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Muscarinic Receptor Function and Characteristics in Cirrhotic Cardiomyopathy

by

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The undersigned certify that they have read, and recommed to the Faculty of Graduate Studies for acceptance, a thesis entitled "Muscarinic Receptor Function and Characteristics in Cirrhotic Cardiomyopathy" submitted by Debbie N. Jaue in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Cirrhotic cardiomyopathy is characterized by blunted contractile responsiveness. Ventricular contractility is dependent on the interplay of stimulatory β-adrenergic and inhibitory muscarinic (M_2) receptors, we aimed to clarify a possible role for M_2 receptor overactivity in the pathogenesis of cirrhotic cardiomyopathy in rats.

Cirrhosis was induced by chronic bile duct ligation of 4-wk duration, while controls underwent sham operations. Cardiac contractile performance was assessed in pithed rats and left ventricular papillary muscles. M_2 receptor characteristics were studied using 1-[N-methyl-^{3}H]scopolamine as a radioligand. The $+dP/dt_{max}$ and $-dP/dt_{max}$ responses to carbachol were blunted in the cirrhotic rats. Papillary muscle contractile responses to carbachol stimulation were significantly reduced in cirrhotic rats. M_2 receptor density and binding affinity were not significantly different between the two groups. Carbachol inhibited adenylyl cyclase activity was significantly lower in cirrhotic rats than controls.

This study demonstrates that muscarinic responsiveness is blunted in cirrhotic hearts, and this is not due to receptor downregulation. These changes in M_2 function are likely compensatory and M_2 receptor overactivity is not involved in the genesis of cirrhotic cardiomyopathy.

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Ach	acetylcholine
ATP	adenosine-5'-triphosphate
AV	atrioventricular valves
βARK	β-adrenergic receptor kinase
BDL	bile duct ligated cirrhotic rats
B_{max}	receptor density
cAMP	3'5'-cyclic adenosine monophosphate
CCl_4	carbon tetrachloride
ChAT	choline acetyltransferase
DAG	diacylglycerol
$+dP/dt_{max}$	first time derivative of left ventricular pressure rise
$-dP/dt_{max}$	first time derivative of left ventricular pressure decline
G-protein	guanine nucleotide binding protein
GDP	guanine diphosphate
GTP	guanine triphosphate
HR	heart rate
IP_3	1'4'5'-inositol triphosphate
K_d	receptor dissociation constant
LVET	left ventricular ejection time
LVSP	left ventricular systolic pressure
nM	nanomole

QS_2	total mechanical systole
PEP	pre-ejection period
PKA	cAMP dependent protein kinase
PKC	protein kinase C
Sham	sham-operated rats
SR	sarcoplasmic reticulum

INTRODUCTION

The aim of the present investigation was to clarify the possible role of muscarinic cholinergic receptors in the pathogenesis of cirrhotic cardiomyopathy. The research was focused on the effect of muscarinic agonists on cardiac contractile function, muscarinic cholinergic receptors and its signaling pathway function. Therefore, a brief literature review of liver cirrhosis, cardiovascular complications in cirrhosis, and muscarinic cholinergic receptor signal transduction will set the stage for further description of the project.

1.1. THE LIVER

The liver is the largest gland in the human body, weighing approximately 1200-1500 g (Sherlock, 1993; Conn and Allerbury, 1993). The liver functions both as an exocrine gland, secreting bile and an endocrine gland, synthesizing a variety of substances that are released into the bloodstream. The liver has a dual blood supply. The portal vein delivers venous blood from the intestines and spleen while the hepatic artery supplies the liver with oxygenated arterial blood. Venous blood exits the liver through the right and left hepatic veins. The hepatic veins emerge from the liver and enter the inferior vena cava near the right atrium. Thus the liver is positioned between the intestinal tract and the general venous circulation. All the material absorbed from the intestinal tract enters the liver via the hepatic system. Absorbed products can be metabolized in the liver

or transformed there and returned to the circulation for storage or utilization elsewhere. The liver also acts to cleanse the blood of toxic substances through oxidation, hydroxylation or conjugation reactions. The liver synthesizes a number of very important protein components of blood plasma and maintains tight control over the amount of glucose in the blood by altering the rate of glycogen synthesis or breakdown.

1.2. THE HEART

The four chambered mammalian heart is a complex pump that moves blood through the vascular system. The left heart chambers maintain circulation through the systemic vascular bed, while the chambers on the right perfuse the pulmonary vessels. The atria are thin-walled, low pressure chambers that function like large reservoirs of blood. The ventricles are formed by a continuum of muscle fibers that originate from fibrous skeleton at the base of the heart (Berne & Levy, 1992). Cardiac valves are responsible for unidirectional flow of blood through the heart. There are two types of valves in the heart - the atrioventricular valves (AV) and the semilunar valves. The AV valves are located between the atrium and ventricles while the semilunar valves are located between the ventricles and the greater arteries.

The sympathetic and parasympathetic divisions of the autonomic nervous system are the major extrinsic systems that regulate the heart (Lindemann *et al.*, 1989). Cell bodies of sympathetic preganglionic axons are located in the

interomediolateral columns of the upper thoracic cord. Postganglionic sympathetic fibers originate in the superior cervical, middle cervical, and stellate ganglia or cognate structure, but there are considerable variations among species. Postganglionic sympathetic nerves that innervate the heart release norepinephrine. Norepinephrine interacts with postsynaptic cardiac α- and β-adrenergic receptors. In general, an increase in sympathetic nerve activity increases heart rate, conduction velocity and myocardial contractility (Lindemann *et al.*, 1989). Preganglionic parasympathetic neurons that supply the heart originate mainly from the dorsal motor nucleus and the nucleus ambiguous and travel in the vagus nerve. Postganglionic parasympathetic nerves release acetylcholine that activates postsynaptic cardiac muscarinic cholinergic receptors. Activation of cardiac muscarinic cholinergic receptors regulates pacemaker activity, atrioventricular conduction, and force of contraction. Cardiac sympathetic and parasympathetic fibers innervate atrial muscle, ventricular muscle, sinoatrial node, atrioventricular node, and intraventricular conducting fibers. Neural density is greater in atrial than ventricular tissue (Milnor, 1990).

1.3. INTRODUCTION TO LIVER CIRRHOSIS

Cirrhosis is a common disease of the liver (Sherlock, 1993, Conn and Allerbury, 1993). Cirrhosis is defined as a chronic disease of the liver in which diffuse destruction and regeneration of hepatic parenchymal cells has occurred. Laennec was the first to describe the pathologic features of cirrhosis in 1826 and

since then liver cirrhosis has often been referred to as Laennec's cirrhosis (Conn and Allerbury, 1993). Liver cirrhosis can be caused by several pathogenic factors. Common causes are viral hepatitis B, hepatitis C and alcohol abuse. Some medications and toxins can also inflict hepatic injury resulting in liver cirrhosis.

The obstruction of hepatocytes seen during cirrhosis leads to a diffuse increase in connective tissue resulting in disorganization of hepatic lobular and vascular architecture (Conn and Allerbury, 1993). These pathological changes usually involve the entire liver (Poulsen, 1987). Due to the disturbances of normal hepatic architecture, there is a reduction of functional hepatocyte mass and portal hypertension. The disease has a long latent period, with the appearance of abdominal swelling and pain, indigestion, fatigue, edema, and jaundice. In advanced cirrhosis, ascites, jaundice, portal hypertension, hepatic coma or hepatic encephalophathy may develop. Kidney function is also impaired in cirrhosis, which manifests as sodium and water retention.

As well as the clinical features, there are also two major cardiovascular complications in liver cirrhosis: hyperdynamic circulation and impaired cardiac contractile function. Hyperdynamic circulation also exists in portal hypertension without cirrhosis. The characteristics of hyperdynamic circulation in liver cirrhosis are that cardiac output is increased at baseline; decreased peripheral vascular resistance; and decreased arterial blood pressure (Abelmann, 1994; Abelmann *et al.*, 1955; Kontos *et al.*, 1964). Curiously, despite the

hyperdynamic circulation, it appears that some vascular beds behave as if there is insufficient blood flow. For example, the kidneys retain salt and water, eventually leading to the development of ascites. In addition, some cardiovascular reflexes are also reported to be abnormal, i.e. increased splanchnic blood flow after meals, and responses to exercise.

The appearance of hyperdynamic circulation is variable in humans, due to the heterogeneity of the disease (Murray *et al.*, 1958). However, circulatory changes develop in more than 80% of cirrhotic animals (Fernandez *et al.*, 1985). At baseline, cardiac contractile function is hyperdynamic, and when stressed by different physiological or pharmacological stimuli it is clearly abnormal. Decreased positive inotropic (contractile force) and chronotropic (heart rate) responses to β-adrenergic receptor stimulation can be seen in both human patients with cirrhosis and animal models of cirrhosis (reviewed in Lee, 1989, Ramond *et al.*, 1986). This blunted response has been termed cirrhotic cardiomyopathy (Lee, 1989; Rapaport, 1989).

1.4. Cardiomyopathy in liver cirrhosis

1.4.1. Cirrhotic cardiomyopathy in patients

Studies have documented an impairment in cardiac contractile function in liver cirrhosis (Lee, 1989). In 1953, Kowalski and Abelmann first reported hyperdynamic circulation at baseline. One third of the cirrhotic subjects they examined were found to have elevated cardiac output at baseline (Kowalski and

Abelmann, 1953). Numerous studies since then have confirmed Kowalski and Abelmann's original observations about basal cardiac output.

1.4.1.1. *Impaired cardiac performance under physical stress*

During exercise a series of physiological changes occur that increase oxygen delivery to exercising muscles. An important determinant of exercise capacity is the ability of the cardiovascular system to deliver oxygen to particular organs. One of the responses of the heart to the increased demand for oxygen is to increase cardiac output. During cirrhosis, the heart cannot pump enough blood to meet the body's requirement due to the impairment of heart function. As a result symptoms of heart failure occur when the heart is exposed to exercise or stress. A recent study by Grose *et al.* looked at the effect of exercise on cirrhotic patients. When cirrhotic patients were exposed to maximal exercise, their cardiac output was subnormal. Cardiac output in cirrhotic patients increased by only 97% (Grose *et al.*, 1995) while in normal subjects cardiac output in response to maximal exercise increases by about 300% or more (Vander *et al.*, 1990). Cardiac end systolic and diastolic volumes were also significantly increased in cirrhotic patients indicating the presence of cirrhotic cardiomyopathy (Grose *et al.*, 1995). Another study, which examined the effects of tilting on cardiovascular responses, found tilting induced tachycardia in normal subjects (Bernardi *et al.*, 1983). In contrast, tilting did not induce tachycardia in cirrhotic patients. The cardiovascular system is also stimulated by eating. In

1988, Lee demonstrated that eating decreases rather than increases cardiac output in cirrhotic patients (Lee *et al.*, 1988). More over, Lunzer and colleagues demonstrated that in cirrhotic patients heart rate and forearm blood flow responses were impaired to the Valsalva maneuver, ice cold stimulation on the forehead and stressful mental arithmetic (Lunzer *et al.*, 1975).

Systolic time intervals have been used as a non-invasive tool for measuring ventricular contractile function. Systolic time intervals are determined from simultaneous fast-speed electrocardiogram and phonocardiogram recordings and carotid arterial-pulse tracing (Weissler, 1977). Three important parameters of the phases of systole can be determined from systolic time intervals. They are total mechanical systole (QS_2), left ventricular ejection time(LVET), and pre-ejection period (PEP). As left ventricular performance diminishes, a distinct pattern of interval change occurs: pre-ejection period lengthens, and left ventricular ejection time shortens while total mechanical systole remains unchanged. Pre-ejection period divided by left ventricular ejection time (PEP/LVET) is an important parameter for the determination of left ventricular function. When PEP/LVET increases to 0.44 or greater, this indicates the presence of diminished left ventricular performance.

Kelbaek and colleagues investigated cardiac performance in patients with alcoholic cirrhosis using echocardiograms and radionuclide angiocardiography. At rest no significant differences were found between control and cirrhotic subjects, except that heart rate was elevated in cirrhotic patients. During

exercise, radionuclide angiocardiography demonstrated that left ventricular ejection fraction increased significantly in both groups. However, cirrhotic patients had significantly lower LVEF when compared to controls (Kelbaek *et al.*, 1984). These results suggested impaired cardiac performance in patients with alcoholic cirrhosis.

Bernardi *et al.* studied systolic-time intervals in 22 cirrhotic patients and found that electromechanical systole, pre-ejection period, and PEP/LVET were prolonged at baseline when compared with controls ($p<0.05$). During exercise, most of the systolic interval shortened but the decrease in PEP and PEP/LVET was less in cirrhotic patients than controls (Bernardi *et al.*, 1991). Once again, the results suggested that left ventricular performance is impaired in cirrhotic patients.

Finucci and colleagues used doppler echocardiogram as a tool to study left ventricular diastolic filling dynamics (Finucci *et al.*, 1996). The results from their experiments demonstrated that cirrhotic patients had an impaired left ventricular diastolic filling in the presence of high stroke volume, and is characterized by increased late diastolic flow velocity, decreased E/A ratio, and delayed early diastolic transmitral filling (Finucci *et al.*, 1996)

1.4.1.2. *Impaired cardiac performance under pharmacological stimulation*

Stimulation of the heart by the β-adrenergic agonist isoproterenol stimulates the heart causing an increase in heart rate (positive chronotropic

response) and force of contraction (positive inotropic response). However, many studies on cirrhotic patients have demonstrated that both positive inotropic and chronotropic responses to isoproterenol stimulation are blunted. In one study, cirrhotic patients were found to have increased cardiac output (9.7 L/min) and decreased systemic vascular resistance (825 dynes-sec-cm^{-5}) at baseline. When angiotension was infused to increase diastolic arterial blood pressure by 20 mmHg, they found that pulmonary wedge pressure (a reflection of left ventricular filling pressure) increased, but cardiac output did not change (Limas *et al.*, 1972). The absence of a change in ventricular output despite an increase in filling pressure, suggests an abnormality of cardiac contractile function. In another study where norepinephrine was infused into cirrhotic patients, the mean % change in forearm blood flow was significantly lower in cirrhotic patients than control subjects, again demonstrating a defect in cardiac output in cirrhotic patients (Lunzer *et al.*, 1975).

Positive chronotropic responses to β adrenergic agonist stimulation were also diminished during cirrhosis (Ramond *et al.*,1986). Cirrhotic patients given isoproterenol, required a much higher dose of the agonist to increase the heart rate by 25 beats per minute than control subjects.

1.4.2. Cardiomyopathy in cirrhotic animal models

Cardiomyopathy has also been documented in animal models of cirrhosis. Ingles and colleagues performed a study on cardiac contractile function in the

CCl_4-induced cirrhotic rat. At baseline cardiac output was significantly higher in cirrhotic animals than controls. However, in control rats, increases in right atrial pressure were accompanied by an elevation in cardiac output. In contrast, increases in right atrial pressure were at accompanied by an increase than a dramatic decrease in cardiac output. This study demonstrated that the cardiac preload reserve in cirrhosis is limited. The results also suggested that when the cirrhotic heart requires increased cardiac performance, heart failure results, since in normal healthy rats, there is a parallel increase in cardiac output when blood volume is increased (Ingles *et al.*, 1991). Cardiac function has also been tested in common bile duct-ligated cirrhotic rats by isoproterenol infusion (Lee *et al.*, 1990). A much higher dose of isoproterenol was required to increase heart rate by 50 beats per min in cirrhotic animals than controls. The chronotropic response in cirrhotic rats was 30% lower than controls. When Caramelo and colleagues examined the effect of volume expansion on cardiac output in conscious CCl_4-induced cirrhotic rats, isotonic saline infusion was demonstrated to cause an increase in cardiac output in control rats but in cirrhotic rats cardiac output decreased by 50% (Caramelo *et al.*, 1986).

In other laboratories the effect of isoproterenol stimulation on chronotropic and inotropic responses in portal hypertensive rats (due to prehepatic portal vein stenosis) was investigated. The response of cardiac strips to isoproterenol stimulation was found to be blunted in portal hypertensive rats (Batterbee *et al.*, 1992).

1.4.3. Cardiac histology in cirrhosis

Lunseth and colleagues performed a large histological study on heart tissues from patients with portal cirrhosis. Of one hundred and eight patients autopsied, 37 patients had no historical or pathological evidence of hypertension, valvular disease or artherosclerotic heart disease. Out of these 37 patients, 12 cases exhibited cardiac hypertrophy. Cardiac hypertrophy seems to occur in early and moderate stage of cirrhosis and could be due to chronic hemodynamic overload of the heart because of the hyperdynamic circulation. The pathology of the 12 cases displayed considerable dilation of the ventricles, especially the right. Cardiomyocytes were swollen, myocardial fibrosis, scarring, exudation, nuclear vacuolation and unusual pigmentation were present (Lunseth *et al.*, 1958). From his results Lunseth concluded that cirrhotic patients suffered from idiopathic cardiomyopathy. Other studies have reported similar results (Hall *et al.*, 1953 & Loyke, 1955).

1.4.3. Mechanisms of cirrhotic cardiomyopathy

The pathogenesis of cirrhotic cardiomyopathy is unclear. Much of the research has focused on β-adrenergic receptors density and binding affinity. 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). Lee

et al (1990) demonstrated that β-adrenergic receptor density is decreased with no change in binding affinity in cardiac plasma membrane of a biliary cirrohtic rat model. It was also demonstrated in Lee's investigation that β-adrenergic receptors were desensitized *in vivo.* Other studies reported that sarcolemmal plasma membranes in cirrhotic rats were more rigid than controls, β-adrenergic receptor density was reduced by 21%, and cAMP generation was reduced by 37% (Ma *et al.*, 1994). Zavecz and colleagues reported that the coupling between the β-adrenergic receptor and its agonists appear to be impaired as well since a 3-fold greater concentration of isoproterenol was required to have the same contractile responses in portal hypertensive rats than controls (Zavecz *et al.*, 1995). Contractile responses to isoproterenol are also blunted in papillary muscles of cirrhotic rats (Ma *et al.*, 1996).

1.4.4. Summary of cirrhotic cardiomyopathy

The evidence on cardiac function in liver cirrhosis has demonstrated that cardiac contractile function is impaired, but the mechanisms behind the impairment remain unclear. The heart at baseline appears to be hyperdynamic however, when the heart is exposed to physical exercise, mental stress, blood volume expansion or sympathetic stimulation, cardiac performance is abnormal. The pathogenesis of cirrhotic cardiomyopathy is unclear but it does appear that β-adrenergic receptors are involved.

1.5. SIGNAL TRANSDUCTION

Cell systems communicate with one another and respond to demands of the organism through messages transmitted via the nervous and circulatory systems. Ligands are sent as signals from one group of cells and are received by receptors on another group of responding cells. Once the signal ligand and the receptor interact, the message is transduced by secondary signaling systems into the interior of the responding cell (Figure 1). The second messenger hypothesis of hormone action was first proposed by Sutherland and Rall in 1957 with the discovery of 3',5' cyclic adenosine monophosphate (cAMP).

Cardiac function is regulated by the sympathetic and parasympathetic branches of the autonomic nervous system and occurs predominantly through β_1- and β_2-adrenergic and muscarinic cholinergic receptors. Most of the cardiac plasma membrane receptors are coupled to sarcolemmal G-proteins, which in turn interact with intracellular effector proteins to produce the cellular physiological response. G-protein-regulated signaling systems known to affect cardiac cellular responses include the enzymes adenylate cyclase, which produces the second messenger cAMP, and phospholipase C, which produces second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (Fleming *et al.*, 1992).

1.5.1. β-adrenergic receptors

Two types of adrenergic receptors were described by Alhquist in 1948, α and β-adrenergic receptors. α-adrenergic receptors stimulate vaso-constriction, pupil dilation and uterus contraction (Alhquist, 1948). β-adrenergic receptor stimulation evokes vasodilation and inhibition of uterine contraction. β-adrenoceptors also stimulate heart contraction (Alhquist, 1948). Two major subtypes of β-adreneoceptors are involved in cardiac contraction, β_1-and β_2-adrenergic receptors. In general, in normal heart tissue, β_1-adrenergic receptors account for 80% of the total β-receptor population, whereas β_2-receptors comprise 20% of all β-receptors (Brodde, 1986). Both β_1 and β_2-adrenergic receptors participate in the positive cardiac contractile responses (Brodde *et al.*, 1989). β_2-adrenergic receptors are coupled to adenylyl cyclase more efficiently that β_1-adrenergic receptors (Brodde *et al*, 1984). The structure of the β-adrenergic receptors are similar to other G-protein coupled receptors. It is composed of seven putative transmembrane domains with three extracellular and three intracellular loops. The amino terminus (NH_2) is located on the extracellular side while the carboxyl terminus (COOH) is located on the intracellular side. The intracellular half of the seven transmembrane domains are highly conserved in the β-adrenergic receptor.

1.5.2. Muscarinic cholinergic receptors

In 1951, Riker and Wescoe discovered that muscarinic receptors did not form a homogenous class. However, it was not till the 1980's that the different subtypes of muscarinic receptors were defined. It was first thought that there were only two subtypes, M_1 and M_2, based on their affinity for pirenzepine, an antiulcer drug. Pirenzepine has almost a one hundred-fold lower affinity for cardiac muscarinic receptors than for brain muscarinic receptors. With the introduction of selective antagonists and molecular techniques it became possible to further subdivide muscarinic receptors. Five functional muscarinic receptor subtypes have been discovered with substantial differences in amino acid sequence (reviewed by Hulme *et al.*, 1990; Peralta *et al.*, 1987). All muscarinic receptors share extensive structural homology with other G-protein-coupled receptors. The molecular structure of muscarinic cholinergic receptors is characterized by seven transmembrane domains which are connected by three extracellular and three intracellular loops. The NH_2 terminus is thought to be located extracellularly, while the COOH terminus is thought to protrude into the cytoplasm. The different muscarinic receptor subtypes are well conserved across various mammalian species (Wess *et al.*, 1995; Bonner, 1989). The M_1-M_5 subtypes are also quite similar to each other, with about 145 invariant shared amino acid residues (Wess, 1993). Transmembrane segments two to seven contain the highest degree of sequence homology, in contrast to the third

cytoplasmic loop where virtually no sequence similarity exists (Wess, 1993). Although the cytoplasmic loops for each receptor subtype are different from each other, receptor subtypes that couple to the same signal transduction pathways have greater similarity (i.e., M_1, M_3, and M_5 are more similar to each other than M_2 and M_4, and vice versa). Muscarinic receptors, M_1, M_3, and M_5 are grouped together since they function to mobilize intracellular calcium by activating multiple signaling effectors simultaneously. The M_2 and M_4 subtypes have been shown to augment phospholipase A2 as well as inhibit adenylate cyclase (Felder, 1995).

Small molecule agonists and antagonists bind to a hydrophobic pocket buried in the transmembrane core of the receptor (Strader *et al.*, 1995). It has been proposed that an Asp residue on the third transmembrane helix is involved in the binding of muscarinic agonists. When Asp was replaced with Asn, it resulted in a mutant receptor with reduced ligand binding affinities (Fraser *et al.*, 1989). All muscarinic receptors also contain a series of conserved Ser, Thr, and Tyr residues that are not found in other G-protein coupled receptors. When these conserved residues were replaced by Ala (for Ser or Thr) and Phe (for Tyr), six of the nine mutant receptors showed reduced binding affinities for acetylcholine and carbachol (Wess, 1991). Evidence to date suggests the acetylcholine binding domain on the muscarinic receptor protein is associated with the Asp residue on the third transmembrane segment and Thr and Tyr residues.

Studies on chimeric receptors and various deletion mutants of muscarinic receptor subtypes suggest that the major site for the interaction of the muscarinic receptor with G protein is contained in the cytoplasmic domains. It appears the NH_2 terminal region of the third cytoplasmic loop contains the crucial determinants allowing the receptor to recognize G proteins (Hosey, 1992). When 12 amino acids of this region in the M_1 muscarinic receptor are replaced with the turkey β-adrenergic receptor sequence, it resulted in the muscarinic receptor producing a β-adrenergic response (they stimulated adenylyl cyclase via G_s) (Wong *et al.*, 1990). In another study with chimeras of M_2 and M_3 muscarinic receptors, when small segments of the NH_2 terminal region were transferred, a M_2 muscarinic receptor begins to signal like a M_3 muscarinic receptor and vice versa (Lechleiter *et al.*, 1990).

Muscarinic cholinergic receptors mediate parasympathetic control of the heart. The only muscarinic cholinergic protein detectable on mammalian heart is the M_2 receptor (Caulfield, 1993). Muscarinic regulation of atria and ventricles is different. For example, muscarinic agonists have powerful direct negative chronotropic effects on atrial myocardium and sinoatrial and atrioventricular nodal tissues and a direct negative inotropic effect on atrial myocardium. Muscarinic agonists have minimal direct negative chronotropic effects on ventricular myocardium but they can indirectly modify ventricular myocardial contractility. The primary effect of muscarinic stimulation in ventricular

myocardium is to oppose β-adrenergic stimulation (decrease heart rate and force of contraction).

Acetylcholine (Ach) is the neurotransmitter released from parasympathetic nerves. Acetylcholine is synthesized from the enzymatic transfer of the acetyl moiety from acetyl coenzyme A to choline: choline + acetyl-CoA $\leftrightarrow$ Ach + CoA. Acetylcholine synthesis is catalyzed by choline acetyltransferase (ChAT) in cholinergic neurons but in myocardial cells carnitine acetyltransferase is the enzyme that acetylates choline, but at a much slower rate than ChAT. Acetylcholine is degraded by myocardial cells via acetylcholinesterase (Loffelhotz and Pappano, 1985).

1.5.3. G-proteins

Heterotrimeric GTP-binding proteins are a critical component of muscarinic and adrenergic signal transduction pathways. They carry information from the cell surface to the appropriate cellular effector system. In the heart several different types of G-proteins have been identified. β-adrenoceptors are coupled to a stimulatory G-proteins (G_s) while muscarinic cholinergic receptors are coupled to an inhibitory G-protein (G_i) and G-other (G_o). G_s stimulates adenylate cyclase and the production of the second messenger cAMP. G_s also mediates the β-adrenergic modulation of voltage dependent ion channels (Brown *et al.*, 1990; Wickman *et al.*, 1995). G_i mediates inhibition of β-adrenergic stimulated adenylate cyclase and directly couples muscarinic receptors to atrial

and pacemaker cell K^+ channel activation (in Fleming *et al.*, 1992). G_o is also present in cardiovascular tissue but its exact role in the heart is unclear. G_q is also found in the heart and appears to be involved in muscarinic and α-adrenergic stimulation of phospholipase C and A_2 (in Fleming *et al.*, 1992).

Heterotrimeric G-proteins consists of an α-, β-, and γ-subunit (G_α, G_β, and G_γ). The G_α-subunit contains the guanine nucleotide binding site and a specific GTP hydrolytic enzyme (GTPase). G_β and G_γ-subunits are tightly cohesive complexes that interact with G_α-subunits. $G_{\beta\gamma}$-subunits function as a monomer and can only be dissociated under denaturing conditions (Rens-Domiano *et al.*, 1995).

1.5.3.1. *G-protein function*

G-protein mediated signal transduction begins by the activation of a receptor by an specific agonist (Figure 2). The activated receptor interacts with the heterotrimeric G-protein. The interaction of the activated receptor and G-protein leads to the exchange of GDP for GTP on G_α. Once the guanine nucleotides exchange, G_α-GTP will dissociate from $G_{\beta\gamma}$ subunit , as well as from the activated receptor. G_α-GTP is then free to bind and regulate the appropriate effector system. In the case of β-adrenergic receptors, $G_{\alpha s}$ will stimulate adenylate cyclase, converting ATP to the second messenger 3'5'-cyclic adenosine monophosphate (cAMP). $G_{\alpha i}$ on the other hand, will inhibit adenylate cyclase, thus decreasing cAMP levels in the myocyte. Termination of the cycle

occurs when the intrinsic GTPase activity of G_α subunit hydrolyzes the GTP to GDP and G_α-GDP reassociates with $G_{\beta\gamma}$. $G_{\beta\gamma}$ can also directly inhibit adenylyl cyclase by binding to $G_{s\alpha}$-GTP subunit, before it can stimulate adenylyl cyclase. Reassociation of G_α with $G_{\beta\gamma}$ will inactivate G_α. $G_{\beta\gamma}$ subunits are thought to come form G_i and G_o (Felder, 1995)

1.5.4. Adenylyl cyclase

Adenylyl cyclase is composed of 12 trans-membrane domains. Both the amino terminus (NH_2) and carboxyl terminus (COOH) are located on the intracellular side. The first 6 trans-membrane domains and the last 6 trans-membrane domains are connected by an intracellular loop. The intracellular loop and the carboxyl tail are thought to be the major components of the catalytic domain of the enzyme as these sequences are highly conserved among the different types of adenylyl cyclase (Taussig and Gilman, 1995). A total of 8 different adenylyl cyclase isoforms have been described so far, but in the mammalian heart, types 4-7 are predominant (Ishikawa *et al.*, 1994; Taussig and Gilman, 1995). When adenylyl cyclase is activated by $G_{\alpha s}$, ATP in the cytosol is converted to its second messenger, cAMP. An increase in cAMP levels activates cAMP dependent protein kinase A (PKA). cAMP dependent protein kinase then selectively phosphorylates a number of proteins producing the physiological response.

cAMP dependent protein kinase interactions and associated cellular responses include 1) phosphorylation and activation of phosphorylase kinase leading to mobilization of glycogen stores; 2) phosphorylation of troponin I, which decreases the Ca^{2+} affinity of the protein; 3) phosphorylation of myofibrillar C protein; 4) phosphorylation of phospholamban, which regulates Ca^{2+} ATPase in sarcoplasmic reticulum membranes; 5) phosphorylation of voltage-sensitive Ca^{2+} channels for increased Ca^{2+} influx across the sarcolemma; 6) phosphorylation of sarcolemmal Na^{+} channels to effectively close them when the membranes are depolarized (Figure 3).

Catecholamines will increase the developed force by increasing PKA phosphorylation of L-type Ca^{2+} channels and muscarinic agonists will decrease developed force because it decreases PKA phosphorylation (Figure 3). Catecholamines will also increase the speed of contraction since PKA will allow a greater influx of Ca^{2+} into the cell. G_i protein activation inhibits adenylate cyclase, resulting in decreased levels of cAMP and PKA. Therefore, PKA-catalyzed protein phosphorylation will also decrease. With less phosphorylation of voltage sensitive Ca^{2+} channels, there would be decreased Ca^{2+} influx and decreased Ca^{2+} induced Ca^{2+} release. The lower levels of Ca^{2+} would decrease the developed force since force is dependent on intracellular Ca^{2+} levels.

1.5.5. Cellular mechanisms of contraction of cardiomyocytes

The generation of force in cardiac muscle depends on the interaction between the contractile machinery and the intracellular concentration of the messengers which regulate this interaction. The most important intracellular messenger is calcium. The concentration of intracellular calcium depends of the amount of extracellular calcium entering and extruded from the plasma membrane as well as the release or uptake of calcium into the intracellular stores (the sarcoplasmic reticulum). Calcium influx is mediated by voltage sensitive channels on the plasma membrane (Callewaert, 1992). In cardiac cells, L type and T type Ca^{2+} channels have been identified (Nilius *et al.*, 1985; Bean, 1989). In mammalian cardiac cells the contribution of T type channels to total Ca^{2+} influx is relatively small (Callewaert, 1992). On the other hand the L type channel is the major pathway for voltage gated Ca^{2+} entry in mammalian cardiac cells (Callewaert, 1992).

Membrane potential is determined by the ionic concentration gradient across the cell membrane and its relative permeability to ions according to the Goldman equation and is maintained close to the potassium equilibrium potential by the opening and closing of potassium channels. The membrane potential is also affected by the Na^{+}-K^{+} pump and the Na^{+}-Ca^{2+} exchanger, since 3 Na^{+} ions are transported for each Ca^{2+} ion. During the plateau of the action potential, Ca^{2+} permeability increases and Ca^{2+} enters the cell through the voltage

sensitive channel. It is thought that opening of the channel is caused by phosphorylation of the channel proteins by PKA. The amount of extracellular Ca^{2+} that enters the cell is not sufficient to induce contraction of the myofibrils, but it can trigger the intracellular Ca^{2+} stores to release Ca^{2+}. The amount of Ca^{2+} that is released is proportional to the Ca^{2+} content of the sarcoplasmic reticulum and dictates the force of the cardiac contraction. The released Ca^{2+} binds to the protein troponin C. The Ca^{2+}-troponin complex interacts with another protein tropomyosin to uncover active sites for myosin attachment. This allows for cross bridge cycling and hence contraction of the myofibrils.

1.5.6. Cellular mechanisms of relaxation of cardiomyocytes

In cardiac muscles, the process of relaxation is dependent on the mechanisms that remove calcium from the cytosol. Calcium can be eliminated from the cytosol via three pathways. Calcium released from the SR and Ca^{2+} that entered the cell via Ca^{2+} channels can be resequestered by a Ca^{2+} pump on the sarcoplasmic reticulum (ter Keurs, 1993). This Ca^{2+} pump is stimulated by phospholamban following phosphorylation by PKA (Fleming *et al.*, 1992). The remaining Ca^{2+} is probably extruded by the low affinity, high capacity Na^{+}-Ca^{2+} exchanger on the plasma membrane. This system exchanges three Na^{+} ions for each Ca^{2+} ion and the driving force depends on the electrochemical gradients for Na^{+} and Ca^{2+}. Calcium can also be removed from the cell by an electrogenic pump that utilizes energy to transport Ca^{2+} across the plasma membrane. This

Ca^{2+} pump is considered a high affinity, low capacity Ca^{2+} extrusion system. Calcium efflux through the membrane must balance the influx during the action potential.

Cardiac relaxation is accelerated by catecholamine and adenylate cyclase activation and decelerated by muscarinic stimulation. The phosphorylation of troponin I by PKA inhibits the binding of Ca^{2+} to troponin C thus permitting tropomyosin to cover the sites for interaction of actin and myosin increasing the speed of relaxation.

1.5.7. Inositol triphosphate

Muscarinic (M_2) cholinergic receptors can also be coupled to phosphoinositide turnover through a pertussis toxin-insensitive G protein (Robishaw *et al.*, 1989). Effects through the phosphoinositide pathway are positively inotropic and are opposite to the effects that usually occur when muscarinic receptors are stimulated. Stimulation through the phosphoinositol pathway results in the generation of diacylglycerol (DAG) and inositol triphosphate (IP_3) through the hydrolysis of phosphatidylinositol 4,5 bisphosphonate. IP_3 and DAG are known to enhance intracellular levels of Ca^{2+}. IP_3 releases Ca^{2+} from the sarcoplasmic reticulum and DAG stimulates protein kinase C (PKC) to phosphorylate and activate sarcoplasmic Ca^{2+} channels (Figure 3). The physiological significance of the muscarinic-phophoinositide pathway is unclear since high concentrations of agonists are needed to produce

this positive inotropic effect. The concentrations required are far above the physiological range found in the system.

1.5.8. Possible roles of muscarinic receptor dysfunction in cirrhotic cardiomyopathy

The role of muscarinic cholinergic receptor dysfunction has been researched extensively in many different types of heart disease. Reports of muscarinic receptor function in heart disease are conflicting. In spontaneously hypertensive cardiomyopathy, cardiac contractile response to muscarinic stimulation was unchanged but both β-adrenergic and muscarinic receptor numbers were reduced while myocardial $G_{i\alpha}$ was increased (Bohm *et al.*, 1992). In diabetic cardiomyopathy, muscarinic density and affinity were not significantly changed but G_i-protein expression was decreased. Inhibition of isoproterenol induced cAMP production by carbachol was also not altered (Wichelhaus *et al.*, 1994). Chronic hypoxia is characterized by a blunted cardiac contractile response to endogenous and exogenous stimulation. In this model there is an increase in sympathetic tone, which leads to a down regulation of β-adrenergic receptors and a decreased contractile response to β-adrenergic agonists. Muscarinic receptor density was found to be increased however, there was not a change in dissociation constant (Kacimi *et al.*, 1993). In human ventricular tissue from hearts with end-stage heart failure due to idiopathic dilated cardiomyopathy, the negative inotropic effect of the muscarinic agonists

carbachol was unchanged despite an increase in G_i protein (Schmitz *et al.*, 1991; Bohm *et al.*, 1990). β-adrenergic receptor number was reduced but muscarinic receptor numbers were not. In cardiomyopathic hamsters, a defect in the cardiac contractile response to muscarinic and β-adrenergic stimulation has been described. Muscarinic receptor number was found to be decreased by Wilkinson and colleagues (1994) but unchanged by Chidiac and colleagues (1991). Muscarinic regulation of cAMP was also impaired in cardiomyopathic hamsters (Chidiac *et al.*, 1991). Finally, in a model of chronic left ventricular heart failure in the dog, muscarinic receptor density was reported to be decreased by 36% percent with no change in affinity (Vatner *et al.*, 1988). Adenylyl cyclase stimulated activity was inhibited 15 % in normal left ventricles but only 5% in animals suffering from heart failure suggesting the inhibitory pathway regulating left ventricular adenylyl cyclase is defective in heart failure (Vatner *et al.*, 1988).

The dfferent types of heart disease described above suggest that muscarinic dysfunction is dependent on the disease. Therefore, it is possible that in cirrhotic cardiomyopathy, the muscarinic cholinergic receptor signal transduction pathway is altered. Thus far, systematic studies of the muscarinic receptor signalling pathway function in cirrhotic cardiomyopathy are not available.

1.5.9. Summary of cardiac muscarinic cholinergic receptor function

Cardiac contractile function is modulated by stimulatory β-adrenergic receptors and inhibitory muscarinic receptors. β-adrenergic receptors are coupled to G-proteins that stimulate cAMP production while muscarinic receptors are coupled to G-proteins that inhibit cAMP production. An elevation of cAMP will activate cAMP-dependent protein kinase which will phosphorylate a series of proteins resulting in cardiomyocyte contraction. A decrease in cAMP will decrease cAMP-dependent protein kinase phosphorylation. Alterations in muscarinic cholinergic receptor number and activity have been associated with a number of heart diseases. Therefore, it is possible that altered muscarinic receptor function also plays a role in cirrhotic cardiomyopathy.

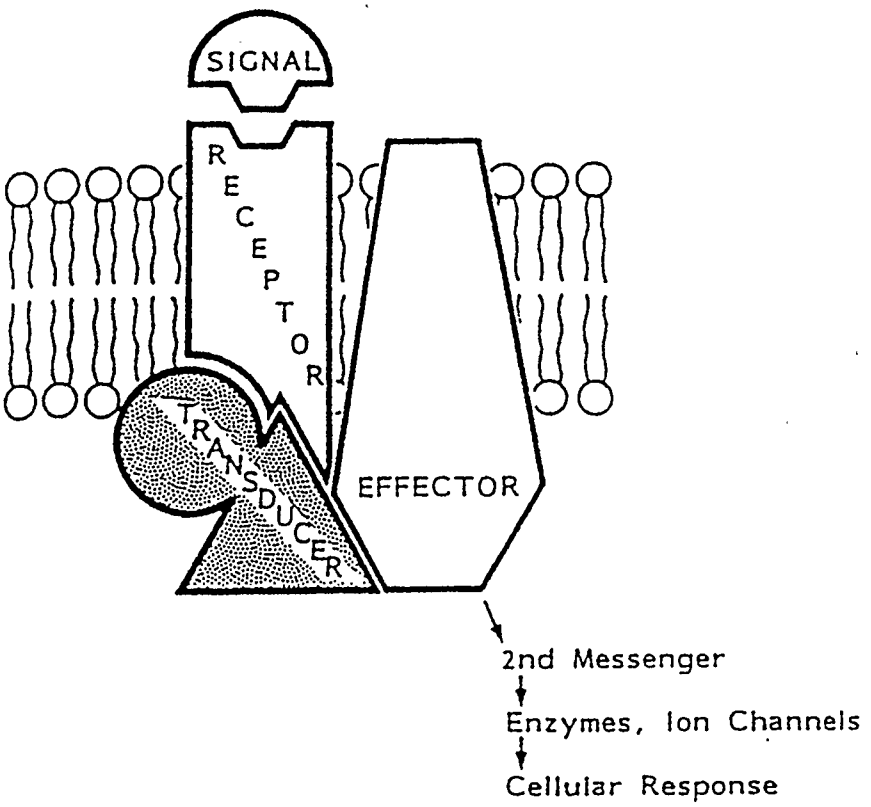

Figure 1. Schematic of signal transduction in cells. A ligand, a signal molecule or hormone, interacts with a receptor molecule, which is often embedded in the cell membrane. The ligand-receptor interaction activates a transducer molecule (exemplified by the G proteins), which, in turn, modulates an effector protein, often an enzyme. The effector may then produce secon-messenger biochemicals that regulate enzymes and ion channels involved in evoking the specialized cellular response.

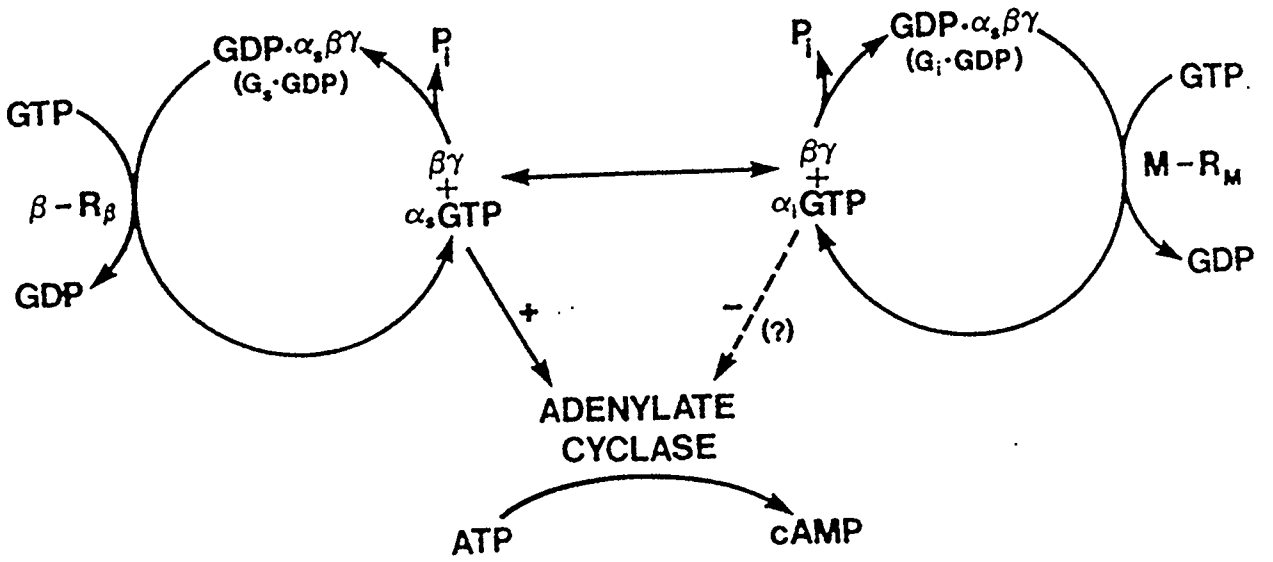

Figure 2. A schematic representation of the mechanism by which G proteins mediate hormonal regulation of adenylate cyclase activity

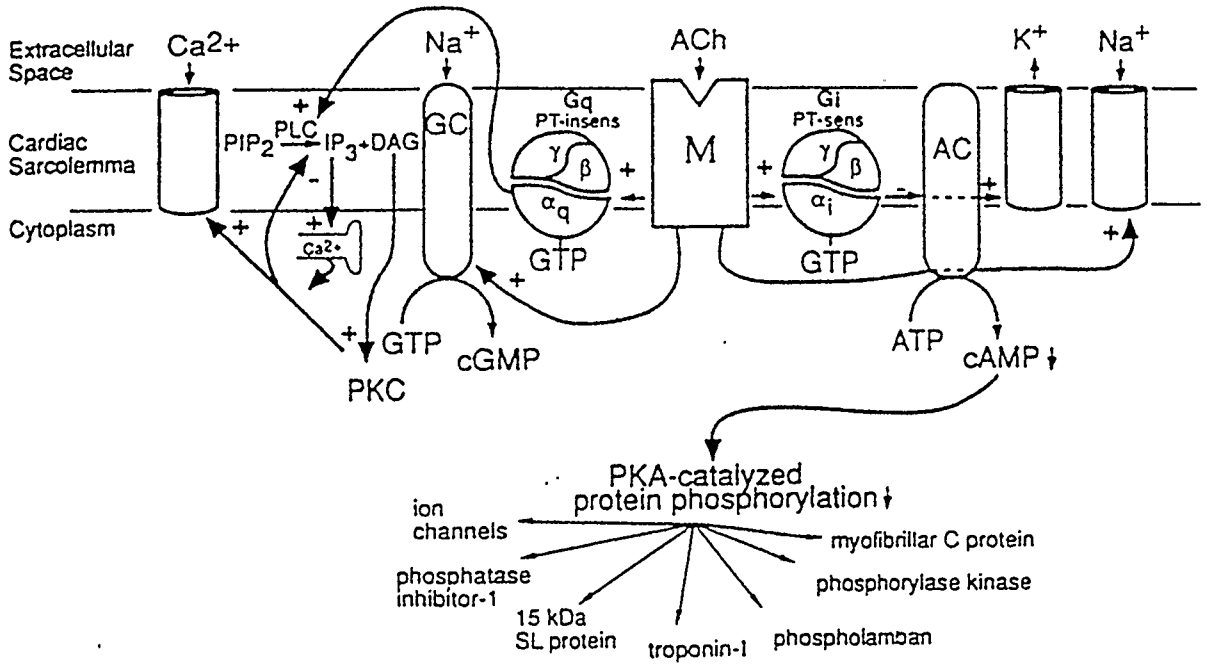

Figure 3. Schematic of muscarinic cholinergic receptor-effector coupling. Acetylcholine (Ach) reacts with a muscarinic receptor (M). The M is coupled to a pertussis toxin insensitive G protein which, in the presence of GTP, dissociates to $G_{q\alpha}$ to stimulate phospholipase C (PLC). This results in the formation of second messengers IP_3 and DAG, which enhance intracellular Ca^{2+} levels. IP_3 and Ca^{2+} from the sarcoplasmic reticulum and DAG stimulates protein kinase C to phosphorylate and activate sarcoplasmic Ca^{2+} channels. M is also coupled to G_i, which in the presence of GTP dissociates its G_i α and $\beta\gamma$ subunits to inhibit adenylate cyclase and subsequent protein kinase A (PKA) phosphorylation of cellular proteins. $G_{i\alpha}$ subunit couples M directly for atrial K^+ channels for their stimulation.

HYPOTHESIS

In cirrhosis cardiac contractile performance is impaired under pharmacological and physiological stimuli. This condition has been termed cirrhotic cardiomyopathy. The pathogenesis for this condition is still unclear.

Cardiac contractile function is regulated by sympathetic and parasympathetic branches of the autonomic nervous system. β-adrenergic and muscarinic cholinergic receptors and their post-receptor signal transduction pathways are the main modulators of cardiac function. The following hypothesis was proposed to clarify the possible role of muscarinic cholinergic receptors in the pathogenesis of cirrhotic cardiomyopathy. Therefore the following hypothesis was tested:

a. Muscarinic cholinergic receptor activity is increased in cirrhotic cardiomyopathy
b. The resulting increase in muscarinic receptor activity in cirrhotic cardiomyopathy will decrease cAMP production leading to decreased contractile function.

To test the above issue, an integrated series of experiments was proposed. Cardiac contractile performance in liver cirrhosis was evaluated by two methods. Myocardial contractility was measured *in situ* using the slope of

the ascending limb of the ventricular pressure curve. The slope indicates the maximum rate of force development by the ventricle ($+dP/dt_{max}$). The slope of the descending limb of the ventricular pressure curve was also measured ($-dP/dt_{max}$) as an indicator of relaxation. Contractility was also measured *in vitro* in isolated left ventricular papillary muscles. Cardiac plasma membrane muscarinic cholinergic receptor density and affinity were also determined by a radioligand binding assay. Finally, muscarinic receptor signal transduction pathway was evaluated by measuring cAMP generation.

AIM

The aim of this thesis is to test the aforementioned hypothesis in cirrhotic cardiomyopathy in a systematic manner, using *in situ* and *in vitro* techniques.

MATERIALS AND METHODS

3.1. CHEMICAL REAGENTS AND ISOTOPES

(-)-Isoproterenol (+)-bistartate salt, carbachol, atropine, forskolin, ATP, GTP and 5' nucleotidase assay kit were purchased from Sigma Chemical Company (St. Louis, USA). Radioligand 1-{n-methyl-^{3}H]scopolamine and cAMP protein binding assay kit were purchased from Amersham Ltd. (Montreal, Quebec, Canada).

3.2. ANIMAL MODEL

Male Sprague-Dawley rats (Bioscience, Calgary, AB, Canada) weighing between 200-250 grams and 350-400 grams were used in this study. The animals were housed in an environmentally controlled vivarium with a 12 hour light/dark cycle. The animals were allowed access to rat chow and water *ad libitium*. The protocol was approved by the University of Calgary Animal Care Committee under the guidelines of the Canadian Council on Animal Care. The animals were divided into two groups. Cirrhosis was induced by ligation of the common bile duct in one group. The other group received a sham operation: they were subjected to all the surgical manipulations except that they did not have their bile ducts ligated. The operation procedure for ligation has been previously described (Kountoutas *et al.*, 1985; Franco *et al.*, 1979). Briefly, under halothane inhalation anesthesia the common bile duct was exposed

through a midline abdominal incision. The common bile duct was doubly ligated with 3-0 silk and sectioned between the ligatures. The incision was then closed with 5-0 silk and Gentamicin was sprayed at the incision site and Penicillin G Benzathine (30,000 IU) was given intramuscularly to prevent infection. The animals were allowed to recover and kept in the vivarium for 4 weeks. Animals that did not show macroscopic and histological changes of cirrhosis were excluded from the final analysis. Cirrhosis was characterized by massive hepatomegaly, splenomegaly, ascites and microscopically by widespread bile ductular proliferation, necrosis and moderate-to-extensive fibrosis.

3.3. TRANSDUCER

Preliminary studies were performed on the fluid filled PE-50 tubing system that was to be measuring the cardiovascular performance in the pithed rat. The fluid filled PE-50 transducer (Viggo-Spectramed, P23XL, Oxnard, California) was compared with the Millar transducer (Millar Inst., SPR-524, Houston Texas). Data was recorded on a VR-16 recorder (Electronics for Medicine/Honeywell, White Plains, NY) and on a Gould chart recorder (Gould Inc., Cleveland, Ohio). Using a custom designed software package (CVSOFT, Odessa Computer System Ltd., Calgary, Alberta) the recorded signals were analyzed on a personal computer (IBM Corporation). Pressure signals were produced by a Multifunction pressure generator WGA-200 (Millar Inst., Houston, Texas).

3.4. PITHED RAT STUDY *IN SITU*

The effects of bolus infusion of muscarinic agonists on the pressor, inotropic and chronotropic responses of the cirrhotic heart was evaluated in the pithed rat preparation as described by Gillespie and Muir (1967). The muscarinic agonists carbachol (Sigma Chemical, St. Louis, MO) was studied. On the day of the experiment, the rat was anesthetized by halothane inhalation. Once the rat was anesthetized, the jugular vein and carotid artery were cannulated and a bilateral vagotomy was performed to block cholinergic activity. Through the carotid artery the left ventricular function and heart rate was recorded via a pressure transducer (Viggo-Spectramed, P23XL, Oxnard, CA) connected to a Gould recorder (Gould Inc., Cleveland, Ohio). The jugular catheter was used for the bolus infusion of the muscarinic agonist. After the cannulation the rat was intubated via tracheotomy and then pithed by passing a stainless steel rod through the right orbit into and down the spinal cord. On completion of the pithing procedure, the animal was maintained by mechanical respiration using a small animal ventilator (Harvard Apparatus, South Natick, MA). Pithing caused vasodilation of all vessels therefore, 1-1.5 mL of saline was injected to increase blood volume. The animal was allowed to stabilize for 30 min. during which the left ventricular pressure and heart rate was continuously monitored. Body temperature was monitored by a rectal probe and controlled by keeping the animal on a warming blanket. During the stabilization period a small

amount of blood was removed for determination of blood pH and gas tensions by a blood gas analyzer (IL-1302, Instrumentation Laboratory). The range of values at which the animal was considered "stable" was pH 7.35-7.45, Po_2 100-120 mmHg and Pco_2 30-35 mmHg. After "stabilization" of the pithed rat, as assessed by the stability of blood pressure, heart rate, and blood pH and gas tensions, increasing doses of the muscarinic agonist (carbachol) was injected into the jugular vein at an injection volume of 100μL over a dose range of 0.9-91.3μg. At the time of injection, the computer system Dataq (Dataq Instrument Inc., Akron, Ohio) recorded the beat-to-beat changes in left ventricular pressure. Dose response curves to the changes in heart rate, left ventricular systolic pressure, maximal time derivative for the development of left ventricular pressure ($+dP/dt_{max}$) (ventricular contraction) and maximal time derivative for the dissipation of left ventricular pressure ($-dP/dt_{max}$) (ventricular relaxation) were constructed. On completion of the recordings, the animals were killed by cervical dislocation. The duration of the experiment was usually between 1.5 and 2 h.

3.5. LEFT VENTRICULAR PAPILLARY MUSCLE CONTRACTILE STUDY

To examine the contractile response to muscarinic agonists in cirrhotic rats, experiments on electrically driven rat papillary muscles were performed. Methods from recently published papers were used in establishing our model of papillary muscle (Bohm *et al.*, 1994; Otani *et al.*, 1988). Rats were sacrificed by

decapitation with a Harvard guillotine. The heart was quickly excised and transferred to an aerated bathing solution for papillary muscle isolation. The bathing solution was a modified Tyrodes solution containing (in mM): NaCl 122.5, KCl 5.4, $CaCl_2$ 1.1, $MgCl_2$ 1.1, $NaHCO_3$ 24, and glucose 10. The solution was continuously gassed with 95% O_2 - 5% CO_2 and maintained at 37°C at a pH of 7.4. The muscles were attached to a bipolar platinum stimulating electrode with 5-0 silk and suspended in 15 ml glass tissue chambers for recording isometric contractions. The force of contraction was measured with a Grass force/displacement transducer (Quincy, MA, USA) attached to a Grass model 75 polygraph recorder (Quincy, MA, USA). All muscles were placed under 0.5 g initial resting tension and electrically paced at 1 Hz with rectangular-wave pulses of 5 ms duration (Grass 88 Stimulator, Quincy, MA). Preliminary studies had shown that at 0.5 g initial tension, maximum contractile force could be obtained. The voltage was about 1.5 fold above threshold (30-40 v). All preparations were allowed to equilibrate in drug-free bathing solution until stabilization (approx. 1.5 hours). After the contractile force reached a steady state, the anti-adrenergic effects of carbachol were studied. Carbachol was applied cumulatively at a concentration range 10^{-6} to 10^{-3} mol/L in the presence of isoproterenol at 10^{-6} mol/L concentration. When a dose reached maximum response, the next higher dose was added. Dose response curves were constructed.

3.6. CARDIAC SARCOLEMMAL PLASMA MEMBRANE PREPARATION

3.6.1. Cardiac plasma membrane isolation

The cardiac sarcolemmal plasma membranes were prepared using a previously reported method (Ma *et al.*, 1994; St. Louis *et al.*, 1976). Briefly, the rats were sacrificed by decapitation with a Harvard Guillotine. Their hearts were rapidly excised and placed in cold 10mM tris(hydroxymethyl)aminomethane.HCl buffer at pH 7.4 (Tris buffer). All procedures were carried out at 4°C. The hearts were trimmed of excess vessels, leaving only the ventricles which were finely minced with scissors. Two ventricles from the same group were pooled and suspended in 10 vol (wt/vol) of the Tris buffer and homogenized using a Brinkmann Polytron PT20ST homogenizer (Brinkmann Instruments, Rexdale, ONT, Canada) and a Polytron PTA 10 ST probe at setting 7. This procedure was repeated twice for 35 seconds. The homogenate was then filtered through 4 layers of Meditron gauze sponges (Meditron Corp. Montreal, Quebec, Canada) pre-wetted with Tris buffer. Potassium chloride (KCl) was added to the filtrate to produce a final concentration of 1.25 M KCl. The filtrate was then placed on ice for 10 min. and centrifuged at 9,000 g for 10 min. The residue was then resuspended in 10 vol of 1.25 M KCl in tris buffer, placed on ice for 10 min., and centrifuged for 10 min. at 4,000 g. The resulting residue was washed twice with Tris buffer and centrifuged for 10 min. at 3,000 g. The residue was then resuspended in 10% sucrose and layered on top of a discontinuous sucrose

gradient (45, 50, 55, and 60%) and centrifuged at 40,000 g for 1 hour. The band between 50 and 55% was taken as the final cardiac plasma membrane preparation. This fraction was washed twice with Tris buffer and centrifuged at 12,000 g for 30 min. between washings. The final pellet was resuspended in the Tris buffer at a protein concentration of 1 mg/ml. Cardiac plasma membranes prepared by this method were stored at -70°C. Studies were carried out within 1 month except for adenosine 3',5'-cyclic monophosphate (cAMP) studies, which were carried out on the same day the plasma membranes were prepared.

3.6.2. Cardiac plasma membrane protein assay

Protein content of the preparations were determined according to the method of Bradford (1976). The core reagent in this assay system is Coomassie brilliant blue. This dye has both red and blue color forms. When the dye combines with proteins, the dye will shift from the red color to the blue color which can be detected at 595 nm with a spectrometer.

3.6.3. Cardiac plasma membrane marker enzyme assay

The cardiac membrane enzyme marker 5'-nucleotidase activity was tested utilizing a previously published method 5'-nucleotidase activity was determined (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), and L-glutamate dehydrogenase (11,000 U/L). The AMP is hydrolyzed

by 5'-nucleotidase to form adenosine and inorganic phosphorus. Adenosine is then 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 is carried out at 30°C with each reaction cuvette containing 1.2 ml of the above reagent and 10 μg of membrane protein. The absorbance values at 5 minutes after the addition of the membranes was recorded as initial A. The absorbance values after another 5 minutes was recorded as final A. Final A minus initial A give 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.

3.7. MUSCARINIC RECEPTOR SIGNAL TRANSDUCTION ASSESSMENT

3.7.1. Muscarinic cholinergic receptor binding assay

[N-methyl-^{3}H]scopolamine (^{3}H-NMS) (Amersham Canada, Oakville, Ontario, Canada) was used as a radioligand according to a previously reported method (Deighton *et al.,* 1990; Fields *et al* 1978; Horackova *et al.*, 1990). Nonspecific binding was measured in the presence of 500 times excess atropine (Sigma Chemical, St. Louis, MO). At room temperature, increasing concentrations of NMS (0.5-20 nM) were incubated in glass tubes with 100μg of plasma membrane for 45 minutes. The addition of 3 ml of ice cold Tris buffer

stopped the binding reaction. Bound and free radioligands were separated by rapid filtration through Whatman microfiber filters (GF/B) on a 12-port Millipore filtration manifold under vacuum pressure of -30 kPa. The tubes and filters were washed three more times with 3 ml of the cold buffer. Radioactivity on the filters were determined in Ecolite (+) scintillation liquid (ICN, Costa Mesa, CA, USA), using a LKB Wallac 1214 RackBeta liquid scintillation counter (LKB, Stockholm, Sweden). Receptor density (B_{max}) and dissociation constant (K_d) were calculated using a commercially available Figure Perfect program. Receptor binding affinity is expressed as binding dissociation constant, K_d. K_d can be illustrated by the following equation :

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

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 K_d value, the lower the receptor binding affinity is.

3.7.2. Cardiac plasma membrane cAMP production measurement

Adenylyl cyclase activity was measured according to previously reported methods (Ma *et al*, 1994; Gilman, 1970). All cAMP assays were carried out on the same day the cardiac plasma membranes were prepared. Briefly, cAMP generation in cardiac plasma membranes under the stimulation of isoproterenol or forskolin and inhibition with carbachol was determined. Under isoproterenol

stimulation GTP was added at a final concentration of 100 μmol/L. Under forskolin stimulation (100 μmol/L) no GTP was added. The cAMP generation assay was carried out in a basic buffer containing 50 mM Tris, 10mM $MgCl_2$, 1mM EDTA, 1mM 3-isobutyl-1-methylxanthine (IBMX), 1mM dithiothreitol, 10mM creatine phosphate, and 1mM ATP at pH 7.4. EDTA and IMBX are needed since they have inhibitory effects on phosphodiesterase to block cAMP degradation. The reaction was carried out at a final volume of 300μl containing 12 μg creatine phosphokinase. For isoproterenol stimulation 100μg of plasma membrane protein was used and for forskolin stimulation 25μg of membrane protein was used. Creatine phosphokinase catalyses the transfer of phosphate from creatine phosphate to ADP, and this process will supply ATP. The reaction was incubated at 37°C for 20 minutes and was stopped by placing the tubes in boiling water for 3 minutes. The precipitate was then removed by centrifugation at 10,000 g. 150μl of the clear supernatant was used to test cAMP content, which was determined by using a commercially available ^3H binding protein assay kit (TRK.432, Amersham Canada, Oakville, Ontario, Canada). The binding proteins in this kit are purified from bovine muscles. The major components of the binding proteins are cAMP dependent protein kinases. Experiments have demonstrated that these proteins bind specifically with cAMP. No cross binding was found between the binding protein and ATP or GTP using the Amersham assay kits. The principle of the cAMP assay is shown in Figure 4. Bound and free ^3H-cAMP were separated by charcoal absorption followed by 2000 g

centrifugation. cAMP concentration was normalized to protein content and expressed as ρmol/mg protein/min.

3.8. Statistics

Statistics were performed by utilizing a commercially available software program InStat (Version 2, Graphpad Software Inc., San Diego, CA, USA) All results are expressed as mean ± SE. The results were analyzed by the Student's *t*-test or a repeated measures ANOVA test for trend with a Tukey correction for multiple comparisons. A probability of < 0.05 was considered significant.

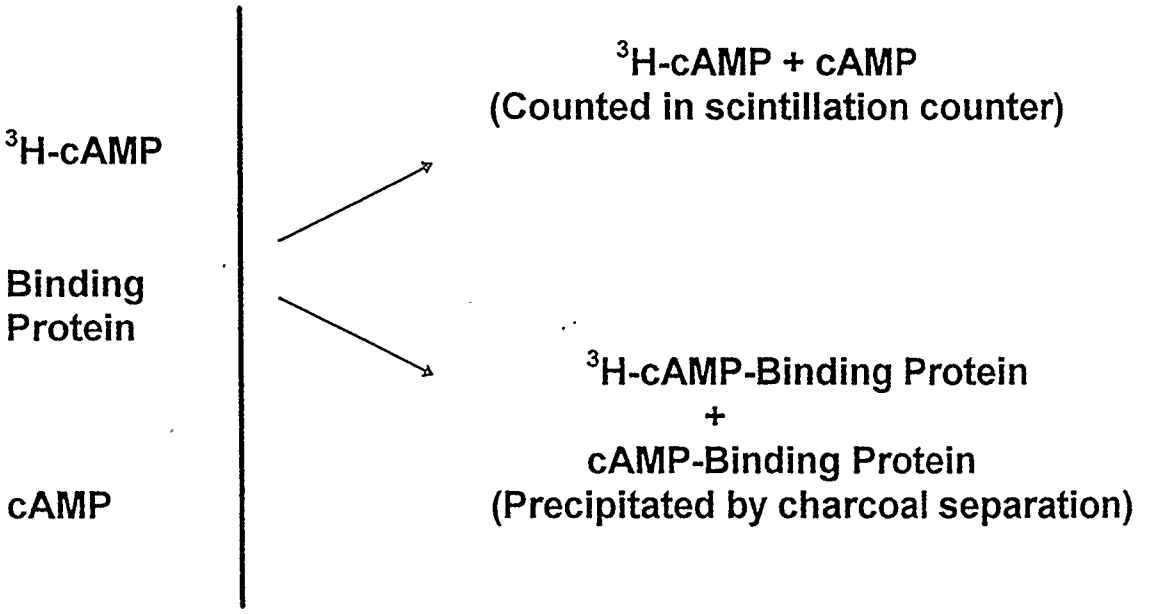

Figure 4. Basic principles of cAMP protein binding assay. cAMP generated from cardiac plasma membrane, ^{3}H-labeled cAMP, and cAMP binding protein are incubated at 4°C for 2 hours. Cold cAMP competes with ^{3}H-labeled cAMP to combine with the binding proteins. The cAMP bound with the binding proteins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

RESULTS

4.1. EVIDENCE OF IMPAIRED VENTRICULAR MUSCLE CONTRACTILITY

Cardiac contractility studies were performed in the BDL model of cirrhosis to confirm the existence of cirrhotic cardiomyopathy and blunted cardiac contractile response to exogenous (carbachol infusion) muscarinic stimulation. An *in situ* pithed rat and an *in vitro* left ventricular papillary muscle model were selected. To confirm the validity of the results obtained in the pithed rat study, the Viggo-Spectramed P23XL transducer used in this study was compared with a Millar SPR-524 transducer in a frequency response test.

4.1.1. Transducer study

The Viggo-Spectramed (P23XL) transducer used in the pithed study was tested to confirm that the response recorded was valid. The result from the study is depicted in Figure 5a and 5b. The Viggo-Spectramed transducer at 10 Hz is 94% of the amplitude ratio and at higher frequencies the amplitude ratio drops steadily (Figure 5b). The frequency response of the Millar (SPR-524) transducer system can be considered 100% at all frequencies.

4.1.2. Pithed rat study

Cardiac function and contractility were measured in the pithed rat to study the effect of carbachol on the cardiovascular system. There was no significant

difference in basal heart rate between Sham and BDL rats. Basal left ventricular systolic pressure was found to be significantly different between Sham and BDL rats (Table 1). Intravenous infusion of carbachol produced decreases in heart rate and left ventricular systolic pressure in both groups, but the magnitude of the decrements were similar (Figure 6 and 7).

At comparable preloads a good index of myocardial contractility can be obtained from the contour of ventricular pressure curves. The maximum slope of the ascending limb of the ventricular pressure curve indicates the maximum rate of force development by the ventricle (maximum rate of change in pressure with time; $+dP/dt_{max}$). The maximum slope of the descending limb of the ventricular pressure curve indicates the maximum rate of force dissipation (maximum rate of change in pressure with time; $-dP/dt_{max}$). At any time of ventricular filling the slope provides an index of the initial velocity of contraction and hence contractility. Both $+dP/dt_{max}$ and $-dP/dt_{max}$ responses to carbachol are presented in Figure 7 and 8. At baseline, BDL rats showed a significant reduction in $+dP/dt_{max}$ and $-dP/dt_{max}$ (Table 1). The reduced rate of force development is an indicator of a hypodynamic heart, which is what is usually found in cardiac failure. As incremental doses (0.9-45.7 μg) of carbachol were infused intravenously to sham-operated rats, a decrease was seen in $+dP/dt_{max}$ and $-dP/dt_{max}$ (Figure 8 and 9). However, in BDL rats the incremental doses of carbachol do not appear to affect $+dP/dt_{max}$ and $-dP/dt_{max}$.

4.1.3. Impaired left ventricular papillary muscle

To verify the impaired contractile response to carbachol stimulation in cirrhosis left ventricular papillary muscle contractile response was measured. Baseline measurements were not found to be significantly different between the two groups. The initial response of the papillary muscle was under 10^{-6} mol/L isoproterenol stimulation. Isoproterenol stimulated force of contraction was sustained throughout the entire experiment in Sham rats. However, in BDL rats isoproterenol stimulated force of contraction decreased over the same time period (Figure 10a).

The effect of incremental doses of carbachol (10^{-5} to 10^{-3}) on isoproterenol stimulated force of contraction is shown in Figure 10b. Carbachol significantly decreased force of contraction in sham-operated papillary muscles. However, carbachol did not significantly decrease force of contraction in BDL heart muscle.

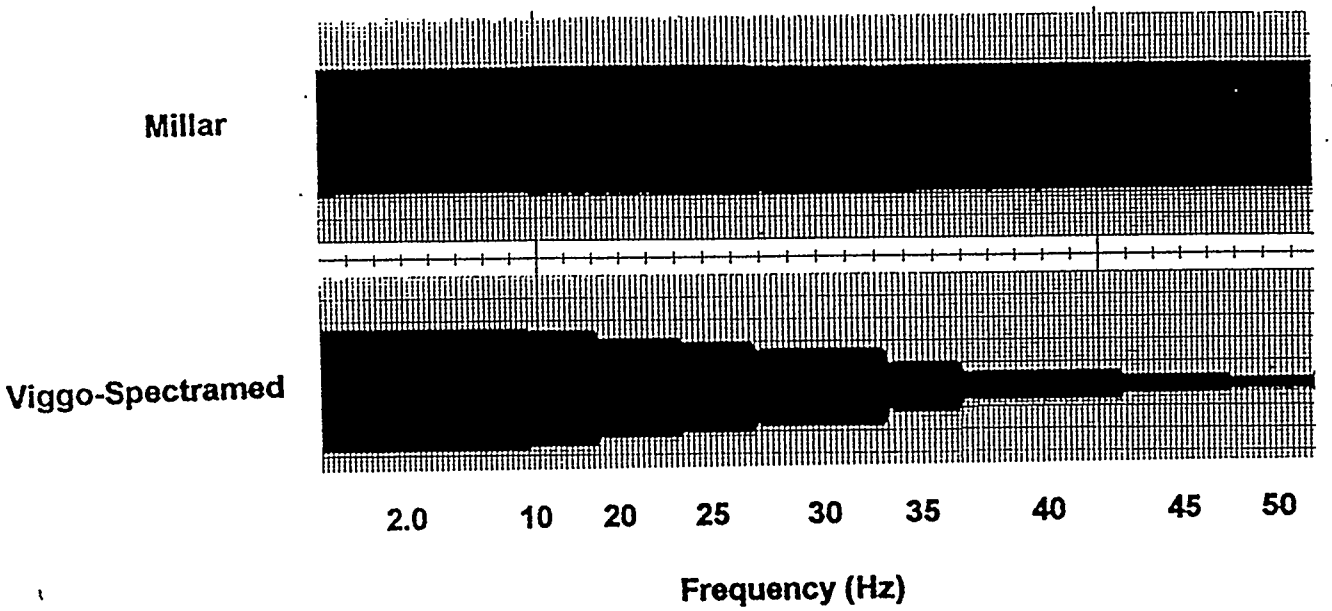

Figure 5a. Representative tracing of the responses from the Viggo-Spectramed P23XL transducer and the Millar SPR-524 transducer.

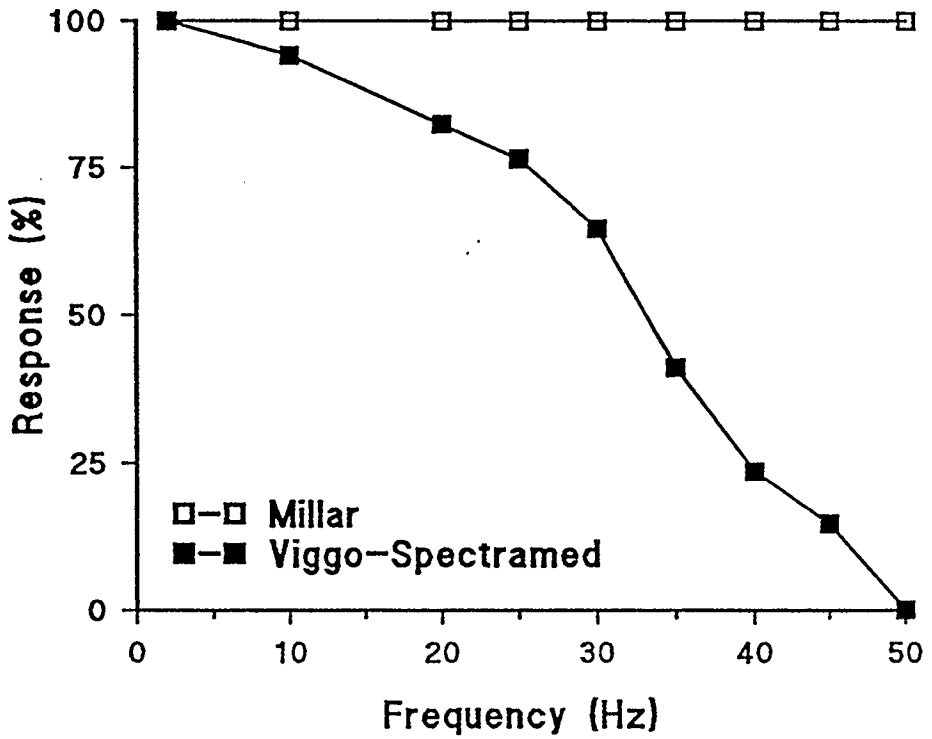

Figure 5b. Comparison of the Viggo-Spectramed P23XL transducer with the Millar SPR-524 transducer in frequency response.

Table 1. Basal cardiac dynamics in pithed sham-operated and cirrhotic rats.

	SO (n=4)	BDL (n=5)	P
HR, beats/min	378±14	356±30	NS
LVSP, mmHg	103±5	64±6	<0.05
$+dP/dt_{max}$, mmHg/s	3599±296	1226±63	<0.01
$-dP/dt_{max}$, mmHg/s	-3040±235	-864±59	<0.01

Values are means ± SE. SO, sham-operated. BDL, bile duct ligated. HR, heart rate. LVSP, left ventricular systolic pressure. $+dP/dt_{max}$, maximal time derivative of left ventricular pressure development. $-dP/dt_{max}$, maximal time derivative of left ventricular pressure dissipation.

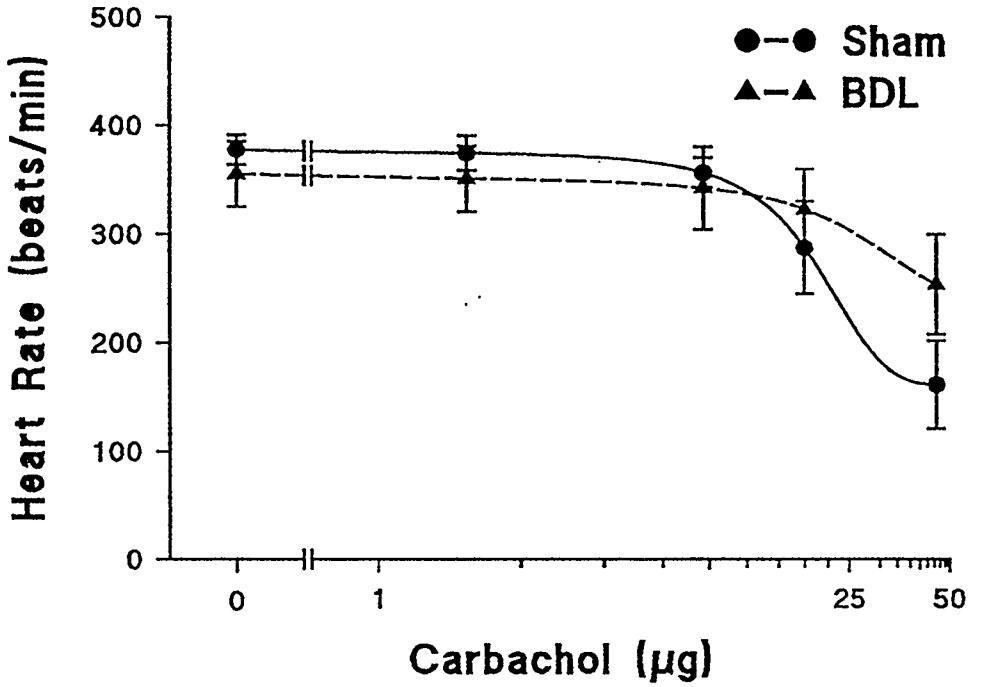

Figure 6. Changes in heart rate in sham-operated (Sham, n=4) and cirrhotic (BDL, n=5) rats during infusion of increasing concentrations of carbachol. Data are presented as mean ± SEM. There was no significant differences between the 2 groups in response to carbachol stimulation.

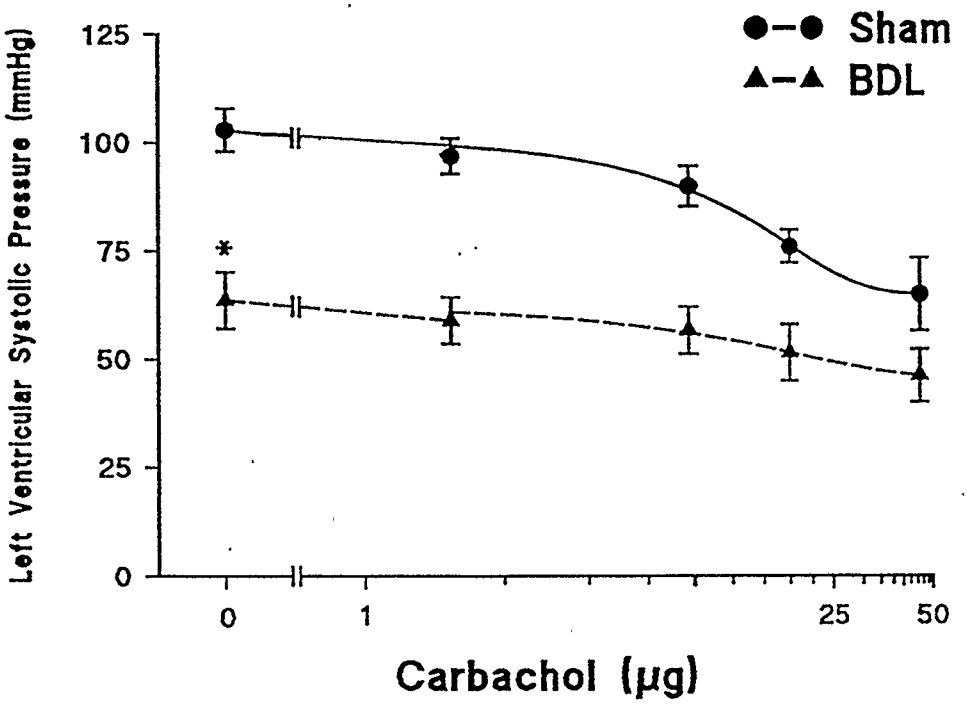

Figure 7. Changes in left ventricular systolic pressure in sham -operated (Sham, n=4) and cirrhotic (BDL, n=5) rats during infusion of increasing concentrations of carbachol. Data are presented as mean ± SEM. * indicates a significant difference between sham and BDL groups.

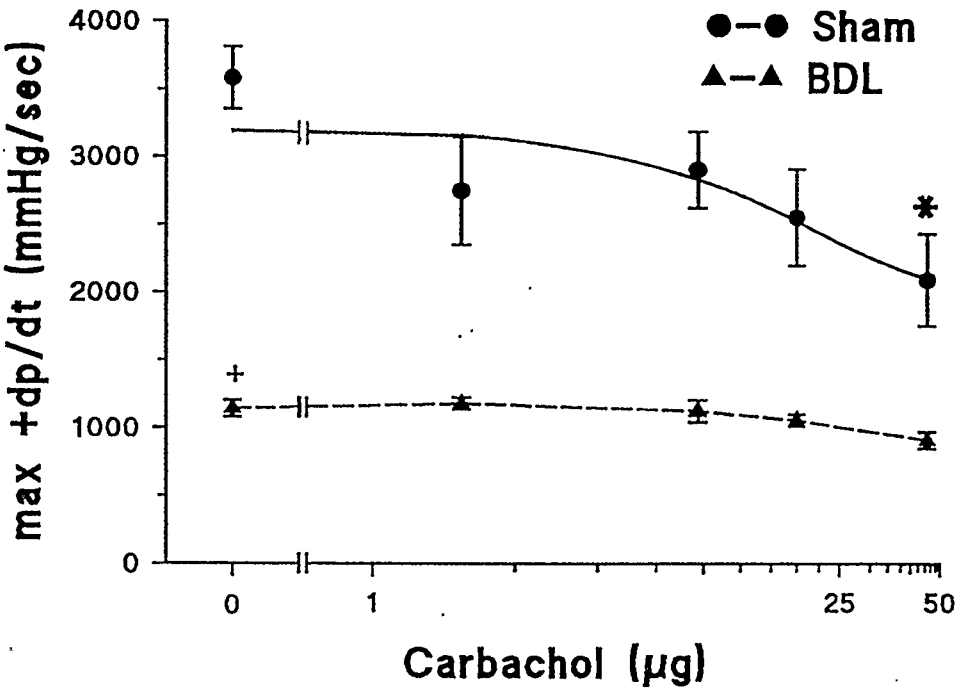

Figure 8. Changes in $+dP/dt_{max}$ in sham-operated (Sham, n=4) and cirrhotic (BDL, n=5) rats during infusion of increasing concentrations of carbachol. Data are presented as mean ± SEM. + indicates a significant difference between sham-operated and cirrhotic rats. * indicates a significant decrease in $+dP/dt_{max}$ in sham-operated rats ($p<0.05$ by repeated measures ANOVA).

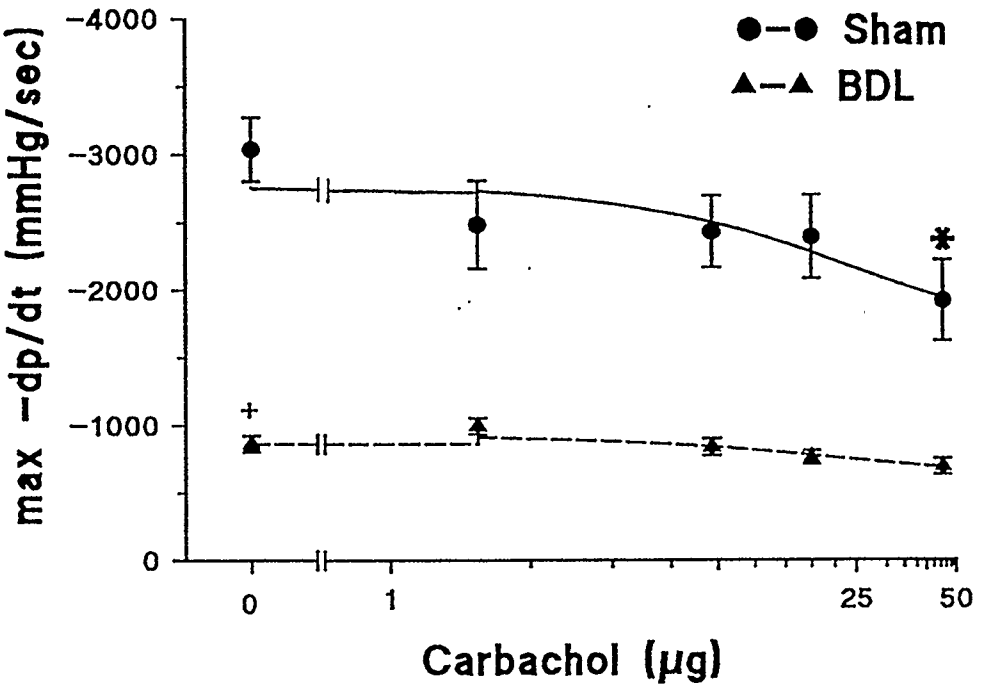

Figure 9. Changes in -dP/dt_{max} in sham-operated (Sham, n=4) and cirrhotic (BDL, n=5) rats during infusion of increasing concentrations of carbachol. Data are presented as mean ± SEM. + indicates a significant difference between sham-operated and BDL rats. * indicates a significant decrease in -dP/dt_{max} in sham-operated rats ($p<0.05$ by repeated measures ANOVA).

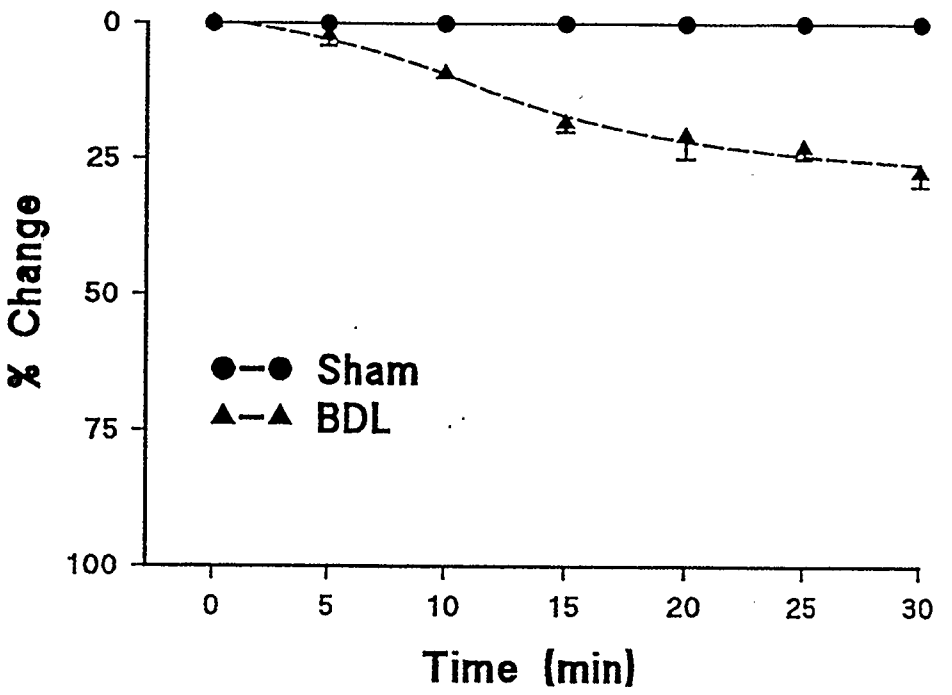

Figure 10a. Time-dependent response to isoproterenol stimulation in isolated left ventricular papillary muscle from cirrhotic (BDL) and sham-operated (Sham) rat hearts. As time increased a decrease in force of contractions occurred in BDL rat heart muscle.

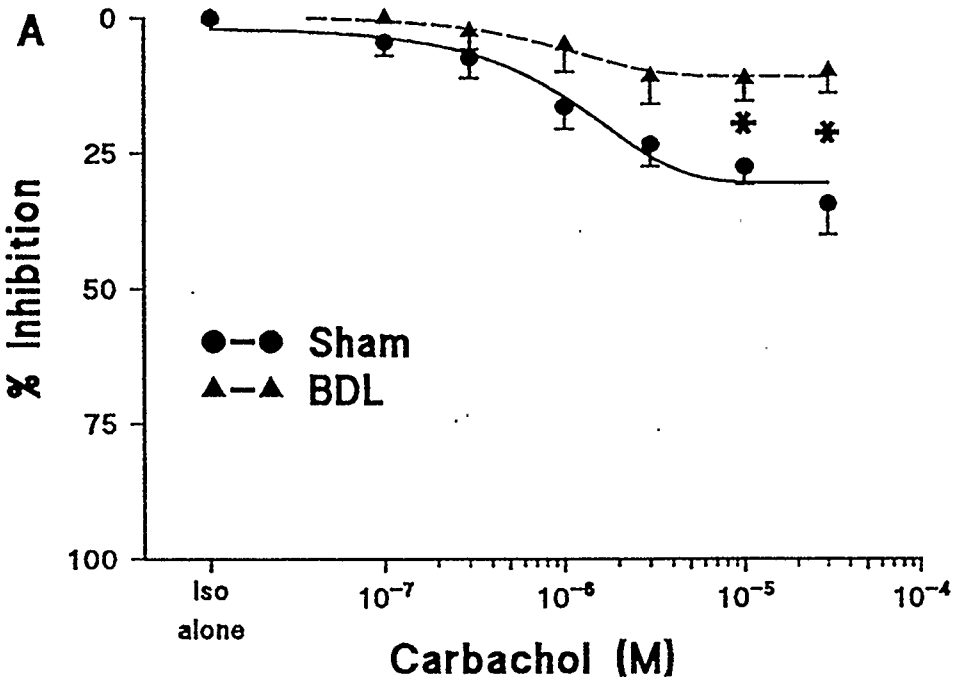

Figure 10b. Cumulative dose-responses to isoproterenol-stimulated and carbachol inhibited contractions in isolated left ventricular papillary muscles from cirrhotic (BDL, n=7) and sham-operated (Sham, n=7) rat hearts. * designates a significant difference between sham-operated and BDL hearts in % inhibition in response to carbachol, $P<0.05$.

4.2. Enrichment of the isolated cardiac plasma membrane vesicles

The cardiac plasma membrane enzyme marker 5'-nucleotidase activity is plotted in Figure11. Approximately 9 to 10 fold enrichment of this enzyme activity was obtained in the final cardiac plasma membrane preparation compared with the initial homogenate. The results are comparable to previous studies by other investigators (Ma *et al*, 1994; Pierce and Dhalla 1980). Sham operated and BDL rats were found to have comparable enrichment.

4.3. Characterization of cardiac muscarinic cholinergic receptors

To assess muscarinic cholinergic receptor function in cirrhotic cardiomyopathy, a muscarinic receptor binding assay was performed using ^{3}H-NMS as a radioligand. The non-specific binding was less than 10% of total binding throughout the binding study. As mentioned in the method section, a computer program was used to calculate receptor number (B_{max}) and dissociation constant (k_d). The saturation curves of specific binding are presented in Figure 12. The Figure illustrates that the specific binding of ^{3}H-NMS to cardiac plasma membrane is saturable.

Muscarinic cholinergic receptor density and binding affinity in sham operated and BDL rats are shown in Figure 12. Cardiac muscarinic receptor density and binding affinity were not found to be significantly different between the two groups.

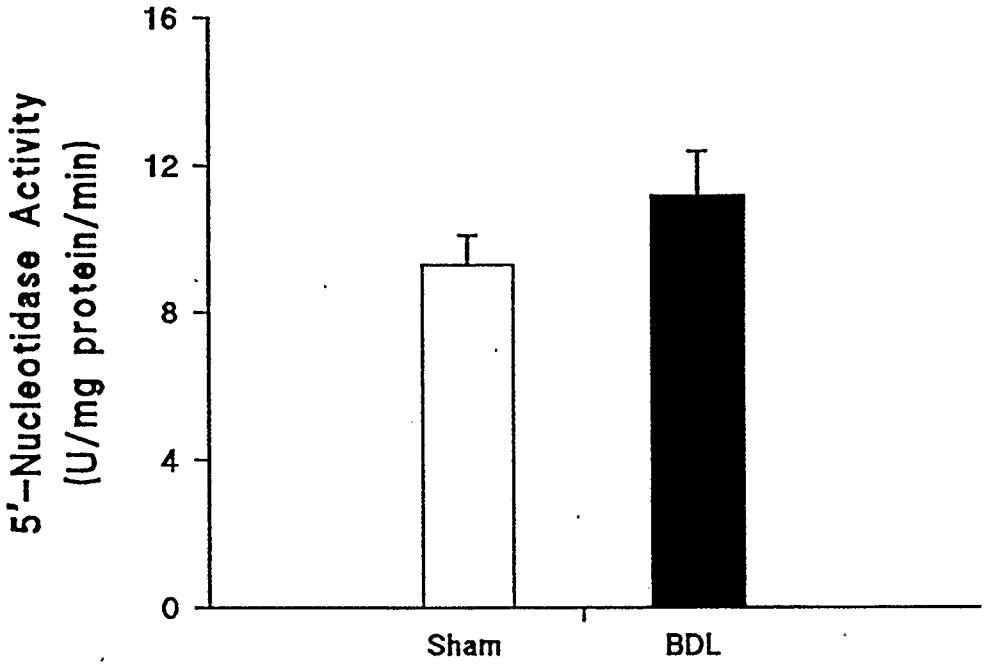

Figure 11. Comparison of 5'-nucleotidase activity in the final cardiac plasma membrane preparation with the initial homogenate. Data are presented as mean ± SEM, obtained from 12 cirrhotic (BDL) and 12 sham-operated (Sham) rats. No purification differences were observed between BDL and Sham rats.

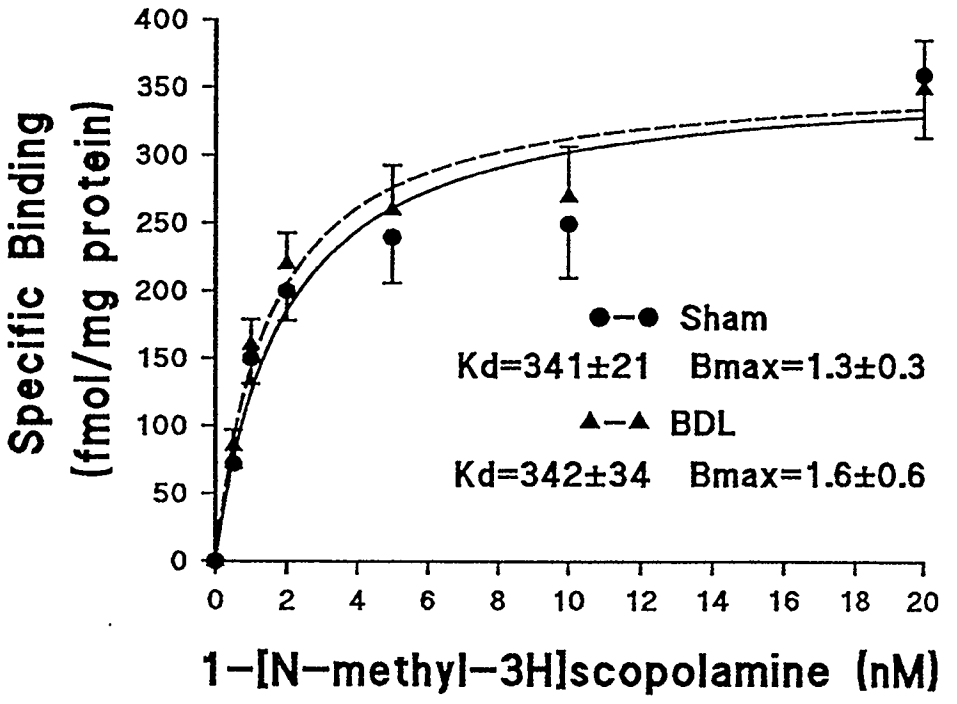

Figure 12. Binding assay saturation curves. The specific binding of the radioligand ^{3}H-NMS, to cardiac plasma membranes from both bile duct ligated cirrhotic rats and sham-operated rats is saturable within the concentration range of ^{3}H-NMS used in the present study. No significant differences were observed in K_d and B_{max} between sham-operated and BDL membranes.

4.4. cAMP production

To examine the muscarinic receptor signal transduction pathway in cirrhotic cardiomyopathy, cAMP production was measured. Adequate amounts of membrane proteins and incubation times were determined in preliminary experiments, so that the amount of cAMP produced in each tube were within the detectable range of the assay system. Figure 13 represents the effect of carbachol on isoproterenol stimulated BDL and sham-operated sarcolemmal membranes.

Carbachol has an inhibitory effect on adenylyl cyclase but its effect is much stronger under β-adrenergic stimulation. Previous studies (Ma *et al.,*1994) have demonstrated that isoproterenol has maximal stimulation at 10^{-4} mol/L, therefore 10^{-4} mol/L was used to stimulate adenylyl cyclase. Increasing doses of carbachol (10^{-5}-10^{-3}) were then added. cAMP stimulated by isoproterenol and inhibited by carbachol was significantly lower in BDL rats.

Since previous studies have shown that isoproterenol stimulation of adenylate cyclase is impaired, forskolin was used as a stimulator. Forskolin has been shown to directly stimulate adenylyl cyclase by acting on the catalytic domains of this enzyme (Seamen and Daly, 1980). The results from the forskolin assay is shown in Figure 14. At baseline, forskolin stimulated cAMP production was significantly decreased in BDL rats. cAMP stimulated by forskolin and inhibited by carbachol was not significantly different between the two groups.

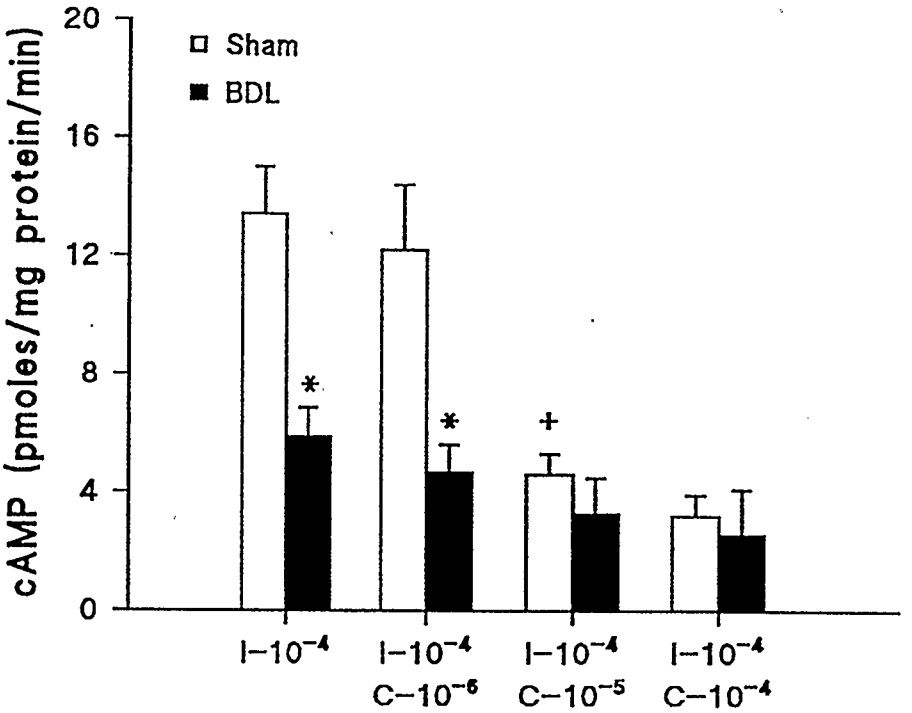

Figure 13. cAMP generation under isoproterenol (10^{-4} mol/L) stimulation and carbachol inhibition at various concentrations in cardiac plasma membranes from cirrhotic rats and sham-operated rats. I-10^{-4} represents the isoproterenol concentration used. C-10^{-x} represents the carbachol concentration used in the study. Data are presented as mean ± SEM on 6 separate membrane preparations in each group. * designates a significant difference between sham-operated and BDL membranes at the specified concentration of isoproterenol and carbachol, $p<0.05$. + designates a significant decrease in cAMP production at the specified carbachol concentration in sham membranes, $p<0.05$.

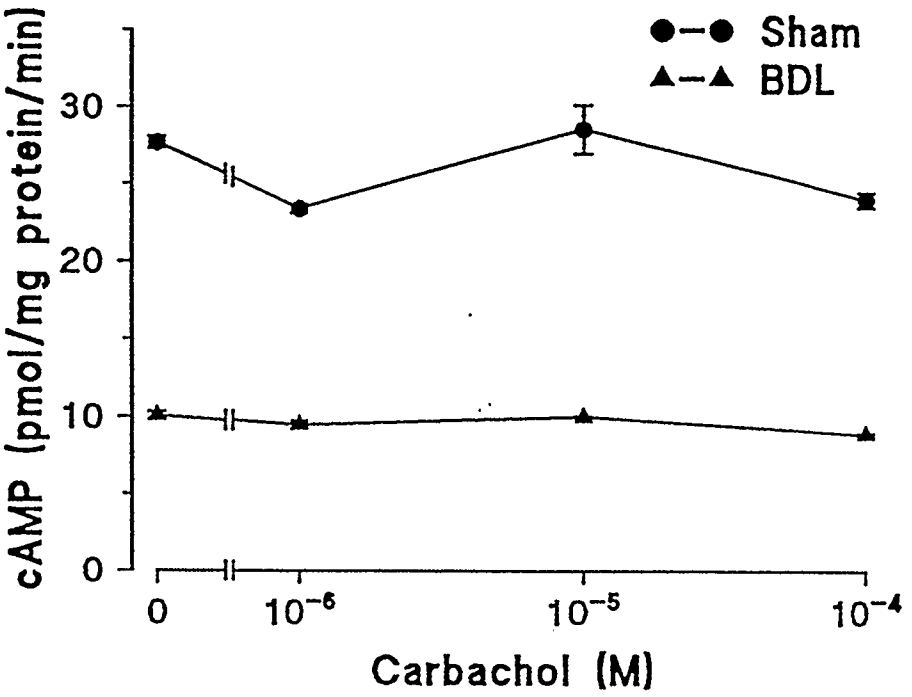

Figure 14. cAMP generation under forskolin (100 μmol/L) stimulation and carbachol inhibition in cardiac plasma membranes from cirrhotic rats and sham-operated rats. Data are presented as mean ± SEM on 6 separate membrane preparations in each group. * indicates a significant difference between sham-operated and BDL membranes.

DISCUSSION

Cardiac contractility is regulated in a coordinated manner by stimulatory β-adrenergic and inhibitory muscarinic receptors. Although receptor function has been shown to be abnormal in various noncirrhotic models of heart failure such as congenitally cardiomyopathic hamsters (Wilkinson *et al.*, 1994; Chidiac *et al.*, 1991) and hypertensive cardiomyopathy (Bohm *et al.*, 1992; Fu *et al.*, 1994), no previous study has investigated the possible role of altered muscarinic function in the genesis of cirrhotic cardiomyopathy. In this study, two different preparations, *in situ* and *in vitro*, were used to investigate cardiac contractile function: the pithed rat model and the isolated left ventricular papillary muscle.

5.1. THE PITHED RAT MODEL

5.1.1. The transducer study

As an index of cardiac contractility, peak positive left ventricular dP/dt_{max} was measured with a fluid-filled PE-50 tubing system. The fluid filled tubing system was tested against the Millar transducer. The Millar transducer is approximately 1 mm in diameter and can be inserted into the ventricle directly bypassing the tubing system. The results show that at 10 Hz the amplitude ratio of the fluid-filled system is 94% and at higher frequencies the amplitude ratio drops steadily (Figure 5b). A previous study by Fry found that the frequency response of his fluid-filled system could be considered flat (± 5%) to 20 Hz (Fry,

1960). The frequency response of our fluid-filled system was not as good as Fry's; thus, this system could introduce some error on the absolute values of dP/dt_{max} because of the high-frequency response needed to measure this variable with accuracy in the rat.

5.1.2. Blunted cardiac dynamics in the pithed rat

The evidence over the last four decades has shown the existence of cirrhotic cardiomyopathy in human patients with cirrhosis and animal models of cirrhosis (reviewed in Lee, 1989). The data from the pithed rat study suggest that when the autonomic nervous system is ablated, cirrhotic rats showed significantly impaired cardiac systolic contractility and diastolic relaxation (Table 1). Baseline left ventricular $+dP/dt_{max}$ and $-dP/dt_{max}$ were both markedly different between the cirrhotic and control rats. In addition, baseline peak systolic pressure was also significantly lower in cirrhotic rats than sham-operated rats. Previous studies have demonstrated that at baseline, cardiac output in cirrhotic rats is increased by 15-30%. This change from a 15-30% increase to a 3-fold decrease in cardiac contractility and peak systolic pressure suggests that autonomic, particularly the sympathetic innervation is critically important to maintain normal or even supranormal ventricular contractility. The only previous study of the cardiac effects of pithing in cirrhosis was conducted by Abergel and colleagues (1992) who examined the same BDL rat model. They found that while cardiac output in pithed rats dropped markedly, compared to pithed control

rats, the values in the cirrhotic rats continued to remain slightly but significantly increased. They therefore suggested that in cirrhotic rats the hyperdynamic circulation is not entirely due to activation of the sympathetic nervous system but also to nonneurogenic factors (Abergel *et al.*, 1992). This apparent discordance likely reflects the different parameters assessed in their study and the present one. Positive dP/dt_{max} measures myocardial contractility and can be affected by many factors, notably ventricular loading conditions (preload and afterload). It is important to mention that cardiac preload was not measured or controlled for in these experiments and might not be the same in both groups. The experimental results from the pithed rat study could then be subject to large errors. Previous studies have indicated that $+dP/dt_{max}$ is influenced by end-diastolic volume (preload), and so cannot distinguish length-dependent properties (Starling's law) from those arising from altered contractility (Parmley *et al.*, 1973; Katz, 1992). Therefore, the difference in contractility between sham-operated and cirrhotic rats at baseline could be due to different preload conditions.

Muscarinic cholinergic receptors and β-adrenergic receptors are the major receptor systems that regulate the normal heart (Brodde *et al.*, 1992; Lindemann *et al.*, 1989). Experimental models of cirrhosis have already established impaired contractile response to β-adrenergic stimulation (Ma *et al.*, 1996; Batterbee *et al.*, 1992; Zavecz *et al.*, 1995) Since cardiac function is regulated in a coordinated manner by both stimulatory β-adrenergic receptors and inhibitory muscarinic cholinergic receptors, the effect of muscarinic stimulation on heart

rate and cardiac contractile function was investigated in the pithed cirrhotic rat. Stimulation of muscarinic cholinergic receptors is known to have negative inotropic and chronotropic effects on the heart (Lindemann *et al.*, 1989; Loffelholz *et al.*, 1985). In the present study the effect of carbachol on heart rate was as expected, heart rate decreased with increasing concentrations of the muscarinic agonists. However, the magnitude of the decrements was found to be similar between the two groups. Three general mechanisms are thought to decrease the discharge frequency of the sino-atrial node. The three possible mechanisms are decreased rate of diastolic depolarization, diastolic hyperpolarization and an increase in the threshold (Katz, 1992). One of the major actions of acetylcholine in the heart is to activate an outward potassium current (Giles *et al*, 1981). In nodal tissue, activation of the outward potassium current will result in hyperpolarization and reduction in the slope of the pacemaker potential. Thus, decreasing heart rate. Since heart rate was found to be similar in both sham-operated and BDL rats it would appear that muscarinic activation of potassium channels are unaffected by liver disease. Left ventricular end-systolic pressure was found to be decreased in cirrhotic rats when compared with sham-operated controls at baseline; however, the response to carbachol in both groups was found to be similar.

The response of cirrhotic rats to muscarinic stimulation was found to be significantly different between the two groups. There was a significant decrease in dP/dt_{max} with increasing concentrations of the muscarinic agonist (carbachol)

in sham-operated rats. However, the cirrhotic (BDL) rats were unresponsive to increasing concentrations of carbachol. Muscarinic agonists regulate myocardial contractility by inhibiting adenylate cyclase, which results in decreased levels of cAMP and cAMP dependent protein kinase (PKA). PKA is known to phosphorylate voltage-sensitive Ca^{2+} channels for increased Ca^{2+} influx across the sarcolemma. Decreased levels of PKA will decrease the Ca^{2+} influx, thus decrease contractility. Muscarinic agonists can also reduce contractility by opening potassium channels which will promote repolarization. This will shorten systole and thus depress contractility. Since cirrhotic rats have a blunted contractile response to muscarinic agonists it suggests that either the muscarinic cholinergic signal transduction pathway is impaired or the acetylcholine-activated potassium channel is impaired. Left ventricular relaxation was determined from the recordings of left ventricular pressure. Carbachol significantly decreased $-dP/dt_{max}$ in pithed sham-operated rats but not in pithed cirrhotic rats. Once again, ventricular contraction and relaxation are altered by changes in temperature (Courtois *et al.*, 1988), catecholamines (Weiss *et al.*, 1976), heart rate (Freeman *et al.*, 1987) and loading conditions (Raff *et al.*, 1981). Several of these factors were taken into consideration in this study. Temperatures in both groups were maintained constant and heart rate was not found to be significantly different between the two groups. Changes in preload or afterload can substantially affect developed force or pressure and, if uncontrolled as in this study, can invalidate any assessment of contractility

changes (Parmley *et al.*, 1969). In this study loading conditions were uncontrolled. Different loading conditions could account for the difference seen between the sham-operated and cirrhotic rats in response to carbachol therefore, we cannot conclude whether or not contractility was depressed in BDL pithed rats.

5.2. BLUNTED VENTRICULAR PAPILLARY MUSCLE CONTRACTILE RESPONSES

The response of the heart to muscarinic stimulation is variable depending on the region of the heart. In the ventricle, muscarinic agonists are known to antagonize the effects of β-adrenergic agonists, and this anti-adrenergic effect is much more pronounced compared to the effect of muscarinic agonists alone (Caulfield 1993; Loffelholz *et al.*, 1985). In other words, it is thought that the physiological role of muscarinic function in the ventricle is to counteract β-adrenergic stimulation of the heart. Accordingly, to investigate the more physiological anti-adrenergic effect of carbachol in cirrhosis, *in vitro* studies using isolated papillary muscles were conducted. At baseline, there was no significant difference between the sham-operated and cirrhotic rats in the force of contraction. This suggests that the apparent altered contractility observed at baseline of the cirrhotic pithed rats are likely due to different loading conditions, since in the papillary muscle experiments preload conditions were the same in both groups. The magnitude of the negative inotropic response to carbachol

was significantly reduced in cirrhotic rats when compared with controls, indicating blunted muscarinic responsiveness (in the presence of isoproterenol, Figure 10b) which supports the pithed rat data. Force of contraction is regulated mainly by changes in calcium fluxes involved in excitation-contraction coupling and relaxation (Katz, 1992). Therefore, any of the mechanisms that modulate calcium influx may be impaired. Such as changes in the behaviour of calcium channels on the plasma membrane, or second messengers which regulate calcium entry could be responsible for the decreased force of contraction observed in the cirrhotic heart. Pak and colleagues (1994) have shown that bile acids at levels found in the cirrhotic rat can interfere with the function of membrane Ca^{2+} channels. Previous studies have established that in cirrhosis β-adrenergic responsiveness is blunted (Ramond *et al.*, 1986; Lee *et al.*, 1990; Ma *et al.*, 1996), and from the present results, it appears that the counteracting muscarinic function is also attenuated. The result obtained in the *in vitro* study is in agreement with other studies of heart disease (Kacimi *et al.*, 1993; Wilkinson *et al.*, 1994). Impaired cardiac contractile response to muscarinic and β-adrenergic stimulation was found in cardiomyopathic hamsters (Horackova *et al.*, 1991). Kacimi and colleagues (1993) also found blunted contractile response to endogenous and exogenous stimulation in chronic hypoxia. The blunted contractile response was attributed to an increase in muscarinic receptors.

Further evidence of diminished β-adrenoceptor function in cirrhosis was noted during the papillary muscle studies: the time-dependent decline in

isoproterenol-stimulated contractility in cirrhotic but not control muscles (Figure 10a). Isoproterenol-stimulation on sham-operated muscles was found to be significantly different from that of cirrhotic muscles. In control muscles 10^{-6} M dose of isoproterenol induced a steady-state contractile response over a 35 minute time period. In BDL muscles the same dose of isoproterenol increased force of contraction for only 15 minutes before it started to decline. Previous studies on β-adrenergic receptors have discovered that β-adrenergic receptors lose their ability to generate a response when a neurotransmitter such as norepinephrine is continually delivered to the heart; this has been termed desensitization (Katz, 1992; Hausdorff *et* al., 1990). It would appear that in cirrhotic rats, β-adrenergic receptors lose their ability to respond much more quickly than sham-operated controls. This observation is in agreement with Lee and colleagues; they also found abnormal responses to isoproterenol stimulation in cirrhotic animals (Lee *et al.*, 1990). Desensitization can occur through three sequential mechanisms: uncoupling, internalization and digestion. The first step in the process of desensitization is uncoupling of β-adrenergic receptors. Uncoupling is mainly due to cAMP-stimulated phosphorylation of the receptor, this is catalyzed by a β-adrenergic receptor kinase (βARK) which is specific for β-adrenergic receptors. The phosphorylated receptor then binds arrestin, which blocks the interaction between the phosphorylated receptor from its G-protein. The second step in desensitization which occurs after phosphorylation uncouples the receptors from the cyclase is the disappearance of the receptor

molecules from the plasma membrane. The phosphorylated receptor-arrestin complex is detached from the plasma membrane and moves into the cytosol. Internalized receptors remain structurally intact even though they have lost their ability to interact with their other components. The internalized receptor can either be destroyed by proteolytic enzymes or dephosphorylated by a phosphoprotein phosphatase. The reasons for the time-dependent attenuation of the isoproterenol effect remains unclear, but relatively rapid dissipation of agonist effect can be observed with a desensitized or downregulated receptor system, and previous studies have documented defects in β-adrenergic receptor density and its signal transduction pathway (Ma *et al.*, 1991; Lee *et al.*, 1990; Ma *et al.*, 1996)

5.3. CARDIAC MUSCARINIC CHOLINERGIC RECEPTOR CHARACTERISTICS AND ADENYLYL CYCLASE ACTIVITY

Since attenuation of receptor effect may be caused by change in receptor density or receptor-ligand affinity, muscarinic receptor characteristics were studied using the nonspecific muscarinic radioligand [N-methyl-^{3}H]scopolamine (NMS). In other forms of cardiomyopathy the research on muscarinic cholinergic receptor characteristics is conflicting. Some investigators have found decreases in muscarinic receptor density while others have found no changes or increases in muscarinic receptor density (Vatner *et al.*, 1988; Kacimi *et al.*, 1993; Wilkinson *et al.*, 1994). The data generated using NMS reflects ventricular M_2

characteristics, as it has been clearly established that only the M_2-subtype of muscarinic receptors are found in ventricles (Fields *et al.*, 1978). In the present study, muscarinic receptor density and affinity were found to be similar in both sham-operated and cirrhotic rats, thus suggesting that the receptor *per se* and the receptor-ligand interaction are not altered and the blunting of the muscarinic effect is due to postreceptor mechanisms.

Since muscarinic cholinergic receptor characteristics were unchanged, the next step was an investigation of the signal transduction pathway. Muscarinic receptor signal transduction system plays an important role in the modulation of cardiac contractility. Previous studies in heart failure and various models of heart failure have observed changes in muscarinic receptors and its signal transduction pathway; receptor number, adenylate cyclase, G proteins, and coupling of muscarinic receptors to adenylate cyclase (Chidiac *et al.*, 1991; Vatner *et al.*, 1988; Schmitz *et al.*, 1991).

Activation of muscarinic receptors will enable it to interact with its inhibitory G-protein which leads to the exchange of GDP for GTP. This results in the dissociation of the α-subunit from the $\beta\gamma$-subunits. $G_{i\alpha}$-GTP inhibits adenylate cyclase from converting ATP to the second messenger cAMP. Decreased levels of cAMP will also mean decreased activation of cAMP dependent protein kinase A (PKA). Adenylyl cyclase activity is dually regulated by the inhibitory G_i and the stimulatory G_s proteins. The effect of carbachol on isoproterenol-stimulated adenylyl cyclase activity was reduced in cirrhotic

membranes, suggesting a defect of the muscarinic signal transduction system (Figure 15). The results obtained from the cAMP production assay is internally consistant with the papillary muscle study. Under isoproterenol stimulation carbachol significantly decreased force of contraction and cAMP production in sham-operated rats but not in bile-duct ligated rats. This observation suggests that there in an impairment of the inhibitory muscarinic receptor system in cirrhosis. $G_{i\alpha}$ status in heart failure remains controversial. Some studies in animal models of heart failure indicate that increased $G_{i\alpha}$ expression may result in reduced heart contractile function (Feldman 1993). However, other studies of heart failure have documented decreased $G_{i\alpha}$ expression, suggesting that the role of reduced $G_{i\alpha}$ expression in heart failure may be simply compensatory rather than pathogenic (Roth *et al.*, 1993). Membrane G_i protein content was not investigated in the present study but a recent study demonstrated that $G_{i2\alpha}$ in cirrhotic cardiomyopathy is decreased in conjunction with reduced G-protein expression (Ma *et al.*, 1996).

In order to test adenylate cyclase activity directly another cAMP assay was performed using forskolin as the stimulatory agent instead of isoproterenol. Forskolin is known to directly stimulate the activity of adenylate cyclase, therefore bypassing the stimulatory β-adrenergic receptors and the stimulatory G-protein. The results from the forskolin stimulated cAMP assay demonstrated no decrease in cAMP production between the sham-operated and the bile-duct ligated groups in response to carbachol inhibition (Figure 16). Inhibition of

adenylate cyclase activity has been proposed to occur by two mechanisms: directly by $G_{i\alpha}$ and/or indirectly via inactivation of $G_{s\alpha}$ by $G_{\beta\gamma}$ released from G_i and possibly G_o (Gilman, 1984). Although the α-subunit of G_i has been shown to inhibit adenylate cyclase activity, it appears to do so weakly and such inhibition by $G_{i\alpha}$ apparently is not regulated by muscarinic receptors in the heart (Lindemann *et al.* 1983). If this is the case, the results of the forskolin study are not unexpected. Forskolin directly stimulates adenylate cyclase, bypassing the stimulatory G protein. Without any $G_{s\alpha}$ subunits the $G_{\beta\gamma}$ released from G_i cannot indirectly inhibite adenylate cyclase, thus there would be no inhibition of cAMP production by carbachol.

5.4. SUMMARY AND CONCLUSIONS

The present study demonstrated that myocardial contractility at baseline is not altered in cirrhosis. In the papillary muscle study, isoproterenol-stimulated and carbachol inhibited force of contraction was significantly less in BDL muscles than sham muscles. Cardiac muscarinic cholinergic receptor density and binding affinity are not affected in cirrhosis which suggests that the impaired negative inotropic effect of carbachol is not due to increased muscarinic receptor number or a change in receptor affinity. Cardiac muscarinic cholinergic receptor signal transduction pathway is also diminished in cirrhosis, suggesting that part of the impaired negative inotropic effect of carbachol is due to impaired signal transduction of muscarinic receptors.

In conclusion, the changes in muscarinic function are likely compensatory (in response to the defect in β-adrenergic system) and muscarinic receptor overactivity is not involved in the genesis of cirrhotic cardiomyopathy.

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