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THE ROLE OF CATECHOLAMINES IN Ca^{2+} HOMEOSTASIS IN FISH HEPATOCYTES

by

Jinrui Zhang

A Thesis presented to the University of Ottawa

in partial fulfilment of the degree of

Masters of Science in Biology

Jinrui Zhang, Ottawa, Canada, 1992

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ISBN 0-315-85793-5

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ABSTRACT

The role of Ca^{2+} in catecholamine actions on hepatocyte metabolism was studied in three fish species: American eel, brown bullhead and rainbow trout. The cellular Ca content, Ca^{2+} fluxes across the cell membrane and cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in Fura-2-loaded single hepatocytes were measured respectively using atomic absorption spectrophotometer, a ^{45}Ca exchange technique and a computer-controlled microspectrofluorimeter technique. The effects of metabolic inhibitors (iodoacetatic acid and potassium cyanide), α- and β-agonists (epinephrine, phenylephrine and isoproterenol), antagonists (phentolamine and propranolol) and external Ca^{2+} were also investigated.

The results indicated the following about Ca^{2+} and hepatocyte metabolism. **First,** fish hepatocytes contain higher Ca content than equivalent mammalian cells (8.25 ± 1.03 (eel) and 10.49 ± 1.26 (bullhead) $\mu moles \cdot g^{-1}$ wet wt., respectively). **Second,** Ca^{2+} uptake is a passive or energy-independent process whereas Ca^{2+} efflux may be an active or energy-dependent process. **Third,** Ca^{2+} uptake was not significantly stimulated by the catecholamines studied. Under control condition, Ca^{2+} uptake into bullhead hepatocytes was about 2-times that seen in eel hepatocytes (212.9 ± 38.9 and 105.9 ± 6.6 $nmoles \cdot g^{-1}$ wet wt., respectively at 30 min point). These higher rates are key to Ca_i^{2+} metabolism in bullhead cells (see seventh below). **Fourth,** Ca^{2+} efflux was significantly stimulated by both epinephrine and phenylephrine in eel hepatocytes and these effects were blocked by the α-antagonist phentolamine. The β-agonist isoproterenol and β-antagonist propranolol

did not affect basal or hormone-stimulated Ca^{2+} efflux. Ca^{2+} efflux was not affected in bullhead hepatocytes by any α- and β-agonist or antagonist tested. Under control conditions, Ca^{2+} efflux in bullhead hepatocytes was not significantly slower than that in eel cells (at 30 min, 68 ± 2% (bullhead) and 61 ± 3% (eel) ^{45}Ca remaining in hepatocytes, respectively). **Fifth**, basal $[Ca^{2+}]_i$ was similar in eel and trout hepatocytes (79.6 ± 14.6 and 75.7 ± 17.4 nM, respectively), but significantly higher in bullhead cells (184 ± 23 nM). **Sixth**, $[Ca^{2+}]_i$ was significantly increased in eel hepatocytes by either epinephrine or phenylephrine at 10^{-7}M (232% and 143% increase, respectively), but not the β-agonist isoproterenol (4% increase); the epinephrine effect was blocked by the α-antagonist phentolamine but not the β-antagonist propranolol. Epinephrine and phenylephrine were found to induce repeated Ca_i^{2+} transients (i.e., Ca^{2+} oscillations) with variable patterns in individual eel hepatocytes. Epinephrine and phenylephrine also increase $[Ca^{2+}]_i$ significantly in bullhead cells (236% and 198% increase, respectively) and the epinephrine was again blocked by phentolamine. Epinephrine, however, did not induce Ca^{2+} oscillations. In these cells epinephrine caused only a transient rise in Ca^{2+} followed by a sustained plateau. Phenylephrine and isoproterenol produced oscillations in these bullhead cells. As opposed to eel and bullhead cells, trout hepatocytes exhibited little sensitivity to epinephrine with less than 20% of the cells tested showing changes in $[Ca^{2+}]_i$ in response to catecholamines. **Seventh**, the initial rise in Ca_i^{2+} induced in eel hepatocytes by epinephrine was independent of external Ca^{2+}, although external Ca^{2+} is required for the long-term maintenance of Ca^{2+} oscillations. Bullhead cells depend totally on external Ca^{2+} for increasing Ca_i^{2+}. It is difficult to make conclusion about trout cells in

this regard because of the low response obtained even in the presence of external Ca^{2+}.

Overall, the results of this thesis support the existence of α-adrenoceptors on fish hepatocyte membranes at least in American eel and brown bullhead and indicate the involvement of a Ca^{2+} messenger system in catecholamine actions. It is clear that there are major differences between fish species with respect to the role of cellular Ca^{2+} in catecholamine action.

SOMMAIRE

Le rôle du Ca^{2+} dans l'action des catécholamines sur le métabolisme hépatique fut étudié chez les hépatocytes de trois espèces de poissons: l'anguille américaine,la barbotte ainsi que la truite arc-en-ciel. Les changements du contenu cellulaire en Ca^{2+}, du flux de ces ions à travers la membrane cellulaire ainsi que de la concentration en Ca^{2+}-libre dans le cytoplasme ($[Ca^{2+}]i$) furent mesurés chez des cellules hépatiques individuelles à l'aide de la spectroscopie d'absorbtion atomique, d'une technique de transfert de ^{45}Ca et d'un spectrophotomètre respectivement. Les effets de substances métaboliques inhibitrices (l'acide iodoacétique et le cyanure de potassium), d'agonistes α et ß (l'adrénaline, la phényléphrine et l'isoprotérénol), d'antagonistes (phentolamine et propranolol) ainsi que du Ca^{2+}externe furent aussi investigués.

Les conclusions de ces expériences sont les suivantes. Premièrement, les hépatocytes des poissons ont des niveaux de Ca^{2+} plus élevés que les hépatocytes mammaliens (8.25 ± 1.03 et 10.49 ± 1.26 µmoles.g^{-1} chez l'anguille et la barbotte respectivement). Deuxièmement, l'entrée de Ca^{2+} est passive ou n'est pas un processus qui requiert de l'énergie, tandis que la sortie de Ca^{2+} est peut-être un processus actif ou qui requiert de l'énergie. Troisièmement, l'entrée de Ca^{2+} n'est pas stimulée de façon significative par les catécholamines que nous avons étudiées et notons que sous nos conditions de contrôle l'entrée de Ca^{2+} chez les hépatocytes de barbotte est deux fois plus élevée que chez les hépatocytes d'anguille (212.9 ± 38.9 versus 105.9 ± 6.6 nmoles.g^{-1} poids mouillé à 30 minutes). Les taux plus élevés observés chez la barbotte sont

importants dans le métabolisme du Ca_i^{2+} (voir notre septième conclusion).

Quatrièmement, la sortie de Ca^{2+} est stimulée de façon significative par l'adrénaline et la phényléphrine chez les hépatocytes d'anguille, tandis que ces effets sont bloqués par la phentolamine, un α antagoniste; le ß-agoniste isoprotérénol et l'antagoniste propranolol n'affectent pas le taux de la sortie de Ca^{2+}, soit-il basal ou stimulé avec des hormones. Le taux de la sortie de Ca^{2+} des hépatocytes de barbotte n'est pas affecté par aucun des agonistes ou antagonistes testés. Sous nos conditions de contrôle, la sortie du Ca^{2+} ne significatiment pas plus lente chez les hépatocytes de barbotte que chez les cellules d'anguille: à 30 minutes 68 ± 2% et 61 ± 3% du ^{45}Ca était toujours présent dans les cellules de ces espèces respectives. Cinquièmement, le $[Ca^{2+}]_i$ basal était similaire chez les hépatocytes d'anguille et de truite (79.6 ± 14.6 et 75.7 17.4 nM respectivement), mais significativement plus élevé chez les cellules de barbotte (184 23 nM). Sixièmement, le $[Ca^{2+}]_i$ augmente de façon significative en présence d'adrénaline et de phényléphrine à $10^{-7}M$ (augmentation de 232% et 143% respectivement), mais l'isoprotérénol n'a aucun effet. L'effet de l'adrénaline est bloqué par la phentolamine, un α-antagoniste, mais pas par le propranolol, un ß-antagoniste. L'adrénaline et la phényléphrine peuvent induire des oscillations répétées du Ca_i^{2+} ayant des patrons différents chez différentes cellules de l'anguille. Ces deux substances augmentent le $[Ca^{2+}]_i$ de façon significative chez les cellules de barbotte (augmentations de 236% et 198% respectivement) et l'effet de l'adrénaline est ici aussi bloqué par la phentolamine. Par contre, l'adrénaline n'induit pas d'oscillations du Ca^{2+} chez ces cellules. Elle n'induit qu'une élévation transitoire suivie par un plateau soutenu; la phényléphrine et

l'isoprotérénol produisent des oscillations, mais ces observations ne furent pas analysées en plus de détails. Les hépatocytes de truite sont peu sensibles à l'adrénaline. Moins de 20% des cellules testées répondirent aux catécholamines par un changement en $[Ca^{2+}]_i$. Septièmement, l'augmentation initiale du Ca_i^{2+} induite par l'adrénaline chez les cellules d'anguille est indépendante de la concentration externe en Ca^{2+}, quoique du Ca^{2+}externe est nécessaire pour le maintien à long terme des oscillations en Ca^{2+}. Les cellules de barbotte dépendent totalement du Ca^{2+} externe pour augmenter le Ca_i^{2+}. Il est difficile de faire des conclusions sur ce qui a trait aux cellules de truite étant donné leur faible réponse même en présence de Ca^{2+} externe.

Nos résultats indiquent que les membranes des hépatocytes de poisson (dans les anguille américaine et la barbotte) contiennent des α-adrénorécepteurs et qu'un système de Ca^{2+} messager est impliqué dans l'action des catécholamines. Il y a de nettes différences nettes dans le métabolisme du Ca^{2+} entre différentes espèces de poissons.

ACKNOWLEDGEMENTS

I would like to give my sincere thanks to my supervisor, Dr. T. W. Moon, for his excellent guidance, kind support, patient help, great encouragement and understanding; without him this thesis would not have been possible.

Thanks from the bottom of my heart should also go to my committee members Drs J. C. Fenwick, S. F. Perry, K. B. Story and M. Désilets, for their critical comments and helpful discussions. Special thanks must go to Dr. M. Désilets for the use of the microspectrofluorimeter which made all of the Fura-2 experiments possible. His patience and help were invaluable to the completion of these experiments. Also, special thanks to Dr. J. C. Fenwick, for his great help and assistance in interpreting the comments of Dr. Moon in Dr. Moon's absence.

I am indebted to all my fellow cubists for their kindness and great help, and I will never forget them.

I also express my thanks to Jacques Hélié for drawing the figures for my thesis and Paul Brunon for photographing them.

Grateful also to the Fenwicks for their warm care during my stay in Ottawa.

Thanks also to my husband, Q. Xue, for his support and computer assistance.

The Funding for this work and my salary were provided by an operating grant from the Natural Sciences and Engineering Research Council, Canada to Dr. T. W. Moon (A6944).

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LIST OF ABBREVIATIONS

β	ratio of fluorescence of Fura-2 at 380 nm in zero and in saturating Ca^{2+} solution or beta
ACT	activity
ANOVA	analysis of variance
BSA	bovine serum albumin
C-KINASE	protein kinase C
Ca_e^{2+}	extracellular Ca^{2+}
Ca_i^{2+}	cytosolic free Ca^{2+}
CAM	calmodulin
cAMP	cyclic adenosine 5'monophosphate
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
E	epinephrine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
F	fluorescence
FIG	figure

FURA-2	1-[2-(5-carboxyoxal-2-2yl)-6-aminobenzofuran-5-oxyl]-2-(2'amino-5'-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acid
FURA-2/AM	acetoxymethyl fura-2
G_i	inhibitory GTP-protein
G_s	stimulating GTP-protein
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
HR	hour
IAA	iodoacetatic acid
IP_3	myo-inositol 1,4,5-trisphosphate
ISO	isoproterenol
KCN	potassium cyanide
K_a	association or activation constant
K_d	dissociation constant
LDH	lactate dehydrogenase
MT	mitochondria
MIN	minute
ND	not done
NE	norepinephrine
P-PROTEIN	phosphorylated protein
PE	phenylephrine
PEG	^{14}C polyethylene glycol
PHE	phentolamine

PIP_2	phosphatidylinositol 4,5-bisphosphate
PKS	protein kinases
PRO	propranolol
R	fura-2 fluorescence ratio at 350 nm : 380 nm
R_{max}	maximal fluorescence ratio at 350 nm : 380 nm
R_{min}	minimal fluorescence ratio at 350 nm : 380 nm
SE	standard error
SEC	second
SPEC	specific
$[Ca^{2+}]_i$	cytosolic free Ca^{2+} concentration
$\Delta[Ca^{2+}]_i$	cytosolic free Ca^{2+} concentration change
$\Delta[Ca^{2+}]_{i(f)}$	initial maximal cytosolic free Ca^{2+} concentration change
$\Delta[Ca^{2+}]_{i(s)}$	cytosolic free Ca^{2+} concentration change at 2 min after the initial response
WT	weight

CHAPTER 1:

INTRODUCTION

INTRODUCTION

I. Calcium Status in Organisms

Calcium is a ubiquitous cation in nature. It is found in all environments including water, soil and rock. In many animals and especially vertebrates, Ca^{2+} is the most abundant cation in the body due to the major constitute of Ca^{2+} in hard tissues. Ca^{2+} is cycled through the environment and organism within the food chain with water as an intervening medium (Dacke, 1979). Terrestrial animals obtain Ca^{2+} primarily from food and store more than 99% of their body Ca in bone in the form of calcium phosphate and carbonate which serve as a mobilizable internal Ca^{2+} reservoir; less than 1% of total body Ca is found intracellularly within soft tissues (Parfitt and Kleerekoper 1980). In contrast, aquatic animals, including fish, directly use the water medium as an external Ca^{2+} reservoir (Dacke, 1979; Fenwick, 1989). About 50% of the total body uptake of Ca^{2+} by a fish is across the gills (Perry and Wood, 1985), with skin, gut and kidney also being important organs for exchange of Ca^{2+}. The hard tissues including bony scales and skeleton (Dacke, 1979) act as Ca^{2+} reservoirs in fishes although to a smaller extent than in mammals. The soft tissues like skeletal muscles of teleost fishes contain about four-times more Ca than mammalian muscles and this tissue may also provide a readily mobilizable internal Ca^{2+} reservoir (see Dacke, 1979).

Calcium is not evenly distributed within tissues and organs and it plays many important but distinct roles in these different tissues and organs. It is abundant in hard tissues such as bone, fish scales, mollusk shell etc., in which the major inorganic

constituents (calcium phosphate and carbonate) give structures rigidity and strength for protection and as an Ca^{2+} reservoir. In plasma of vertebrates, Calcium concentrations are in the range of 2 to 4 mM (Simkiss, 1967; Dacke, 1979) where it is found either free or bound to plasma protein, where it acts as a cofactor for extracellular enzymes and proteins during the blood clotting process. In muscles, Ca^{2+} couples excitation to contraction. In nerve terminals and secretory organs, Ca^{2+} couples excitation to the secretion of neural transmitters and some hormones. Some organs such as kidney, bone, intestine, salivary glands and other secretory glands have higher Ca concentrations than most tissues because of specialized mechanisms for sequestering Ca^{2+} (Rubin, 1974). Comparatively, the liver lacks the specialized mechanisms for Ca^{2+} transport found in tissues with irritability, contractility, or a specific role in Ca^{2+} conservation; however, liver has a principle metabolic role and Ca^{2+} has been found to play a key role in these functions in mammals (Exton, 1985; 1988).

Calcium is not evenly distributed at the cellular level. Ca is primarily sequestered in organelles such as the endoplasmic reticulum, mitochondria, the nucleus (Exton, 1988) and possibly in specialized organelles called "calciosomes" (Volpe et al., 1988). It is also found bound to cell and cell organelle membranes but only a small part of the Ca is present in the cytoplasm. In the cytoplasm, Ca^{2+} is found either in the free or bound forms (bound to membranes, macromolecules or other ions). Cytosolic free Ca^{2+} in the resting cell is about 0.1 μM, while the Ca^{2+} concentration of the extracellular medium is at least a 10000-fold higher. In comparison, the concentration gradients for K^+, Na^+ and Mg^{2+} are very much lower, being in the range of 30 to 100. Thus, any small increase in

the permeability of the cell membrane to Ca^{2+} would cause a very large fractional change in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), whereas the fractional change in K^+, Na^+ and Mg^{2+} would be small in comparison. This specific control over cellular $[Ca^{2+}]$ is critical to maintain the integrity of the cell. It is the lower cytosolic free Ca^{2+} concentration, the larger concentration gradient across the cell membrane and the ability of Ca^{2+} for tighter and more specific binding than other common ions which make Ca^{2+} one of the most important cations in the body. In addition, Ca^{2+} along with cyclic AMP (cAMP) have been called cellular "Second Messengers". These so-called "Second Messengers" are relative to the "First Messenger", or a physical, electrical or chemical stimulus coming to the cell surface which leads to a change in the concentration of Ca^{2+} and/or cAMP within the cell. Ca^{2+} and/or cAMP are internal signals or "Second Messengers" triggering an appropriate biochemical response within the cell to the stimulus. This is a vast field, involving neural and endocrine systems which ensure the physiological integrity of the organism.

Cytosolic free Ca^{2+} concentration is tightly regulated. It is generally accepted that there are cellular membrane channels specific for Ca^{2+} entry or influx (Exton, 1988; Crofts and Barritt, 1990), Ca^{2+}-pumps (Exton, 1988) and $3Na^+$-Ca^{2+} exchange (Blaustein and Nelson 1982) for Ca^{2+} extrusion or efflux. Other transport mechanisms also control Ca^{2+} flux across the cell and organelle membranes. In the resting cell, Ca^{2+} influx and efflux across the cell membrane are at steady-state, as it is across the mitochondria membrane. Mitochondria acts as an intracellular Ca^{2+} buffer (Borle, 1973) or as a high capacity safety apparatus (Meldolesi et al., 1990); i.e., mitochondria take up Ca^{2+} when

$[Ca^{2+}]_i$ rise to levels that may ultimately endanger cellular integrity. In activated cells, Ca^{2+} permeability of the plasma membrane increases 4- to 5-fold (Borle, 1981a), the cytosolic free Ca^{2+} concentrations may rise by 10- to 100-fold because of the increased Ca^{2+} uptake across the cell membrane and increased Ca^{2+} release from the intracellular Ca^{2+} pools (Exton, 1988) such that Ca^{2+} reaches a new steady-state. Although early studies suggested that mitochondria represented the major intracellular Ca^{2+} pool (Carafoli, 1987; Exton, 1988), more recent investigations indicate that the intracellular Ca^{2+} pool is non-mitochondria and most likely endoplasmic reticulum or an associated specialized organelle the "calciosome" (Exton, 1988; Meldolesi et al., 1990).

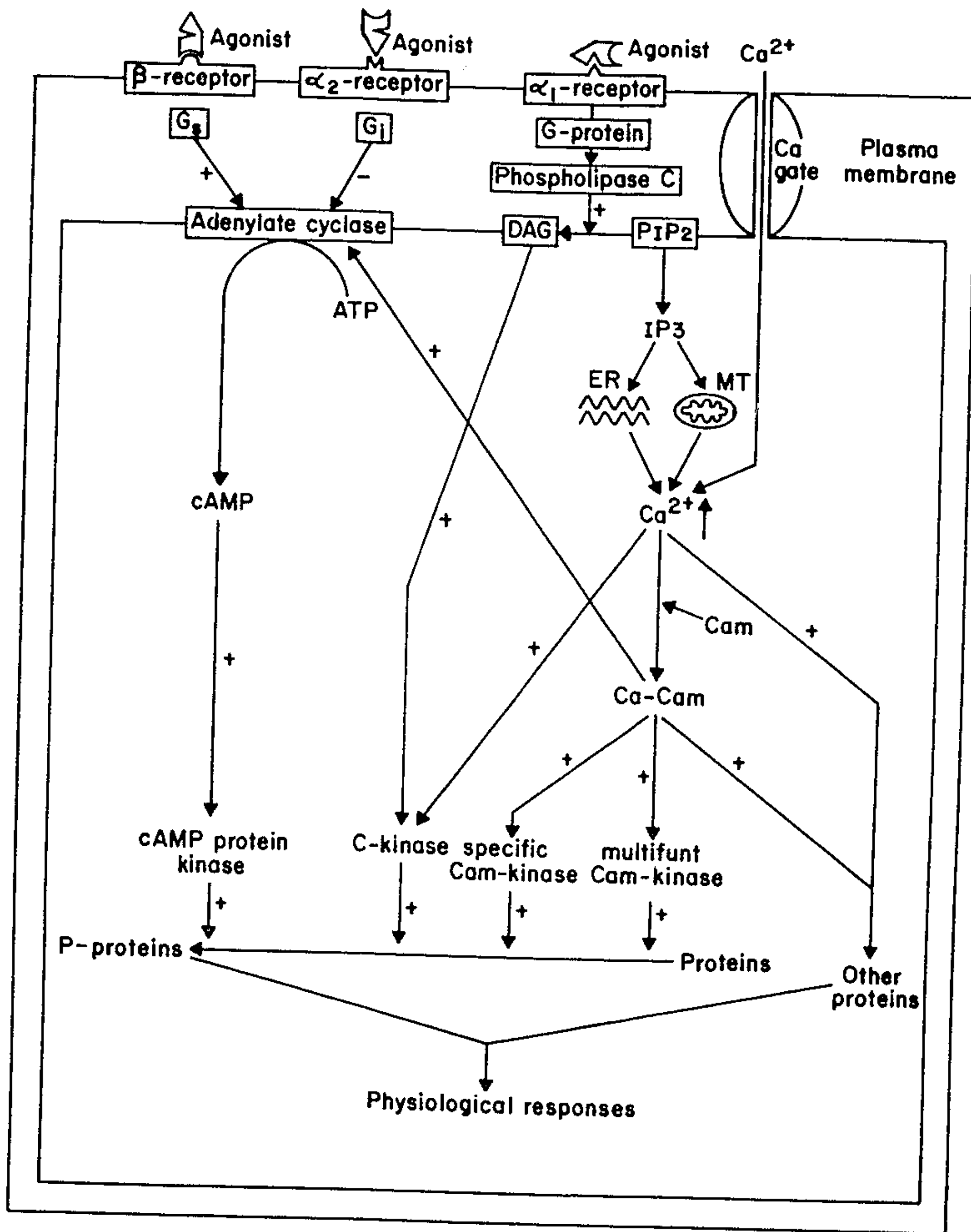
II. Relationship among Ca^{2+}, Catecholamines and Hepatocyte Metabolism

Epinephrine and norepinephrine are catecholamine hormones of vertebrates produced and released into the blood stream from the adrenal medulla or from adrenergic nerve endings. One of the catecholamine target organs is the liver; the principal physiological response to catecholamine-induction in liver cells or hepatocytes is the stimulation of glycogenolysis and gluconeogenesis (Exton, 1985; 1988). This induction is initiated by the catecholamine binding to various subtypes of specific adrenoceptors (Lefkowitz and Caron, 1986; Exton, 1988). These adrenoceptors are classified as either α or β. These 2 groups are further pharmacologically subdivided into α_1-, α_2- and β_1-, β_2-adrenoceptors; α_1- and β_2-subtypes are the main adrenoceptors on hepatic membranes (Exton, 1984). Epinephrine stimulates both α- and β-adrenoceptors, but the actions of the hormone on α- receptors dominate, at least in rats (Exton, 1985; Lefkowitz and Caron,

1986). The synthetic analogues of the natural catecholamines, phenylephrine and isoproterenol, are considered specific α_1- and β-agonists, respectively (Weiner, 1985). The potency ranking for α_1-adrenoceptors in the rat is epinephrine (E) >= norepinephrine (NE) >> phenylephrine (PE) >> isoproterenol (ISO); the potency ranking for β-adrenoceptors is ISO > E > NE > PE (Lefkowitz and Caron, 1986). The synthetic antagonists, phentolamine and propranolol, act to block the actions of α- and β-agonists, respectively, at the receptor. For specific identification of α- and β-adrenoceptors, these agonists and antagonists are employed individually and in combination.

The physiological responses of the liver to catecholamines are mediated by α- and/or β-adrenoceptors (Exton, 1979; 1985, 1988) and their corresponding signal transduction systems which include the intracellular messengers Ca^{2+} and CAMP, respectively. These two transduction systems co-exist in many cell types (Nishizuka, 1986). The relationship between intracellular messengers, catecholamines and catecholamine-induced hepatocyte metabolism is outlined in Fig. 1-1 which shows the cAMP system mediated by β- and α_2-adrenoceptors and the Ca^{2+} system mediated by α_1-adrenoceptors. The description of the cAMP system has changed little since its original formulation. The interaction of an agonist with a β-adrenoceptor stimulates the membrane bound adenylate cyclase through a G_s-protein system; this action leads to the accumulation of intracellular cAMP and to the activation of cAMP-dependent protein kinases (PKs) (Rasmussen et al., 1984; Lefkowitz and Caron, 1986; Exton, 1988). These activated PKs phosphorylate specific enzymes within glycolysis and gluconeogenesis, leading to enhanced blood glucose by activating glycogen breakdown (glycogenolysis)

Fig. 1-1: Mechanism by which α- and β-adrenergic agonists produce their physiological Responses. cAMP acts as a "Second Messenger" mediating α_2- and β-adrenoceptor responses and Ca^{2+} acts as a "Second Messenger" mediating α_1-adrenoceptor responses (Rasmussen et al., 1984; Exton, 1988). Abbreviations: G_s - stimulating GTP-protein, G_i - inhibiting GTP-protein, DAG - diacylglycerol, PIP_2 - phosphatidylinositol 4,5-bisphosphate, IP_3 - myo-inositol 1,4,5-trisphosphate, ER - endoplasmic reticulum, MT - mitochondria, Cam - calmodulin, C-kinase - protein kinase C.



Agonist
Agonist
Agonist
Ca^{2+}
β-receptor
α2-receptor
α1-receptor
Gs
Gi
G-protein
Phospholipase C
Ca gate
Plasma membrane
Adenylate cyclase
DAG
PIP2
ATP
IP3
ER
MT
cAMP
Ca^{2+}
Cam
Ca-Cam
cAMP protein kinase
C-kinase
specific Cam-kinase
multifunt Cam-kinase
P-proteins
Proteins
Other proteins
Physiological responses

and glucose synthesis (gluconeogenesis) (Exton, 1985).

The Ca^{2+} system is induced by the binding of a hormone (α_1-agonist) to the α_1-adrenoceptor which leads to increases in $[Ca^{2+}]_i$ which then acts as a "Second Messenger". The events are complicated and there appears to be an interaction between the cAMP- and the Ca^{2+}-systems. The binding of agonist to the α_1- adrenoceptor leads to the activation of a GTP-bound G-protein and in turn the activation of phospholipase C which hydrolyses membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) with the generation of diacylglycerol (DAG) in the plasma membrane and myo-inositol 1,4,5-trisphosphate (IP_3) in the cytosol (Rasmussen et al., 1984; Lefkowitz and Caron, 1986; Exton, 1988). The rise in $[IP_3]$ results in the mobilization of Ca^{2+} from either extracellular sources or intracellular pools (Exton, 1988) located in the endoplasmic reticulum (ER), mitochondria (MT) or a specialized Ca^{2+} storage organelle (calciosomes). The endoplasmic reticulum is an important intracellular Ca^{2+} pool and the time course of its activation is such that the mobilization of this store is thought to be the primary Ca^{2+} source (Charest et al., 1983). The confirmation that IP_3 specifically releases Ca^{2+} from these stores comes from many studies (see Kraus-Friedmann, 1990; Meldolesi et al., 1990). When $[Ca^{2+}]_i$ increases, Ca^{2+} directly activates enzymes including protein kinase C or complexes with calmodulin (Cam) (Exton, 1988). Calmodulin is a Ca^{2+} receptor, a major and ubiquitous Ca^{2+}-binding protein with no enzymatic activity. The Ca^{2+}-calmodulin complex activates certain Ca^{2+}-calmodulin-dependent protein kinases and the Ca^{2+} pump (Exton, 1988) mainly through the regulation of protein phosphorylation. Some phosphorylase kinases can be activated by both a Ca^{2+}-calmodulin dependent protein

kinase (Cohen et al., 1978) and a cAMP-dependent protein kinase (Rasmussen et al., 1984; Exton, 1988). For example, phosphorylase b kinase actually contains calmodulin as one subunit of this multi-subunit enzyme; thus, this kinase can be activated directly by increased $[Ca^{2+}]_i$ and can bind additional Ca^{2+}-calmodulin subunits to further increase its activity (Exton, 1981; Williamson et al., 1981). Phosphorylase b kinase can also be activated by cAMP-dependent protein kinase. These actions lead to the phosphorylation of phosphorylase b, converting it to the more active form (phosphorylase a) which catalyses glycogen breakdown or glycogenolysis increasing the availability of glucose.

There are many similarities as well as interactions between the two "Second Messenger" generating pathways (Rasmussen et al., 1984). For example, both pathways activate the phosphorylation of protein kinases (such as phosphorylase b kinase) cooperatively and synergistically; both pathways interact through the regulation between Ca^{2+} and cAMP metabolism. Much evidence exists that α_1-adrenoceptor agonists and other Ca^{2+}-mobilizing hormones (e.g., vasopressin, angiotensin II) induce activation of type II Ca^{2+}-calmodulin-dependent protein kinase, which leads to the phosphorylation and inactivation of pyruvate kinase in liver and stimulation of mitochondrial pyruvate carboxylation which in turn stimulates gluconeogenesis (see Exton, 1988). Evidence also exists that glycogen synthase is inactivated by a Ca^{2+}-dependent or calmodulin-dependent protein kinase (Exton, 1988), thereby inhibiting glycogen synthesis.

The effect of catecholamines on hepatocyte metabolism has been studied in a number of species. Results confirmed that catecholamines stimulate both glycogenolysis and gluconeogenesis in mammalian liver (Hems and Whitton, 1980; Arinze and Kawai,

1983; Exton, 1985; 1988). In lower vertebrates, the stimulating effect of catecholamines has also been reported in amphibians (Wong and Hanke, 1977; Farrar and Frye, 1979; Janssens and Grigg, 1984) and in teleost fish (Young and Chavin, 1965; Nakano and Tomlinson, 1967; Birnbaum et al., 1976; Ottolenghi et al., 1984; Brighenti et al., 1987; Sheridan, 1987; Mommsen et al., 1988; Wright et al., 1989; Michelsen and Sheridan, 1990; Danulat and Mommsen, 1990). In our lab, phenylephrine, an α_1-agonist in mammalian liver modulated hepatic glycogenolysis and gluconeogenesis in rainbow trout, brown bullhead and American eel, but with significant quantitative differences between species; interestingly, these effects were blocked by propranolol, a β-antagonist (Moon and Mommsen, 1990). Other authors have also found that catecholamine-induced hepatocyte metabolism is fish species dependent and may not follow the same pharmacological schemes noted for mammals (Morata et al., 1982; Ottolenghi et al., 1985; 1986).

Many investigations have undertaken to investigate the existence of α- and β-adrenoceptors on hepatocyte membranes, especially in mammalian liver. There is much evidence that α_1-receptors are important mediators of the glycogenolytic action of catecholamines in the livers of rat (Sherline et al., 1972; Exton, 1979), dog, cat and guinea pig (see Brighenti et al., 1987); β-adrenoceptors, however, are predominant in rabbit and human hepatocytes (see Brighenti et al., 1987). It is clear that the mechanism of catecholamine action in mammalian livers is as described in Fig. 1-1. Both α- and β-adrenoceptors are present and both signal transduction pathways operate although there are some animal differences in receptor density and thus responsiveness to hormones.

These receptors have been quantified primarily in rat liver (Schmelck and Hanoune, 1980).

The mechanism involved in adrenergic-induction of lower vertebrate liver metabolism, however, is less well understood. Two studies one with the urodele amphibian, *Ambystoma mexicanum* (Janssens et al., 1983) and the other with the anuran amphibian, *Xenopus laevis* (Janssens and Grigg, 1984) have demonstrated that catecholamines activate glycogenolysis via a β-adrenoceptor mechanism and that α-adrenoceptors play no role in the physiological response. The same conclusion has been reported in goldfish (Birnbaum et al., 1976) and carp (Janssens and Lowrey, 1987). Brighenti et al. (1987) could not exclude the possible existence or the physiological action of α-adrenoceptors in catfish hepatocytes, but concluded that β-adrenoceptors were the dominant mediator of catecholamine action in this species. A study by Moon and Mommsen (1990) reported that increases in both glycogenolysis and gluconeogenesis in trout, bullhead and eel hepatocytes induced by phenylephrine could be completely blocked by the β-antagonist propranolol and only partially by α-antagonists. Additionally, phenylephrine induced an increase in hepatocyte cAMP concentrations, indicative of a β-adrenoceptor pathway. Based on such studies, some researchers have suggested that the regulation of hepatic glycogenolysis in a number of non-mammalian vertebrates does not involve α-adrenoceptors that use Ca^{2+} as an intracellular signal but involve β-adrenoceptors and cAMP as the sole "Second Messenger" (Janssens and Lowrey, 1987; Moon and Mommsen, 1990).

III. Questions and Hypothesis

The understanding of membrane adrenoceptors and messenger systems is important for future researches in catecholamine-induced hepatocyte metabolism. The information collected to date on lower vertebrates suggests that β-adrenoceptors are the sole mediators of catecholamine action in hepatic tissue. Is this in fact true for all lower vertebrates? Are α_1-adrenoceptors present on hepatocyte membranes of fish? Is the mechanism of catecholamine action in lower vertebrate livers distinct from that in mammalian livers? Given the great diversity of species of lower vertebrates, the significant species difference observed in catecholamine induced physiological responses and the statement of Simkiss (Dacke, 1979, pp.90) that " fish were the first and in many ways the last of the great modifiers of calcium metabolism in the vertebrates", it was deemed necessary to study in more depth the Ca^{2+} modulation by catecholamines in fish hepatocytes. This thesis, therefore, tests if Ca^{2+} is regulated by catecholamines and the possible involvement of α-adrenergic/Ca^{2+} signalling system in fish hepatic metabolism. Three teleost fish species, the American eel, the brown bullhead and the rainbow trout were chosen for comparative study in this aspect as they are commonly available and have been shown to respond differently to the classic α-agonist phenylephrine (Moon and Mommsen, 1990). Hepatocytes were isolated and Ca^{2+} fluxes and changes in $[Ca^{2+}]_i$ as affected by specific catecholamines were examined.

IV. Catecholamine Action Studies

There are many ways to study catecholamine actions in hepatocytes. These

include studying the β-adrenoceptor mediated pathway and increases in intracellular cAMP; studying the α_1-adrenoceptor mediated pathway by examing IP_3 and Ca^{2+} regulation; studying the effect of catecholamines on the final physiological response by monitoring glycogen content and glucose production; and, studying the effect of catecholamines on enzyme activities such as glycogen phosphorylase and pyruvate kinase. Each of these have been used to varying degrees in diverse vertebrate hepatocytes. One way to explore α_1-adrenoceptors and the role of Ca^{2+} in the hormonal control of hepatocyte metabolism is the direct measurement of Ca^{2+} flux and intracellular Ca^{2+} concentrations or transients induced by α- and β-agonists and antagonists. In this way, one can study the catecholamine-induced changes in the rate of uptake and release of Ca^{2+} across the cell membrane and across the membranes of intracellular organelles. The study of uptake and efflux of Ca^{2+} provides information on how the intracellular Ca^{2+} concentration is regulated. It is essential to measure cytosolic free $[Ca^{2+}]$ to obtain definitive evidence for the regulatory role of intracellular Ca^{2+}. An extensive literature is available on mammalian livers, especially rat livers, reporting the effect of catecholamines on Ca^{2+} flux (Mauger et al., 1984; Altin and Bygrave, 1987; Duddy et al., 1989), Ca^{2+} distribution (Barritt et al., 1981) and Ca^{2+} transients (Woods et al., 1986; 1987; Kawanishi et al., 1989; Rooney et al., 1989, 1990; Reber et al., 1990). Unfortunately, this research area remains unexplored in lower vertebrate livers including fish hepatocytes. Ca^{2+} metabolism and Ca^{2+} transport has been studied in several fish species, in preparations including the whole body, the gill (see Fenwick, 1989) and the perfused head (Perry and Flik, 1988),but not in the liver. Thus, intracellular Ca^{2+}

regulation by hormones in fish hepatocytes is a new area of investigation. So in this study, Ca^{2+} uptake and efflux will be measured by a ^{45}Ca exchange technique and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) changes will be monitored by computer-controlled microspectrofluorimeter using the Ca^{2+} indicator Fura-2/AM and loading techniques previously used for rat hepatocytes. The calcium content will also be estimated by atomic absorption spectrophotometer. Viable hepatocytes are prepared routinely in this laboratory from American eel, brown bullhead and rainbow trout by a modified collagenase perfusion technique (see Moon et al., 1985). To identify α- and β-adrenergic mechanism of catecholamine action, epinephrine plus synthetic α-(phenylephrine) and β-(isoproterenol) agonists, and α- (phentolamine) and β-(propranolol) antagonists will be used individually and in combination.

CHAPTER 2:

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Animals and Holding Conditions

Sexually immature American eels *Anguilla rostrata* (LeSueur) were obtained from the St. Lawrence River at the Saunders Hydroelectric Dam, Cornwall, Ontario in August. Brown bullheads, *Ictaluras nebulosus*, were captured from the Ottawa River by a local fishermen during the summer. Rainbow trout, *Oncorhynchus mykiss*, were purchased from Thistle Springs Trout Farm (Ashton, Ont.) or Linwood Acres Trout Farm (Campbellcroft, Ont.). All fish were kept indoors in tanks supplied with flowing, dechlorinated, and vigorously aerated Ottawa tap water at seasonally fluctuating water temperatures (15°C max, 4°C min); photoperiod was 12L : 12D throughout. Eels were not fed, but bullheads and trout were fed daily to satiety with commercial pellets. Fish for experiments were randomly selected from stock fish tanks following at least three weeks of laboratory acclimation.

II. Solutions

All solutions used in the preparation of hepatocytes and in each experiment have been used routinely in this laboratory over the past decade to produce fish hepatocytes with more than 95% cell viability (Moon and Mommsen, 1990; Moon et al., 1985). Their composition is listed on Table 2-1. Special solutions used in the Fura-2/AM experiments are listed on Table 2-2.

Table 2-1: Composition of salines used in this study.

Identification	Purpose	Components in mM
Medium A	Liver perfusion and first separation	125 NaCl, 3.1 KCl, 0.6 $MgSO_4$, 1.0 $MgCl_2$, 1.25 KH_2PO_4, 10 HEPES[1], 5.0 $NaHCO_3$; gased with 0.5% CO_2 remainder O_2 for 15 min; pH adjusted to 7.8 at room temperature after HCO_3^- addition.
Medium B	Liver digestion and cell isolation	Medium A + 140 units collagenase·mL^{-1} + 1.5 $CaCl_2$
Medium C	Cell washing and incubation	Medium A + 2.0 $CaCl_2$[2] ± 0.1% BSA[3]
Medium D	Washing solution for total calcium analyses	138 N-methyl-D-glucamine, 3.0 HEPES, adjusted to pH 7.8 as above.

[1] HEPES: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid.

[2] 2mM $CaCl_2$ was chosen based on the experiment results (Table 2-3).

[3] BSA (Bovine serum albumin, essentially fatty acid-free) was not added to Medium C for ^{45}Ca flux studies because it inhibited Ca^{2+} uptake (Fig. 2-1). For Fura-2/AM loading of hepatocytes, 0.1% BSA was added for improved loading (Tsien, 1989).

Table 2-2: Composition of salines used in Fura-2 calibration experiments.

Identification	Purpose	Components in mM
Medium E[1]	in vitro calibration of Fura-2 for K_d	Stock 1: 130 KCl, 5 HEPES[2], 5 EGTA[3], pH adjusted to 7.6 at 10°C Stock 2: 130 KCl, 5 HEPES, 1 $CaCl_2$, pH adjusted to 7.6 at 10°C pCa — Stock 2 µL 9.00 — 17.2 8.00 — 131.4 7.50 — 264.9 7.00 — 390.4 6.50 — 459.3 6.00 — 486.4 5.75 — 492.4 5.50 — 495.9 5.25 — 499.6 4.00 — 509.9 3.00 — 600.0 Stock 1 was added to each of the above to a final volume of 100 mL.
Medium F	in vivo calibration of Fura-2 for R_{min}	Medium A + 2 $MgCl_2$ + 10 EGTA + 6 µM ionomycin
Medium G	in vivo calibration of Fura-2 for R_{max}	Medium A + 2 $CaCl_2$ + 6 µM ionomycin

[1] Medium E was made according to Fabiato, 1988.

[2] HEPES: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid.

[3] EGTA: ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

III. Hepatocyte Preparation

Eels were decapitated while bullheads and trouts were sacrificed by a sharp blow to the head. The procedure for hepatocyte isolation was identical for all three species. Briefly, the hepatic portal vein was cannulated and perfused with Medium A (Table 2-1) for 5 min or until all blood was cleared from the liver. The perfusion solution was then switched to Medium B which included collagenase (type IV, Sigma) and the perfusion was continued in a re-circulating mode for a further 45 min. When the liver became soft, the gall bladder was carefully removed, the liver minced with a razor blade on a watch glass, and the resulting suspension poured through two successive plankton netting screens (73 and 253 μm, respectively); the suspension was gently massaged through the screens. Hepatocytes were collected by centrifugation at 1000 rpm for 1 min (Sorval RC28S refrigerated centrifuge; 4°C). The resulting supernatant was carefully decanted. The hepatocyte pellet was washed three times with Medium C using the same centrifugation procedures. The cells were finally resuspended in a volume of Medium C to give a hepatocyte concentration ranging from 10 to 50 mg wet cell weight per mL of suspension medium, depending upon the experiments to be undertaken. The cell concentration was determined by estimating the wet cell weight of a given volume of the suspension after centrifugation (13,000xg for 2 min; Fisher 235B microfuge), removal of the supernatant, and wiping of the tube walls with an absorbent tissue.

IV. Total Hepatocyte Calcium Concentration

Hepatocytes were pre-incubated in Medium C at 10°C in a shaking waterbath for

1.5 hr to ensure steady-state conditions (Borle, 1981b); this time period was experimentally established to ensure stable calcium concentration (see Table 2-3). Cell concentrations ranged from 30 to 50 mg cell wet wt$\cdot$mL^{-1} of incubation medium. Following pre-incubation, 1 mL of the cell suspension was removed, and pipetted into 40 mL of ice-cold isotonic N-methyl-D-glucamine solution (Medium D; see Table 2-1) contained in a 50 mL centrifuge tube. The hepatocytes were spun through this medium immediately by centrifugation at 3000 rpm (Sorval RC28S; 4°C) for 1 min; the supernatant was rapidly decanted. After the tubes were thoroughly drained and the walls wiped dry with an absorbent tissue, 2 mL of deionized water was added to the pellet. The pellet was totally disrupted after adding 20 µL concentrated H_2SO_4 and sonicated with an ultrasonic probe (Kontes Cell Disruptor). One mL of the homogenate was transferred to each of two 1.5 mL tubes for duplicate analyses, and centrifuged to remove denatured materials (13,000xg for 1 min). The supernatant was appropriately diluted with deionized water and analyzed for total Ca concentration by atomic absorption spectrophotometer (Varian Spectr AA 10). Ca^{2+} contamination was minimized by using plastic containers, fresh solutions, and deionized water as a solvent. In addition, the amount of Ca^{2+} trapped amongst pelleted hepatocytes was estimated by using ^{14}C polyethylene glycol (PEG) as an extracellular marker; i.e., measuring total ^{14}C in the cell incubation and the ^{14}C among the cell pellet which was got as described above so that the percentage of ^{14}C trapped was known. Based on this information, Ca^{2+} trapped amongst cells was calculated. This residual contamination was subtracted from each sample before calculating the total calcium concentration in the hepatocytes. Calcium concentrations are presented as

Table 2-3: Searching for appropriate Ca^{2+} concentrations in incubation and pre-incubation periods to ensure Ca^{2+} equilibration, determined by atomic absorption spectrophotometer.

Species	$[Ca^{2+}]$ in incubation medium (mM)	µmoles $Ca^{2+} \cdot g^{-1}$ wet cell wt		
		0 min Pre-incubation	1 hr Pre-incubation	2 hr Pre-incubation
Eel (n = 2)	0 mM	7.1	4.9	2.6
	1 mM		7.3	3.2
	2 mM		6.4	7.3
	5 mM		14.9	10.1
Bullhead (n = 3)	0 mM	7.4	6.4	5.4
	1 mM		7.6	8.0
	2 mM		7.6	8.9
	5 mM		8.5	7.4

Hepatocytes were pre-incubated or incubated under standard conditions (10°C) at the indicated $[Ca^{2+}]$ in Medium A (Table 2-1).

μmoles·g^{-1} wet cell wt.

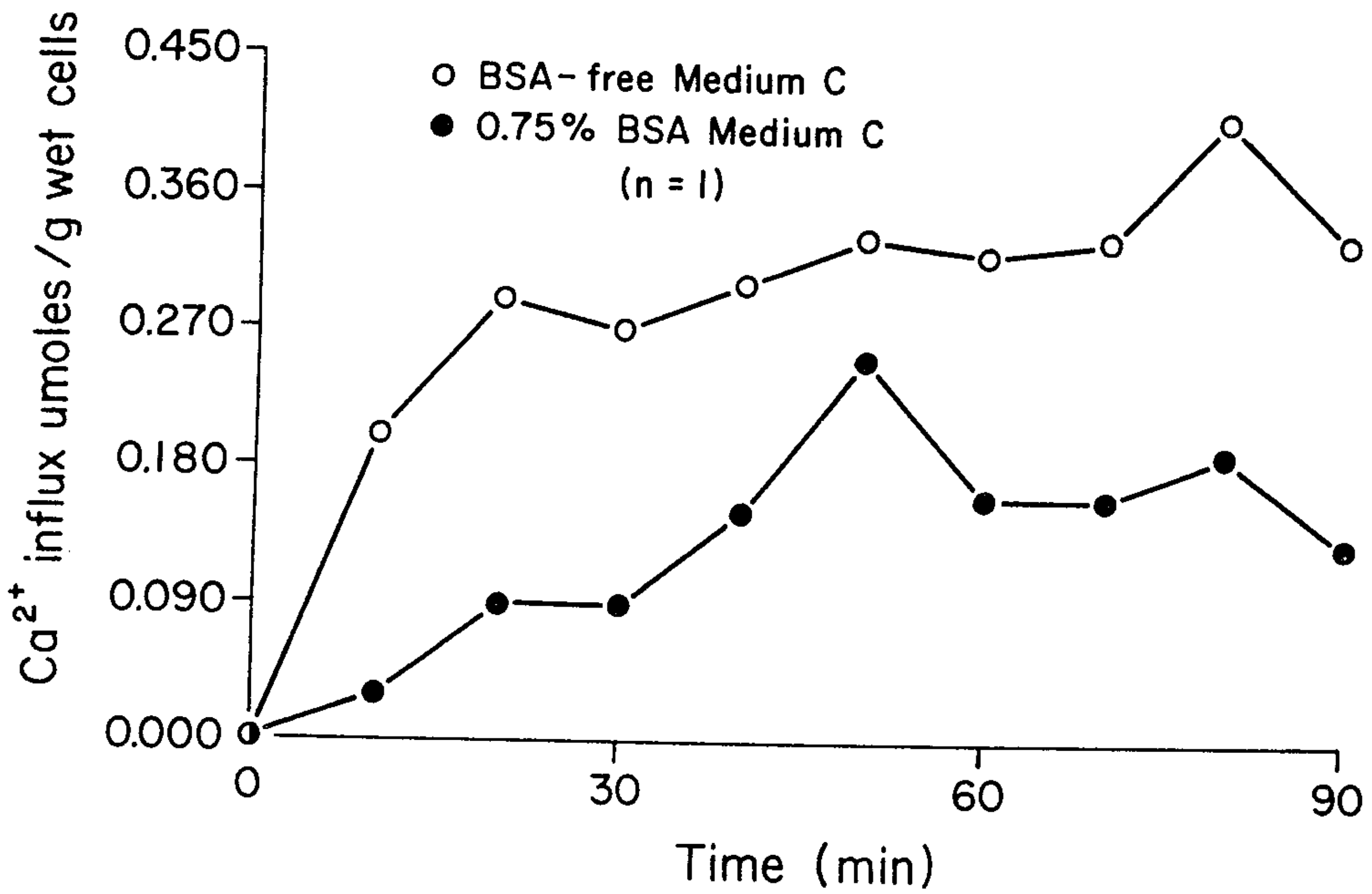
V. ^{45}Ca Flux Studies

A) ^{45}Ca Uptake (Influx)

Hepatocytes were suspended in Medium C less BSA (Table 2-1; a preliminary experiment shown in Fig. 2-1 suggested BSA inhibited Ca^{2+} uptake) and were pre-incubated at 10°C in a shaking waterbath for 1.5 hr to ensure that Ca^{2+} was at steady state across the membrane (Borle, 1981b). After this pre-incubation period, ^{45}Ca (NEN Canada Ltd; Spec. Act. = 10 mCi·mL^{-1}) was added to give a final radioactivity of approx. 1 μCi ^{45}Ca per mL of incubate. When the effects of alpha- and/or beta-adrenergic agonists, antagonists or metabolic inhibitors were investigated, the agonists were added with the isotope at 0 time while the antagonists or metabolic inhibitors were added 15 min before ^{45}Ca addition. An equivalent volume of saline was added to the control incubates. A 0.2 mL sample was removed from each incubation at 0 min and at 3 min intervals thereafter (or as otherwise indicated) for 30 min and added to 8 mL of cold washing medium (Medium C) in a 16 x 100 mm test tube, immediately centrifuged at 3000 rpm for 1 min (Sorval RC28S; 4°C), decanted and drained on paper towels for 30 min. At this point, 0.6 mL of deionized water was added to the pellet, the tube vortexed and the mixture disrupted with the ultrasonic probe. A 0.4 mL aliquot was added to 6 mL of Aquasol-2 (Du Pont) scintillation cocktail. Radioactivity in the sample was determined by liquid scintillation counting (LKB Rac Beta 1211) as an indicator of ^{45}Ca uptake. An aliquot of

Fig. 2-1: Effect of BSA (bovine serum albumin) on Ca^{2+} uptake in eel hepatocytes.

Isolated hepatocytes were suspended in BSA-free Medium C (Table 2-1) or 0.75% BSA-containing Medium C at a cell concentration of 30 to 40 $mg \cdot mL^{-1}$. Cells were pre-incubated for 1.5 hr, ^{45}Ca was then added at 1 $\mu Ci \cdot mL^{-1}$. Samples (0.2 mL) were removed from 0 to 90 min at the 10 min intervals, washed in 8 mL rinsing solution (Medium C). Deionized water (0.6 mL) was added to the pellet, vortexed and the cells were burst using an ultrasonic probe. The aliquot was counted as an estimate of ^{45}Ca uptake, and the radioactivity in the original incubate was also counted to give medium specific activity. Values are expressed as µmoles $Ca^{2+} \cdot g^{-1}$ cell wet wt.


○ BSA- free Medium C
● 0.75% BSA Medium C
(n = 1)
0.450
0.360
0.270
0.180
0.090
0.000
Ca2+ influx umoles/g wet cells
0
30
60
90
Time (min)

the initial incubate was also counted to determine medium Ca^{2+} specific activity.

Calculation of ^{45}Ca Uptake: The ^{45}Ca found in the pellet at time 0 is considered to be that due to contamination or non-specific ^{45}Ca adherence to the hepatocyte membranes. This value was, therefore, subtracted from the radioactivity found at each time point to give an indication of specific ^{45}Ca uptake. ^{45}Ca uptake was expressed as nmoles $Ca^{2+} \cdot g^{-1}$ wet cell wt using the following formula:

$$\text{Calcium uptake in nmoles} \cdot g^{-1} \text{ wet cell wt} = \frac{\text{Cell } ^{45}\text{Ca radioactivity in dpm} \cdot g^{-1} \text{ wet cells}}{\text{Medium specific activity in dpm } ^{45}\text{Ca} \cdot \text{nmoles}^{-1}\ \text{Ca}^{2+}}$$

B) ^{45}Ca Efflux

The procedure used to establish efflux was basically the same as that for ^{45}Ca uptake, except for the following. After the 1.5 hr pre-incubation period, ^{45}Ca was added to the hepatocyte suspension at a final concentration of approx. 2 $\mu Ci \cdot mL^{-1}$. Cells were allowed to load with ^{45}Ca at 10°C for 1 hr in a shaking waterbath. Following this period, the radioactive medium was removed by centrifugation at 1000 rpm for 1 min (Sorval RC28S; 4°C), and the cells were resuspended in the same volume of ^{45}Ca-free Medium C to which was added any hormone or drug to be investigated. As above, inhibitors or

antagonists were added 15 min before the end of loading while agonists were added at the time of adding the ^{45}Ca-free Medium C. Sampling began immediately and cell ^{45}Ca was determined in the same way as that described for the ^{45}Ca uptake experiments.

Calculation of ^{45}Ca Efflux: ^{45}Ca efflux was expressed as ^{45}Ca dpm remaining in the cell pellet per mg wet cell wt. To standardize the estimate of efflux, the ratio of radioactivity at 6 min compared to that at 0 min was used for comparison between hormone treatments. This was necessitated due to differences in the extent of ^{45}Ca-loading between experiments.

VI. Cytosolic Free Ca^{2+} Concentration Studies By Fura-2/AM

A) Fura-2/AM Loading of Hepatocytes

Hepatocytes were suspended in Medium C plus 0.1% bovine serum albumin (BSA) at a concentration of 10 mg wet cell wt per mL; cells were pre-incubated on ice for 1.5 hr as before. Loading was initiated by adding 1 mM stock Fura-2/AM (Molecular Probes; Eugene, Ore.) dissolved in undiluted dimethyl sulfoxide (DMSO) with 0.02% pluronic F-127 to the hepatocyte suspensions to give a final concentration of 5 μM Fura-2/AM; loading proceeded for 1.5 hr on ice with swirling from time to time. The cells were then centrifuged at 1000 rpm for 1 min (IEC Clinical centrifuge), the extracellular medium containing dye was decanted from the pellet, and the pellet was resuspended in the same volume of Medium C without BSA. All procedures were performed in the dark

in foil-wrapped containers due to the light sensitivity of Fura-2/AM. BSA and pluronic F-127 were used as dispersing agents for improved loading (Tsien, 1989).

B) Microspectrofluorometric Studies

Studies were carried out on single hepatocytes attached to a glass cover slip which was mounted in a persplex chamber and superfused with chilled (10°C) medium. This chamber was mounted on the stage of a Nikon inverted epifluorescence microscope equipped with 40x UV objective. In addition, illuminator, photometer and detection modules were equipped for isolating individual cells on the microscope stage so that the fluorescence from centralized individual hepatocytes could be monitored. The system is also equipped with a SPEX DM 3000-CM spectroscopy computer (IBM PC/AT compatible) supplied with a Cation Measurement System (Model CM1T11I) so that the instrument can be controlled automatically, the data processed, saved and printed by the computer. The rate of superfusion was approx. 2 $mL \cdot min^{-1}$ in order that the entire medium in the chamber could exchange rapidly (less than 10 sec). Fura-2-loaded hepatocytes were excited in the wavelength ranged of 320 - 400 nm using a SPEX Excitation Scan System (Model CM1T11I, Rayonics Scientific Inc.). Ca^{2+} binding by Fura-2 (i.e., from calcium-free Fura-2 to calcium-bound Fura-2) shifts the excitation spectrum about 30 nm to shorter wavelengths; i.e., from 362 to 335 nm. The highest dynamic range for Ca^{2+} measurement is obtained by comparing the ratio of maximum chelator fluorescence at 500 to 510 nm with excitations near 350 and 380 nm. The change in fluorescence (F) at these two wavelengths is opposite; thus a rise in F_{350} is

accompanied by a lowering at F_{380} with Ca^{2+} binding so that the ratio of excitation intensities obtained at 350 nm : 380 nm is a good measure of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Fura-2 fluorescence, which was monitored by a photomultiplier tube, was determined at excitation wavelengths of 350 nm and 380 nm with emission centred at 505 nm. Excitation beams were alternated at a frequency of 4 Hz using a computer controlled dual-beam fluorescence spectrometer (Model CM1T11I Cation Measurement System; SPEX Industries). The fluorescence spectra of 5 to 10 Fura-2-unloaded cells were also subjected to excitation and emission analyses at these two wavelengths with the average value as an estimate of non-specific fluorescence (autofluorescence). Autofluorescence was not affected in these cells by the superfusion conditions or changes in these conditions in my study. To provide an absolute measure of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$), the fluorescence ratio (the ratio of fluorescence at 350 and 380 nm) was determined after the non-specific fluorescence was subtracted from the fluorescence of the Fura-2-loaded cells at the corresponding excitation wavelengths (Grynkiewicz et al., 1985). The cells were kept for about 6 hrs after loading, there being no obvious difference in Ca^{2+} response over this time period.

C) Data Analysis

The fluorescence ratio (R) was converted to cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \cdot \beta \cdot [(R - Rmin)/(Rmax - R)]$$

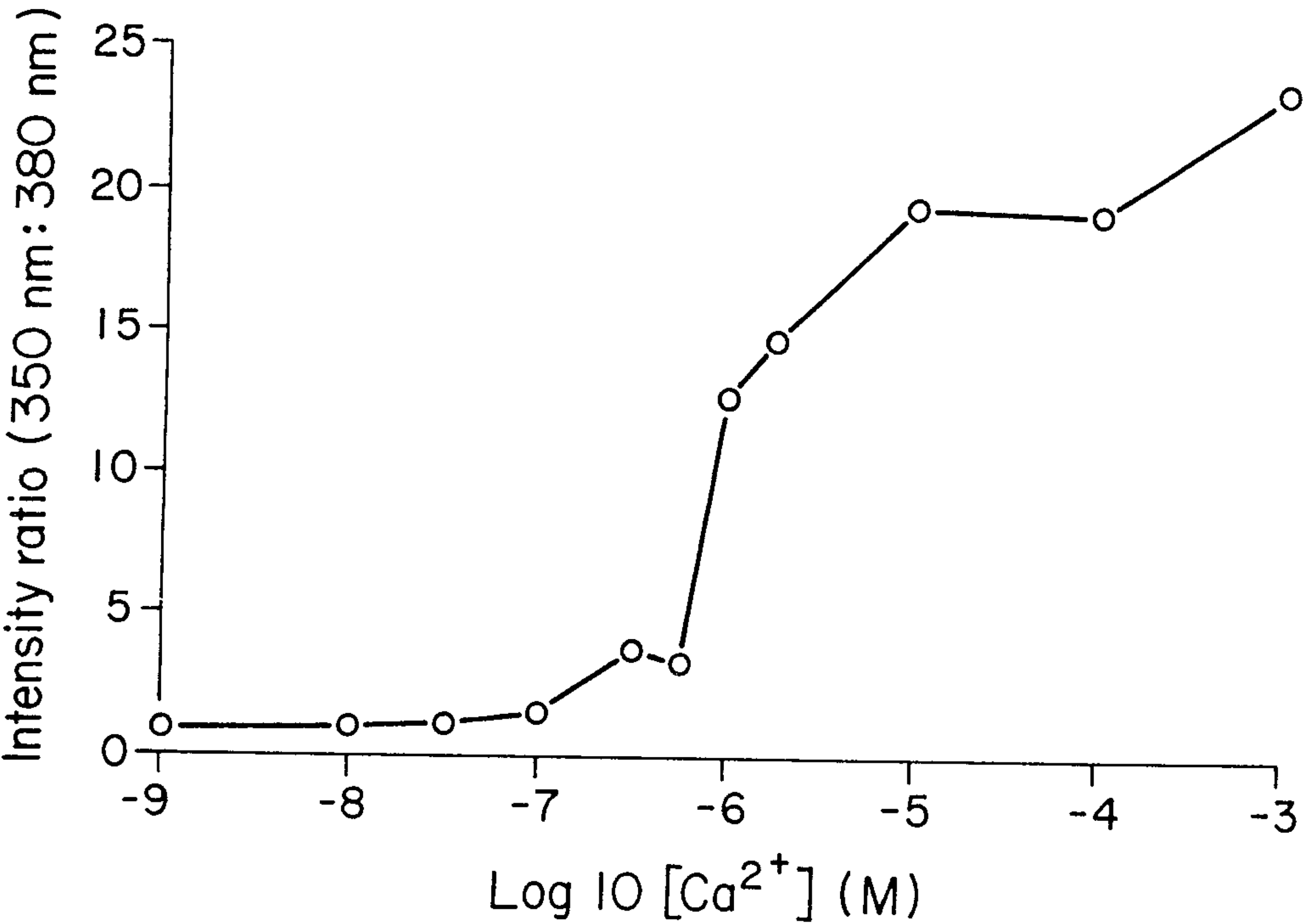
Where R is the fluorescence ratio (ratio of fluorescence at 350 nm and 380 nm) obtained

for the particular experiment. R_{min} and R_{max} represent these ratios for Fura-2 in nominally zero Ca^{2+} and in the presence of saturating Ca^{2+}, respectively. These were obtained by in vivo calibration as described below. K_d is the dissociation constant of Fura-2. β is the ratio of fluorescence of Fura-2 at 380 nm in zero and in saturating Ca^{2+} solution. $K_d \cdot \beta$ is the apparent dissociation constant of Fura-2, which was obtained from in vitro calibration as noted below. All data were processed using the Asyst program (Asyst technologies) and an IBM-compatible computer. Ratio data here were digitally smoothed at a cut-off frequency of 0.2 Hz before analysis.

D) Calibration of Fura-2

To measure $[Ca^{2+}]_i$ using Fura-2, it is essential to calibrate the Fura-2 fluorescence signal and relate it to free Ca^{2+} concentration. In this study, in vitro calibration (without hepatocytes) was accomplished by measuring the fluorescence ratio (350 nm : 380 nm) through the microscope optics in calcium-EGTA buffers containing 3 μM Fura-2 and a range of free $[Ca^{2+}]$ from 10^{-9} to 10^{-3}M (Medium E, see Table 2-2; solutions were prepared according to Fabiato, 1988). A standard curve relating the fluorescence ratio values to free Ca^{2+} concentrations can then be plotted (Fig. 2-2). From this curve, apparent K_d (i.e., $K_d \cdot \beta$) was determined to be 1.07×10^{-6}M. To ensure a precise measurement of $[Ca^{2+}]_i$, R_{min} and R_{max} were determined from in vivo calibration (Scanlon et al., 1987). In vivo calibration (with hepatocytes) was performed by using ionomycin (6 μM) permeabilized cells in the presence of either a Ca^{2+}-free buffer (Medium F, Table 2-2) (R_{min}) or a 2 mM Ca^{2+} buffer (Medium G, Table 2-2) (R_{max}). Ionomycin is a Ca^{2+}-

Fig. 2-2: Fura-2 in vitro calibration curve. The fluorescence ratio (350 nm : 380 nm) was estimated through the microscope optics without hepatocytes in Ca^{2+}-EGTA buffers containing 3 μM Fura-2 and a range of free [Ca^{2+}] (Medium E, see Table 2-2). The resulting values are plotted as the fluorescence ratio against free Ca^{2+} concentrations. The apparent K_d (i.e., $K_d \cdot \beta$), 1.07 x 10^{-6}M, was determined after this curve regression (i.e., the free Ca^{2+} concentration needed to achieve half maximal response).


Intensity ratio (350 nm : 380 nm)
25
20
15
10
5
0
-9
-8
-7
-6
-5
-4
-3
Log 10 [Ca2+] (M)

ionophore which allows the equilibration of intra- and extracellular $[Ca^{2+}]$s (Liu and Hermann, 1978). R_{min} and R_{max} values between the three fish species were not significantly different, so an average value of R_{min} and R_{max} was used for the conversion of fluorescence ratios to $[Ca^{2+}]_i$ from experiments with eel, bullhead and trout hepatocytes. The average values were: R_{min}, 0.925 ± 0.102 (n = 29); R_{max}, 5.5 ± 0.375 (n = 27).

VII. Chemicals

Catecholamines, alpha- and beta-adrenergic agonists and antagonists, metabolic inhibitors, collagenase, digitonin and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO) or Boehringer-Mannheim (Lachine, PQ). ^{45}Ca was purchased from NEN Canada Ltd. Fura-2/AM was obtained from Molecular Probes, Inc. (Eugene, Ore). All other reagents were obtained from local supplies and were of the highest available purity. All alpha- and beta-adrenergic agonists and antagonists were made as stock solutions at 10^{-4} to 10^{-2}M in physiological saline (Medium C, Table 2-1). When the stock solution were frozen for later use, 0.4% ascorbic acid was added as an antioxidant; epinephrine stock was made fresh daily; Fura-2/AM stock was made at 1 mM in undiluted dimethyl sulfoxide (DMSO) with 0.02% of pluronic F-127. When epinephrine or Fura-2 were used, wrapped containers and dark experimental conditions were necessary to reduce photodegradation of these compounds.

VIII. Statistics

Comparisons were made by first determining the significant difference between two groups or among three or more groups by one-way or two-way ANOVA (analysis of variance) (Zar, 1974). To find difference amongst groups, Student's t-test were performed; a $P < 0.05$ was considered significant and $P < 0.01$ as highly significant. All values are expressed as the mean ± SE of N number of independent experiments.

CHAPTER 3:

RESULTS

RESULTS

I. Calcium Status in Fish Hepatocytes

A) Total Calcium Content of Fish Hepatocytes

Using the centrifugation procedures outlined and ^{14}C-polyethylene glycol as an extracellular marker, it was determined that 0.11 ± 0.005% (n = 6) of the Ca^{2+} in the incubate was trapped within the hepatocyte pellet. Since the [Ca^{2+}] of the incubation (i.e., [Ca^{2+}] in Medium C) was 2 mM, 2.2 ± 0.1 µM Ca^{2+} was trapped and this value was subtracted before calculating the total calcium concentration of the hepatocytes. Table 3-1 presents the total Ca content of eel and bullhead hepatocytes, expressed as $\mu moles \cdot g^{-1}$ cell wet wt. In eel hepatocytes, the calcium content was 8.52 ± 1.03 $\mu moles \cdot g^{-1}$ wet wt. (n = 12), and that of the bullhead hepatocytes was 10.49 ± 1.26 $\mu moles \cdot g^{-1}$ wet wt. (n = 8).

B) Calcium Adhering to the Hepatocyte Membrane

To assess the amount of Ca^{2+} adhering to the cell membrane, "coated" and "naked" hepatocytes were prepared by using different rinsing solutions. Based on Borle (1968), two rinsing solutions were used: Medium A (Table 2-1) was used as a rinsing solution to prepare "coated" cell; and, Medium A + 0.2% trypsin + 1 mM EGTA for the "naked" cell preparation. Hepatocytes were exposed to these rinsing solutions for 10 min before centrifugation (3000 rpm; 1 min) followed by the estimation of total [Ca^{2+}] using

Table 3-1: The calcium status of fish hepatocytes.

Contents and Units		Eel	Bullhead	Rainbow trout
Total calcium content[1]	μmoles·g^{-1} wet wt	8.25 ± 1.03 (n = 12)	10.49 ± 1.26 (n = 8)	ND
Calcium adhering to the hepatocyte membrane	Amount adhering μmoles·g^{-1}	ND	1.88[2] (n = 5)	ND
	% of total calcium	ND	20%[2] (n = 5)	ND
Cytosolic free calcium concentration[3]	nM	79.6 ± 14.6 (n = 16) (m = 109)	183.9 ± 23.0 (n = 19) (m = 88)	75.7 ± 17.4 (n = 6) (m = 27)

[1] Eels were not fed while bullheads and trout were fed daily. Total Ca^{2+} contents were estimated by atomic absorption spectrophotometer. The cells were pre-incubated in Medium C (Table 2-1) for 1.5 hr in 10°C waterbath, at time 0, the cells were separated from the external Ca^{2+} by centrifuging cells through a 40-fold dilution of isotonic methyl-glucamine solution (Medium D, Table 2-1). Ca^{2+} trapped within the hepatocyte pellet was measured using ^{14}C-polyethylere glycol; this value was subtracted.

[2] Ca^{2+} adhering to the hepatocyte membrane was obtained by measuring the difference in Ca^{2+} content of "coated" cells (Medium A as rinsing solution) and "naked" cells (0.2% trypsin + 1 mM EGTA in Medium A as rinsing solution). The trypsin-EGTA rinsing solution was not suitable for eel cells.

[3] Cytosolic free-Ca^{2+} concentrations were measured on single Fura-2-loaded hepatocytes by microspectrofluorimeter. The hepatocytes were superfused with chilled (10°C) Medium C (less BSA, Table 2-1) and $[Ca^{2+}]_i$ determined as stated in the Materials and Methods. The cytosolic free $[Ca^{2+}]_i$ of eel and trout are different from that of bullhead ($p < 0.01$, ANOVA). m = cells studied; n = preparations.

atomic absorption spectrometer. The procedures were basically the same as those described for total calcium content except for the rinsing solutions and the exposure time of the cells to these rinsing solutions. The difference between total [Ca] of "coated" and "naked" cells would provide an estimate of membrane adhering Ca^{2+}. The results with bullhead hepatocytes indicated that total Ca content in "coated" cells was 9.42 ± 1.47 $\mu moles \cdot g^{-1}$ wet wt., while in "naked" cells it was 7.54 ± 1.55 $\mu moles \cdot g^{-1}$ wet wt. (n = 5); the difference, 1.88 $\mu moles \cdot g^{-1}$ (Table 3-1) is the amount of Ca^{2+} adhering to the cell membranes. This amount accounts for 20% of total Ca^{2+} content measured in the bullhead hepatocytes.

The percentage of membrane adhering Ca^{2+} which is part of the exchangeable Ca^{2+} pool was also investigated by using ^{45}Ca as a marker. Bullhead hepatocytes were pre-incubated in Medium C (Table 2-1) in a 10°C shaking waterbath for 1.5 hr for Ca^{2+} equilibrium. At time 0, ^{45}Ca was added at a final concentration of 1 $\mu Ci \cdot mL^{-1}$ incubate. The cells were incubated for another 1 hr for ^{45}Ca loading. At the end, "coated" and "naked" cells were prepared as described above and ^{45}Ca radioactivity was counted according to the methods described in the Ca^{2+} uptake experiments (see II.A). As presented in Table 3-2, the amount of Ca^{2+} uptake in 1 hr by "coated" cells was 428.2 ± 28 $nmoles \cdot g^{-1}$ wet wt. while in "naked" cells it was 132.8 ± 28.8 $nmoles \cdot g^{-1}$ wet wt. (n = 5). These values were statistically different ($p < 0.01$). This difference, 295.4 $nmoles \cdot g^{-1}$ wet wt., represents 69% of the exchangeable Ca^{2+} adhering to the bullhead hepatocyte membranes. Thus, the hepatocyte membrane adhering Ca^{2+} accounts for 20% of total calcium content or 69% of total exchangeable Ca^{2+}, suggesting that Ca^{2+} adhering

Table 3-2: Estimate the importance of membrane adhering Ca^{2+} in Ca^{2+} exchange across fish hepatocyte membranes.

Contents and Units		Eel (n = 6)	Bullhead (n = 5)
EGTA rinsing method	Coated cell Ca^{2+} uptake ($nmoles \cdot g^{-1}$)	212.9 ± 17.1	302.4 ± 25.4
	Naked cell Ca^{2+} uptake ($nmoles \cdot g^{-1}$)	158.4 ± 9.2 *	217.9 ± 12.8 *
	Cell coat Ca^{2+} ($nmoles \cdot g^{-1}$)	54.5	84.5
	Ca^{2+} adhering to cell membrane (% of exchangeable Ca^{2+})	25.6	27.9
Trypsin EGTA rinsing method	Coated cell Ca^{2+} uptake ($nmoles \cdot g^{-1}$)	ND	428.2 ± 28
	Naked cell Ca^{2+} uptake ($nmoles \cdot g^{-1}$)		132.8 ± 28.2 **
	Cell coat Ca^{2+} ($nmoles \cdot g^{-1}$)		295.4
	Ca^{2+} adhering to cell membrane (% of exchangeable Ca^{2+})		69

Eel and bullhead hepatocytes were pre-incubated in Medium C (Table 2-1) for 1.5 hr and ^{45}Ca was then added to give a final concentration of 1 $\mu Ci \cdot mL^{-1}$. ^{45}Ca uptake was estimated in "coated" cells and "naked" cells after a 1 hr incubation at 10°C (cells were prepared using either 5 mM EGTA rinsing solution or trypsin-EGTA rinsing solution). * $p < 0.05$, ** $p < 0.01$ significantly different from "coat" cell uptake by a Student's t-test.

to the membrane plays an important role in the Ca^{2+} status of the cells in these studies.

These rinsing solutions were unsuitable for eel hepatocytes since 0.2% trypsin killed the cells. As an alternative, an isotonic solution consisting of 130 mM NaCl + 5 mM HEPES, pH 7.8 was used to prepare "coated" cells and 130 mM NaCl + 5 mM HEPES + 5 mM EGTA to prepare "naked" cells. The hepatocytes were exposed to the rinsing solutions and immediately centrifuged (3000 rpm; 1 min). The results (Table 3-2) indicated that 25.6% of the exchangeable Ca^{2+} was removed by 5 mM EGTA. To compare the two methods, this second method (5 mM EGTA rinsing method) was also applied to the bullhead hepatocytes; 27.9% of exchangeable Ca^{2+} of bullhead hepatocytes was removed by 5 mM EGTA. The value is similar to that for the eel hepatocytes, but well below that using the first method.

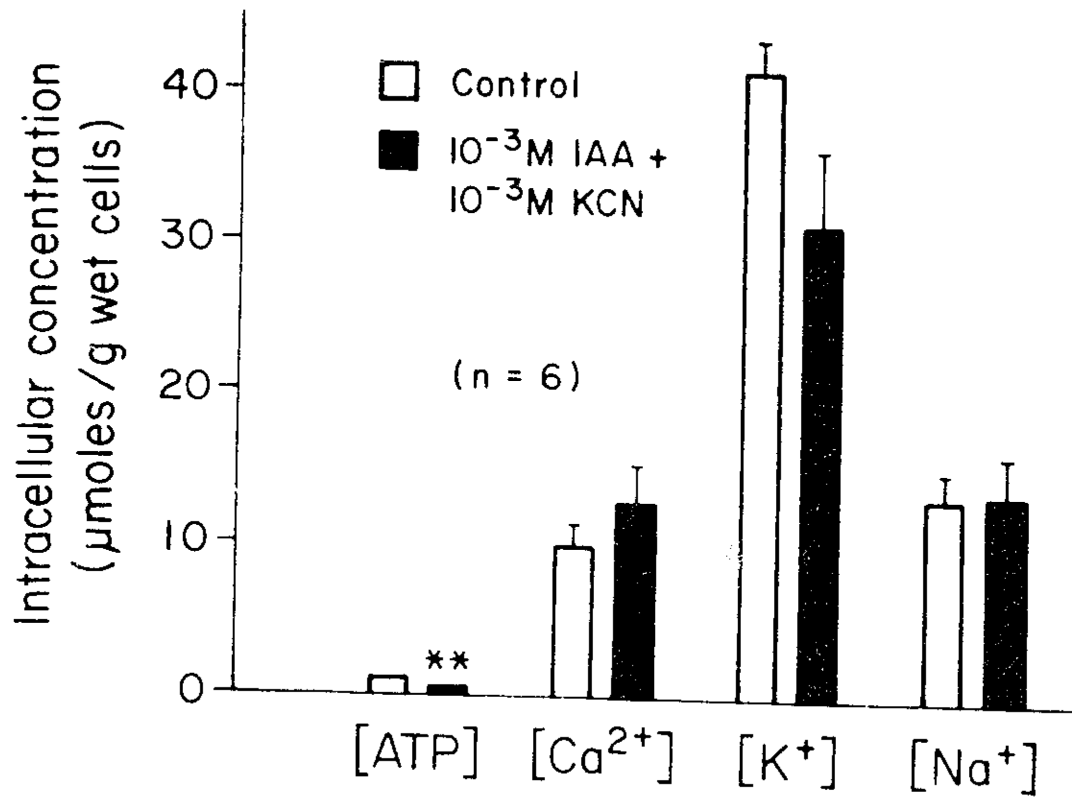
C) Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_i$)

Cytosolic free Ca^{2+} concentration was measured on Fura-2-loaded single hepatocytes by microspectrofluorimeter (see Materials and Methods) and using Medium C (less BSA, Table 2-1) as a superfusion solution. Table 3-1 indicates that eel and rainbow trout hepatocytes had similar free $[Ca^{2+}]_i$; i.e., 79.6 ± 14.6 nM (n = 16) and 75.7 ± 17.4 nM (n = 6), respectively. Values for bullhead hepatocytes were 183.9 ± 23 nM (n = 19), significantly higher ($p < 0.01$) than that of eel or trout cells.

D) Influence of Metabolic Inhibitors on Cytosolic [ATP], [Na^+], [K^+] and [Ca^{2+}] of Eel Hepatocytes

Eel hepatocytes were pre-incubated in Medium C for 1.5 hr, divided into two batches, and the metabolic inhibitors iodoacetatic acid (IAA) and potassium cyanide (KCN) were added to a final concentration of 10^{-3}M to one batch as a experimental group or an equal volume of Medium C to the second batch as a control group. The incubation continued for another 45 min before sampling; this time period was chosen as it corresponded exactly to the period used in Ca^{2+} flux studies. Energy content ([ATP]), energy-dependent cation transport ([Na^+], [K^+]), and total [Ca^{2+}] were estimated in both batches of cells. [Na^+], [K^+] and [Ca^{2+}] were all measured by atomic absorption spectrometer in the same manner reported for calcium content in the Materials and Methods. The concentrations of Na^+, K^+ and Ca^{2+} in Medium C were 130 mM, 4.35 mM and 2 mM, respectively. As noted previously, 0.11% of the ^{14}C was trapped in the hepatocyte pellet; if a similar amount of these cations were also trapped, this would mean, 140 μM, 4.7 μM and 2.2 μM, respectively. These values were subtracted as contamination. The very high extracellular sodium contamination probably masked any differences in cytosolic [Na^+] between the control and the metabolic inhibitor groups (Fig. 3-1), [K^+] decreased 25% (from 41.4 ± 2.0 to 31.2 ± 5.0 $\mu moles \cdot g^{-1}$ wet wt.), which suggested that the metabolic inhibitors may have slowed active Na^+_{out} : K^+_{in} exchange. [Ca^{2+}] did not change much (from 10.0 ± 1.3 to 12.8 ± 2.4 $\mu moles \cdot g^{-1}$ wet wt.). [ATP], analyzed according to Bergmeyer (1974), significantly decreased from 1.1 ± 0.1 to 0.62 ± 0.05 $\mu moles \cdot g^{-1}$ wet wt. ($p < 0.01$), which indicated that total cellular energy decreased

Fig. 3-1: Effects of the metabolic inhibitors on [ATP], [Ca^{2+}], [K^+] and [Na^+] of eel hepatocytes. Hepatocytes were pre-incubated in Medium C at 10°C for 1.5 hr; the metabolic inhibitors iodoacetatic acid (IAA) and potassium cyanide (KCN) were added to give 10^{-3}M or an equivalent volume of Medium C was added to control. The cells were incubated for another 45 min, then a 1 mL aliquot was removed and washed in 40 mL isotonic methyl-glucamine solution (Medium D, Table 2-1) by centrifugation at 3000 rpm for 1 min. Deionized water (2 mL) was added to the pellet, vortexed and burst using an ultrasonic probe. Aliquots were used for [Ca^{2+}], [K^+] and [Na^+] assays with an atomic absorption spectrophotometer. [ATP] was analyzed according to Bergmeyer (1974). Values are expressed as μmoles·g^{-1} cell wt ($n = 6$). ** $p < 0.01$ significantly different from control value.


Intracellular concentration
(μmoles/g wet cells)
40
30
20
10
0
Control
10⁻³M IAA +
10⁻³M KCN
(n = 6)
**
[ATP]
[Ca2+]
[K+]
[Na+]

with the inhibitors, but this decrease did not significantly modify any cation pattern.

II. ^{45}Ca Flux Studies

A) ^{45}Ca Uptake (Influx)

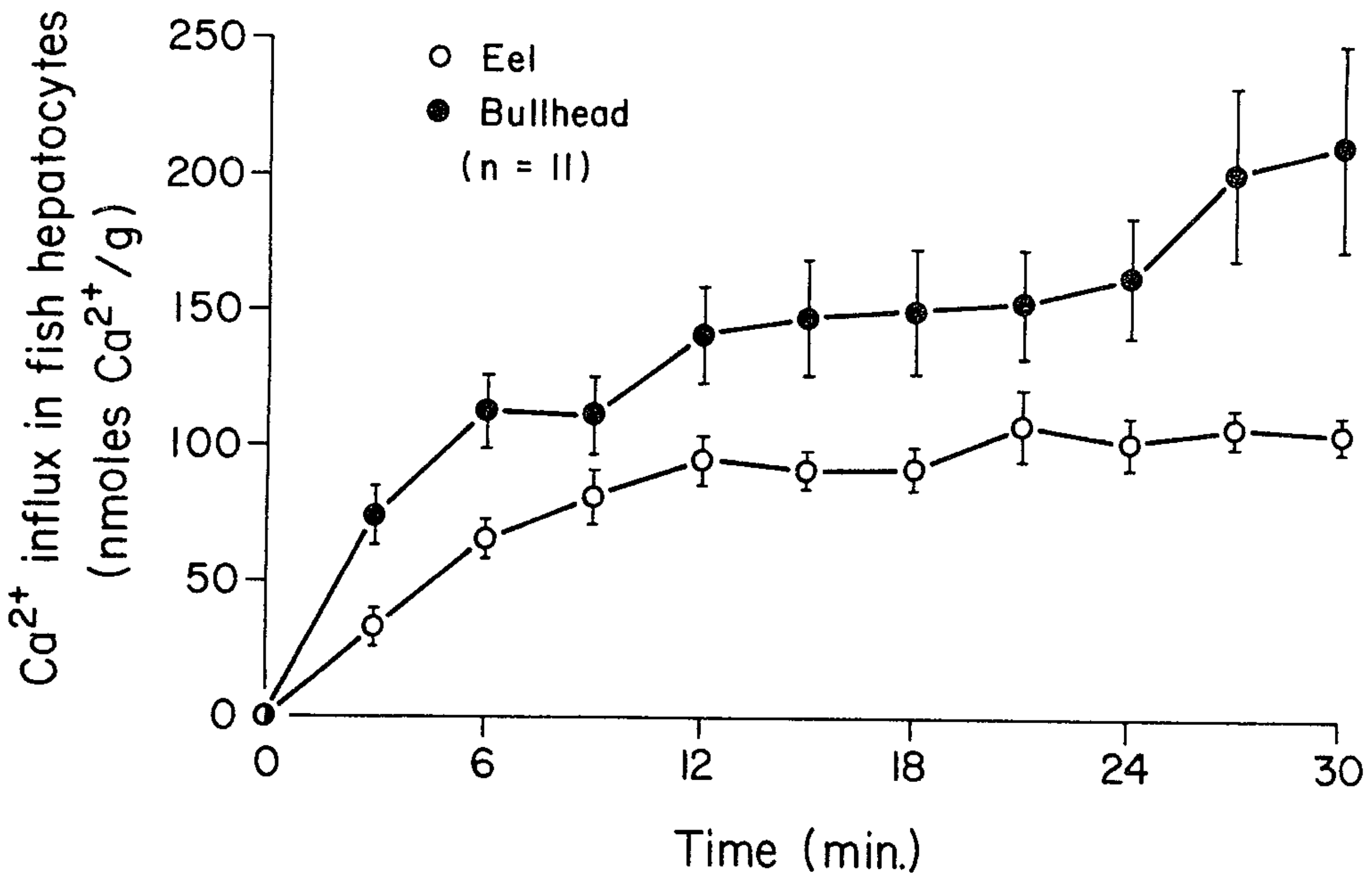
(1) The shape of Ca^{2+} uptake curves in fish hepatocytes

Fig. 3-2 shows time course curves of Ca^{2+} uptake in both eel and bullhead hepatocytes under normal conditions. The Ca^{2+} uptake curve is hyperbolic, so that during the first 6 to 9 min, the rate of Ca^{2+} uptake was rapid, after which the rate slowed to a plateau. ^{45}Ca flux from 12 to 30 min, achieved equilibrium across the cell membrane. For this reason, 30 min was chosen as a time period in the Ca^{2+} uptake studies of fish hepatocytes. In addition, when hormonal effects were studied, Ca^{2+} uptake at 6 min showed the greatest difference between control group and experimental groups; therefore, 6 min Ca^{2+} uptake results were selected for comparative studies. When basal Ca^{2+} uptake was considered, Ca^{2+} uptake in bullhead hepatocytes was about 2-times that in eel hepatocytes although each showed a similar hyperbolic Ca^{2+} uptake curve (Fig. 3-2).

(2) Influence of metabolic inhibitors on Ca^{2+} uptake in fish hepatocytes

The energy dependence of Ca^{2+} uptake was estimated by examining the effects of metabolic inhibitors on Ca^{2+} uptake in eel (Fig. 3-3A) and in bullhead (Fig. 3-4A) hepatocytes. The inhibitors (IAA + KCN, final concentration 10^{-3}M) were added 15 min before sampling commenced and samples were taken at 3 min intervals for 30 min. These

Fig. 3-2: Ca^{2+} uptake in fish hepatocytes under normal conditions. Isolated eel and bullhead hepatocytes were pre-incubated, incubated in 1 μCi $^{45}Ca \cdot mL^{-1}$, sampled between 0 to 30 min at 3 min intervals, rinsed and burst as noted in Fig. 2-1. Values are expressed as nmoles $Ca^{2+} \cdot g^{-1}$ wet wt (n = 11). Ca^{2+} uptake in eel cells significantly differed from that in bullhead cells by two-way ANOVA.



Eel
Bullhead
(n = 11)
Ca^{2+} influx in fish hepatocytes (nmoles Ca^{2+}/g)
0
50
100
150
200
250
0
6
12
18
24
30
Time (min.)

Fig. 3-3: Effects of metabolic inhibitors on Ca^{2+} uptake (panel A) and efflux (panel B) in eel hepatocytes. Hepatocytes were suspended in Medium C, pre-incubated at 10°C for 1.5 hr, followed by the addition of ^{45}Ca to give a final radioactivity of approx. 1 $\mu Ci \cdot mL^{-1}$ (uptake) and 2 $\mu Ci \cdot mL^{-1}$ (efflux) respectively. Samples for Ca^{2+} uptake were taken immediately after ^{45}Ca addition, while samples for Ca^{2+} efflux were taken following a 1 hr ^{45}Ca loading and resuspending the cells in Medium C. Metabolic inhibitors iodoacetatic acid (IAA) and potassium cyanide (KCN) or an equal volume of saline (control) were added 15 min before the sampling began, and sampling was identical to other uptake and efflux experiment. Ca^{2+} uptake values are expressed as nmoles $Ca^{2+} \cdot g^{-1}$ wet cell wt (n = 6), and Ca^{2+} efflux as the ratio of Ca^{2+} remaining in cells relative to 0 min (n = 5).

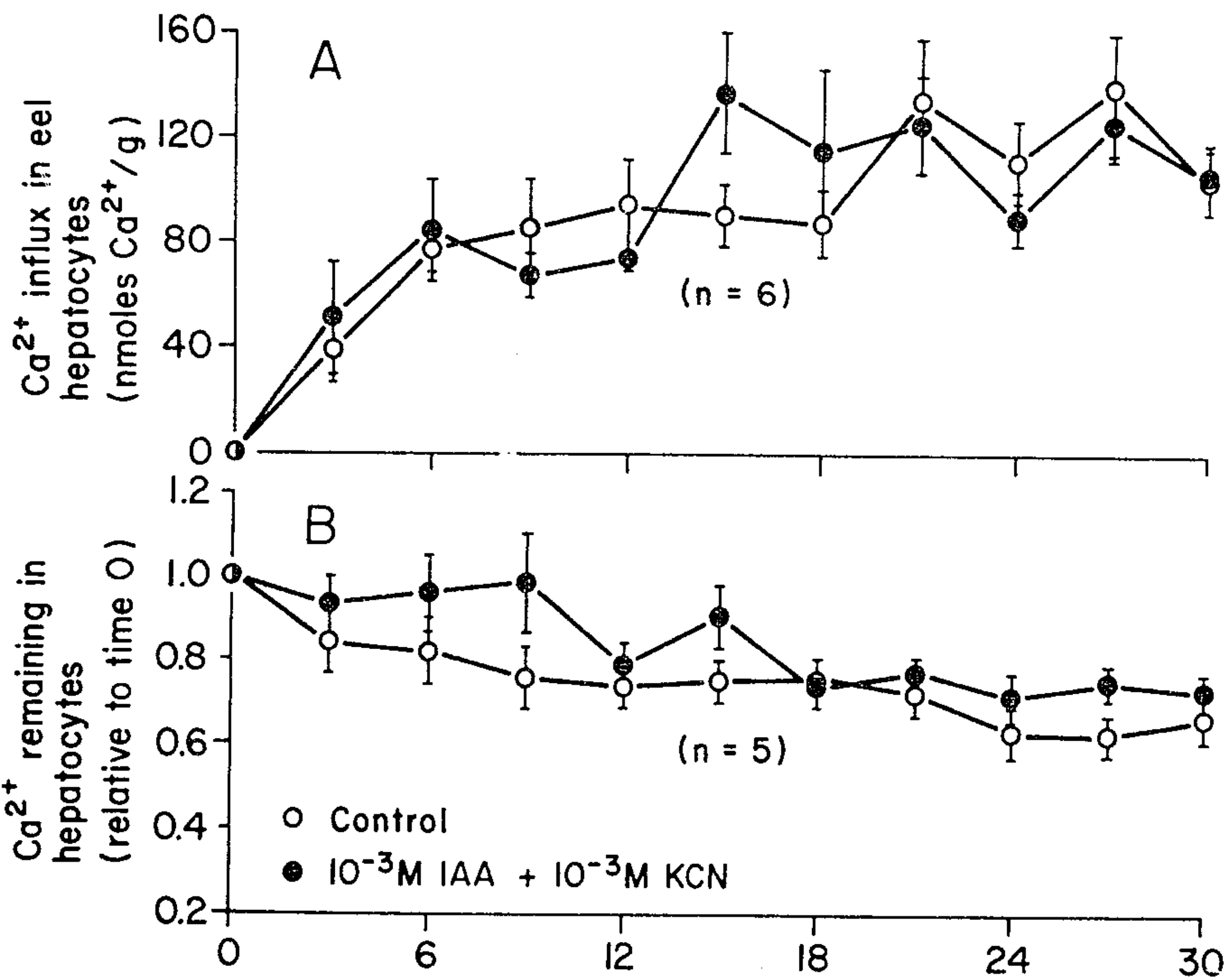
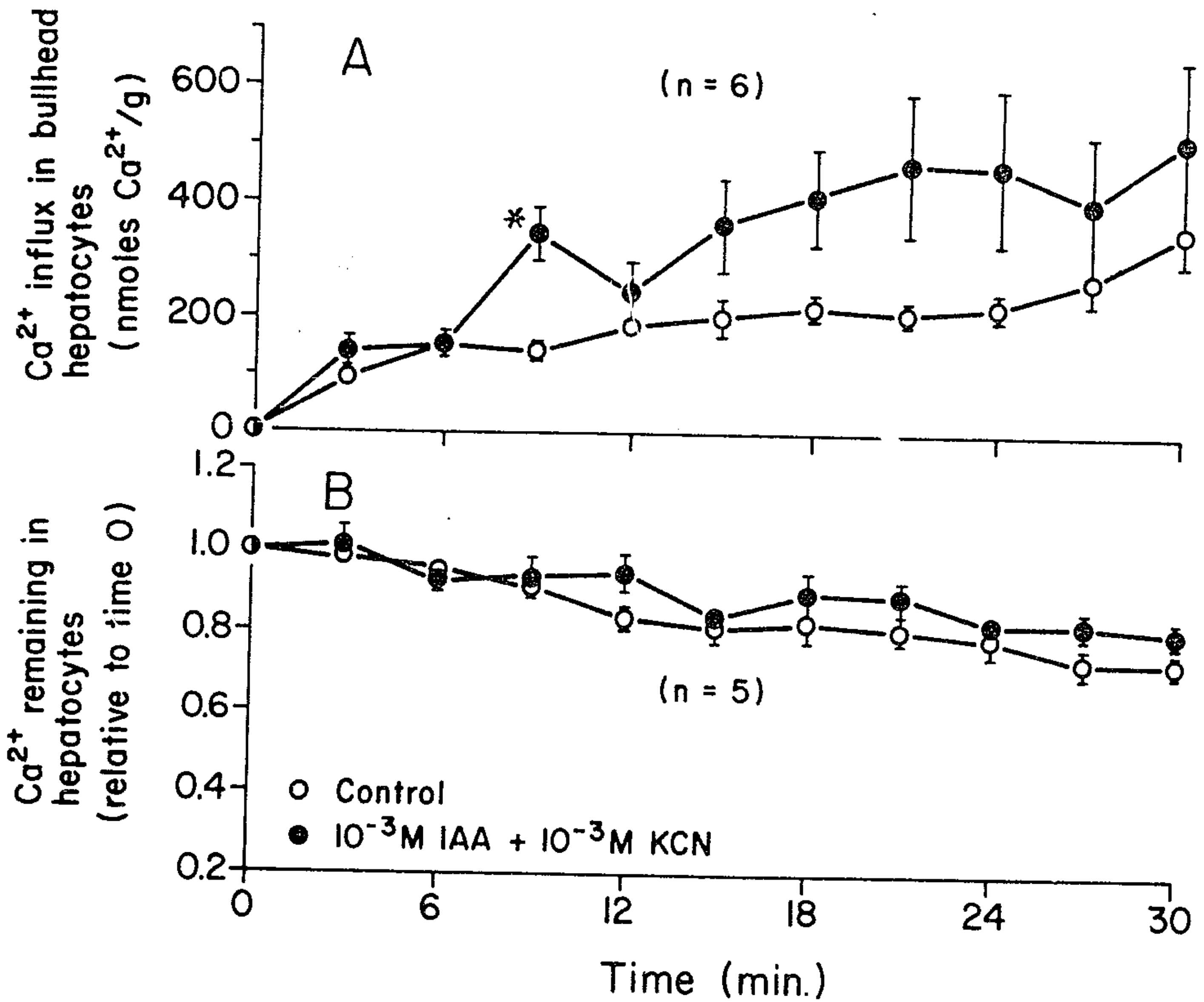

A
B
Ca²⁺ influx in eel hepatocytes (nmoles Ca²⁺/g)
Ca²⁺ remaining in hepatocytes (relative to time 0)
(n = 6)
(n = 5)
Control
10⁻³M IAA + 10⁻³M KCN
Time (min.)
0
6
12
18
24
30
160
120
80
40
0
1.2
1.0
0.8
0.6
0.4
0.2

Fig. 3-4: Effects of metabolic inhibitors on Ca^{2+} uptake (panel A) and efflux (panel B) in bullhead hepatocytes. Conditions as in Fig. 3-3. * $p < 0.05$ significantly different from control value at the same time by a Student's t-test. n = independent determinations.


A
(n = 6)
600
400
200
0
Ca^{2+} influx in bullhead hepatocytes (nmoles Ca^{2+}/g)
*
B
1.2
1.0
0.8
0.6
0.4
0.2
Ca^{2+} remaining in hepatocytes (relative to time 0)
(n = 5)
○ Control
● 10^{-3}M IAA + 10^{-3}M KCN
0
6
12
18
24
30
Time (min.)

metabolic inhibitors did not affect Ca^{2+} uptake in eel hepatocytes, but generally stimulated Ca^{2+} uptake in bullhead hepatocytes although only one point (9 min) was statistically different ($p < 0.05$).

(3) Effect of epinephrine and phenylephrine on Ca^{2+} uptake in fish hepatocytes

Epinephrine (E, 10^{-5}M) and phenylephrine (PE, 10^{-5}M) when added to hepatocyte incubations at time 0, inclined to increase Ca^{2+} uptake but the effect was not statistically significant when compared with the control at 6 min (Fig. 3-5). Again, as was shown in Fig. 3-2, Ca^{2+} uptake in bullhead hepatocytes was much more rapid than that in eel cells.

B) ^{45}Ca Efflux

(1) The shape of ^{45}Ca efflux curves in fish hepatocytes

The Ca^{2+} efflux time course curve is an inverted hyperbolic response, thus during the first 3 min, the rate of Ca^{2+} efflux was more rapid, but decreased thereafter (Fig. 3-6). As with the Ca^{2+} uptake studies, a 30 min time period was used for efflux studies, and the ratio of results at 6 min compared to 0 min was used for comparative purposes. Fig. 3-6 shows that Ca^{2+} efflux in bullhead hepatocytes was slower than that in eel cells, which is opposite from that of Ca^{2+} uptake (Fig. 3-2). These differences in Ca^{2+} uptake and efflux values between eel and bullhead are consistent with the results presented later (see Fig. 3-10) on the source of the mobilized Ca^{2+} in these two species.

Fig. 3-5: Effect of epinephrine (E) and phenylephrine (PE) on Ca^{2+} uptake in eel and bullhead hepatocytes. Hepatocytes were suspended in Medium C, pre-incubated at 10°C for 1.5 hr, and ^{45}Ca (final radioactivity of approx. 1 $\mu Ci \cdot mL^{-1}$) and epinephrine (10^{-5}M), phenylephrine (10^{-5}M) or saline (control) were added to begin the incubation. A 0.2 mL sample was removed from each incubation at 0 min and 6 min, washed in 8 mL of cold washing medium (Medium C) by centrifugation and disrupted as noted in other figure legends. Ca^{2+} uptake was expressed as nmoles $Ca^{2+} \cdot g^{-1}$ wet cells at 6 min after subtracting 0 min value. No statistical difference was found by ANOVA. n = independent determinations.

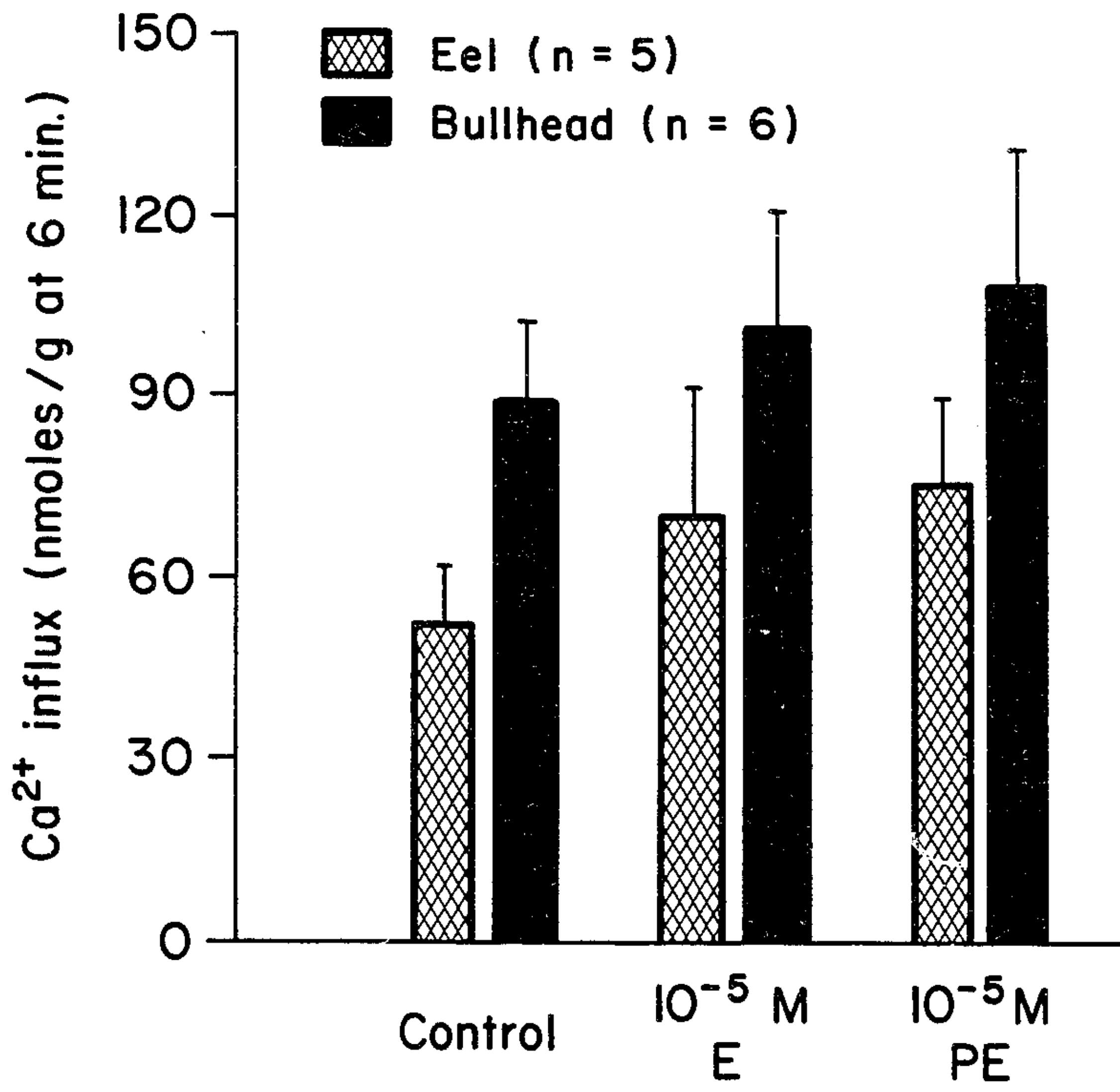
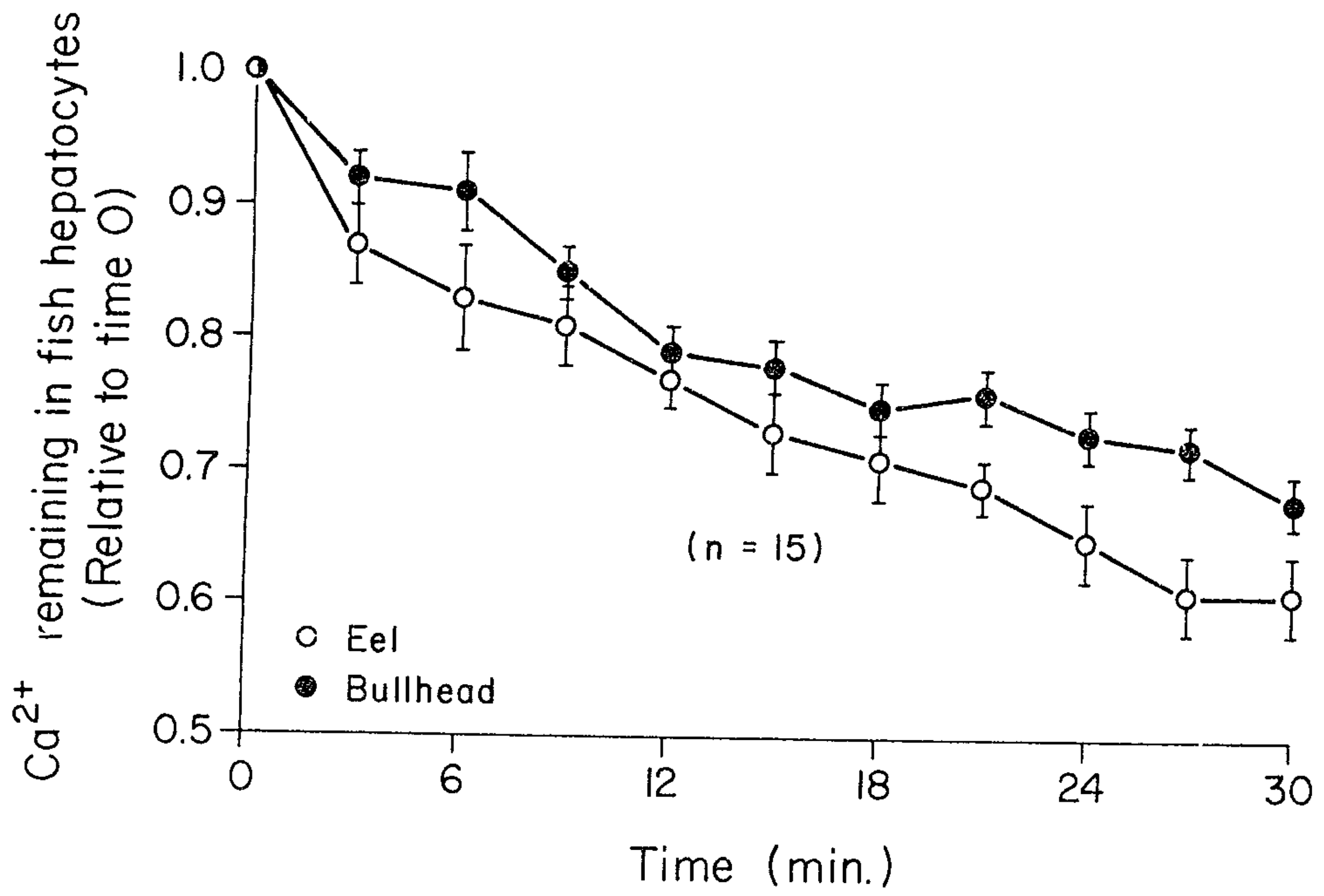

Eel (n = 5)
Bullhead (n = 6)
Ca^{2+} influx (nmoles/g at 6 min.)
150
120
90
60
30
0
Control
10^{-5} M
E
10^{-5} M
PE

Fig. 3-6: Ca^{2+} efflux in fish hepatocytes under normal conditions. Isolated hepatocytes were pre-incubated in Medium C at 10°C for 1.5 hr, ^{45}Ca was added to give 2 $\mu Ci \cdot mL^{-1}$ and loading proceeded for 1 hr. Cells were centrifuged and resuspended in the same volume of ^{45}Ca-free Medium C. Samples (0.2 mL) were removed at 3 min intervals between 0 and 30 min, and washed in 8 mL rinsing solution (Medium C) as before. The resulting pellet was disrupted in 0.6 mL deionized water, and ^{45}Ca was estimated as described previously. Values are expressed as ^{45}Ca remaining relative to 0 min (n = 15).



Ca^{2+} remaining in fish hepatocytes
(Relative to time 0)
1.0
0.9
0.8
0.7
0.6
0.5
0
6
12
18
24
30
Time (min.)
(n = 15)
Eel
Bullhead

(2) Influence of metabolic inhibitors on Ca^{2+} efflux in fish hepatocytes

The metabolic inhibitors (IAA + KCN, final concentration 10^{-3}M) had a trend to reduce Ca^{2+} efflux in fish hepatocytes (Figs. 3-3B and 3-4B) but at no time point was the effect statistically significant.

(3) Effect of α- and β-adrenergic agonists and/or antagonists on ^{45}Ca efflux in eel hepatocytes

The α-antagonist phentolamine (PHE, 10^{-5}M) and the β-antagonist propranolol (PRO, 10^{-5}M) had no effect on Ca^{2+} efflux (Fig. 3-7A) when added alone or together. The agonists, epinephrine (E, 10^{-5}M) and phenylephrine (PE, 10^{-5}M) did not cause significant hepatocyte leakage or volume change as noted by monitoring both LDH leakage and hepatocyte wet to dry weight ratio (Table 3-3). Thus, any changes seen in efflux may be interpreted as a result of changes in flux rather than changes in the condition of the hepatocytes themselves.

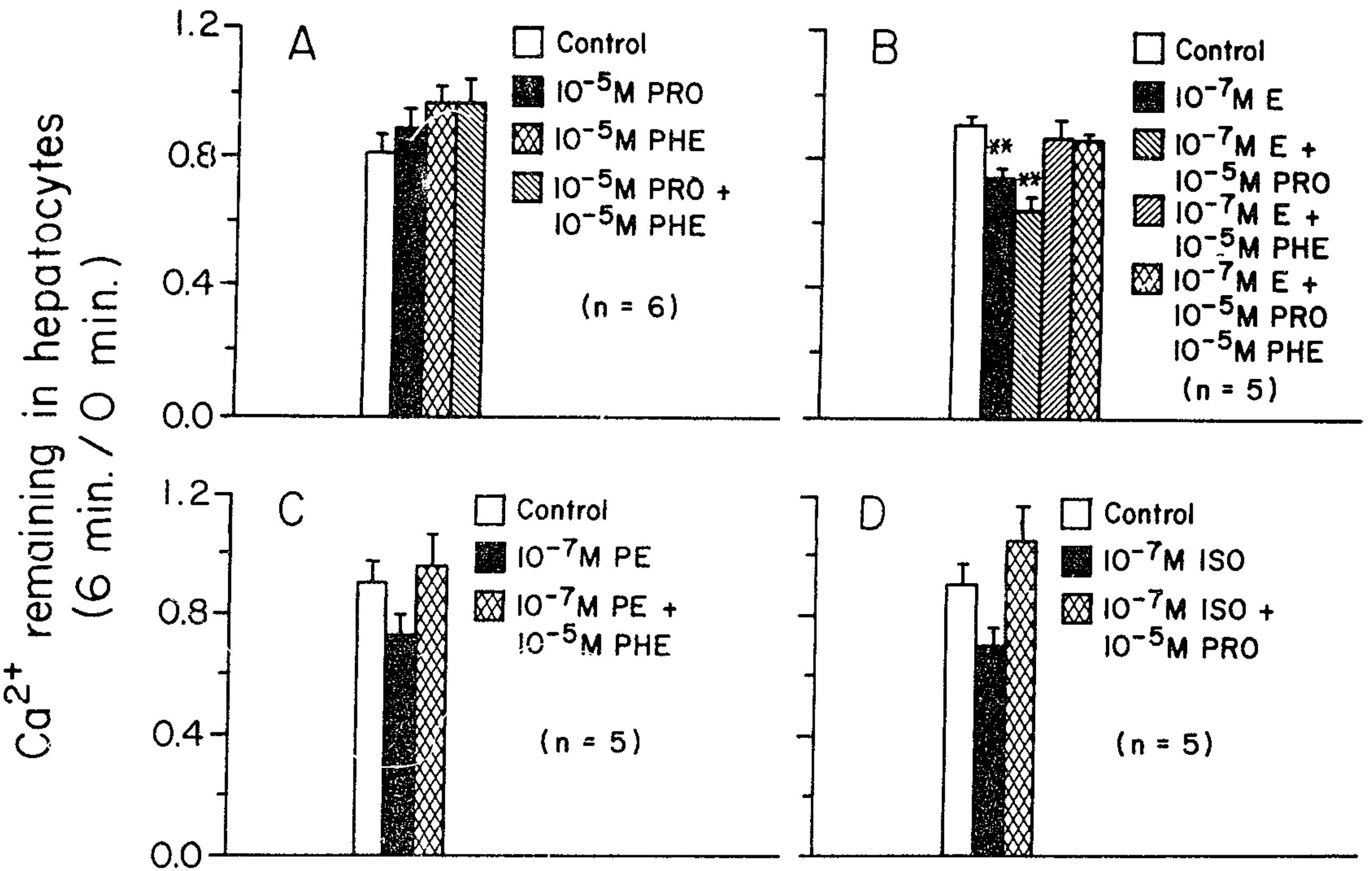
Epinephrine significantly stimulated Ca^{2+} efflux at 6 min compared to controls, and the β-antagonist propranolol did not block this effect (Fig. 3-7B; Table 3-4). To confirm this result, epinephrine concentrations at both 10^{-5}M and 10^{-7}M with a 10- or 100-fold higher concentration of the antagonist propranolol (PRO) were used (Table 3-4; Figs. 3-7B and 3-8D); results were similar under all conditions and an epinephrine dose response was noted. Phentolamine (PHE), an α-antagonist, when added at 10^{-5}M, completely abolished the effect of 10^{-7}M epinephrine on Ca^{2+} efflux, as did both antagonists when added together (Table 3-4; Fig. 3-7B). Thus, it is likely that the Ca^{2+}

Table 3-3: Study of phenylephrine and epinephrine effects on hepatocytes leakage and volume change.

Treatment	LDH rate (6 min : 0 min) (n = 6)		Hepatocytes weight ratio (wet : dry) (n = 6)
	in extracelullar medium	in hepatocytes	
Control	1.011 ± 0.128	1.083 ± 0.080	4.976 ± 0.322
10^{-5}M PE	0.883 ± 0.064	1.041 ± 0.062	5.135 ± 0.277
10^{-5}M E	1.091 ± 0.211	1.092 ± 0.040	4.938 ± 0.317

Cell leakage and cell volume change were monitored by measuring LDH leakage and hepatocyte wet to dry weight ratio, respectively in eel hepatocytes, pre-incubated in Medium C (Table 2-1) for 1.5 hr and incubated with phenylephrine (PE) or epinephrine (E) at 10^{-5}M. Lactate dehydrogenase (LDH) activity was measured in both extracellular medium and hepatocytes, and cell wet and dry (dried in an oven at 55°C until no weight change) weight were assessed at 0 min and 6 min. Values (mean ± SE) represent ratios at 6 min to 0 min. Values do not differ from controls (ANOVA).

Fig. 3-7: Effect of α- and β-adrenergic agonists (10^{-7}M) and antagonists (10^{-5}M) on Ca^{2+} efflux in eel hepatocytes. Hepatocytes were pre-incubated, ^{45}Ca-loaded as noted previously (Fig. 3-6). Epinephrine (E, α- and β-agonist), phenylephrine (PE, α-agonist) and isoproterenol (ISO, β-agonist) were added to give 10^{-7}M at the time of resuspending the loaded cells while propranolol (PRO, β-antagonist) and phentolamine (PHE, α-antagonist) were added 15 min before the end of loading at a final concentration of 10^{-5}M. Samples were prepared as previously noted. Values are expressed as the ratio of ^{45}Ca remaining in cells at 6 min relative to that at 0 min (n = determinations). ** $p < 0.01$ significantly different from the corresponding control value.



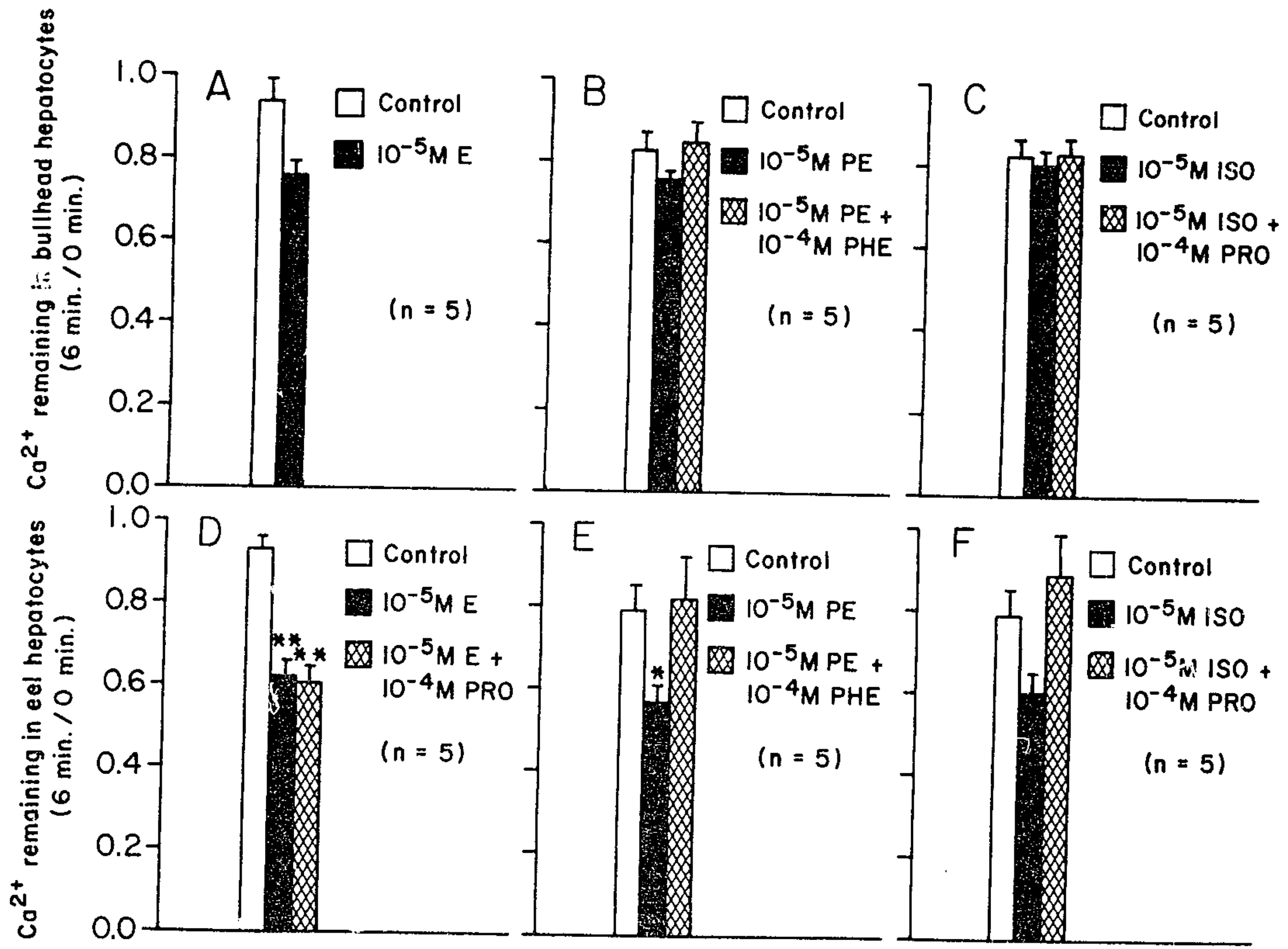
Ca2+ remaining in hepatocytes (6 min. / 0 min.)
1.2
0.8
0.4
0.0
A
Control
10-5M PRO
10-5M PHE
10-5M PRO + 10-5M PHE
(n = 6)
B
Control
10-7M E
10-7M E + 10-5M PRO
10-7M E + 10-5M PHE
10-7M E + 10-5M PRO 10-5M PHE
(n = 5)
**
**
C
Control
10-7M PE
10-7M PE + 10-5M PHE
(n = 5)
D
Control
10-7M ISO
10-7M ISO + 10-5M PRO
(n = 5)

Table 3-4: Effect of epinephrine (10^{-5}M or 10^{-7}M) in the presence and absence of antagonist(s) (10^{-4}M or 10^{-5}M) on Ca^{2+} efflux in eel hepatocytes.

Ca^{2+} remaining in cells at 6 min : 0 min		
Treatments	10^{-5}M Epinephrine with/without 10^{-4}M antagonist(s) (n = 5)	10^{-7}M Epinephrine with/without 10^{-5}M antagonist(s) (n = 6)
Control	0.929 ± 0.025	0.906 ± 0.027
Epinephrine	0.622 ± 0.034 **	0.740 ± 0.027 **
Epinephrine + Propranolol	0.606 ± 0.036 **	0.640 ± 0.039 **
Epinephrine + Phentolamine	ND	0.865 ± 0.059
Epinephrine + Propranolol + Phentolamine	ND	0.861 ± 0.022

Eel hepatocytes were pre-incubated, ^{45}Ca-loaded for 1 hr and efflux determined as noted previously (Fig. 3-6). The antagonists, propranolol and phentolamine, were added 15 min before the end of the loading to a final concentrations 10- to 100-fold that of epinephrine. Ca^{2+} efflux is expressed as the ratio of ^{45}Ca remaining in cell at 6 min to that at 0 min. ** $p < 0.01$ compared with the corresponding controls by one-way ANOVA followed by a Student's t-test.

Fig. 3-8: Effect of α- and β-adrenergic agonists (10^{-5}M) and antagonists (10^{-4}M) on Ca^{2+} efflux in eel and bullhead hepatocytes. Experiments proceeded as noted in Fig. 3-7. Values are expressed as the ratio of ^{45}Ca remaining in cells at 6 min relative to that at 0 min (n = determinations). * $p < 0.05$, ** $p < 0.01$ significantly different from the corresponding control value.



Ca2+ remaining in bullhead hepatocytes (6 min. / 0 min.)
1.0
0.8
0.6
0.4
0.2
0.0
A
Control
10-5M E
(n = 5)
B
Control
10-5M PE
10-5M PE + 10-4M PHE
(n = 5)
C
Control
10-5M ISO
10-5M ISO + 10-4M PRO
(n = 5)
Ca2+ remaining in eel hepatocytes (6 min. / 0 min.)
D
Control
10-5M E
10-5M E + 10-4M PRO
(n = 5)
E
Control
10-5M PE
10-5M PE + 10-4M PHE
(n = 5)
F
Control
10-5M ISO
10-5M ISO + 10-4M PRO
(n = 5)

efflux pathway is mediated in some way by a pathway sensitive to a classic mammalian α-adrenergic antagonist (PHE) rather than a β-adrenergic antagonist (PRO). The fact that epinephrine significantly stimulated Ca^{2+} efflux but not Ca^{2+} uptake may be related to the observation that epinephrine significantly increased cytosolic free Ca^{2+} concentration (see Fig. 3-16), and the direct relationship which has been reported between $[Ca^{2+}]_i$ and Ca^{2+} efflux (Freudenrich and Borle, 1988; Studer and Ganas, 1989) and the inverse relationship between $[Ca^{2+}]_i$ and Ca^{2+} uptake (Poggioli et al., 1985).

The α-agonist phenylephrine (PE, 10^{-5}M) also significantly stimulated Ca^{2+} efflux, and this effect was blocked by the α-antagonist phentolamine at 10^{-4}M (Table 3-5; Fig. 3-8E). Lower concentrations of phenylephrine (10^{-7}M) also inclined to stimulate Ca^{2+} efflux (Table 3-5; Fig. 3-7C), although not statistically significant. It must be noted, however, that the classification of phenylephrine as an α-adrenoreceptor agonist in fish liver has been questioned by Moon and Mommsen (1990).

The β-agonist isoproterenol (ISO) at either 10^{-7}M or 10^{-5}M (Table 3-6; Figs. 3-7D and 3-8F) had a trend to increase Ca^{2+} efflux too, but the effect was not statistically significant; these increases were blocked by propranolol (Table 3-6; Figs. 3-7D and 3-8F). These data provide further evidence for the apparent absence of a β-adrenergic control of Ca^{2+} efflux in eel hepatocytes.

(4) Effect of α- and β-adrenergic agonists and antagonists on ^{45}Ca efflux in bullhead hepatocytes

Comparatively, Ca^{2+} efflux in bullhead hepatocytes (Table 3-7; Fig. 3-8A, B, C)

Table 3-5: Effect of phenylephrine (α-agonist) in the presence and absence of phentolamine (α-antagonist) on Ca^{2+} efflux in eel hepatocytes.

Ca^{2+} remaining in cells at 6 min : 0 min (n = 5)		
Treatments	10^{-5}M Phenylephrine with/without 10^{-4}M Phentolamine	10^{-7}M Phenylephrine with/without 10^{-5}M Phentolamine
Control	0.790 ± 0.06	0.907 ± 0.067
Phenylephrine	0.568 ± 0.04 *	0.733 ± 0.065
Phenylephrine + Phentolamine	0.818 ± 0.10	0.958 ± 0.100

Eel hepatocytes were pre-incubated, ^{45}Ca-loaded and efflux determined as noted previously (Fig. 3-6). Efflux proceeded in a ^{45}Ca-free Medium C containing phenylephrine with or without phentolamine which was added 15 min before the end of the loading at a concentration of 10- to 100-fold that of phenylephrine. * $p < 0.05$ compared with the corresponding control by one-way ANOVA followed by a Student's t-test.

Table 3-6: Effect of isoproterenol (β-agonist) in the presence and absence of propranolol (β-antagonist) on Ca^{2+} efflux in eel hepatocytes.

Ca^{2+} remaining in cells at 6 min : 0 min (n = 5)		
Treatments	10^{-5}M Isoproterenol with/without 10^{-4}M Propranolol	10^{-7}M Isoproterenol with/without 10^{-5}M Propranolol
Control	0.790 ± 0.06	0.907 ± 0.067
Isoproterenol	0.602 ± 0.05	0.702 ± 0.066
Isoproterenol + Propranolol	0.886 ± 0.10	1.058 ± 0.109

Conditions as in Table 3-5, except isoproterenol and propranolol used. Values do not differ from controls (ANOVA).

Table 3-7: Effect of α- and β-adrenergic agonists in the presence and absence of antagonists on Ca^{2+} efflux in bullhead hepatocytes.

Ca^{2+} remaining in cells at 6 min : 0 min (n = 5)			
Agonists and Antagonists Used	Control	10^{-5}M Agonist	10^{-5}M Agonist + 10^{-4}M Antagonist
Epinephrine	0.911 ± 0.05	0.757 ± 0.03	ND
Phenylephrine (+ Phentolamine)	0.832 ± 0.04	0.758 ± 0.02	0.848 ± 0.05
Isoproterenol (+ Propranolol)	0.832 ± 0.04	0.806 ± 0.04	0.836 ± 0.04

Conditions as noted on Tables 3-5 and 3-6, Values do not differ from controls (ANOVA).

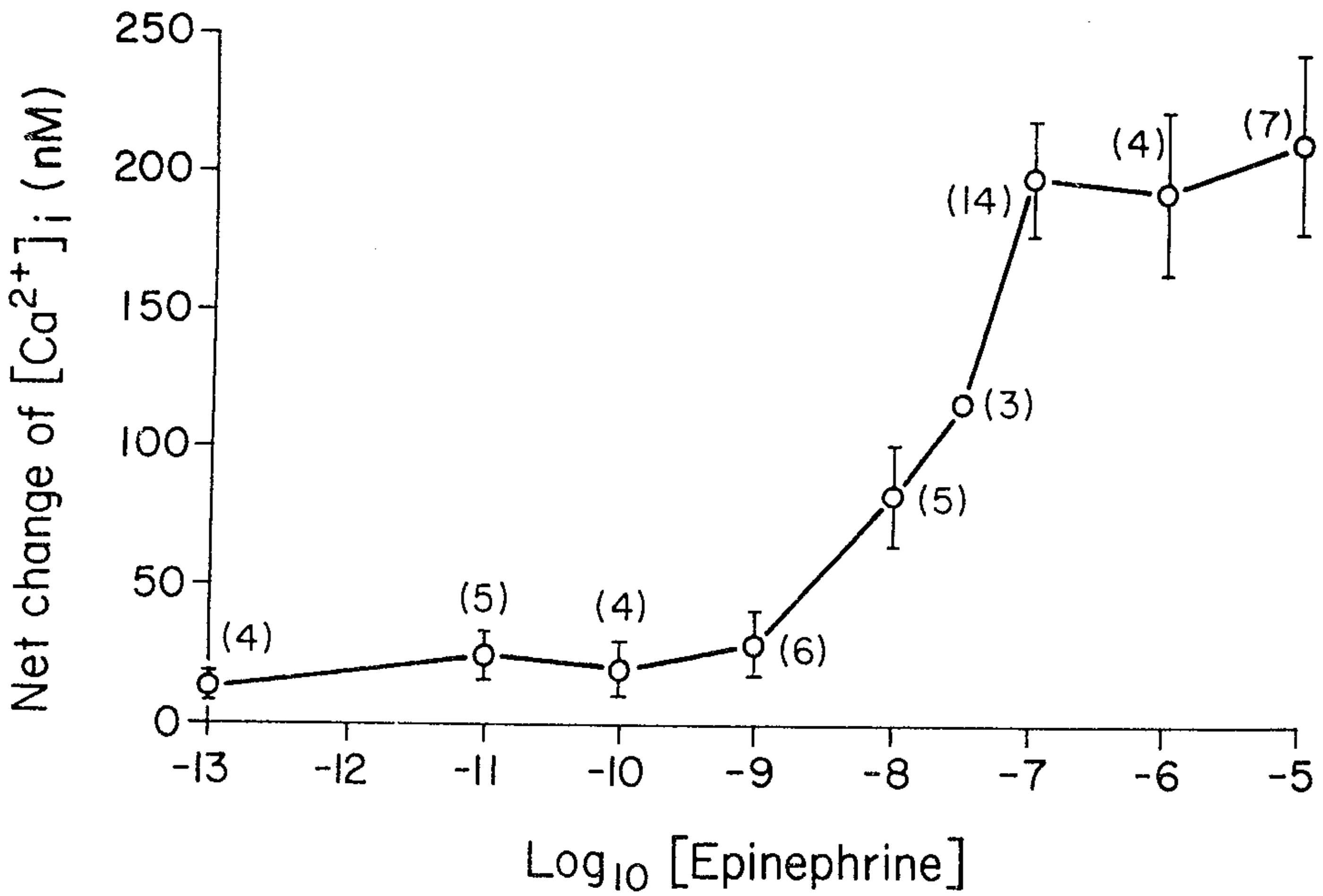
was slower than that in eel hepatocytes (compare with Fig. 3-8D, E, F). Epinephrine did not significantly stimulate Ca^{2+} efflux in bullhead hepatocytes, while epinephrine stimulated Ca^{2+} efflux from eel hepatocytes. Likewise, the α-agonist (10^{-5}M phenylephrine) and the β-agonist (10^{-5}M isoproterenol) in the presence and absence of their appropriate antagonists had little effect on Ca^{2+} efflux in bullhead hepatocytes.

III. Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_i$) Studies

A) $[Ca^{2+}]_i$ - epinephrine dose response in eel hepatocytes

Cytosolic free Ca^{2+} concentration was measured on single Fura-2-loaded hepatocytes by microspectrofluorimeter. Different epinephrine concentrations were tested on the initial peak rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in individual hepatocytes. An epinephrine dose response curve (Fig. 3-9) was obtained from these studies by plotting $\Delta[Ca^{2+}]_i$ against applied epinephrine dose. Eel hepatocytes were highly sensitive to epinephrine, with 10^{-13}M epinephrine inducing a $[Ca^{2+}]_i$ response (Fig. 3-14). 10^{-5}M epinephrine gave a maximum response, although the $[Ca^{2+}]_i$ response appeared to plateau at 10^{-7}M epinephrine. The epinephrine concentration required to achieve half-maximal response, or K_a, was approximately 3 x 10^{-8}M.

Fig. 3-9: $[Ca^{2+}]_i$ - epinephrine dose response curve in Fura-2-loaded eel hepatocytes. Cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured on single Fura-2-loaded hepatocytes by microspectrofluorimeter. The initial peak rise in Ca_i^{2+} compared to the absence of epinephrine ($\Delta[Ca^{2+}]_i$) was determined at a number of applied epinephrine doses. The number in parentheses means the number of independent determinations. The epinephrine concentration needed to achieve half-maximal response, or K_a, was approximately 3 x 10^{-8}M.



Net change of $[Ca^{2+}]_i$ (nM)
250
200
150
100
50
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log_{10} [Epinephrine]
(4)
(5)
(4)
(6)
(5)
(3)
(14)
(4)
(7)

B) Effect of external Ca^{2+} on $[Ca^{2+}]_i$ induced by epinephrine in eel, bullhead, and rainbow trout hepatocytes

(1) Effect of external Ca^{2+} on $[Ca^{2+}]_i$ in eel hepatocytes

Cytosolic free Ca^{2+} concentration was measured under two conditions: the first condition was 10^{-7}M epinephrine with 2 mM external Ca^{2+} (i.e., the superfusion solution contained 10^{-7}M epinephrine + 2 mM $CaCl_2$ in Medium A); and the second condition was 10^{-7}M epinephrine without external Ca^{2+} (i.e., the superfusion solution contained 10^{-7}M epinephrine + 2 mM $MgCl_2$ + 0.5 mM EGTA in Medium A). Changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by epinephrine were measured and compared between these two conditions at two time points. The first time point was at the maximal $\Delta[Ca^{2+}]_i$ of the initial peak ($\Delta[Ca^{2+}]_{i(f)}$) and the second was the $\Delta[Ca^{2+}]_i$ measured 2 min after the initial peak response ($\Delta[Ca^{2+}]_{i(s)}$). The results (Table 3-8; Fig. 3-10) showed that the presence and absence of external Ca^{2+} did not affect the epinephrine induced cytosolic free Ca^{2+} concentration change during either time period measured. These data support conclusion that the epinephrine-induced cytosolic free Ca^{2+} concentration increase in eel hepatocytes was accomplished by the mobilization of Ca^{2+} from intracellular Ca^{2+} pools at least during the time period measured.

(2) Effect of external Ca^{2+} on $[Ca^{2+}]_i$ in bullhead hepatocytes

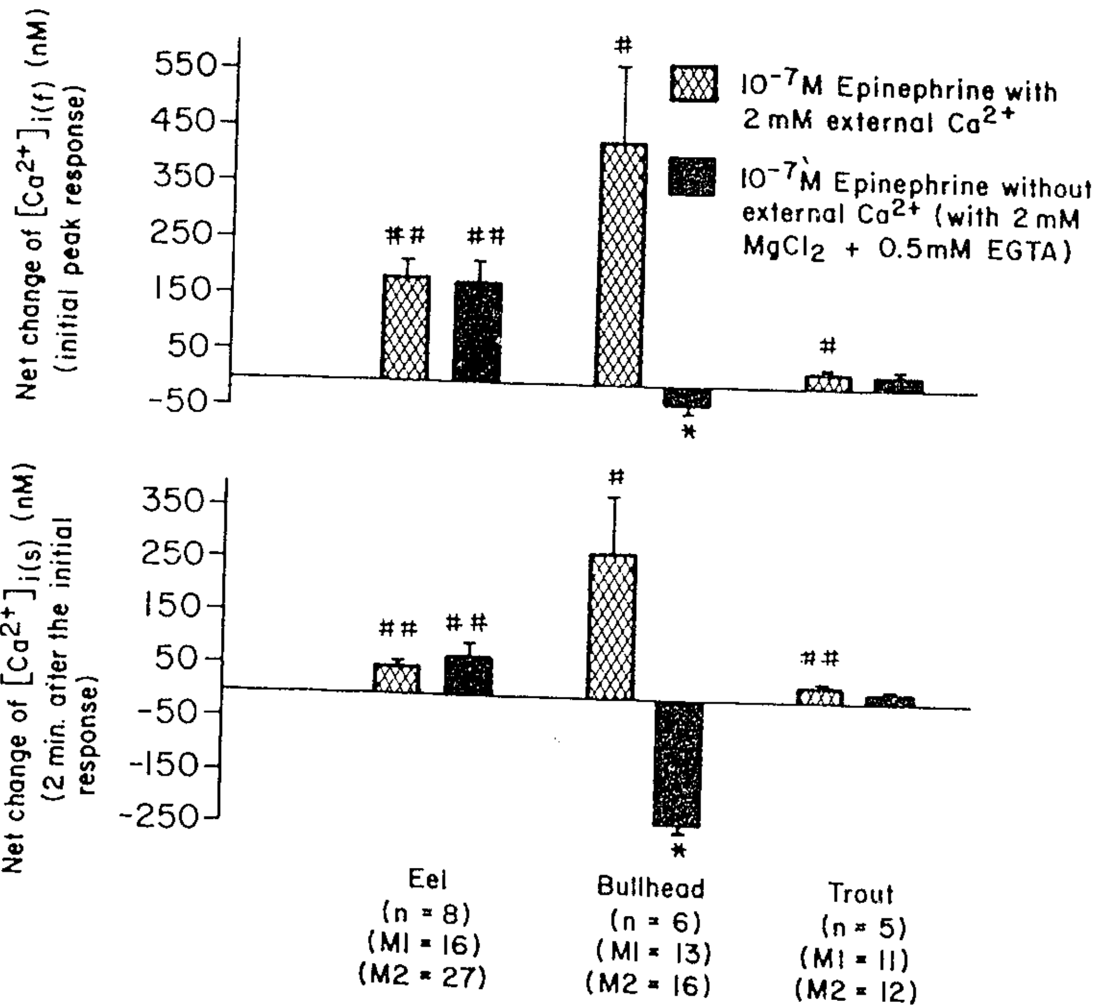
Distinct from eel hepatocytes, external Ca^{2+} greatly affected the epinephrine-induced increase in $[Ca^{2+}]_i$ in bullhead hepatocytes (Table 3-8; Fig. 3-10). With 2 mM

Table 3-8: Effect of epinephrine in the presence and absence of external Ca^{2+} on $[Ca^{2+}]_i$ of Fura-2-loaded hepatocytes in three fish species.

Species	$\Delta[Ca^{2+}]_i$ of initial response ($\Delta[Ca^{2+}]_{i(f)}$ nM)		$\Delta[Ca^{2+}]_i$ of 2 min after initial response ($\Delta[Ca^{2+}]_{i(s)}$ nM)	
	10^{-7}M E + 2 mM Ca^{2+}	10^{-7}M E + 0.5 mM EGTA	10^{-7}M E + 2 mM Ca^{2+}	10^{-7}M E + 0.5 mM EGTA
Eel (n = 8) (m_1 = 16) (m_2 = 27)	184.3 ± 30.0 ##	176.7 ± 36.1 ##	51.7 ± 8.5 ##	72.4 ± 23.3 ##
Bullhead (n = 6) (m_1 = 13) (m_2 = 16)	433.5 ± 135.6 #	-36.5 ± 124.4 *	273 ± 105.9 #	-241 ± 110 *
Trout (n = 5) (m_1 = 11) (m_2 = 12)	27.2 ± 7.1 #	22.3 ± 8.6	27.6 ± 4.2 ##	16.2 ± 6.0

10^{-7}M epinephrine-induced cytosolic free-Ca^{2+} concentration changes ($\Delta[Ca^{2+}]_i$) were measured on single Fura-2-loaded hepatocytes by microspectrofluorimeter in the presence and absence of external Ca^{2+}. Changes in $[Ca^{2+}]_i$ were compared at Ca_i^{2+} peak rise of initial response ($\Delta[Ca^{2+}]_{i(f)}$) and $\Delta[Ca^{2+}]_i$ of 2 min after the initial response ($\Delta[Ca^{2+}]_{i(s)}$). Values are expressed as mean ± SE of n independent preparations using m_1 and m_2 total number of cells studied in the presence and absence of external Ca^{2+}, respectively. A Student's t-test was performed: # $p < 0.05$, ## $p < 0.01$ compared with 0; * $p < 0.05$ compared with the results at the same time but with external Ca^{2+}.

Fig. 3-10: Effect of epinephrine (10^{-7}M) on Ca^{2+} transients in the presence and absence of external Ca^{2+} in Fura-2-loaded hepatocytes of three fish species. Medium C (Table 2-1) either contained normal external [Ca^{2+}] (2 mM) or no external Ca^{2+} but replaced by 2 mM $MgCl_2$ and 0.5 mM EGTA. $\Delta[Ca^{2+}]_i$ was measured at the two time periods noted on Table 3-8. Significant differences determined by one-way ANOVA followed by a Student's t-test: * $p < 0.05$, significantly different from the same time but with external Ca^{2+}; # $p < 0.05$, ## $p < 0.01$ compared with 0. m_1 and m_2 = cells studied in the presence and absence of external Ca^{2+} respectively; and n = independent preparations.



Net change of $[Ca^{2+}]_{i(f)}$ (nM) (initial peak response)
550
450
350
250
150
50
-50
10^{-7}M Epinephrine with 2 mM external Ca^{2+}
10^{-7}M Epinephrine without external Ca^{2+} (with 2 mM $MgCl_2$ + 0.5mM EGTA)
##
#
*
#
Net change of $[Ca^{2+}]_{i(s)}$ (nM) (2 min. after the initial response)
350
250
150
50
-50
-150
-250
##
#
*
##
Eel
(n = 8)
(M1 = 16)
(M2 = 27)
Bullhead
(n = 6)
(M1 = 13)
(M2 = 16)
Trout
(n = 5)
(M1 = 11)
(M2 = 12)

external Ca^{2+}, 10^{-7}M epinephrine significantly induced a 433.5 ± 135.6 nM (n = 6) net increase in $[Ca^{2+}]_{i(f)}$. In contrast, the absence of external Ca^{2+} decreased $[Ca^{2+}]_{i(f)}$ below basal levels to -36.5 ± 124.4 nM (n = 6; $p < 0.05$) even though 10^{-7}M epinephrine was still present. At 2 min after the initial response, $[Ca^{2+}]_{i(s)}$ was further decreased; $\Delta[Ca^{2+}]_{i(s)}$ in the absence of external Ca^{2+} decreased so much that it significantly differed from $\Delta[Ca^{2+}]_{i(s)}$ in the presence of external Ca^{2+}. It can be concluded, therefore, that the epinephrine-induced cytosolic free Ca^{2+} concentration increase in bullhead hepatocytes was contributed mainly by Ca^{2+} influx from external Ca^{2+} sources, not internal stores.

(3) Effect of external Ca^{2+} on $[Ca^{2+}]_i$ in trout hepatocytes

This phenomena in trout hepatocytes differed from those of both the eel and bullhead hepatocytes. Rainbow trout hepatocytes exhibited a very weak Ca_i^{2+} response to epinephrine. 10^{-7}M epinephrine induced a minor or no peak rise in $[Ca^{2+}]_i$; Figs 3-11 and 3-12 are examples of the effect of 10^{-7}M epinephrine on $[Ca^{2+}]_i$ in individual trout hepatocytes in the presence and absence of external Ca^{2+}, respectively. In only 18% (3 of 17) of individual cells tested was there a Ca^{2+} peak induced by epinephrine similar to that shown in the middle panel of Fig. 3-11. This response was much retarded in magnitude and time compared to the other two species; however, as seen in Table 3-8, the Ca_i^{2+} increase induced by epinephrine in the presence of external Ca^{2+} was statistically different from 0 change (i.e., the control) and the Ca_i^{2+} response in the absence of external Ca^{2+} was not significantly different from the control. Given the extant of this response, this significant value seems without physiologic importance.

Fig. 3-11: Examples of epinephrine-induced Ca_i^{2+} responses in individual trout hepatocytes in the presence of external Ca^{2+} (2 mM). The addition of epinephrine (10^{-7}M), indicated at the arrow, induced a small peak Ca_i^{2+} rise in less than 20% of the cells as demonstrated by the centre panel, while the other cells exhibited not clear responses as shown by the lateral panels.

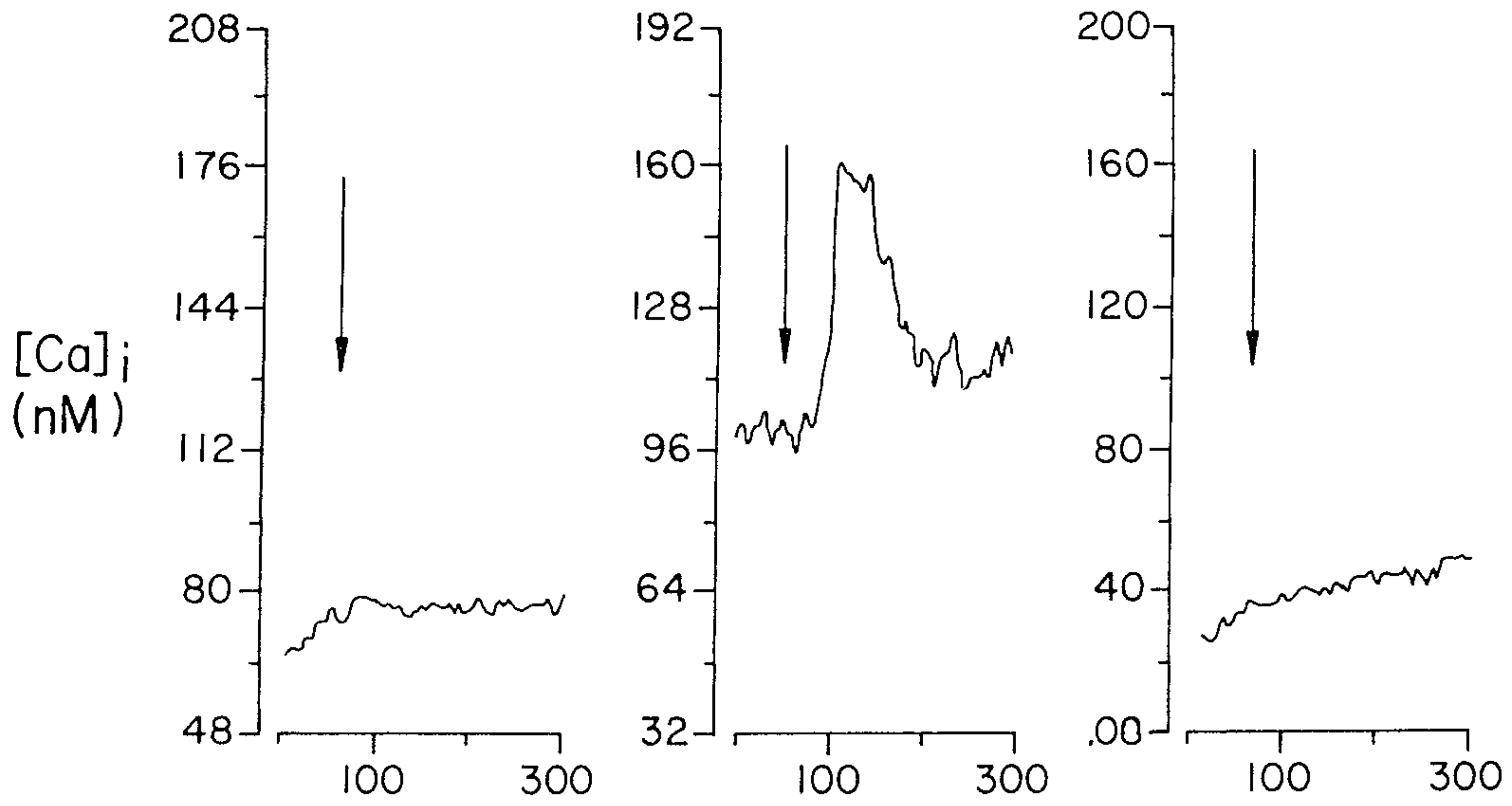
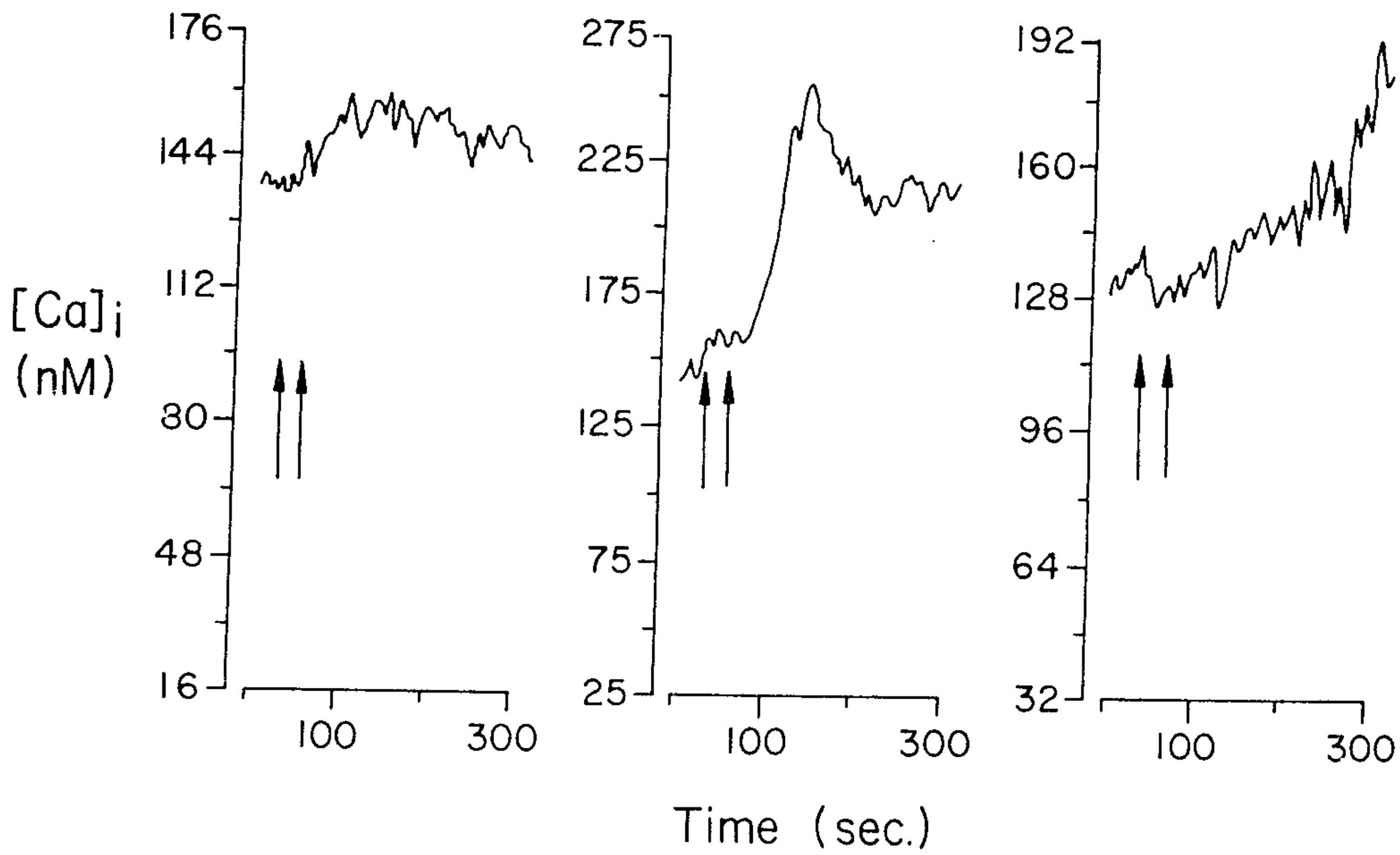

[Ca]i
(nM)
208
176
144
112
80
48
192
160
128
96
64
32
200
160
120
80
40
.00
100
300
100
300
100
300
Time (sec.)

Fig. 3-12: Examples of epinephrine-induced Ca_i^{2+} responses in Fura-2-loaded trout hepatocytes in the absence of external Ca^{2+}. The initial arrow indicates that the superfusion solution was changed from Ca^{2+}-containing solution (Medium C, Table 2-1) to Ca^{2+}-free solution to wash out external Ca^{2+}. The compositions of the Ca^{2+}-free solution was that noted on Fig. 3-10. The second arrow indicates the addition of 10^{-7}M epinephrine. An epinephrine-induced peak rise in Ca_i^{2+} was observed in only a few cells (as shown in centre panel), while most cells exhibited no response (lateral panels).


[Ca]i
(nM)
176
144
112
80
48
16
100
300
275
225
175
125
75
25
100
300
192
160
128
96
64
32
100
300
Time (sec.)

C) Additional Evidence for the Site of Ca^{2+} Mobilization in Eel and Bullhead Hepatocytes

(1) Depletion of intracellular Ca^{2+} pools in eel hepatocytes

Consistent with the results that epinephrine mobilized Ca^{2+} from intracellular Ca^{2+} pools in eel hepatocytes, Tables 3-9 and 3-10 provide evidence for the depletion of the intracellular Ca^{2+} pool(s). When eel hepatocytes were exposed to 10^{-7}M epinephrine three separate but sequential times (i.e., cells exposed to epinephrine, Ca_i^{2+} spike induced, then epinephrine is washed out until $[Ca^{2+}]_i$ reached the basal level, followed by this protocol repeated 2 additional times), the $\Delta[Ca^{2+}]_i$ induced by this same concentration of epinephrine significantly decreased with the number of exposures (Table 3-9; Fig. 3-13). The decreased peak $[Ca^{2+}]_i$ with epinephrine exposures may be due to two reasons: first, hepatocytes are desensitized to epinephrine by successive short exposures; or, second, the depletion of intracellular Ca^{2+} pool(s). To identify which of these maybe the most reasonable explanation, different epinephrine concentrations from low to high were tested on single hepatocytes. Fig. 3-14 provides two examples of epinephrine concentration-induced Ca_i^{2+} responses in eel hepatocytes. At submaximal epinephrine concentrations (e.g., 10^{-13}M, 10^{-11}M, 10^{-9}M), $\Delta[Ca^{2+}]_i$ increased with doses; however, when large doses of epinephrine (e.g., 10^{-7}M, 10^{-5}M) were used, a large $\Delta[Ca^{2+}]_i$ was induced but this was no longer dose dependent. These results support the idea that epinephrine induces depletion of intracellular Ca^{2+} pools in eel hepatocytes. Results using other epinephrine concentrations provide additional support for this conclusion (Table 3-10). Moreover, this

Table 3-9: Effect of three repeated exposures to epinephrine (10^{-7}M) in the presence of external Ca^{2+} (2 mM) on $[Ca^{2+}]_i$ in eel hepatocytes.

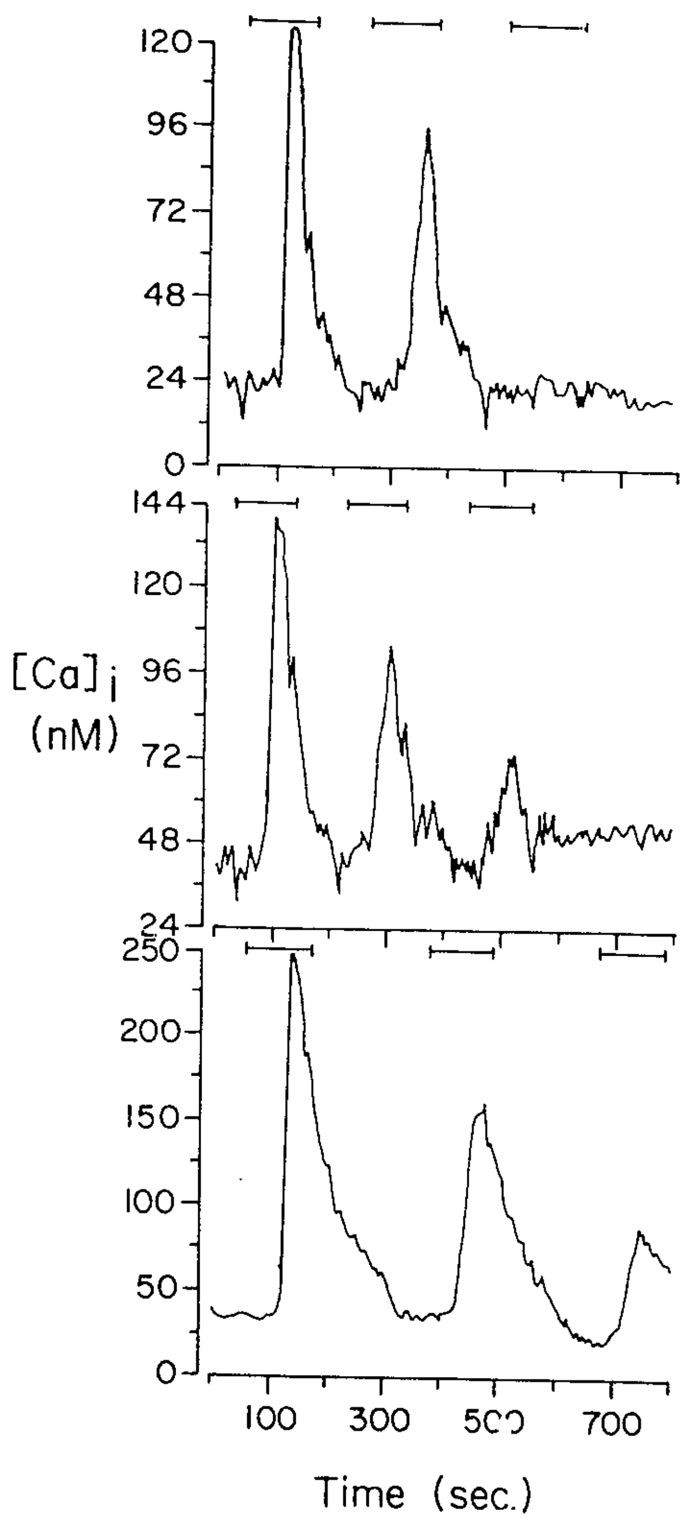
$\Delta[Ca^{2+}]_i$ nM (n = 11, m = 14)		
10^{-7}M epinephrine 1st exposure	10^{-7}M epinephrine 2nd exposure	10^{-7}M epinephrine 3rd exposure
237.9 ± 42.0	83.1 ± 16.2 **	39.6 ± 15.4 **

Table 3-10: Effect of epinephrine concentrations and order of exposures in the presence of external Ca^{2+} (2 mM) on $[Ca^{2+}]_i$ in eel hepatocytes.

[epinephrine]	The order of exposures to hepatocytes	$\Delta[Ca^{2+}]_i$ nM (n = 4, m = 6)
10^{-11}M	1st exposure	12.1 ± 2.0 **
10^{-9}M	2nd exposure	23.2 ± 18.8 **
5 x 10^{-8}M	3rd exposure	158 ± 20.1
10^{-6}M	4th exposure	126 ± 16.6

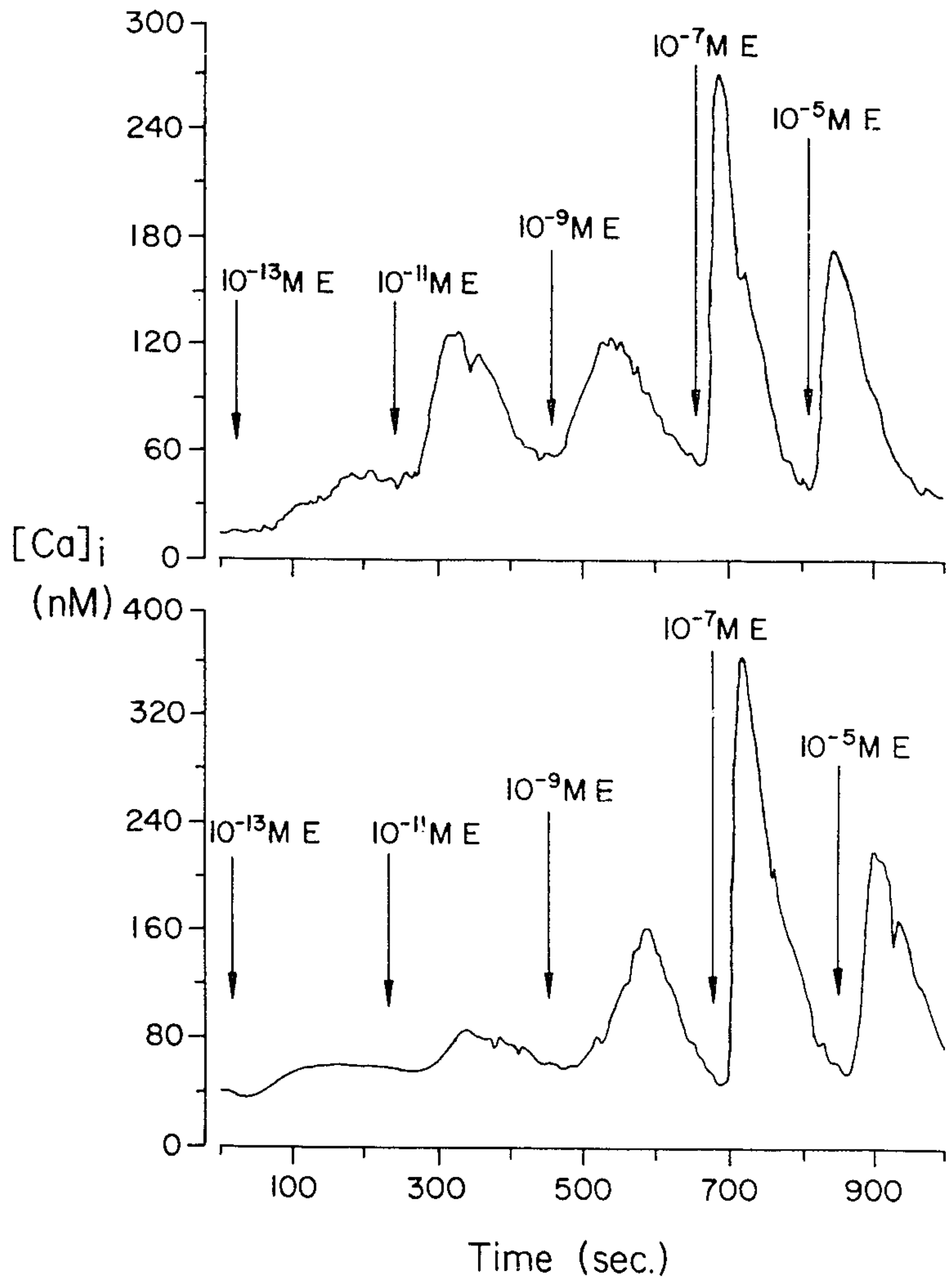
Cytosolic free-Ca^{2+} concentration changes were measured on individual Fura-2-loaded eel hepatocytes by microspectrofluorimeter. Each exposure was separated (Table 3-9) or not separated (Table 3-10) by a wash-out with Medium C (Table 2-1) so that $[Ca^{2+}]_i$ returned to basal levels. Values are expressed as mean ± SE of n independent preparations and m cells studied. ** $p < 0.01$ compared with value of first exposure (Table 3-9) or values of higher epinephrine doses (5 x 10^{-8}M or 10^{-6}M) (Table 3-10) by one-way ANOVA followed by a Student's t-test.

Fig. 3-13: Ca^{2+} transients in three individual Fura-2-loaded eel hepatocytes induced by three repeated epinephrine exposures in the presence of external Ca^{2+} (2 mM). The horizontal bar indicates the period during exposure to 10^{-7}M epinephrine. The cells were superfused with Medium C (less BSA, Table 2-1) throughout.



120
96
72
48
24
0
144
120
96
72
48
24
[Ca]i
(nM)
250
200
150
100
50
0
100
300
500
700
Time (sec.)

Fig. 3-14: The effects of different epinephrine doses on the $[Ca^{2+}]_i$ resp onse in Fura-2-loaded eel hepatocytes. They are recorded in 2 individual hepatocytes by microspectrofluorimeter in the presence of external Ca^{2+} (2 mM). The arrows indicate the addition of different epinephrine doses.



300
240
180
120
60
0
10^{-13}M E
10^{-11}M E
10^{-9}M E
10^{-7}M E
10^{-5}M E
[Ca]i
(nM)
400
320
240
160
80
0
10^{-13}M E
10^{-11}M E
10^{-9}M E
10^{-7}M E
10^{-5}M E
100
300
500
700
900
Time (sec.)

conclusion can be further supported by the observation that epinephrine-induced Ca^{2+} oscillations exist longer with an external Ca^{2+} supply than those without an external Ca^{2+} supply (compare Figs. 3-18 with 3-19).

(2) External Ca^{2+} sources maintain epinephrine-induced cytosolic free Ca^{2+} concentration increase in bullhead hepatocytes

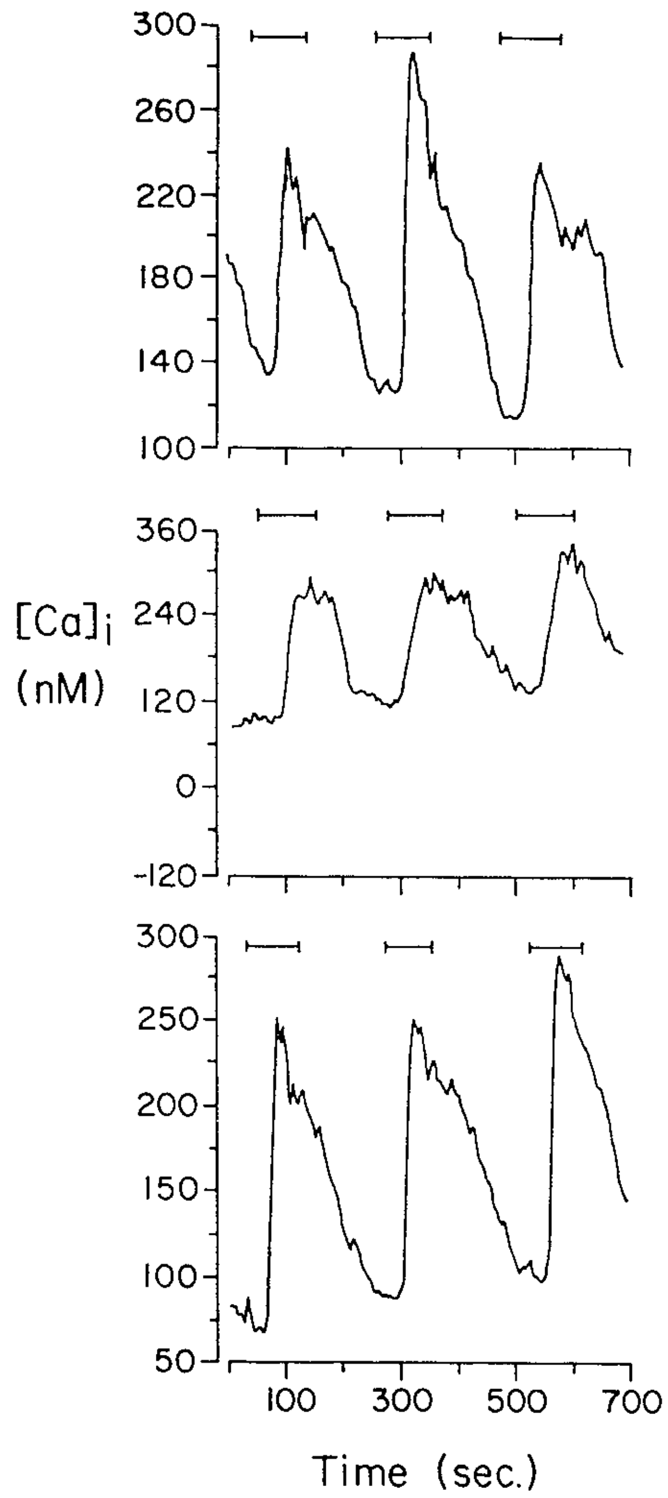
Distinct from those eel hepatocyte studies, bullhead hepatocytes exposed to epinephrine three-times showed no statistically significant decrease in induced $\Delta[Ca^{2+}]_i$ with epinephrine exposures (Table 3-11). The three peaks of $[Ca^{2+}]_i$ induced by epinephrine exposure were nearly identical as shown graphically in Fig. 3-15. No evidence for intracellular Ca^{2+} pool depletion was observed as seen in eel hepatocytes. These experiments had a 2 mM external Ca^{2+} source always presence, thus, depletion would be negligible. The results are consistent with the bullhead hepatocytes depending mainly on external Ca^{2+} sources. These differences in $[Ca^{2+}]_i$ peaks induced by epinephrine exposure between eel and bullhead hepatocytes once again confirmed that Ca^{2+} from intracellular pools was mobilized by epinephrine in eel hepatocytes and Ca^{2+} from external Ca^{2+} pools was mobilized in bullhead hepatocytes.

Table 3-11: Effect of three repeated exposures of epinephrine (10^{-7}M) in the presence of external Ca^{2+} (2 mM) on $[Ca^{2+}]_i$ in bullhead hepatocytes.

$\Delta[Ca^{2+}]_i$ nM (n = 5)		
10^{-7}M Epinephrine 1st exposure	10^{-7}M Epinephrine 2nd exposure	10^{-7}M Epinephrine 3rd exposure
291.4 ± 126.6	252.2 ± 75.8	244.4 ± 63.7

Conditions as on Table 3-9. Values are expressed as mean ± SE (n = 5). Values do not differ from first exposure (ANOVA).

Fig. 3-15: Examples of Ca^{2+} transients induced by three repeated epinephrine (10^{-7}M) exposures in the presence of external Ca^{2+} (2 mM) in bullhead hepatocytes. They are recordings from 3 Fura-2-loaded bullhead cells by microspectrofluorimeter under conditions noted in Fig. 3-13.


300
260
220
180
140
100
360
240
120
0
-120
[Ca]$_i$
(nM)
300
250
200
150
100
50
100
300
500
700
Time (sec.)

D) Effects of α- and β-adrenergic Agonists and/or Antagonists on Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_i$) of Fish Hepatocytes

(1) Effect of α- and β-antagonists (phentolamine, propranolol) alone on $[Ca^{2+}]_i$ of fish hepatocytes

Propranolol and phentolamine (at 10^{-5}M) had no effect by themselves on $[Ca^{2+}]_i$ in eel and bullhead hepatocytes (Table 3-12).

(2) Effect of epinephrine in the presence and absence of α- and β-antagonists on $[Ca^{2+}]_i$ of fish hepatocytes

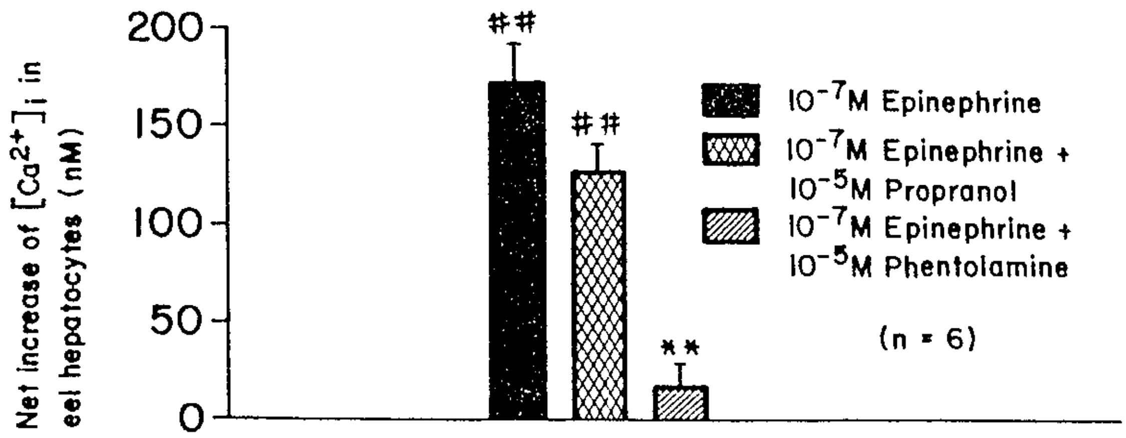
Fig. 3-16 shows the similar effect of epinephrine with or without its antagonist on $[Ca^{2+}]_i$ of both eel and bullhead hepatocytes. In eel hepatocytes, 10^{-7}M epinephrine significantly induced a 172.6 ± 20.7 nM (n = 6) cytosolic free Ca^{2+} concentration increase; the β-antagonist propranolol (10^{-5}M) although blocked 26.4% of this epinephrine effect, the inhibition was not statistically significant. The α-antagonist phentolamine (10^{-5}M) blocked 89.9% of the epinephrine stimulating effect ($p < 0.01$). In bullhead hepatocytes, 10^{-7}M epinephrine significantly induced a 394 ± 84.7 nM (n = 6) $[Ca^{2+}]_i$ increase, which was about two-times the $[Ca^{2+}]_i$ increase of the eel hepatocytes. A similar percentage of the epinephrine effect was blocked by propranolol (24.8%) and phentolamine (89.6%) as shown for the eel. Again these results support an α-adrenergic role in the Ca_i^{2+} response of these two fish hepatocyte systems.

Table 3-12: Effect of individual α- and β-adrenergic antagonist on $[Ca^{2+}]_i$ in the presence of external Ca^{2+} (2 mM) in fish hepatocytes.

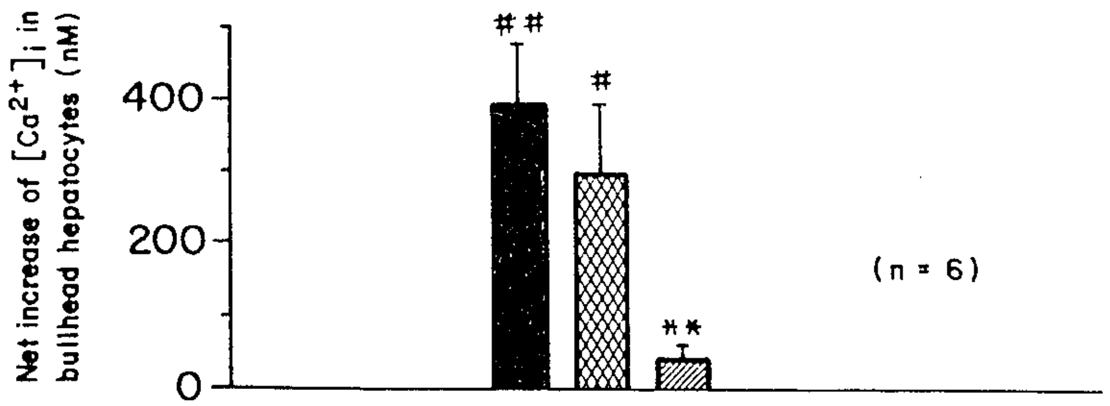
Species	$\Delta[Ca^{2+}]_i$ nM	
	10^{-5}M Propranolol	10^{-5}M Phentolamine
Eel (n = 10, m = 17)	-0.1 ± 0.7	-0.7 ± 1.1
Bullhead (n = 7, m = 20)	9.2 ± 13.1	1.5 ± 4.6

$[Ca^{2+}]_i$ was estimated in Fura-2-loaded eel and bullhead hepatocytes by microspectrofluorimeter. Values are mean ± SE (n = number of determinations; m = number of cells). Values do not differ from 0 (ANOVA).

Fig. 3-16: Effect of epinephrine in the presence and absence of propranolol (β-antagonist) and phentolamine (α-antagonist) on $[Ca^{2+}]_i$ in eel and bullhead hepatocytes. Ca^{2+} transients were measured by microspectrofluorimeter on single Fura-2-loaded cells in the presence of external Ca^{2+} (2 mM). $\Delta[Ca^{2+}]_i$ was taken from the initial maximal Ca_i^{2+} increase. # $p < 0.05$, ## $p < 0.01$ significantly different from 0; ** $p < 0.01$ different from epinephrine group by one-way ANOVA followed by a Student's t-test.



200
150
100
50
0
Net increase of $[Ca^{2+}]_i$ in eel hepatocytes (nM)
##
##
**
10^{-7}M Epinephrine
10^{-7}M Epinephrine + 10^{-5}M Propranol
10^{-7}M Epinephrine + 10^{-5}M Phentolamine
(n = 6)



400
200
0
Net increase of $[Ca^{2+}]_i$ in bullhead hepatocytes (nM)
##
#
**
(n = 6)

(3) Effect of phenylephrine or isoproterenol on $[Ca^{2+}]_i$ of fish hepatocytes

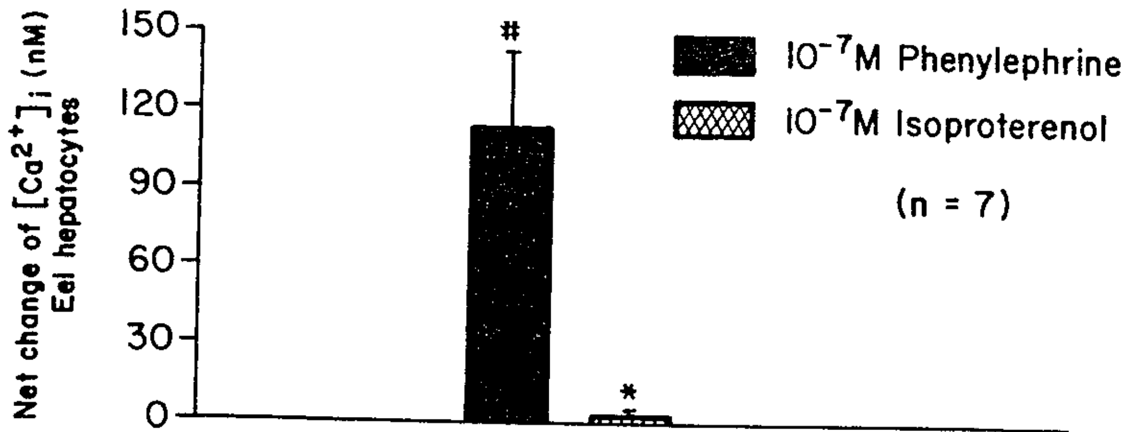
The effect of phenylephrine (10^{-7}M) and isoproterenol (10^{-7}M) on $[Ca^{2+}]_i$ in the presence of external Ca^{2+} was also tested (Fig. 3-17). Phenylephrine increased $[Ca^{2+}]_i$ of eel hepatocytes (114.1 ± 29.0 nM (n = 6); $p < 0.05$ compared with control), but the effect was lower in magnitude than the effect of epinephrine at the same concentration, but it was about as thirty-five-times that of the isoproterenol effect. Isoproterenol actually had an effect on only approx. 50% of the cells tested. This finding supports an α-adrenergic role in eel hepatocytes found with the epinephrine studies. Although the difference between the effects of phenylephrine and isoproterenol were not as great in the bullhead hepatocytes as that in the eel hepatocytes, the effect of phenylephrine on $\Delta[Ca^{2+}]_i$ (364.1 ± 118.6 nM (n = 6); $p < 0.05$ compared with control) was only just less than the effect of an equivalent concentration of epinephrine. The effect of isoproterenol (not statistically different from control), was about 1.6 times less than the phenylephrine effect, which may indicate that both an α- and β-adrenoceptors function in increasing $[Ca^{2+}]_i$, but with the α-receptor pathway predominating.

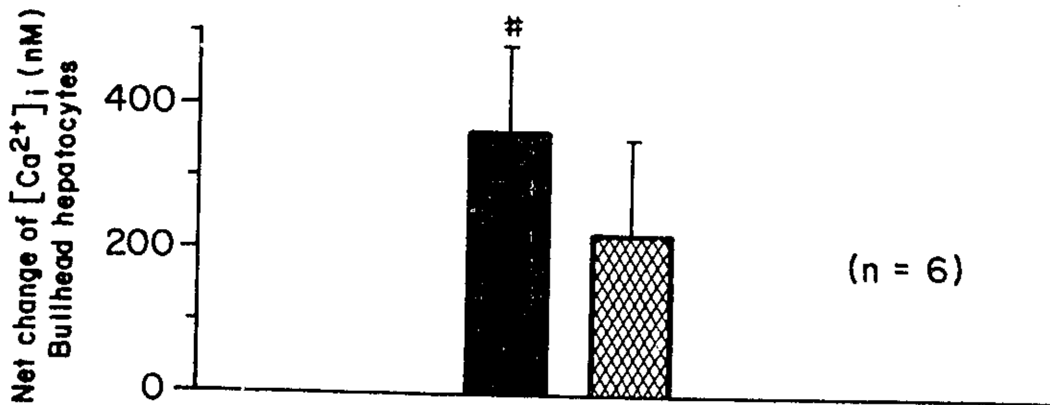
E) The Patterns of Ca_i^{2+} Response to α- and β-agonists in Fish Hepatocytes

(1) The patterns of Ca_i^{2+} response to epinephrine, phenylephrine and isoproterenol in eel hepatocytes

Changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in response to α- and β-agonists were studied in single Fura-2-loaded eel hepatocytes. Agonist-induced Ca^{2+} oscillations were

Fig. 3-17: Effect of phenylephrine (α-agonist) and isoproterenol (β-agonist) on $[Ca^{2+}]_i$ in eel and bullhead hepatocytes. Conditions as on Fig. 3-16. Only 50% eel cells tested responded to isoproterenol. # $p < 0.05$ compared with 0; * $p < 0.05$ compared with phenylephrine group using a one-way ANOVA followed by a Student's t-test.

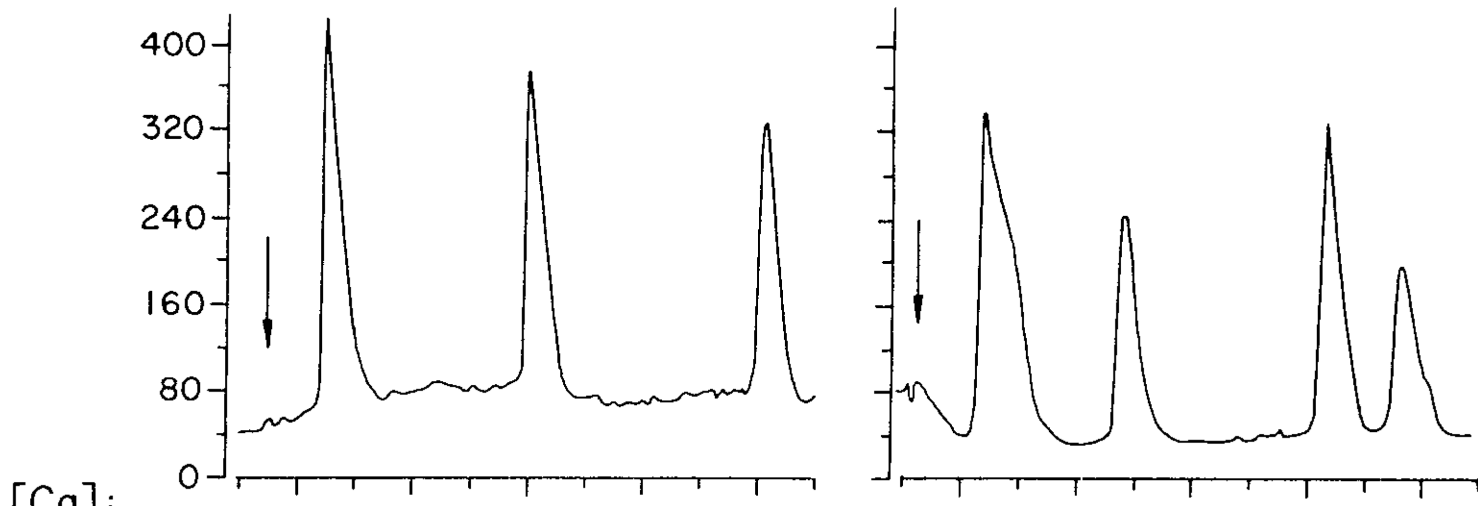

Net change of [Ca2+]i (nM)
Eel hepatocytes
150
120
90
60
30
0
#
*
10-7M Phenylephrine
10-7M Isoproterenol
(n = 7)


Net change of [Ca2+]i (nM)
Bullhead hepatocytes
400
200
0
#
(n = 6)

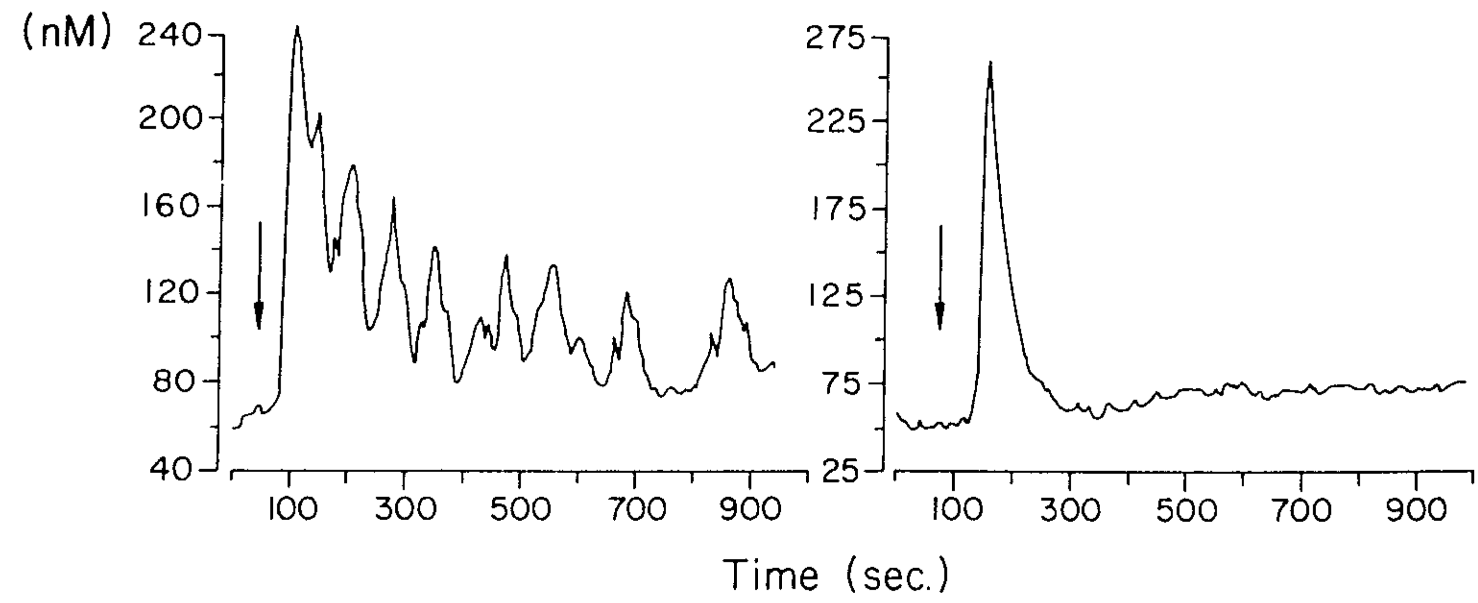
studied in 22 hepatocytes from 9 eel livers in the presence of 10^{-7}M epinephrine and external Ca^{2+} (2 mM); 18 cells from 7 livers (82%) exhibited obvious Ca^{2+} oscillations, and the other 4 cells had either no or no clear Ca^{2+} oscillations. When external Ca^{2+} was absent, 10^{-7}M epinephrine could still induce Ca^{2+} oscillations, but they disappeared more quickly than those induced by epinephrine in the presence of external Ca^{2+}. Of 14 hepatocytes from 7 eel livers studied in the absence of external Ca^{2+}, 12 cells of 5 independent preparations (86%) had Ca^{2+} oscillations. Not all Ca^{2+} oscillation patterns observed were identical; they differed from cell to cell. Figs 3-18 and 3-19 presented several typical Ca^{2+} oscillation patterns induced by 10^{-7}M epinephrine in the presence and absence of external Ca^{2+}, respectively. The effect of different epinephrine concentrations (10^{-9} to 10^{-5}M) on Ca^{2+} oscillations was studied; all epinephrine concentrations studied could induce Ca^{2+} oscillations, but no specific relationship between agonist concentration and the frequency of Ca^{2+} oscillations could be discerned as shown previously in mammals (Exton, 1988; Rooney et al., 1989; 1990).

The α-agonist phenylephrine and the β-agonist isoproterenol induced Ca_i^{2+} response patterns were also investigated. Phenylephrine (10^{-7}M) induced Ca^{2+} oscillations in 5 of 6 eel hepatocytes (representing 3 separate preparations) studied. In contrast, isoproterenol (10^{-7}M) did not induce Ca^{2+} oscillations in 5 of 6 eel hepatocytes studied; only 1 of 6 had Ca^{2+} oscillations. The oscillation patterns induced by phenylephrine were not obviously different from those induced by epinephrine (not shown).

Fig. 3-18: Examples of epinephrine-induced Ca^{2+} oscillations in the presence of external Ca^{2+} (2 mM) in Fura-2-loaded eel hepatocytes. The addition of 10^{-7}M epinephrine (indicated by the arrow) initiated clear Ca^{2+} oscillations with a variable pattern (first three panels). Only a few cells exhibited a single spike, and such an example is given in the last panel. These recordings are from 4 different cells in the presence of external Ca^{2+}.

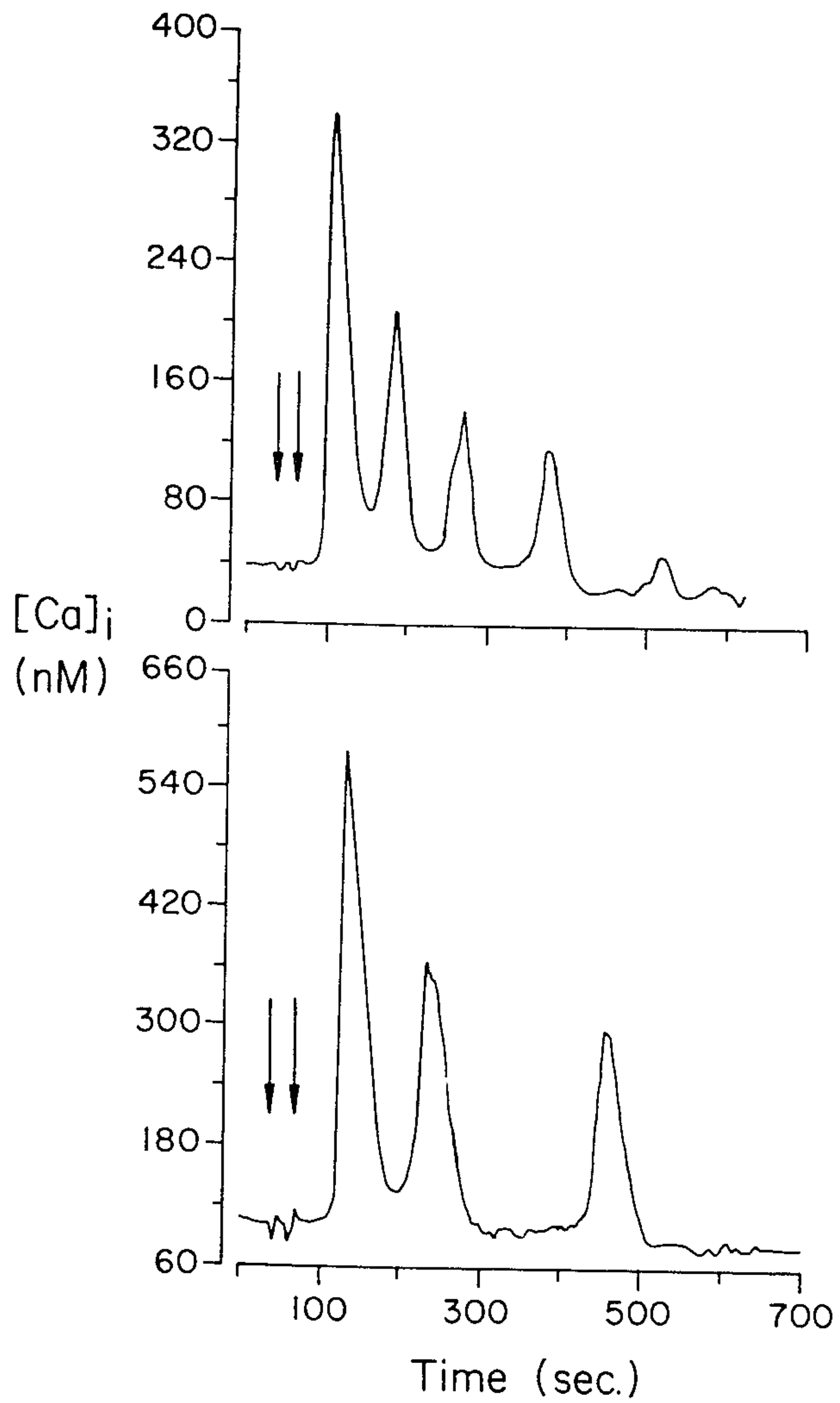


[Ca]i
(nM)
400
320
240
160
80
0



240
200
160
120
80
40
275
225
175
125
75
25
100
300
500
700
900
Time (sec.)

Fig. 3-19: Examples of Ca^{2+} oscillations induced by epinephrine (10^{-7}M) in the absence of external Ca^{2+} in eel hepatocytes. The control superfusion solution was Medium C (Table 2-1). The first arrow indicates that the superfusion solution was changed to a Ca^{2+}-free solution (see Fig. 3-10) to wash out external Ca^{2+}. The second arrow indicates the addition of 10^{-7}M epinephrine. The magnitude of Ca^{2+} spikes decreased and the latent time between spikes increased regularly with the cell exposure time.



400
320
240
160
80
0
[Ca]$_i$
(nM)
660
540
420
300
180
60
100
300
500
700
Time (sec.)

(2) The patterns of Ca_i^{2+} response to epinephrine, phenylephrine and isoproterenol in bullhead hepatocytes

The Ca_i^{2+} response to epinephrine (10^{-7}M) was quite different in bullhead cells compared with the eel hepatocytes. No Ca^{2+} oscillations or no obvious Ca^{2+} oscillations were observed in 15 hepatocytes from 6 independent bullhead preparations with epinephrine and external Ca^{2+} present. All cells exhibited the initial increase in $[Ca^{2+}]_i$ which was usually two-times that of eel hepatocytes, and then a sustained elevated Ca_i^{2+} level was observed. Figs 3-20 and 3-21 show several examples of the Ca_i^{2+} responses induced by 10^{-7}M epinephrine in the presence and absence of external Ca^{2+}. The initial increase of $[Ca^{2+}]_i$ and the maintenance of the sustained elevated Ca_i^{2+} level depended on external Ca^{2+}. Of 16 bullhead hepatocytes (n = 6) studied in the absence of external Ca^{2+}, half induced an initial increase in $[Ca^{2+}]_i$ which was lower than that induced by epinephrine with external Ca^{2+}; the other half had no initial increase in $[Ca^{2+}]_i$, but decreased below basal level. All of these cells showed further decreases until no cytosolic free Ca^{2+} remained.

Epinephrine did not induce Ca^{2+} oscillations in bullhead hepatocytes, but 10^{-7}M phenylephrine and 10^{-7}M isoproterenol did. From 4 hepatocytes of 3 bullhead livers studied, 3 cells showed phenylephrine-induced Ca^{2+} oscillations in the presence of external Ca^{2+}. Of 10 hepatocytes from 6 livers, 7 cells of 4 preparations showed Ca^{2+} oscillations induced by 10^{-7}M isoproterenol. The Ca^{2+} oscillation patterns were similar to those observed in eel hepatocytes.

Fig. 3-20: Examples of Ca^{2+} transients induced by epinephrine in the presence of external Ca^{2+} (2 mM) in bullhead hepatocytes. Conditions as noted previously. The addition of 10^{-7}M epinephrine, indicated by the arrow, caused a rapid rise of $[Ca^{2+}]_i$ followed by a sustained elevated Ca_i^{2+} level. All bullhead cells studied (n = 15) exhibited the similar pattern.

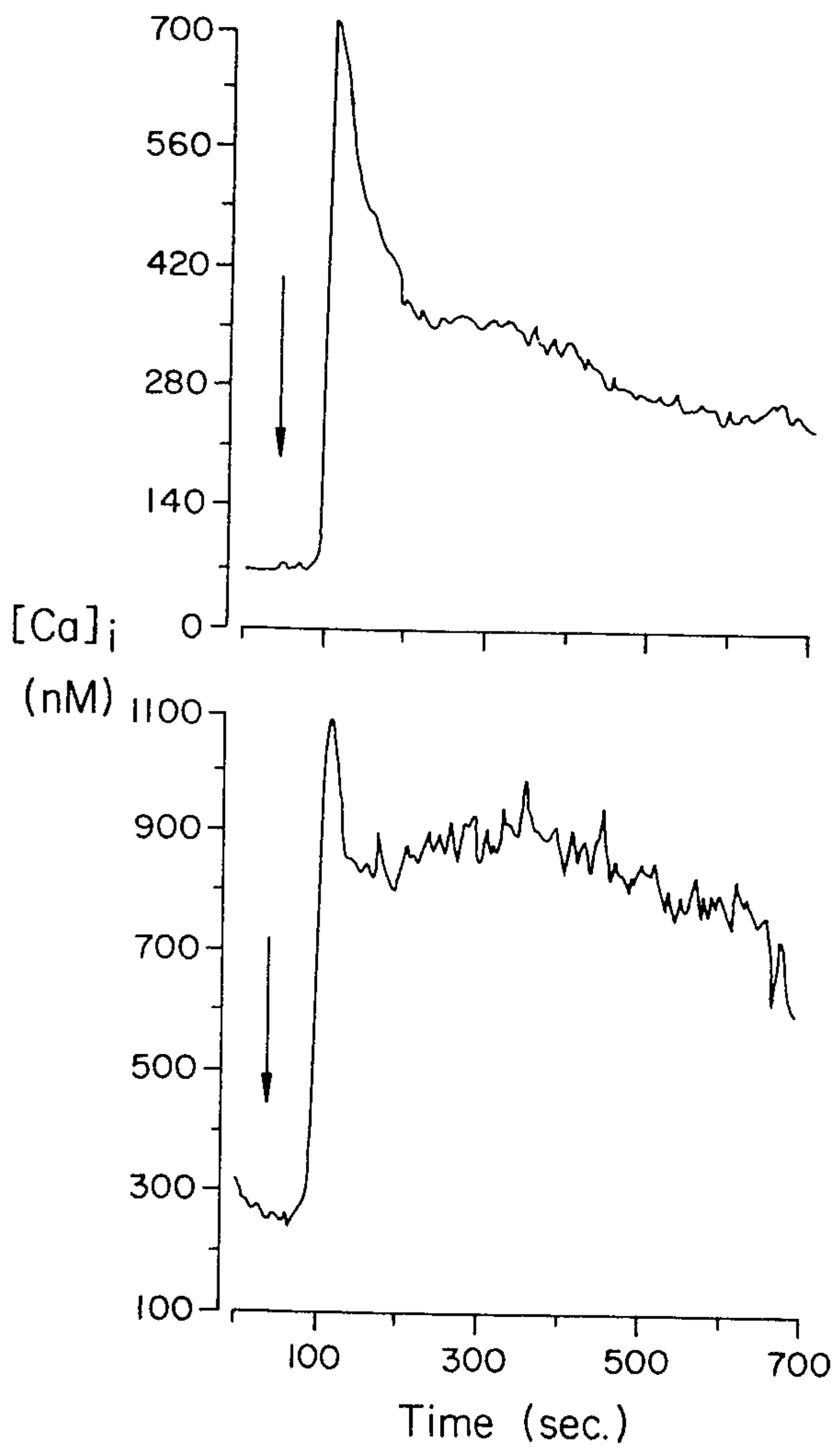
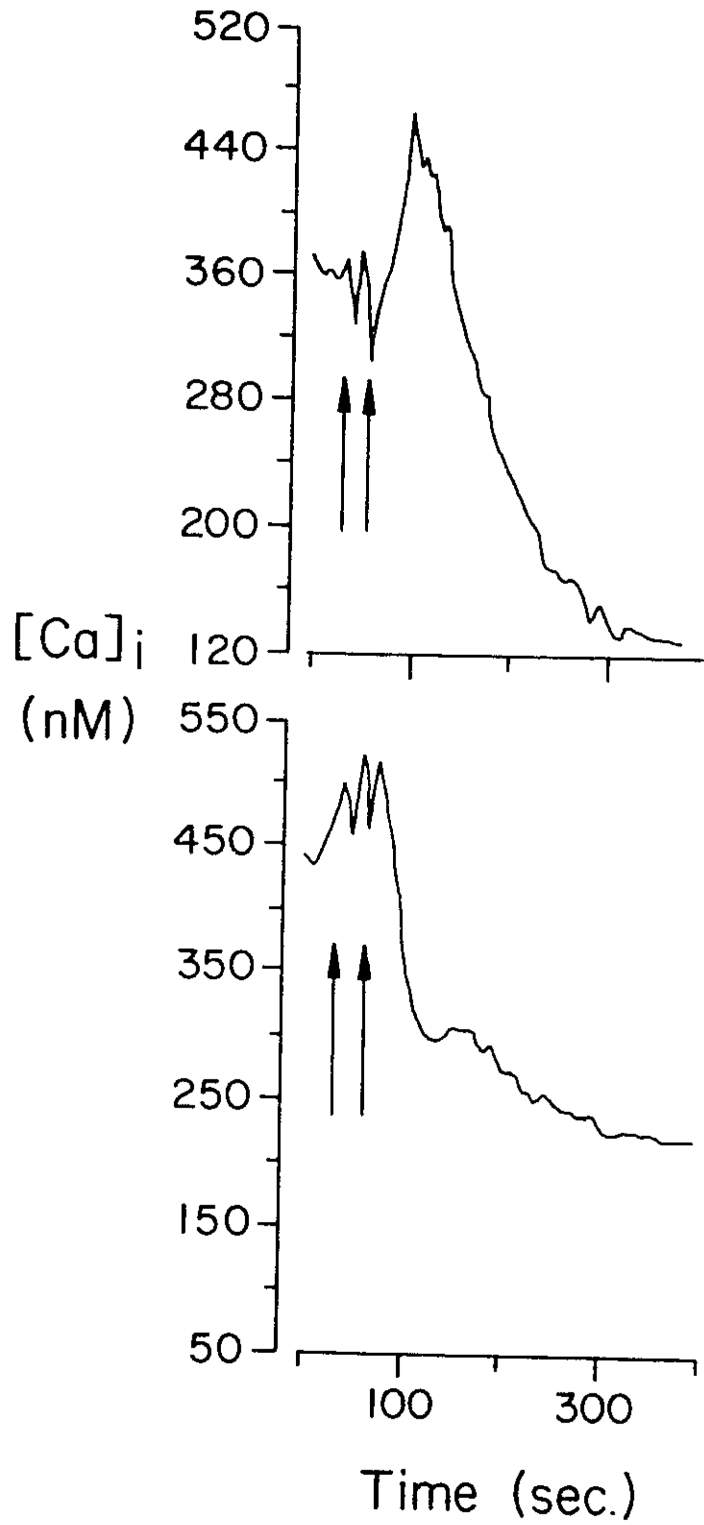

700
560
420
280
140
0
[Ca]i
(nM)
1100
900
700
500
300
100
100
300
500
700
Time (sec.)

Fig. 3-21: Examples of Ca^{2+} transients induced by epinephrine in the absence of external Ca^{2+} in bullhead hepatocytes. The first arrow indicates that the superfusion solution was changed from Ca^{2+}-containing (Medium C, see Table 2-1) to Ca^{2+}-free (2 mM $MgCl_2$ + 0.5 mM EGTA replacing 2 mM $CaCl_2$ in Medium C) solution. The second arrow indicates the addition of 10^{-7}M epinephrine. Exposure to epinephrine induced a rapid decline of $[Ca^{2+}]_i$. In some cells, a small transient rise was initiated (upper panel) while this spike was virtually absence in others (lower panel).



[Ca]i
(nM)
520
440
360
280
200
120
550
450
350
250
150
50
100
300
Time (sec.)

(3) The patterns of Ca_i^{2+} response to epinephrine in rainbow trout hepatocytes

Epinephrine did not induce Ca_i^{2+} responses or induced small increases in $[Ca^{2+}]_i$ in the presence or absence of external Ca^{2+} as previously seen in Figs 3-11 and 3-12. 17 trout hepatocytes from 7 livers were studied with 10^{-7}M epinephrine and external Ca^{2+} present; only 3 cells had a clear but small $[Ca^{2+}]_i$ peak response, while the other cells had no clear response. The presence and absence of external Ca^{2+} did not affect the patterns of cytosolic free Ca^{2+} concentration changes but did affect the magnitude of the $[Ca^{2+}]_i$ peak (Table 3-8) in trout hepatocytes. Phenylephrine and isoproterenol were not assessed in this regard.

CHAPTER 4:

DISCUSSION

DISCUSSION

I. Calcium Status in Fish Hepatocytes

To study Ca^{2+} metabolism in fish hepatocytes, it is essential to know the basal calcium status of the cells, including cellular calcium concentration, free Ca^{2+} concentration and Ca^{2+} adhering to the membranes. In the literature, there is much information about rat hepatocytes and other mammalian cells, but little about fish hepatocytes.

Total cellular calcium concentrations range from 0.4 to 5 $\mu moles \cdot g^{-1}$ of cell water in a variety of animal cells (see Borle, 1968), and a value of around 1 $\mu mole \cdot g^{-1}$ wet wt is considered the calcium concentration of most tissues (Rubin, 1974). Comparatively, fish hepatocyte total calcium concentrations of 8.5 $\mu moles \cdot g^{-1}$ wet weight (eel) and 10.5 $\mu moles \cdot g^{-1}$ wet weight (bullhead) (Table 3-1) are higher, closer to the calcium concentration reported in certain tissues that have specialized mechanisms for sequestering calcium, such as kidney (cortex, 8.9 $\mu moles \cdot g^{-1}$; medulla, 16.9 $\mu moles \cdot g^{-1}$) and salivary gland (16.9 $\mu moles \cdot g^{-1}$) (Rubin, 1974). This high cellular calcium concentration in fish hepatocytes may be due to several reasons. First, species differences. Fish are distinct from mammals in calcium metabolism and calcium sources (see Introduction). Mammals get calcium from their diet and use bone as an internal calcium reservoir. On the other hand, fish get calcium from their environment (water) and their reserves are more easily lost. Fish muscles, for example, contain about four-times more

calcium than mammalian muscles (see Dacke, 1979), thus it may act as an internal Ca^{2+} reservoir besides using the less developed bones. It seems, therefore, reasonable that fish hepatocytes may have higher calcium content than that of the same mammalian cells. Second, calcium contamination. Although efforts were made to remove calcium contamination, it is difficult to remove the contamination completely. Third, extracellular calcium. A significant amount of calcium adhering to the cell membrane, and variable quantities can be removed by different washing procedures used to separate the cells from the extracellular medium, which in turn will lead to variable measured total calcium concentration. In my study, a 40-times isotonic methyl-glucamine solution was used to separate the cells from the extracellular calcium, but since there was no Ca^{2+} ligand added to the solution (e.g., EGTA), the Ca^{2+} adhering to the cell membrane was probably not removed at all. The calcium concentration measured in the fish hepatocytes actually represented the total calcium concentration of intracellular calcium and extracellular calcium adhering to the cell surface. Fourth, the higher $[Ca^{2+}]$ (2 mM) in incubation medium in my study than that (1.3 mM) used in other people's studies in mammalian cells may also lead to a higher calcium content in fish hepatocytes than that in mammalian cells.

According to Borle (1968), the cell coat is the layer of glycoprotein which is present outside the phospholipid layer of the plasma membrane to which Ca^{2+} adheres, and the presence of this cell coat or glycocalyx is a common feature of vertebrate cells. The cell coat can be removed by trypsin-EDTA treatment without affecting the viability or the growth potential of cultured HeLa cells (Borle, 1968). The greater chelation of

Ca^{2+} by EGTA, rather than EDTA, lead to the use of a modified trypsin-EGTA solution in my study to remove the cell coat of bullhead hepatocytes. The membrane adhering Ca^{2+}, 1.88 $\mu moles \cdot g^{-1}$, represented 20% of the total cell calcium or 69% of the exchangeable Ca^{2+} (Tables 3-1, 3-2), which suggested that Ca^{2+} exchange rapidly between the extracellular medium and the surface adhering sites of the cell membrane, in agreement with the so-called fast exchangeable Ca^{2+} pool (Borle, 1969a). The 69% of the exchangeable Ca^{2+} that was in or attached to the cell coat of bullhead hepatocytes, is a value similar to that reported in rat hepatocytes (63%, Claret-Brethon et al., 1977; 70%, Gish et al., 1990), but lower than that in HeLa cells (87%, Borle, 1968). When only EGTA was used in the washing solution, only 27.9% of exchangeable Ca^{2+} was removed from bullhead hepatocytes and 25.6% from eel hepatocytes (Table 3-2). This result supports that of Claret-Brethon et al. (1977) that only part of the extracellular Ca^{2+} is EGTA removable, the other part being trypsin or La^{3+} (lanthanum)-displaceable. The trypsin-EGTA rinse could not be used for eel hepatocytes which were extremely sensitive to this treatment.

Cytosolic free Ca^{2+} concentrations in fish hepatocytes (Table 3-1) were 79.6 nM (eel), 75.7 nM (rainbow trout) and 183.9 nM (bullhead), well within the range of 60 and 200 nM reported in the literature (Carafoli, 1987; Tsien, 1989), but somewhat lower than the 150 to 250 nM, range reported for rat hepatocytes (Charest et al., 1983; Woods et al., 1986; Kawanishi et al., 1989). The higher basal $[Ca^{2+}]_i$ in bullhead hepatocytes compared with those of eel and trout hepatocytes maybe related to the high Ca^{2+} uptake reported in the bullhead hepatocytes compared with the eel (Figs. 3-2, 3-3, 3-4, 3-5). As in other

cells analyzed, fish hepatocyte cytosolic free Ca^{2+} concentrations account for only a minute fraction of the total cellular Ca^{2+} content (Carafoli, 1987; Tsien, 1989).

II. Metabolic Inhibitor Studies

Many investigations have been made on the energy dependence of the Ca^{2+} flux process. Three general trends have been found in the Ca^{2+} influx data as summarized by Borle (1981b). First, metabolic inhibitors are found to depress Ca^{2+} uptake, supporting the proposal that calcium uptake is energy dependent. Second, most investigators have reported that metabolic inhibitors have no effect on Ca^{2+} uptake and concluded that uptake was a passive transport process; the data obtained in this thesis (Fig. 3-3A) with eel hepatocytes is in excellent agreement with these reports. Third, some studies have shown that specific inhibitors stimulated Ca^{2+} uptake, which maybe due to the inhibition of the plasma membrane (Ca^{2+}-Mg^{2+}) ATPase pump and, therefore, Ca^{2+} efflux. My results with bullhead hepatocytes (Fig. 3-4A) are similar to these observations. The effect on the bullhead cells Ca^{2+} influx, however, was significant at only one time point. Overall, my studies seem that Ca^{2+} uptake in fish hepatocytes (i.e., eels and bullheads) is a passive or energy-independent process.

Most data support the proposal that Ca^{2+} efflux is an energy-dependent, active transport process at least in HeLa cells and rat hepatocytes (Borle, 1969b; Chan and Junger, 1983; Lin, 1985a; 1985b). A recent study by Sulakhe (1990) also demonstrated an active Ca^{2+} efflux process in plasma membranes from the livers of a wide variety of vertebrate species from amphibians to mammals. My study with fish hepatocytes found

that Ca^{2+} efflux was not significantly blocked and intracellular Ca^{2+} concentration did not increase as much as expected in the presence of metabolic inhibitors (Figs. 3-1, 3-3B, 3-4B). This result may be due to the action of potassium cyanide. Cyanide, although decrease [ATP], is reported to stimulate Ca^{2+} efflux in exchange for external Na^+ (Blaustein and Hodgkin, 1968). ATP significantly decreased in the presence of the metabolic inhibitors (IAA + KCN) (Fig. 3-1), K^+_{in} : Na^+_{out} active exchange was inhibited, leading to a decrease in intracellular [K^+] and an increase in intracellular [Na^+]; however, my result did not show such a [Na^+] change because of the large Na^+ contamination noted in the results. Given a "calcium pump" for Ca^{2+} efflux (Exton, 1988; Kraus-Friedmann, 1990), an increased intracellular Ca^{2+} concentration should be observed in the presence of metabolic inhibitors. The lack of a significant increase in intracellular [Ca^{2+}] (Fig. 3-1) in my study may be due to two reasons: 1) increased Ca^{2+} efflux driven by downhill Na^+ influx caused by cyanide (Blaustein and Hodgkin, 1968); and/or, 2) increased Ca^{2+} efflux driven by an increased intracellular Ca^{2+} concentration caused by the inhibition of active Ca^{2+} uptake by mitochondria (Borle, 1968). These two reasons also give a possible explanation for the fact that Ca^{2+} efflux was not significantly blocked in the presence of metabolic inhibitors (IAA + KCN) in my study. Although the results are equivocal, I have provided some evidence that Ca^{2+} efflux in fish hepatocytes maybe an active transport mechanism.

III. Relationship between Ca^{2+} Fluxes and $[Ca^{2+}]_i$ in Fish Hepatocytes

Ca^{2+} uptake was not significantly stimulated by epinephrine and phenylephrine in

eel and bullhead hepatocytes (Fig. 3-5). In comparison of Ca^{2+} influx in these two species, it was found that Ca^{2+} uptake in bullhead hepatocytes was more than two-times higher than that of eel cells (Figs. 3-2, 3-3A, 3-4A, 3-5). On the other hand, Ca^{2+} efflux was significantly increased by epinephrine (Figs. 3-7B, 3-8D) and phenylephrine (Fig. 3-8E) in eel hepatocytes, but not in bullhead hepatocytes (Fig. 3-8A, B). These observations that Ca^{2+} efflux rather than Ca^{2+} uptake was significantly stimulated by α-agonists and other Ca^{2+} mobilizing hormones are agreed upon by most workers using the perfused liver or isolated hepatocytes (Efflux: Chen et al., 1978; Althaus-Salzmann et al., 1980; Blackmore et al., 1978; 1982; 1983. Uptake: Morgan et al., 1982). The stimulated Ca^{2+} efflux can be explained by the activation of the Ca^{2+} pump located on the plasma membrane resulting from increased cytosolic Ca^{2+} (Exton, 1988; Kraus-Friedmann, 1990). That bullhead cell Ca^{2+} efflux was not stimulated by catecholamines and increased cytosolic $[Ca^{2+}]_i$ may be due to a low densities of Ca^{2+} pumps on the cell membranes or an inhibitory effect of strong Ca^{2+} influx trend. The insignificant increases noted in Ca^{2+} uptake in this study may have been due to two reasons 1) an inhibitory effect on the concomitant rise in $[Ca^{2+}]_i$. 2) a stimulated Ca^{2+} influx may be masked, given the higher ^{45}Ca background value. Although not significant, Ca^{2+} uptake was stimulated as shown by others (Blackmore et al., 1984; Mauger et al., 1984; Joseph et al., 1985; Poggioli et al., 1985; Altin and Bygrave, 1987). A stimulated Ca^{2+} uptake depends on an activation of membrane receptors by the hormones (Mauger et al., 1984), an increased Ca^{2+} transport through the fixed number of resting channels (Poggioli et al., 1985), or an increased permeability of the plasma membrane for external Ca^{2+} (Joseph et al., 1985). Comparing

Ca^{2+} flux between eel and bullhead hepatocytes, Ca^{2+} influx was more dominate in bullhead than in eel, but Ca^{2+} efflux was more dominate in eel than in bullhead hepatocytes (compare above Figures). The inward Ca^{2+} movement in bullhead and outward movement in eel hepatocytes reflect the major sources from which Ca^{2+} was mobilized to increase $[Ca^{2+}]_i$ as demonstrated in Fig. 3-10. This hypothesis was supported by the study which demonstrated that external Ca^{2+} did not affect the $[Ca^{2+}]_i$ increase (Table 3-8; Fig. 3-10), and that Ca^{2+} from intracellular Ca^{2+} pools was mobilized in eel hepatocytes (Table 3-9). In contrast, bullhead hepatocytes were dependent on external Ca^{2+} to increase $[Ca^{2+}]_i$ (Table 3-8; Fig. 3-10). It can be concluded that in eel hepatocytes, Ca^{2+} efflux was stimulated secondarily to the $[Ca^{2+}]_i$ rise and reflected the increased activity of the plasma membrane Ca^{2+} pump due to this rise, which collaborates reports by other authors (Studer and Borle, 1984; Joseph et al., 1985; Freudenrich and Borle, 1988; Studer and Ganas, 1989) that Ca^{2+} efflux was directly related to the rise in $[Ca^{2+}]_i$. In bullhead hepatocytes, the phenomena was quite different from that of eel hepatocytes, as the rise in $[Ca^{2+}]_i$ was secondary to Ca^{2+} influx; i.e., the increased $[Ca^{2+}]_i$ was mainly from external Ca^{2+} sources although the mobilization of Ca^{2+} from intracellular Ca^{2+} pool(s) can not be excluded.

IV. Sources from Which Ca^{2+} Was Mobilized by Epinephrine

My study with Fura-2-loaded eel hepatocytes supported the hypothesis that the initial increase in $[Ca^{2+}]_i$ was the result of Ca^{2+} mobilization from some intracellular Ca^{2+} pools as reported by others (see Exton, 1988). The mobilization of the internal stores was

confirmed by two observations. 1) initial Ca^{2+} transients were not affected by external Ca^{2+} (Fig. 3-10). 2) repeated exposures of eel hepatocytes to epinephrine at 10^{-7}M lead to a reduction in the amount of free $[Ca^{2+}]_i$ (Table 3-9). The possibility of the desensitization of the cell to epinephrine was excluded by the results that showed successive exposures of hepatocytes to submaximal epinephrine dose could induce $[Ca^{2+}]_i$ increases (i.e., mobilization of Ca^{2+} from intracellular stores), but larger doses could not maintain this linear increase in $[Ca^{2+}]_i$ (Table 3-10). In addition, other authors (Morgan et al., 1982; Charest et al., 1983) have reported the depletion of internal Ca^{2+} stores in rat hepatocytes, without desensitization occurring. In contrast to eel hepatocytes, bullhead hepatocytes depend upon extracellular Ca^{2+} sources (Ca^{2+} influx) to increase $[Ca^{2+}]_i$ (Fig. 3-10). This dependence upon external Ca^{2+} has also been observed in rat pinealocytes (Sugden et al., 1987). This dependence upon Ca_e^{2+} was confirmed by repeated exposures of bullhead hepatocytes to large dose of epinephrine (10^{-7}M) which always induced a similar increase in $[Ca^{2+}]_i$ (Table 3-11) providing no evidence for intracellular store depletion as observed in eel hepatocytes. It can be concluded, therefore, that the Ca^{2+} source (intracellular or extracellular) from which Ca^{2+} is mobilized by epinephrine are fish species dependent, as well as cell-type dependent.

V. Dose Response of Epinephrine on Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_i$) in Eel Hepatocytes

Ca_i^{2+} responded to epinephrine administration in eel hepatocytes in a manner similar to that reported in rat hepatocytes. The epinephrine concentration required to

achieve half-maximal activation or K_a, was 3 x 10^{-8}M in eel (Fig. 3-9) which was the same as that reported in rat hepatocytes (Studer and Ganas, 1989). The response curve (Fig. 3-9) indicates that even 10^{-11}M to 10^{-9}M epinephrine induced a small Ca_i^{2+} response, while 10^{-7}M to 10^{-5}M epinephrine saturated this response. Eel hepatocytes demonstrated high sensitivities to epinephrine in the range of 10^{-9}M to 10^{-7}M; i.e., a small change in epinephrine concentration caused a large change in cytosolic free $[Ca^{2+}]$. This condition reflects the role that epinephrine and $[Ca^{2+}]_i$ **may** play in the control of eel hepatocyte metabolism in vivo. The following three points are important. First, epinephrine concentrations as low as 10^{-13}M can induce a detectable rise of $[Ca^{2+}]_i$ in 50% of the cells tested (Fig. 3-14). Epinephrine concentrations in the range 10^{-11}M to 10^{-9}M induced a similar response in $[Ca^{2+}]_i$, and these are within the physiological range of epinephrine concentrations reported to be 2.5 x 10^{-10}M in the plasma of American eels, *Anguilla rostrata* (Epple et al., 1982). Thus, eel hepatocytes have the ability to respond metabolically during physiological fluctuations in epinephrine concentrations. Second, when epinephrine concentrations increase to the 10^{-9}M to 10^{-7}M range or that range experienced during stress in some fish (Dashow et al., 1982; Plisetskaya et al., 1984; Perry and Wood, 1989), the $[Ca^{2+}]_i$ response increases sharply with epinephrine concentration indicating the possibility of a major metabolic increase. Third, as epinephrine concentrations increased further to 10^{-7}M or even higher to 10^{-5}M, the Ca_i^{2+} response reaches a maximal level; this response level maybe set to protect the hepatocytes against the exhaustion of $[Ca^{2+}]_i$, but more likely by the saturation of the system which triggers changes in $[Ca^{2+}]_i$ (see VIIC).

Epinephrine at 10^{-7}M induced a net increase in $[Ca^{2+}]_i$ of about 200 nM in eel hepatocytes (Tables 3-8, 3-9; Figs. 3-10, 3-16), which was about 80 nM less than that reported in rat hepatocytes at the same epinephrine concentration (Studer and Ganas, 1989). However, a similar epinephrine concentration increased $[Ca^{2+}]_i$ by about 430 nM in bullhead hepatocytes (Table 3-8) which was much higher than reported for rat hepatocytes (Studer and Ganas, 1989) and similar to the change in $[Ca^{2+}]_i$ induced by 10^{-6}M epinephrine in rat hepatocytes (Charest et al., 1983; Studer and Ganas, 1989). These species differences may reflect the hormone sensitivity of the Ca^{2+} pool and/or its size. In eel hepatocytes, the intracellular Ca^{2+} pool size may be smaller than that in rat hepatocytes, while in bullhead hepatocytes, the extracellular Ca^{2+} sources are mobilized and these can be very high (see Introduction).

VI. α- and β-adrenergic Responses in Ca^{2+} Fluxes and Cytosolic Free Ca^{2+} Concentration

A) α- and β-adrenergic responses in eel hepatocytes

The α-adrenergic response dominates the control of rat liver metabolism (Sherline et al., 1972; Exton, 1979; 1985; 1988). There is, however, much species variability as to which adrenergic system dominates mammalian liver metabolism (see Moon, 1988; Moon and Mommsen, 1990). My study using eel hepatocytes is the first to provide evidence that an α_1-adrenergic system is present in any fish hepatic system. This statement is supported by the following four results. First, the classic generalized β-antagonist

propranolol at 10^{-5}M did not block epinephrine at 10^{-7}M from increasing $[Ca^{2+}]_i$, but the classic generalized α-antagonist phentolamine at 10^{-5}M did (Fig. 3-16). In addition, 10^{-7}M phenylephrine (α-agonist) also increased $[Ca^{2+}]_i$, but 10^{-7}M isoproterenol (β-agonist) had little effect on $[Ca^{2+}]_i$ (Fig. 3-17). These results suggest that α_1-adrenoceptors are present and that epinephrine can act via an α_1-adrenergic pathway to produce a Ca_i^{2+} response. Second, consistent with the above results, the significant epinephrine-induced Ca^{2+} efflux was not blocked by propranolol, but the effect was totally blocked by phentolamine (Table 3-4; Figs. 3-7B, 3-8D). In addition, phenylephrine, an α-agonist in rat hepatocytes (Exton, 1985), at 10^{-5}M also stimulated Ca^{2+} efflux and this effect was abolished by the α-antagonist phentolamine (10^{-4}M) (Table 3-5; Figs. 3-7C, 3-8E); the β-agonist isoproterenol (10^{-5}M or 10^{-7}M) in the presence and absence of its antagonist propranolol (10^{-4}M or 10^{-5}M) had no significant effect (Table 3-6; Figs. 3-7D, 3-8F). These results support an α_1-adrenergic role in Ca^{2+} efflux and the presence of α_1-adrenoceptors on eel hepatocyte membranes. Third, the Ca^{2+} efflux (Figs. 3-7 and 3-8) and $\Delta[Ca^{2+}]_i$ (Figs. 3-16 and 3-17) results demonstrate that the potency ranking of the α- and β-adrenergic agonists tested in eel hepatocytes was epinephrine (E) > phenylephrine (PE) >> isoproterenol (ISO). This ranking is consistent with that for the α_1-adrenoceptor system in rat (Lefkowitz and Caron, 1986). This indicates an α-adrenergic response in eel cells. Fourth, the agonists epinephrine and phenylephrine both induced Ca^{2+} oscillations in eel hepatocytes (Fig. 3-18). A recent review by Berridge (1990) indicated that most transient Ca^{2+} oscillations found in non-excitable cells are controlled by activation of the phosphoinositol pathway, which is generally considered in mammals to be linked to the

α- rather than the β-adrenergic system. Thus, the observed Ca^{2+} oscillations in Fura-2-loaded eel hepatocytes again support the involvement of an α_1-adrenergic response.

It is important to note that neither propranolol nor phentolamine when added alone had any effect on the parameters studied (Table 3-12; Fig. 3-7A).

B) α- and β-adrenergic responses in bullhead hepatocytes

Similar to the Ca_i^{2+} responses in eel hepatocytes, epinephrine at 10^{-7}M significantly increased $[Ca^{2+}]_i$ in bullhead hepatocytes and this effect was blocked by phentolamine but not propranolol (Fig. 3-16). Again this result provides evidence for the existence of an α-adrenergic system in bullhead hepatocytes. Given the different Ca^{2+} sources mobilized by epinephrine between eel and bullhead cells, it may be that the α_1-adrenergic response can trigger both a mobilization of Ca^{2+} from intracellular stores (in eel hepatocytes) and an increase in Ca^{2+} uptake from extracellular medium (in bullhead hepatocytes). The evidence that uptake is a possible site for epinephrine effects is that 1) Ca^{2+} uptake is much higher in bullhead than eel cells (Figs. 3-2, 3-3, 3-4, 3-5) although it is not significantly affected by hormones (Fig. 8), and 2) Ca^{2+} efflux is generally slower (Fig. 3-6) and unaffected by hormones (Fig. 3-8A, B, C), unlike the eel. Although these results are equivocal, they do support the membrane route for Ca^{2+} inward movements being key in this species. It should also be noted that uptake values are more difficult to assess given the high background Ca^{2+} values, which could have masked any effects of hormones on this parameter.

To support the hypothesis that stimulated Ca^{2+} influx in increasing cytosolic Ca_i^{2+}

is an α-adrenergic response in bullhead hepatocytes, two relevant reports by other authors studying rat hepatocytes can be cited. First, Mauger et al. (1984) reported that noradrenaline, vasopressin and angiotensin stimulate unidirectional Ca^{2+} influx which is directly related to the activation of α_1-receptors by these hormones since the α-adrenergic antagonist phentolamine can abolish this stimulating effect. An increased Ca^{2+} influx contributed to the cytosolic [Ca^{2+}] increase (Exton, 1988; Kraus-Friedmann, 1990), although the contribution of internal Ca^{2+} release could not be excluded. Second, Woods et al. (1986) and Rooney et al. (1989) reported that vasopressin at a high dose (5 nM) induced one Ca_i^{2+} peak with a sustained after peak Ca_i^{2+} level (although Ca^{2+} oscillations were induced at lower doses). The pattern is similar to that induced by epinephrine in bullhead hepatocytes (Fig. 3-20) (although in my experiments Ca^{2+} oscillations were induced by phenylephrine and isoproterenol rather than lower doses of epinephrine). Considering that both rat and bullhead hepatocytes can exhibit a single-peak Ca_i^{2+} response with sustained Ca_i^{2+} levels and Ca^{2+} oscillations which are agonist- and agonist-concentration-dependent, an exhaustible internal Ca^{2+} pool and the dominate α-adrenergic response in rat hepatocytes, it is possible that this single-peak response in both rats and bullhead hepatocytes is maintained by a stimulated Ca^{2+} influx by increased agonist concentration acting via α-receptor (rat hepatocytes, high dose vasopressin case; bullhead hepatocytes, epinephrine case). This is supported by other authors (Reinhart et al., 1984; Mauger et al., 1984; 1985) that report an increased cytosolic Ca^{2+} concentration in hepatocytes in the presence of agonists such as vasopressin, adrenaline and angiotensin II requires the stimulation of Ca^{2+} influx by the agonists.

C) The adrenergic response of trout hepatocytes

Trout hepatocytes responded weakly to epinephrine doses which resulted in large changes in $[Ca^{2+}]_i$ in both eel and bullhead hepatocytes, but the epinephrine-induced cytosolic Ca^{2+} increase was significantly different from controls (Figs. 3-11, 3-12). This lack of an effect is surprising given that epinephrine does cause a hepatic metabolic effect in vivo and in vitro (e.g., Morata et al., 1982; Wright et al., 1989; Moon and Mommsen, 1990; Michelsen and Sheridan, 1990). This may mean, however, that the epinephrine-effect in the trout liver is entirely a β-adrenergic effect. In agreement with this hypothesis, these authors found that the metabolic effects of epinephrine are blocked by the β-antagonist propranolol. Michelsen and Sheridan (1990) recently reported that epinephrine-stimulated hepatic glycogenolysis in rainbow trout via β-adrenergic system and cAMP, but that incubating the liver pieces in a Ca^{2+}-medium stimulated the magnitude and the time of the glycogenolytic response. This extends the reports by both Birnbaum et al. (1976) and Janssens and Lowrey (1987) that Ca^{2+} modulates fish metabolism; the mode of modulation is unknown at this time.

D) The α-adrenergic-metabolic paradox in fish hepatocytes

The studies reported in this thesis support the existence of an α-adrenergic system mediating changes in hepatic $[Ca^{2+}]_i$ and indirectly the existence of α-adrenoceptors on hepatic membranes of both the American eel and the bullhead. Is there a metabolic effect of these changes in $[Ca^{2+}]_i$? There is strong evidence in at least some mammals that α-adrenoceptor action modify both glycogenolysis and gluconeogenesis (see reviews by

Exton, 1979; 1985; 1988). No such evidence exists in lower vertebrates (fish and amphibians), at least in those species tested to date (see Introduction).

Previous studies in teleosts have shown that epinephrine-induced increases in hepatic glycogenolysis can be blocked by the classic generalized β-antagonist propranolol. This is true in vivo (Wright et al., 1989-trout) and in hepatocytes in vitro (Birnbaum et al., 1976-goldfish; Brighenti et al., 1987-catfish; Janssens and Lowrey, 1987-carp; Danulat and Mommsen, 1990-rockfish). Where propranolol was used, it was found to block the epinephrine effect entirely, supporting a β-adrenergic mode of action for epinephrine in these species. A recent report by Moon and Mommsen (1990) reported that the phenylephrine induced glycogenolytic response of the bullhead was also blocked by propranolol. In no case did phentolamine, where used, block these effects.

Binding studies using both goldfish (Birnbaum et al., 1976) and carp (Janssens and Lowrey, 1987) hepatocytes and displacement assays with α- and β-agonists/antagonists failed to support the existence of α-adrenoceptors; only β-adrenoceptors could be conclusively demonstrated. Brighenti et al. (1987) using catfish and Moon and Mommsen (1990) using eels and bullheads could not eliminate the possibility of an α-adrenergic component to the glycogenolytic response, but if one did exist, it was minor compared to the β-adrenergic effect.

Some of the differences noted between my studies and those in the literature may be partially explained by the use of different species. In no case have the same hepatocytes from the same fish been examined for metabolic and Ca^{2+} effects in the same lab. More likely, however, the differences observed may result from three possibilities.

First, the metabolic experiments generally run for 1 to 2 hrs using a suspension of millions of hepatocytes, while the changes in $[Ca^{2+}]_i$ use single cells and time periods of 10 to 20 min. Second, changes in $[Ca^{2+}]_i$ may have no metabolic role and simply represent noise in the system; this conclusion is supported by the studies of Janssens' group in both amphibians (Janssens et al., 1983; Janssens and Crigg, 1984) and carp (Janssens and Lowrey, 1987). Janssens' group supports the primary role of cAMP as a 2nd messenger of hormone action in lower vertebrates with Ca^{2+} not acting as an intracellular signal. Given the importance of Ca^{2+} as an intracellular signal in mammalian tissues, it is unlikely that this system had not involved somewhere before being "tried" in mammals. Ca^{2+} does play some role in metabolic processes in insects, such as Ca^{2+} activating phosphorylase kinase in cockroach fat body (Pallen and Steele, 1988), and Ca^{2+} activating protein kinase C in the pupal brain of the tobacco hornworm (Qiu et al., 1990); in no case; however, has Ca^{2+} been unequivocally shown to be a second messenger in insects. Third, many cellular effects of cAMP may actually be due to a rise in cytoplasmic $[Ca^{2+}]_i$ (Rasmussen et al., 1984), which may be affected by the interaction between the Ca^{2+} and cAMP transduction systems (see Introduction). There are a number of papers supporting such interactions. First, cAMP levels in *Paramecium* increasing in a dose-dependent manner with a stepped increase in $[Ca^{2+}]$ in the incubation (Schultz et al., 1984). Second, glucagon is traditionally considered to stimulate cAMP-pathway, but it does increase $[Ca^{2+}]_i$ and Ca^{2+} flux in liver at low and presumably physiological doses (Blackmore and Exton, 1986; Altin and Bygrave, 1987). This suggest that Ca^{2+} plays some role in glucagon modulating hepatic function. Third, Michelsen and Sheridan (1990)

reported that epinephrine induce hepatic glycogenolysis via a β-receptor pathway in rainbow trout but Ca^{2+} modulated the cAMP effect; Birnbaum et al. (1976) reported a similar result in goldfish. Fourth, Mommsen and Moon (1989) found no direct relationship between the amount of glucagon-induced cAMP and the physiological response in eel bullhead and trout hepatocytes, suggesting a possible existence of another signal transduction system. Fifth, Moon and Mommsen (1990) reported that phenylephrine increases glycogenolysis and gluconeogenesis in trout, eel and bullhead hepatocytes via a cAMP-pathway and phenylephrine induced a rise in [cAMP]. I have shown in this thesis (Fig. 3-17) that phenylephrine induced an increase in $[Ca^{2+}]_i$ in eel and bullhead hepatocytes. This suggests that the phenylephrine-stimulation of hepatic metabolism maybe a complex interaction between both the cAMP and the Ca^{2+} transduction pathways.

This metabolic paradox does exist, and more studies are needed to unravel the role of Ca_i^{2+} in fish hepatic metabolism.

VII. Calcium Oscillations

Calcium oscillations is a recent subject area. They were first observed by Cuthbertson et al. (1981; 1985) in mouse oocytes during fertilization using the aequorin technique, followed by reports of Ca^{2+} oscillations induced by α_1-adrenergic and other Ca^{2+}-mobilizing agonists in rat hepatocytes with the same technique (Woods et al., 1986; 1987; Schöfl et al., 1991). Ca^{2+} oscillation studies have been expanding since 1985 when it was found that Fura-2 could provide a useful indicator of Ca^{2+} transients and

oscillations in single cells. Ca^{2+} oscillations induced by phenylephrine and vasopressin have be reported in Fura-2-loaded rat hepatocytes (Kawanishi et al., 1989; Rooney et al., 1989; 1990; Reber et al., 1990) and in cells including pituitary cells (Schlegel et al., 1987), pituitary gonadotropes (Shangold et al., 1988; Iida et al., 1991), somatotropes (Holl et al., 1988), endothelial cells (Jacob et al., 1988), muscle cells (Ambler et al., 1988), tumor mast cells (Millard et al., 1988), rat glomerulosa cells (Connor et al., 1987) and REF52 fibroblasts (Harootunian et al., 1991). There have been no reports of Ca^{2+} oscillations in lower vertebrate cells, so my study provides the first evidence for the phenomena of Ca^{2+} oscillations induced by catecholamines in any fish hepatocyte system.

A) Calcium Oscillations in Eel Hepatocytes

Ca^{2+} oscillations were induced in eel hepatocytes consistently by epinephrine (10^{-7}M) (Fig. 3-18) and phenylephrine (10^{-7}M); isoproterenol (10^{-7}M) did induce oscillations but in only 1 cell out of 6 investigated. The oscillation patterns differed from cell to cell, even using cells from the same preparation and using the same agonists. This large cell to cell variation in Ca_i^{2+} oscillation patterns have also been observed by others in Fura-2-loaded hepatocytes (Monck et al., 1988; Rooney et al., 1989; Kawanishi et al., 1989) and in other cell types (Connor et al., 1987; Ambler et al., 1988; Jacob et al., 1988; Millard et al., 1988; Prentki et al., 1988). Rooney et al. (1989) suggested that this heterogeneity in the Ca^{2+} response must be due to a common thing; i.e., differences amongst individual cells including differences in cell sensitivities to agonists, in Ca^{2+} pool size and in Ca^{2+} transport systems such as the densities of Ca^{2+}-pump sites and Ca^{2+}-

channels. I have no evidence that other factors may be operating in this fish system.

External Ca^{2+} also affected Ca^{2+} oscillations. Comparing the oscillations induced by 10^{-7}M epinephrine in the presence (Fig. 3-18) and absence of external Ca^{2+} (Fig. 3-19), external Ca^{2+} appeared not to affect the magnitude of the initial Ca_i^{2+} peak but did result in the Ca^{2+} oscillations disappearing more quickly than in the presence of external Ca^{2+}. An interesting observation was that the magnitude of Ca_i^{2+} peaks decreased regularly and the period between each peak increased regularly until the peaks vanished, which was not the case when external Ca^{2+} was present. It can be concluded that in eel hepatocytes the initial Ca_i^{2+} peak was independent of external Ca^{2+} since Ca^{2+} mobilization was from intracellular Ca^{2+} pools; however, the later Ca^{2+} peaks were dependent upon the refilling of the intracellular pool(s). The source of the Ca^{2+} to refill this pool(s) would be from that Ca^{2+} influxed and released to the cytosol. In the absence of external Ca^{2+}, the Ca^{2+} may efflux from the cell rather than be taken back up into the intracellular pool. Thus, Ca^{2+} pool(s) failed to be refilled. In the presence of external Ca^{2+}, the Ca^{2+} concentration gradient would favour the uptake of Ca^{2+} into the pool(s) rather than its loss. It may also be that external Ca^{2+} does play a role in $[Ca^{2+}]_i$ at these later oscillation periods (see VII C). These observations are similar to those for rat hepatocytes (Rooney et al., 1989) and HeLa cells (Sauvé et al., 1990) that the availability of Ca^{2+} to maintain and to refill the intracellular Ca^{2+} pools is an important determinant of the periodicity of the oscillations. In addition, my observation that epinephrine caused a loss of Ca^{2+} from intracellular stores and an increased Ca^{2+} influx has also been reported by Barritt et al. (1981) in isolated rat liver parenchymal cells.

The effect of agonist concentration on oscillation frequency was also studied, but no direct relationship was found between agonist concentration and oscillation frequency which has been reported by others (Woods et al., 1986; Exton, 1988; Rooney et al., 1989; 1990; Schöfl et al., 1991). The rate of the Ca_i^{2+} rise was independent of agonist dose and the Ca_i^{2+} response induced by different agonists was not obviously different. This may be the case in fish hepatocytes or it may be a result of two things. First, even using the same epinephrine concentration, the frequency and the pattern of the Ca^{2+} oscillations differed from cell to cell, so it is simply too difficult to quantify the frequency of oscillations induced by different epinephrine concentrations and to compare the oscillation patterns induced by different agonists. Second, epinephrine caused the depletion of intracellular Ca^{2+} pools in eel hepatocytes making it impossible to study oscillations using different epinephrine concentrations in one cell for extended time period. It is clear, however, that as in the rat hepatocyte system, eel hepatocytes demonstrate hormone-induced Ca^{2+} oscillations.

By comparing the Ca^{2+} oscillation frequencies between eel and rat hepatocytes, it is found that the frequency in eel cells is generally lower (more than 1 min for eel and 20 to 30 sec for rat (Schöfl et al., 1991)), which may lead to a slow and delayed metabolic response to hormones in fish compared to mammals, and/or to the low experimental temperature (10°C) used for eel compared to the high temperature (37°C) used for rat experiments.

B) Calcium Oscillations in Bullhead Hepatocytes

Results with bullhead hepatocytes indicated that the Ca_i^{2+} response was specific to the agonists used, supporting the concept that each hormone has a specific signature with respect to $[Ca^{2+}]_i$ change (Berridge et al., 1988). Epinephrine induced a single Ca_i^{2+} peak followed by a sustained elevated Ca^{2+} level (Fig. 3-20); no Ca^{2+} oscillations were observed. My results indicated that the changes in Ca_i^{2+} found in bullhead hepatocytes were dependent upon external Ca^{2+}, so in the absence of external Ca^{2+} even this sustained level declines (Fig. 3-21) suggesting a significant efflux of Ca^{2+} occurs. Given the large Ca^{2+} influx necessary to trigger changes in Ca_i^{2+}, Ca^{2+} oscillations do not occur (Kawanishi et al., 1989). The sustained elevated Ca_i^{2+} level was maintained by both a large Ca^{2+} influx and small Ca^{2+} efflux. Berridge (1990) in his review states that oscillations can arise from either fluctuations in Ca^{2+} influx or Ca^{2+} release from internal stores. Although the former occurs primarily in excitable cells, it seems also the case in bullhead hepatocytes where Ca^{2+} oscillations can be induced by phenylephrine or isoproterenol; however, these studies were not undertaken in the absence of external Ca^{2+} so it is premature to say that fluctuations in Ca^{2+} influx are responsible. It may be that the Ca_i^{2+} response induced by different agonists may involve different mechanisms in the same or different species.

C) A Model to Interpret Ca^{2+} Oscillations in Fish Hepatocytes

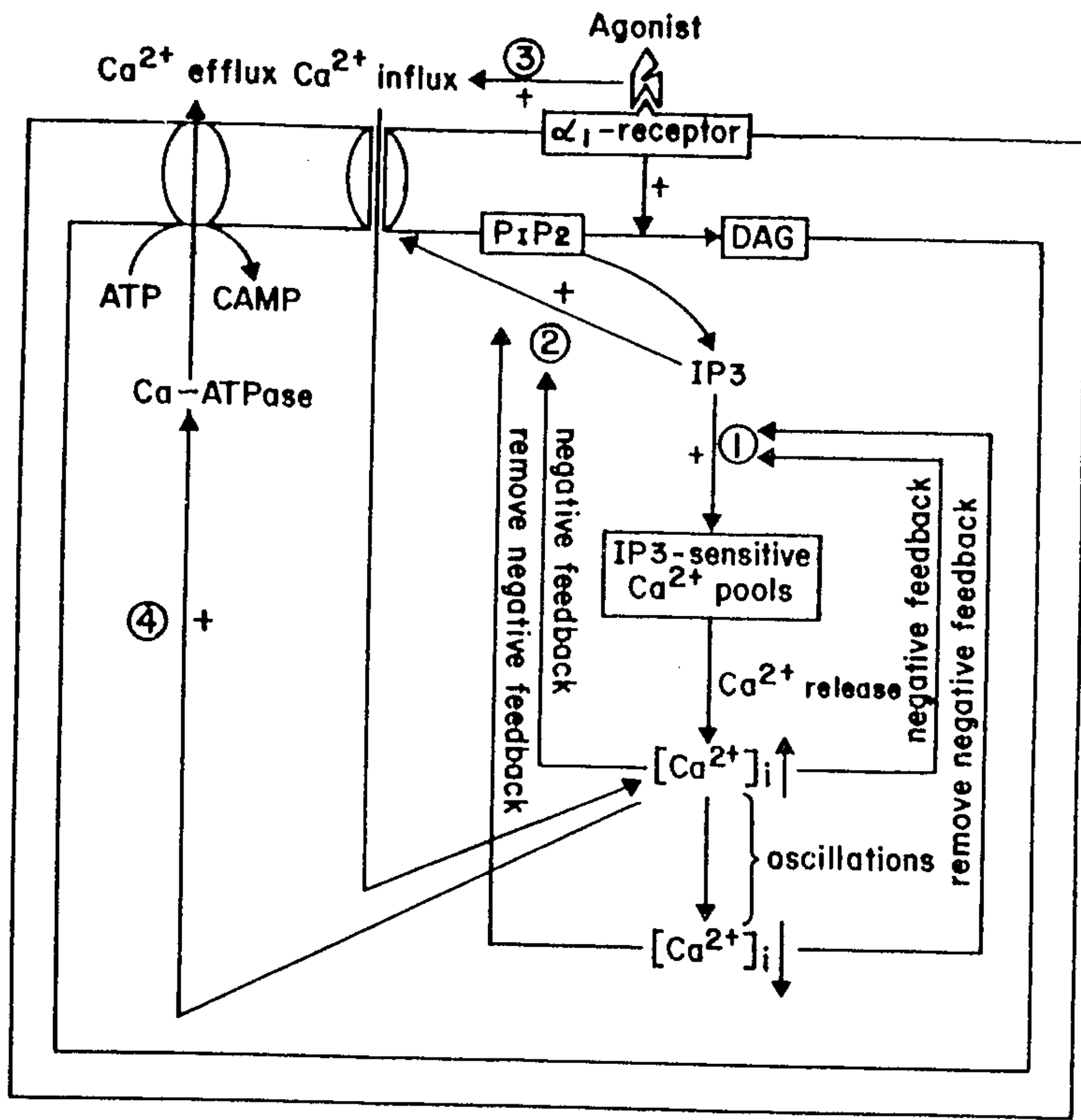
Several models have been proposed to explain the existence of Ca^{2+} oscillations in cells. Each model involves cellular inositol trisphosphate (IP_3) levels. These are divided

into two main classes: IP_3 constant or IP_3 oscillate; $[Ca^{2+}]_i$ feedback positively or negatively on itself. Each class can be further subdivided into two subclasses. With IP_3 constant, one model is increased $[Ca^{2+}]_i$ negative feedback IP_3-induced Ca^{2+} release (Parker and Ivorra, 1990; Payne et al., 1990); the other model is increased $[Ca^{2+}]_i$ positive feedback its release; such as a two-pool model where IP_3 stimulates Ca^{2+} entry and transfers Ca^{2+} from an IP_3-sensitive internal pool to IP_3-insensitive pools from which Ca^{2+} is released by Ca^{2+} induced Ca^{2+} release (Berridge and Irvine, 1989; Rooney et al., 1989; Goldbeter et al., 1990; Berridge, 1991; Dupont et al., 1991). When IP_3 oscillates, one model is that the released Ca^{2+} acts as a negative feedback on IP_3 formation (in which protein kinase C is involved), such that IP_3 oscillations lead to oscillations in the release of Ca^{2+} (Woods et al., 1987; Berridge et al., 1988; Cobbold et al., 1991); or the other model is a positive feedback of released Ca^{2+} on IP_3 production which releases Ca_i^{2+} until Ca^{2+} stores were essentially depleted (Meyer and Stryer, 1988).

In the absence of data on cytosolic $[IP_3]$, it is impossible to know which of these models is the most appropriate explanation of hormone-induced Ca^{2+} oscillations in eel and bullhead hepatocytes. Fig. 4-1 presents a model which fits my data, which is related to the model of constant IP_3 and increased $[Ca^{2+}]_i$ decreasing IP_3-induced Ca^{2+} release (Parker and Ivorra, 1990; Payne et al., 1990) and which leads to testable hypotheses for further study.

The agonist and IP_3 are related in that a given agonist dose results in a specific increase in cytosolic $[IP_3]$ since IP_3 generation is proportional to receptor occupancy (Lynch et al., 1985); the $[IP_3]$ and $[Ca^{2+}]_i$ are related in that certain $[IP_3]$s induce specific

Fig. 4-1: A model to interpret Ca^{2+} transients in fish hepatocytes. The number in parentheses indicate the routes of Ca^{2+} mobilization and Ca^{2+} transport. For eel hepatocytes, route (1) is dominate, route (2) and (3) also may operate, and route (4) maybe significant. For bullhead cells, route (3) is dominate, route (1) and (2) may operate, and route (4) is insignificant. This represents only a proposal and many studies are needed to be done to confirm or reject it. Abbreviation: DAG - diacylglycerol, PIP_2 - phosphatidylinositol 4,5-bisphosphate, IP_3 - myo-inositol 1,4,5-trisphosphate.


Agonist
Ca2+ efflux
Ca2+ influx
③
+
α1-receptor
+
PIP2
DAG
ATP
CAMP
+
②
IP3
Ca-ATPase
negative feedback
remove negative feedback
+
①
IP3-sensitive
Ca2+ pools
④
+
Ca2+ release
negative feedback
remove negative feedback
[Ca2+]i
oscillations
[Ca2+]i

increases in $[Ca^{2+}]_i$ (Eugling et al., 1991). This direct relationship between agonist, $[IP_3]$ and $[Ca^{2+}]_i$ can be drawn from the epinephrine - $[Ca^{2+}]_i$ dose response curve (Fig. 3-9), which demonstrates a direct relationship between agonist doses (submaximal doses) and $[Ca^{2+}]_i$ and which also indirectly indicates a positive relationship between $[IP_3]$ and $[Ca^{2+}]_i$. The induced increase in $[Ca^{2+}]_i$ is from both Ca^{2+} release from IP_3-sensitive pools and stimulated Ca^{2+} influx. The stimulated Ca^{2+} influx includes both IP_3-dependent Ca^{2+} influx supported by the findings that IP_3 activates transmembrane Ca^{2+} channels in T-lymphocytes (Kuno and Gardner, 1987) and *Xenopus* oocytes (Parker and Miledi, 1987), and the IP_3-independent Ca^{2+} influx is supported by the observations that cytosolic Ca^{2+} is elevated by certain agonists with no detectable IP_3 increase (Merritt et al., 1986; Tashjian et al., 1987; Merritt and Rink, 1987) which may be cAMP linked and partly explain the paradox mentioned in VI D. A rise in $[Ca^{2+}]_i$ induces Ca^{2+}-uptake back into cellular Ca^{2+} pools and/or Ca^{2+} efflux (Exton, 1988) and decreases the sensitivity of the IP_3-sensitive pools to IP_3 (i.e., decrease Ca^{2+} release) and Ca^{2+} channels to IP_3 (i.e., decrease IP_3-dependent Ca^{2+} influx, which can be confirmed by reports of an inverse relationship between Ca^{2+} influx and $[Ca^{2+}]_i$ (see III)). As $[Ca^{2+}]_i$ decreases, the negative feedback is removed, and IP_3 further increases Ca^{2+} release and Ca^{2+} influx. Ca^{2+} oscillations in eel hepatocytes may result from this mechanism predominating; given the relative high Ca^{2+} efflux and low influx in these cells, Ca_i^{2+} depletion would result which would decrease the magnitude of the successive Ca^{2+} oscillation peaks. In the absence of external Ca^{2+}, this depletion could be exaggerated.

Hormone-induced Ca^{2+} oscillations are more complex as phenylephrine and

isoproterenol but not epinephrine resulted in oscillations in bullhead hepatocytes. The epinephrine-induced a single Ca^{2+} peak with a sustained after-peak Ca_i^{2+} response may be due to the following four aspects. First, epinephrine stimulated increased $[Ca^{2+}]_i$ by increasing Ca^{2+} influx by mainly an IP_3-independent mechanism, which always exists when the agonist is present (Mauger et al., 1984). Second, the effect of IP_3-dependent Ca^{2+} influx and Ca^{2+} release on increasing $[Ca^{2+}]_i$ was so small that the negative feedback of increased $[Ca^{2+}]_i$ on the IP_3 effect caused little changes of $[Ca^{2+}]_i$. Third, as there was no change in cytosolic Ca^{2+} stores, uptake into these stores would be small. Fourth, Ca^{2+} efflux was relatively slow as observed in the ^{45}Ca studies. Phenylephrine and isoproterenol must act via a mechanism similar to that in the eel for epinephrine, or $[Ca^{2+}]_i$ must modify the influx system.

It must be remembered that this model is only a proposal and other studies must be undertaken before any conclusive model can be developed.

VIII. Conclusion

This study has provided new information on the role of Ca^{2+} in fish hepatocytes. Specifically, the following is a listing of significant contributions.

1) The lack of information concerning Ca^{2+} and the role of Ca^{2+} in lower vertebrates has been observed. Changes in $[Ca^{2+}]_i$ do occur at least in American eel and bullhead hepatocytes to applied hormones; the metabolic consequences of these changes are poorly understood at this time.

2) The proposal that lower vertebrate liver cells employ only cAMP as a sole

intracellular messenger must be re-evaluated given the finding that Ca^{2+} plays a role in catecholamine action in fish hepatocytes.

3) Ca^{2+} metabolism and its cellular role is cell type-dependent, species-dependent and agonist-dependent. There are significant species differences in Ca^{2+} metabolism among American eel, brown bullhead and rainbow trout.

4) The significant species difference in Ca^{2+} metabolism may provide a new way to look at and to explain the significant species difference in physiological responses to applied hormones.

5) This thesis provides the first evidence for the possible existence of α-adrenoceptors on fish hepatocyte membranes.

6) This thesis is the first to show agonist-induced Ca^{2+} oscillations in Fura-2-loaded fish hepatocytes.

7) Eel hepatocyte Ca^{2+} responses show many similarities to those of rat hepatocytes, which suggest that the mechanism of Ca^{2+} mobilization by catecholamines between these species may be similar.

8) Ca^{2+} plays an important role in catecholamine actions as noted by the observations that external Ca^{2+} supply is required for epinephrine to exert its action (bullhead) or for the maintenance of the hormone action (eel).

9) Evidence indicates that Ca^{2+} influx may be a passive process and Ca^{2+} efflux may be an active process in fish hepatocytes.

CHAPTER 5:

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