ in eel and bullhead, respectively. In eel hepatocytes the displacement of ^{3}H-IP$_3$ was always obtained at 10-100 times lower IP$_3$ concentrations than in bullhead. This difference probably reflects the higher affinity of IP$_3$ for its specific binding sites in eel than in bullhead sites (Table 3-2). Scatchard analysis revealed heterogeneity in IP$_3$-binding sites in both species, and a two-site binding model gave the "best fit" for these data (as statistically determined). A similar two state IP$_3$ binding model has been reported for membrane fractions from rat liver by Mauger *et al.* (1989) showing K_Ds at 3.8 and 87 nM and binding capacities of 38 and 162 fmoles/mg

protein, with the low affinity binding sites accounting for about 85% of the specific binding. Studies using bovine adrenal cortex (Poitras *et al.*, 1993) suggested that the two affinity states may switch from one to the other in order to modulate intracellular Ca^{2+} oscillations. IP_3-binding sites in guinea pig hepatocytes (Spät *et al.*, 1986) and the microsomal fraction of bovine adrenal cortex (Palmer *et al.*, 1989) fit instead a single-site model. The two studies report K_D and B_{max} values of 220 nM and 194 fmoles/mg protein and 6.82 nM and 370 fmoles/mg protein, respectively. Another study on bovine adrenal cortex (Challiss *et al.*, 1990) showed again a homogeneous receptor population with a K_D of 3.7 nM and a B_{max} of 872 fmoles/mg protein.

The only other similar fish study used channel catfish olfactory ciliar and brain membranes (Kalinoski *et al.*, 1992). A one-site model apparently was more appropriate than the two-site model with a "best-fit" K_D of 1.1 µM and B_{max} of 17.6 pmoles/mg protein. The K_D and B_{max} values obtained by Scatchard analysis for eel and bullhead in my study (Table 3-2) showed the highest affinity for IP_3 of the studies mentioned and a density in the range reported for the mammalian receptors. The high and low affinity sites account for 35 and 65%, and for 30 and 70% of the specific binding in eel and bullhead, respectively. A comparison of the data gathered for the two species shows that the eel binding sites for IP_3 have a higher affinity and lower density for both classes of sites than in bullhead.

As noted in the Introduction, Zhang *et al.* (1992a) reported significant differences in the EPI-induced change in hepatocyte $[Ca^{2+}]_i$ between eel and bullhead. Changes in $[Ca^{2+}]_i$ were rapid and independent of extracellular Ca^{2+} ($[Ca^{2+}]_e$) for eel, but bullhead hepatocytes demonstrated a rapid increase in $[Ca^{2+}]_i$ which was prolonged and dependent upon $[Ca^{2+}]_e$. Although both hepatocytes produce about the same absolute change in intracellular IP_3 at 10 µM EPI (Fig. 3-6A, B), the higher basal IP_3 levels found in bullheads implies that their hepatic Ca^{2+} signalling mechanism may be less sensitive to IP_3. The higher K_Ds found for bullhead IP_3 binding than those for eel support this hypothesis.

These are the first studies which have demonstrated a link between EPI binding at

α_1-adrenoceptors, IP_3 production, IP_3binding and changes in $[Ca^{2+}]_i$ in the liver of any fish species. Although the physiological significance of such changes are yet to be deduced (Moon *et al.*, 1993), these studies support a strong evolutionary conservativeness within vertebrate hepatic tissue with respect to catecholamines activities.

C. Glucagon Stimulation of Second Messengers in Eel and Bullhead Hepatocytes

The stimulation of cAMP and IP_3 synthesis through specific glucagon receptors has been extensively investigated in mammalian liver, where the structure and the characteristics of the receptors and the transduction mechanisms are well known (Rodbell *et al.*, 1971; Pohl *et al.*, 1972; Heyworth *et al.*, 1983; Friedmannn, 1976; Horwitz *et al.*, 1985; Houslay, 1986; Iwanij and Vincent, 1990; Bharucha and Tager, 1990).

As for the adrenergic-mediated pathways, studies on the second messengers involved in GLU stimulation in the more primitive vertebrates are still unresolved. To date, the presence and characterization of glucagon receptors and the down-regulation phenomenon have been reported only in the hepatocytes of two teleost species, eel and bullhead (Navarro and Moon, 1994). It is already known that in reptiles (Janssens and Grigg, 1992), amphibians (Kleineke and Janssens, 1993) and teleost fish (Birnbaum *et al.*, 1976; Janssens and Lowrey, 1987; Petersen *et al.*, 1987; Mommsen and Moon, 1989; Foster and Moon, 1990), GLU regulates hepatic gluconeogeneis, glycogenolysis and glucose release through cAMP synthesis triggering the same enzymic modulation of activities that catecholamines do through the β_2-adrenoceptor mechanism. The IP_3-mediated pathway following glucagon stimulation seems to be excluded in lower vertebrates, so that in these classes the hepatic metabolism is regulated only through the cAMP pathway. The increases in $[Ca^{2+}]_i$ concentration induced by glucagon in axolotl hepatocytes (Kleineke and Janssens, 1993) was explained as a hormone-induced inflow of Ca^{2+} from the extracellular milieu, due to a cAMP-dependent regulation of Ca^{2+} channels at the level of the plasma membrane. Studies on carp liver culture could not correlate hormone-regulated glycogenolysis with changes in cytosolic Ca^{2+}(Janssens and Lowrey, 1987). My experiments

provide evidence that GLU stimulation of eel and bullhead hepatocytes induces the synthesis of both second messengers, thus supporting the view that, as in mammals, the cAMP and the IP_3 transduction systems operate side by side to regulate liver metabolism.

GLU (0.1 µM) stimulated eel hepatocyte cAMP production by 19-fold compared with only 2.3-fold in bullhead (Fig. 3-3A, B); this result confirms that of Mommsen and Moon (1990) using the same species and the same GLU concentrations. The time dependency of cAMP production was the same observed for EPI stimulation (compare Fig. 3-3 with 3-2). A lower GLU concentration (0.01 nM) poorly stimulated the hepatocytes (Fig. 3-3A, B), although both responded with a significant increase in cAMP production compared with control values.

Time course experiments in carp liver (Janssens and Lowrey, 1987) showed increasing cAMP production at least up to 10 min following 1 µM GLU stimulation, but the levels of cAMP achieved were 10- and 100-fold lower than those I reported for bullhead and eel, respectively. Lahlou *et al.* (1988) reported peak cAMP production in trout hepatocytes within 5 min and values at 0.1 µM GLU in between those I reported for eel and bullhead (data recalculated considering the protein content of 1g of cell wet wt as 8% of its weight). These findings support a more sensitive system in eel, trout and bullhead than in carp and underline a species-dependent sensitivity to hormone stimulation. Measurements of cAMP production at 10 min using 1 µM GLU in turtles (Janssens and Grigg, 1992) showed an increase of the same order of magnitude as in eel and bullhead. Similar experiments using canine hepatocytes gave basal and GLU (0.5 µM)-stimulated levels of cAMP of 4.8 and 120 nmoles/g wet wt; this value was reached after 45 min of incubation and in the presence of theophylline (Grady *et al.*, 1987). These authors showed clearly that the peak cAMP concentration achieved was directly related to the concentration of theophylline. Stimulation of rat hepatocytes with 10 nM GLU reported by Heyworth *et al.* (1983) and Irvine *et al.* (1993) show the same time-dependency as for cAMP production in eel and bullhead but peak levels that are 10-fold higher than that observed in the two fish species studied. The higher effectiveness of GLU on mammalian hepatocytes than on fish hepatic tissue may relate to a GLU receptor in mammals that has a 10-fold higher affinity

for GLU (Bharucha and Tager, 1990) than that reported by Navarro and Moon (1994) for eel and bullhead high affinity sites, even though the number of receptors/cell is within the same range.

GLU demonstrated a dose-response effect with cAMP, although the eel cells resulted in a much higher stimulation than in bullhead hepatocytes (Fig. 3-5). Both species showed a peak cAMP production at 1 μ M GLU, but the bullhead absolute cAMP production was 4 times lower than in eel. Control values were similar in the two species.

The K_a (GLU) for the cAMP effect in each species is about 10 and 100-times higher than that reported for rat (Wakelam *et al.*, 1986) and canine (Grady *et al.*, 1987) hepatocyte cAMP production, respectively, indicating a major difference in sensitivity of the transduction pathways between the different species. As with rat and canine cells, very high GLU concentrations inhibited cAMP production. Grady *et al.* (1987) suggested this inhibition involves two GLU receptor classes, with the low affinity group involved in the inhibition. Navarro and Moon (1994) showed two classes of GLU receptors in both eel and bullhead, so again the inhibition noted in Fig. 3-5 may result from the action of the low affinity receptor class. These K_a values for cAMP production are about 10 times higher than the K_D for GLU binding to the hepatic high affinity GLU receptors of either species.

ACase activities were induced by increasing concentrations of mammalian GLU in both species (Fig. 3-16A). The poor response induced by GLU in bullhead hepatic membranes (Fig. 3-16A) is similar to that reported by Ottolenghi *et al.* (1988) for this same species. This response did not exceeded 29% from basal levels at high hormone concentrations (Fig. 3-16B). In contrast the activity increased by 74% above control in eel membranes (Fig. 3-16B). In gill epithelium Guibbolini and Lahlou (1987) reported a 30% increase in ACase activity at 0.57 nM GLU, considered as a "maximally stimulating concentration". At the same GLU concentration, the % activity increase in eel and bullhead liver crude membranes were approximately 58 and 23%, respectively. As for EPI, the % change from control ACase activities with GLU (Fig. 3-16 B) is higher in the eel than in the bullhead. ACase activities in rat liver plasma membranes

exposed to the same range of GLU concentrations (Pohl *et al.*, 1972; Ottolenghi *et al.*, 1988) showed values that are 100-fold higher than in fish. As noted above, rat hepatic membrane ACase is stimulated more by GLU than by EPI.

A comparison between the effects of EPI and GLU stimulation on ACase activity shows that in both species EPI is more effective than GLU and that eel ACase is more activated by both the hormones, reaching higher values than the bullhead membrane-bound enzyme. These findings agree with the results of a higher cAMP synthesis in eel than in bullhead hepatocytes stimulated by EPI and GLU.

These results clearly indicate that GLU stimulates the cAMP signalling pathway as has been reported in other fish and mammalian hepatic studies. Studies using similar preparations of rat liver plasma membranes showed more ACase sensitivity to GLU than to EPI and activity levels 100-fold higher to both hormones than those found in eel and bullhead membranes (Pohl *et al.*, 1971, 1972).

The stimulation of eel and bullhead hepatocytes with 0.1 µM GLU also induced the accumulation of IP_3 in these cells following the same time course as for EPI (Fig. 3-7A, B). A peak IP_3 level was achieved between 30 and 45 sec, but the effect was greater in bullhead than in eel cells. The absolute IP_3 production above control was 2.8 and 4.2 nmoles/g for eel and bullhead, respectively, at 0.1 µM GLU. In contrast, a lower GLU concentration (0.01 nM) induced an absolute IP_3 production of 1.8 and 0.8 nmoles/g in eel and bullhead, respectively (Fig. 3-7A, B). These small differences, are not sufficient to infer a species difference with respect to GLU-induced IP_3 changes. Rat hepatocytes incubated with vasopressin (230 nM) achieved a peak IP_3 production 600% above controls at approximately 10 sec (Palmer *et al.*, 1989). In my experiments, 0.1 µM GLU induced a 235 and 172% increase above basal levels in eel and bullhead, respectively, in less than 50 sec. Studies with carbachol-stimulated rat cerebral cortex and tracheal smooth-muscle slices by Challiss *et al.* (1990) showed IP_3 production reaching maximal at 20 and 5 sec, respectively. After reaching these levels, IP_3 synthesis in the cerebral cortex remained elevated for at least 60 sec, while in muscle slices IP_3 control values

were reached within 30 sec. It is apparent that the hepatic IP_3 production in eel and bullhead is as rapid as in other tissues.

A weak dose-response was noted for GLU on IP_3 production (Fig. 3-9) even though bullhead cells responded with approximately 1.5 times more IP_3 than eel (referring to their control values). However, it must be taken in consideration that after 30 sec incubation IP_3 production is already stimulated also in the controls. Similar dose-response experiments are not found in the literature, possibly due to the odd results I demonstrated. These bimodal curves must reflect the rapid pattern of synthesis-degradation of IP_3 in the hepatocytes or the activation of the two affinity classes of GLU receptors found in these species by Navarro and Moon (1994). It is important to note, however, that even at 0.01 nM GLU, a significant elevation of IP_3 was noted (Fig. 3-9), which is not the case for cAMP (Fig. 3-5). This is discussed below.

The experiments performed on eel and bullhead hepatocytes support the hypothesis that GLU modulates fish liver metabolism through both the cAMP and IP_3 transduction mechanisms.

D. Comparison between Epinephrine and Glucagon Stimulation of Second Messenger

It is clear that 10 µM EPI (Fig. 3-2A, B) and 0.1 µM GLU (Fig. 3-3A, B) induced higher cAMP responses in eel than in bullhead. The effectiveness of EPI over GLU is seen to hold at all concentrations higher than 0.1 µM for EPI and 1 nM GLU tested in the bullhead (compare Fig. 3-4 with Fig. 3-5). The eel hepatocytes are actually more sensitive to lower concentrations of GLU (< 0.1 µM) than EPI, and only above 0.1 µM hormone does EPI induce greater levels of cAMP than GLU. Plasma concentrations of EPI and GLU have been determined in eels (< 1 nM: Reid and Perry, 1994; 0.037 nM: Navarro and Moon, 1994), but only GLU values are available for bullhead (0.66 nM: Navarro and Moon, 1994). Obviously these plasma values are well below the K_a for cAMP production (Fig. 3-4 and 3-5; approximately 100 nM) and ACase activation (Fig. 3-15A and 3-16A; approximately 100 nM). Such discrepancies between binding constants and plasma titers are seen in all *in vitro* studies and may simply reflect subtle damage to the membrane during preparation (see Moon *et al.*, 1985) or desensitization of the receptors at

pharmacological hormone concentrations. GLU is known to desensitize GLU receptors in eel and bullhead hepatocytes, but only at very high concentrations (Navarro and Moon, 1994).

It is interesting that GLU is a more potent cAMP inducer than EPI in both rat (Exton, 1979; Ottolenghi *et al.*, 1988) and reptile (Janssens and Grigg, 1992) livers, which is the opposite of what I found in eels and bullheads. Given that GLU is a plasma hormone while catecholamines may be either of plasma or nervous origin (see Introduction), this difference may reflect the relative importance of the system of delivery of these hormones to the liver in these species.

GLU and EPI induce the same absolute IP_3 synthesis in both species (Figs 3-6 and 3-7). Control levels of IP_3 are lower in eel than in bullhead hepatocytes, but the absolute production of this second messenger is comparable in the two species. Low EPI concentrations do not seem to stimulate IP_3 synthesis in either species (Fig. 3-8), whereas the IP_3 system seems to be more sensitive to low GLU concentrations (Fig. 3-9), in accordance with the model proposed by Wakelam *et al.* (1986) that GLU stimulates IP_3 at low, not high concentrations.

The global view suggests cAMP as the second messenger preferentially produced by eel hepatocytes under both EPI and GLU stimulation, while in the bullhead EPI induces similar cAMP and IP_3 production, but GLU stimulates more IP_3 than cAMP synthesis (compare Figs 3-2 with 3-6 and 3-3 with 3-7). A species-dependent regulating system of the synthesis of these two intracellular mediators is at the basis of the modulation of this cellular response and may balance the glucose output of the two species by using to a different extent the two available transduction mechanisms. Given that nothing is known of the linkages between each component of these two systems in fish, it is quite likely that these elements could differ and account for some of the diversities between species.

My results showing that both EPI and GLU stimulate IP_3 production in fish hepatocytes link this hormone stimulation with the Ca^{2+}-mediated transduction pathway hypothesized by Zhang *et al.* (1992a) and Moon *et al.* (1993). Moreover by detecting the presence of cytosolic IP_3, it is possible to say that the inositol phosphate-mediated pathway is present at least in the

hepatocytes of these two fish species as it is in mammals (Exton, 1985, 1988a, b). The tendency to consider the α-adrenergic pathway as a species feature of the liver of the more evolved vertebrates does not agree with my studies. Even though studies on amphibians and reptiles (Sulakhe *et al.*, 1988; Janssens and Grigg 1988, 1992) tend to demonstrate that at least in those species of these two classes assayed the α_1-subtype is not represented on the membranes of liver cells, and EPI plays no role in the Ca^{2+}-mediated regulation of hepatic glycogenolysis, my studies support the existence of the IP_3-mediated pathway at least in some fish species. Phylogenetic differences and adaptive strategies may be at the basis of different genetic expressions that result in the great diversity observed between and within the vertebrate classes.

E. Conclusions

This study provides new informations on the transduction mechanisms present in eel and bullhead hepatocytes.

First, it provides evidence that the cAMP system is not the only mechanism by which EPI and GLU can stimulate metabolic responses of hepatic cells in at least eels and bullheads..

Second, it shows that cAMP synthesis induced by the two hormones achieves different levels in the two species. This fact confirms the study of Mommsen and Moon (1990) on GLU stimulation of eel and bullhead liver cells. GLU in both species is less potent than EPI in stimulating cAMP synthesis, except at low concentrations in eels, and cAMP in eel hepatocytes seems to be more sensitive than the bullhead cells to both hormones.

Third, it provides the first evidence that the intracellular mediator IP_3 is produced in fish hepatocytes, at least in the two species studied, following exposure to both hormones. EPI does show more absolute effectiveness than GLU in terms of the quantity of IP_3 produced, but eel and bullhead produce the same absolute amount of IP_3. The existence of IP_3 confirms the presence of the Ca^{2+}-mediated transduction pathway which may modulate some metabolic responses of liver, as has been reported in some mammals.

Fourth, the use of the α- and β-adrenergic antagonists PHE and PROP in this study

demonstrates that EPI-induced IP_3 and cAMP production involves the presence of α_1- and β_2-adrenoceptors, respectively, on eel and bullhead hepatocytes.

Fifth, the specific IP_3-binding sites assessed in membrane preparations from eel and bullhead liver homogenates provide support for the Ca^{2+} transduction mechanism.

Sixth, the existence of α_1-adrenoceptors was demonstrated on liver membranes of both species. The existence of the α_1-subtype in fish livers demonstrates that the α-adrenergic receptors do not appear only in the more evolved vertebrates like birds and mammals as suggested by Sulakhe *et al.* (1988), but they exist also in at least some less evolved vertebrates. It is possible, however, that these receptors are not expressed in all vertebrate classes, and within a class the α- and β-adrenoceptors may be differently expressed in relation to some characteristics of catecholamines or the tissues of these organisms which have yet to be defined.

Seventh, the characteristics of the binding sites observed in the IP_3 and α_1-adrenoceptors studies suggest a strong inter-specific variability in hormone responsiveness that could explain the lack of the Ca^{2+}-mediated pathway in other teleosts (goldfish: Birnbaum *et al.*, 1976; carp: Janssens and Lowrey, 1987).

Since the hepatocyte stimulation of cAMP and IP_3 production was carried out at concentrations of agonists and antagonists that induced maximal responses, these results do not necessarily correspond to the levels of second messengers produced under physiological conditions.

Further studies are required to confirm that both the cAMP- and IP_3-mediated pathways modulate the metabolic responses of the hepatic tissue of other fish species and to unravel the species differences which are seen with respect to these two transduction pathways.

CHAPTER 5:
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APPENDIX

A. Rainbow Trout and Adrenergic Pathways

Preliminary experiments (not reported in this thesis) on rainbow trout (*Oncorhynchus mykiss*) hepatocytes treated under the same experimental conditions as for eel and bullhead show less sensitivity to EPI than the other two species in terms of cAMP synthesis. Trout hepatocytes were more sensitive to 0.1 µM GLU than bullhead but less than eel liver cells. These same hepatocytes produced no significant changes in IP_3 following EPI and GLU exposure. Even though no changes in IP_3 were observed, a single class of IP_3-binding sites with much lower affinity [K_D (PRZ) = 18.7 nM] for the specific ligand than bullhead and a density of sites within the range of values found for eel and bullhead (107.8 fmoles/mg) were observed. These findings support a predominant β-adrenergic pathway for metabolic effects in trout liver, but do not exclude an α-mediated pathway. Additional studies by E. Fabbri (unpublished) found α_1-adrenoceptors on trout hepatic membranes, but the K_D (PRZ) is about 40 times higher than that for the β-adrenoceptor. Further studies using this comparative strategy are necessary to understand more fully the adrenergic systems of this species.

B. Protein Kinase A

A complementary study in connection with the activity of the enzyme ACase was carried out on isolated bullhead hepatocytes (approximately 230 mg/ml wet weight) testing the activation of the cAMP-dependent protein kinase A (PKA). PKA is activated by cAMP (Exton, 1979a) and act as "a major enzyme" which phosphorylates target proteins in cells, directly influencing the rate-limiting stages of metabolic responses affected by cAMP (Exton, 1979a; Summers and McMartin, 1993). The purpose of the study was to further probe the cAMP transduction pathway. Each experiment consisted of a time course and a dose response carried out at 10°C. PKA activity was tested at 0, 1, 5, and 10 min. Cells were exposed to 10 µM EPI or 1 µM GLU and centrifuged at 13,000 xg for 30 sec to terminate the incubation. The supernatant

was quickly removed and samples were frozen in liquid nitrogen. In the dose response experiments, cells treated with 0.001 nM EPI or GLU acted as controls, as at this hormone concentration cAMP production is similar to that of non-stimulated cells (see Figs 3-4 and 3-5). Other samples were treated with 1 µM and 10 µM EPI or GLU and the incubation was terminated after 5 min of exposure as in the time course experiment.

The activity of the enzyme was measured by detection of the radioactivity of the synthetic peptide substrate kemptide following its phosphorylation by [γ-^{32}P] ATP by the activated PKA. Measurements were undertaken by Clark Holden (PhD student, Carleton University), in collaboration with Dr. K. Storey. Following preliminary trials, the experimental conditions for the assay chosen were: 10 µl of cell homogenate, incubated for 20 min with the radioactive tracer. The enzyme activity was expressed in % of activation, calculated as the ratio between the radioactivity measured in the assay in the absence and in the presence (100%) of cAMP. Two independent experiments were assayed.

In the time course (Fig. App-1) control PKA activities were found in the range of 20-30%. EPI exposure at 0 time already activated the enzyme to 68.5%, with activities stabilizing at approximately 83% for the rest of the time course. GLU stimulated the enzyme less intensively yielding 50% at 0 time and approximately 64% for the rest of the time course. The major activation of the enzyme took place in the first minute of hormone incubation. Further experiments are required to examine more closely these initial time periods, when the enzyme activation is rapid. Obviously activation of this enzyme is a very rapid event.

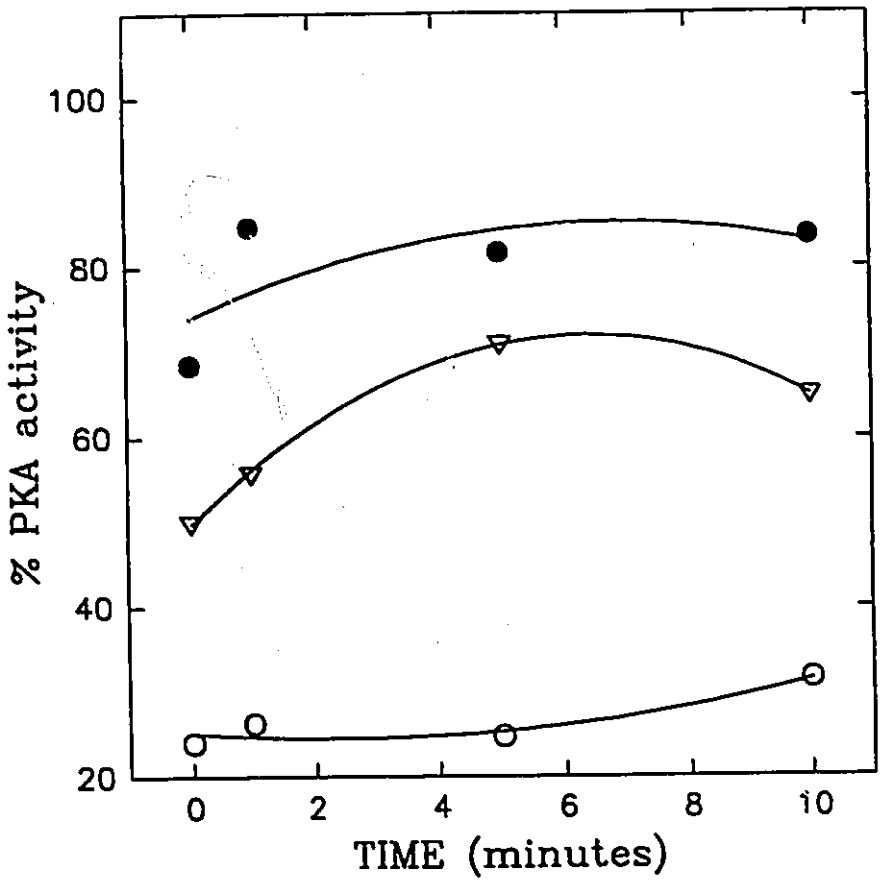
Incubation of hepatocytes with 1 and 10 µM EPI and GLU again suggests that EPI is a more potent activator of PKA (Table App-1) just as I observed for cAMP accumulation and ACase activities. These preliminary experiments provide support for the stimulation of the cAMP signalling pathway in bullhead hepatocytes by both EPI and GLU.

Table App-1. Dose response of PKA activities to epinephrine (EPI) and glucagon (GLU) in bullhead hepatocytes.

TREATMENT	% ACTIVITY
CONTROL (1 pM EPI)	30.3 - 49.4
1 μM EPI	73.8 - 83.4
10 μM EPI	91.4 - 92.4
CONTROL (1pM GLU)	34 - 52.5
1 μM GLU	65.5 - 72.3
10 μM GLU	59.9 - 71.3

Incubation time, 5 min. The values are the range of 2 separate experiments. For each sample 4 trials were carried out.

Fig. App-1: Time course for epinephrine (EPI) and glucagon (GLU) effects on the activation of the enzyme protein kinase A (PKA) in bullhead hepatocytes. Hepatocytes (230 mg/ml), prepared as described in Materials and Methods, were incubated in the absence (open circle) or in the presence of 10 µM EPI (close circle) and 1 µM GLU (open triangle), at 10°C. Incubations were terminated at the chosen time by centrifuging the samples at 13,000 xg for 30 sec at 10°C. Values are average of 2 separate experiments.


100
80
60
40
20
% PKA activity
0
2
4
6
8
10
TIME (minutes)