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Cardiac β-Adrenergic Receptor Function & Plasma Membrane Physical Properties in Cirrhotic Cardiomyopathy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a dissertation entitled "Cardiac β-Adrenergic Receptor Function & Plasma Membrane Physical Properties in Cirrhotic Cardiomyopathy" submitted by Zenghua Ma in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

To clarify the underlying mechanisms for cirrhotic cardiomyopathy, cardiac β-adrenergic receptor signal transduction pathway function and plasma membrane physical and chemical properties were studied in a cirrhotic rat model. Cardiac β-adrenergic receptor function was correlated with membrane physical properties.

Cirrhosis was induced by bile duct ligation (BDL), while controls were sham-operated (Sham). Cardiac performance was assessed by measuring left ventricular papillary muscle contractility. Cardiac β-adrenergic receptor characteristics were evaluated by a receptor binding assay using ^{3}H-dihydroalprenolol as a radioligand. β-Adrenergic receptor signalling was determined by measuring cAMP production via stimulating the receptor with isoproterenol, Gs-protein with fluoride ions, and adenylyl cyclase with forskolin. Cardiac plasma membrane G-protein expression was assayed by Western blot analysis to detect Gsα, Gi2α, and Gcommonβ, respectively. The membrane physical properties were evaluated by determining membrane fluidity using a fluorescent depolarization method. Cardiac plasma membrane total cholesterol and phospholipid levels were determined by colorimetric analysis, and fatty acid components by gas-chromatography.

Maximum cardiac papillary muscle contractile responses and β-adrenergic receptor density were significantly reduced in BDL without significant changes in the ED_{50} and Kd values. cAMP production was significantly lower in BDL than Sham. The expression of Gsα and Gi2α was decreased in BDL compared with Sham. Gcommonβ expression did not differ between the groups. The cardiac plasma

membrane was more rigid in the BDL group, apparently caused by an increase in the cholesterol/phospholipid ratio since there was no gross changes in fatty acid composition. When the membranes from BDL were fluidized to Sham values, cAMP generation stimulated at the receptor level was restored. However, membrane fluidization had no effect on cAMP production stimulated at G-protein and adenylyl cyclase levels

In conclusion, this study demonstrated that myocardium contractile function was attenuated in cirrhosis, confirming the existence of cirrhotic cardiomyopathy. Blunted cardiac performance was associated with diminished β-adrenergic receptor signalling function and alteration in the receptor density and G-protein expression. Changes in membrane fluidity may affect the receptor and G-protein coupling.

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To my family,

whose understanding, support, and sacrifice made this work possible

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

LIST OF TABLES

CHAPTER FOUR

CHAPTER SIX

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
A_2C	2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate
ALT/GPT	alanine aminotransferase
16-AP	16-(9-anthroyloxy)palmitic acid
AST/GOP	aspartate aminotransferase
ATP	adenosine triphosphate
βAR	β-adrenergic receptor
βARK	β-adrenergic receptor kinase
BDL	bile duct ligated cirrhotic rats
Bmax	receptor density
cAMP	3':5'-cyclic adenosine monophosphate
CCl_4	carbon tetrachloride
CICR	calcium induced calcium release
ED_{50}	half maximal effective concentration
EDRF	endothelium-derived relaxing factors
Emax	maximum contractile responses
G$\beta\gamma$	$\beta\gamma$ subunits of G-protein
Giα	α-subunit of inhibitory G-protein
G-protein	guanine nucleotide binding protein
Gsα	α-subunit of stimulatory G-protein
GTP	guanosine triphosphate

Kd	receptor dissociation constant
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LVET	left ventricular ejection time
msec	millisecond
n-AS	*n*-(9-anthroyloxy)-stearic fatty acid
nmol	nanomole
NNA	N^{ω}-nitro-L-arginine
PEP	pre-ejection period
PKA	cAMP dependent protein kinase
r_s	anisotropy parameter
Sham	sham-operated rats
SR	sarcoplasmic reticulum
pmol	picomole
μmol	micromole

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The aim of the present investigation was to clarify the possible underlying mechanisms of cirrhotic cardiomyopathy. The research was focused on cardiac β-adrenergic receptor signalling pathway function and its relationship with plasma membrane physical and chemical properties. Therefore, a brief literature review on liver cirrhosis, cardiovascular complications in cirrhosis, β-adrenergic receptor signal transduction, and plasma membrane physical properties will set the stage for the further description of the project.

1.1. INTRODUCTION OF LIVER CIRRHOSIS

Cirrhosis is a common chronic liver disease (Sherlock, 1993, Conn and Allerbury, 1993). Some of the present knowledge about liver cirrhosis should be credited to the early work of Laennec who first thoroughly described the pathological features of liver cirrhosis in 1826 (Conn and Allerbury, 1993). Since then, liver cirrhosis has often been referred as Laennec's cirrhosis to acknowledge his contribution in the field. Multiple pathogeneses can lead to liver cirrhosis. The most common ones are viral hepatitis B, hepatitis C, and alcohol abuse. Others include biliary cirrhosis and Wilson's disease, as well as α_1-antitrypsin deficiency. Hepatic injury caused by some medications and toxins can also result in liver cirrhosis. Different organs and systems in the human body are inter-related. The dysfunction of one organ could influence others. In this regard, chronic heart failure can cause cardiac cirrhosis. Vice versa, liver diseases can also lead to cardiac function impairment. This will be the main theme of the present research.

Pathological characteristics of liver cirrhosis are fibrosis and nodule formation (MacSween, 1980). During the process of the disease, parenchymal tissue necrosis and regeneration co-exist. Following hepatic cell necrosis, fibrotic tissues fill in, forming the basis of fibrosis. Compensatory hepatocyte regeneration leads to nodule formation. This unique pathological process causes hepatic architecture distortion. Because of the fibrosis and nodule formation, the vasculature is distorted, which forms the anatomical basis for portal hypertension.

Symptoms in cirrhosis can be attributed to two major aspects, hepatocellular insufficiency and portal hypertension. Hepatocyte inflammation and necrosis account for the liver function insufficiency, while fibrosis and distorted liver architecture lead to portal hypertension. Because of hepatic insufficiency, patients usually suffer from fatigue, indigestion, edema, bleeding, elevations of bilirubin and aminotransferases. Ascites and coma or hepatic encephalopathy often occur in advanced cirrhosis. Esophageal varices, portal-systemic shunting, and neutropenia are common consequences of portal venous hypertension. Kidney function is also impaired, manifested as sodium and water retention.

Besides the above common clinical presentations, there are two major cardiovascular complications observed in liver cirrhosis, hyperdynamic circulation and impairment of cardiac contractile performance. Hyperdynamic circulation also exists in portal hypertension without cirrhosis. While these complications are closely interrelated, in order to cogently describe their characteristics, the research work about these two aspects will be reviewed separately as follows.

1.2. HYPERDYNAMIC CIRCULATION IN CIRRHOSIS

1.2.1. General characteristics of hyperdynamic circulation in cirrhosis

The characteristics of the hyperdynamic circulation in liver cirrhosis are that the cardiac output is increased at baseline; systemic and regional blood flows are increased; peripheral vascular resistance is decreased; and arterial blood pressure is decreased (Abelmann, 1994; Abelmann *et al.*, 1955; Kontos *et al.*, 1964). In spite of the hyperdynamic circulation, several vascular beds behave as if circulation or blood flow is insufficient to meet their needs. For example, the kidneys retain salt and water, eventually leading to the development of ascites. In addition, some cardiovascular reflexes, such as increased splanchnic blood flow after meals, and responses to exercise are abnormal. Cardiac contractile function, although hyperdynamic at baseline, is noted to be distinctly abnormal when stressed by different physiological or pharmacological stimuli. In both cirrhotic humans and animal models, there is evidence of decreased positive inotropic (contractile force) and chronotropic (heart rate) responses to β-adrenergic receptor stimulation (reviewed in Lee, 1989; Ramond *et al.*, 1986). These blunted chronotropic and inotropic responses have been termed cirrhotic cardiomyopathy (Rapaport 1989; Lee, 1989). Impairment of cardiac contractile function will be discussed in detail in cirrhotic cardiomyopathy section.

1.2.2. Possible mechanisms for hyperdynamic circulation in cirrhosis

It remains unclear what causes hyperdynamic circulation in cirrhosis and

portal hypertension. Several lines of inquiry have been carried out to clarify the underlying pathogenic mechanisms. These mainly include humoral factors, neural factors, and plasma volume expansion.

1.2.2.1. Humoral factors

Humoral factor effects on hyperdynamic circulation in cirrhosis and portal hypertension have been under intensive research during the past decades. A cross-perfusion study by Benoit *et al* demonstrated that humoral factors play an important role in the genesis of hyperdynamic circulation in portal hypertensive rats. In that study, normal rats were cross-perfused with the blood from portal hypertensive rats. The results showed that the recipient rats developed hyperdynamic circulation during the cross-perfusion period (Benoit *et al.*, 1984). Many vasodilatory humoral factors have been investigated such as nitric oxide, bile salts, adenosine, glucagon, vasoactive intestinal peptide (VIP), histamine, prostaglandins, and endogenous opioids. It is not difficult to understand the role that humoral factors play in the cirrhotic hyperdynamic circulation if one considers the existence of portal systemic shunting in cirrhosis. Because of systemic shunting, some humoral factors with vasoactive properties by-pass liver inactivation and enter into the systemic circulation leading to vasodilation. Alternatively, hepatocyte function is insufficient in cirrhosis, therefore, vasodilators may not be inactivated properly by the liver. In the following sections, several important humoral factors will be briefly reviewed to illustrate their effects on hyperdynamic circulation in cirrhosis and portal hypertension.

Nitric oxide

The importance of nitric oxide (NO) has been appreciated in the last few years. Nitric oxide was selected as the molecule of the year in 1992 (Koshland, 1992; Gulotta and Koshland, 1992). Nitric oxide is a gas with a half-life of about 3-4 seconds *in vitro*. It is synthesized by NO synthase using L-arginine as a substrate. Nitric oxide is produced in many different tissues including endothelial cells of the vascular system. It is believed that NO is one of the major endothelium-derived relaxing factors (EDRF) with strong vasodilatory effects. Nitric oxide dilates blood vessels mainly by activating cytosolic guanylyl cyclase which leads to an increase in cGMP production. cGMP activates the cGMP receptors in the cytosol. cGMP receptors have been identified as cGMP-dependent protein kinase (Lincoln and Cornwell, 1993). cGMP-dependent protein kinase further causes a series of protein phosphorylations resulting in a decrease of cytosolic calcium concentration, subsequently leading to vasodilation. The exact mechanisms by which cGMP-dependent protein kinase reduce cytosolic calcium are still inconclusive. According to the available literature, the following major mechanisms are involved. First, cGMP-dependent protein kinase phosphorylates a regulatory protein, phospholamban, on the sarcoplasmic reticulum (SR). This process removes the inhibition on the calcium pump on the SR, hence increasing calcium sequestration leading to a decrease of cytosolic calcium content (Lincoln *et al.*, 1991). Secondly, cGMP-dependent protein kinase phosphorylates the IP_3 receptors on the SR, which leads to a reduction of calcium release from the SR (Jin *et al.*, 1993; Komalavilas and

Lincoln, 1994). Thirdly, cGMP-dependent protein kinase activates the calcium pump on the smooth muscle plasma membrane extruding calcium out of the cells (Furakawa *et al.*, 1988). Finally, cGMP-dependent protein kinase phosphorylates potassium channels on the plasma membranes which leads to potassium efflux, thus hyperpolarizing the cell. When the cells are hyperpolarized, voltage dependent calcium channels are inhibited resulting in calcium influx reduction (Thornbury *et al.*, 1991, Robertons *et al.*, 1993). It should be emphasized that there is also evidence that NO-induced vessel relaxation is independent of cell hyperpolarization (Garland and McPherson, 1992).

In cirrhosis and portal hypertension, systemic vasodilation and blunted responses to vasoconstrictors are part of the characteristics of hyperdynamic circulation. Since NO has strong vessel dilatory effects, it has been hypothesized as a potential vasodilator producing systemic vasodilation and blunted responses to vasoconstrictive drugs in cirrhosis and portal hypertension. In this regard, much research has been done in the last decade. Sieber *et al* perfused the mesenteric arterial beds of normal control and portal hypertensive rats with an α-adrenoceptor agonist methoxamine. It was found that in portal hypertensive rats, the vasoconstrictive response was sharply blunted. However, if mesenteric arterial beds were pre-perfused with a specific NO biosynthesis inhibitor N^{ω}-nitro-L-arginine (NNA), then perfused with methoxamine, constrictive responses of mesenteric arterial beds from both normal and portal hypertensive rats were not significantly different (Sieber and Groszmann, 1992). Lee *et al* studied the hypothesis of NO

over-production leading to decreased peripheral vascular resistance in portal hypertension. It was found that the total peripheral vascular resistance was significantly lower in portal hypertensive rats than the control animals. However, if the portal hypertensive rats were pretreated with NNA, no significant differences in peripheral vascular resistance were observed between the two groups (Lee *et al.*, 1992). In order to further confirm the effects of NO on hyperdynamic circulation in portal hypertension *in vivo*, NNA was given to the control and portal hypertensive rats for 4 days. The results demonstrated that the blood pressure was increased, the cardiac index was reduced and the hyperdynamic circulation was reversed (Lee *et al.*, 1993). Castro *et al* explored the role of NO on hyperdynamic circulation in carbon tetrachloride-induced cirrhotic rats. It was found that in cirrhosis, total peripheral vascular resistance was significantly reduced and isolated aortic ring responses to angiotensin II perfusion were blunted. The blunted responses were reversible by endothelium denudation or NO synthesis inhibition with NNA (Castro *et al.*, 1993). Intravenous injection of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO biosynthesis, to CCL_4-induced cirrhotic rats was performed by Pizcueta and colleagues. This treatment significantly decreased cardiac output and blood flow to splanchnic organs, increased systemic blood pressure, and increased systemic and splanchnic vascular resistance (Pizcueta *et al.*, 1992). Very recently, Cahill *et al* demonstrated that NO synthase activity in the mesenteric arteries and thoracic aorta is increased in portal hypertensive rabbits. This is accompanied by an increment in cGMP content. Therefore, their results also support the notion that NO plays a role

in hyperdynamic circulation in cirrhosis (Cahill *et al.*, 1995).

Although the above results strongly suggest that NO could be responsible for the hyperdynamic circulation in cirrhosis or in portal hypertension, some studies have found contrary results. Sogni and colleagues infused cirrhotic and control rats with L-arginine (NO substrate). It was found that heart rate was increased and arterial blood pressure was decreased by the same magnitude in both the cirrhotic and control groups. Infusion of L-NMMA deceased cardiac output, increased portal territory resistance, and decreased portal blood flow in the two groups by the same magnitude. Thus, they concluded that NO did not play a role in hyperdynamic circulation in cirrhosis (Sogni *et al.*, 1992). Pak *et al* added the NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), into rat drinking water for a four week period. However, their results showed that addition of L-NAME to the rat drinking water had no effects on the hyperdynamic circulation in liver cirrhosis except for an increase in arterial blood pressure in cirrhotic rats (Pak *et al.*, 1994). Kanwar and colleagues measured NO synthase activity in the liver, jejunum, stomach, and kidney in cirrhotic and portal hypertensive rats. Total NO synthase, inducible NO synthase and constitutive NO synthase activities were not increased in those tissues in cirrhosis or portal hypertension. Ironically, constitutive and inducible NO synthase activities in the kidneys of cirrhotic rats were significantly decreased. Therefore, their results did not support the NO hypothesis in hyperdynamic circulation in cirrhosis (Kanwar *et al.*, 1994)

Taken together, the results concerning the role of NO in hyperdynamic

circulation in cirrhosis remain controversial. More research work before any conclusion can be drawn is still needed.

Glucagon

Glucagon is a 29-amino acid peptide secreted by pancreatic α cells and the oxyntic mucosa of the stomach. Glucagon is inactivated in the liver. Besides its role in carbohydrate metabolism, it has profound effects on the cardiovascular system (Farah *et al.*, 1983). The effects of glucagon on hyperdynamic circulation in cirrhosis and portal hypertension have received broad research in the last decade. In cirrhosis and portal hypertension, blood glucagon levels are increased to about 3-6 fold (Marco *et al.*, 1973; Levy, 1978). The exact mechanisms of glucagon increment are not clear. Interestingly, in cirrhosis and portal hypertension, pancreatic mass is increased, which might explain the elevation of glucagon levels in cirrhosis. Data has shown that when cirrhotic patients receive exogenous glucagon, the plasma half-life of this exogenous glucagon is prolonged. This indicates that glucagon inactivation in cirrhosis was reduced (Silva *et al.*, 1990). It has been confirmed that glucagon has vasodilatory effects. The mechanisms of vasodilatory effects of glucagon remain to be explored. One possibility is that it may dilate the blood vessel by reducing the vascular sensitivity to endogenous vasoconstrictors (Kock *et al.*, 1971). Benoit *et al* (1984) intravenously infused specific glucagon antiserum into portal hypertensive rats. Following the above treatment, mesenteric hyperaemia was reduced by 25% This experiment suggests that glucagon plays an important role in hyperdynamic

circulation in portal hypertension. However, other research leads us to question the role of glucagon in hyperdynamic circulation in cirrhosis and portal hypertension. For example, Sikuler *et al* showed that blood glucagon levels did not correlate with cardiac output. Furthermore, it was found that hyperdynamic circulation disappeared in long-term portal hypertensive rats (6 months), despite the persistence of high plasma glucagon levels (Sikuler *et al.*, 1985; Kikuler *et al.*, 1986).

Bile salts

Blood plasma bile salts are increased both in cirrhotic patients and animal models of cirrhosis (Clain *et al.*, 1977; LaRusso *et al.*, 1975). Many experiments demonstrated that high plasma bile salts may lead to hyperdynamic circulation in cirrhosis. In an *in vivo* study, the rat common bile duct was ligated for three days to assess the bile salt effects on the cardiovascular system function. It was found that the blood pressure significantly dropped and cardiac output increased in those cholestasis animals. These results strongly support the notion that bile salts play an important role in hyperdynamic circulation in cirrhosis since bile salt levels were increased in cirrhotic human and animal models (Bomzon *et al.*, 1990). The mechanisms by which bile salts cause vascular dilation remain to be explored. Pak *et al* did *in vitro* experiments to explore the possible role of bile salts in blood vessel relaxation. Their results suggest that bile salts (taurodeoxycholic acid, TDC) inhibit calcium entry into the blood vessel smooth muscle cells through both voltage-operated and receptor-operated calcium channels (Pak *et al.*, 1994).

Prostaglandins

It is known that prostaglandins have vasodilatory effects, but whether these vasodilatory effects play any role in cirrhosis or portal hypertension is not yet clear. Bruix *et al* studied the role of prostaglandins on systemic hyperdynamic circulation in 18 cirrhotic patients. The patients received the prostaglandin synthesis inhibitor, indomethacin (50mg/per 8 hours for 24 hours). Following the above treatment, urine prostaglandin secretion was reduced, indicating prostaglandin synthesis inhibition. At the same time, hemodynamic results demonstrated that cardiac output was reduced and the total peripheral resistance was increased but without any changes in arterial blood pressure (Bruix *et al.*, 1985). Pique *et al* studied the relationship between prostaglandin and regional hyperdynamic circulation in cirrhosis. After prostaglandin synthesis was inhibited by indomethacin, blood flow in the stomach was reduced (Pique *et al.*, 1988). The two studies mentioned above suggest prostaglandins might play a role in hyperdynamic circulation in liver cirrhosis both systemically and regionally.

However, other studies obtained opposite results. Blanchart *et al* treated portal hypertensive rats with indomethacin for 24 days. The urinary secretion of prostaglandin from these rats was significantly reduced , but no improvement in hyperdynamic circulation was observed in these portal hypertensive animals (Blanchart *et al.*, 1985).

1.2.2.2. Neural factors

It is well known that sympathetic nervous activity is enhanced in cirrhosis (Henriksen *et al.*, 1984; Bomzon *et al.*, 1985). An increase in sympathetic tone has been proposed as a mechanism to cause hyperdynamic circulation in cirrhosis and portal hypertension. Moreau *et al* showed that the central α_2-adrenergic receptor agonist clonidine treatment could decrease hyperdynamic circulation in cirrhotic patients. Six cirrhotic patients were treated with clonidine and another 6 patients with placebo. The clonidine treatment significantly reduced plasma noradrenaline levels, reduced cardiac output by 17% and improved hyperdynamic circulation. It was concluded that sympathetic over-activity contributes to the circulatory derangements in patients with cirrhosis (Moreau *et al.*, 1987). Abergel's experiments demonstrated that when portal hypertensive rats were pithed to ablate central sympathetic tone, hyperdynamic circulation was markedly diminished (Abergel *et al.*, 1992). Primary afferent innervation might also play a role in producing hyperdynamic circulation in cirrhosis. Lee and Sharkey studied rats with their primary afferent nerves permanently destroyed at the neonatal stage by capsaicin treatment. These rats were made cirrhotic and portal hypertensive after reaching adulthood. It was found that in the rats treated with capsaicin hyperdynamic circulation was significantly improved. These results suggest that primary afferent innervation plays a role in producing hyperdynamic circulation in cirrhosis (Lee and Sharkey, 1993). The mechanisms by which the afferent nervous system leads to hyperdynamic circulation in cirrhosis and portal hypertension are not clear. Possibly, visceral congestion, which is a common phenomenon in cirrhosis and portal

hypertension, activates afferent nerves with resultant reflex increases in sympathetic efferent activity (Kostreva *et al.*, 1980).

1.2.2.3. Plasma volume expansion

Evidence also exists that shows plasma volume expansion in both cirrhotic patients and animal models of cirrhosis (Eisenberg *et al.*, 1956, Rector *et al.*, 1988). Two major mechanisms are suggested to explain plasma volume expansion in cirrhosis and portal hypertension. First, plasma volume expansion is caused by splanchnic and splenic sequestration of fluid due to portal venous obstruction. Second, plasma volume expansion is also the consequence of increased sodium retention by the renin angiotensin aldosterone system acting on the kidneys, leading to sodium and water over-absorption. Cardiac output could be increased by augmentation of plasma volume. Recent experiments have confirmed that plasma volume expansion was directly involved in hyperdynamic circulation in portal hypertension. Genecin *et al* demonstrated that when sodium intake was restricted in portal hypertensive rats for three weeks, the plasma volume in these animals returned to normal. At the same time, hyperdynamic circulation was consequently ameliorated (Genecin *et al.*, 1990). Recently, questions were raised about whether plasma volume expansion causes the hyperdynamic circulation, or if it is the hyperdynamic circulation which results in plasma volume expansion in cirrhosis and portal hypertension. Albillos *et al* made a portal hypertensive rat model by calibrated portal vein stenosis. Plasma volume, total peripheral resistance, and

cardiac output were continuously measured after surgery. It was found that plasma volume expansion occurred 24 hours following the appearance of hyperdynamic circulation (Albillos *et al.*, 1992).

1.2.3. Summary of hyperdynamic circulation in cirrhosis

Liver cirrhosis or portal hypertension are associated with hyperdynamic circulation characterized as an increase of cardiac output at baseline, elevation of regional and systemic blood flow, and a decrease of peripheral vascular resistance and arterial blood pressure. The causes for this hyperdynamic circulation are comprehensive, and may include humoral factors, neural factors, and plasma volume expansion.

1.3. CARDIOMYOPATHY IN LIVER CIRRHOSIS

1.3.1. Cirrhotic cardiomyopathy in patients

1.3.1.1. Cardiac function at baseline

Not only is the vascular function deranged as described previously, but the cardiac function is also altered in liver cirrhosis. Much research performed in the last four decades has documented an impairment of cardiac contractile function in cirrhosis (reviewed in Lee, 1989). Abnormal cardiac hyperdynamic status at baseline in cirrhosis was first reported by Kowalski and Abelmann in 1953. In that study, 22 cirrhotic patients were studied. One third of the patients had elevated baseline cardiac output (Kowalski and Abelmann, 1953). Since then, numerous studies have

confirmed and extended Kowalski and Abelmann's observation. Murray *et al* studied cardiac index in 24 cirrhotic patients and 14 normal subjects. It was found that cardiac output at rest in liver cirrhosis was significantly higher than that in control subjects. Some patients showed ventricular enlargement on the chest x-ray examination. Electrocardiography also demonstrated left ventricle enlargement. Two of the cirrhotic patients were found to be suffering from severe cardiomyopathy. One patient died of heart failure as liver function deteriorated (Murray *et al.*, 1958).

Increased baseline cardiac output could be caused by decreased peripheral vascular resistance and increased blood volume (Ingles *et al*, 1991). Usually heart failure cannot be observed at baseline in cirrhosis; only when patients are challenged by physical or mental stress, or pharmacological stimulation, do the symptoms of impaired cardiac contractile function become apparent.

1.3.1.2. Impaired cardiac function under physical and mental stresses

During exercise or stress, the tissue oxygen consumption is increased. A variety of physiological responses take place in the body to meet the oxygen consumption increment. Among these, an increase in cardiac output is an important one. However, in cirrhosis, because of impairment of the heart function, the heart cannot pump enough blood to meet the body's needs. Consequently, the heart failure symptoms occur when the heart is exposed to exercise or stress. A very recent study by Grose and colleagues demonstrated that when cirrhotic patients were exposed to maximal exercise, the cardiac output increased only by 97% (Grose *et al.*,

1995). However, a previous study demonstrated that when healthy subjects were exposed to the same amount of exercise, cardiac output increased by 300% (Poliner *et al.*, 1980). Cardiac end systolic and diastolic volumes were also increased significantly in cirrhosis compared with normal subjects. Grose's study indicates the presence of cirrhotic cardiomyopathy. In another study, Gould and colleagues found that when the cirrhotic patients were exposed to exercise, the left ventricular diastolic pressure and pulmonary arterial pressure were increased. However, cardiac stroke index did not change, and even decreased in some patients, which supports the contention of cirrhotic cardiomyopathy (Gould *et al.*, 1969). Bernardi and colleagues recruited 20 cirrhotic patients and 10 healthy subjects to investigate cardiovascular responses to tilting. Tilting induced tachycardia responses in normal subjects. However, no tachycardia was observed in cirrhotic patients (Bernardi *et al.*, 1983). Eating is another physiological stimulation to the cardiovascular system. Lee and colleagues demonstrated that after eating, cardiac output was not increased in cirrhotic patients. In contrast, it was decreased (Lee *et al.*, 1988). Lunzer *et al* investigated cardiovascular responses to the Valsalva maneuver, ice cold stimulation on the forearm, and mental arithmetic stress in cirrhotic patients (Lunzer *et al.*, 1975). The results showed that the heart rate and forearm blood flow responses to the above stimuli were all diminished in cirrhosis.

Impaired cardiac performance during exercise was not only observed clinically, but also documented by cardiovascular testing. Measurement of systolic-time intervals has been used as a non-invasive tool to evaluate the ventricular contractile

performance. This procedure has been validated by cardiac catheterization (Weissler, 1976). The pre-ejection period over left ventricular ejection time ratio (PEP/LVET) is an important parameter for the determination of left ventricular contractile function. In cardiomyopathy, PEP is prolonged and LVET is shortened, thus, PEP/LVET is lengthened (Weissler, 1976). Bernardi and colleague studied systolic-time intervals in 22 cirrhotic patients. Their results showed that both at baseline and after muscle exercise, PEP/LVET in cirrhotic patients were prolonged compared with healthy subjects (Bernardi *et al.*, 1991). Kelbaek and colleagues used echocardiograms and radionuclide angiocardiography to investigate cardiac performance in cirrhotic patients. It is important to mention that in this study, very strict exclusion criteria were established. Patients with any sign or history of ischemic heart disease, hyper or hypothyroidism, and diabetes mellitus were excluded. At rest, no significant differences were discovered between the cirrhotics and control subjects except that the heart rate in the cirrhotic patients was significantly increased. However, during exercise, cirrhotic patients showed impaired cardiac performance. The echocardiography demonstrated that left atrium dimensions in the cirrhotic patient group was significantly larger (36 mm) than controls (31 mm). The increment in left atrial dimension suggests blood retention in the left atria, indicating left ventricular contractile insufficiency. The echocardiography also demonstrated that ventricular wall compliance in cirrhotic patients was reduced. Radionuclide angiocardiography demonstrated that at baseline, left ventricular ejection fraction (LVEF) in cirrhotic patients and control subjects were not significant different.

However, when patients and healthy subjects were exposed to the same amount of exercise, LVEF in cirrhotic patients only increased by 6%, yet 14% LVEF increment was observed in the control group, $p<0.01$ (Kelbaek *et al.*, 1984).

1.3.1.3. Blunted cardiac responses to inotropic pharmacological stimulation

When the heart is stimulated by agonists such as isoproterenol, the contractile responses are expected to increase both inotropically (contractile force) and chronotropically (heart rate). However, many reports have demonstrated that both inotropic and chronotropic responses to β-adrenergic agonist stimulation in cirrhotic patients are diminished, which indicates cirrhotic cardiomyopathy. Limas *et al* (1992) investigated cardiac performance in cirrhotic patients and found that blood pressure in cirrhosis was significantly lower than normal. Using angiotensin infusion to elevate the blood pressure back to the normal range, it was observed that the pulmonary wedged pressure increased tremendously (almost doubled), but the cardiac output was not increased (Limas *et al.*, 1972). Failure to increase left ventricular output despite the increase in filling pressure is grossly abnormal. In the same study, when ouabain, a cardiac glycoside, was infused into the cirrhotic patients, no increase in cardiac output was observed. Limas' results demonstrated cardiac contractile function impairment in cirrhotic patients. Mikulic and colleagues infused dobutamine, a synthetic β_1-adrenergic receptor agonist, into cirrhotic patients. The cardiac stroke volume was expected to increase after dobutamine infusion. However, their results showed that cardiac stroke volume before and after dobutamine infusion

was 79 *vs.* 74 mL, $p>0.05$ (Mikulic *et al.*, 1983). Lunzer and colleagues infused cirrhotic patients with norepinephrine to evaluate the cardiovascular performance. The increase in the forearm blood flow was significantly lower in cirrhosis than control subjects. The above data indicate that cardiac output in the cirrhotic patients under agonist simulation is lower than the control subjects (Lunzer *et al.*, 1975).

Not only are the inotropic cardiac contractile responses blunted to agonist stimulation in cirrhosis, but the chronotropic responses are also diminished. Ramond *et al* (1982) demonstrated that when cirrhotic patients were given isoproterenol, the dose required for the heart rate to increase 25 beats per min (CD_{25}) was significantly higher in cirrhotic patients (4.47 μg) than the control subjects (1.34 μg). A tripled CD_{25} value suggests that the cirrhotic hearts were desensitized to β-adrenergic receptor stimulation (Ramond *et al.*, 1986).

1.3.2. Cardiomyopathy in cirrhotic animal models

Cardiomyopathy has been documented in different cirrhotic animal models. To test cardiac function in cirrhosis, Lee and colleagues infused isoproterenol into common bile duct-ligated cirrhotic rats. The doses required for the heart rate to increase 50 beats per min in cirrhosis were four times higher than that observed in their sham control counterparts. The maximal chronotropic responses in cirrhotic rats were 30% lower than controls. These abnormal responses to isoproterenol stimulation strongly suggest cardiac β-adrenergic receptor desensitization in cirrhotic animals (Lee *et al.*, 1990). Ingles and colleagues performed a comprehensive study

to investigate cardiac contractile performance in a cirrhotic rat model induced by CCl_4 inhalation. It was found that cardiac output and first time derivative peak positive left ventricular pressure (dp/dt) were all significantly decreased when the right atrial pressure was increased by dextran infusion. As expected, left ventricular end-diastolic pressure was also increased in cirrhotic animals. The above data demonstrated that the cardiac preload reserve in cirrhosis was limited. These results suggest that in a situation where an increase in cardiac performance is needed, heart failure will occur in cirrhosis (Ingles *et al.*, 1991). In the normal healthy circulatory status, when blood volume is increased, cardiac output is expected to be increased. However, because of cardiac contractile function impairment in cirrhosis, the above phenomena do not apply. Caramelo and colleagues expanded blood volume by isotonic saline infusion to test the sodium handling ability of the kidneys in CCl_4-induced cirrhotic rats. Their results demonstrated that when blood volume was expanded, the cardiac output in control animals increased significantly. In contrast, the cardiac output in cirrhotic rats decreased by 50% (Caramelo *et al.*, 1986). This is a very abnormal response. Battarbee's *in vitro* studies demonstrated that the cardiac muscle strip chronotropic and inotropic responses to isoproterenol stimulation in portal hypertensive rats were significantly blunted, an indication of heart failure (1992). Portal hypertension is one of the major complications in liver cirrhosis. Hyperdynamic circulation and chronic overload of the heart also exist in portal hypertension. However, it should be emphasized that cardiac β-adrenergic receptor density and affinity in portal hypertension alone are not altered (Lee *et al.*,

1990). These results suggest that portal hypertension alone cannot induce any changes in cardiac β-adrenergic receptor characteristics.

1.3.3. Cardiac histology in cirrhosis

One of the largest histological studies on heart tissues in cirrhosis was done by Lunseth and colleagues (1958). A total of 108 cirrhotic patients were autopsied. In this group of patients, 37 cases had no historical or pathological evidence of hypertension, valvular disease or atherosclerotic heart disease. Among these 37 patients, 32% had cardiac hypertrophy. Cardiac hypertrophy seems to occur in the early and moderate stage of cirrhosis. In advanced cirrhosis, less cardiac hypertrophy was found in this group of patients. The cardiac hypertrophy could be caused by chronic hemodynamic overload of the heart because of the hyperdynamic circulation in liver cirrhosis. Ventricular dilation, especially right ventricle dilation, was obvious. Further histological studies on those patients with cardiac hypertrophy demonstrated less cardiac tissue consistency. Cardiomyocytes were swollen. Myocardial fibrosis, scaring, exudation, nuclear vacuolation, and unusual pigmentation were observed. It was concluded that cirrhotic patients tended to suffer from idiopathic cardiomyopathy (Lunseth *et al.*, 1958). Early histological investigations by Spatt (1949), Hall (1953), and Loyke (1955) demonstrated similar findings.

The study by Caramelo and colleagues demonstrated that normalized heart weight in cirrhotic rats induced by CCl_4 inhalation was significantly higher than that in the controls. Increased heart weight was attributed to cardiac hypertrophy caused

by chronic heart overload. However, heart histological section examination did not discover any abnormality in these cirrhotic animals (Caramelo *et al.*, 1986).

1.3.4. Mechanisms of cirrhotic cardiomyopathy

Unlike the research on vasculature abnormalities in hyperdynamic circulation in liver cirrhosis, the mechanisms of cirrhotic cardiomyopathy are far from clear. Gerbes *et al* studied β_2-adrenergic receptor density and binding affinity on blood lymphocytes from cirrhotic patients. It was found that the receptor density was significantly reduced in cirrhotic patients with ascites (Gerbes *et al.*, 1986). Given the difficulty of obtaining heart tissue from humans, to our knowledge, no β-adrenergic receptor binding assay has been done in cardiac tissues from cirrhotic patients. It was speculated based on Gerbes' results that cardiac β-adrenergic receptor may also be down-regulated in cirrhotic patients. Indeed, Lee *et al* demonstrated that in a biliary cirrhotic rat model, cardiac plasma membrane β-adrenergic receptor density was reduced without changes in receptor binding affinity (1990). It was also demonstrated in Lee's investigation that β-adrenergic receptors were desensitized *in vivo*. Zavecz and colleagues reported that the coupling between the β-adrenergic receptor and agonist was impaired since a 3-fold greater concentration of isoproterenol are required to have the same contractile responses for cardiac papillary muscle in portal hypertensive rats than in controls (Zavecz *et al.*, 1995b).

It is possible that the cardiac impairment in liver cirrhosis is caused by toxic

effects of alcohol on heart tissue since many cardiac performance studies were done on alcoholic cirrhotic patients. However, evidence from both humans and animal models demonstrated that cardiomyopathy also occurs in non-alcoholic cirrhosis. In Bernadi's study, an increase in the ratio of PEP/LVET was observed both in alcoholic cirrhotic patients and non-alcoholic cirrhotic patients (1991). In both Grose's (1995) and Lewis' (1980) investigations, impairment of ventricular contractile function was observed both in alcoholic and non-alcoholic cirrhotic patients. Others have reported impairment of cardiac function in non-alcohol induced cirrhotic animal models (Caramelo *et al.*, 1986; Ingles *et al.*, 1991). Therefore, it is the cirrhosis *per se* which induces cirrhotic cardiomyopathy. Ethanol toxicity could be just one of the co-existing factors in some cases.

1.3.5. Summary of cardiomyopathy in cirrhosis

Voluminous evidence has demonstrated that cardiac contractile performance in liver cirrhosis is impaired, yet the mechanisms remain unclear. At baseline, cardiac output is increased which is one of the characteristics of hyperdynamic circulation in cirrhosis. However, when the heart is exposed to physical exercise, mental stress, blood volume expansion, or challenged by agonist infusion, the symptoms of impaired cardiac function occur. The mechanisms of cirrhotic cardiomyopathy remain unclear. Possibly, impairment of β-adrenergic receptor function plays an important role.

1.4. CARDIAC β-ADRENERGIC RECEPTOR SIGNAL TRANSDUCTION

1.4.1. Historical aspects of the adrenergic receptors

The receptor concept was first proposed by Langley at the beginning of the century (Langley, 1904). It was documented in his early study that nicotine causes certain skeletal muscle contraction by acting on the "receptive substances". These "receptive substances" then interact with "contractive substances" to induce muscle contraction. Alhquist first described that there are two different types of adrenergic receptors, α and β-adrenergic receptors. According to his early experimental observations, α-adrenergic receptor activation leads to excitatory responses such as vaso-constriction, pupil dilation, and uterus contraction. α-Adrenergic receptor stimulation could also induce intestinal muscle relaxation. On the other hand, β-adrenergic receptor stimulation mainly invokes inhibitory responses such as vasodilation and inhibition of uterine contraction. Furthermore, one of the important features of the β-adrenergic receptor is that it simulates heart contraction (Alhquist, 1948). It was Langer who demonstrated that there are two subtypes of α-adrenergic receptors, post-junctional (α_1) and pre-junctional (α_2) receptors (Langer, 1974; Langer, 1977). Subsequently, the α-adrenergic receptors were more properly classified as α_1 and α_2 adrenergic receptors. This is based on the receptor function and responses to specific pharmacological agents, rather than their anatomical locations (Bylund *et al.*, 1994). Based on the relative potency of the sympathomimetic amines on the different tissues, Lands and colleagues proposed that β-adrenergic receptors could be further subclassified as β_1 and β_2-adrenergic

receptors. Their experimental data suggest that β_1-adrenergic receptors are more potent on cardiac contraction and lipolytic activity. On the other hand, β_2-adrenergic receptors are more potent on bronchodilation and vascular dilation. Furthermore, one of the most important differences between β_1- and β_2-adrenergic receptors is the relative potency of epinephrine and norepinephrine on those receptors. Norepinephrine has almost the same potency on β_1- and β_2-adrenergic receptors. However, epinephrine is 100 times more potent on the β_2-adrenergic receptor than the β_1-adrenergic receptor (Lands *et al.*, 1967). Subsequently, the β_3-adrenergic receptor was identified. β_3-Adrenergic receptors are predominantly distributed in the adipose tissue. The major function of the β_3-adrenergic receptors is lipolysis (Arch *et al.*, 1984; Giacobino, 1995). Classification and subclassification of adrenergic receptors has been updated in detail in the recent reviews by the International Union of Pharmacology (Bylund *et al.*, 1994; Hibele *et al.*, 1995). In the last decade, much research work has been done in the field of the β-adrenergic receptor and its related signal transduction pathway. The following sections are devoted to the β-adrenergic receptor system in the heart.

1.4.2. β-Adrenergic receptor in the heart

The cardiac β-adrenergic receptors and their related post-receptor signal transduction pathway play an important role in modulating the cardiac contractile function (Fleming *et al.*, 1992). Two major subtypes, β_1-and β_2-adrenergic receptors, are involved in cardiac contraction. Although there are some species differences in

terms of the β_1 and β_2-adrenergic receptor ratio in the heart, in general, β_1-adrenergic receptors account for 80% and β_2-adrenergic receptors account for 20% of the total β-adrenergic receptor population in heart tissue (Brodde, 1991). Both β_1 and β_2-adrenergic receptors participate in the positive cardiac contractile responses because specific β_1 and β_2-adrenergic receptor antagonists shift the cardiac contractile response curve (stimulated by isoproterenol) to the right (Brodde *et al.*, 1989; Kaumann and Lemoine, 1987). Both β_1 and β_2-adrenergic receptors are coupled to adenylyl cyclase in the heart. However, β_2-adrenergic receptors couple to adenylyl cyclase more efficiently than β_1-adrenergic receptors, both in the atria and in the ventricles. This statement is based on the observations that cAMP production stimulated by a specific β_2-adrenergic receptor agonist, procaterol, is equivalent to 60-70% of cAMP production stimulated by isoproterenol which acts on both β_1- and β_2-adrenergic receptors (Brodde *et al.*, 1984; Bristow *et al.*, 1989). However, as stated above, the β_2-adrenergic receptors only account for 20% of the total β-adrenergic receptor population in the heart.

The structure of the β-adrenergic receptor is relatively clear (Strader *et al.*, 1995; Schwinn *et al.*, 1992). The cardiac β-adrenergic receptor is, like other G-protein coupled receptors, composed of seven putative transmembrane domains with three extracelluar and three intracellular loops as presented in figure 1.1. The amino terminus (NH_2) is located on the extracelluar side. The carboxyl terminus (COOH) is located on the intracellular side. Examination of the structure of adrenergic receptors and other G-protein coupled receptors reveals that the structure of the

intracellular half of the seven trans-membrane domains are relatively conserved. In contrast, the outside half of the seven trans-membrane domains show obvious variations. Thus, it is possible that the outside half of the trans-membrane domains may be the sites for binding of different ligands (O'Dowd *et al.*, 1989b). Recently, it has been proposed that Asp133, located on the third transmembrane helix, Ser204 and Ser207 on the fifth transmembrane helix, and Phe290 on the fifth transmembrane helix form the pocket in the β-adrenergic receptor that binds with catecholamines (Strader *et al.*, 1995). The third intracellular loop between the fifth and sixth transmembrane domains plays an important role in coupling with G-proteins (O'Dowd *et al.*, 1988; Strader *et al.*, 1987). A chimeric receptor study has demonstrated that the fourth transmembrane domain plays a major role in the differentiation between β_1 and β_2-adrenergic receptors (Frielle *et al.*, 1988).

1.4.3. β-Adrenergic receptor activation and cardiac cell contraction

The effects of β-adrenergic receptor activation on cardiomyocyte contraction are depicted in figure 1.1. When a β-adrenergic receptor is occupied by an agonist such as isoproterenol, the receptor is activated. The activated receptor further interacts with stimulatory G-protein. The G-protein is a heterotrimer composed of α, β, and γ subunits. The $\beta\gamma$-subunits are tightly associated. They function as a monomer. $\beta\gamma$-Subunits cannot be separated unless the proteins are denatured (Gilman, 1987; Helper and Gilman, 1992). Previous studies suggest that multiple sites on the COOH terminus of Gαs interact with the receptors (Conklin and

Bourne, 1993). The interaction between the activated receptor and G-proteins leads to guanine nucleotide exchanges. That is, GTP binds to the αs subunit of stimulatory G-protein and GDP dissociates from it. This has been termed as a switch-on process. After guanine nucleotide exchange, the receptor will dissociate from the receptor-G-protein-GTP complex, and stimulatory G-protein dissociates to αs-GTP subunit and $\beta\gamma$ subunits. αs-GTP further stimulates adenylyl cyclase, converting ATP to the second messenger 3'5'-cyclic adenosine monophosphate (cAMP). It has been demonstrated that multiple regions at the C-terminus of αs subunit activate adenylyl cyclase (Berlot and Bourne, 1992). αs-GTP also has intrinsic GTPase activity. Intrinsic GTPase hydrolyses GTP to GDP, which leads to αs inactivation. This is a switch-off process (Neer, 1995; Rens-Domiano and Hamm, 1995).

A total of 8 different types of adenylyl cyclase have been discovered thus far, and 6 of them (type 1-6) have been completely cloned. Type 7-8 have been partially cloned. In the mammalian heart, type 4-7 are predominant (Iyengar, 1993; Taussig and Gilman, 1995). Adenylyl cyclase is composed of 12 trans-membrane domains. Both amino terminus (NH_2) and carboxyl terminus (COOH) are located on the intracellular side. The first 6 trans-membrane domain and last 6 trans-membrane domain are connected by an intracellular loop as presented in figure 1.1. This intracellular loop and the carboxyl tail are speculated to be the major components of the catalytic domains of the enzyme since their structures are very similar in the different types of adenylyl cyclase (Taussig and Gilman, 1995). All adenylyl cyclases can be activated by the active αs subunit of stimulatory G-protein.

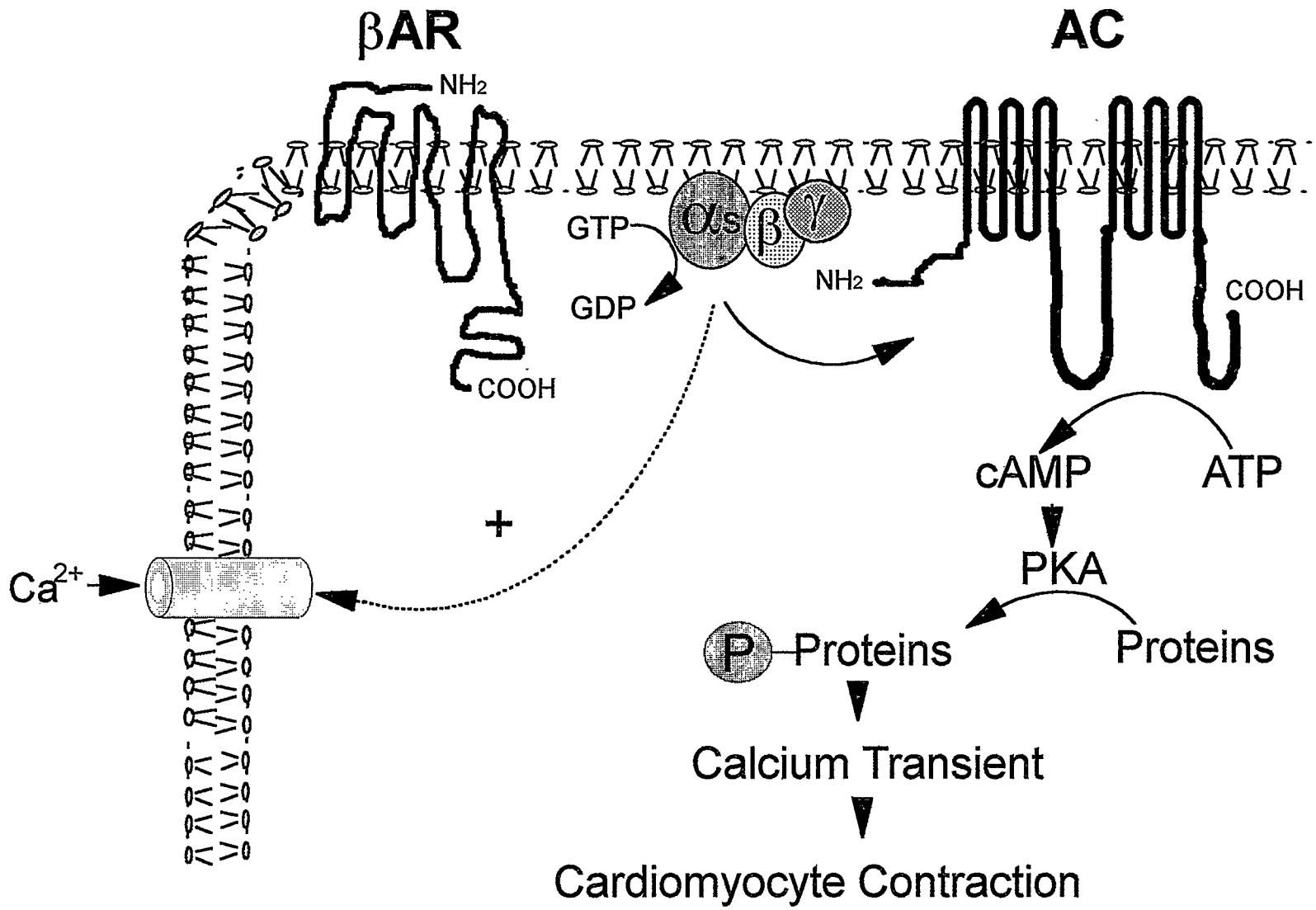
While adenylyl cyclase is activated, ATP in the cytosol is converted to the second messenger, cAMP. Elevation in cAMP content further activates cAMP-dependent protein kinase A (PKA). cAMP-dependent protein kinase will selectively phosphorylate a number of proteins to continue the process of signal transduction. Briefly, PKA phosphorylates L-type calcium channels on the cardiac plasma membrane and enhance the inward calcium current (Reuter *et al.*, 1983). The L-type calcium channels are composed of α_1, $\alpha_2\delta$, β subunits. The α_2 and δ subunits are linked by a disulphide bond. The fifth subunit is dependent on tissues where the L-type calcium channels locate. For example, the fifth subunit is a γ subunit in skeletal muscle. However, no γ subunits have been identified thus far in heart L-type calcium channels (Birnbaumer *et al.*, 1994; Norman and Leach, 1994; Spedding and Paoletti, 1992). The α_1 subunit forms the pores for the L-type calcium channels. Evidence suggests that PKA-induced phosphorylation of L-type calcium channels occurs on both α_1 (Sculptoreanu *et al.*, 1993) and β subunits (Haase *et al.*, 1993). An increase in inward calcium current plays a major role in strengthening the cardiomyocyte contraction. cAMP-dependent protein kinase also phosphorylates troponin I, facilitating the conformation change of troponin complex so that Ca^{2+} cannot bind with troponin C. Therefore, myosin cannot interact with actin. This process accelerate relaxation. It has also been demonstrated that the serine-20 of troponin I is phosphorylated by PKA in rabbit heart (Solaro *et al.*, 1976; Moir *et al.*, 1980). It has been documented that a protein called phospholamban is phosphorylated by PKA during the process of β-adrenergic receptor activation (Fujii

et al., 1989). Phospholamban is a protein located on the membrane of the sarcoplasmic reticulum (SR). Phospholamban is a pentamer and its gene has been cloned (Simmerman *et al.*, 1989). At unphosphorylated status, phospholamban inhibits calcium ATPase (calcium pump) on the SR membrane. When phospholamban is phosphorylated by PKA, phospholamban loses its inhibition on the calcium pump by reducing calcium pump's K_m for Ca^{2+} (Simmerman *et al.*, 1989). Thus, the pumping activity of calcium ATPase is enhanced. More calcium ions are pumped back into the SR. This process leads to a reduction in relaxation time and an increase in the heart rate.

β-Adrenergic receptor activation also increases positive chronotropic contractile responses through pacemaker cells in the mammalian heart. For example, when the β-adrenergic receptors in the SA node are activated by epinephrine, inward current is augmented. Thus, the slope of pacemaker potential (phase four) is increased, which leads to an increase in heart rate (Giles, 1989; Berne and Levy, 1993).

Evidence demonstrates that activated αs subunits of Gs proteins can directly act at the L-type calcium channels on the cardiac plasma membrane to increase calcium influx (Yatani *et al.*, 1989). The direct effects of Gsα on the plasma membrane calcium channels enable the cardiomyocytes to respond to the adrenergic nerve stimulatory signals much faster than the cAMP pathway discussed above. This direct stimulatory function of G-proteins on the membrane ion channels has been defined as membrane delimited effects (reviewed in Brown, 1993).

Figure 1.1. Schematic description of β-adrenergic receptor signal transduction pathway. The proteins involved in positive contractile responses and phosphorylated by PKA mainly include: calcium channels in the cardiac plasma membrane, phospholamban on the sarcoplasmic reticulum, and troponin I. βAR, β-adrenergic receptor; AC, adenylyl cyclase; PKA, cAMP-dependent protein kinase.


βAR
AC
NH2
GTP
αs
β
γ
GDP
NH2
COOH
COOH
cAMP
ATP
+
PKA
Ca2+
P
Proteins
Proteins
Calcium Transient
Cardiomyocyte Contraction

1.4.4. Possible roles of β-adrenergic receptor dysfunction in cirrhotic cardiomyopathy

The cardiac β-adrenergic receptor signalling pathway function has been intensively researched in many different types of cardiomyopathy. In heart disease, the diminished β-adrenergic receptor signalling function involves multi-elements in this signal transduction pathway. In human dilated cardiomyopathy, the β-adrenergic receptor density was documented to be reduced. cAMP production was decreased. G-protein expression was altered (Feldman, 1993). Previous studies in heart aortic and mitral valvular diseases demonstrated comparable results (Steinfath *et al.*, 1993). A variety of cardiomyopathy animal models have been established and the findings were similar to that in humans. For example, Vatner *et al* demonstrated that in a chronic norepinephrine-induced canine cardiomyopathy, the cardiac β-adrenergic receptors shift from high binding affinity to low binding affinity, associated with decreased cAMP production (Vatner *et al.*, 1989). In an ischemic cardiomyopathy animal model, cardiac G-protein and adenylyl cyclase-mediated cAMP production were impaired (Yamamoto *et al.*, 1994). Ishikawa and colleagues recently demonstrated that the adenylyl cyclase type V and VI activity was severely damaged in a pacing-induced canine cardiomyopathy (Ishikawa *et al.*, 1994). A study in pulmonary artery banding-induced congestive heart failure revealed a decrease in the β-adrenergic receptor density and impairment of the post-receptor signal transduction pathway (Fan el al., 1987).

A close examination of these different kinds of cardiomyopathy described above reveals that two major pathological factors are considered important: the

cardiac hemodynamic overload and an elevation in blood plasma catecholamine levels. Both of them are commonly observed in cirrhosis (Lee, 1989; Ring-Larsen *et al.*, 1982). Therefore, it is possible that like other types of cardiomyopathy, the β-adrenergic receptor signal transduction pathway function is altered in cirrhosis, consequently leading to impaired cardiac contractile performance. Thus far, systematic studies of the β-adrenergic receptor signalling pathway function in cirrhotic cardiomyopathy are not available.

1.4.5. Summary of cardiac β-adrenergic receptor function

Cardiac contractile function is modulated by β-adrenergic receptors. When a β-adrenergic receptor is occupied by an agonist, the receptor will in turn activate the stimulatory G-proteins. The G-proteins further act on adenylyl cyclase leading to an elevation of cAMP content and activation of cAMP-dependent protein kinase. cAMP-dependent protein kinase phosphorylates a series of proteins, inducing calcium transients and cardiomyocyte contraction. It has been demonstrated that the β-adrenergic receptor and its related signal transduction pathway function are altered in different types of cardiomyopathy. Therefore, it is possible that diminished β-adrenergic receptor function also plays an important role in cirrhotic cardiomyopathy.

1.5. PLASMA MEMBRANE FLUIDITY AND PROTEIN FUNCTIONS

1.5.1. Outline of plasma membrane structure

The cell plasma membrane encloses the constitutive intracellular components. Signals going from one cell to another, between the cells and the external environment are transferred through the plasma membrane. Therefore, normal plasma membrane properties are essential for the cells to function properly. The plasma membrane is a lipid bilayer. The major lipids in the plasma membranes are phospholipids, glycolipids, and sterols of which cholesterol is a major component. The phospholipids account for the major constitutive lipid elements of the membrane bilayer. A phospholipid molecule consists of three parts: a headgroup, a glycerol backbone, and fatty acyl hydrocarbon chains. Majority polar headgroups contain phosphate, therefore, these lipids are termed phospholipids. In the membrane bilayer, the hydrophillic region, or the polar headgroup of the phospholipids, orients toward the outside of the membranes (aqueous face). The hydrophobic fatty acyl hydrocarbon chains remain inside the membrane bilayer. The polar headgroup and fatty acyl chains are esterified to the glycerol backbone. The fatty acyl chains with 16, 18, and 20 carbons are most common in the membrane bilayer. The majority of the cholesterols in the plasma membrane bilayer are free cholesterols. A variety of proteins are embedded in the membrane bilayer, such as β-adrenergic receptors and adenylyl cyclase. When these proteins exert their functions, a protein to protein or/and protein to lipid interaction occurs in the membrane bilayer. Therefore, changes in the membrane physical and chemical properties could influence the membrane protein functions.

1.5.2. Membrane fluidity

Membrane fluidity is a term often used to illustrate the membrane physical properties. It has been used widely for many years in membrane physical property studies (Brasitus and Schachter, 1980; Cogan and Schachter, 1981; Meddings *et al.*, 1991). Fluidity is a general term. However, when applied to the anisotropic biological membranes, it refers to the relative motional freedom of lipid molecules or substituents in the membrane lipid bilayer. It is difficult to define fluidity precisely because of the complexity of motional freedom available to lipid or protein molecules in the lipid bilayer. The most common motional forms of molecules in the membrane bilayer include rotation, wobbling, and flip-flop which are depicted in figure 1.2. Additional complexities arise because estimates of physical properties depend on the type of probes used, the probe location in the membrane, and the extent to which the probe itself perturbs the membrane structure. In the following section, a steady-state fluorescence depolarization method is used as an example to further elaborate this widely used term of "membrane fluidity".

A biological membrane is an anisotropic fluid mosaic. The resistance to the movement for the lipid molecules or proteins embedded in the membrane depends on the force and motional modes. As shown in figure 1.2, many forms of movement exist in the membrane bilayer. Therefore, the ideal and accurate definition of membrane fluidity should be the total effects of all these motional modes. The measurement of membrane fluidity depends heavily on experimental techniques. Unfortunately, thus far, there is no single procedure capable of accurately

characterizing the complexity of structural arrangements and dynamic motions inherent in each membrane component. The available methods used to detect membrane fluidity cannot provide a parameter which is the weighted effects of all the above motional modes. Due to the restriction described above, it is not difficult to understand that when different methods and different probes are used to measure the "membrane fluidity", the results have their own distinct meaning. For example, by using 1,6-diphenyl-1,3,5,-hexatriene (DPH) as a probe in fluorescence polarization studies, the packing order of the membrane bilayer or the wobbling movement of the DPH probes is determined. This is because the absorption or excitation dipole and emission dipole of DPH are almost parallel. Therefore, when the probe exhibits a rotational movement, depolarization could hardly be detected since depolarization only occurs when the direction of emission dipole alters. In contrast, when the *n*-(9-anthroyloxy)-stearic fatty acids or 9-anthroyloxy-palmitic fatty acid are used as probes, the results indicate the rotational freedom of the probes in the membrane bilayer. Taken together, once again, we loosely define membrane fluidity as the motional freedom of lipid molecules or substituents in the membrane bilayer. It is important to bear in mind that with different methods and probes, the term fluidity has its own distinct meaning.

1.5.3. Membrane fluidity and protein function

Many experiments have demonstrated that the function of certain proteins embedded in the membrane lipid bilayer is fluidity dependent. For example, when

turkey erythrocyte plasma membranes were fluidized, the β-adrenergic receptor-mediated cAMP production was increased, but without changes in the β-adrenergic receptor binding affinity or density. These results suggest that β-adrenergic receptor function is modulated by membrane fluidity (Hanski *et al.*, 1979). Furthermore, observations by Miyamoto and colleagues demonstrated that in aged rats, IP_3 production stimulated by α_1-adrenergic receptor activation in the parotid membranes was reduced. The reduction in IP_3 levels was correlated with changes in membrane fluidity. These results indicate the α_1-adrenergic receptor function is regulated by membrane fluidity (Miyamoto *et al.*, 1993). Sodium-dependent glucose transporters are proteins located on the brush border membranes. Experimental data demonstrated that when brush border membranes were fluidized with a fluidizing agent, A_2C, glucose transporter activity was increased (Jourd'Heuil *et al.*, 1993).

The plasma lipid profile must play an important role in regulating the membrane fluidity. For example, a previous study has demonstrated that when the cardiomyocytes were cultured in a medium containing high cholesterol content, the cardiomyocyte plasma membrane became more rigid (Bastiaanse *et al.*, 1993). In cirrhosis, lipid metabolism is highly abnormal (Zambon *et al.*, 1995). It has been documented that plasma polyunsaturated long chain fatty acids are significantly reduced in both cirrhotic patients and animal models compared with controls (Cabre *et al.*, 1988; Moreira *et al.*, 1995). A decrease in polyunsaturated long chain fatty acid levels reduces the membrane fluidity. The plasma cholesterol and liver microsomal membrane cholesterol levels have been documented to be increased in

cirrhosis (Moreira *et al.*, 1995). An increase in cholesterol content also decreases the membrane fluidity. Since alterations in plasma lipid profiles are evident, it is logical to speculate that the cardiac plasma membrane lipid composition and fluidity could be changed in cirrhosis As stated above, changes in the membrane fluidity are usually correlated with alterations in protein function. This background forms the basis for further speculation that changes in cardiac plasma membrane fluidity lead to alterations of β-adrenergic receptor function in cirrhotic cardiomyopathy.

1.5.4. Summary of membrane fluidity and protein functions

Membrane fluidity defines the motional freedom of lipid molecules or substituents in the membrane lipid bilayer. It has been demonstrated that changes in the membrane fluidity can alter membrane protein function. Cell plasma membrane fluidity is regulated by the blood milieu. In cirrhosis, blood lipid metabolism is highly abnormal. Therefore, it is possible the cardiac plasma membrane fluidity is changed. Alterations in cardiac plasma membrane fluidity may play an important role in diminished β-adrenergic receptor function in cirrhosis.

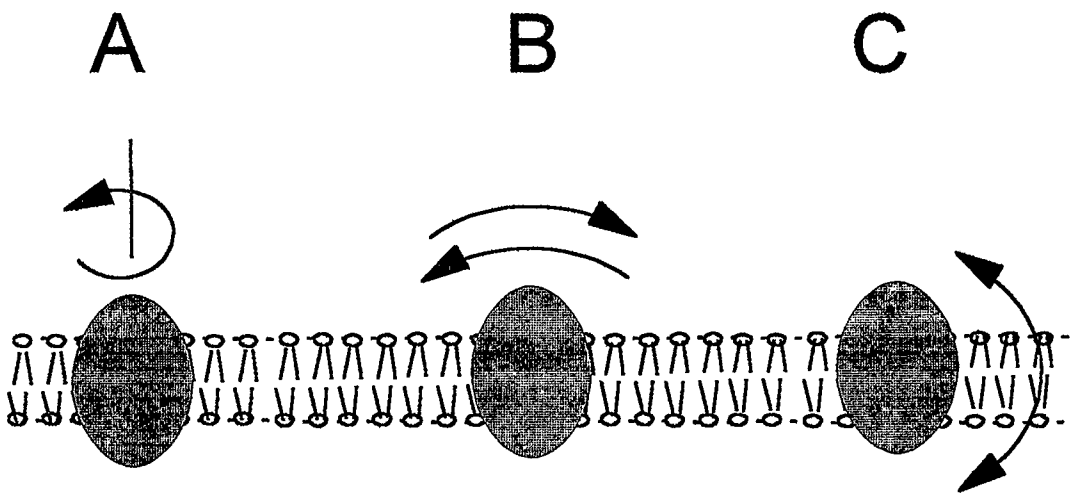


Figure 1.2. Motional freedom of molecules in the membrane bilayer. A) Rotational movement; B) Wobbling movement; and C) Flip-flop movement.

CHAPTER TWO

WORKING HYPOTHESES AND EXPERIMENTAL APPROACHES

In liver cirrhosis, cardiac performance is impaired which is characterized by increased baseline cardiac output and impaired ventricular performance under pharmacological stimulation and physiological and mental stress. This has been termed "cirrhotic cardiomyopathy". The pathogenic mechanisms for cirrhotic cardiomyopathy remain unclear. To elucidate the possible mechanisms underlying cirrhotic cardiomyopathy, the following two major hypotheses were proposed and adequate experimental approaches were pursued:

1) Cardiac contractile function is modulated by β-adrenergic receptors and their post-receptor signal transduction pathway. Therefore, it was hypothesized that the cardiac β-adrenergic receptors and their signal transduction pathway function are altered. Furthermore, this alteration may play an important role in blunted cardiac performance in cirrhosis. To test the above contention, cardiac performance in liver cirrhosis was evaluated by measuring left ventricular papillary muscle contractility in a rat model of cirrhosis induced by common bile duct ligation. Cardiac plasma membrane β-adrenergic receptor density and binding affinity were determined by a receptor binding assay using ^{3}H-dihydroalprenolol as a radioligand. Cardiac β-adrenergic receptor signalling was functionally evaluated by measurement of cAMP generation after stimulating the β-adrenergic receptor signal transduction pathway at the receptor level with isoproterenol, G-protein level with fluoride ions, and adenylyl cyclase level with forskolin. G-protein expression was determined with Western blot analysis using RM/1, AS/7, and SW/1 antibodies to detect Gsα, Gi2α, and Gcommonβ subunits, respectively, followed by laser densitometry quantification.

2) Previous experiments have demonstrated that alterations in membrane physical properties or membrane fluidity could impair the function of some proteins located in the membrane milieu. The β-adrenergic receptor and adenylyl cyclase are proteins with multiple transmembrane domains. G-proteins are localized to the inner hemileaflet of the plasma membrane by lipid modification as shown in figure 1.1. During the process of β-adrenergic receptor activation, protein-protein interaction and possibly protein-lipid interaction occur in the plasma membrane lipid bilayer. Hence, a normal cardiac plasma membrane lipid environment is essential for the β-adrenergic receptor system to function properly. Since liver cirrhosis is associated with a host of lipid metabolic disorders, it was hypothesized that cardiac plasma membrane physical and chemical properties are altered. These alterations may play a role in the diminished β-adrenergic receptor function in cirrhosis. To test the validity of the above hypothesis, membrane fluidity was measured by steady-state fluorescent polarization using either DPH, *n*-AS, or 16-AP as probes to detect the static and dynamic components of membrane fluidity respectively. Cardiac plasma membrane total phospholipid and cholesterol contents were measured by colorimetric methods. The cardiac plasma membrane fatty acid composition was analyzed by gas-chromatography. The effects of membrane fluidity on the β-adrenergic receptor density and affinity were evaluated by measuring these variables before and after fluidizing cardiac plasma membranes with a fluidizing agent, 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate (A_2C). The relationship between membrane fluidity and β-adrenergic receptor function was assessed by

measurement of cAMP generation stimulated at different levels of the β-adrenergic receptor signal transduction pathway, before and after the cardiac plasma membrane fluidization.

CHAPTER THREE

MATERIALS AND METHODS

3.1. CHEMICAL REAGENTS AND ISOTOPES

Fatty acid standard, 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate (A_2C), (-)-Isoproterenol(+)-bitartrate salt, sodium fluoride (NaF), forskolin, ATP, and GTP were purchased from Sigma Chemical Company (St. Louis, USA). Radioligand ^{3}H-dihydroalprenolol (^{3}H-DHA) and cAMP protein binding assay kit were purchased from Amersham Ltd. (Montreal, QC, Canada). Fluorescent probes 1,6-diphenyl-1,3,5,-hexatriene (DPH), *n*-(9-anthroyloxy)-stearic acids (*n*-AS) and 16-(9-anthroyloxy)palmitic acid (16-AP) were purchased from Molecular Probes (Junction City, OR). Polyclonal antibody RM/1 for Gsα, AS/7 for Gi2α, and SW/1 for Gcommonβ were purchased from Dupont, New England Nuclear (Boston, MA, USA). The ECL Western Blot Detection system and Hyperfilm-ECL were obtained from Amersham. Other reagents were purchased either from Sigma Chemical Company or Fisher Scientific (Pittsburg, PA, USA), and they were the highest grade available on the market.

3.2. ANIMAL MODELS

3.2.1. Animal surgery

Male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada or Bioscience, Calgary, AB, Canada) weighing between 200-250 grams and 350-400 grams were used in the present investigation. The animals were housed in an environmentally controlled vivarium with a 12 hr light/dark cycle and allowed access to rat chow and water *ad libitum*. The protocol was approved by the Animal Care

Committee of the University of Calgary Faculty of Medicine under the guidelines of Canadian Council on Animal Care. Half the rats were subjected to common bile duct ligation (BDL) to induce cirrhosis. For the sham control group (Sham), rats were subjected to all the surgical manipulation except that the bile duct was not ligated. Previous evidence suggested that after about 4 weeks of surgery, the BDL rats showed reliable cirrhotic changes. Therefore, we chose 4 weeks duration of BDL for the experiments. The details of the bile duct ligation have been described previously (Kountouras *et al.*, 1985, Lee and Sharkey, 1993), but briefly, under halothane inhalation anaesthesia the common bile duct was exposed through a midline abdominal incision and doubly ligated with 3-0 silk thread, then sectioned between the ligatures. The incision was closed with 5-0 silk thread. After surgery, all the rats were given Penicillin G Benzathine (30,000 U i.m.) and Gentamicin spray at the incision site to prevent infection.

3.2.2. Portal pressure measurements

To confirm the existence of portal hypertension in the present cirrhotic animal model, 11 rats from both groups were anesthetized with pentobarbital (40mg/kg i.p.) and the portal pressure was measured (Lee and Sharkey, 1993). Portal venous pressure was measured by superior mesenteric vein cannulation with a 23-gauge needle connected to a Statham PD-23 pressure transducer (Gould, Oxnard, CA, USA) and a Gould 2400S recorder. Although pentobarbital has been shown to have strong effects on some hemodynamic parameters, previous experiments demonstrated

that no changes in portal pressure were observed in pentobarbital anesthetized rats (Lee *et al.*, 1985).

3.2.3. Organ histology and wet weight

To verify the existence of pathological cirrhotic changes in the BDL model, hepatic histological examination was performed. Liver tissue was fixed in 10% formalin and embedded with paraffin. Three micron thick tissue sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Liver and spleen wet weight were determined and normalized to 100 g of body weight.

To search for the histological evidence of any changes in cardiac architecture, 5 hearts from each group (BDL and Sham) were examined via the above histological processes. Heart and ventricular wet weights were also measured and normalized to 100 g of body weight as detailed in membrane preparation section.

3.2.4. Liver biochemistry tests

To ensure that there was liver function damage in the present cirrhotic animal model, liver biochemistry tests were performed. Truncal blood was collected after decapitation. The blood was placed on ice for half an hour, then centrifuged at 1000g. The serum was harvested. The serum samples were kept at -70°C and all the tests were done within three weeks after the serum collection. Total bilirubin, aspartate amino-transferase (AST), alanine amino-transferase (ALT), alkaline

phosphatase activity, bile salts, and total cholesterol were determined.

Total bilirubin was measured by previously reported methods (Hillmann *et al.*, 1967). The assay contained 0.1 ml of serum, 1 mmol/L of diazotized sulfanilic acid, and 0.2 mol/L of hydrochloric acid with surfactant at a final volume of 2.6 ml. The direct or conjugated bilirubin reacts with sulfanilic acid to form a pink colour compound. Indirect bilirubin or unconjugated bilirubin also reacts with diazotized sulfanilic acid in the presence of surfactant to form the same coloured compound. The intensity of the coloured compound is proportional to the bilirubin content. The reaction mixture was incubated at 37°C for 5 min and detected at a wavelength of 540 nm.

AST (EC:2.6.1.1) activity was measured by the methods of Bergmeyer and colleagues (1978). The assay is composed of 0.1 ml of serum, 200 mmol/L of l-aspartate, 12 mmol/L of 2-oxoglutarate, 600 U/L of malate dehydrogenase, 0.25 mmol/L of nicotinamide adenine dinucleotide (NADH) in a final volume of 1.3 ml at 30°C. AST transfers amino groups from aspartate to 2-oxoglutarate to produce oxalacetate. Malate dehydrogenase reduces oxalacetate to l-malate and at the same time NADH is oxidized to NAD. Because of NADH oxidation, absorbance at wavelength of 340 nm decreases, which is proportional to the activity of AST. The results were expressed as Sigma units. Each unit represents 1 μmole of NADH oxidized per min.

ALT (EC 2.6.1.2) was assayed using the same principal as AST measurement. Each assay tube contained 0.1 ml of serum, 400 mmol/L of L-alanine, 12 mmol/L of

2-oxoglutarate, 2000 u/L lactate dehydrogenase, and 0.25 mmol/L of NADH in a final volume 1.3 ml. ALT transfers amino groups from alanine to 2-oxoglutarate to produce pyruvate and l-glutamate. Lactate dehydrogenase reduces pyruvate to lactate and NADH oxidized to NAD. ALT activity is expressed as Sigma units. Each unit represents 1 μmol of NADH oxidized per min.

Bessey and colleagues' methods were employed to determine serum alkaline phosphatase activity (1947). *p*-Nitrophenyl phosphate was used as a substrate. Alkaline phosphatase hydrolyses *p*-nitrophenyl phosphate to *p*-nitrophenol and inorganic phosphate. When sodium hydroxide is added into the reaction mixture *p*-nitrophenol changes to a yellow colour compound detectable at the wave length of 400-420 nm. Each assay tube contained 0.5 ml of substrate, 0.5 ml of 2-amino-2-methy-1-propanol (1.5 mol/L) alkaline buffer and 0.1 ml of serum. After incubation at 37°C for 15 minutes, 10 ml of 0.05 N NaOH was added to stop the reaction. The samples were detected at a wavelength of 410 nm. Alkaline phosphatase activity was expressed as Sigma units. Each Sigma unit represents 1 μmol of inorganic phosphate hydrolysed from *p*-nitrophenyl phosphate per hour.

Bile acids were determined by the methods of Mashige and colleagues (1981). The reaction involves oxidation of bile acids to 3-oxo bile acids catalyzed by 3α-hydroxysteroid dehydrogenase. When bile acids are oxidized, NAD is reduced to NADH. Then NADH is oxidized to NAD and nitro blue tetrazolium salt is reduced to formazan. The maximum absorbance for formazan is at 530 nm. The intensity of the absorbance is proportional to the concentration of bile acids.

Serum total cholesterol content was tested using the same method as cardiac plasma membrane total cholesterol content analysis, which will be described in the later sections.

All the liver biochemistry assays were performed utilizing commercially available Sigma kits.

3.3. LEFT VENTRICULAR PAPILLARY MUSCLE CONTRACTILE FORCE STUDY

In order to verify the existence of cardiomyopathy in our cirrhotic rat model, left ventricular papillary muscle contractile force was studied. Recently published methods were employed in the present investigation (Bohm *et al.*, 1994, Otani *et al.*, 1988). Briefly, rats were sacrificed by decapitation with a Harvard guillotine. The hearts were quickly dissected and placed in a modified Tyrode's buffer which contains (in mmol/L): NaCl 122.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 24, and Glucose 10 at pH 7.4. Papillary muscles from left ventricles were isolated in a 95% O_2 and CO_2 saturated Tyrode's buffer. The papillary muscles were suspended with a 5-0 thread in an organ bath containing Tyrode's buffer at pH 7.4, 37°C saturated with 95% O_2 and 5% CO_2. After being equilibrated in the Tyrode's buffer for 1.5 hours, the papillary muscles were subjected to electrical field stimulation with a rectangle pulse at frequency of 1 Hz, voltage 1.5-fold of threshold (30-40 v), and a duration of 5 msec. The electric signal was generated by a Grass 88 Stimulator (Quincy, MA, USA). The isometric tension or contractile force was detected with a Grass force displacement transducer (Quincy, MA, USA) and recorded by a Grass

amplifier, Grass Model 75 Polygraph (Quincy, MA, USA). The initial tension for the papillary muscles was around 500 mg. Preliminary results demonstrated that at this initial tension maximum contractile force could be obtained. After contractile force reached steady state under electrical stimulation, cumulative doses of the β-adrenergic receptor agonist, isoproterenol, were added into the organ bath at a concentration range from 10^{-10} to 10^{-6} mol/L. When a dose reached maximum response, the next higher dose was added. Dose response curves were constructed. Maximum contractile force (E_{max}) and dosages needed for half maximum responses (ED_{50}) were calculated by nonlinear regression with a commercially available software Figure Perfect program (Biosoft Inc., Ferguson, MO, USA)

3.4. CARDIAC SARCOLEMMAL PLASMA MEMBRANE PREPARATION

3.4.1. Cardiac plasma membrane isolation

The cardiac plasma membranes were isolated according to methods reported previously (St. Louis *et al.*, 1976). In brief, rats were sacrificed by decapitation with a Harvard Guillotine. The hearts were quickly dissected and placed in a ice cold 10 mmol/L tris(hydroxymethy)aminomethane (Tris) buffer at pH 7.4. Blood was washed out. Fat, connective tissue, and large vessels were trimmed off. The hearts were blotted with Kimwipes tissue and wet weight was determined. Then, the atria were trimmed off and ventricular wet weight was measured. The ventricles were minced with scissors and homogenized with a homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) and a Polytron PTA 10 ST probe (Brinkmann) at setting 6

for 45 seconds twice. The homogenate was filtered through 4 layers of Meditron gauze sponges (Meditron corp. Montreal, QC, Canada) pre-wetted with Tris buffer. Potassium chloride (KCl) was added into the filtrate at a final concentration of 1.25 mol/L to extract contractile protein for 10 min. The filtrates were centrifuged at 9,000 g for 10 min. The pellets were extracted again with KCl at the above concentration for another 10 min and centrifuged at 4,000 g for 10 min. The resulting pellets were washed in Tris buffer twice, suspended in a 10% sucrose and layered on the top of a discontinuous 45%, 50%, 55%, 60% sucrose gradient. The constructed gradient was centrifuged at 40,000 g for 1 hour. The band between 50% and 55% sucrose was harvested and washed twice in Tris buffer as final membrane preparation. The membrane preparation was subjected to 12,000 g centrifugation for 30 min between each washing. The membranes prepared with the above methods were used for the β-adrenergic receptor binding assay, cAMP generation measurement, Western blot analysis of G-protein subunit expression, determination of membrane physical properties, and membrane chemical composition studies.

3.4.2. Cardiac plasma membrane protein assay

Bradford's method was used to determine membrane protein content (1976). Coomassie Brilliant Blue is the core reagent in this protein assay system. This dye has both red and blue colour forms. When Coomassie Brilliant combines with proteins, the dye shifts from red colour to blue colour which could be detected at 595 nm with a spectrometer.

3.4.3. Cardiac plasma membrane marker enzyme assay

To ensure the relative purity of the cardiac plasma membrane preparation, 5'-nucleotidase activity was tested as a plasma membrane enzyme marker. Since mitochondria are common cardiomyocyte cellular organelles, cytochrome-*c* oxidase which is dominant in mitochondria was chosen to exclude gross contamination by other cellular organelles.

3.4.3.1. Measurement of 5'-nucleotidase activity (EC 3.1.3.5)

A previously published method was used to determine the 5'-nucleotidase activity (Arkesteijin *et al.*, 1976). The assay contains AMP (3.2 mmol/L), bovine adenosine deaminase (400 U/L), 2-oxoglutarate (3.7 mmol/L), NADH (0.2 mmol/L), L-glutamate dehydrogenase (11,000 U/L). AMP is hydrolysed by 5'-nucleotidase to form adenosine and inorganic phosphorus. Adenosine is deaminated by adenosine deaminase to produce inosine and NH_4. NADH is oxidized to NAD in the presence of NH_4, 2-oxoglutarate, and L-glutamate dehydrogenase. NAD production produces a decrease of absorbance at 340 nm which is directly proportional to 5'-nucleotidase activity. The assay was carried out at 30°C. Each reaction cuvette contained 1.2 ml above reagent and 10 μg of membrane protein. The absorbance value at 5 minutes after adding the membranes was recorded as initial A. After incubation for another 5 minutes, the absorbance was recorded as final A. Final A minus initial A produces delta A. The activity of 5'-nucleotidase was calculated from the delta A value and expressed as units. Each unit represents 1 μmol of NADH consumed per minute.

3.4.3.2. Measurement of cytochrome-c oxidase (EC 1.9.3.1)

Wharton and Tzagoloff's method was used for cytochrome-*c* oxidase activity measurement (Wharton and Tzagoloff, 1967). Cytochrome *c* (100 mg) was reduced in 8 ml of 10 mmol/L potassium phosphate at pH 7 by adding 5 mg of sodium ascorbate. The excess ascorbate was removed by dialysing the cytochrome *c* in a Spectrapor dialysis tubing against the potassium phosphate buffer for 24 hours at 4°C with 3 changes of the buffer. The cut-off molecular weight for the dialysis tubing was approximately 3,500 and dry cylinder diameter was 11.5 mm (Spectrummedical Industries, INC, LA, USA). After dialysis, potassium phosphate buffer was added to the dialysis tubing to make a final volume of 10 ml. Cytochrome *c* oxidase activity assay was carried out at 37°C. Each assay tube contained 2.7 ml of potassium phosphate buffer, 0.2 ml of the above reduced cytochrome *c*, and 100 μg (0.1 ml) of membrane protein. The absorbance at a wavelength of 550 nm was recorded for 10 minutes at 1 min intervals. The activity of cytochrome *c* oxidase was calculated using these absorbance values. The cytochrome *c* oxidase activity was expressed as units, and each unit oxidized 1 μmol of reduced cytochrome *c*.

3.5. β-ADRENERGIC RECEPTOR SIGNAL TRANSDUCTION FUNCTION ASSESSMENT

3.5.1. β-Adrenergic receptor binding assay

^{3}H-dihydroalprenolol (^{3}H-DHA) (TRK.649, Amersham, Montreal, QC, Canada) was used as a radioligand. The specific activity of the radioligand was 86

ci/mmol. The receptor binding assay was performed according to previously reported methods (Jacob *et al.*, 1993). The assay contained, in a total volume of 300 μl, 10 mmol/L of Tris pH 7.4, 2 mmol/L $MgCl_2$, 1% bovine serum albumin, 1-80 nmol/L of ^{3}H-DHA and 100 μg of protein. Total binding was determined in the absence of propranolol. Nonspecific binding was measured in the presence of 10^{-5} mol/L of propranolol. Specific binding was calculated by subtraction of non-specific binding from total binding. The mixture was incubated at 37°C for 45 min in a shaking water bath, and the reaction was stopped by quenching with 3 ml of ice cold water. Bound and free radioligands were separated by rapid filtration through Whatman microfiber filters (GF/C) on a 12 port Millipore filtration manifold under vacuum pressure of -80 kPa. The tubes and the filters were washed twice with 3 ml of ice cold water. Radioactivity on the filters was determined in Ecolite (+) scintillation liquid (ICN, Costa Mesa, CA, USA), using a LKB Wallac 1214 RackBeta liquid scintillation counter (LKB, Stockholm, Sweden).

Receptor density and binding affinity can be obtained by different methods. Commonly used ones are Scatchard plot (Eadie-Hofstee plot) and non-linear regression. The principal of Scatchard plot is by a linear transformation of the Langmuir isotherm (non-linear regression) to yield the following equation and to plot bound/free as a function of bound:

$$B/F = -B/K_A + Bmax/K_A$$

On a Scatchard plot, B/F (bound/free) is the Y axis. B (bound) is the X axis. $-1/K_A$ is the slope. K_A is binding affinity. When B/F equals zero, B equals Bmax.

Therefore, the Bmax is the intercept on the X axis. On the other hand, non-linear regression directly uses raw data to calculate receptor Bmax and Kd. It has been demonstrated that direct calculation of Bmax and Kd by non-linear regression is more accurate than Scatchard plot (Meddings *et al.*, 1991). Therefore, it was chosen to use direct non-linear regression to acquire Bmax and Kd values. The non-linear regression equation used in the present study is:

$$RL^* = (Bmax \cdot L^*)/(Kd + L^*)$$

Where RL^* is receptor bound with radioactive ligand. Bmax is receptor density. L^* is free radioactive ligand. Kd is dissociation constant. Measurement of bound ligand d.p.m was converted to molar concentration is by following equation (Hulme, 1990):

$$RL^* = 10^{-9} \cdot B/(V \cdot SA \cdot 2220)\ [M]$$

Where, RL^* is ligand bound with receptor, B is the bound d.p.m., V is the assay volume, SA is specific activity. The results were further normalized to (divided by) the protein concentration and expressed as pmol/mg protein. Receptor density (Bmax) and receptor dissociation constant (Kd) were calculated using a commercially available LIGAND program (Bio-soft, version 3).

3.5.2. Cardiac plasma membrane cAMP production measurement

cAMP content was determined according to the methods developed by Gilman (1970) utilizing a protein binding assay kit from Amersham (TRK.432, Amersham, Montreal, QC, Canada). The binding proteins are purified from bovine muscles. The major components of the binding proteins are cAMP dependent

protein kinases. Experiments have demonstrated that these proteins specifically bind with cAMP. No cross binding was found between the binding protein and ATP or GTP using Amersham assay kits. All the cAMP assays were carried out on the same day the cardiac plasma membranes were prepared.

cAMP generation in the cardiac plasma membranes under the stimulation of isoproterenol (10^{-4} mol/L), NaF (10 mmol/L), and forskolin (100 μmol/L) was determined. When isoproterenol was used as a stimulator, GTP at a final concentration of 100 μmol/L was added. $AlCl_3$ was added at a final concentration of 30 μmol/L when NaF was used as an agonist. The cAMP generation assay was carried out in a basic buffer containing Tris 50 mmol/L, EDTA 1 mmol/L, 3-isobutyl-1-methylxanthine (IMBX) 1 mmol/L, and $MgCl_2$ 10 mmol/L at pH 7.4. Both EDTA and IMBX have inhibitory effects on phosphodiesterase to block cAMP degradation. To ensure enough substrate for the reaction, ATP, creatine phosphate, and creatine phosphokinase were added into each reaction tube at the final concentration of 1 mmol/L, 10 mmol/L and 12 μg/per tube, respectively. As shown in the figure 3.1, creatine phosphokinase catalyses the transfer of phosphate from creatine phosphate to ADP, and this process will supply ATP. The total volume of each cAMP generation assay was 300 μl. The assays were started with 100 μg membrane protein for isoproterenol stimulation and 25 μg membrane protein for NaF, and forskolin stimulation. The preliminary experiments demonstrated that at the above membrane concentration, cAMP generation was within the detectable range of the cAMP protein binding assay system. After incubation with the above agonists for 20 min,

the reactions were stopped by boiling the incubation mixture for 3 min (Severson *et al.*, 1972). Then, the mixtures were centrifuged at 10,000 g for 10 min.

A total of 100 μl of supernatant from each cAMP generation tube was assayed for cAMP content as per the Amersham protocol. The principle of the cAMP assay is shown in figure 3.2. Bound and free ^{3}H-cAMP were separated by charcoal adsorption followed by 2000g centrifugation. cAMP concentration was normalized to protein content and expressed as pmol/mg protein/min.

Figure 3.1. ATP regeneration system. Creatine phosphate kinase catalyses transfer of high-energy phosphate from creatine phosphate to ADP to produce ATP.

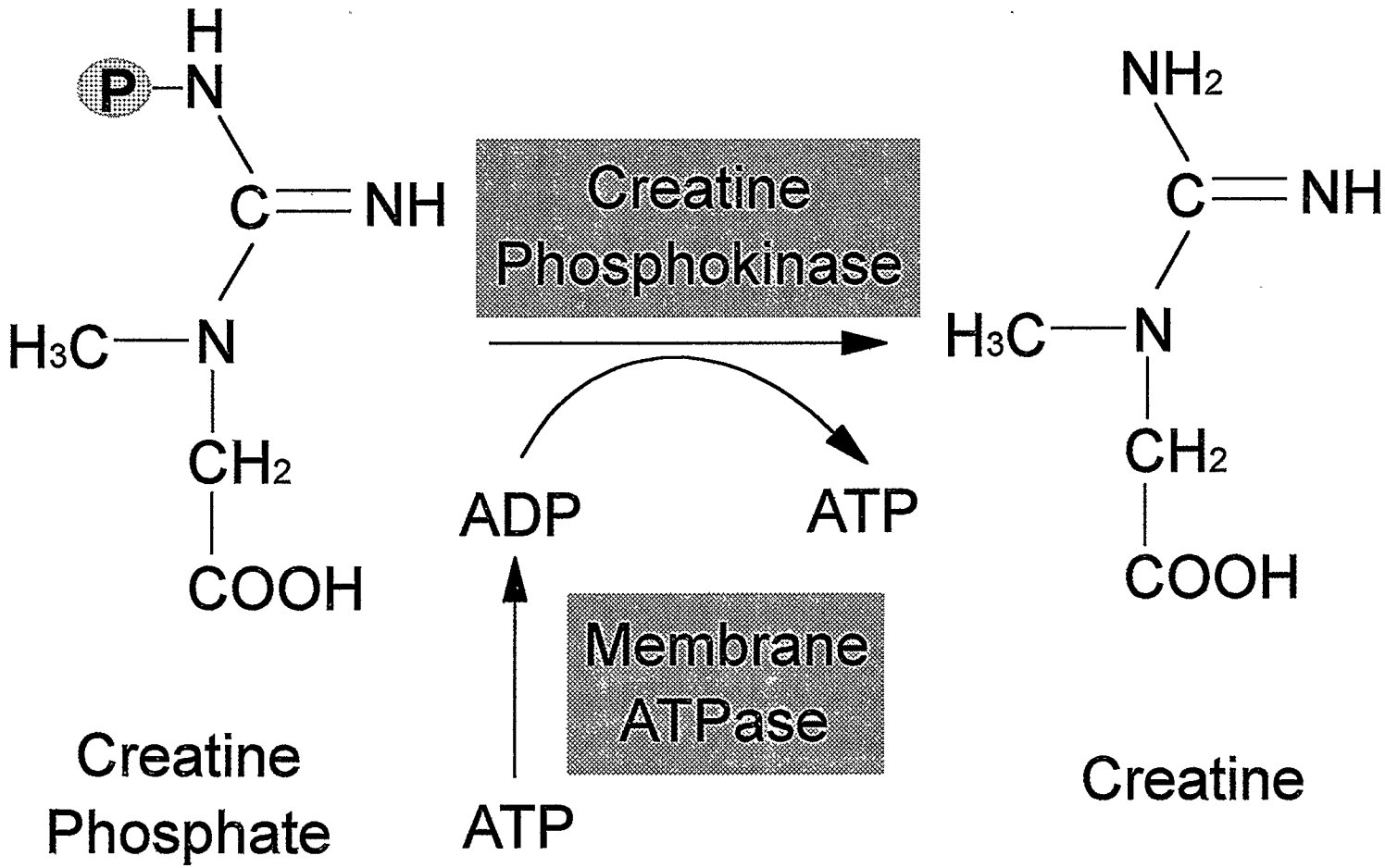
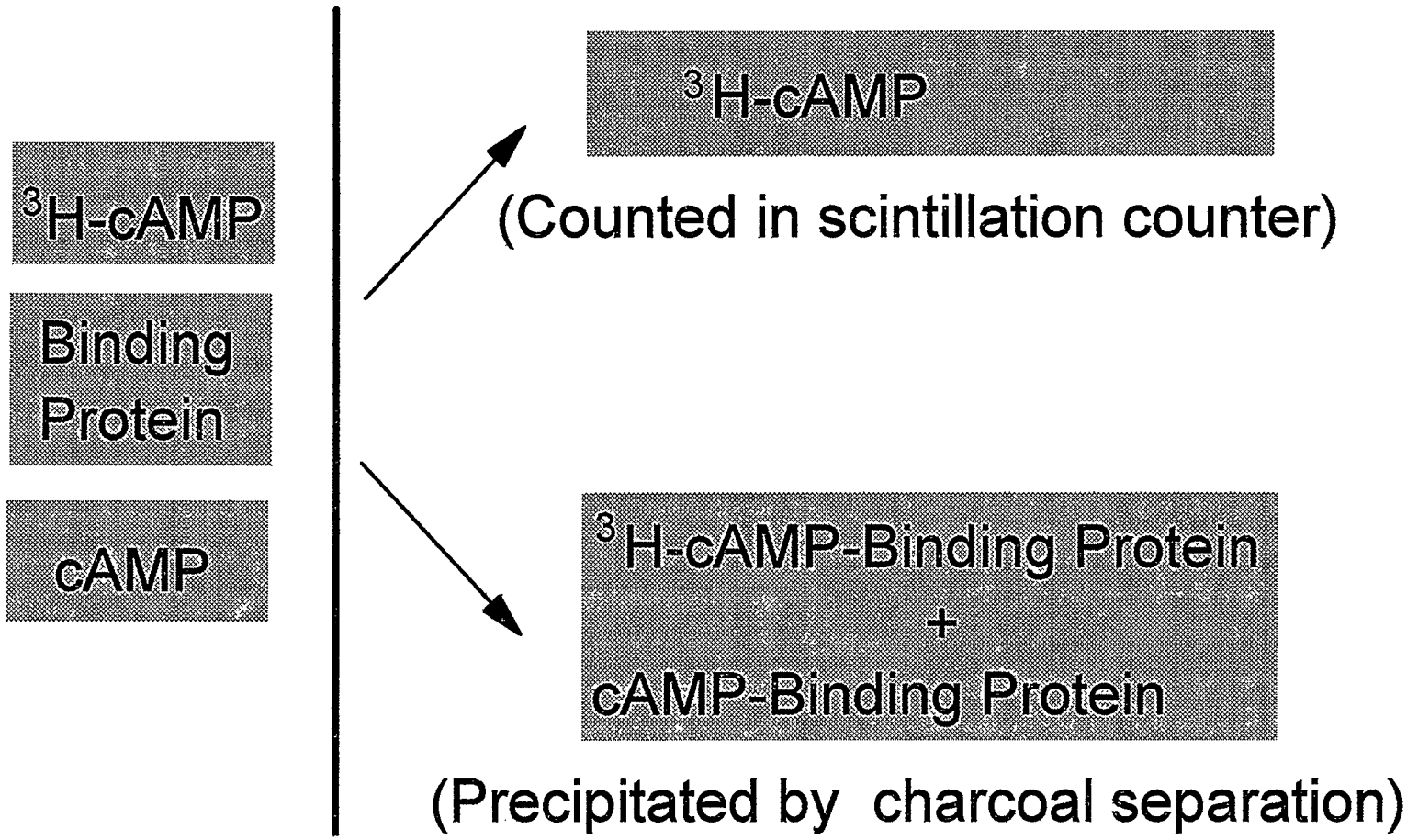

H
P
N
C
NH
H3C
N
CH2
COOH
Creatine Phosphokinase
ADP
ATP
Membrane ATPase
ATP
Creatine Phosphate
NH2
C
NH
H3C
N
CH2
COOH
Creatine

Figure 3.2. Basic principles of cAMP protein binding assay. cAMP generated from cardiac plasma membrane, ^{3}H-labelled cAMP, and cAMP binding protein are incubated at 4°C for 2 hours. Cold cAMP competes with ^{3}H-labelled cAMP to combine with the binding proteins. The cAMP bound with the binding proteins is adsorbed by charcoal and separated from free cAMP by centrifugation. The supernatant is counted in a scintillation counter.


^{3}H-cAMP
Binding Protein
cAMP
^{3}H-cAMP
(Counted in scintillation counter)
^{3}H-cAMP-Binding Protein
+
cAMP-Binding Protein
(Precipitated by charcoal separation)

3.5.3. Western blot analysis of G-protein expression

Western blot analysis was performed by Dr. Miyamoto at the Sapporo Medical University in Japan using previously published methods (Miyamoto *et al.*, 1994; Miyamoto *et al.*, 1992). In brief, cardiac plasma membrane proteins were boiled for 5 min in a buffer containing 62.5 mmol/L Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 0.0025% bromophenol blue at pH 6.8. The membrane proteins (5μg/per lane) were applied to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) with 10% polyacrylamide for 100 min at 40 mA. Following electrophoresis, the gel was soaked in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. Then, proteins were transferred to a pre-wet nitrocellulose membrane at 180 mA for 90 min. Nitrocellulose membranes were incubated in an incubation buffer containing 10 mmol/L Tris, 0.9% NaCl, and 3% bovine serum at pH 7.4 for 2 hours. Specific rabbit antisera RM/1, AS/7, SW/1 at a dilution factor of 1:2000 were used to detect Gsα, Gi2α, and Gcommonβ respectively. After incubation with the above antibodies in the incubation buffer at room temperature overnight, the nitrocellulose membranes were washed 5 times within 30 mins and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at a dilution factor of 1:1000 for 2 hours in the above incubation buffer. The immunoactivity was assessed by utilizing enhanced chemiluminescence (ECL) Western Blot Detection system (Amersham). In the ECL Western Blot Detection System, horseradish peroxidase/hydrogen peroxide oxidize a luminescent agent luminol (cyclic diacylhydrazides). After

oxidation, luminol is excited and the light is emitted. In the presence of an enhancement agent phenol, the light output was greatly increased. Followed by exposure to the films (Hyperfilm-ECL), the intensity of the emitted light was recorded. The preliminary experiments have demonstrated that the relationship between the intensity of immunoblotting signals recorded on the Hyperfilm-ECL and the concentration of the membrane proteins loaded onto the SDS gel was linear. The expression of G protein subunits was quantified via scanning each band on the hyperfilm-ECL by computerized laser scanning densitometry using a MasterScan Intensive Densitometer (Scanalytics, CSP Inc., Billerica, MA, USA). The results were expressed as arbitrary optical density units.

The antisera used to detect αs subunits were RM/1 which were raised in rabbits using a synthetic decapeptide RMHLRQYELL as an antigen. This antigen corresponds to the C-terminus of Gsα predicted from cDNA sequence. It has been confirmed that RM/1 can block isoproterenol stimulated cAMP production. It has also been demonstrated that no cross-reaction occurs between RM/1 and Gi1α, Gi2α, Gi3α, Goα. Therefore, these antisera are very specific to bind with Gsα (Simonds *et al.*, 1989b).

The rabbit antisera used to detect Giα in the present study were AS/7 which were raised against a synthetic decapeptide with a sequence of KENLKDCGLF. This sequence corresponds to the C-terminus of transducin-α, Gtα. This antigen decapeptide, KENLKDCGLF, has only one residue which is different from the C-terminal sequence of Gi1α and Gi2α. The C-terminal sequence of Gi1α and Gi2α

is KNNLKDCGLF (Goldsmith *et al.*, 1987). Immunoblot and ELISA studies have demonstrated that antisera, AS/7, react specifically with Gi1α and Gi2α and transducin-α. In the heart, no transducin α has been detected thus far. Very weak reaction between the antisera, AS/7, and Gi3α was detected. No cross reaction was observed between AS/7 and Goα, which also belongs to Gi protein family (Goldsmith *et al.*, 1987). This is because that the C-terminal sequence of Goα is ANNLRGCGLY which has five residues being different from the antigen decapeptide used to raise AS/7 (Goldsmith *et al.*, 1988).

Rabbit antibodies, SW/1, were used in the present study to detect G-protein β-subunit expression. The antisera, SW/1, were raised against a synthetic peptide antigen which corresponds to the amino acid sequence of 330-340 in β1 subunit of G-proteins. The antisera, SW/1, recognize 35- and 36-kDa β (Spiegel, 1990). Since the structures β-subunits in the different G-proteins are highly conserved, at the present no antibodies are available to differentiate between 5 different β-subunits.

3.6. MEMBRANE FLUIDITY MEASUREMENT

Membrane fluidity was determined by steady-state fluorescent polarization methods (Meddings *et al.*, 1990). Fluorescent molecules emit fluorescent light when excited by the light at a certain wavelength. In the situation where fluorescent probes are embedded in a fixed or rigid environment, the emission light of fluorophore is in the same direction as exciting light because no fluorescent probe movement occurs during the life time of the fluorophore excitation. However,

biological membrane is a fluid mosaic. Fluorescent probes are free to move and have different modes of movement such as rotating, wobbling and flip-flop in the membrane bilayer. Therefore, the emission of the fluorophore in the membranes is depolarized when excited. The extent of depolarization is dependent on the membrane physical properties or membrane fluidity. By measuring the fluorescent depolarization, the membrane physical properties or membrane fluidity could be extrapolated.

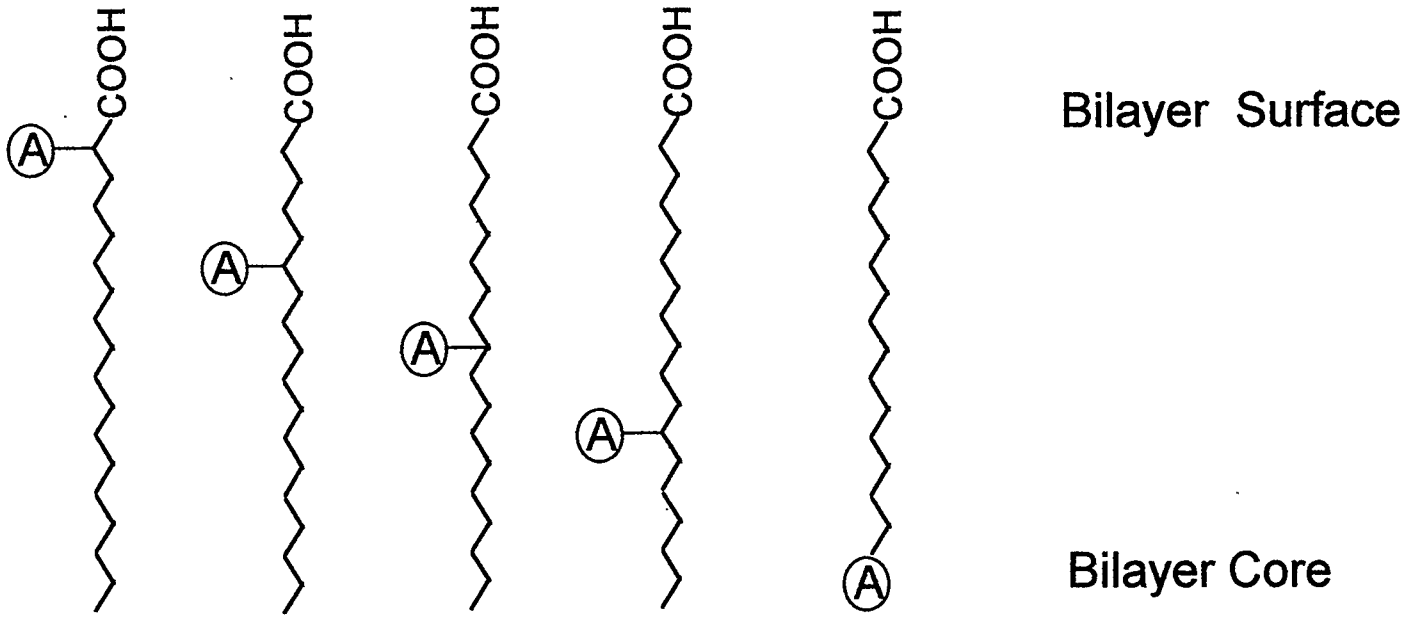
Six fluorescent probes were used for this study: 1,6-diphenyl-1,3,5,-hexatriene (DPH); four isomers of stearic acid labelled with 9-anthroyloxy groups at either carbon numbers 2, 6, 9, or 12 (*n*-AS); and 16-(9 anthroyloxy) palmitic acid (16-AP).

DPH was used to determine the static component of membrane fluidity. The location of DPH probes in the membrane bilayer is not completely clear. However, experimental data demonstrated that the long axis of DPH is parallel to the lipid fatty acid chains. The measurement represents the wobbling movement of the probe molecules in the membrane bilayer. DPH was dissolved in tetrahydrofuran (THF) at the concentration of 1 mmol/L used as stock solution and kept in 20°C. On the day of fluorescent depolarization measurements, DPH was further diluted in THF in 1:150 ratio to constitute the working solution. Twelve μl of the above working solution was added to 100 μg of membrane protein and incubated in a total volume of 4.75 ml of 10 mmol/L Tris buffer at pH 7.4 in dark. The incubation time was 1 hour to let the probe incorporate into the membrane vesicles.

The fatty acid probes are sensitive to the dynamic component of membrane

fluidity (Meddings and Theisen, 1989). Furthermore, since the fluorescent anthroyloxy group can be placed on any of the base carbons on the stearic acid, this series of probes has been found useful to determine fluidity gradients within membranes (Meddings and Theisen, 1989; Tilley *et al.*, 1979). As shown in the figure 3.3, 2-AS places the fluorescent group just underneath the phospholipid head group while 12-AS and 16-AP localizes the fluorescent group close to the core of the bilayer. Anthroyloxy stearic acids and anthroyloxy palmitic acid were dissolved in 80% ethanol (v/v) containing 10 mmol/L tris at pH7.4 to make 0.25 mmol/L fluorescent stock solution and stored in -20°C. On the day of fluorescent measurements, 5 μl of stock solution was added to 50 μg of membrane protein. The mixture was incubated at room temperature for 20 min in dark.

Figure 3.3. *n*-AS and 16-AP probe position at the different depths of membrane bilayer. The 2-AS probe positions close to the surface of membrane bilayer and 16-AP locates in the membrane bilayer core. Because fluorescent group 9-anthroyloxy group can be placed at different depths of the membrane bilayer, membrane fluidity gradient can be determined.



2-AS 6-AS 9-AS 12-AS 16-AP

Ⓐ = 9-Anthroyloxy molecule

n-AS=n-(9-anthroyloxy)Stearic acid 16-AP=16-(9-anthroyloxy)Palmtic acid

For the purpose of this study, the term membrane fluidity is used to refer to the relative motional freedom of these probes within the membrane bilayer, which could be extrapolated to represent the motional freedom of lipid or protein molecules in the membrane bilayer. It should be kept in mind that the methods used here only indirectly represent lipid or protein molecule motional freedom. A more extensive explanation of the term membrane fluidity has been described elsewhere (Brasitus *et al.*, 1986).

For DPH probe measurement, the excitation wavelength was 360 nm and emission wavelength was 450 nm. *n*-AS and 16-AP probes were excited at a wavelength of 385 nm and recorded at 450 nm. Steady-state polarization measurements were performed using a SLM-4800C spectrofluorometer (SLM-Aminco, Urbana, IL, USA). This is a T format spectrofluorometer. The principles for the measurement are shown in figure 3.4. The intensity of horizontal and vertical components of fluorescent emission were detected simultaneously. These results are used to further calculate steady-state fluorescence anisotropy parameter, r_s, according to the following equation (Sawyer, 1988):

$$r_s=(I_V - I_H)/(I_V + 2I_H)$$

Where r_s is anisotropy parameter; I_V and I_H represent the fluorescent emission intensity detected by photomultiplier detectors vertically and horizontally as shown in figure 3.4. The anisotropy parameter r_s is used to quantitatively represent the membrane physical properties. The higher the r_s values, the more rigid the membranes are.

Figure 3.4. Schematic description of the principles of T-format fluorescent depolarization measurement. LS, light source; M, monochromator which selects the light wavelength; PE, excitation polarizer which polarizes excitation light in horizontal and vertical direction in each measurement; C, sample cuvette compartment; P_H and P_V, horizontal and vertical emission polarizers which polarize the fluorescent light in horizontal and vertical direction respectively; PM_H and PM_V, photomultiplier detectors which detect horizontal and vertical component of fluorescent emission respectively.

LS
M
PE
C
PMH
M
PH
PV
M
PMV

3.7. CARDIAC PLASMA MEMBRANE LIPID COMPOSITION ANALYSIS

In order to determine the lipid composition of the cardiac plasma membrane preparations, total phospholipid, cholesterol, and fatty acid composition were analyzed. All lipid contents were normalized to protein concentration of the membrane preparations. The following procedures were used.

3.7.1. Folch extraction

For accuracy of lipid analysis, the cardiac plasma membrane preparations were first subjected to extraction in an organic solvent by the method of Folch *et al* (1957). To avoid lipid autolysis and degradation, lipids were extracted from the membrane preparations on the same day when the membrane vesicles were prepared. One hundred μg of membrane protein in 1 ml of isotonic sodium chloride was added to 20 volumes of organic solvent containing chloroform and methanol at a ratio of 2:1 (v/v) and extracted overnight. To remove nonlipid contamination in the extract, aqueous wash was employed. A total of 4 ml of isotonic sodium chloride was added to each Folch extraction tube and mixed well. The extract was centrifuged at 1000 g for 20 min. The lower phase was aspirated and dried down under nitrogen. Total phospholipid, cholesterol, and fatty acid contents were tested after the above processing.

3.7.2. Total phospholipid analysis

Previously published methods were used to determine total phospholipid

content in the membrane preparation (Rouser *et al.*, 1970). The principle of this assay is the reaction of inorganic phosphorus with ammonium molybdate to form phosphomolybdate complex. In the presence of ascorbic acid, phosphomolybdate is reduced to form a yellow colour compound which could be directly detected at a wavelength of 800 nm (Chen *et al.*, 1956). To each Folch extracted tube, 260 μl of 70% perchloric acid ($HClO_4$) was added. These tubes were heated at 180°C for 75 mins to digest the phosphorus-ester bonds of the phospholipids and produce inorganic phosphorus. The tubes were cooled at room temperature. A total of 920 μl of double distilled water, 400 μl of 1.25% ammonium molybdate and 400 μl of 5% ascorbic acid were added to each tube. The tubes were then incubated at 100°C for 5 mins and absorbance was read at a wavelength of 800 nm. Inorganic phosphate (K2HPO4) was used to construct standard curves. All the samples were analyzed in triplicate. The results of total phospholipid content were expressed as nmol per milligram of protein.

3.7.3. Total Cholesterol measurement

After Folch extraction, 100 μl of 10% Triton in 95% ethanol was added into each tube. Cholesterol was measured by an enzymatic colorimetric method using a Boehringer kit (Boehringer Mannheim Canada, Montreal, QC, Canada) and a standard curve was constructed. The principle of the assay is as follows: cholesterol esterase hydrolyzes cholesterol ester to free cholesterol. Free cholesterol is oxidized to Δ^4-cholestenone and hydrogen peroxide (H_2O_2) by cholesterol oxidase. H_2O_2 and

peroxidase further oxidizes 4-aminophenazone and phenol to a red-colored compound 4-(*p*-benzoquinone-monoimino)-phenazone which can be detected by a spectrophotometer at a wavelength of 485 nm. Every sample was assayed in duplicate. The results of total cholesterol content were expressed as nmol per mg protein.

3.7.4. Fatty acid composition measurement

Following Folch extraction, lipid fatty acids were methyltransesterified by utilizing modified methods (Lepage and Roy, 1986) and quantified by gas chromatography. Briefly, 200 μl of acetyl chloride was gently added into each tube containing Folch extracted lipids. Three ml of benzene-methanol in a ratio of 1:4 (v/v) was pipetted into the tubes. The lipids were then subjected to methanolysis at 100°C for 1 hour. After methyl-transesterification, 5 ml of 6% K_2CO_3 was added into the tube to stop the reaction and neutralize the tube contents. Following a brief vortex and centrifugation at 1000 g for 20 min, the upper solvent phase was collected and dried under nitrogen. Each sample was then reconstituted in 1 ml of hexane and transferred to gas chromatography vials for analysis. The resulting fatty acid methyl esters were separated by a Hewlett-Packard 5890 GLC system (Hewlett-Packard, Avondale, PA, USA). Supelcowax 10 capillary column with a diameter of 0.25 mm was employed. The samples were injected with an inlet temperature at 280°C and a flow rate of 30 ml/min. Nitrogen was used as a carrier gas and hydrogen as a make-up gas. The column temperature was increased in a step-wise fashion 5°C/min

from 90°C to 170°C. The eluted fatty acids were quantitated using a flame ionization detector maintained at 350°C. Fatty acids over the range of 14:0 and 22:6 were clearly separated using the above temperature gradient technique (Meddings and Dietschy, 1989). Identification of each methyl ester was established from the known standards. The results of fatty acid composition were expressed as weight percent.

3.8. EVALUATION OF THE RELATIONSHIP BETWEEN MEMBRANE FLUIDITY AND β-ADRENERGIC RECEPTOR FUNCTION

In another series of experiments, cardiac plasma membrane fluidity was manipulated using a fluidizing agent 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)octanoate (A_2C) at graded concentrations (0-500 nmol/mg protein) to determine the effects of change in cardiac plasma membrane fluidity on β-adrenoceptor function. Three series of experiments were performed.

1) The effects of A_2C on membrane fluidity were tested by adding different concentrations of A_2C to the membrane vesicle preparations. Both time and concentration kinetics were determined. A_2C is a fatty acid-like compound with two alkoxy groups and one cyclopropyl group as shown on the figure 3.5. Since the cyclopropyl group mimics the function of fatty acid double bonds, it can change the packing order of the membranes and fluidize the membrane lipid environment.

2) The effects of membrane fluidity on the β-adrenergic receptor density and binding affinity were determined by measuring those parameters before and after

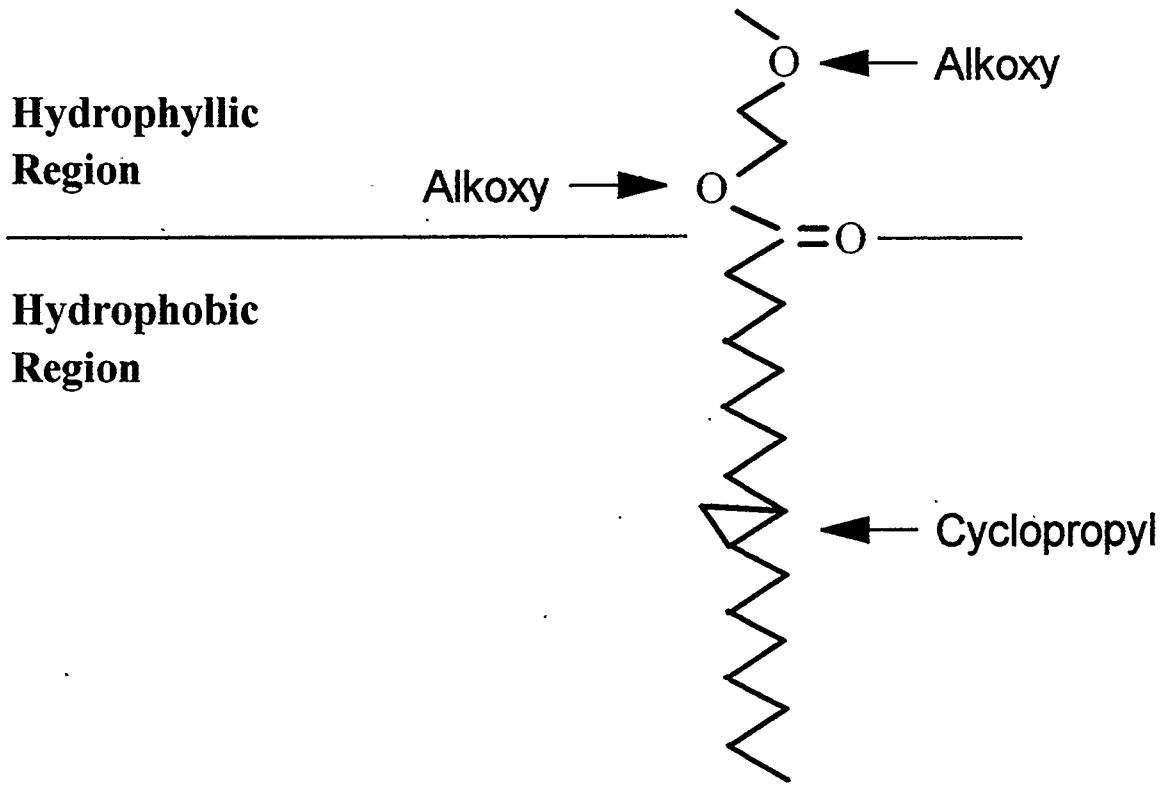
fluidizing the membrane vesicles with A_2C.

3) The effects of membrane fluidity on the β-adrenergic receptor function were assessed by detecting cAMP production before and after adding A_2C to the membranes. When the membranes were fluidized, cAMP production stimulated at different levels of the β-adrenergic receptor pathway was tested. To exclude the effects of A_2C *per se* on cAMP generation independent of its fluidizing effects, A_2C at concentrations of 100, 300, and 500 nmol/mg protein were added to six separate membrane preparations to monitor the cAMP generation without any agonists. The results showed that A_2C *per se* did not induce any changes in cAMP production.

3.9. STATISTICS

Statistics were performed by utilizing a commercially available software, InStat (Version 2, GraphPad Software Inc., San Diego, CA, USA). An unpaired student *t* test was employed in the present investigation. A p value of < 0.05 was considered significantly different between the groups.

Figure 3.5. A_2C molecular structure. A_2C is a fatty acid-like molecule with two alkoxy groups and one cyclopropyl group, hence it is abbreviated as A_2C. Cyclopropyl group has the effects of double bonds, therefore, A_2C has fluidizing effects.


Hydrophyllic Region
Hydrophobic Region
Alkoxy
O
O
Alkoxy
=O
Cyclopropyl

A_2C

CHAPTER FOUR

RESULTS I

ANIMAL MODEL CHARACTERISTICS

4.1. HEPATOSPLENOMEGALY AND PORTAL HYPERTENSION

The basic anatomical measurements are tabulated in table 4.1. Four weeks after the surgery, the body weight gain doubled in sham-operated rats compared with cirrhotic BDL rats. Decreased body weight gain in BDL rats could be the result of malnutrition in cirrhosis. Because of bile duct ligation, normal bile salt entero-hepatic circulation is interrupted. Therefore, levels of some nutrients, such as fat and certain vitamins absorbed from the intestine are decreased since their absorption is heavily dependent on bile acids. Other mechanisms could be the hepatocyte damage in this cirrhotic model. As the consequence of liver function insufficiency, protein and fatty acid synthesis is impaired. The normalized liver weight is doubled in cirrhotic rats compared with sham-operated rats. Liver enlargement is the result of liver inflammation, fibrosis, and portal hypertension. Spleen enlargement is evident by tripled normalized spleen weight in cirrhotic BDL rats. Portal hypertensive congestion is the major cause of splenomegaly.

Comparison of portal venous pressure in cirrhotic BDL rats with sham-operated rats is illustrated in figure 4.1. It is obvious that portal venous pressure in cirrhotic group was significantly higher than that in the sham-operated control group. Many pathological changes in liver cirrhosis lead to portal hypertension. Two major theories, "backward flow" and "forward flow", were used to explain increased portal venous pressure in liver cirrhosis. The backward flow theory emphasizes that the increase in portal pressure is induced by elevated portal venous resistance. Increased portal resistance is the consequence of distorted hepatic vasculature. The forward

flow theory focuses on the hyperaemia in the portal tributary area. This theory claims that the blood flow back to liver is more than the liver could handle, therefore, the portal pressure increases. It was estimated that forty percent of the increase in portal pressure is caused by forward flow and sixty percent is caused by backward flow (Benoit *et al.*, 1985).

Ascites is a common clinical manifestation in liver cirrhosis, especially in decompensated cirrhosis. In the present study, it was found that about 60% of the cirrhotic BDL rats developed ascites. The pathogenesis of ascites formation in the cirrhosis is a complicated issue and has been under intensive research in the last decade (Gentilini *et al.*, 1994; Wong and Blendis, 1994). Primary renal sodium and water retention may be a major pathological cause of ascites formation in cirrhosis. Alternatively, liver function insufficiency as shown in the next section leads to a reduction in the blood albumin level, which in turn decreases the plasma osmotic pressure. The decline of blood plasma osmotic pressure facilitates fluid leaking from the vessels into the peritoneal cavity. Portal hypertension as shown in figure 4.1. precipitates ascites development.

Table 4.1. Basic anatomical measurements. Data represent mean ± SEM. Four weeks after surgery, body weight gain in cirrhotic rats (BDL) was significantly lower than sham-operated rats (Sham). The liver and spleen wet weight were determined when the animals were sacrificed for cardiac plasma membrane preparation. Normalized liver weight is doubled in BDL compared with Sham. Normalized spleen weight is tripled in BDL rats compared with Sham.

Table 4.1. Basic anatomical data (n presented as Sham/BDL)

	Body weight before surgery (g)	Body wt., 4 weeks after surgery (g)	Liver wt./Body wt. (g/100g)	Spleen wt./Body wt. (g/100g)
Sham	371±5	464±8	3.7±0.1	0.22±0.01
BDL	357±4	401±7	6.7±0.2	0.66±0.02
n	21/14	21/14	11/18	11/18
p	>0.05	<0.01	<0.01	<0.01

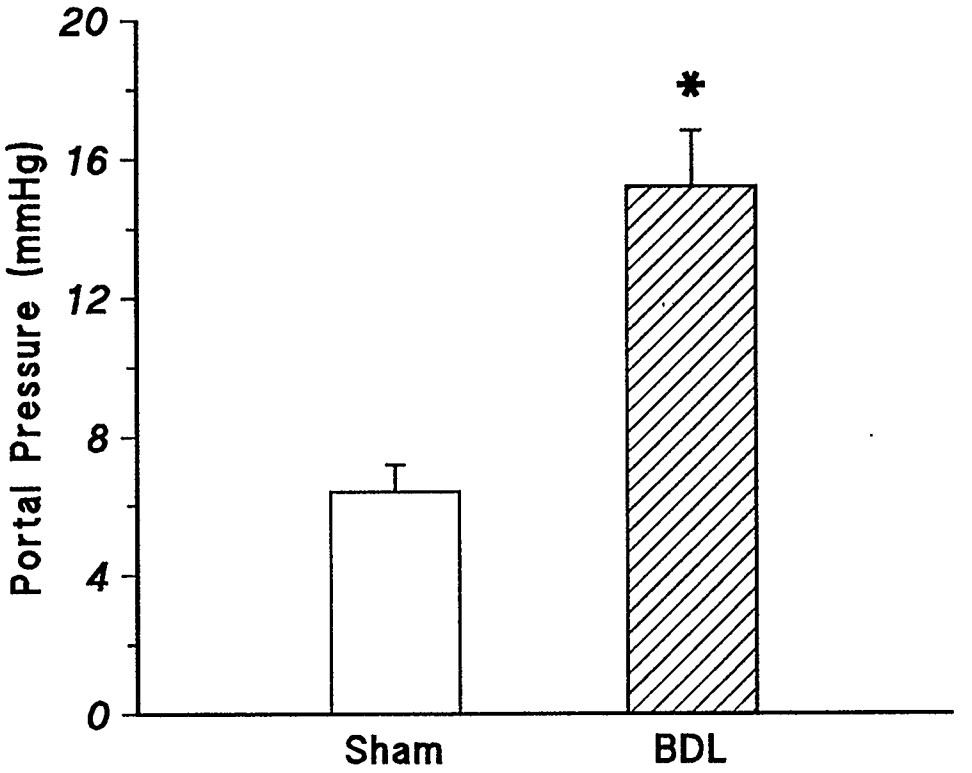


Figure 4.1. Comparison of portal venous pressures in cirrhotic rats (BDL) with sham-operated rats (Sham). The data were obtained from 11 animals in each group and expressed as mean ± SEM. Portal venous pressure was measured by superior mesenteric vein cannulation with a 23-gauge needle connected to a pressure transducer. * Significantly different between the two groups ($p<0.01$).

4.2. HEPATOCYTE FUNCTION INSUFFICIENCY

Liver biochemistry test results were tabulated in table 4.2. ALT and AST catalyze transfer of α-amino groups from alanine or aspartate to α-ketoglutarate. When liver cells are injured such as in fulminant hepatic failure and viral hepatitis, AST and ALT levels are increased. AST activity is also elevated in the conditions such as cardiac infarction and muscle injury. In the present BDL cirrhotic animal model, significant augmentation of ALT and AST activities was observed as presented in the table 4.2. The data herein demonstrated liver cell injury and hepatocyte function insufficiency in cirrhotic BDL rats.

Phosphatases are widely distributed in the different tissues in the body including the liver, intestine, bone, and kidneys. Serum alkaline phosphatases are the enzymes that hydrolyse the phosphate esters at alkaline pH conditions. A variety of disorders can cause alkaline phosphatase activity increment, such as bone disease, intestine injury, liver disease, *etc.* In liver disease, alkaline phosphatase activity elevation indicates liver cell damage and cholestasis. As presented in the table 4.2, the alkaline phosphatase activity is increased in the cirrhotic BDL rats, which is direct evidence of bile duct obstruction and liver cell injury.

Increased bile acid content shown in the table 4.2 could be caused by liver damage and portal systemic shunting. Bilirubin produced from heme degradation is secreted by the liver. Increment of the total bilirubin content presented in the table 4.2 was also the result of hepatocyte damage and bile duct obstruction.

The plasma total cholesterol level was significantly increased in the present

cirrhotic animal model. Cholesterols are the precursors for bile salt and other steroid hormone synthesis. One of the major routes of elimination of cholesterol from the body is by secretion into the bile in the form of either cholesterol or bile salts. As the consequence of bile duct obstruction, cholesterol secretion by the liver is blocked, leading to an elevation in blood cholesterol level. Because of bile duct ligation, plasma bile salt levels increase. Elevation in plasma bile salts could inhibit bile salt synthesis by feedback mechanisms. Consequently, cholesterol consumption decreases. On the other hand, lecithin-cholesterol acyltransferase activity is decreased in cirrhosis (Imai *et al.*, 1992) which also accounts for part of the elevation in the plasma cholesterol content, which will be elaborated in detail in the discussion chapter.

4.3. LIVER HISTOLOGY

Liver histology examination demonstrated that liver structure was damaged in cirrhotic BDL rats. In the portal area, fibrosis and lymphocyte infiltration were observed. Massive liver architecture derangement and bile duct proliferation were also obvious in BDL cirrhotic animals. Hepatocyte necrosis was observed. These histological changes were in good agreement with a report by Kountouras *et al* (1985).

Taken together, the evidence of hepatosplenomegaly, ascites, liver histological changes, increased portal venous pressure, and altered liver biochemistry test results suggest that a successful biliary cirrhotic rat model has been established.

Table 4.2. Liver biochemistry tests. Data represent mean ± SEM. Aspartate amino-transferase and alanine amino-transferase activity was expressed as Sigma units (U). Each unit represents one μmole of NADH oxidized per min. Alkaline phosphatase activity was expressed as Sigma units (U). Each unit hydrolysed 1 μmole of p-nitrophenyl phosphate per hour. Total bilirubin is expressed as an absolute value of μmol/L. The total cholesterol content is expressed as mmol/L. AST, Aspartate amino-transferase; ALT, Alanine amino-transferase.

Table 4.2. Liver biochemistry test (n presented as Sham/BDL)

	AST (U/L)	ALT (U/L)	Alkaline phosphatase (U/mL)	Total Bilirubin (μmol/L)	Bile Acid (μmol/L)	Total Cholesterol mmol/L
Sham	85±7	38±3	6.8±0.4	3.0±0.3	8±2	1.21±0.04
BDL	519±29	95±5	15.2±1.0	132.7±3.4	303±19	1.77±0.11
n	23/25	23/30	13/15	21/13	16/17	13/19
p	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

CHAPTER FIVE

RESULTS II

LEFT VENTRICULAR PAPILLARY MUSCLE CONTRACTILITY AND β-ADRENERGIC RECEPTOR FUNCTION IN CIRRHOSIS

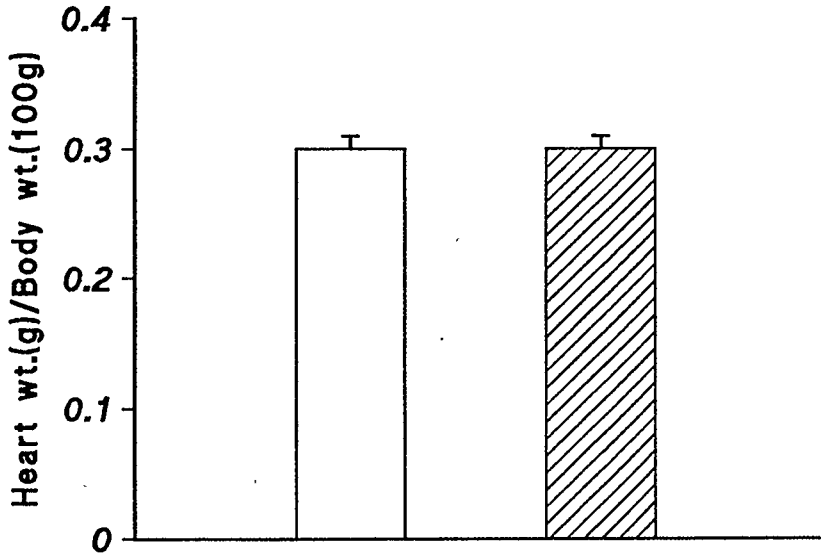
5.1. CHARACTERIZATION OF NORMALIZED HEART WEIGHT AND CARDIAC HISTOLOGICAL EXAMINATION

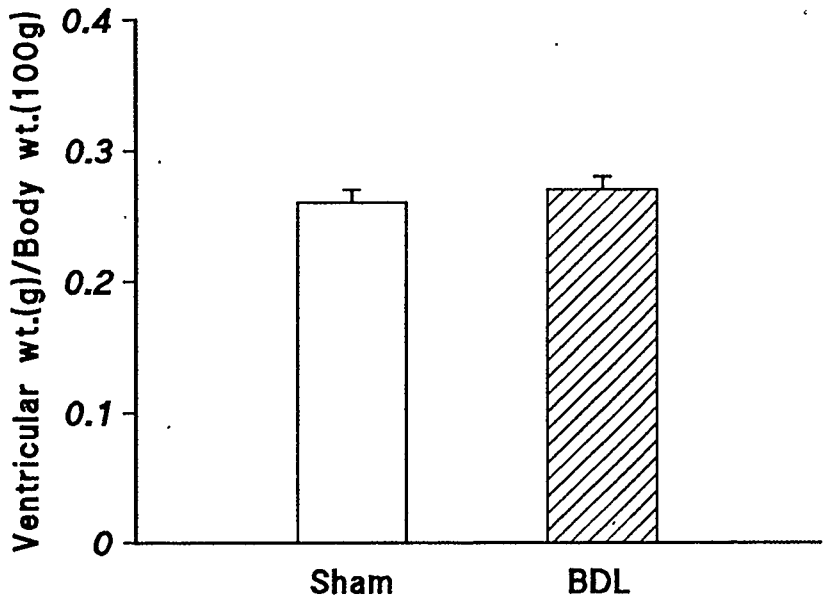
To clarify if cardiac hypertrophy exists in the present cirrhotic model, the heart and ventricular wet weights were measured and normalized to 100 g of body weight. The data are presented in figure 5.1. No significant differences were observed between the two groups in these parameters. In this regard, the data herein are in contrast with Caramelo's observation (Caramelo *et al.*, 1985). In that study, cardiac hypertrophy was observed in a CCl_4-induced cirrhotic rat model. The mechanisms for these discordant observations remain unclear. Reviewing the two studies, it was found that the cirrhotic model used here and the one in Caramelo's study are different. CCl_4 inhalation takes 10 to 12 weeks to produce cirrhosis. The CCl_4-induced cirrhotic rats are exposed to a much longer period of hyperdynamic circulation than the BDL cirrhotic rats. The present model only takes 4 weeks to produce cirrhosis. Therefore, prolonged exposure the heart to hyperdynamic circulation in CCl_4-induced cirrhosis may account for the discordance between the two studies.

Heart histological examination under light microscopy did not discover any abnormal changes in cardiac muscle structure or cardiac cell size and shape. Neither was any interstitial abnormality observed.

The results herein suggest that hypertrophic cardiomyopathy is not the case in the present cirrhotic rat model.

Figure 5.1. Comparison of normalized heart and ventricular wet weight in cirrhotic group (BDL) with sham-operated group (Sham). Data represent mean ± SEM obtained from 27 BDL and 15 Sham rats. The upper panel is normalized heart weight and lower panel is normalized ventricular weight. No significant differences were observed between the two groups.


Heart wt.(g)/Body wt.(100g)
0.4
0.3
0.2
0.1
0


Ventricular wt.(g)/Body wt.(100g)
0.4
0.3
0.2
0.1
0
Sham
BDL

5.2. EVIDENCE OF IMPAIRED VENTRICULAR PAPILLARY MUSCLE CONTRACTILITY

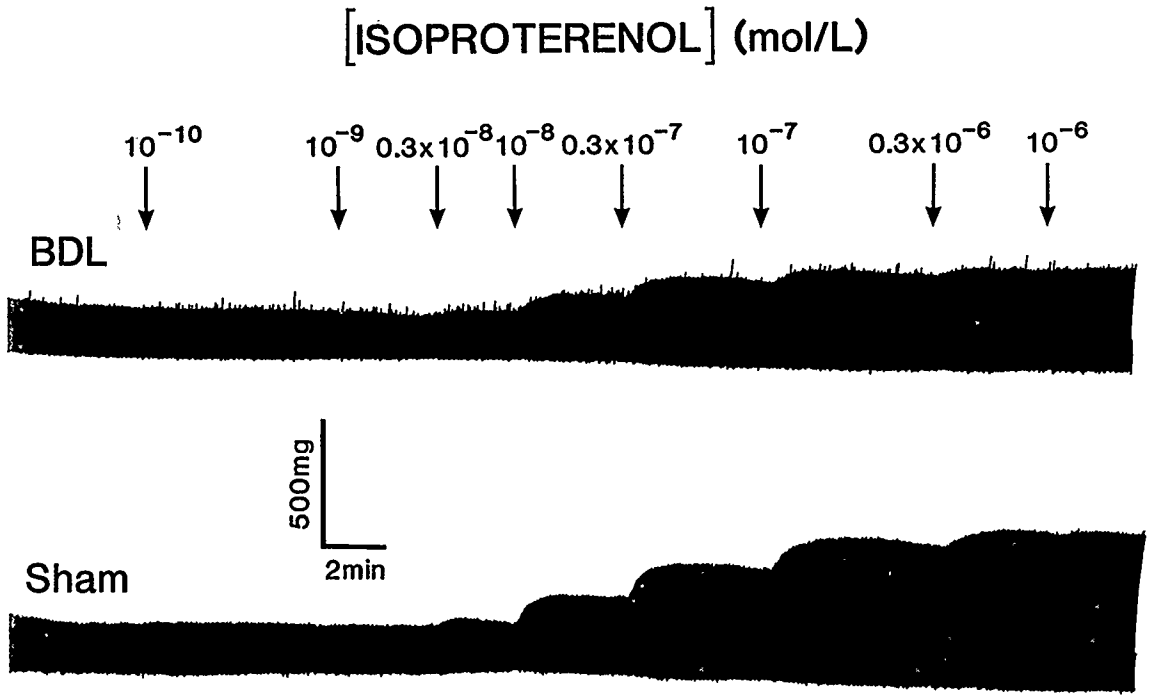
To confirm the existence of cirrhotic cardiomyopathy in the present biliary cirrhotic rat model, left ventricular papillary muscle contractile experiments were performed. Original representative tracings of left ventricular papillary muscle contractile forces are presented in figure 5.2. The paper speed was set at 5 mm/min to continuously record contractile responses. The upper tracing is from a cirrhotic BDL rat and the lower tracing represents a sham-operated rat. When the data were normalized to the papillary muscle mass, at the baseline, no significant differences were observed between the two groups. However, as incremental doses of isoproterenol from 10^{-9} to 10^{-6} mol/L were added to the organ bath, significantly blunted contractile responses were observed in BDL rats, especially at the higher concentrations of isoproterenol. Figure 5.3 compares maximum contractile responses of the left ventricular papillary muscles under isoproterenol stimulation between the BDL rats and sham control rats. The dose response curves were constructed by non-linear regression. From this figure, it is instantly clear that the maximum contractile responses of cirrhotic BDL rats are significantly lower than sham-operated rats. Calculated by non-linear regression, it was found that maximum contractile responses for cirrhotic BDL and sham-operated control rats were 70±2% and 113±3% of basal respectively, $p<0.01$. No significant differences were observed in ED_{50} values between cirrhotic BDL and sham-operated groups, which were 13.6±1.6 *vs.* 9.7±1.3 nmol/L respectively.

Blunted left papillary muscle contractile responses to the β-adrenergic receptor stimulation suggest that impairment of the β-adrenergic receptor function plays a crucial role in cirrhotic cardiomyopathy. The data herein are in good agreement with previous *in vivo* studies by Lee and colleagues (1990) and *in vitro* investigation by Ingles *et al* (1991). These results further support and confirm the contention of cirrhotic cardiomyopathy.

5.3. PURIFICATION OF THE ISOLATED CARDIAC PLASMA MEMBRANE VESICLES

The cardiac plasma membrane enzyme marker 5'-nucleotidase activity is plotted in figure 5.4. Approximately 5 to 6-fold enrichment of this enzyme activity was obtained in the final cardiac plasma membrane preparations compared with the initial homogenate. The results were comparable to previous studies by other investigators (Pierce and Dhalla 1980; Kidwai *et al.*, 1979). The mitochondrial enzyme cytochrome *c* oxidase activity is illustrated in figure 5.5. The cytochrome *c* oxidase activities were not increased in the final cardiac plasma membrane preparations compared with the initial homogenate, thus excluding gross contamination by intracellular organelles.

Figure 5.2. Representative tracing of left ventricular muscle contractile responses from cirrhotic (BDL) and sham-operated (Sham) rats. The papillary muscle wet weight was 8.2 mg for the BDL rat and 8.4 mg for the Sham rat. At baseline, no significant differences were observed between the two tracings. However, when the ventricular papillary muscles were exposed to cumulative isoproterenol stimulation, blunted contractile responses were observed in the BDL ventricular papillary muscle.


[ISOPROTERENOL] (mol/L)
10^-10
10^-9
0.3x10^-8
10^-8
0.3x10^-7
10^-7
0.3x10^-6
10^-6
BDL
500mg
2min
Sham

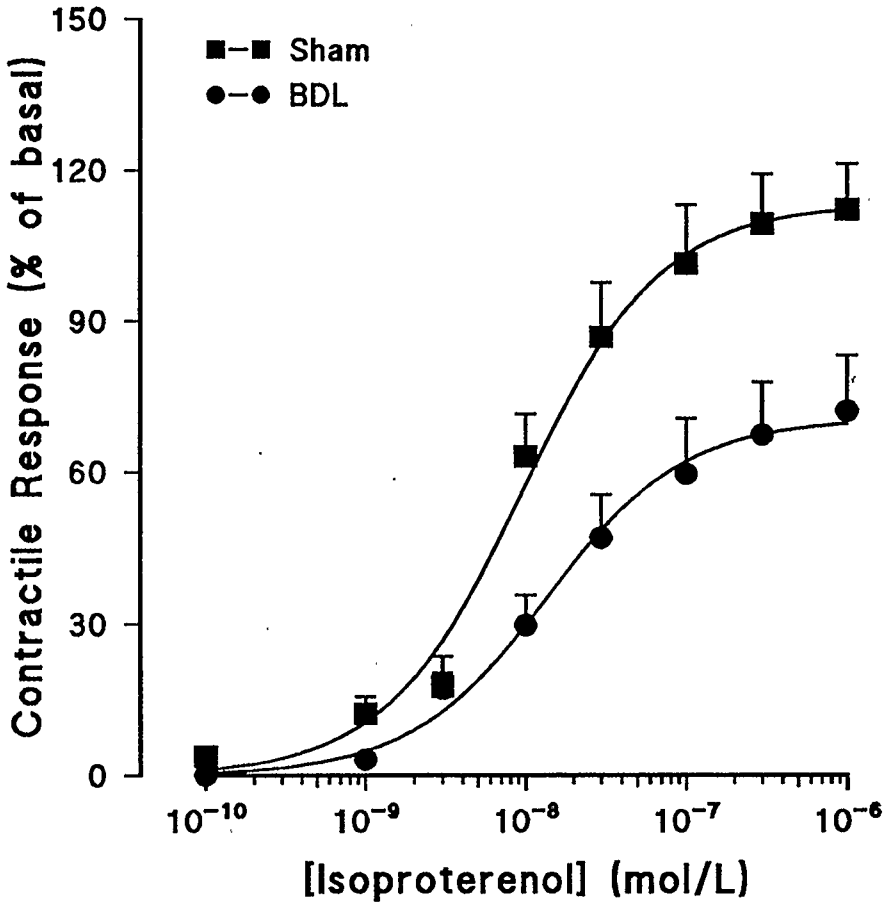


Figure 5.3. Cumulative dose-responses to isoproterenol in isolated left ventricular papillary muscles from cirrhotic (BDL, n=14) and sham-operated (Sham, n=15) rat hearts. Maximal responses (Emax) for BDL and Sham rats were 70±2% and 113±3% of basal respectively, $p<0.01$. No significant differences were observed in ED_{50} values between BDL and Sham rats (9.7±1.3 *vs* 13.6±1.6 nmol/L).

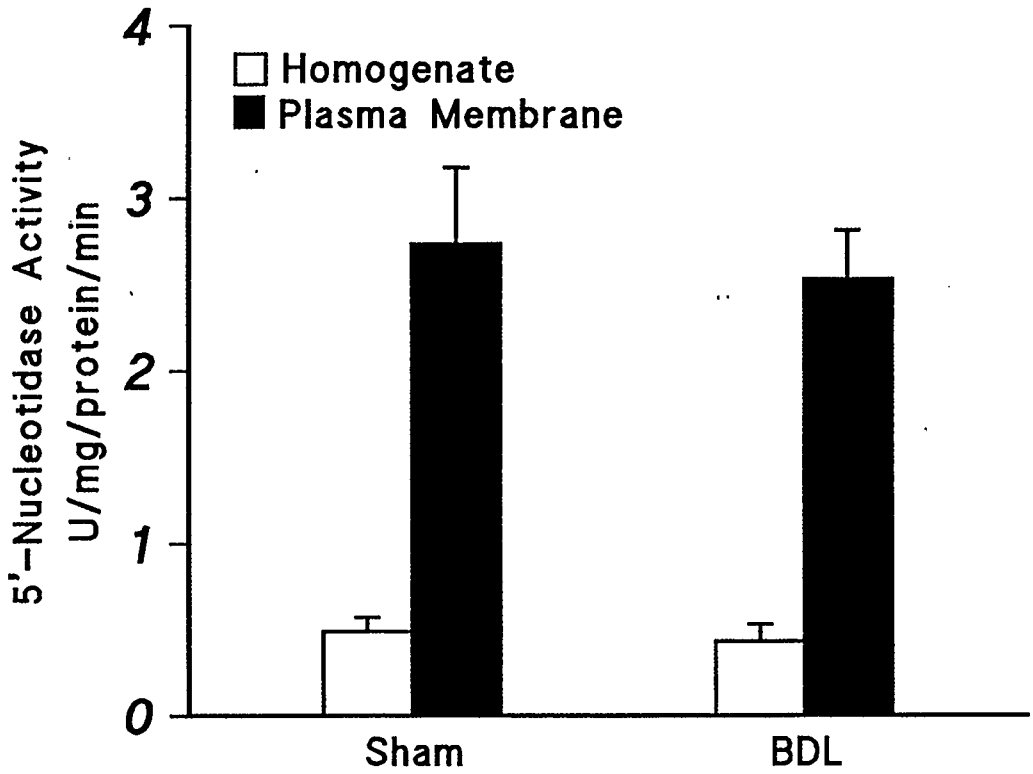


Figure 5.4. Comparison of 5'-nucleotidase activity in the final cardiac plasma membrane preparation with the initial homogenate. Data are presented as mean ± SEM, obtained from 23 cirrhotic (BDL) and 28 sham-operated (Sham) rats. Approximately 5 to 6-fold enrichment of 5'-nucleotidase activity was observed in the final membrane preparations. No purification differences were observed between BDL and Sham rats.

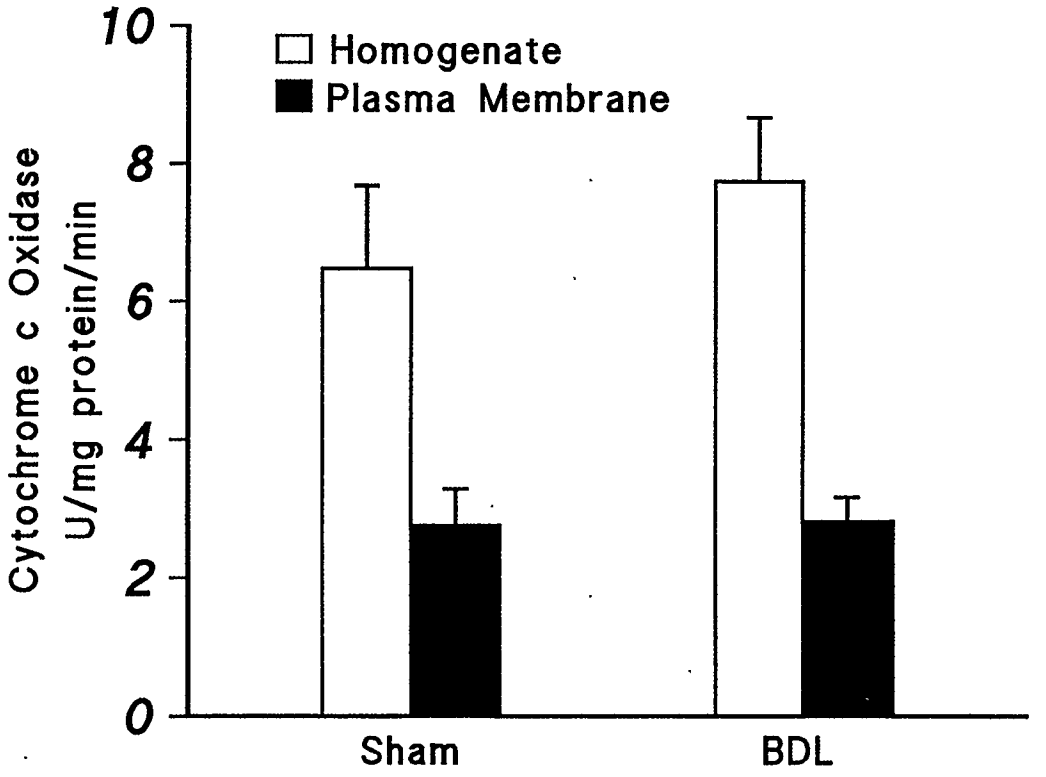


Figure 5.5. Comparison of cytochrome *c* oxidase activity in the final cardiac plasma membrane preparation with the initial homogenate. Data are presented as mean ± SEM, obtained from 23 cirrhotic (BDL) and 28 sham-operated (Sham) rats. Cytochrome *c* oxidase activities were not enriched in the final cardiac plasma membrane preparation, thus excluding gross contamination by other cellular organelles.

5.4. CHARACTERIZATION OF β-ADRENERGIC RECEPTOR SIGNAL TRANSDUCTION PATHWAY FUNCTION

5.4.1. Cardiac β-adrenergic receptor characteristics

To further search for direct evidence of impaired β-adrenergic receptor function in liver cirrhosis, β-adrenergic receptor binding assay was performed using ^{3}H-dihydroalprenolol (^{3}H-DHA) as a radioligand as detailed in the method section. The non-specific binding is around 10% of total binding throughout the binding study. As described in the method section, direct non-linear regression was employed to acquire receptor number (Bmax) and dissociation constant (Kd) values. The saturation curves of specific binding raw data are presented in figure 5.6. This figure demonstrates that the specific binding of ^{3}H-DHA to cardiac plasma membrane preparations is saturable at the ^{3}H-DHA concentration range used in the present study. Furthermore, the data were best fit for one binding site regression. This is because both ^{3}H-dihydroalprenolol and propranolol are non-selective β-adrenergic receptor antagonists. Therefore, β-adrenergic receptor subtypes cannot be differentiated by the present binding assay.

Comparison of cardiac β-adrenergic receptor density (Bmax) between the cirrhotic BDL and sham-operated rats is presented in figure 5.7. Cardiac β-adrenergic receptor density in BDL rats was significantly lower than that in the sham-operated control group. These results suggest cardiac β-adrenergic receptor down-regulation in cirrhosis. The data herein are in accordance with previous reports by Gerbes and colleagues (1986), Lee *et al* (1990), and a recent report by

Battarbee and colleagues in CCl_4-induced cirrhotic rats (Battarbee *et al.,* 1995).

The receptor binding affinity is expressed as binding dissociation constant, Kd, in the present study. Kd can be illustrated by the following equation:

$$Kd=(k-1)/k=[R][L]/[RL]$$

Where k-1 and k represent the rates of dissociation and association at equilibrium, respectively. [R] and [L] represent uncoupled receptor and free ligand respectively. [RL] represents the receptor bound with ligand. Therefore, the higher the Kd value, the lower the receptor binding affinity is. Comparison of the binding affinity between the BDL rats and sham-operated rats is presented in figure 5.8. No significant differences were observed in these variables between the two groups.

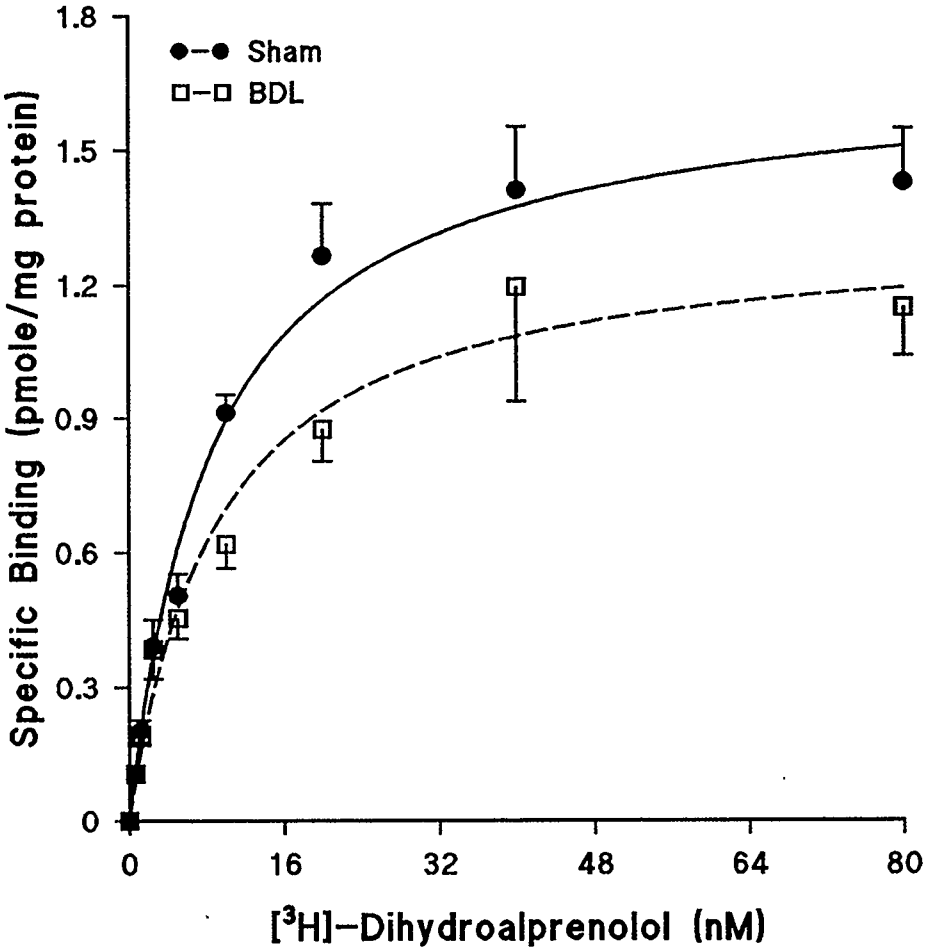


Figure 5.6. Binding assay saturation curves. The specific binding of the radioligand, ^{3}H-dihydroalprenolol (^{3}H-DHA), to cardiac plasma membranes from both bile duct ligated cirrhotic rats (BDL) and sham control rats (Sham) is saturable within the concentration range of ^{3}H-DHA used in the present study.

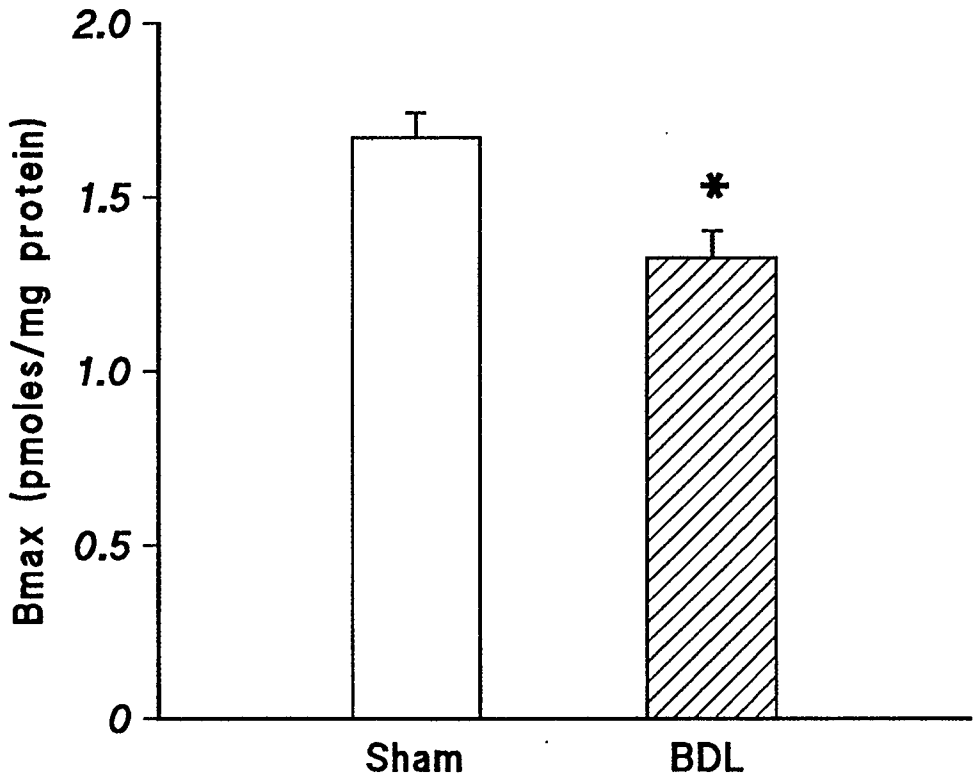


Figure 5.7. Comparison of cardiac β-adrenoceptor density (Bmax) in cirrhotic rats (BDL) with sham-operated rats (Sham). Data are mean ± SEM obtained from 5 separate membrane preparations. * Significantly different from the sham-operated group, $p < 0.05$.

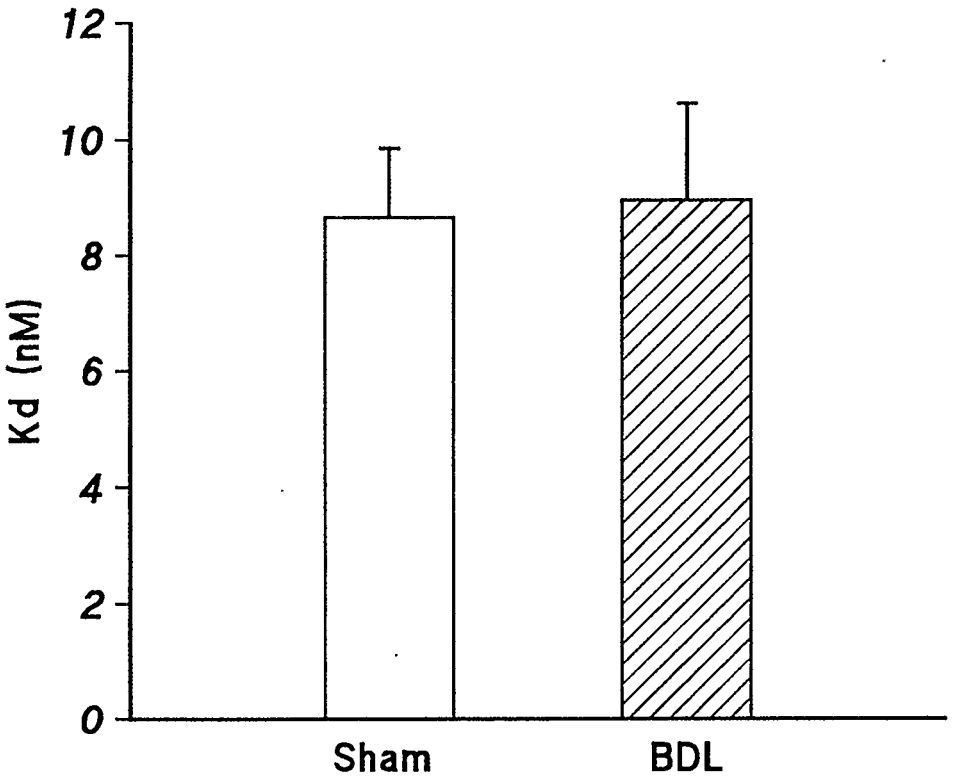


Figure 5.8. Comparison of cardiac β-adrenergic receptor binding affinity (Kd) of cirrhotic rats (BDL) with sham-operated rats (Sham). Data are mean ± SEM obtained from 5 separate membrane preparations. No significant differences were observed between the two groups in Kd values.

5.4.2. Decreased cAMP production

To illuminate the cardiac β-adrenergic receptor signal transduction pathway function in cirrhosis, cAMP production stimulated by a variety of agonists was measured. A typical standard curve of cAMP assay using Amersham cAMP protein binding assay kits in our laboratory is shown in figure 5.9. The maximum cAMP content that could be detected by this protein binding assay system is 16 pmol/per tube. Therefore, adequate amounts of membrane proteins and incubation times were determined in the preliminary experiments, so that the amount of cAMP produced in each tube is within the detectable range of the assay system.

An initial experiment was performed to verify cardiac β-adrenergic receptor signal transduction pathway function at the receptor level in cirrhosis by stimulating the receptor with isoproterenol at concentration of 10^{-4} mol/L. The results are presented in figure 5.10. cAMP production stimulated by isoproterenol was significantly decreased in BDL rats by 37% compared with sham-operated rats. However, our receptor binding assay shows that the receptor density was only reduced by 21%. Therefore, the discrepancy between the receptor binding assay and cAMP data suggest that the decreased cAMP production is not only caused by reduced receptor density. Other factors such as post-receptor signal transduction impairment are probably involved.

To further illuminate the β-adrenergic receptor signal transduction pathway function at the G-protein level, cAMP production was measured using fluoride ions (NaF) as a stimulator at a final concentration of 10 mmol/L. The fluoride ions in

the presence of aluminum play a role of γ-phosphate in GTP molecule. The interaction between GDP, AlF_4^-, and αs subunit of G-proteins has been clarified in the last few years. Aluminum combines with β-phosphate on GDP molecule. Four fluoride ions interact with aluminum to form the αs-GDP-AlF_4 complex which directly stimulates adenylyl cyclase with much stronger potency compared with β-adrenergic receptor stimulation (Rens-Domiano and Hamm, 1995). The results of fluoride ion stimulation are also shown in figure 5.10. Significant differences were observed between the two groups.

To clarify if there is any functional alteration at adenylyl cyclase level, forskolin at a concentration of 100 μmol/L was used as a stimulator. It has been documented that forskolin directly stimulates adenylyl cyclase by acting on the catalytic domains of this enzyme (Iyengar, 1993). The magnitude of cAMP production stimulated by forskolin is even higher than that observed in the fluoride ion experiments. Comparison of cAMP production stimulated by forskolin in cardiac plasma membranes between the two groups is plotted in figure 5.10. Significantly reduced cAMP production in the BDL group was recorded in this figure.

Taken together, the above data suggest that the cardiac β-adrenergic receptor signal transduction pathway function is impaired at the receptor and post-receptor levels in cirrhosis.

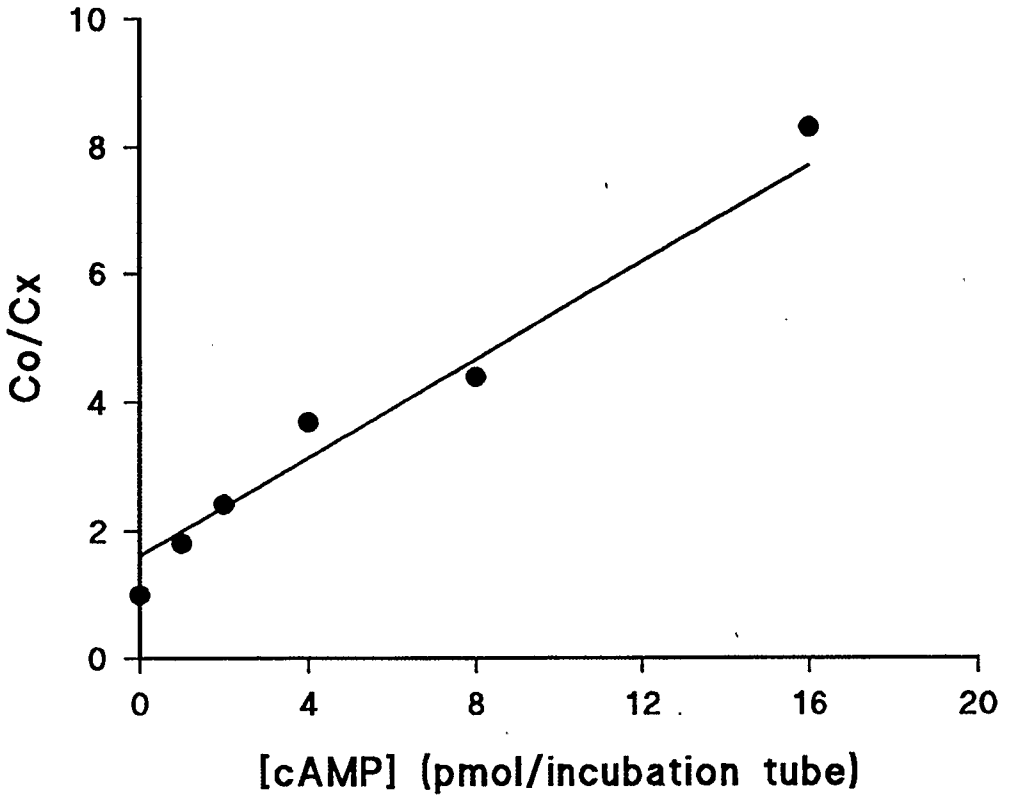


Figure 5.9. A typical standard curve of cAMP assay using an Amersham cAMP protein binding assay kit in our laboratory. The detectable range of cAMP production is from 0 to 16 pmol/per tube. Co represents the radioactive counts per minute (cpm) bound with binding proteins in the absence of unlabelled cAMP; Cx represents radioactive cpm bound with binding proteins in the presence of standard or unknown unlabelled cAMP.

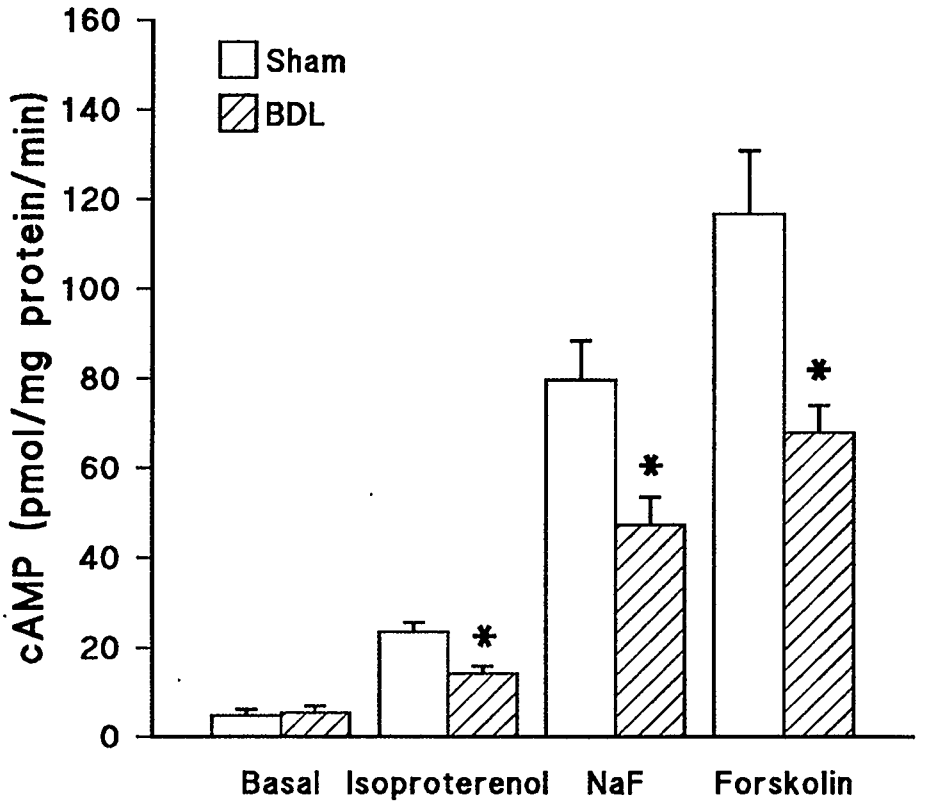


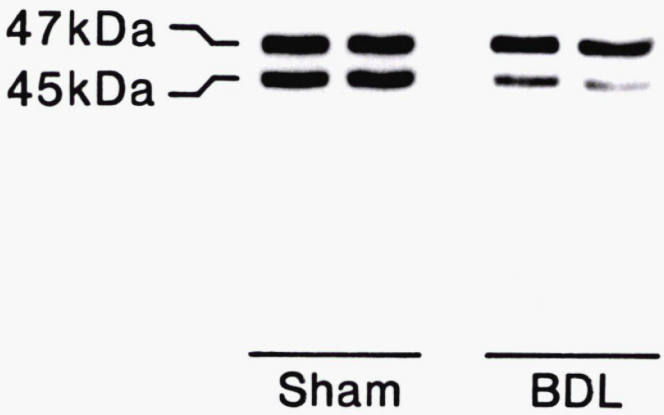
Figure 5.10. cAMP generation at baseline and under isoproterenol (10^{-4} mol/L), sodium fluoride (10 mmol/L) and forskolin (100 μmol/L) stimulation in cardiac plasma membranes from cirrhotic rats (BDL) and sham-operated rats (Sham). Data are presented as mean ± SEM, based on 5 separate membrane preparations in each group. * Significantly different from controls, $p<0.05$.

5.4.3. Characterization of G-protein expression in cirrhosis

To further illuminate the underlying mechanism(s), G-protein expression was determined by Western blot analysis followed by laser densitometry scanning quantification. Gsα expression data are presented in figure 5.11, Gi2α presented in figure 5.12, and Gcommonβ presented in figure 5.13. In all those G protein expression figures, the upper panels are the representatives of Western blot analysis and lower panels are the arbitrary optical density units obtained by laser densitometry scanning of each G-protein subunit band. Two bands of Gsα at the molecular weight of 45 and 47 kDa were detected. The lower panels of Gsα expression figure are pooled data of optical density from 45 and 47 kDa bands. The Gsα expression in BDL rats was significantly reduced compared with sham-operated control group. Similar results were obtained in Gi2α expression. In contrast, no significant differences were observed in Gcommonβ expression between the two groups.

Taken together, the above Western blot analysis data demonstrated that cardiac G-protein expression was altered in cirrhosis which may play an important role in diminished β-adrenergic receptor signal transduction function in cirrhotic cardiomyopathy.

Figure 5.11. Western blot analysis of Gsα expression. The cardiac plasma membranes (5 μg/per lane) were resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immuno-blotted using rabbit antisera RM/1 as first antibodies. The upper panel is representative of Hyperfilm-ECL of Gsα immunoblot. The lower panel shows computerized optical density scanning analysis of the combined 45 and 47 kDa bands. The results are expressed as mean ± SEM, based on 7 cirrhotic (BDL) and 8 sham-operated (Sham) membrane preparations. * Significantly different from Sham, $p<0.05$.


47kDa
45kDa
Sham
BDL

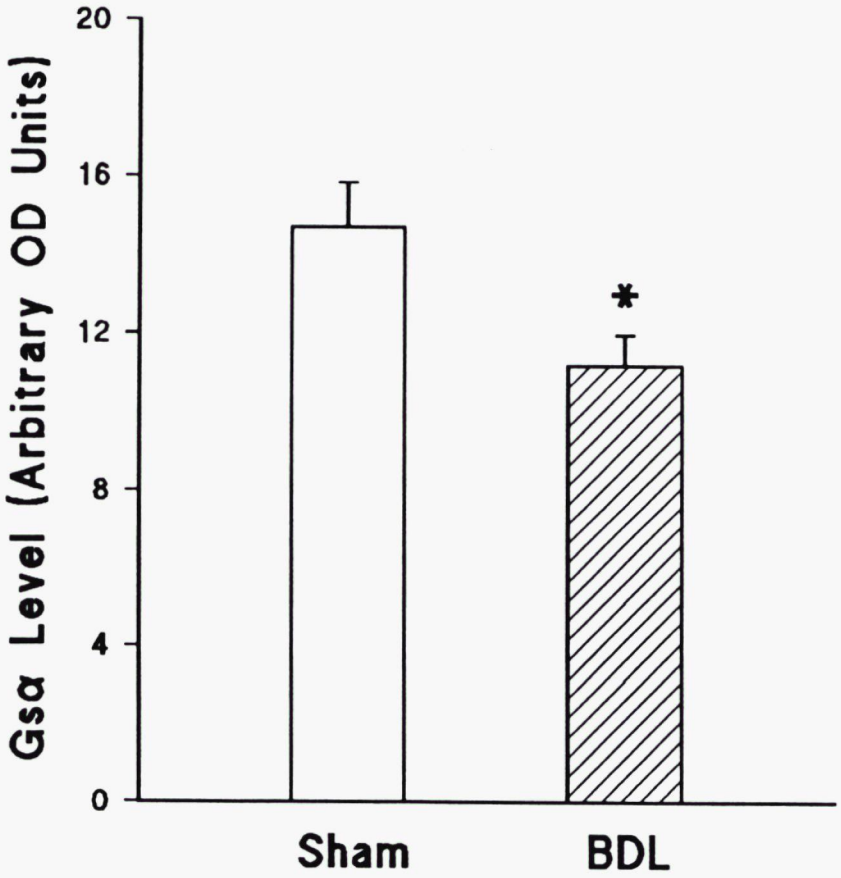
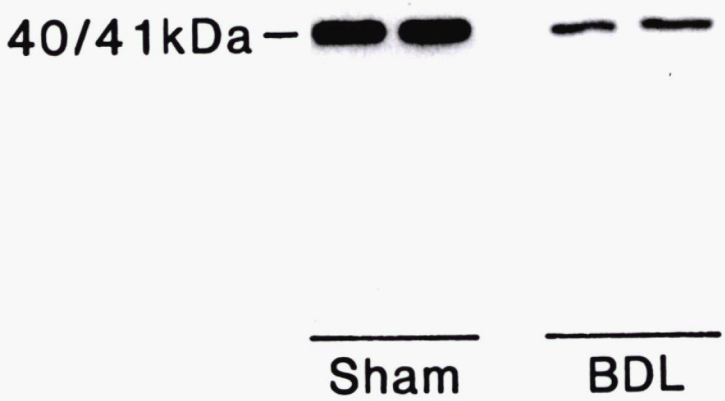

Gsα Level (Arbitrary OD Units)
20
16
12
8
4
0
*
Sham
BDL

Figure 5.12. Western blot analysis of Gi2α expression. The cardiac plasma membranes (5 μg/per lane) were resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immuno-blotted using rabbit antisera AS/7 as first antibodies. The upper panel is representative of Hyperfilm-ECL of Gi2α immunoblot. The lower panel shows computerized optical density scanning analysis of the blots. The results are expressed as mean ± SEM, based on 7 cirrhotic (BDL) and 8 sham-operated (Sham) membrane preparations. * Significantly different from Sham, $p<0.01$.


40/41kDa
Sham
BDL

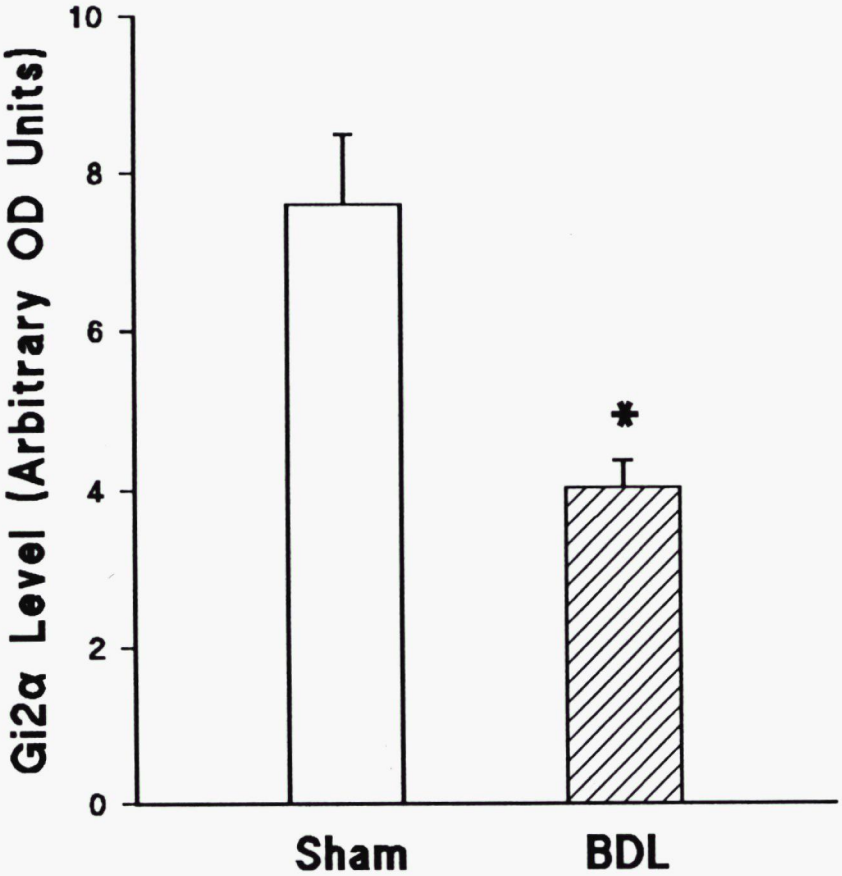
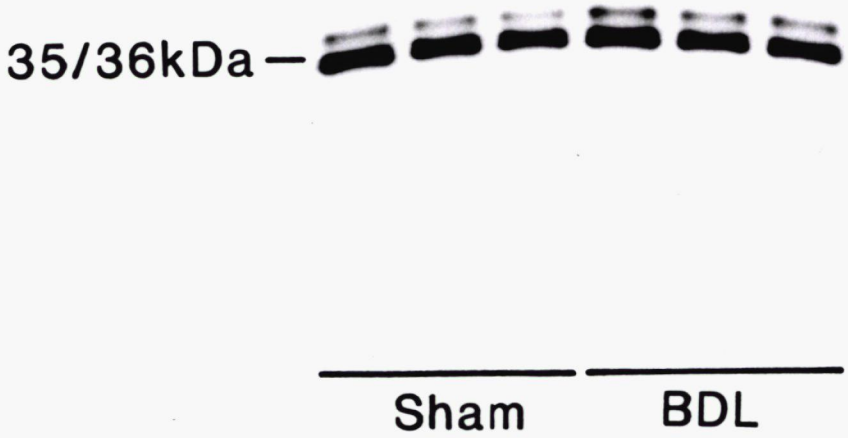
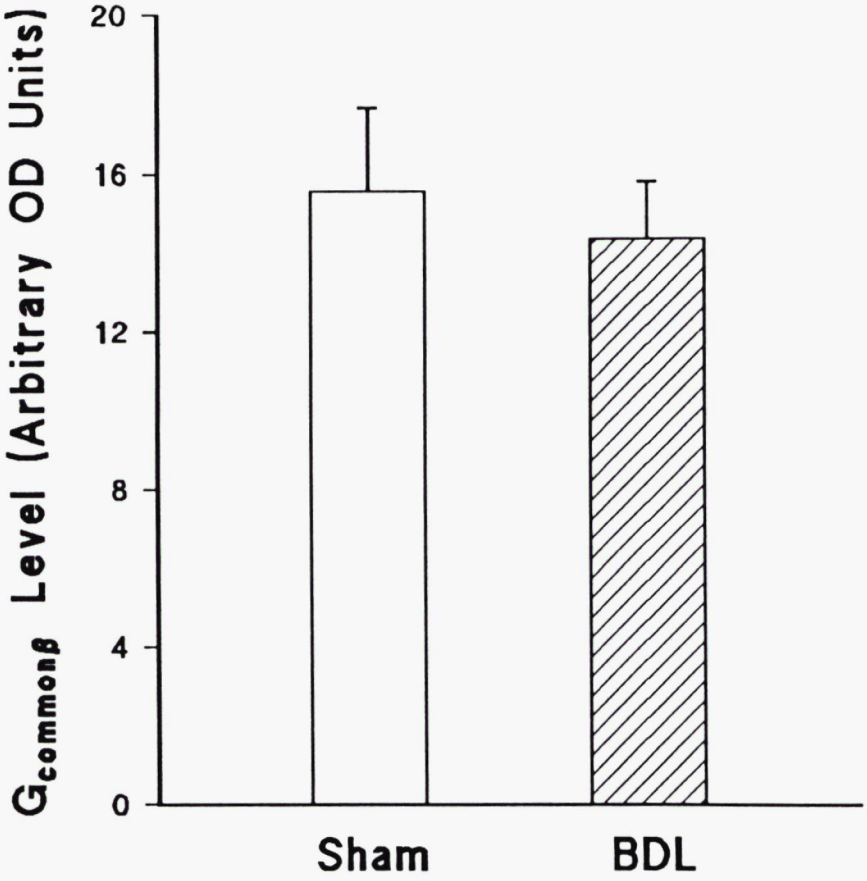

Gi2α Level (Arbitrary OD Units)
10
8
6
4
2
0
*
Sham
BDL

Figure 5.13. Western blot analysis of Gcommonβ expression. The cardiac plasma membranes (5μg/per lane) were resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immuno-blotted using rabbit antisera SW/1 as first antibodies. The upper panel is representative of Hyperfilm-ECL of Gcommonβ immunoblot. The lower panel shows computerized optical density analysis of the blots. The results are expressed as mean ± SEM, based on 6 cirrhotic (BDL) and 8 sham-operated (Sham) membrane preparations. No significant differences were observed between the groups in Gcommonβ expression.


35/36kDa
Sham
BDL


$G_{common\beta}$ Level (Arbitrary OD Units)
20
16
12
8
4
0
Sham
BDL

CHAPTER SIX

RESULTS III

CARDIAC PLASMA MEMBRANE PHYSICAL PROPERTY AND ITS RELATIONSHIP WITH β-ADRENERGIC RECEPTOR FUNCTION

6.1. CHARACTERIZATION OF MEMBRANE FLUIDITY

The static components of the cardiac plasma membrane fluidity detected using DPH probes are presented in figure 6.1. The anisotropy parameter in the BDL rats is significantly higher than that in their sham-operated counterparts. Since the total fluorescence is not changed, we interpret the higher value of anisotropy parameter to imply a more rigid membrane lipid environment. The increased anisotropy parameter indicates the wobbling movement hindrance for the DPH probes in the membrane lipid bilayer, which could be extrapolated to represent the lipid and protein molecule movement restriction in the membrane bilayer (Brasitus and Schachler, 1980; Meddings *et al.*, 1990).

The dynamic components of cardiac plasma membrane fluidity are plotted in figure 6.2. It was found that at regions close to the membrane bilayer surface (from n=2 to 6), anisotropy parameters in BDL rats membranes are significantly higher than the sham-operated membranes. Once again, since the total fluorescence is not changed, the results are interpreted as a more rigid membrane environment. These data suggest that the rotational movement of the fluorescent probes is restricted, which could be extrapolated to indicate that the rotational movement of the lipid and protein molecules are impaired (Cogan and Schachter, 1980; Schachter, 1984).

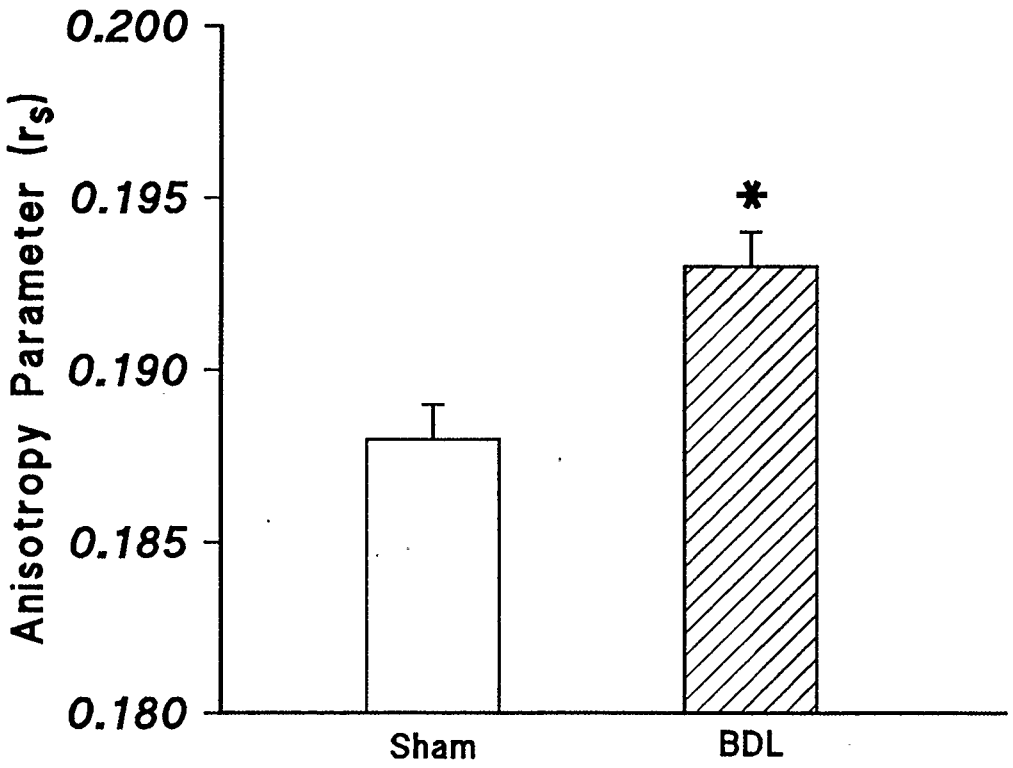


Figure 6.1. Static component of membrane fluidity in cirrhotic (BDL) and sham-operated (Sham) rats. Data represent mean ± SEM of at least 5 determinations from 5 separate membrane preparations. * Significantly different from the sham-operated group, $p < 0.01$.

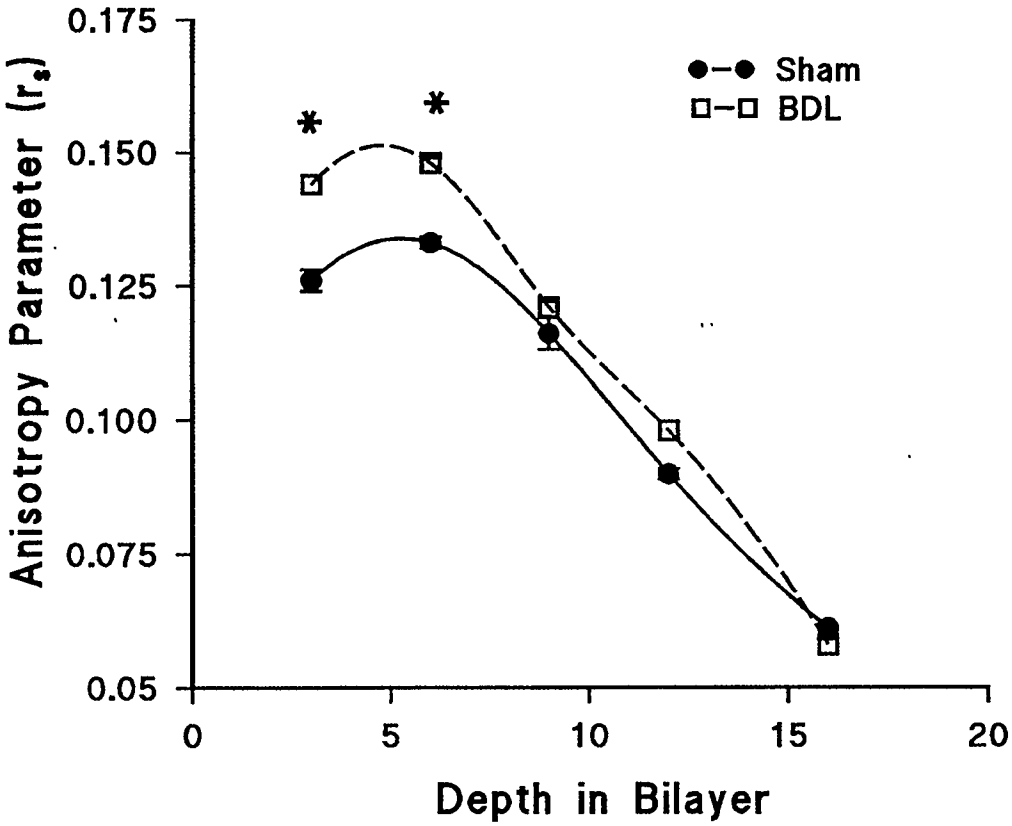


Figure 6.2. Dynamic component of membrane fluidity in cirrhotic (BDL) and sham-operated (Sham) rats. Data represent mean ± SEM of at least 6 determinations from 5 separate membrane preparations. Where error bars are absent, they fell within the limits of the symbols. * Significantly different from the sham-operated group, $p < 0.01$.

6.2. CHARACTERIZATION OF CARDIAC PLASMA MEMBRANE LIPID COMPOSITION

Since it was found that the cardiac plasma membrane from BDL rats became rigid (higher r_s values), the next series of experiments were carried out to elucidate the mechanisms which lead to the increase in anisotropy parameters in cirrhosis. The membrane fluidity is dominantly modulated by the cholesterol and phospholipid molar ratio, saturation of the fatty acids, and length of the fatty acid chains (Schachter, 1984). Therefore, the above parameters were determined and the cardiac plasma membrane chemical composition was assessed.

Total cholesterol and phospholipid contents and cholesterol/phospholipid molar ratio from the cardiac plasma membranes are presented in table 6.1. Total cholesterol content was significantly increased in the BDL rats. No significant differences were observed in total phospholipid contents between the two groups. Therefore, the cholesterol to phospholipid molar ratio was significantly increased. Increase in cholesterol/phospholipid ratio could be the major cause that rigidizes the cardiac plasma membranes in the BDL cirrhotic rat group.

The fatty acid composition of the cardiac plasma membrane is also tabulated in table 6.1. The results are expressed as weight percentage. Ten major fatty acids were analyzed from each sample. No gross alteration in membrane fatty acid composition was observed, other than a slight increase in arachidonic acid (20:4) in the cirrhotic BDL group.

Table 6.1. Lipid composition of cardiac plasma membrane preparations. Total cholesterol and phospholipids were analyzed using colorimetric methods. Fatty acid contents were determined by gas-chromatography. Values are mean ± SEM from 5 separate membrane preparations. The membrane total cholesterol content and cholesterol/phospholipid ratio were significantly increased in cirrhotic rats (BDL) compared with sham-operated control rats (Sham). No gross significant differences in fatty acid composition were observed except that arachidonic acid content was elevated in BDL rats. * Significantly different between the two groups, $p<0.05$.

Table 6.1. Lipid and fatty acid composition of cardiomyocyte plasma membrane

	Sham	BDL
Cholesterol (nmol/mg protein)	134.46±10.73	178.05±6.74*
Phospholipid (nmol/mg protein)	410.21±16.15	339.94±28.07
Cholesterol/Phospholipid	0.34±0.02	0.46±0.04*
Fatty Acid, wt%		
14:0	0.24±0.03	0.34±0.04
15:0	0.19±0.02	0.21±0.02
16:0	17.80±0.69	17.45±0.97
16:1	0.34±0.02	0.68±0.08
18:0	26.81±0.37	27.14±0.45
18:1	4.76±0.20	4.37±0.22
18:2	22.60±1.58	21.38±1.11
20:0	0.37±0.01	0.31±0.08
20:4	17.58±0.35	20.28±0.51*
22:6	9.30±0.84	7.83±0.35

6.3. FLUIDIZING EFFECTS OF A_2C ON CARDIAC PLASMA MEMBRANE

It was found that the cardiac plasma membranes from cirrhotic BDL rats were more rigid (higher r_s value) than sham-operated control animals as shown in the above sections. In order to correlate those membrane physical property changes with cardiac β-adrenergic receptor function, the next series of experiments were performed to test if the cardiac plasma membranes could be fluidized back to normal by a known fluidizing agent A_2C. The fluidizing agent, A_2C, at concentrations from 0 to 500 nmol/mg protein was incubated with the cardiac plasma membrane preparations. The results were presented in figure 6.3. It was found that at the above A_2C concentration range, the cardiac plasma membranes from both cirrhotic BDL rats and sham-operated control rats could be fluidized. Time kinetics study presented in figure 6.4 indicates that the fluidizing effects of A_2C on the cardiac plasma membranes are maintained for about 15 min after A_2C addition. Therefore, A_2C was added to the cardiac plasma membrane at every 15 min interval if necessary. The mechanisms by which A_2C loses its fluidizing effects on the cardiac plasma membrane after 15 min in the incubation mixture are not clear. Since the fluidizing effects of A_2C are dependent on the cyclopropyl group (figure 3.5), possibly some enzymes in the cardiac plasma membranes break cyclopropyl groups.

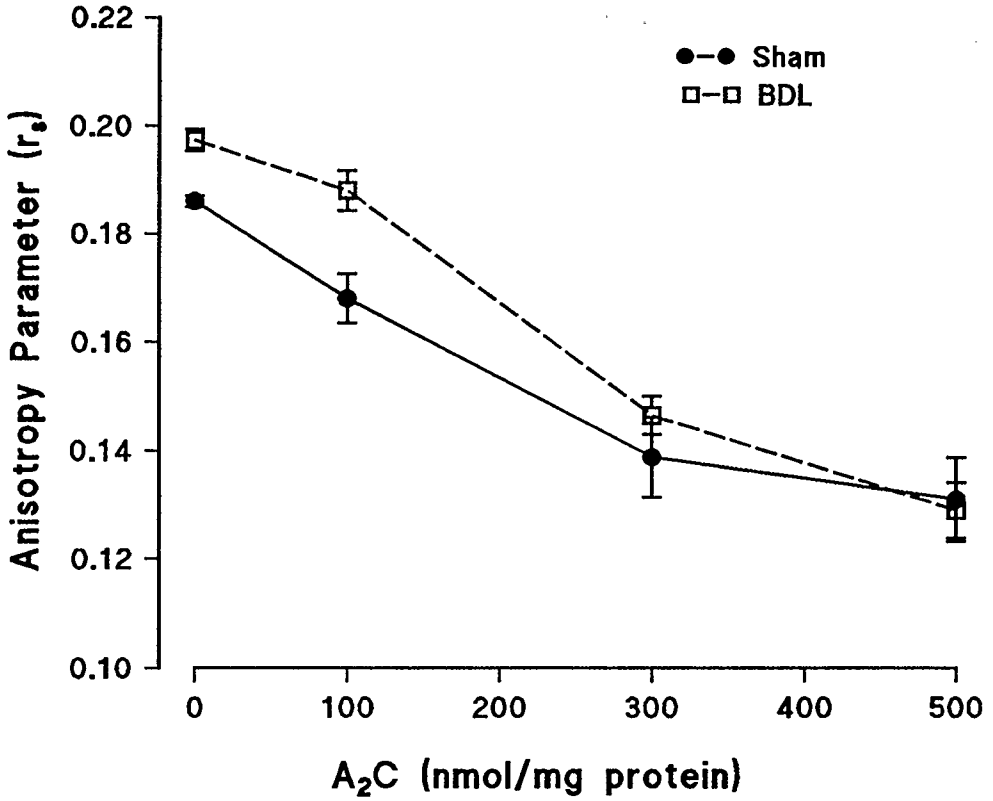


Figure 6.3. Concentration kinetics of 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C) on cardiac plasma membranes. Each data point represents mean ± SEM from 5 separate membrane preparations. The values at each point were obtained 10 min after adding A_2C. The membranes from both cirrhotic (BDL) and sham-operated (Sham) rats could be fluidized at the indicated A_2C concentrations.

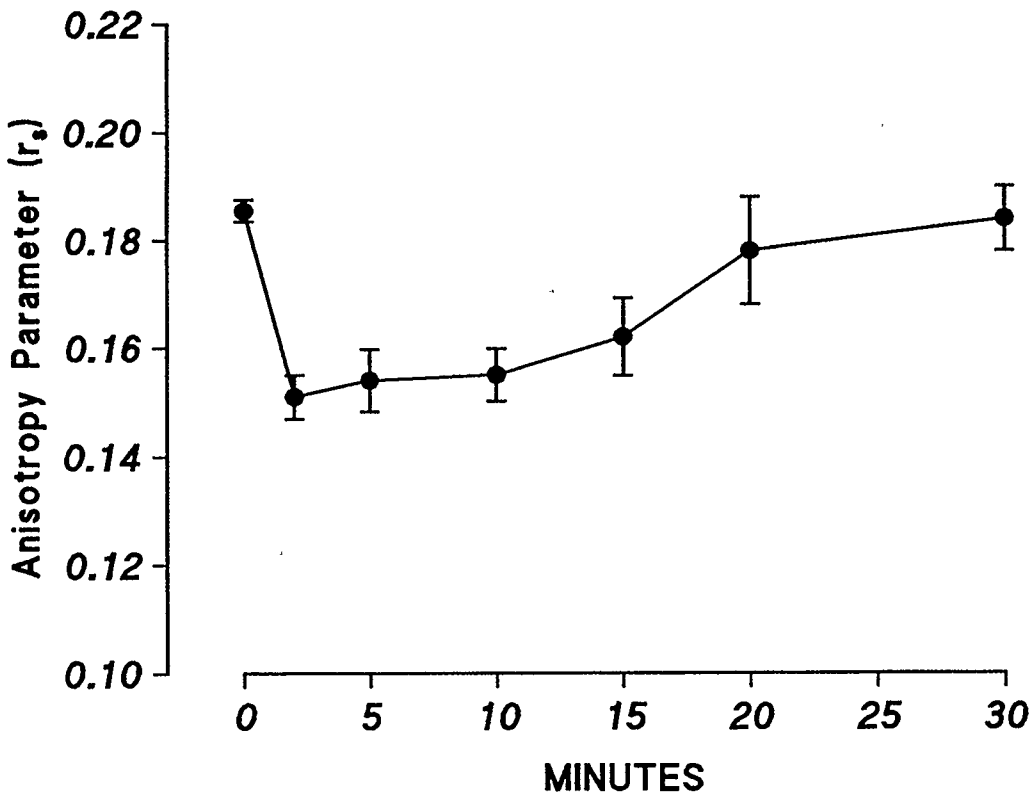


Figure 6.4. Time kinetics of 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C) on cardiac plasma membranes. Each data point represents mean ± SEM from 5 separate sham-operated membrane preparations. A_2C concentration was 200 nmol/mg protein. The fluidizing effect of A_2C was observed 2-3 min after its addition. However, at about 15 mins after adding A_2C, the fluidizing effects of A_2C waned.

6.4. EFFECTS OF FLUIDIZING CARDIAC PLASMA MEMBRANE ON β-ADRENERGIC RECEPTOR BINDING CHARACTERISTICS

While the cardiac plasma membranes from both cirrhotic BDL rats and sham-operated control rats were fluidized with A_2C, β-adrenergic receptor density (Bmax) and binding affinity (Kd) were determined. The effects of fluidizing cardiac plasma membranes on cardiac β-adrenergic receptor density are presented in figure 6.5. No significant differences were observed in the receptor density before and after the membranes were fluidized. The effects of membrane fluidity on the receptor binding affinity is presented in figure 6.6. Similarly, no alteration in binding affinity was observed before and after the addition of A_2C to the cardiac plasma membrane preparation.

Therefore, the data herein suggest that the cardiac β-adrenergic receptor density and binding affinity are not fluidity dependent.

6.5. EFFECTS OF FLUIDIZING CARDIAC PLASMA MEMBRANE ON cAMP PRODUCTION

The next series of experiments were performed to further assess the relationship between cardiac β-adrenergic receptor function and plasma membrane fluidity by measuring cAMP generation before and after the membrane fluidization. In these series of experiments, initially it was necessary to exclude direct stimulatory effect of A_2C on adenylyl cyclase, independent of its fluidizing effects. cAMP production was measured in control rat cardiac plasma membrane preparations

incubated with A_2C in the absence of any β-adrenergic receptor, G-protein, and adenylyl cyclase stimulators. The results are presented in figure 6.7. No changes in cAMP production were observed when graded concentrations of A_2C were added into the membrane incubation mixture. Therefore, these preliminary experiments exclude a direct stimulatory effect of the A_2C on adenylyl cyclase.

The effects of membrane fluidity on β-adrenergic receptor function were first investigated by stimulating the receptor with isoproterenol and measuring cAMP generation before and after membrane fluidization. Despite the fact of that change in membrane fluidity has no effects on β-adrenergic receptor density and binding affinity, cAMP generation was found to be a fluidity dependent process when stimulated at β-adrenergic receptor level. It was noted that as the cardiac plasma membrane from cirrhotic BDL rats were fluidized from r_s value around 0.197 back to the the sham value range, 0.184, cAMP production in the fluidized cirrhotic membrane was significantly higher than that in the native membranes from cirrhotic BDL rats. Furthermore, no significant differences in the cAMP production were observed between the membranes from sham-operated control rats and fluidized membrane from BDL cirrhotic rats. The results were presented in figure 6.8.

The data herein suggest that alteration in cardiac plasma membrane fluidity in cirrhotic rats plays an important role in diminished β-adrenergic receptor function.

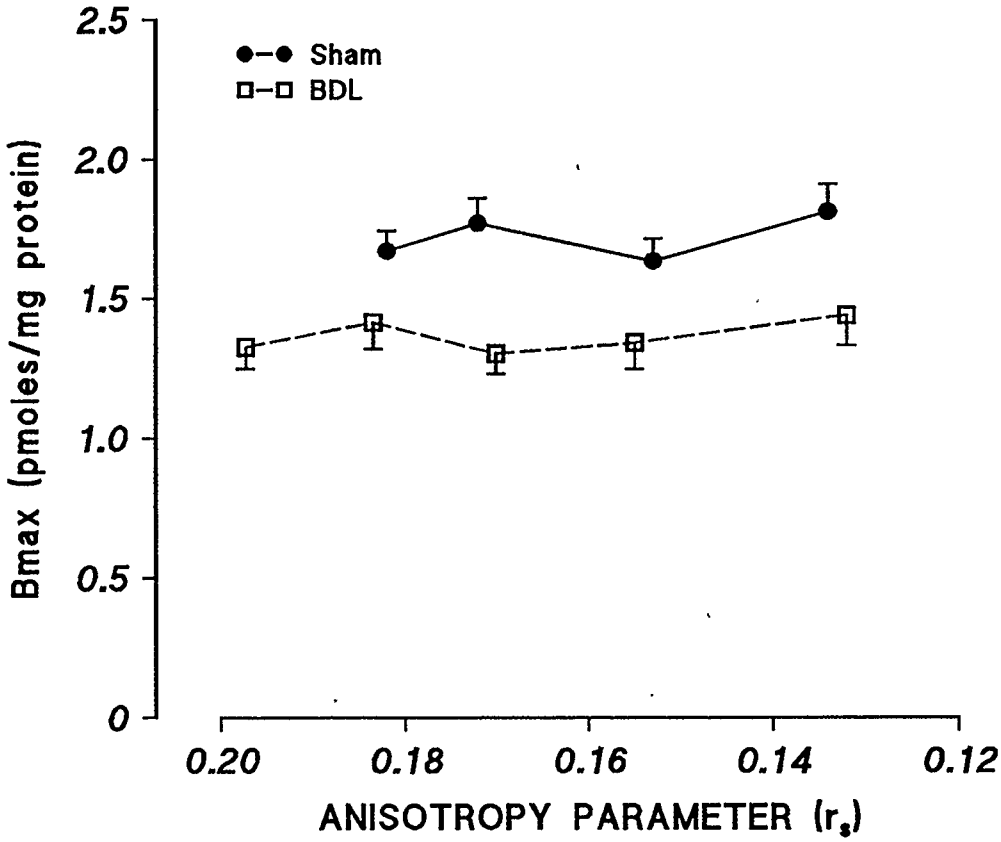


Figure 6.5. Effects of graded concentrations of a fluidizing agent, 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C), on β-adrenoceptor density (Bmax). For clarity, error bars of anisotropy parameters are not shown. Each data point represents mean ± SEM obtained from 5 separate membrane preparations. No significant differences were observed in Bmax values when membranes were fluidized with A_2C.

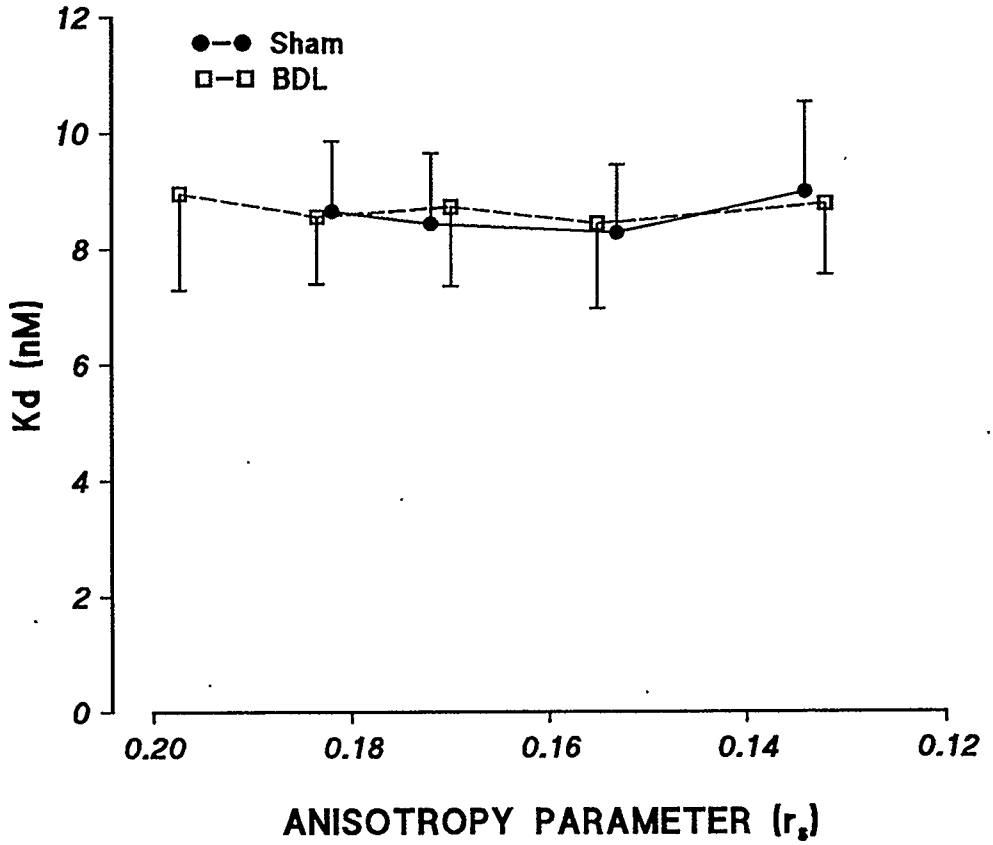


Figure 6.6. Effects of graded concentrations of a fluidizing agent, 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C), on β-adrenoceptor density binding affinity (Kd). For clarity, error bars of anisotropy parameters are not shown. Each data point represents mean ± SEM obtained from 5 separate membrane preparations. No significant differences were observed in Kd values when membranes were fluidized with A_2C.

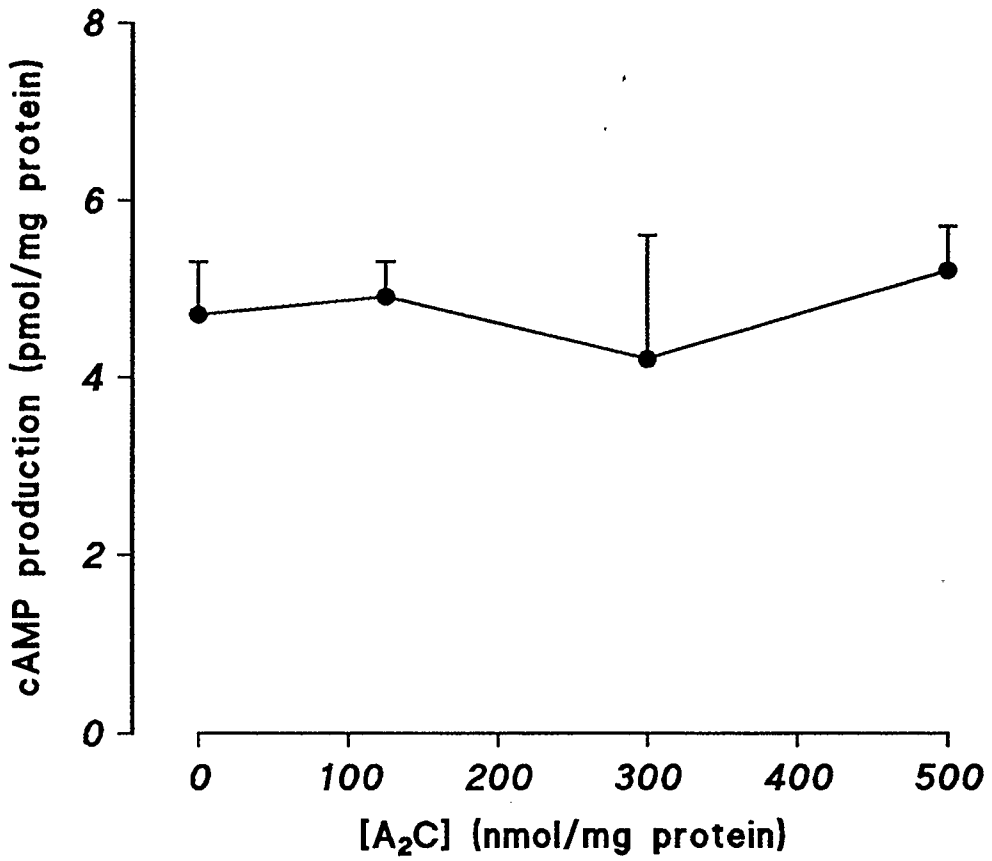


Figure 6.7. Effects of graded concentrations of a fluidizing agent, 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C), on the cAMP production without adding any β-adrenergic receptor signal transduction pathway stimulator. Data are presented as mean ± SEM obtained from 6 separate cardiac plasma membrane preparations of sham-operated rats. No significant differences in cAMP production were observed when A_2C were added to the membrane preparations alone.

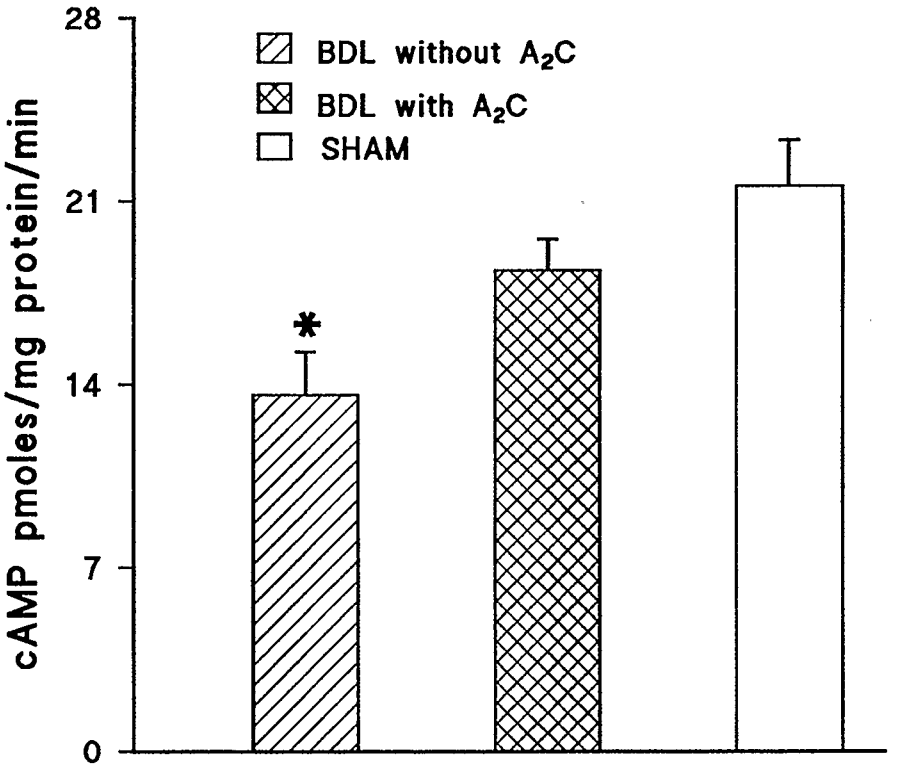


Figure 6.8. Comparison of cAMP content generated under isoproterenol stimulation (10^{-4} mol/L) while fluidizing cardiac plasma membranes of cirrhotic BDL rats with a fluidizing agent, 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate (A_2C, 125 nmol/mg protein), to reduce anisotropy parameter in cirrhotic (BDL) membranes to the same range as sham-operated (Sham) group, with the native BDL and Sham membranes that did not have added A_2C. * Significantly different from both Sham and BDL with A_2C groups, $p<0.05$. There is no significant difference between BDL with A_2C group and Sham group. Data were collected from 5 separate membrane preparations.

In order to further explore to what extent membrane fluidity can influence cAMP production when stimulated at the receptor level, cardiac plasma membranes from both cirrhotic BDL and sham-operated control rats were further fluidized to a r_s value around 0.13. In these series of experiments, it was found that cAMP production curves were in a bell shape as shown in figure 6.9. Three points should be emphasized upon analysing the data in figure 6.9. First, when the membranes from the cirrhotic rats were fluidized to the normal r_s value (first point in the sham-operated group and second point in the cirrhotic BDL group), cAMP production was not different between the two groups, which suggests that decrease in membrane fluidity (higher r_s value) plays a role in diminished cAMP production in cirrhosis. Secondly, when the membranes from both groups were further fluidized, cAMP production was increased first and then decreased. However, the magnitude of increase in cAMP production in BDL rats was smaller than that in sham-operated rats, which suggests that besides membrane fluidity alteration, other defects in cAMP production pathway also exist. Indeed, cardiac β-adrenergic receptor density was decreased (figure 5.7) and Gs-protein expression was reduced (figure 5.11) in cirrhotic BDL rats. Thirdly, optimal cAMP production is not at native r_s value, instead it lies at more fluidized points.

It was found that when cardiac plasma membranes were fluidized to the r_s around 0.16, cAMP production reaches the highest point (figure 6.9). Therefore, the effect of membrane fluidization on cAMP production stimulated at G-protein and adenylyl cyclase level was evaluated by fluidizing the membrane to the r_s values

around 0.16.

Effects of membrane fluidity on cAMP production at G-protein level were tested by stimulating cAMP production with fluoride ions and fluidizing the membranes. The results are presented in figure 6.10. No significant changes were observed before and after the addition of A_2C to the membrane preparations.

Effects of membrane fluidity on cAMP generation at adenylyl cyclase level under forskolin stimulation are presented in figure 6.11. No significant changes were observed in cAMP production before and after the membranes were fluidized.

Taken together, the results herein suggest that the cAMP production stimulated at the receptor level is fluidity dependent but not at G-protein and adenylyl cyclase levels. Alteration of cardiac plasma membrane fluidity may play a crucial role in the diminished β-adrenergic receptor signal transduction pathway function.

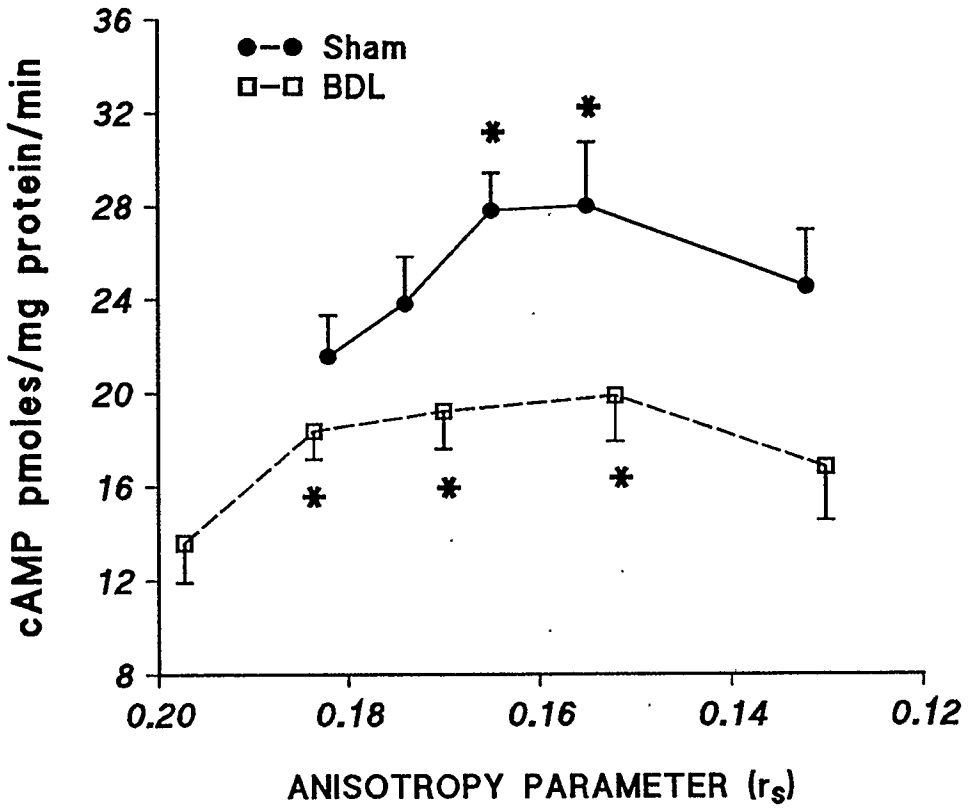


Figure 6.9. Effects of graded concentration of the fluidizing agent 2-(2-methoxyethoxy)ethyl 8-(cis-n-octylcyclopropyl) octanoate on cAMP production under isoproterenol (10^{-4} mol/L) stimulation. Each data point represents mean ± SEM obtained from 5 membrane preparations. For clarity, the error bars of anisotropy parameters were not shown. * Significantly different compared with initial value in each group, $p<0.05$. Note that first point in the sham-operated group (Sham) and second point in the cirrhotic (BDL) group are not significantly different from each other.

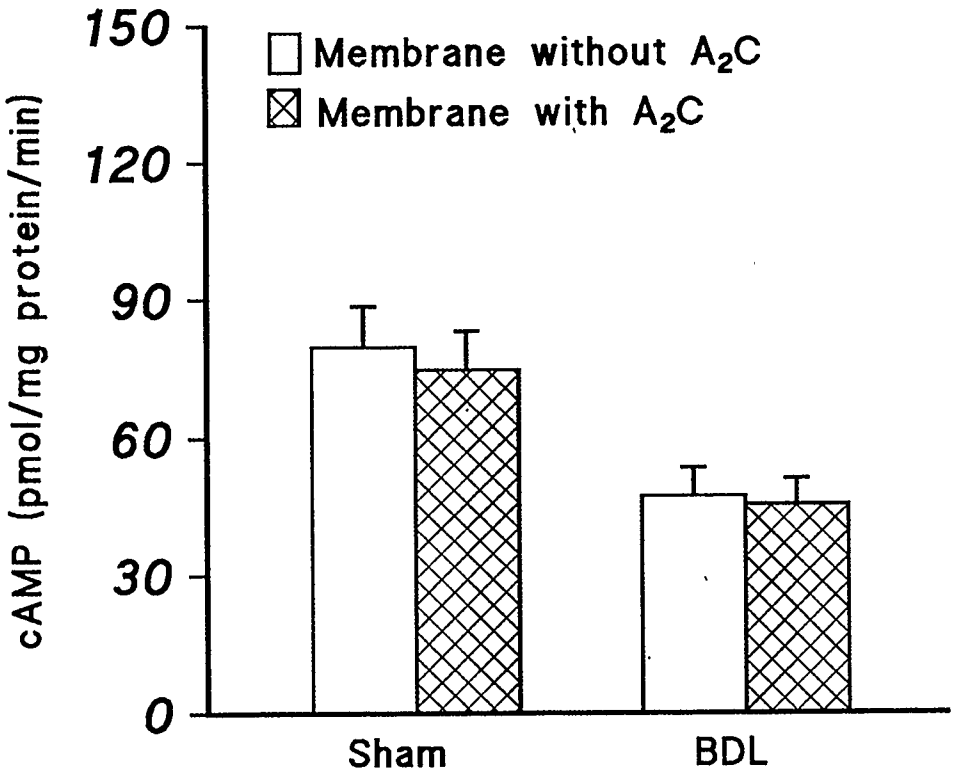


Figure 6.10. Effect of membrane fluidization on cAMP production under fluoride ion stimulation (10 mol/L). Data are presented as mean ± SEM obtained from 5 separate membrane preparations. The r_s values for cirrhotic membrane (BDL) before and after adding A_2C (200 nmol/mg protein) were 0.197±0.001 *vs.* 0.163±0.011; for sham-operated membrane (Sham) before and after adding A_2C (120 nmol/mg protein) were 0.186±0.001 *vs.* 0.159±0.08. No significant differences in cAMP production were observed before and after the membranes fluidization.

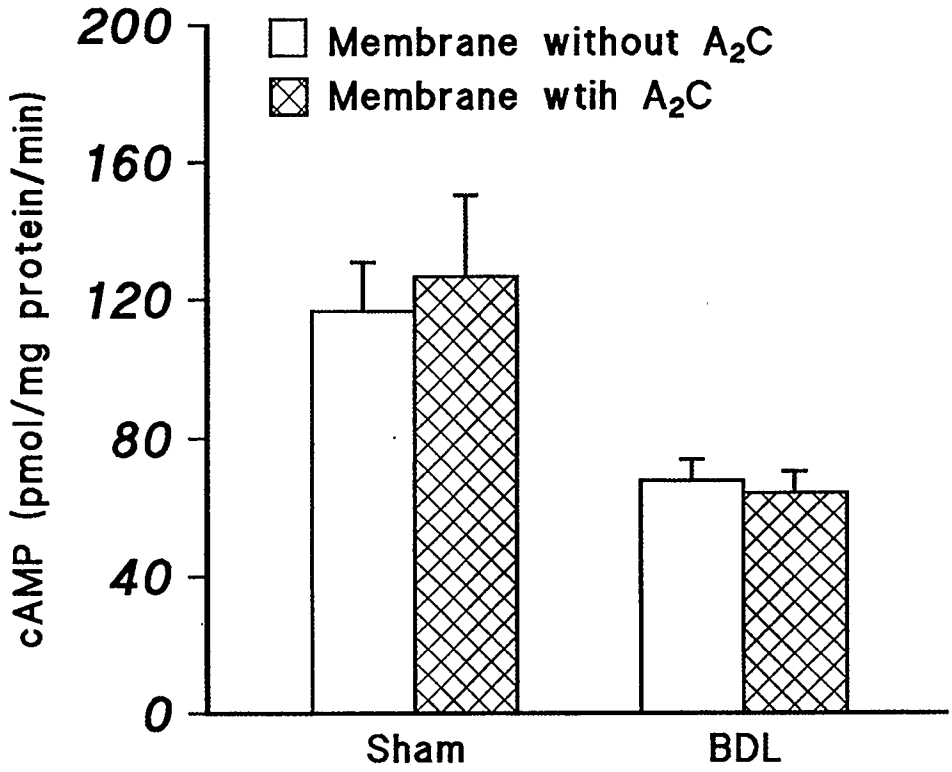


Figure 6.11. Effects of membrane fluidization on cAMP production under forskolin (100 μmol/L) stimulation. Data are presented as mean ± SEM obtained from 5 separate membrane preparations. The r_s values for cirrhotic membranes (BDL) before and after adding A_2C (200 nmol/mg protein) were 0.196±0.002 *vs.* 0.161±0.009; for sham-operated membrane (Sham) before and after adding A_2C (120 nmol/mg protein) were 0.185±0.001 *vs.* 0.157±0.01. No significant differences in cAMP production were observed before and after the membranes fluidization.

CHAPTER SEVEN

DISCUSSION

7.1. ANIMAL MODEL AND MEMBRANE PREPARATIONS

7.1.1. Animal models

Given the difficulty of obtaining heart tissue from cirrhotic patients, the first experimental approach in the present investigation was to set up a reliable cirrhotic animal model. To date, no perfect cirrhotic animal models are available. The most commonly used ones are CCl_4-induced cirrhosis and common bile duct ligation-induced biliary cirrhosis. In the CCl_4-induced model, the advantages are that the liver histology, if successful, is more close to the cirrhotic changes observed in humans. However, the time required to induce cirrhosis is very long (10 weeks) and the mortality rate is high. Most importantly, histological changes are variable. Furthermore, a fairly high percentage of rats do not present cirrhotic changes after long exposure to CCl_4 (Tamayo, 1983). Chronic oral administration of dimethylnitrosamine to induce cirrhosis was reported by Madden *et al* (1970). This process takes about four weeks to produce cirrhosis. Toxic effects of dimethylnitrosamine on humans prevent its wide use. A choline-deficient diet is another method used to induce cirrhosis, however, this method requires about 25 weeks, therefore, it is not widely used (Murray *et al.*, 1992).

It was decided to use a BDL rat model in the present study because this model has many advantages such as low mortality rate, short time needed to induce histological cirrhotic changes (only 4 weeks), and a relatively simple surgical intervention. The bile duct-ligated cirrhotic rat model was first reported by Cameron and Oakley six decades ago (1932). Since then, this animal model has been used

extensively. It has been documented in numerous articles that at around 4 weeks following bile duct ligation, the histological features of biliary cirrhosis, portal hypertension, ascites, jaundice, hepatocellular failure, hepatosplenomegaly, and muscle wasting are reproduced. Cardiovascular abnormalities in BDL rats mirror those reported in human cirrhosis. Indeed, recent studies even showed gastric mucosal lesions similar to the portal hypertensive gastropathy reported in human cirrhosis (Beck *et al.*, 1992).

The results herein, such as hepatocyte insufficiency (table 4.2), elevated portal pressure (figure 4.1), and liver microscopic changes, confirm previous observations. Furthermore, the left ventricular papillary muscle contractile data confirms the existence of cirrhotic cardiomyopathy in our BDL cirrhotic rats (figure 5.2 , 5.3).

It should be pointed out that the present animal model is not perfect. The major drawback of this experimental model is that the effects of chronic cholestasis cannot be separated from the effects due to cirrhosis *per se*, because both conditions are found in this model. The data obtained from this model mainly represent biliary cirrhosis. It must be with caution when extrapolating the data from this model to explain situation in other types of cirrhosis and clinical situations.

7.1.2. Cardiac plasma membrane preparations

Two major objectives of the present study were to investigate the cardiac plasma membrane β-adrenergic receptor signal transduction pathway function and to clarify the relationship between cardiac β-adrenergic receptor function and plasma

membrane physical and chemical properties. Therefore, it was essential to monitor the relative purity of the final cardiac plasma membrane preparations. A variety of cardiac plasma membrane isolation methods are available (Dhalla and Pierce, 1984). The one used here involves salt extraction and sucrose gradient centrifugation separation. The salt extraction process is an important procedure for membrane purification in any muscle tissue in order to extract contractile proteins. However, it should be emphasized that prolonged membrane exposure to high concentrations of salt leads to inactivation of adenylyl cyclase since this enzyme is very fragile (Drummond and Duncan, 1969). Therefore, short term salt extraction procedures were used in the present study (10 min twice). The cAMP data herein demonstrated that the adenylyl cyclase in the present cardiac plasma membrane preparations remains active, suggesting that the present methods are valid. The cardiac plasma membrane enzyme marker, 5'-nucleotidase, has been extensively utilized to evaluate the purity of plasma membrane preparations. The final membrane preparations herein had a 5 to 6-fold enrichment of the specific activity of 5'-nucleotidase compared with the initial homogenate. These results are comparable to the data from other groups isolating similar membrane preparations (Kidwai *et al.*, 1971; Pierce *et al.*, 1980). Gross contamination by other intra-cellular organelles is unlikely since the mitochondrial membrane enzyme marker, cytochrome *c* oxidase, was not enriched in the final plasma membrane preparations. The qualities of the cardiac plasma membrane preparations from cirrhotic BDL rats and sham-operated controls are similar since the enrichment of 5'-nucleotidase and cytochrome *c* oxidase is not

different between the two groups. Based on the above data, the membrane preparations isolated using the above methods are suitable for the present investigation.

7.2. CARDIAC β-ADRENERGIC RECEPTOR FUNCTION IN LIVER CIRRHOSIS

7.2.1. Blunted left ventricular papillary muscle contractile responses

Compelling evidence in the past four decades has demonstrated the existence of cardiomyopathy in both cirrhotic patients and animal models of cirrhosis (Lee, 1989). The characteristics of cirrhotic cardiomyopathy are that at baseline, the cardiac output is increased and impairment of cardiac contraction usually cannot be observed. It is quite possible that at baseline, because of reduced peripheral resistance, the blunted cardiac contractile function is masked. Only under physical and mental stress, pharmacological stimulation, or when the heart is overloaded with extra plasma volume, is the abnormal cardiac contractile function revealed (Grose *et al.*, 1995; Limas *et al.*, 1970; Ingles *et al.*, 1991; Caramelo *et al.*, 1986). The data of left ventricular papillary muscle contractility (figure 5.2, 5.3) from the present study further demonstrated directly that myocardium contractile function was blunted in cirrhotic rats. Therefore, the results support and confirm the existence of cirrhotic cardiomyopathy.

From the data presented in figure 5.1 (normalized heart and ventricular weight) as well as heart histological examination, it is clear that cardiac tissues in our cirrhotic rats are not hypertrophic. Histological examination under light microscopy

did not discover any abnormal changes in the heart structures. These results indicate that blunted cardiac contractile responses in cirrhosis were not caused by alterations in cardiac tissue mass.

7.2.2. β-adrenergic receptor characteristics and adenylyl cyclase activity

We were particularly interested in β-adrenoceptor function since this is the prime determinant of ventricular contractility (Fleming *et al.*, 1992; Stiles and Lefkowitz, 1984). In this regard, only two previous studies have directly examined β-adrenergic receptor characteristics in cirrhosis. Gerbes and colleagues demonstrated that cirrhotic patients with ascites had decreased lymphocyte β-adrenoceptor density (Gerbes *et al.*, 1986). There are no reports in the literature concerning β-adrenergic receptor status in cirrhotic human heart tissue. However, it has been demonstrated that lymphocyte β-adrenergic receptor density is positively correlated with both the heart β-adrenergic receptor density and cardiac contractility (Brodde *et al.*, 1986). Therefore, the β-adrenergic receptor characteristics on the lymphocytes could represent the β-adrenergic receptors in the heart tissue. Indeed, a reduced β-adrenergic receptor density in the cardiac membrane was also found in cirrhotic rats, and this was associated with blunted chronotropic responses to isoproterenol stimulation (Lee *et al.*, 1990). In accordance with these previous observations, the present study demonstrated a comparable decrease in β-adrenoceptor density (figure 5.7), despite using a different method of membrane preparation and radioligand. Whether the reduction in β-adrenergic receptor density

plays any pathological role in blunted cardiac contraction in cirrhosis merits further discussion. The heart is a special organ in the body. In contrast to other tissues, previous research has demonstrated that spare β-adrenergic receptors are rare in the heart (Port and Bristow, 1988). Maximum cardiac contractile responses generally require participation of all the receptors (Brodde, 1991). A reduction in β-adrenergic receptor density in the heart always leads to the impairment of β-adrenergic receptor signal transduction (Brodde *et al.*, 1991). Evidence also suggests that a reduction in β-adrenergic receptor number causes a proportional decrease in isoproterenol stimulated cAMP production (Port and Bristow, 1988). Therefore, we contend that the reduced cardiac β-adrenergic receptor density in the BDL rats presented in the present study is partially responsible for diminished β-adrenergic receptor function as demonstrated by our papillary muscle contractile data.

β-Adrenergic receptor signal transduction system plays an important role in modulation of cardiac contractility. In this signal transduction pathway, cAMP generation is a core event (Fleming *et at.*, 1992). The cAMP data from the present investigation demonstrate that β-adrenergic receptor signal transduction pathway function is diminished. Figure 5.10 illustrates that cAMP production was decreased when stimulated at the receptor, G-protein, as well as adenylyl cyclase levels. Multiple causes are considered to be responsible for decreased cAMP production, such as a reduction in β-adrenergic receptor density, Bmax, (figure 5.7), alterations in G-protein expression (figure 5.11), and functional impairment of adenylyl cyclase *per se*. Other factors which can lead to a reduction in cAMP generation include

changes in cardiac plasma membrane fluidity which will be discussed in more detail in the later sections. Since cAMP production was impaired at multi-levels of the signal transduction pathway, a generalized impairment in cardiac β-adrenergic receptor signalling is the characteristics of cirrhotic cardiomyopathy.

The cAMP generation data herein concur with a previous report by Cahill and colleagues. In that study, it was found that in portal hypertensive rats cAMP generation in the blood vessels was significantly reduced (Cahill *et al.* 1994). Wu and Benoit recently showed that G-protein related signal transduction in the blood vessels of portal hypertensive rats was blunted (1994). Taken together, the results from the present investigation and these previous reports suggest that a decrease in cAMP generation in cirrhosis and portal hypertension may not only involve the heart, but the whole cardiovascular system as well.

7.2.3. G-protein expression in cirrhotic cardiomyopathy

It has been demonstrated that there are four types of Gsα. They are encoded by the same gene and are the products of alternative splicing of mRMA post-transcription. On SDS gel, they are distributed as 42 to 45 and 47 to 52 kDa bands (Holmer and Homcy, 1991; Bray *et al.*, 1986). In the present investigation 45 and 47 kDa bands were detected. It was found that Gsα expression in BDL rats was significantly reduced compared with sham-operated control rats. The antibodies used to detect Gsα in the present investigation were RM/1. The antisera, RM/1, were raised against the deduced 10 amino acid sequence on the carboxyl terminus of αs

subunit of G-proteins (Simonds *et al.*, 1989b). Previous experiments have demonstrated that RM/1 antisera can block the stimulatory effect of Gsα on adenylyl cyclase (Simonds *et al.*, 1989b). Traditionally, it was considered that a significant reduction in Gsα expression plays an important role for the impairment of cardiac contractile function (Feldman, 1993). However, very recently, this idea was challenged by β-adrenergic receptor, Gs-protein, and adenylyl cyclase stoichiometric studies. Current stoichiometric investigation reveals that Gs-proteins are present in large excess relative to β-adrenergic receptor and adenylyl cyclase content. The β-adrenergic receptor:Gs-protein:adenylyl cyclase ratio is 1:167:3 (Post *et al.*, 1995). Based on the above stoichiometric data, it should be cautious to explain the relationship between reduced cAMP production and Gsα expression observed in the present study. It might be possible that impairment of Gsα function is more important than a moderate reduction in Gsα expression. But unfortunately, direct Gsα function studies such as measurement of GTPase activity have not been performed in the present investigation. This topic is warranted for further research.

As described in the introduction, cAMP production is regulated by stimulatory G-protein (Gs) coupled receptors such as β-adrenergic receptors. cAMP generation is also modulated by inhibitory G-protein (Gi) coupled receptors such as M_2 muscarinic receptors in the heart (Felder, 1995). When a M_2 receptor is occupied by an agonist such as carbachol, the receptor is activated. The activated receptor will interact with Gi protein which results in guanine nucleotide exchange and αi subunit dissociation from $\beta\gamma$ subunits. Two mechanisms have been proposed to explain M_2

receptor mediated adenylyl cyclase inhibition (Linder and Gilman, 1992): 1) αi subunit directly inhibits adenylyl cyclase; 2) $\beta\gamma$ subunits bind with the activated αs subunit of Gs-protein, which will remove the stimulatory effect of αs on adenylyl cyclase and lead to a decrease in cAMP production. Data concerning the expression of Giα in heart failure remains controversial. Some reports suggest that an increase in Giα expression may result in blunted heart contractile function (reviewed by Feldman, 1993). Others documented that Giα expression was decreased, and their data argued that an increase in Giα expression was not necessary in heart failure (Roth *et al.*, 1993). Giα subunit has three isoforms which are coded by distinct genes. Gi1α was cloned by Nukada *et al* (1986), Gi2α by Itoh and colleagues (1986), and Gi3α by Jones and Reed (1987). Giα subunit is expressed in almost all tissues (Kaziro *et al.*, 1991). However, in the heart, Gi2α is predominant. The expression of Gi1α in the heart is very low and Gi3α is even less. In contrast, Gi1α is vastly distributed in the brain and Gi3α in the liver (Holmer and Homcy, 1991; Holmer *et al.*, 1989). In the present study, the antisera, AS/7, was used. The reaction between AS/7 and 3 different Giα was Gi1α=Gi2>>Gi3α. No cross reaciton was observed between AS/7 and Goα (Simonds *et al.*, 1989a). Therefore, the results here represent the expression of Gi2α in heart tissue. The data herein demonstrated that in cardiomyopathy, or at least in cirrhotic cardiomyopathy, Gi2α expression was decreased. The pathological roles of a decrease in Gi2α expression in cirrhotic cardiomyopathy merit further discussion. Since reduced Giα expression could lead to an increase in cAMP production in the situation where the inhibitory pathway is

activated, it is possible that the inhibitory pathway function is also blunted in cirrhotic cardiomyopathy.

The $\beta\gamma$-subunits of G-proteins are tightly associated. The antibodies used to detect $\beta\gamma$-subunit expression are SW/1. At present, 5 different types of β subunit have been identified (Watson *et al.*, 1994; Rens-Domain and Hamm, 1995). The antibodies, SW/1, were raised against the C-terminus of G-protein β-subunit. Since SW/1 cannot differentiate between the various types of β-subunits, the results are termed Gcommonβ. Data from the present investigation demonstrated that Gcommonβ expression was not different between the cirrhotic BDL rats and sham-operated control rats. Since Gsα expression was decreased, with no changes in Gcommonβ, the $\beta\gamma/\alpha$s ratio is increased. In the last few years, it has been appreciated that G-protein $\beta\gamma$ subunits play comprehensive roles in G-protein related signal transduction, especially in cAMP generation (Clapham and Neer, 1993; Muller and Lohse, 1995). The role of an increase in $\beta\gamma/\alpha$s ratio in cirrhotic cardiomyopathy remains unclear. However, the following speculations could be considered. A previous report by Tang and Gilman demonstrated that $\beta\gamma$-subunits of G-proteins can activate both type II and type IV adenylyl cyclase (Tang and Gilman, 1991). Type IV-VII adenylyl cyclases are commonly distributed in heart tissue (Iyengar, 1993). Therefore, an increase in Gcommonβ/Gsα ratio could be another compensatory effect in the face of the reduced cAMP generation in cirrhosis. On the other hand, it has been demonstrated that $\beta\gamma$-subunits of G-proteins facilitate movement of β-adrenergic receptor kinase (βARK) from the cytosol to plasma

membrane. βARK phosphorylates β-adrenergic receptors leading to receptor desensitization (Pitcher *et al.*, 1992). Therefore, an increase in $\beta\gamma/\alpha s$ ratio in cirrhotic cardiomyopathy leads to a subtle tuning in cAMP generation.

A generalized decrease in G-protein expression in cirrhotic cardiomyopathy is unlikely since Gcommonβ expression was not different between the groups. It should also be emphasized that from the present data, it cannot be concluded whether changes in G-protein subunit expression were caused by a decrease in G-protein synthesis or an increase in G-protein degradation. This topic merits further research.

7.3. ALTERATION OF CARDIAC PLASMA MEMBRANE PHYSICAL AND CHEMICAL PROPERTIES IN LIVER CIRRHOSIS

The plasma membrane plays an important role in cardiomyocyte contractile function since it forms the lipid environment for the β-adrenergic receptor and its associated second messenger generation system. Almost all previous studies of cirrhotic cardiomyopathy have been descriptive in nature, and the underlying membrane mechanisms responsible for altered contractile function have remained unstudied. To our knowledge, the present study is the first to investigate the basic biochemical and biophysical membrane mechanisms underlying the abnormal β-adrenergic receptor function in cirrhosis.

In the present investigation it was found that the cardiac plasma membrane became more rigid in biliary cirrhotic rats than sham-operated control rats. Whether

these changes are specific to biliary cirrhosis or, if they represent the common characteristics of liver cirrhosis caused by different pathological origins, should be discussed. The data herein and a very recent report by Kakimoto and colleagues (1995) support the latter contention. Kakimoto's investigation demonstrated that red blood cell membranes became rigid in patients with cirrhosis due to chronic viral hepatitis. Furthermore, it was demonstrated that the decrease in the red blood cell membrane fluidity was positively correlated with the severity of liver cirrhosis. The red blood cell membranes from Child-Pugh class C patients (more severe liver failure) are more rigid than the membranes from Child-Pugh class A (less severe). The membrane fluidity data from different animal models are also in good agreement with our observation. It was demonstrated that liver microsomal membranes from CCl_4-induced rats were more rigid than their control counterparts (Buters *et al.*, 1993). Taken together, the fact that the membranes are rigid could be a common feature in liver cirrhosis, irrespective of its pathogenesis.

Changes in membrane fluidity in liver cirrhosis seem to be a generalized pathological phenomenon which involves multiple systems in the body. Here we demonstrated that the plasma membranes from heart tissue became rigid. Previous studies demonstrated that microsomal membranes from the liver in cirrhosis were rigid (Reichen *et al.*, 1992; Nakashima *et al.*, 1993). It was also reported that the brush border membranes in the kidneys were more rigid in cirrhotic rats than their control counterparts (Imai *et al.*, 1992; Kawata *et al.*, 1987). Evidence showed that the red blood cell membranes were also rigid (Owen *et al.*, 1982). A recent report

by Lang and colleagues demonstrated that peripheral white blood cell membranes became rigid in BDL rats (Lang *et al.*, 1995). These generalized changes in membrane physical properties in different tissues in cirrhosis are correlated to some extent with alterations in the function of proteins embedded in the membrane bilayer. Therefore, the data herein and previous studies support the contention that the membrane physical properties play an important role in modulating the related membrane protein function.

Many factors influence plasma membrane physical properties. Among them, cholesterol content and the cholesterol:phospholipid molar ratio in the plasma membrane are perhaps the most important ones (Shinitzky and Barenbholz, 1978). The sterol ring in the cholesterol molecule is rigid which interacts with fatty acyl chains, thus, reducing the membrane fluidity. A previous study by Bastiaanse and colleagues directly demonstrated that when cardiomyocytes were cultured in a medium containing a high content of cholesterol analogue, dehydroergosterol, the cardiac plasma membrane quickly became rigid (Bastiaanse *et al.*, 1993). It has also been demonstrated that a decrease in cellular free cholesterol level, induced by metabolic inhibition in cultured neonatal cardiomyocytes, leads to an increase in the cardiomyocyte plasma membrane fluidity, that is the membrane became less rigid (Bastiaanse *et al.*, 1995). In accordance with these previous observations, in the present study it was found that the cholesterol:phospholipid ratio increased following bile duct ligation, with significant differences between the BDL group and the sham-operated group primarily due to an increase in cholesterol content. This was

associated with an increase in the anisotropy parameters for the fluorescent probes, DPH and AS in the superficial regions of the lipid bilayer of the plasma membrane in cirrhotic rats. These results indicate that the cardiac plasma membrane of BDL rats is significantly less fluid than their sham-operated counterparts.

The mechanisms of the increase in the cardiac plasma membrane total cholesterol levels in liver cirrhosis merit further discussion. It should be noted that the alteration of membrane fluidity in cirrhosis in the different tissues reported previously was associated with an elevation in cholesterol content and cholesterol/phospholipid ratio. In our cirrhotic animal model, it has been shown that the total blood cholesterol content was increased (table 4.2). The increase in the blood cholesterol level facilitates incorporation of more cholesterol into the cardiac plasma membranes. Furthermore, it has been demonstrated that the blood lecithin-cholesterol acyltransferase activity in cirrhosis was decreased (Tahara *et al.*, 1993; Simon, 1971). Lecithin-cholesterol acyltransferase catalyzes esterification between free cholesterol and long chain fatty acyl residues from lecithin. Because of the functional impairment of lecithin-cholesterol acyltransferase, the free cholesterol in the blood is increased. The above metabolic abnormalities also facilitate incorporation of cholesterol into the cell plasma membranes.

Other factors that have effects on plasma membrane physical properties include membrane fatty acid composition. The degree of saturation and the length of the fatty acid side chains of phospholipids are important in regulating membrane physical properties. *Cis* double bonds create angles along the fatty acid axis and

introduce "kinks" which prevent close packing and thus increase molecular specific volume (Ladbrooke and Chapman, 1969). These double bonds allow greater molecular freedom for other lipid molecules and, hence, increase membrane fluidity. The first double bonds in the fatty acids are more important in fluidizing the membranes (Stubbs *et al.*, 1981). The longer the fatty acid chain, the more chain-chain interactions will occur, and this serves to reduce membrane fluidity (Ladbrooke and Chapman, 1969). In our study we did not find gross differences in fatty acid composition between the two groups. Only arachidonic acid (20:4) content was increased in the BDL group, and the increase was very modest. Obviously, this alteration does not contribute to a reduction in membrane fluidity but may possibly represent a compensatory response to the increased cholesterol content and altered membrane physical properties. Regardless of the mechanisms, the cardiomyocyte plasma membranes of the cirrhotic rats are more rigid than controls.

7.4. RELATIONSHIP BETWEEN CARDIAC PLASMA MEMBRANE PHYSICAL PROPERTIES AND β-ADRENERGIC RECEPTOR FUNCTION

In the present investigation, it was found that when the cardiac plasma membranes from cirrhotic rats were fluidized back to normal, cAMP generation could be restored when stimulated at the receptor level with isoproterenol (figure 6.8). However, when β-adrenergic receptor signal transduction pathway was stimulated at G-protein and adenylyl cyclase levels, fluidization of cardiac plasma membranes has no effect on cAMP production (figure 6.10, 6.11). Furthermore, the

results from figure 6.5 and figure 6.6 clearly show that the effects of change in membrane fluidity on cAMP generation are not through an increase in the β-adrenergic receptor density (Bmax), or changes in the receptor binding affinity (Kd), because these parameters were not significantly different before and after membrane fluidization.

The lipid membrane bilayer properties are critical for the β-adrenergic receptor and G-protein to function properly. The plasma membrane closely interacts β-adrenergic receptors and G-proteins. For example, the β-adrenergic receptor is palmitoylated. Without palmitoylation, isoproterenol stimulated cAMP production is impaired (O'Dowd *et al.*, 1989a). The functional importance of G-protein lipid modification has been greatly appreciated in the past few years (Casey, 1994). The αs subunits are palmitoylated, αi-subunits are myristoylated or/and palmitoylated, and γ-subunits are prenylated. One of the important characteristics of lipid modification is to facilitate the anchoring of G-protein in the lipid bilayer. Furthermore, it has been demonstrated that without lipid modification, the G-proteins cannot function properly (Wedegaertner *et al.*, 1995).

In the β-adrenergic receptor signalling pathway, when the receptor is occupied or activated by an agonist, the next step is the coupling process between the receptor and the G-protein. Therefore, the present results suggest the interaction between the receptor and G-protein in the lipid bilayer could be a fluidity-dependent process. The data herein support our hypothesis that membrane fluidity plays a role in the diminished β-adrenergic receptor function. The present data are also in good

agreement with the previous report by Hanski and colleagues. They demonstrated that cAMP generation in turkey erythrocytes was fluidity dependent when the β-adrenergic receptor is stimulated with isoproterenol (Hanski *et al.*, 1979).

It is also intriguing to observe that the fluidization of cardiac plasma membranes has no effect on cAMP generation when stimulated at the G-protein and adenylyl cyclase levels. It remains unclear why change in membrane fluidity has distinct effects on cAMP production while β-adrenergic receptor signal transduction pathway is activated at differenct levels. One explanation could be that when stimulated by fluoride ion and forskolin, adenylyl cyclase reaches its maximum activity. On the other hand, the effect of membrane fluidity on cAMP production is relatively subtle. Therefore, no further elevation in cAMP production could be observed when the membranes were fluidized.

Although it is possible that the fluidizing agent, A_2C, could exert direct stimulatory effects on cardiac plasma membrane cAMP generation independent of its effects of fluidizing the membrane preparations, we believe that this is unlikely. Previous work has shown that A_2C has no direct effects on other membrane-bound enzymes such as sucrase, maltase, gamma glutamyl transpeptidase, alkaline phosphatase and leucine aminopeptidase (Dudeja *et al.*, 1991). Figure 6.7 directly demonstrates that A_2C, at three different concentrations, has no direct stimulatory effects on cAMP generation. Furthermore, close examination of figure 6.9 reveals bell-shaped curves, i.e, that the highest doses of A_2C used to decrease anisotropy parameters actually lead to a decrease in cAMP generation. These results suggest

that A_2C *per se* does not directly stimulate adenylyl cyclase.

7.5. POSSIBLE PATHOLOGICAL FACTORS WHICH LEAD TO CARDIOMYOPATHY IN CIRRHOSIS

Liver cirrhosis is a complex clinical condition associated with many different pathological changes which could affect the cardiovascular system. Among these, metabolic and hemodynamic alterations may be the important ones. The question about which factors in cirrhosis cause blunted myocardium contractility and diminished β-adrenergic receptor signalling remains unclear. However, the following points should be considered.

First, in the present cirrhotic animal model, it is evident from our data presented in table 4.2 that liver function is damaged. Because of hepatocyte insufficiency, toxic substances absorbed from the intestines could not be inactivated properly. Furthermore, the portal venous pressure is elevated in the liver cirrhosis as shown in figure 4.1. Portal hypertension leads to portal systemic shunting. The portal systemic shunting could also allow some toxic substances to bypass the liver inactivation directly into the systemic circulation. Those toxic substances may exert inhibitory effects on cardiac tissues. Another phenomenon often observed in cirrhosis is that cirrhotic patients are more prone to suffer from bacterial infection because of a diminished immune resistance and bacterial translocation (Sorell *et al.*, 1993). As a consequence of bacterial infection, endotoxin levels have been documented to be elevated in cirrhosis (Fukui *et al.*, 1993; Lin *et al.*, 1995). It has

been shown that endotoxin inhibits cardiac contractile function (Sosa *et al.*, 1994). Tanaka and colleagues have demonstrated that endotoxin could cause cardiac cell degeneration (Tanaka *et al.*, 1994). It has also been documented that cytokine levels are elevated in cirrhosis (Napoli *et al.*, 1994; Tilg *et al.*, 1992). The cytokines can stimulate the inducible NO synthase, leading to nitric oxide over-production in heart tissue, which in turn causes blunted cardiac contractility (Schulz *et al.*, 1995; Ungureanu-Longrois *et al.*, 1995). In fact, there is evidence showing that plasma from cirrhotic patients contained some "depressant factors" which cause blunted contractile responses of neonatal rat heart cells (de Mikulic *et al.*, 1987). These "depressant factors" could be the toxic substances discussed above.

Secondly, it has been well recognized that blood norepinephrine levels are elevated both in cirrhotic patients (Braillon *et al.*, 1992; Ring-Larsen *et al.*, 1982; Bichet *et al.*, 1982) and animal models of cirrhosis including the model used in the present study (Gaudin *et al.*, 1989; Abergel *et al.*, 1992). The norepinephrine level at baseline is correlated to the severity of cirrhosis. The more severe the cirrhosis, the higher the norepinephrine level (Grose *et al.*, 1995). Sustained increases in norepinephrine have been reported to cause myocardium injury (Baue *et al.*, 1968) and β-adrenergic receptor desensitization (Hausdorff *et al.*, 1990). When β-adrenergic receptors are exposed to the agonists such as norepinephrine for a short period of time, the receptors are phosphorylated by the β-adrenergic receptor kinase and cAMP dependent kinase. The phosphorylated receptor will be uncoupled from the G-proteins, which results in decreased contractile responses. Short term

desensitization recovers very quickly and does not need receptor protein re-synthesis. Long term exposure of β-adrenergic receptors to norepinephrine leads to receptor internalization, sequestration, and downregulation. This results in a decrease of β-adrenergic receptor density on the plasma membrane (Hausdorff *et al.*, 1990; Pitcher *et al.*, 1992; Premont *et al.*, 1995). In agreement with the previous observations, it was found in the present study that cardiac contractile responses are blunted and β-adrenergic receptor density is decreased in cirrhotic rats (figure 5.2, 5.3, and 5.7). These abnormalities can be partially explained by β-adrenergic receptor desensitization due to plasma norepinephrine elevation. However, it is worth mentioning that β-adrenergic receptor desensitization alone cannot explain all the diminished β-adrenergic receptor signal transduction data observed in the present investigation. This is because previous studies demonstrated that in a desensitized β-adrenergic receptor model, although cAMP generation was severely impaired when stimulated at the receptor level, cAMP generation stimulated at G-protein level and at adenylyl cyclase level were almost intact (Sibley *et al.*, 1985; Hausdorff *et al.*, 1990). From figure 5.10, it is evident that cAMP production was not only impaired at the receptor level, but also at G-protein and adenylyl cyclase levels. Therefore, the data herein indicate that besides β-adrenergic receptor desensitization, other factors in liver cirrhosis also have negative effects on G-protein and adenylyl cyclase function. The mechanisms of diminished cAMP production when stimulated at the G protein level could be partially explained by alterations in G-protein expression in cirrhosis as discussed previously. However, the exact mechanisms of impaired

cAMP production at adenylyl cyclase level remain unknown. A recent study by Zavecz and colleagues may provide some explanation (1995a). Endogenous bile from bile duct fistulae of portal hypertensive rats could inhibit cAMP generation by 30% and 29% in control cardiac plasma membranes when stimulated by GTPγs (G-protein stimulator) and forskolin, respectively. In contrast, synthetic bile salts, hyodesoxycholate and cholate, increase cAMP production in the cardiac plasma membrane (Zavecz *et al.*, 1995). Therefore, Zavecz's study suggests that some components in the bile of the cirrhotic or portal hypertensive rats can inhibit cAMP production in cardiac plasma membranes at both G-protein and adenylyl cyclase levels.

Thirdly, increases in plasma bile salt concentration may lead to a decrease in cardiac contractility. Jacob *et al* recently demonstrated that cardiac contractility was diminished in the 3-day bile duct ligated jaundiced rats. But there were no changes in β-adrenergic receptor characteristics in these rats (Jacob *et al.*, 1993). Lumlertgol *et al* reported that in jaundiced patients, the heart contractile performance was impaired (Lumlertgol *et al.*, 1991). Previous research demonstrated *in vitro* that cardiac papillary muscle contraction responses were blunted when different bile salts were added to the tissue bath (Binah *et al.*, 1985). Pak and colleagues demonstrated that bile salts may interfere with the calcium channels in blood vessels (Pak *et al.*, 1994). It has been documented that plasma bile salt levels are increased in cirrhotic patients (Clain *et al.*, 1977; Larusso *et al.*, 1975). In the present cirrhotic model, bile salts increased about 40-fold (table 4.2). Therefore, increases in bile acids in

cirrhosis could be one of the pathogenetic factors leading to cirrhotic cardiomyopathy.

Finally, besides the liver function damage and metabolic alterations mentioned above, another related complication in cirrhosis is hyperdynamic circulation. In the hyperdynamic circulation status, the heart is overloaded. Increases in the heart workload could be induced by expansion of blood plasma volume and sodium retention in cirrhosis. Available reports showed that prolonged hemodynamic overload of the heart leads to impairment of cardiac contractility (Holtz *el al.*, 1992). Evidence also suggests that the hyperdynamic circulation *per se* could possibly play a role in the blunted cardiac contractile performance in cirrhosis (Battarbee and Zavecz, 1992). Hyperdynamic circulation exists in portal hypertension without liver diseases. To test the possible role of hyperdynamic circulation on cardiac contractile function, a recent study by Zavecz and colleagues demonstrated that in portal hypertensive rats without obvious liver damage, cardiac contractile function was impaired both chronotropically and inotropically (Zavecz *et al.*, 1995b). These results are consistent with a previous report by the same group (Battarbee and Zavecz, 1992). However, it should be emphasized that in portal hypertension alone, without hepatocyte function insufficiency, cardiac β-adrenergic receptor density and binding affinity are not changed (Lee *et al.*, 1991; Ma *et al.*, 1995; Zavecz *et al.*, 1995b).

In view of the above considerations, it is quite possible that the pathogenesis of cirrhotic cardiomyopathy could be multifactorial. At present, no single factor can convincingly explain all the phenomena of cirrhotic cardiomyopathy.

7.6. SUMMARY AND CONCLUSION

The data herein demonstrated that myocardial contractility was blunted under β-adrenergic receptor stimulation *in vitro* in cirrhotic rats. This directly confirms the existence of cirrhotic cardiomyopathy. Cardiac β-adrenergic receptor density was reduced without changes in receptor binding affinity. The cardiac β-adrenergic receptor signal transduction pathway function was impaired at the receptor, G-protein, and adenylyl cyclase levels as manifested by diminished cAMP generation when stimulated by isoproterenol, fluoride ion, and forskolin. The expression of G-protein was altered with a decrease in Gsα and Gi2α levels. However, no changes were observed in Gcommonβ expression.

The present data also demonstrated that cardiac plasma membranes became more rigid in BDL rats compared with Sham rats, which apparently was caused by an increase in the membrane cholesterol/phospholipid ratio. By fluidizing the membrane to sham control levels with A_2C, the production of cAMP stimulated at the receptor level could be restored. However, the fluidization of cardiac plasma membrane had no effects on cAMP production when stimulated at either G-protein or adenylyl cyclase levels. Taken together, the above results suggest that the coupling process between the β-adrenergic receptor and G-protein is fluidity dependent.

Impairment of the β-adrenergic receptor signal transduction pathway function, alterations in G-protein expression, and changes in the membrane physical properties may play crucial roles in the pathogenesis of cirrhotic cardiomyopathy.

CHAPTER EIGHT

FUTURE PERSPECTIVES

Cirrhotic cardiomyopathy is a complex clinical situation and many questions remain to be answered. Based on the present investigation, following studies can be performed to further clarify the underlying mechanisms which lead to blunted cardiac contraction in cirrhosis.

1) Stimulatory G-protein function study:

In the present research, it was demonstrated that cAMP production was decreased when stimulated at G-protein level associated with a decrease in Gsα expression. Recent stoichiometric studies have demonstrated that in β-adrenergic receptor signal transduction pathway, G-proteins are in large excess relative to β-adrenergic receptor and adenylyl cyclase contents. Therefore, a moderate decrease in Gs-protein expression presented in this dissertation cannot explain the entire picture of diminished cAMP generation stimulated at G-protein level. It is possible that Gs-protein function is impaired in cirrhotic cardiomyopathy. In order to approve this hypothesis, GTPase activity in cardiac plasma membrane can be detected. Cardiac plasma membrane will be incubated with [γ-^{32}P]GTP for 20 min at 25°C. The intrinsic GTPase of Gsα in the membrane hydrolizes [γ-^{32}P]GTP to produce GDP and ^{32}P. The incubation mixture will be centrifuged at 25,000 g for 20 min to precipitate the membranes. GDP associated with G-protein stays in the membranes. Supernatant containing ^{32}P will be counted and GTPase activity is evaluated (Sun *et al.*, 1995).

2) Assessment of adenylyl cyclase quantity:

One of the important findings in the present study was that cAMP production was decreased when stimulated at adenylyl cyclase level. But it is not known if this decrement is due to the functional impairment of adenylyl cyclase or due to a reduction in adenylyl cyclase content. It has been demonstrated in stoichiometric studies that adenylyl cyclase number is crucial in β-adrenergic receptor signal transduction. Therefore, quantification of adenylyl cyclase content in cirrhotic cardiomyopathy is warranted. This can be done by a binding assay using ^{3}H-labelled forskolin as a radioligand (Post *et al.*, 1995).

3) cAMP dependent protein kinase (PKA) function study:

cAMP dependent protein kinase is another important component in β-adrenergic receptor signal transduction pathway. No studies have been performed to investigate PKA function in cirrhotic cardiomyopathy thus far. It is possible that impairment of PKA function plays an important role in cirrhotic cardiomyopathy. To approve this hypothesis, PKA function will be determined by following phosphorylation experiments. Supernatant from heart ventricle homogenate containing PKA will be added to a reaction mixture with histone and [γ-^{32}P]ATP, in the presence or in the absence of cAMP. cAMP dependent protein kinase catalyzes transfer of the terminal phosphate from [γ-^{32}P]ATP to histone. The amount of histone phosphorylated in the presence cAMP minus the amount in the absence of cAMP represents PKA activity (Schaffer *et al.*, 1991).

4) Evaluation of calcium transients:

In the present study, assessment of β-adrenergic receptor signal transduction pathway function was focused on cAMP measurements. The next messenger after cAMP production in this signal transduction pathway is Ca^{2+}. Calcium transits during β-adrenergic receptor stimulation are crucial in the signal transduction. Measurement of calcium movement will help to illustrate the mechanisms whereby cardiac contractile function becomes blunted.

Two series of experiments can be performed. First, it is necessary to determine if calcium influx is abnormal. Patch clamp is the best method to test calcium influx via L-type calcium channels on cardiac plasma membranes. After Ca^{+2} currents are measured at baseline, Ca^{2+} influx under isoproterenol stimulation will be further determined. If Ca^{2+} influx is found to be decreased under isoproterenol stimulation, experiments will be performed to determine if reduced Ca^{2+} currents are caused by decreased cAMP production. This can be testified by superfusing cardiac cells with membrane permeable cAMP analogue, 8-br-cAMP. It also can be done by dialysis of cardiomyocyte with cAMP through patch clamp pipette. These experiments will verify if reduced cAMP production is responsible for proposed alteration in Ca^{2+} influx via L-type calcium channels.

The second series of experiments involve measurement of calcium induced calcium release from sarcoplasmic reticulum (SR). Following Ca^{2+} influx by L-type calcium channels into cytosol, the influxed Ca^{2+} ions bind to ryanodine receptors on SR membranes to induce calcium release. This process is termed calcium induced

calcium release (CICR). It has been demonstrated that influxed Ca^{2+} accounts for 10% and Ca^{2+} released from SR accounts for 90% of total cytosolic free Ca^{2+} which trigger cardiomyocyte contraction. It is hypothesized that CICR is abnormal in cirrhotic cardiomyopathy. Impairment of CICR leads to blunted cardiac contractile responses. This hypothesis can be tested by a fluorescent polarization method. Briefly, fluorescent probes, Indo 1, will be loaded into isolated ventricular cells through a patch clamp pipette. Ventricular cells are illuminated at a wavelength of 630 nm. Indo 1 bounded with Ca^{2+} released from SR emits fluorescent light at wavelength 410 nm, while free Indo 1 emits fluorescent light at a wavelength of 500 nm. Therefore, the ratio of fluorescent intensity at wavelength of 410 to 500 nm (410/500) will increase after cell excitation. This measurement represents Ca^{2+} released from SR (Bouchard *et al.*, 1995).

CHAPTER NINE

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