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THYROID STATUS AND THE MODULATION OF MYOCARDIAL CONTRACTILITY BY DIETARY FATTY ACIDS

BY

E.M. KOSI AWUMEY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

FACULTY OF DENTISTRY

EDMONTON, ALBERTA

FALL, 1993

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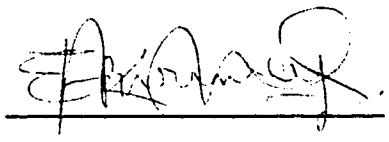

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DEDICATION

To

My wife Janet,

My children Fred, Vida and Emmanuel Jr.

and

My Mother for all her efforts.

ABSTRACT

Thyroid hormone regulates cardiac performance by modulating chronotropic and inotropic responses. The decreased responsiveness to ß-adrenergic stimulation in hypothyroidism may involve alterations in the lipid environment of the ß-adrenoceptor/adenylate cyclase complex induced by inadequate levels of thyroid hormone. In this study, dietary fatty acids which have the potential to alter membrane lipid structure were fed to euthyroid and hypothyroid animals and the responsiveness of isolated papillary muscles to inotropic agents determined. The fatty acid profiles of phospholipids in cardiac sarcolemma were also determined to ascertain the influence of thyroid hormone and dietary lipids on myocardial contractility and to determine if thyroid status modifies fatty acid metabolism.

The results indicate that increasing membrane levels of n-3 fatty acids enhanced contractility in papillary muscles, irrespective of thyroid status, compared to n-6 fatty acids. This effect is apparently not due to increased ß-adrenoceptor density or binding affinity. Responses to forskolin were attenuated by n-3 fatty acids in euthyroid animals suggesting that enhancement of contractiltiy may be due to increased efficiency of coupling of receptors to post-receptor mechanisms not involving adenylate cyclase.

The sensitivity of papillary muscles to extracellular calcium was enhanced by n-3 relative to n-6 fatty acids but the maximal response was reduced compared to n-6 and saturated fatty acids. These effects apparently are not due to differences in myofilament calcium sensitivity in different thyroid states alone but may also involve

changes in membrane lipid environment induced by dietary fats. Increasing membrane levels of n-3 fatty acids enhanced the sensitivity of dihydropyridine-sensitive calcium channels to nifedipine. While the affinity of nitrendipine binding to these channels did not differ significantly in euthyroid animals, the binding site densities were dependent on thyroid status and dietary fat. These results suggest that n-3 fatty acids may affect the kinetics of L-type calcium channels, probably as a consequence of altered membrane lipid environment.

Thyroid state influenced the metabolism of n-6 fatty acids but not the incorporation of n-3 fatty acids into membrane phospholipids indicating that thyroid hormone plays an important role in determining the fatty acid composition of cardiac membrane phospholipids. Therefore, altered membrane lipid environment may be responsible for the depressed contractility associated with hypothyroidism.

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I. GENERAL INTRODUCTION

A. Thyroid hormone: Synthesis and actions

The thyroid gland, located below the larynx and anterior to the trachea, secretes thyroxine and triiodothyronine which are essential for growth and development as well as regulation of energy metabolism. Thyroid secretion is about 90% thyroxine and 10% triiodothyronine, the latter being present in blood in smaller quantities, has a shorter half-life and is about four times as potent as thyroxine. Thyroid hormone[1] is synthesized and stored in sufficient quantities within a glycoprotein (thyroglobulin) in the follicles of the thyroid gland to meet body requirements (Genuth, 1988). A deficiency in thyroid hormone causes significant reduction in basal metabolic rate while an excess results in elevation of this rate. Generally, thyroid hormone exerts its effect at nuclear receptors causing transcription of a large number of genes and increasing protein synthesis in virtually all cells of the body (Samuels *et al.*, 1988; Rohrer and Dillmann, 1988; Balkman *et al.*, 1992). The majority of the actions of thyroid hormone is believed to result from the enzymatic (Effron *et al.*, 1987; Hagiwura *et al.*, 1988; Syrovy, 1988), receptor (Ross *et al.*, 1988; Bahouth, 1991; Birk *et al.*, 1992), transport (Kim and Smith, 1985; Famulski *et al.*, 1988; Marsh, 1990) and other functions of the new proteins. In addition to the

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[1]In this context, thyroid hormone will refer to the iodinated thyronines, triiodothyronine (T_3) and thyroxine (T_4), synthesized by iodination of tyrosines and coupling of the resulting iodothyronines. T_4 is converted to T_3 intracellularly hence both are considered as one hormone.

nuclear effect, thyroid hormone also exerts direct effects on membranes (Davis *et al.*, 1989; Beleznai *et al.*, 1989). Hormonal receptors are present in plasma membranes and these mediate direct membrane effects independent of nuclear receptors. Such effects, which are prompt in onset and independent of new protein synthesis, include those on the transcellular fluxes of ions and substrates. The mechanism involves stimulation of adenylate and subsequent increase in the function of carriers in the plasma membrane. The membrane effects are intertwined with the action of adrenergic agents whose effects are also mediated at the plasma level. Multiple sites with high affinity for T_3 and T_4 have been found in membranes, nuclei as well as mitochondria (Segal and Sidney, 1986).

An important action of thyroid hormone is the promotion of general growth and development of the organism and general retardation can ensue from insufficient secretion of the hormone by the fetus. The hormone affects the fundamental processes of oxygen consumption and heat production, as well as the metabolism of carbohydrates, fats and proteins. Hence availability is determined by the body's changing caloric and thermal status. The cardiovascular system is particularlly sensitive to thyroid hormone action and increases in heart rate and force of contraction are observed at increased hormone levels above that required to maitain basal metabolic rate while the opposite effects are associated with reduced levels (Sharp *et al.*, 1985; Holubarsch *et al.*, 1985). The action of thyroid hormone is made complex by the fact that it exerts a permisive function, being necessary for and/or

synergistic with actions of other hormones, such as those of catecholamines[2] (Malbon *et al.*, 1988). Thus, potentiation of ß-adrenergic effects occur in the hyperthyroid state while reduction in these effects is associated with the hypothyroid state. This pattern of response to ß-adrenergic agents is demonstrable in the heart and changes in receptor properties correlate well with changes in sensitivity observed *in vivo*. In addition to these effects, there is evidence which indicates that the wholesale gene transcription induced by thyroid hormone results in increased synthesis of some lipogenic enzymes and their modifiers which cause changes in membrane lipid composition and subsequent modulation of enzyme-, transporter- or receptor-mediated activities (Mueller and Seitz, 1984). The metabolism of essential fatty acids and the thyroid state of animals have been shown to be related. Similarly, the basal metabolic rate of animals vary with the type of dietary fat fed and thyroid state (Hoch, 1988). Thus, thyroid hormone acts at multiple sites within the cell to produce a coordinated cellular response in which enhanced substrate availability proceeds simultaneously with increased energy metabolism and increased synthesis of specific proteins (Segal and Sidney, 1986).

B. Thyroid status and cardiac performance

The general mechanisms responsible for the cardiac effects of thyroid hormone include a direct and primary effect on heart cells which is mediated through

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[2]The catecholamines exert their stimulatory effects in heart, liver and adipose tissue by binding to cell-surface receptors and activating effector systems coupled via G proteins.

nuclear T_3 receptors, an interaction between the hormone and the sympathoadrenal system which enhances the sensitivity of the adrenergic system of the heart, and hormone-induced stimulation of oxygen utilization by peripheral tissues and the resultant hemodynamic demand which enhances cardiac contractility (Dillman, 1989). The positive inotropic effect is partly due to induction of myosin isoenzyme with high adenosine triphosphatase (ATPase) activity resulting primarily from the control of expression of genes for ventricular myosin isoenzymes (Effron *et al.*, 1987; Morkin, 1989). In addition to these, an increase in the number of Na^+/K^+-ATPase pump sites and subsequent increase in oxygen consumption, stimulation of Ca^{2+} uptake by the sarcoplasmic reticulum as well as stimulation of sarcolemmal Ca^{2+}-ATPase are also implicated (Kim and Smith, 1984; Rudinger *et al.*, 1984; Segal and Sidney, 1986).

Changes in the pattern of growth and development of the organism caused by thyroid dysfunction profoundly affects the functioning of the cardiovascular system. In the hyperthyroid state, the excessive secretion of thyroid hormone leads to increases in heart size as well as the rate and force of contraction (Feldman *et al.*, 1986). The enhancement of the chronotropic and inotropic effects may be due to an increase in the number or affinity of ß-adrenoceptors (Stiles and Lefkowitz, 1981; Stiles *et al.*, 1984) or changes in catecholamine-stimulated adenylate cyclase activity (Birk *et al.*, 1992; Pracyk and Slotkin, 1992). Consequently, output failure ensues probably as a result of the inefficient use of the chemical energy of adenosine triphosphate (ATP). On the other hand, the hypothyroid state produces a decreased heart size, rate and force of contraction as a result of the exposure of the heart to

subnormal amounts of thyroid hormone due to inadequate secretion by the thyroid gland (McDonough *et al.*, 1987; Liu and Gerdes, 1990). In addition to these effects, the hypothyroid state enhances responses to α-adrenergic agents while responses to ß-adrenergic agents are reduced (McNeill, 1987). Thus, changes in inotropy and chronotropy can be related to the hypertrophy or atrophy of the myocardium (Sanford *et al.*, 1978; Lompre *et al.*, 1984), altered ATPase activity resulting from different isoenzymic distribution patterns (Schwartz *et al.*, 1981; Dillman *et al.*, 1984) and biochemical changes that occur throughout the heart (Effron *et al.*, 1987; Morkin, 1989). In the latter case, the actual mechanism(s) responsible for these changes are unclear. However, mechanical and biochemical observations suggest that thyroid hormone may affect the mechanisms that trigger and control contraction (Mylotte *et al.*, 1985; Kim *et al.*, 1987; Rohrer and Dillman, 1988). Alteration in *in vivo* thyroid hormone levels can have remarkable effects on the basic physiological and mechanical properties of isolated muscle fibres measured *in vitro* (Di-Meo *et al.*, 1991).

C. Influence of thyroid status on lipid metabolism

Although the main role of thyroid hormone in metabolism is calorigenic[3], paradoxically, it also exerts major anabolic effects such as stimulation of lipogenesis (Hoch, 1988). The primary effect of the hormone triggers the expression of genes

[3]Thyroid hormone accelerates catabolic, exergonic oxidative reactions in most cells.

encoding a number of proteins involved in lipid metabolism and transport. In hyperthyroid rats, the plasma concentration of free and esterified cholesterol decreases compared to euthyroid controls accompanied by increases in total phospholipids and decreased cholesterol as well as cholesterol/phospholipid molar ratio in liver organelles (Ruggiero *et al.*, 1984). The fatty acyl composition of phospholipids are found, consistently to be thyroid hormone-dependent and changes in the fatty acyl proportions of one or more phospholipid classes or a shift in the phospholipid classes that have distinctive fatty acyl compositions have been reported (Hoch *et al.*, 1981). Phospholipids from the liver of hypothyroid rats, for example, have been shown to have lower contents of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) compared to controls and thyroid hormone treatment of such animals or euthyroid animals increased the percentage content of 20:4 while that of 18:2 is decreased, indicating an increase in Δ^6-desaturation activity (Faas and Carter, 1981). In other studies, the contents of 16:0, 18:1 and 18:2n-6 found in phospholipids from hypothyroid rat liver were above normal while that of 20:4n-6 was below normal with a concomitant appearance of dihomo-γ-linolenic acid (20:3n-6) suggesting defects in Δ^6- and Δ^5-desaturase activities (Faas and Carter, 1982). The content of 18:2n-6 in cardiac lipids is reduced in hyperthyroid rats compared to euthyroid controls while that of 22:6n-3 is increased, an indication that thyroid hormone affects desaturative biosynthesis of longer chain fatty acids.

Fatty acid desaturation is one mechanism by which membrane lipid composition can be regulated. The Δ^9-desaturase enzyme, for instance, converts

saturated fatty acids into monounsaturated species. The most important enzymes in the metabolism of n-6 and n-3 polyunsaturated fatty acids, however, are the Δ^6- and Δ^5-desaturases, which respectively convert 18:2n-6 into γ-linolenic acid (18:3n-6), the rate-limiting step in the conversion of 18:2n-6 to 20:4n-6 and, 20:3n-6 to 20:4n-6, the last step of arachidonic acid synthesis. These enzymes also catalyze the conversion of α-linolenic acid (18:3n-3) into eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) thus, n-6 and n-3 fatty acids compete for the same metabolic enzymes. A number of factors, such as saturated fat, trans-fatty acids, cholesterol and alcohol, as well as low energy and protein intakes, aging and diabetes are known to reduce Δ^6-desaturase activity (Brenner, 1989). The activity of this enzyme in humans is adequate for the conversion of α-linolenic acid to eicosapentaenoic acid and subsequently to docosahexaenoic acid.

Generally, thyroid hormone occupancy of specific receptor sites triggers a cascade of events such as expression and modification of specific enzymes involved in lipid mobilization and metabolism, alterations in membrane cholesterol content and fatty acyl composition of membrane phospholipids and the subsequent modulation of a host of enzyme, transporter and receptor systems (Hoch, 1988). The major determinants of biomembrane dependence of enzyme activity are cholesterol/ phospholipid molar ratio, phospholipid composition, degree of fatty acyl unsaturation and lipid/protein ratio (Ruggiero *et al.*, 1984). Changes in fatty acid composition of membrane phospholipids can result in alterations in bulk membrane lipid motional properties and hence changes in properties of membrane enzymes (Stubbs and

Kisielewski, 1990). Similarly, membrane permeability to cations also changes leading to the regulation of intracellular effectors such as cell membrane adenylate cyclase as well as cell and organelle Ca^{2+} pumps. Calcium transport is believed to be dependent on the type of phospholipid head group and fatty acyl composition of the phospholipid molecule as well as the fluidity of the membrane (Navarro *et al.*, 1984; Stubbs and Smith, 1984). Thyroid hormone influences the fatty acid composition of phospholipids in a number of organelles in different tissues and hypothyroidism has been shown to alter this and the properties of the Ca^{2+} pump in rat skeletal muscle sarcoplasmic reticulum (Simonides and van Hardeveld, 1987). This effect is thought to be related to the fatty acyl composition of membrane phospholipids since this has been shown to be influenced by thyroid hormone in organelles from a number of tissues (Hoch, 1982; Ruggiero *et al.*, 1984).

D. Antithyroid agents

The thyroid gland, under normal conditions, secretes sufficient amounts of thyroid hormone to maintain basal metabolic rate and to facilitate normal growth and development. Reduction in the activity of the gland under euthyroid and hyperthyroid conditions can be achieved by blocking hormone synthesis with antithyroid agents, by modification of tissue responses to the hormone itself or by total destruction[4] of the gland. For experimental purposes, the antithyroid agents are most widely used to suppress thyroid activity in order to investigate the physiological

[4]Radiation treatment or surgical removal.

changes that ensue (Pracyk and Slepetis, 1984; Pracyk and Slotkin, 1992). The thionamides, propylthiouracil[5] and methimazole, are the major drugs used clinically to supress thyroid gland activity. These drugs prevent the synthesis of thyroid hormone by inhibiting the thyroid peroxidase-catalyzed reactions, thus blocking iodine organification as well as the coupling of iodinated tyrosines to form the iodothyronines (Engler *et al.*, 1982; Cooper, 1984). Since synthesis rather than the release of the hormone is affected, the onset of the action of these agents is slow and depletion of the stores of iodinated thyroglobulin requires three to four weeks.

E. Mechanism of myocardial contraction

Excitation-contraction coupling

The cardiac sarcolemma, a complex structure composed of two distinct layers[6], is the locus of several ion and substrate transport systems and is therefore intimately involved in the regulation of cardiac contraction and relaxation. It contains different hormone and drug receptors and provides signals for the modulation of metabolism and function, particularly the control of Ca^{2+} influx/efflux in cardiac cells. Myocardial contraction and relaxation is related to the regulation of intracellular calcium which depends on the excitation of the cell in a complex manner

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[5]This drug also inhibits the peripheral deiodination of T_4 to T_3.

[6]An external layer, made up of glycocalyx, forms the basement membrane and the plasma membrane on the cytoplasmic side.

(Kirby *et al.*, 1989; Bers, 1991).

Force generation in the myocardium occurs when trigger Ca^{2+} enters the cell to induce Ca^{2+} release from the sarcoplasmic reticulum, which then activates troponin C to cause interaction between actin and myosin filaments utilizing the energy from ATP hydrolysis (Winegrad, 1986). The sensitivity of the contractile system to Ca^{2+} is influenced by the size of the muscle fibre and tension is directly related to the interfilament surface between the sets of thick and thin filaments (Brady, 1989). Depolarization of the sarcolemma causes calcium influx via slow channels to trigger calcium release from the subsarcolemmal cisternae of the sarcoplasmic reticulum and raising the cytosolic Ca^{2+} concentration (Gibbons, 1986; Lullman and Ziegler, 1987; Bers, 1991). Thus, Ca^{2+} from a source which is in immediate contact with the extracellular calcium ion concentration is required for the initiation of contraction. Calcium binding to troponin C and the subsequent activation of the sliding system by the Ca^{2+}-troponin C complex then leads to contraction whereas relaxation results from the active uptake of Ca^{2+} from the cytosol by the sarcoplasmic reticulum (Winegrad, 1986; Noble and Drake-Holland, 1987; Ford, 1991). Influences of passive or active transport of ions through the sarcolemma may therefore affect cellular calcium metabolism and hence the inotropic state of cardiac muscle (Reiter, 1988). Regulation of calcium release from the sarcoplasmic reticulum in the intact cell is complex but very important since this event is probably the largest source of calcium contributing to the activation of the myofilaments in mammalian cardiac muscle (Bers, 1991). The amount of Ca^{2+} released from the sarcoplasmic

reticulum upon excitation is graded with the amount of "trigger" calcium. However, at high calcium concentration, the calcium-induced calcium release is inhibited or inactivated. Since contraction is governed by calcium transients, modification of the strength of contraction can be achieved by controlling the concentration of free Ca^{2+} in the cytosol, obtained after excitation or changing the calcium sensitivity of myofilaments. Hence, the main sites of contractile control are the sarcolemma which participates in Ca^{2+} uptake (via slow channels) and elimination (via the Na^{+}/Ca^{2+} exchanger) as well as intracellular compartments such as sarcoplasmic reticulum (Ca^{2+} uptake and storage), mitochondria (Ca^{2+} buffer), Ca^{2+}-binding "modulator" proteins (e.g. calmodulin) and myofilaments (Wier, 1990).

The ß-adrenergic receptor pathway

The contractile effects of catecholamines in the myocardium are mediated by ß-adrenergic receptors present in the sarcolemma with subsequent modulation of ion channels or direct phosphorylation of contractile proteins (Bristow *et al.*, 1990). The ß-adrenergic pathway mediate the powerful positive inotropic effects of norepinephrine, released from nerve terminals and circulating epinephrine as well as therapeutically administered ß-agonists. The biological signal produced, when catecholamines occupy the ß-adrenoceptor, is transduced, amplified and regulated by a family of guanine nucleotide-binding (G) proteins which serve both stimulatory and inhibitory functions. The major biochemical effector of ß-adrenoceptors is adenylate cyclase, however, in myocardial cells, receptors may be coupled through

G proteins directly to ion channels that regulate inotropic and electrophysiological effects (Birnbaumer *et al.*, 1990). Thus, although G proteins modulate ion channels via cyclic adenosine 5'-monophosphate (cAMP), they can also modulate specific channels via non-cAMP mechanisms (Bristow *et al.*, 1990; Katz, 1990). Generally, stimulation of ß-adrenergic receptors results in increased levels of cAMP which exerts complex actions, such as phosphorylation of sarcolemmal proteins (Presti *et al.*, 1985), on myocardial cell function thus enhancing the force and rate of contraction.

Cyclic AMP influences excitation-contraction coupling, tension development and shortening by contractile proteins and relaxation from augmented contraction (Sperelakis, 1984). In human ventricular myocardium, augmentation of contractility is exclusively dependent on stimulation of ß-adrenergic receptors since α-adrenergic receptor density is extremely low. On the other hand, rat myocardium has a mixture of both receptors which are coupled to adenylate cyclase and muscle contraction (Katz, 1990). Thus, any variations in the properties of the contractile proteins as well as cytoplasmic Ca^{2+} levels produced by action potentials, activity of the ß-adrenoceptor/adenylate cyclase system or the sarcoplasmic reticulum will affect tension development in the myocardium.

F. The influence of dietary fatty acids on cardiovascular function

Essential fatty acids

Polyunsaturated fatty acids of the n-6 and, to a greater extent, the n-3 classes have been suggested to be useful in protecting against certain cardiovascular diseases (Knapp, 1989). Humans are unable to synthesize fatty acids with double bonds more distal from the carboxyl end of the acid than the ninth carbon atom and cannot transform n-3 and n-6 fatty acids into one another. As a consequence linoleic acid (18:2) and linolenic acid (18:3) must be ingested and are therefore termed "essential" fatty acids. The most widely available n-3 fatty acid (α-linolenic) in common diets comes from plants and is available in adequate quantities in vegetable oil products, however, those from marine sources, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA), have longer carbon chains and are more highly polyunsaturated. Some fish oils contain higher total n-3 (20-25% of total fatty acids) fatty acids compared to canola oil which typically contains α-linolenic at only 10% of totai fatty acids (Hunter, 1990). Ingested essential fatty acids can undergo elongation and desaturation to other fatty acids which are subsequently incorporated into membrane phospholipids[7] where they have structural functions. Such fatty acids affect membrane viscosity and permeability hence the activity of membrane proteins. They are metabolized to eicosanoids such as prostaglandins, leukotrienes or to docosanoids, the oxidized metabolites of C_{22} fatty acids (Drevon, 1992). Eicosanoids

[7]The fatty acids are found mostly in position 2 (*sn-2*).

derived from EPA are less potent than the corresponding compounds derived from arachidonic acid, hence the vascular and inflammatory effects of the former are less severe (Bjørneboe *et al.*, 1987). In addition to these functions, the polyunsaturated fatty acids are important modulators of triglyceride and cholesterol metabolism. The n-3 and n-6 fatty acids share common enzymes for some of their metabolic pathways, however, the n-3 fatty acids usually have higher affinities for the elongation and desaturation enzymes than do the n-6 fatty acids (Drevon, 1992). Since n-3 and n-6 fatty acids compete for the same metabolic enzymes, large amounts of n-6 fatty acids in diet could interfere with the formation of EPA and DCHA from α-linolenic acid. It has therefore been suggested that the intake of essential fatty acids should be 3% of total energy intake and an optimal ratio of n-6:n-3 fatty acids of 4-10:1 has been recommended (Neuringer and Connor, 1986). Diets containing higher ratios (e.g. 50:1) could therefore result in n-3 fatty acid defficiency.

Vascular effects of dietary fatty acids

Epidemiological data from a number of studies indicate a low rate of myocardial infarction among Greenland Eskimos compared to Danes (Bang *et al.*, 1976; Dyerberg and Bang, 1979; Dyerberg and Jorgensen, 1982). Associations between these findings and diets have been established and it was noted that Eskimos consumed diets rich in long-chain n-3 polyunsaturated fatty acids, mostly EPA and DCHA, whereas the Danish diets were rich in saturated fatty acids similar to those found in Western diets. Similar studies in Japanese fishing communities have

also established a link between low mortality rates from coronary heart disease and high intake of fish (Hirai *et al.*, 1980). Based on these early studies, it has been suggested that polyunsaturated fatty acids have beneficial effects in some cardiovascular disease states (Harris, 1989). Until recently, most of the studies elucidating the role of such fatty acids in modulating cardiovascular activity have been limited to the effects of n-6 fatty acids found in vegetable oils. In particular, blood pressure lowering (Iacono *et al.*, 1982; Heagerty *et al.*, 1986) and reduction in events leading to vascular occlusion (Simpson *et al.*, 1982) have been reported. Kudo *et al.* (1988) have demonstrated that n-6 fatty acid deficiency can lead to increased vascular sensitivity to vasoconstrictor stimuli as a result of augmentation of cyclooxygenase activity in vessel wall, indicating the importance of this fatty acid in maintaining the normal integrity of blood vessels. Reports on n-3 fatty acids, derived from marine oils, have also shown similar beneficial effects (Norris *et al.*, 1986; Dyerberg *et al.*, 1986; Rodgers *et al.*, 1987; Fann *et al.*, 1989). The reduction in platelet aggregation by n-3 fatty acids has been reported (Kristensen *et al.*, 1989). Engler (1992) has demonstrated relaxant effects of n-3 fatty acids in rat thoracic aorta and suggested the involvement of intracellular calcium mechanisms. Other studies, however, have failed to demonstrate any significant effects of either n-3 or n-6 fatty acids on blood pressure (Sanders *et al.*, 1981; von Houwelingen, 1987). An extensive review of the effects of fish oil on plasma lipid and lipoprotein metabolism has been provided by Harris (1989).

A number of human studies, employing large amounts of fish oil, support the

hypolipidemic and ultimately the antiatherogenic properties of n-3 fatty acids. However, the overall effect on blood pressure varied from one population to the other (Knapp, 1989). Thus, it appears the effects of dietary fatty acids are not specific. Factors such as other dietary constituents, genetics and environment may also be important. Despite the widespread variations in data obtained from human studies, laboratory studies have shown that n-3 and n-6 fatty acids play some role in modulating cardiovascular function. There is abundant evidence to support the purported beneficial effects of dietary lipids in thrombosis, atherosclerosis and coronary heart disease (Harris, 1989). However, their effect on myocardial contraction is of interest since changes in membrane fatty acid composition induced by these fatty acids may influence membrane events leading to force generation in the heart.

Membrane lipid composition and adrenergic function

The inotropic effect is essentially a membrane phenomenon so that any changes that occur in the composition of cardiac membranes will affect force development in the myocardium (Wince and Rutledge, 1982). The release of norepinephrine from sympathetic nerves to the heart increases both inotropic and chronotropic actions which are the result of interaction of this catecholamine with ß-adrenoceptors located in myocardial cell membranes. The termination of norepinephrine effects occurs partially by uptake into sympathetic nerve terminals[8]

[8]Neuronal uptake or uptake$_1$.

and into effector cells[9], processes which are mediated by mobile carriers present in the lipid bilayer (Paton, 1976; Trendelenburg, 1991), and may be subject to modification. Alteration in the acyl composition of membrane phospholipids could occur by an indirect[10] or direct exposure to diets supplemented with saturated and unsaturated fatty acids. Perturbations in the membrane environment influences the properties of membrane-bound proteins hence dietary fatty acid treatment can produce changes in the function of certain membrane-associated enzymes and other proteins involved in the sequence of events linked with the cardiac actions of catecholamines. Myocardial sympathetic neurons are poorly developed at birth (Tanaka and Shigenobu, 1990), and therefore such functions as storage and release of norepinephrine, actions that involve interaction of the transmitter with a macromolecule oriented within the lipid environment of the neuronal membrane, must progress through stages of postnatal development. Thus, adrenergic function could be modified as a consequence of changes in membrane lipid composition. It has been observed that chronic exposure to high levels of either saturated or unsaturated fatty acids can modify norepinephrine content, storage and release processes as well as postjunctional effects (Semafuko *et al.*, 1987).

Effects of dietary fatty acids on cardiac inotropy and chronotropy

Wince *et al.* (1984; 1985) have studied the effect of sunflower (n-6 fatty acid)

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[9]Extraneuronal uptake or $uptake_2$.

[10]Placental transfer and maternal milk.

and coconut (saturated fatty acid) oils on contractile responses in isolated paced left atria from rats fed diets rich in these fatty acids and concluded that modification of adrenergic responsiveness by these diets was due to functional alteration in the adenylate cyclase/cAMP system distal to the enzyme. In a similar study, the neuronal accumulation of [^{3}H]norepinephrine was found to be hampered by sunflower oil while chronotropic responses to norepinephrine and epinephrine were increased by coconut oil indicating that sympathetic nerve regulation of myocardial function can be modified during development by changing the dietary fatty acid composition (Wince and Rutledge, 1982). A number of possible mechanisms by which such changes can be effected include (i) changes in the density and/or properties of ß-adrenoceptors in cardiac cell membranes (McLennan *et al.*, 1987b), (ii) changes in fatty acyl composition of myocardial cell membranes which can alter the catalytic ability of adenylate cyclase or its coupling with ß-adrenoceptors (McMurchie *et al.*, 1987), (iii) changes in the membrane potential as a consequence of alteration in the tissue distribution of Na^+ and K^+ through effects on Na^+/K^+-ATPase, (iv) changes in the Ca^{2+} translocation mechanism in the myocardium through effects on Ca^{2+}-dependent ATPase, Na^+/Ca^{2+} exchange or the alteration in the number or state of slow calcium channels in the sarcolemma which will reduce or increase the influx/efflux of Ca^{2+} into myocardial cells or alteration in the ability of the sarcoplasmic reticulum to regulate cytosolic Ca^{2+} concentration, (v) changes in the size of the ATP pool available for contraction through effects on mitochondrial membranes, or (vi) alteration of the activity of G proteins in receptor coupling.

Using isolated atria and whole heart, it has been shown that linoleic acid-rich diet produced age-dependent increases in contractile force, coronary flow rate and prostaglandin release as well as reduction in heart rate compared to hearts from animals on linoleic acid-deficient diet (Hoffman *et al.*, 1982; Wince *et al.*, 1984; 1985). In a similar study by Charnock *et al.* (1985), it was shown that a saturated fat diet increased positive inotropic responses to Ca^{2+} and the incidence of spontaneous tachyarrhythmias under catecholamine stress in papillary muscles from adult rats whereas sunflower seed oil (contain n-6 fatty acids) prevented these changes. Long-term feeding of sheep fat (saturated), sunflower seed oil or tuna fish oil (polyunsaturated fatty acids) significantly altered Ca^{2+}-induced contractility and susceptibility to isoproterenol-induced dysrhythmias in rat papillary muscles (McLennan *et al.*, 1987a). Similar studies in marmoset monkeys suggest a possible modification of stimulus-response coupling and therefore nutritional interventions can modify responses to cardioactive drugs (McLennan *et al.*, 1987b). These and other studies have shown that dietary fat is an important modulator of cardiac function and polyunsaturated fatty acids, in particular, exert protective effects against experimentally-induced cardiac diseases in laboratory animals (McLennan *et al.*, 1989 and 1990).

Effects of dietary fatty acids on adenylate cyclase

The influence of dietary lipids on the activity of adenylate cyclase has been studied in both the rat and the marmoset monkey and it was found that a saturated

fat diet significantly increased the cholesterol to phospholipid ratio in cardiac membranes as well as the basal and catecholamine-stimulated adenylate cyclase activity (McMurchie *et al.*, 1987 and 1988; Wince *et al.*, 1987). Binding studies employing ^{125}I-labelled (—)iodocyanopindolol revealed a significant reduction in the number of ß-adrenoceptors in heart ventricular membranes from animals fed the saturated fat diet compared to those fed polyunsaturated fat diets. These findings imply a modulation of the ß-adrenergic receptor/adenylate cyclase system of the heart as a consequence of alteration in the membrane cholesterol to phospholipid ratio. The mechanism probably involves down-regulation of receptors in the plasma membrane. The increase in catecholamine-stimulated adenylate cyclase activity was shown to have a positive correlation with the membrane cholesterol/phospholipid ratio while the reduction in ß-adrenergic receptor number had a negative correlation with same (McMurchie *et al.*, 1987). Thus, the activity of the membrane-associated receptor-enzyme complex can be influenced by dietary lipids particularly those that alter membrane cholesterol to phospholipid ratio and presumably membrane physico-chemical properties.

Using forskolin[11], 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) and dibutyryl cAMP (a derivative of cAMP that penetrates myocardial cells and mimics the biolgical effects of cAMP), it was deduced that the effect of dietary

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[11]A diterpine of the Indian plant *Coleus forskohlii* is a positive inotropic agent at low doses. This effect seems to be due to a direct stimulatory action at the catalytic unit of the sarcolemmal adenylate cyclase. The exact mechanism is unknown but may be related to a cAMP-dependent increase in Na^+ permeability that results in an indirect augmentation of Ca^{2+} release.

fatty acids is at a level proximal to the formation of cAMP (McMurchie *et al.*, 1987).

J. Muscarinic cholinoceptor-mediated inotropy in the heart

In the mammalian myocardium, different biochemical effects and mechanical responses to muscarinic agonists are mediated by a homogeneous population of M_2 muscarinic cholinergic receptors present in the sarcolemma (Doods *et al.*, 1987). In the rat and guinea pig, cardiac muscarinic receptor activation elicits both negative and positive inotropic effects (Korth and Kuhlkamp, 1987; Eglen *et al.*, 1988; Kenakin and Boselli, 1991). The negative inotropic effect, which is sensitive to pertusis toxin and is accompanied by inhibition of adenylate cyclase activity, is due to activation of potassium channels by a G protein (Yatani *et al.*, 1987; Birnbaumer *et al.*, 1990; Breitwieser, 1991). The positive inotropic effect, on the other hand, which is insensitive to pertusis toxin and mediated via a distinct G protein is apparently due to an enhanced hydrolysis of phosphoinositides in the plasma membrane (Mizushima *et al.*, 1987; Kohl *et al.*, 1990). The exact mechanism involved in this process is unclear but thought to be dependent on the elevation of cytosolic levels of inositol-1, 4, 5-trisphosphate (IP_3) and the subsequent release of Ca^{2+} from sarcoplasmic reticulum (Nosek *et al.*, 1986; Lochner and Bester, 1989). Such findings have led to the suggestion that two subtypes of M_2 ($M_{2\alpha}$ and $M_{2\beta}$) cholinoceptors which mediate adenylate cyclase inhibition and phosphoinositide hydrolysis, respectively exist in the myocardium (Woodcock *et al.*, 1987; Mizushima *et al.*, 1987; Zhou *et al.*, 1988; Ford *et al.*, 1992). Thus, the different muscarinic receptor-mediated myocardial responses

may be attributable to the presence of different receptors or the differential regulation of the same receptor by different G proteins. The dual responses are concentration-dependent with negative inotropy observed only at low agonist concentrations while the positive inotropic effect is elicited at higher concentrations indicating that the agonists may be acting at a single receptor site but with different intrinsic efficacies (Kenakin and Boselli, 1991).

Hawthorne and Simmonds (1989) have suggested that muscarinic receptor-mediated inhibition of adenylate cyclase is more important in the heart than phosphoinositide hydrolysis since the latter is slow in onset and occurs only at much higher agonist concentrations. They argued that since muscarinic drugs have negative inotropic effects, induction of an increase in cytosolic calcium concentration by these agents would be an anomally and suggested instead the involvement of protein kinase C activation by diacylglycerol, released from phosphoinositides, as a more plausible mechanism. Thus, the muscarinic "PI" effect may also lead to the activation of protein kinase C which in turn can modulate myocardial contractility. There are several protein kinase C substrates in the heart but the low activity of the enzyme may not be sufficient to support such a mechanism. However, the hydrolysis of phosphoinositide-1,4-bisphosphate by phospholipase C is a primary event in the mechanism of action of hormones, neurotransmitters or other regulatory molecules and therefore seems most likely to be responsible for the positive inotropic effect (Berridge, 1987).

K. Papillary muscles

The papillary muscles are a group of muscles attached to the vanes of the atrio-ventricular valves and contract when the ventricular walls contract. These muscles pull the vanes of the valves inward, toward the ventricles, to prevent their bulging too far backward toward the atria during ventricular contraction (Berne and Levey, 1988). Paralysis in the papillary muscles causes the atrio-ventricular valves to bulge backwards and this may result in leaks or lethal cardiac incapacity.

In order to relate results from experiments on isolated cardiac tissues to *in situ* myocardial dynamics, it is useful to obtain direct measurements of the capacity for linear force development of the ventricular musculature. This will ensure that forces recorded from isolated muscle preparations compare reasonably with those developed by the intact muscle. In the heart, derived linear force per unit cross-sectional area is complicated by distribution in muscle fibre orientation and length as well as other factors which may vary from one myocardial site to another. The excised papillary muscle preparation is therefore most suitable for studies of mechanical function due to its simple shape and approximately parallel alignment of muscle fibres (Myeburg *et al.*, 1985). The extremely thin muscle preparation allows for constant force development which roughly approximates that developed by the muscle *in situ*. Large oxygen and diffusion gradients exist across the tissue thus, adequate perfusion of cells at the core of the preparation can be achieved. A unique advantage of this preparation is that variables of afterload and of muscle length can be controlled by isometric recording techniques and the length can be set at an

optimal value for force development (Abel and McCutcheon, 1979).

L. Background to the present study

Thyroid hormone is an important regulator of cardiac performance, modulating both chronotropic and inotropic responses (Gay *et al.*, 1988; Liu and Gerdes, 1990). Hypothyroidism depresses myocardial contractility partly because of decreased responsivenes to ß-adrenergic stimulation (Stiles *et al.*, 1984; Birk *et al.*, 1992; Pracyk and Slotkin, 1992). This effect may be due to alteration in the lipid environment of the ß-adrenoceptor/adenylate cyclase complex induced by inadequate levels of thyroid hormone.

While dietary fatty acids are determinants of cardiac membrane fatty acyl composition (Charnock *et al.*, 1985) and appear to modulate contractility (Wince *et al.*, 1987; McLennan *et al.*, 1990), it is not clear how n-6 and n-3 polyunsaturated fatty acids alter the physico-chemical properties of these membranes. Moreover, to date information regarding the role of thyroid hormone in modulating the incorporation of these fatty acids into cardiac membranes is lacking. The present study was therefore undertaken to determine the extent to which thyroid hormone regulates membrane lipid metabolism and, ultimately the lipid composition of the sarcolemma. It is hypothesized that "the influences of thyroid state on myocardial contractility are due to changes in fatty acid metabolism and consequently membrane fatty acid composition".

The approach taken was to first challenge cardiac membranes with dietary

fatty acids that have the potential to alter membrane structure. Inotropic responses to various ß-agonists were then determined in euthyroid and hypothyroid animals to ascertain the effect of altered membrane environment on the ß-adrenoceptor/ adenylate cyclase complex. Furthermore, the influence of such change on myocardial calcium sensitivity and dihydropyridine-sensitive calcium channels was examined. Finally, the effect of thyroid hormone in regulating the lipid composition and hence the lipid environment of cardiac plasma membrane was determined to see if the changes could provide an explanation for altered myocardial contractility associated with hypothyroidism.

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II. THYROID STATUS AND DIETARY FATTY ACIDS AFFECT TENSION DEVELOPMENT IN RAT MYOCARDIUM

A. Introduction

The heart is a major target organ for thyroid hormone action and changes in thyroid status may modulate cardiac function by a combination of direct hormonal effects, alterations in the responsiveness of the cardiac sympathoadrenal system and hemodynamic effects generated in the periphery (Dillmann, 1989). Chronic changes in thyroid hormone levels result in alterations in myocardial contraction and relaxation (Sharp *et al.*, 1985; McDonough *et al.*, 1987; Gay *et al.*, 1988; Liu and Gerdes, 1990), with hypothyroidism reducing various indices of cardiac performance, such as size, chronotropy and inotropy (Sharp *et al.*, 1985; Holubarsch *et al.*, 1985;).

Thyroid hormone is known to modulate tension development in myocardium primarily via effects at the cell nucleus and subsequent protein synthesis (Klein and Levey, 1984; Balkman *et al.*, 1992). As a result, depressed performance in the hypothyroid state is due in part to the predominance of V_3 myosin isoenzyme with low ATPase activity which results in a decreased velocity of contraction (Morkin, 1989). At the same time, the hypothyroid heart also shows enhanced responses to α-adrenergic agents and reduced responses to ß-adrenergic agents (McNeill, 1987). These changes do not appear to be the result of altered ß-adrenoceptor number or interconversion between ß-adrenoceptors and α-adrenoceptors (Kunos, 1981). Thus, the influence of thyroid hormone on cardiac function cannot be due to a single factor

and a satisfactory mechanism to explain the altered adrenergic activity is still lacking. A possible explanation may be the influence of membrane fatty acyl composition on the ß-adrenoceptor/adenylate cyclase complex. Alterations in thyroid hormone levels induce changes in fatty acyl composition of membrane phospholipids in heart (Shaw and Hoch, 1977), liver (Hoch *et al.*, 1981) and skeletal muscle (Simonides and van Hardeveld, 1987). In the heart, hypothyroidism reduces levels of oleic acid and increases those of linoleic acid but does not alter the total unsaturation index. On the other hand, thyroid hormone appears to influence fatty acyl desaturation and elongation in liver resulting in changes to total unsaturation in the hypothyroid state. In rabbit cardiac sarcolemma, hyperthyroidism decreases while hypothyroidism increases the cholesterol to phospholipid molar ratio (Szymanska *et al.*, 1991).

Several studies have demonstrated that dietary fatty acids can modulate both inotropic and chronotropic events in the heart, presumably by altering membrane lipid composition (McLennan, 1987b; McLennan *et al.*, 1989; 1990). Diets high in n-6 fatty acids enhance the activity of the cyclic AMP/adenylate cyclase system with no change in ß-adrenergic receptor binding charateristics (McMurchie *et al.*, 1987; 1988). Thus, regulation of the ß-adrenoceptor-mediated mechanical responses of the heart by thyroid hormone may be related to alterations in membrane fatty acid composition. Dietary fatty acids are determinants of membrane fatty acyl composition, however, their effect on membrane properties in cardiac muscle is unclear. In addition, there is inadequate information on the role of thyroid hormone in modulating the fatty acid composition of cardiac membranes.

The present study was therefore undertaken to determine how altered membrane fatty acid composition, produced by dietary manipulation, affects the ß-adrenoceptor/adenylate cyclase complex hence tension development in rat myocardium and how this process is modulated in the hypothyroid state.

B. Materials

Forskolin, 6-*n*-propyl-2-thiouracil and the bitartrate salts of (—)-isoproterenol, (—)-norepinephrine, (—)-epinephrine and (±)-propranolol were purchased from Sigma Chemical Co., St. Louis, MO and (—)-[Propyl-1,2,3-^{3}H]-dihydroalprenolol (2234.8 GBq/mmol) was from NEN, Boston, MA. All other chemicals used in the study were of the purest grade available commercially. Basal mix diet was from Tekland Diets, Madison, WI. Safflower oil, linseed oil, olive oil and beef tallow were purchased on the open market, and fish oil (EP-28) was from Nishin Co., Tokyo, Japan.

C. Methods

Animals and diets

Weanling male Sprague-Dawley rats (40-50 gm), obtained from the University of Alberta Health Sciences Laboratory Animal Services were divided randomly into two groups and fed diets high in n-6 or n-3 fatty acids for four weeks. Each diet group was divided into two with one group serving as euthyroid controls, while the second group was rendered hypothyroid by continuous administration of 6-*n*-propyl-2-thiouracil (PTU, 0.05% solution) in drinking water. The fatty acid composition of the diets was varied to include, either predominantly n-6 polyunsaturates from vegetable oils or n-3 polyunsaturates derived from fish oils (Table II-1). Animals were fed *ad libitum* and body weights monitored on a bi-weekly basis. Diets were stored at 4 °C

Table II-1. Fatty acid composition of diets.

Fatty acid [C:d(n-x)]*	Composition of diet (mol %)	
	n-3	n-6
14:0	4.0	1.5
16:0	15.8	14.5
16:1	5.6	-
17:0	1.7	0.6
18:0	12.8	18.9
18:1(n-7)	26.6	11.1
18:1(n-9)	2.3	0.6
18:2(n-6)	12.7	51.8
18:3(n-3)	2.6	1.4
20:5(n-3)	11.0	-
22:6(n-3)	4.3	-
Σ *Polyunsaturates*	*30.6*	*53.1*
Σ *Saturates*	*34.9*	*35.2*
Σ *n-6*	*12.7*	*51.8*
Σ *n-3*	*17.9*	*1.4*
P/S	*0.9*	*1.5*
n-6/n-3	*0.7*	*37.0*

**This nomenclature refers to the chain length of the fatty acid (C), the number of double bonds (:d) and the position of the first double bond (n-x) relative to the carbon attached to the methyl group of the molecule.*

and feeding cups replenished with fresh diet daily after discarding any uneaten portion.

All diets contained a fat-deficient basal mix of the following composition (gm/kg): starch, 408; casein, 270; non-nutritive cellulose, 50; vitamin mix, 10; mineral mix, 50.85; inositol, 6.25; choline chloride, 2.75 and L-methionine, 2.5. The lipid composition of the diets was achieved by the addition of 20% lipid (w/w) formulated from combinations of beef tallow, safflower oil, linseed oil, olive oil or fish oil to the basal mix. The polyunsaturated (P) to saturated (S) ratio of the n-3 diet reflects the Canadian Heart Association recommendation for human fat consumption (i.e P/S = 1.0).

Contractility studies

Animals were injected intraperitoneally with sodium pentobarbital (50 mg/kg) to induce anesthesia. The thoracic cavity was then opened and the heart rapidly excised and placed in freshly-oxygenated, ice-cold Krebs-Henseleit medium of the following composition (in mM): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4$, 0.6; KH_2PO_4, 1.2; $CaCl_2$, 2.5; D-glucose, 11 (pH 7.4, 22 °C). Papillary muscles were dissected from the left ventricle and placed in fresh medium which was continuously aerated with 95% O_2:5% CO_2. Muscle preparations were then mounted in 4-ml organ baths containing continuously oxygenated medium at 37 °C. One end of the tissue was attached to a tissue holder and the other end to a *Grass FT.03* force displacement transducer connected to a *Grass Model 7E Polygraph* four-channel

recorder and paced at 1 Hz (*Grass SD9 stimulator*) through bipolar platinum electrodes. Each stimulus consisted of a square wave pulse (8 V) at 10 msec duration. Preparations were maintained under resting tension of 500 mg and equilibrated for 30 min, after which they were stretched until the muscle length that gave maximal developed tension (L_{max}) was attained. Muscle length was then reduced until developed tension was equal to 70% of the L_{max} and the tissues allowed to equilibrate for a further 30 min. During the equilibration period, the medium was replaced at 10 min intervals. The basal tension developed, in response to electrical stimulation, was recorded before obtaining inotropic responses to agonists.

Responses to cummulative concentrations of drugs were obtained by increasing the concentrations in the baths by a factor of 3 whiles the previous concentration remained in contact with the tissue. Each concentration (10^{-9} - 4.4 x 10^{-5} M) was left in contact with preparations for 2-3 min. Since more than one drug was tested on each preparation, tissues were allowed to rest for 15 min after each assay. At the end of the experiment, the portion of papillary muscle between the securing ligatures was cut, blotted and weighed. Contractions are expressed as per cent of basal developed tension after normalizing data to force per tissue weight (N/mg). Responses of tissues to isoproterenol (ISO), epinephrine (E), norepinephrine (NE) and forskolin (FSK) were obtained for comparison. Fresh solutions of catecholamines (10 mM) were prepared daily in 0.9% saline, containing 0.05% sodium metabisulfite as an anti-oxidant, and diluted serially to desired concentrations. A stock solution of 100 mM FSK was made up in dimethylsulfoxide

(DMSO)/ethanol (50:50) solution and diluted serially to obtain the working solutions. Higher concentrations of FSK (10 and 1 mM) were prepared by diluting the stock in DMSO/ethanol solution and lower concentrations were prepared by diluting in saline. The vehicle in the former case had no measurable contractile effect on the tissues. The stock solution of FSK was stored at -20 °C.

Preparation of cardiac plasma membrane

Crude cardiac plasma membranes were prepared from ventricles by a modification of the method described by Tanaka and Shigenobu (1990). After i.p. injection of sodium pentobarbital (50 mg/kg), hearts were rapidly removed and rinsed in Krebs-Henseleit medium. The ventricles were minced in 10 volumes (w/v) of ice-cold Tris-HCl buffer of the following composition (in mM): sucrose, 250; EDTA(Na_2), 1; $MgCl_2$, 1 and Tris-HCl, 20 (pH, 7.4) and then homogenized (Kinematica polytron; 50% maximum speed, 10 sec). The homogenate was centrifuged at 1,000 x g for 3 min and the supernatant filtered through four layers of gauze. The resultant pellet was resuspended in 5 ml of Tris-HCl buffer and centrifuged at 800 x g for 10 min. The supernatants from the two centrifugation procedures were combined and centrifuged at 8,700 x g for 15 min. The resulting supernatant was then centrifuged at 48,000 x g for 30 min and the crude plasma membrane pellet resuspended in 50 mM Tris-HCl buffer (pH, 7.4) and stored at -70 °C and used for ligand binding assays.

[^{3}H]Dihydroalprenolol binding assay

The site-specific, equilibrium binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA), a ß-adrenergic receptor antagonist, was measured by a filtration method similar to that described by Tanaka and Shigenobu (1990). Briefly, crude plasma membrane preparations were incubated with graded concentrations of [^{3}H]DHA in the presence or absence of 10 μM propranolol in a total volume of 1 ml Tris-HCl (50 mM; pH, 7.4, 22 °C). Each assay consisted of duplicate incubation mixtures of 100 μl of [^{3}H]DHA (0.05 - 2.0 nM), 500 μl of crude plasma membrane (100 - 200 μg protein) and 400 μl of Tris-HCl (total binding) or propranolol (non-specific binding) in 1.5-ml microcentrifuge tubes and incubated at 22 °C for 1 hr. Protein determination was carried out by the method described by Markwell *et al.* (1978). The binding reaction was terminated by filtering under pressure through Whatman GF/A glass microfibre filters, and immediately rinsing filters three times with 3 ml ice-cold Tris-HCl buffer. Tissue-bound radioactivities were extracted from filters overnight in 6 ml of scintillation fluid (ScintiVerse^{TM}) and samples assayed by liquid scintillation counting.

Data analysis

The pD_2 (-Log EC_{50}) values were determined by regression analysis of Log (concentration) - effect data between 20% - 80% of E_{max} using a computer program (Tallarida and Murray, 1986). Comparisons were made using analysis of variance (ANOVA) and *t-test* analysis for grouped data at 95% and 99% confidence limits. Binding constants, K_D (equilibrium dissociation constant) and B_{max} (maximum binding

capacity) were determined by nonlinear, least-squares analysis of untransformed radioligand binding data using the "LIGAND" computer program (Munson and Rodbard, 1980).

D. Results

Influence of thyroid status and dietary lipids on heart size and body weight

The variation in heart size and body weight of euthyroid and hypothyroid animals resulting from dietary treatment is shown in Table II-2. After four weeks, body weights of euthyroid animals fed the n-6 diet were greater than those fed the n-3 diet ($p < 0.01$). Similarly, body weights of hypothyroid animals on the n-6 diet were greater than those fed the n-3 diet ($p < 0.01$) and these were less than their euthyroid controls fed the same diets. Heart weight to body weight ratios were not different for euthyroid and hypothyroid animals on either diet regimen. The data reflect suppressed growth resulting from PTU administration and is consistent with the known consequences of hypothyroidism (Gay *et al.*, 1988; Liu and Gerdes, 1990).

Effects of thyroid status and dietary lipids on the inotropic response to ß-agonists

The data in Table II-3 show the basal force developed in papillary muscles from euthyroid and hypothyroid animals fed the n-3 diet or the n-6 diet. There are no significant differences between basal tensions developed in medium containing 2.5 mM Ca^{2+}. The inotropic responses of papillary muscles to the ß-adrenoceptor agonists, ISO, E, and NE show that both thyroid state and dietary lipid affect tension development. In papillary muscles from euthyroid animals fed the n-3 diet, the maximal response to ISO was about 400% greater than the basal developed tension and about 300% more than the response elicited in tissues from hypothyroid animals

Table II-2. Effect of thyroid status and diet on body weight and heart weight of animals.

Thyroid state	Diet	Body weight (gm)		Heart weight (gm)	HW/BW ($\times 10^{-3}$)
		Initial	Final[12]		
Euthyroid	n-3	49.1 ± 0.9	257.8 ± 3.7	1.15 ± 0.04	4.4
	n-6	39.2 ± 2.2	293.9 ± 6.2[a]	1.17 ± 0.04	4.3
Hypothyroid	n-3	49.6 ± 0.7	122.5 ± 2.0[b,c]	0.51 ± 0.02[b]	4.5
	n-6	43.2 ± 2.2	157.5 ± 5.0[b]	0.54 ± 0.04[b]	4.1

Values are means (± S.E.); n = 12.

[a]*Significantly different from euthyroid on n-3 diet ($p < 0.01$).*

[b]*Significantly different from euthyroid ($p < 0.01$).*

[c]*Significantly different from hypothyroid on n-6 diet ($p < 0.01$).*

BW = Body weight; HW = Heart weight.

[12]Body weights of animals at the end of the four week feeding period.

Table II-3. Effect of thyroid status and diet on basal tension developed in papillary muscles upon electrical stimulation.

Thyroid state	Diet	Tension ($\times 10^{-6}$ N/mg tissue)
Euthyroid	n-3	24.3 ± 3.7
	n-6	21.9 ± 2.1
Hypothyroid	n-3	22.3 ± 3.8
	n-6	27.6 ± 2.5

Values are means (± S.E); n = 6 animals.

Differences between means are not significant (ANOVA).

on the same diet (Figure II-1). The maximal increase in contractile force in tissues from euthyroid animals fed the n-6 diet was about 160% over basal tension and in tissues from hypothyroid animals on same diet the increase was only 54%. Similar diet-related changes in maximal inotropic responses were observed with NE (Figure II-2) and E (Figure II-3). Responses to NE were increased by about 600% over basal tension in tissues from euthyroid animals fed the n-3 diet and about 500% more than that observed in tissues from hypothyroid animals (only 100% increase). Epinephrine produced higher responses than NE in these tissues. The tension increased by greater than 700% and 90%, over basal, in tissues from euthyroid and hypothyroid animals respectively (Table II-4). On the other hand, tissues from euthyroid animals fed the n-6 diet showed far less maximal responses to NE and E (about 290% and 160% increase in tension, respectively). A comparison of the maximum developed force (E_{max}) in tissues from euthyroid animals shows that the n-3 diet produced substantially higher increases in tension than the n-6 diet. Similarly, in tissues from hypothyroid animals, the n-3 diet had a greater effect than the n-6 diet.

Tissue sensitivity to the inotropic agents used, as measured by pD_2, also appears to be affected by thyroid status and diet (Table II-5). The pD_2 values for all drugs (except ISO) were similar in tissues from animals fed the n-3 diet. However, in tissues from animals fed the n-6 diet, pD_2 values were lower in euthyroid animals than in hypothyroid animals. Again except for ISO, the values obtained in tissues from hypothyroid animals were one order of magnitude higher than those in tissues from euthyroid animals indicating a higher sensitivity to the drugs. The pD_2 values

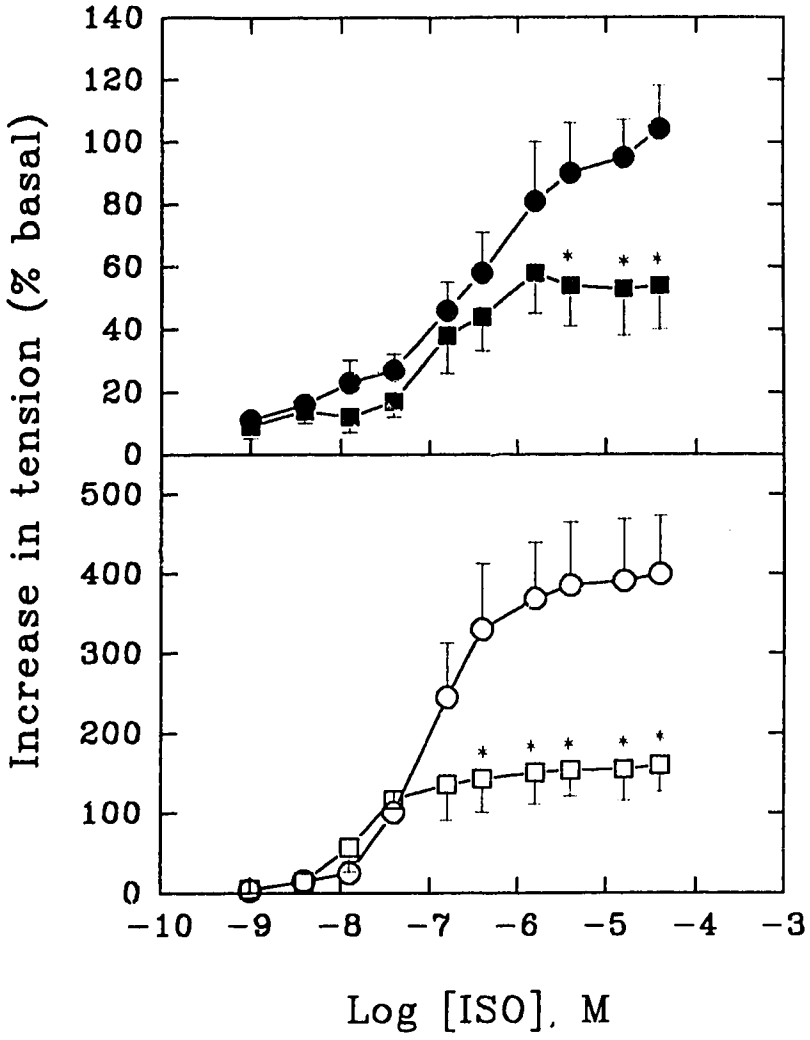

Figure II-1. Inotropic response of papillary muscles to isoproterenol. Contractile responses, to cummulative concentrations of drug, in tissues from euthyroid (□, ○) and hypothyroid (■, ●) animals fed the n-6 diet (□, ■) or the n-3 diet (○, ●). Each data point is a mean (± S.E); n = 4-6 animals ($^{*}p < 0.05$).

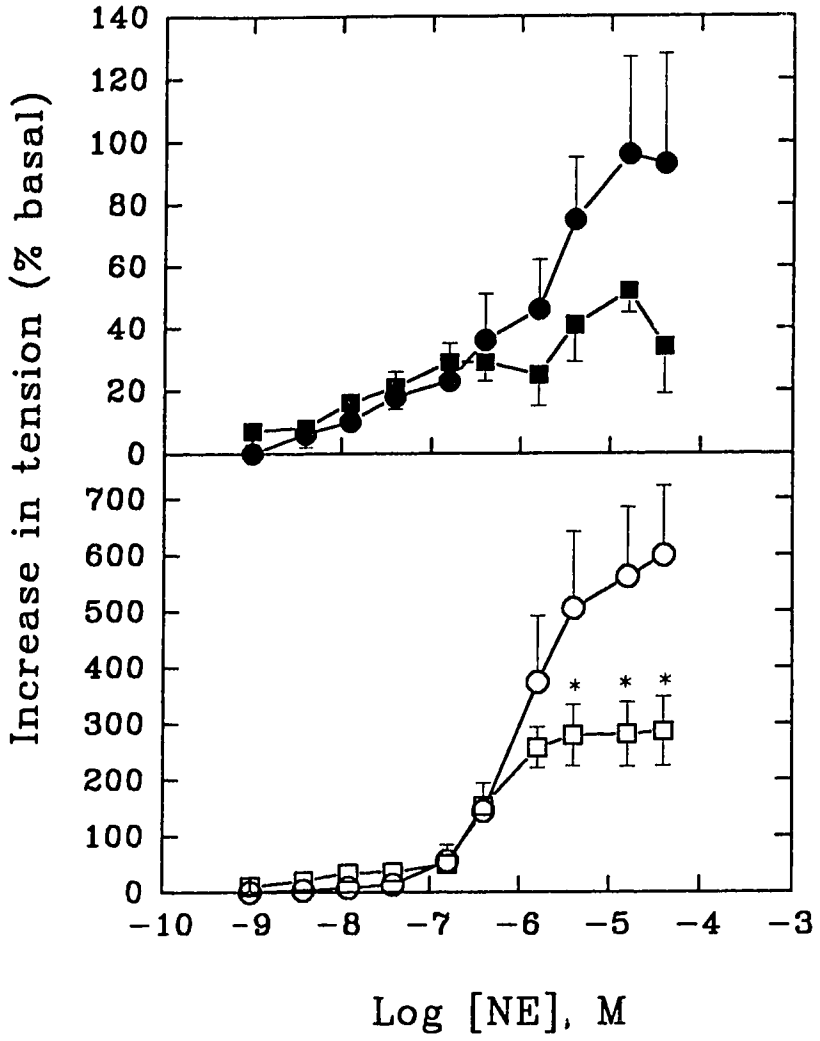

Figure II-2. Inotropic response of papillary muscles to norepinephrine. Contractile responses, to cummulative concentrations of drug, in tissues from euthyroid (□, ○) and hypothyroid (■, ●) animals fed the n-6 diet (□, ■) or the n-3 diet (○, ●) are shown. Each point is a mean (± S.E); n = 4-6 animals ($^{*}p < 0.05$).

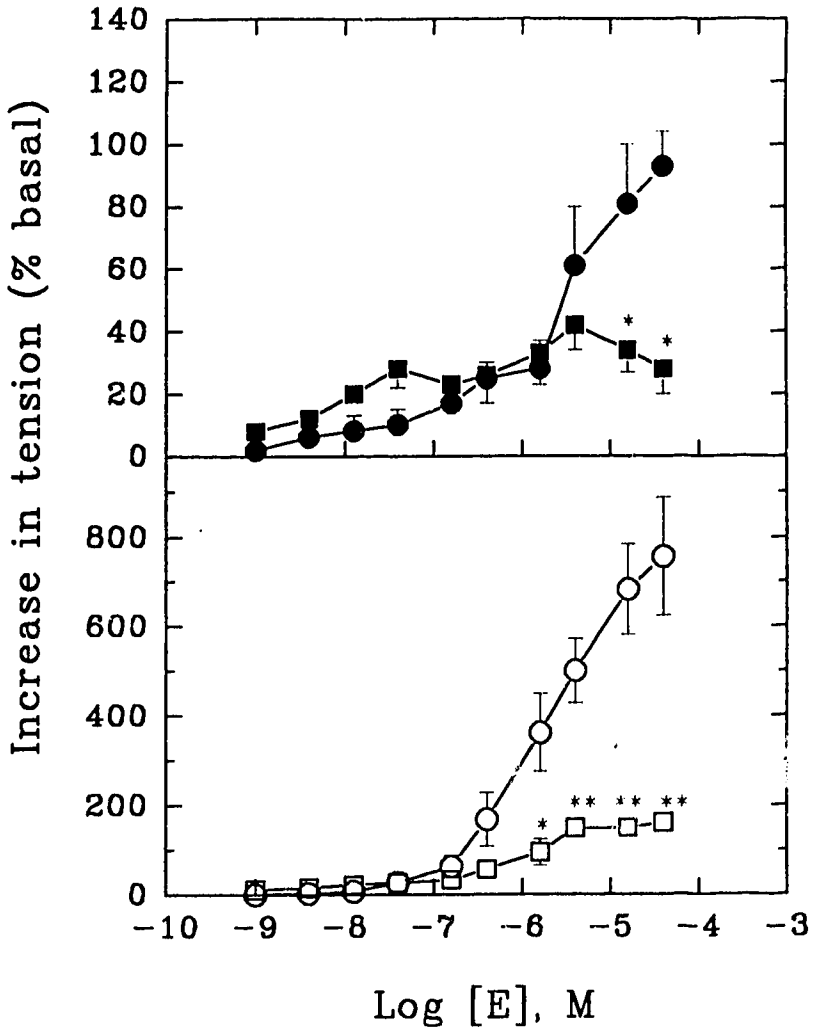

Figure II-3. Inotropic response of papillary muscles to epinephrine. Contractile responses, to cummulative concentrations of drug, in tissues from euthyroid (□, ○) and hypothyroid (■, ●) animals fed the n-6 diet (□, ■) or the n-3 diet (○, ●) are shown. Each point is a mean (± S.E); n = 4-6 animals ($^{*}p < 0.05$; $^{**}p < 0.01$).

Table II-4. Effect of thyroid status and diet on maximum developed tension (% basal), due to positive inotropic agents, in left ventricular papillary muscles.

Thyroid state	Diet	Increase in tension (% basal)			
		ISO	E	NE	FSK
Euthyroid[a]	n-3	399 ± 68	756 ± 132	598 ± 125	49 ± 13
	n-6[b]	160 ± 26	161 ± 24	286 ± 61	201 ± 46
Hypothyroid	n-3	105 ± 15	93 ± 11	96 ± 27	105 ± 24
	n-6	54 ± 14[b]	52 ± 14[b]	51 ± 12	132 ± 15

Values are means (± S.E.) of 4 to 6 determinations.

[a]*Significantly different from hypothyroid ($p < 0.01$).*

[b]*Significantly different from n-3 diet ($p < 0.01$).*

obtained in tissues from euthyroid animals fed the n-6 diet were similar to those in tissues from euthyroid animals fed the n-3 diet. Thus, NE and E had similar concentration-effects in tissues from animals fed the n-3 diet. However, ISO elicited a reduced maximal increase in tension as well as a higher pD_2 value, indicating a higher sensitivity but lower efficacy. In tissues from animals fed the n-6 diet, the maximal increases in tension produced by all three agonists were lower than those seen in tissues from animals fed the n-3 diet. ISO and NE produced similar maximal responses but different pD_2 values. A comparison of the effects of the three ß-adrenoceptor agonists in tissues from hypothyroid animals indicate that n-6 fatty acids attenuated the inotropic responses of hypothyroid tissues to ISO, E, and NE. On the other hand, n-3 fatty acids appear to substantially offset the reduction in efficacies of these agonists.

In obtaining ISO concentration-effect data, it was observed that some tissues developed spontaneous dysrhythmias between driven beats. The threshold concentration for induction of such extra twitches was as low as 10^{-9} M in some tissues while others were completely resistant to ISO stress over the concentration range examined. Increasing the concentration of drug led to pronounced dysrhythmia. The percentage of tissues from euthyroid and hypothyroid animals becoming dysrhythmic at any concentration of ISO up to and including 4.4×10^{-5} M is shown in Figure II-4. The data indicate that the n-3 diet was more effective than the n-6 diet in reducing the incidence of dysrhythmia in euthyroid and hypothyroid tissues.

Table II-5. Effect of thyroid status and diet on pD_2 values for catecholamine- and forskolin-induced contractions in papillary muscles.

Thyroid state	Diet	pD_2			
		ISO	E	NE	FSK
Euthyroid	n-3	6.9 ± 0.1[a] (7.4 — 5.4)	5.7 ± .01[a] (5.8 — 5.6)	5.8 ± .02 (6.0 — 5.6)	5.8 ± 0.3 (6.5 — 5.1)
	n-6	7.6 ± 0.2 (8.9 — 6.3)	6.8 ± 0.1[b] (7.5 — 5.1)	6.2 ± 0.5[b] (7.4 — 5.7)	5.8 ± 0.1[b] (6.0 — 5.4)
Hypothyroid	n-3	6.7 ± 0.3 (7.4 — 6.0)	5.5 ± 0.3[a] (6.7 — 4.9)	6.0 ± 0.1[a] (6.6 — 5.5)	5.3 ± .04[a] (5.8 — 4.8)
	n-6	7.1 ± 0.2 (7.6 — 6.5)	7.9 ± 0.2 (8.4 — 7.4)	7.4 ± 0.3 (8.2 — 6.7)	6.5 ± .02 (6.7 — 6.2)

Values are means (± S.E.) of 4-6 separate determinations.

The confidence limits are indicated in parenthesis.

[a]*Significantly different from n-6 ($p < 0.01$).*

[b]*Significantly different from hypothyroid on same diet ($p < 0.01$).*

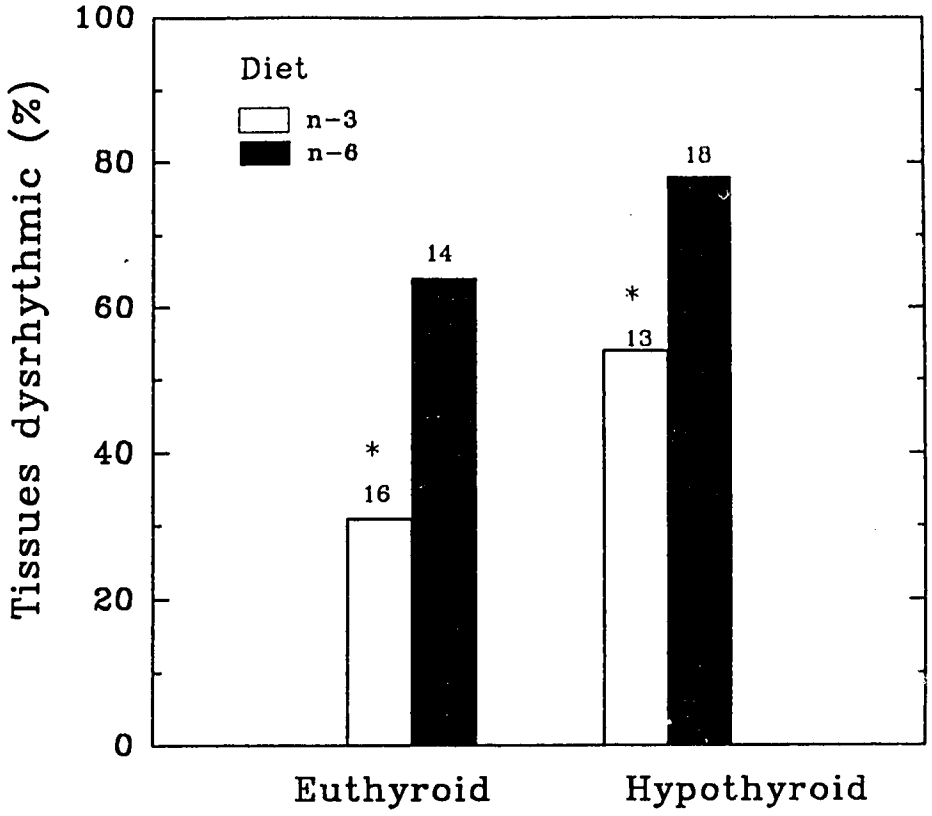

Figure II-4. Histograms showing the percentage of isolated papillary muscles developing spontaneous tachyarrhythmias under isoproterenol stress. The number of tissues in each group is shown at the head of each column ($^{*}p < 0.05$, χ^2 test).

Effect of thyroid status and dietary lipid on inotropic response to forskolin

The effect of FSK in increasing tension was more pronounced in tissues from euthyroid animals fed the n-6 diet compared to those from animals fed the n-3 diet (Figure II-5b). In the latter case, the increase in tension did not exceed 50% of basal tension over the concentration range examined indicating little activation of adenylate cyclase. In hypothyroid tissues, the concentration-effect curve for FSK was shifted to the right by the n-3 diet relative to the n-6 diet (Figure II-5a) while the maximal increase in tension remained the same. The pD_2 values for FSK were similar in all tissues except those from hypothyroid animals fed the n-6 diet in which higher values were obtained indicating higher sensitivity to the drug (Table II-5).

Ligand binding assay

Binding of [^{3}H]DHA to ventricular membranes was saturable and of high affinity (K_D < 1 nM) in both euthyroid (Figure II-6) and hypothyroid (Figure II-7) animals. Straight line fits of the data suggest the presence of a single class of binding sites. There are no apparent differences between the Scatchard plots of data obtained in membranes from euthyroid animals fed the n-3 diet or the n-6 diet. In membranes from hypothyroid animals, the plots indicate that the n-3 diet slightly increased binding compared to the n-6 diet. The mean binding constants, K_D and B_{max}, determined by non-linear least squares analysis of untransformed binding data, however, indicate that binding affinities and binding site densities were similar in all groups (Table II-6). The binding affinity measured in membranes from hypothyroid

animals fed the n-6 diet was significantly lower (high K_D) compared to those from euthyroid controls fed the same diet, and euthyroid and hypothyroid animals fed the n-3 diet suggesting that n-6 fatty acids influence binding to some extent.

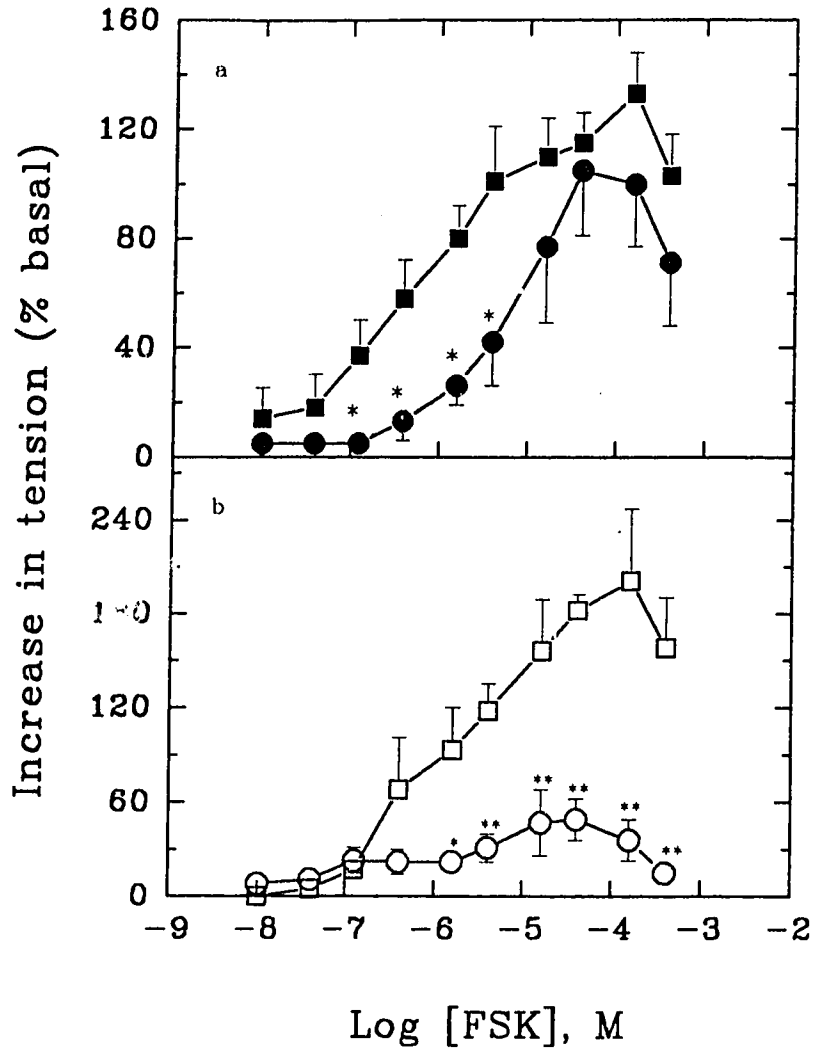

Figure II-5. Inotropic response of papillary muscles to forskolin. Contractile responses, to cummulative concentrations of drug, in tissues from euthyroid (□, ○) and hypothyroid (■, ●) animals fed the n-6 diet (□, ■) or the n-3 diet (○, ●) are shown. Each point is a mean (± S.E); n = 4-6 animals ($^{*}p < 0.05$; $^{**}p < 0.01$).

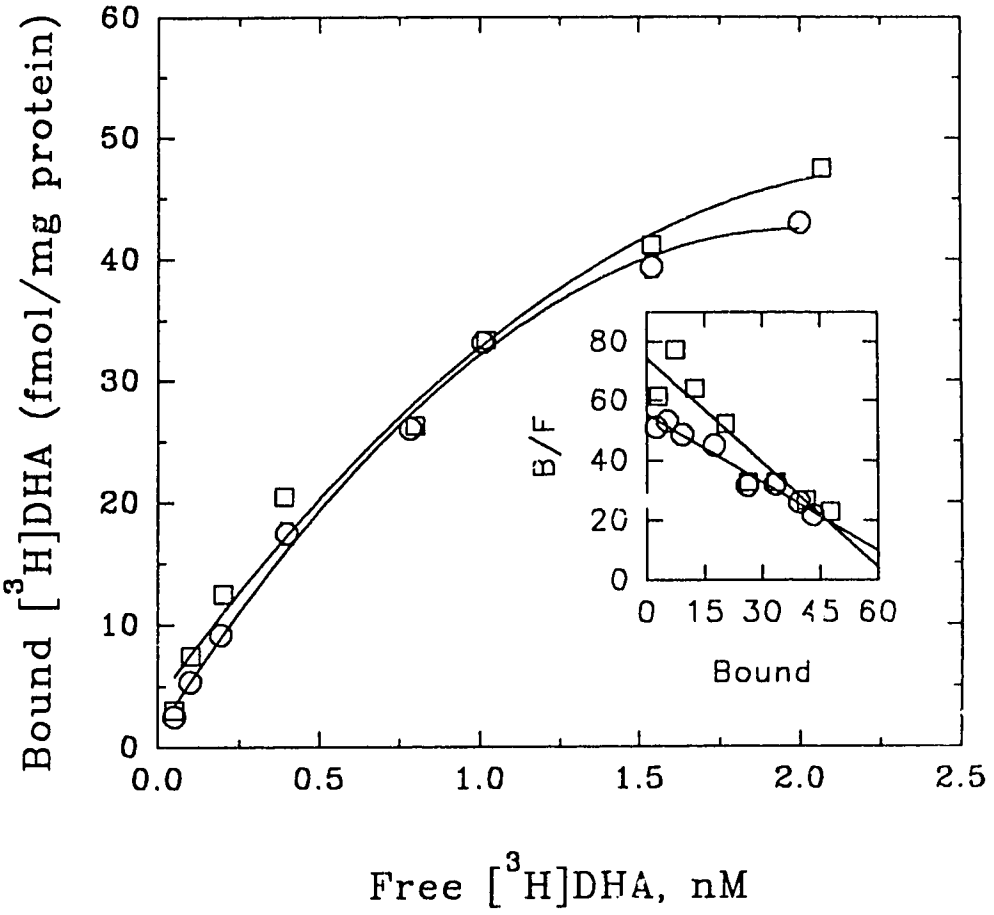

Figure II-6. Site-specific binding of [^{3}H]DHA to crude ventricular plasma membranes prepared from the hearts of euthyroid animals fed the n-3 diet (○) or the n-6 diet (□). Inset are the Scatchard plots of the binding data. Data are means of duplicate determinations in each animal (n = 6).

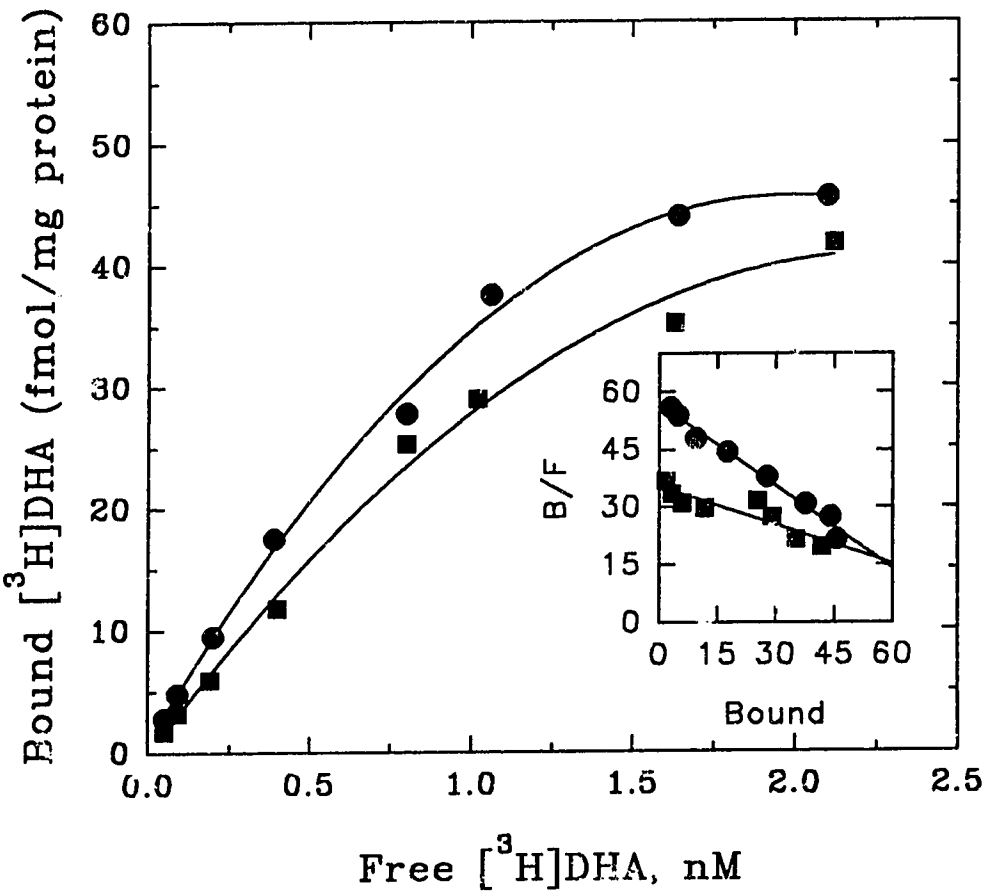

Figure II-7. Site-specific binding of [^{3}H]DHA to crude ventricular plasma membranes prepared from the hearts of hypothyroid animals fed the n-3 diet (●) or the n-6 diet (■). Inset are the Scatchard plots of the binding data. Data are means of duplicate determinations in each animal (n = 6).

Table II-6. Effect of thyroid status and diet on K_D and B_{max} values for site-specific binding of [^{3}H]DHA to crude plasma membranes prepared from ventricles determined by mass-l.. :' analysis of untransformed radioligand binding data.

Thyroid state	Diet	K_D (nM)	B_{max} (fmol/mg protein)
Euthyroid	n-3	0.46 ± 0.09	21.02 ± 4.56
	n-6	0.52 ± 0.08	27.93 ± 3.36
Hypothyroid	n-3	0.53 ± 0.08	20.94 ± 4.23
	n-6	0.96 ± 0.11[a]	30.50 ± 8.28

Values are means (± S.E.); n = 6.

[a]*Significantly different from n-3 and euthyroid ($p < 0.05$).*

E. Discussion

Thyroid hormone causes wholesale nuclear transcription of a large number of genes leading to increased synthesis of enzymes, structural and transport proteins as well as receptors. Ultimately functional activity is increased throughout the body. This multiplicity of effects of the hormone presents difficulties in explaining the altered adrenergic activity observed in the myocardium since the actions of catecholamines are also complex (Kunos, 1981). A number of factors may be responsible for the changes in inotropic responses to catecholamines. For example, changes in the ß-adrenoceptor density in the sarcolemma, stimulus-response coupling, the activity of membrane-associated ß-adrenergic receptor/adenylate cyclase system (McMurchie *et al.*, 1987 and 1988; Wince *et al.*, 1987), membrane physico-chemical properties, transmembrane signalling and membrane enzyme activity (Alam *et al.*, 1989), can all result in altered tension development in the myocardium. Although the major biochemical effector of the ß-adrenoceptor is adenylate cyclase, in myocardial cells, this receptor may be coupled through G proteins directly to ion channels that regulate inotropic and electrophysiologic effects (Katz, 1990). Thus, alteration in the activity of G proteins in receptor coupling to adenylate cyclase subsequent to agonist binding may also affect tension development. Uncoupling of receptors can occur as a result of conformational change in the receptor itself or partial activation of receptors. Thus, if receptor density remains unchanged, reduction in tension could be due to uncoupling of some receptors or to

some post-receptor mechanism. There is almost universal agreement that the hyper-adrenergic state associated with hyperthyroidism is due to increased ß-adrenoceptor number and possibly agonist affinity (Williams *et al.*, 1977; Tsai and Chen, 1978) whereas hypothyroidism is associated with decreased number of receptors (Stiles and Lefkowitz, 1981) in the heart, shown *in vivo* and *in vitro*.

In this study, the inotropic effects of catecholamines were markedly altered by thyroid state and by the fatty acid composition of diets. In euthyroid animals, papillary muscle responses to catecholamines were enhanced by the n-3 diet compared to the n-6 diet indicating the involvement of additional mechanism(s). The maximal increase in tension observed with isoproterenol was smaller relative to epinephrine and norepinephrine and this may be explained by the fact that isoproterenol is not a substrate for neuronal uptake and may not have been transported extraneuronally either. Thus, isoproterenol has a higher potency but lower efficacy compared to norepinephrine and epinephrine. Tissue desensitization, probably resulting from down regulation of receptors, may have occurred. Reduced inotropic effects of isoproterenol have also been shown in perfused hearts (Hoffman *et al.*, 1982), papillary muscles (Charnock *et al.*, 1985) and atria (Wince *et al.*, 1987) of rats fed diets high in n-6 polyunsaturated fatty acids compared to saturated fatty acids. The increase in tension, elicited by forskolin, in papillary muscles from animals fed the n-6 diet compared to those fed the n-3 diet suggests that the latter rendered the catalytic unit of adenylate cyclase in ventricular plasma membranes less sensitive to activation. Wince *et al.* (1987) have reported a similar effect of an unsaturated fat

diet on rat atrial membrane adenylate cyclase compared to a saturated fat diet. The present results indicate that similar variations exist between the effects of n-6 and n-3 fatty acids on adenylate cyclase. Thus, the enhanced inotropic response could not be due to increased adenylate cyclase activity but may be the result of changes at a level proximal to adenylate cyclase in the ß-adrenoceptor/adenylate cyclase pathway, probably in the receptor itself, the G protein or their coupling to adenylate cyclase. The binding experiments have revealed no significant changes in ß-receptor density or affinity in ..uthyroid animals fed the n-3 diet or the n-6 diet. This finding is partly in agreement with the report by McMurchie *et al.* (1987) which indicates that the K_D for ligand binding to the ß-adrenoceptor is unaffected by the nature of the dietary lipid supplement. The enhanced inotropic responses to catecholamines caused by the n-3 diet cannot therefore be due to changes in receptor number but may involve alterations in some other mechanism(s).

The inotropic responses of papillary muscles, from hypothyroid animals, to catecholamines were much lower than those measured for euthyroid animals. All three ß-agonists, isoproterenol, epinephrine and norepinephrine, showed lower efficacies (lower maximum responses) with no apparent change in sensitivity (pD_2 values) in hypothyroid compared to euthyroid animals fed the n-3 diet suggesting partial agonist effects of these drugs in hypothyroid animals. Since ß-adrenoceptor number is the same regardless of thyroid state and diet treatment, the effect of n-3 could not be due to changes in receptor properties (i.e. density or affinity). Although receptor affinity was lower in animals fed the n-6 diet compared to the n-3 diet,

ligand binding was still of high affinity suggesting only subtle effect of this diet on binding. A number of studies have shown that ß-adrenoceptor density is reduced in hypothyroid myocardium (Brodde *et al.*, 1980; Stiles and Lefkowitz, 1981; Whitsett *et al.*, 1982) which is in contrast to the present results. This discrepancy can be explained by the differences in experimental design. These studies employed adult rats which were made hypothyroid by propylthiouracil treatment and no diet treatment was carried out. In all the above studies as well as this study, however, hypothyroidism did not alter receptor affinity for ligand. The K_D values in these studies compare reasonably and are similar to that measured in rat cardiac membranes by Kojima *et al.* (1990). Alam *et al.* (1989) have reported K_D and B_{max} values, in rat heart membranes, which were an order of magnitude higher than those reported in the present study. The differences are probably due to the handling of binding data. Computerized, non-linear, least-squares analysis of untransformed radioligand binding data has some advantages over conventional Scatchard analysis which tends to amplify errors in measurement and therefore results in higher binding constants (Schwarz, 1986). The attenuation of maximum responses to catecholamines in euthyroid and hypothyroid animals by the n-6 diet, compared to the n-3 diet, suggests partial activation of receptors or differences in coupling efficiency of the ß-adrenoceptor to adenylate cyclase.

Birk *et al.* (1992) have shown that the development of the cardiac ß-adrenoceptor in newborn lambs is under the control of thyroid hormone. Similarly, the development of the ß-adrenoceptor and programming of the ontogeny of

transduction factors, such as G proteins linking the receptors to adenylate cyclase, have been shown to be dependent on thyroid hormone (Pracyk and Slotkin, 1992). Thus, thyroid hormone can induce changes at any point in the complex chain of biochemical events occurring between central stimulation of pre-ganglionic neurons and the eventual biological sequelae (Williams and Lefkowitz, 1983). Alterations can also occur at any point between receptor stimulation and activation of adenylate cyclase or in other biochemical processes downstream in the cascade of intracellular events, such as activation of protein kinases and phosphorylation of proteins mediating [illegible]. Therefore, other mechanisms distinct from the ß-adrenergic receptor, [illegible] those involving ionic channels which affect membrane potentials or intracellular cation concentrations may be important in the modulation of myocardial contractility observed in the present study.

Since dietary fatty acids influence the fatty acid composition of cardiac phospholipids (Charnock *et al.*, 1984; McMurchie *et al.*, 1987; 1988) it is possible that the enhanced inotropic responsiveness of papillary muscles induced by the n-3 diet may be related to membrane lipid changes. The fact that inotropic responses to catecholamines were much larger than those elicited by forskolin in the same treatment group suggests that the effect was not due to changes in the catalytic subunit of adenylate cyclase (Seamon and Daly, 1981). Adenylate cyclase is known to be lipid-dependent (Ross and Gilman, 1980) and alterations in the lipid composition of cardiac sarcolemma, where this enzyme is located, may affect its activation (McMurchie *et al.*, 1987). In hypothyroid animals, the sensitivity of

adenylate cyclase to activation by forskolin was decreased by the n-3 diet relative to the n-6 diet. This finding implies that the enhancement in maximum tension caused by this diet was not due to increased ß-adrenoceptor/adenylate cyclase activity but may involve modulation of coupling or of some post-receptor mechanism. This modulation is probably related to dietary fatty acid-induced changes in quantity or the composition of phospholipids of cardiac membranes such as the sarcolemma or the sarcoplasmic reticulum. Neelands and Clandinin (1983) found an apparent correlation between membrane lipid composition and glucagon-stimulated adenylate cyclase activity in liver plasma membranes. Adenylate cyclase undergoes lateral diffusion in contrast to membrane-spanning enzymes and may therefore be more susceptible to bulk membrane lipid changes (Bennett *et al.*, 1975; Abeywardena *et al.*, 1984). The present findings therefore suggest that the effects of the diets may be the consequence of altered membrane fatty acid composition influencing the properties of receptors, transduction factors and, possibly transport proteins in the plasma membrane. Membrane dynamics depend on the degree of saturation or unsaturation of the fatty acid side chain and head groups of membrane phospholipids (van Ginkel *et al.*, 1989) and these can exert strong influences on the activity of membrane proteins (Steck and Fox, 1972).

McMurchie *et al.* (1987) have shown that diets rich in saturated and n-6 polyunsaturated fatty acids did not change ß-adrenoceptor site density or the receptor affinity in rat ventricular membranes. However, the presence of cholesterol in these diets reduced receptor site density concomitant to increase in adenylate cyclase

activity suggesting down regulation of receptors (Lefkowitz *et al.*, 1983). Hormone-sensitive adenylate cyclase are believed to be influenced by membrane physico-chemical properties as a result of *in vivo* modification of membrane lipid fatty acid composition by dietary supplementation (Needham *et al.*, 1985; Morson and Clandinin, 1986). Although the modulation of ß-adrenoceptor number in rat heart by thyroid hormone has been reported (Stiles and Lefkowitz, 1981), other modes of receptor regulation is possible. In the present study, the feeding of the n-3 diet did not change ß-adrenoceptor site density or receptor affinity significantly suggesting that the change in inotropic response was not due to changes in receptor number but may be related to changes in an alternate pathway leading to force generation. The alteration in ligand binding affinity in membranes from hypothyroid animals fed the n-6 diet may be due to conformational change in the receptor itself or to receptor-membrane interactions which may have influenced the accessibility of the binding site to ligand.

Decrease in inotropic effect in hypothyroid rat, however, cannot be solely attributed to a decrease in ß-adrenoceptor density since this effect is also seen in pithed hypothyroid animals as well as those treated with (6-OH)dopamine in which ß-adrenoceptors are only slightly activated by the influence of the adrenergic pathway (Gross and Lues, 1985). Other mechanisms such as direct action on ion channels may therefore be involved (Yin *et al.*, 1992). Forskolin effect in tissues from euthyroid animals fed the n-3 diet is low therefore the increased inotropic response to catecholamines may be related to changes in the Ca^{2+} handling properties of the

myocardium induced by the different diets. In rat myocardium, ß-adrenoceptor site density and adenylate cyclase activity increase progressively from the post-gestation period until adulthood (Kojima *et al.*, 1990; Tanaka and Shigenobu, 1990), therefore developmental changes in the receptor complex can be achieved during this period. Unlike other studies which employed adult rats, in the present study weanling rats were used to ensure developmental modification of the ß-adrenoceptor/adenylate cyclase system by thyroid state and dietary fatty acids.

The effect of diet on isoproterenol-induced dysrhythmia can be explained on the basis of modification of stimulus-response coupling or altered eicosanoid production in cardiac tissue (McLennan *et al.*, 1985; Chardigny *et al.*, 1988). Improved coupling of ß-adrenoceptors to adenylate cyclase, increased adenylate cyclase activity or enhanced Ca^{2+} conductance may be involved. Association between cyclooxygenase and lipoxygenase products and ischaemic dysrhythmias is well established (Parrat *et al.*, 1988; Reidal *et al.*, 1988). It is possible that, in animals fed the n-3 diet, eicosapentaenoic and docosahexaenoic acids may have interfered with incorporation of arachidonic acid into membrane phospholipids thus reducing the level of this fatty acid and hence the synthesis of cyclooxygenase and lipoxygenase products from arachidonic acid (Lee *et al.*, 1984; Abeywardena *et al.*, 1991). An alternative explanation could be that the n-3 diet ultimately altered the Ca^{2+} fluxes in the sarcolemma or the function of the sarcoplasmic reticulum in sequestering or releasing Ca^{2+}. McLennan *et al.* (1987a) have shown that dietary polyunsaturated fatty acid supplementation in the rat provides significant protection against isoproterenol-

induced dysrhythmia in isolated papillary muscles concomitant to reduction in contractility and inotropic responses to Ca^{2+}. It has also been suggested, in another study, that the protective effects of n-3 fatty acids result from the modulation of nitrendipine-sensitive L-type calcium channels (Hallaq *et al.*, 1992).

In summmary, this study has shown that the enhancement of the inotropic effects of catecholamines, in muscles from animals in different thyroid states, by n-3 fatty acids relative to n-6 fatty acids was not due to changes in the density or binding affinity of the ß-adrenoceptor but may be due to increased coupling of the receptor to post-receptor mechanisms, possibly calcium influx/efflux or its sequestration and release by the sarcoplasmic reticulum.

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III. MODULATION OF CALCIUM INOTROPY BY DIETARY FATTY ACIDS IN EUTHYROID AND HYPOTHYROID RAT MYOCARDIUM

A. Introduction

The modulation of myocardial contraction and relaxation by thyroid hormone has been well documented (McDonough *et al.*, 1987; Gay *et al.*, 1988; Liu and Gerdes, 1990). The marked changes in contractile properties of the myocardium induced by thyroid hormone are considered, primarily to be dependent on effects on contractile filaments. Thyroid hormone acts directly on the myocardium, presumably via specific receptors to induce an increase in contractile state as well as in protein synthesis. Although the mechanism by which modulation of contractility occurs is unclear, the involvement of membrane-associated events such as regulation of Ca^{2+} influx/efflux have been suggested (Morkin, 1989). Calcium influx into mammalian heart cells occurs predominantly via slow channels on depolarization (major pathway) and efflux from cells by Na^{+}/Ca^{2+} exchange which depends on the activity of the sodium pump (Kirby *et al.*, 1989). Contraction occurs when extracellular Ca^{2+} enters myocardial cells to trigger Ca^{2+} release from sarcoplasmic reticulum (SR) into the cytosol to activate the troponin-tropomyosin system associated with actin filaments thereby allowing ATP-dependent actin-myosin interaction (Noble and Drake-Holland, 1987; Lullman and Ziegler, 1987; Morkin, 1989). Relaxation is achieved through increase in the rate of Ca^{2+} removal from the cytosol. The binding of Ca^{2+} to troponin is therefore the signal that initiates cardiac contraction, and changes in the

Ca^{2+} translocation mechanisms will affect tension development.

The relationship between changes in the speed of relaxation and altered Ca^{2+}-ATPase activity in the sarcolemma and SR have been demonstrated, and the activity of Ca^{2+}-ATPase shown to increase in the hyperthyroid state (Kim and Smith, 1985; Mylotte *et al.*, 1985; Rohrer and Dillmann, 1988). It has been suggested that improved Ca^{2+} transport associated with thyroid hormone-dependent increased myocardial contractility and increased speed of diastolic relaxation observed in rat heart may be partly related to specific alterations in the level of messenger RNA coding for Ca^{2+}-ATPase (Rohrer and Dillmann, 1988). Thyroid hormone stimulates Ca^{2+} influx into myocardial cells via voltage-dependent, slow channels (Kim *et al.*, 1987; Beekman *et al.*, 1990), as well as transmembrane Na^+ and K^+ exchange via effects on the synthesis of Na^+/K^+-ATPase located in the sarcolemma (Kim and Smith, 1984). Using freshly-isolated rat heart slices, Segal (1990) has shown calcium to be the first messenger for the action of thyroid hormone at the plasma membrane. In addition to these effects, time-dependent and concentration-dependent control of the rate of transsarcolemma Ca^{2+} movement as well as the size of the Ca^{2+} pool associated with the sarcoplasmic reticulum are affected by thyroid hormone levels (Kim and Smith, 1985). Thus, the regulation of cytosolic Ca^{2+} by extrusion from cell or sequestration in SR may be an important mechanism in thyroid hormone control of myocardial contraction and relaxation.

Dietary fatty acids influence the lipid composition of cardiac membranes and such changes can modify sarcolemmal transporters, enzymes and receptors

(McMurchie *et al.*, 1987; 1988; Hoch, 1988). Alterations in membrane lipids can also lead to changes in ion fluxes which may affect tension development in the myocardium (Alam *et al.*, 1989). Hyperthyroidism increased while hypothyroidism decreased cholesterol to phospholipid molar ratio in the sarcolemma of rabbit cardiac muscle, compared to euthyroid animals (Szymanska *et al.*, 1991). Hypothyroidism has also been shown to increase the stoichiometric amount of Ca^{2+} transported per mole of ATP hydrolyzed by Ca^{2+}/Mg^{2+}-ATPase in rat skeletal muscle (Simonides and van Hardeveld, 1987). The modulation of dihydropyridine-sensitive calcium channels in cultured cardiac myocytes by n-3 fatty acids have been recently described (Hallaq *et al.*, 1992). Thus, changes in the calcium translocation mechanism in the myocardium through effects on Ca^{2+}-ATPase, Ca^{2+}/Na^{+} antiporter or the number and/or kinetics of the voltage-dependent, slow calcium channels may be involved in the regulation of cardiac performance by thyroid hormone.

In an earlier study, the enhancement of the inotropic effects of catecholamines by dietary polyunsaturated n-3 fatty acids relative to n-6 fatty acids was found to be dependent on thyroid state and such changes might be related to differences in the coupling mechanism of ß-adrenoceptor to adenylate cyclase. Receptor density and binding affinity remained the same regardless of thyroid status and the type of dietary fatty acid (Chapter II). The present study describes the effects of dietary fatty acids on the Ca^{2+}-sensitivity of papillary muscles obtained from euthyroid and hypothyroid rats.

B. Materials

Nifedipine and 6-*n*-propyl-2-thiouracil were purchased from Sigma, St. Louis, MO, O-acetylcholine hydrochloride was from BDH, and [5-methyl-^{3}H]nitrendipine (2701 GBq/mmol) was from NEN, Boston, MA. All other chemicals used in the study were of the purest grade available commercially. Basal diet was from Tekland Diets, Madison, WI. Safflower oil, linseed oil, olive oil and beef tallow were purchased on the open market, and fish oil (EP-28) was from Nishin Co., Tokyo, Japan.

C. Methods

Animals and diets

Weanling male Sprague-Dawley rats (50-55 gm), obtained from the University of Alberta Health Sciences Laboratory Animal Services, were divided randomly into three groups and fed diets enriched with n-3, n-6 or saturated (SAT) fatty acids for two weeks. The SAT diet was included as a reference. Each group was then subdivided into two, one group serving as euthyroid controls while the other was rendered hypothyroid by continuous administration of 6-*n*-propyl-2-thiouracil (PTU; 0.05% solution) in drinking water. Animals were then fed, *ad libitum*, the diet formulations for a further four weeks. Body weights were monitored on a bi-weekly basis.

All diets contained a fat-deficient basal mix of the following composition (in

gm/kg): starch, 408; casein, 270; non-nutritive cellulose, 50; vitamin mix, 10; mineral mix, 50.85; inositol, 6.25; choline chloride, 2.75 and L-methionine, 2.5. The lipid composition of the diets was achieved by the addition of 20% lipid (w/w) in the form of beef tallow, safflower oil, linseed oil, olive oil or fish oil to the basal mix. The fatty acid composition of diets employed in this study are presented in Table III-1. Diets were stored at 4 °C and feeding cups replenished with fresh diet daily after discarding any uneaten portion.

Contractility studies

After six weeks of dietary treatment, papillary muscles were isolated and contractility studies carried out as previously described (Chapter II). Briefly, animals were injected i.p. with sodium pentobarbital (50 mg/kg) to induce anesthesia and the heart rapidly excised and placed in freshly-oxygenated, ice-cold Krebs-Henseleit medium of the following composition (in mM): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4$, 0.6; KH_2PO_4, 1.2; $CaCl_2$, 2.5 and D-glucose, 11 (pH 7.4; 22 °C). Papillary muscles were dissected from the left ventricle and placed in fresh medium continuously aerated with a mixture of 95% O_2:5% CO_2. Muscle preparations were mounted in 4-ml organ baths containing medium which, was continuously oxygenated, at 37 °C and tissues paced at 1 Hz (8 V, 10 ms) through bipolar platinum electrodes. Contractions were measured with a *Grass FT.03* force displacement transducer connected to a *Grass Model 7E Polygraph*. Preparations were maintained under resting tension of 500 mg and equilibrated for 30 min, after which they were

Table III-1. Fatty acid composition of diets.

Fatty acid	Composition of diet (mol %)		
	n-3	n-6	SAT
14:0	4.5	1.7	5.7
16:0	14.6	15.1	30.9
16:1	5.0	-	-
17:0	2.1	0.7	2.0
18:0	12.6	20.5	48.5
18:1(n-7)	27.0	10.4	2.2
18:1(n-9)	2.1	-	-
18:2(n-6)	11.7	50.5	11.1
18:3(n-3)	2.5	1.2	1.2
20:5(n-3)	13.0	-	-
22:6(n-3)	5.0	-	-
Σ Poyunsaturates	*32.2*	*51.7*	*11.1*
Σ Saturates	*31.7*	*38.0*	*84.7*
Σ n-6	*11.7*	*50.5*	*11.1*
Σ n-3	*20.5*	*1.2*	*1.2*
P/S	*1.0*	*1.4*	*0.1*
n-6/n-3	*0.6*	*42.0*	*9.3*

stretched until the muscle length that gave maximal developed tension (L_{max}) was attained. Muscle length was then reduced until developed tension was equal to 70% of the L_{max} and the tissues allowed to equilibrate for a further 30 min. During the equilibration period, the medium was replaced at 10 min intervals. The basal tension developed, in response to electrical stimulation, in each muscle preparation was recorded before obtaining inotropic responses to drugs. Contractions are expressed, as per cent of basal developed tension after normalizing data to force per tissue weight (N/mg). Stock solutions of 10 mM nifedipine (NIF), made up in ethanol (98%) and 10 mM acetylcholine (ACh) in 0.9% saline were diluted serially with saline to obtain the working solutions. Ca^{2+} was used as a 1 M $CaCl_2$ solution in saline and was well-aerated with 95% O_2:5% CO_2 before use. Contractions to cummulative concentrations of Ca^{2+} were determined relative to contractions induced in Ca^{2+}-free medium.

Preparation of skinned papillary muscles

To investigate any variation in calcium sensitivity of myofilaments, papillary muscles from euthyroid and hypothyroid animals fed standard laboratory chow were chemically skinned and the calcium concentration-effect relationship determined. After equilibrating tissues for 1 hr as described in the previous section, the medium was replaced with an ethylene glycol-bis(ß-aminoethyl ether)-N,N,N^1,N^1-tetraacetic acid (EGTA) relaxation solution (10 mM; pH, 7.0) containing 2% Triton X-100 (v/v) and preparations incubated for 40 min. The tissues were then washed with fresh

EGTA solution without Triton X-100 and myofilament calcium sensitivity determined in an ATP-generating medium of composition (in mM): KCl, 100; $MgCl_2$, 5; EGTA, 10; MOPS[13], 10; Na_2ATP, 5; Na_2-creatine phosphate, 10; creatine phosphokinase, 15 U/ml and various concentrations of Ca^{2+}. Once steady-state was attained, the accumulative tension development of the skinned muscles in different Ca^{2+}-EGTA solutions were recorded with a polygraph as described in the previous section. The concentration of Ca^{2+} in each solution was calculated using a computer program based on the equations by Fabiato (1988).

Preparation of cardiac plasma membranes

Crude cardiac plasma membranes were prepared from ventricles by a previously described method (Chapter II). Ventricles were minced in 10 volumes (w/v) of ice-cold Tris-HCl buffer of the following composition (in mM): sucrose, 250; EDTA(Na_2), 1; $MgCl_2$, 1 and Tris-HCl, 20 (pH 7.4; 22 °C) and homogenized with a polytron homogenizer (Kinematica; 50% maximum speed, 10 sec). The homogenate was initially centrifuged at 1,000 x g for 3 min and the supernatant filtered through four layers of gauze. The pellet was resuspended in 5 ml of Tris-HCl buffer and centrifuged at 800 x g for 10 min, the supernatants from the two centrifugation procedures combined and centrifuged at 8,700 x g for 15 min. The resulting supernatant was then centrifuged at 48,000 x g for 30 min to obtain a crude plasma

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[13]3-(N-morpholino)propanesulfonic acid.

membrane which was stored at -70 °C (in 50 mM Tris-HCl; pH 7.4) and used for ligand binding assays.

Dihydropyridine receptor site ligand binding assay

The site-specific, equilibrium binding of [^{3}H]nitrendipine ([^{3}H]NTD), a calcium channel blocker, was measured by a filteration method previously described for [^{3}H]DHA (Chapter II). Crude plasma membranes were incubated with graded concentrations of [^{3}H]NTD in the presence or absence of 10 μM nifedipine in a total volume of 1 ml Tris-HCl (50 mM; pH, 7.4). Each assay consisted of duplicate mixtures of 100 μl portions of [^{3}H]NTD (0.05 - 2.0 nM), 500 μl of crude plasma membrane (120 - 160 μg protein) and 400 μl of Tris-HCl (total binding) or nifedipine (non-specific binding) in 1.5-ml microcentrifuge tubes and incubated at 22 °C for 90 min. The binding reaction was terminated by filtering, under pressure, through Whatman GF/A glass microfibre filters, and immediately rinsing filters three times with 3 ml ice-cold Tris-HCl buffer. Tissue-bound radioactivities were extracted from filters overnight in 5 ml of scintillation fluid (ScintiVerse^{TM}) and counted.

Data analysis

The pD_2 values were determined by regression analysis of -Log (concentration) - effect data between 20% - 80% of E_{max} using a computer program (Tallarida and Murray, 1986). Comparisons within the same diet group and between different diet groups were made using *t-test* analysis for grouped data and analysis of

variance (ANOVA) at 95% and 99% confidence limits. The binding constants, K_D (equilibrium dissociation constant) and B_{max} (maximum binding capacity) were determined as before (Chapter II).

D. Results

Body weight and heart size

The growth curves for euthyroid and hypothyroid animals over the six weeks feeding period are shown in Figure III-1 and Figure III-2, respectively. The rate of growth of PTU-treated animals was reduced by 24%, 18% and 30%, respectively for animals fed the n-3, n-6 or SAT diets compared to euthyroid controls during this period. The data presented in Table III-2 is a summary of the change in both body weight and heart weight of animals observed under the various diet regimens. The differences between the mean body weights of euthyroid and hypothyroid animals at the end of this period were significant ($p < 0.01$; ANOVA). No significant differences in body weights were found between animals fed the n-3 or the n-6 diets, but the SAT diet produced significant differences when compared with the n-6 diet ($p < 0.05$). The mean heart weights of euthyroid and hypothyroid animals fed the SAT diet were significantly smaller than those of age-matched animals fed the n-3 diet ($p > 0.05$) or the n-6 diet ($p < 0.01$). However, no significant differences were found between the heart weights of animals fed the n-3 or the n-6 diets. Similarly, the heart weight to body weight ratios were not significantly different from each other.

Calcium sensitivity of papillary muscles

The basal tension developed in papillary muscles from euthyroid and

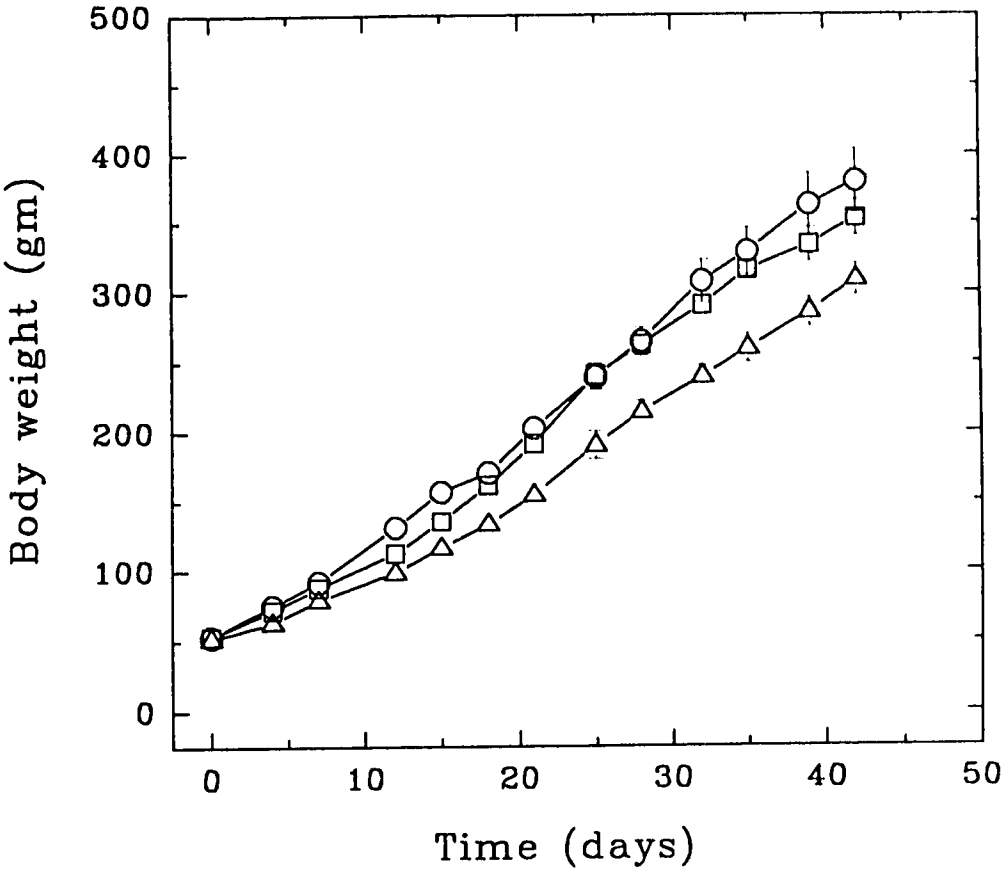

Figure III-1. The growth rate of euthyroid animals fed the n-3 diet (○), the n-6 diet (□) or the SAT diet (△) over the six weeks. Data are means (± S.E.); n = 5 animals.

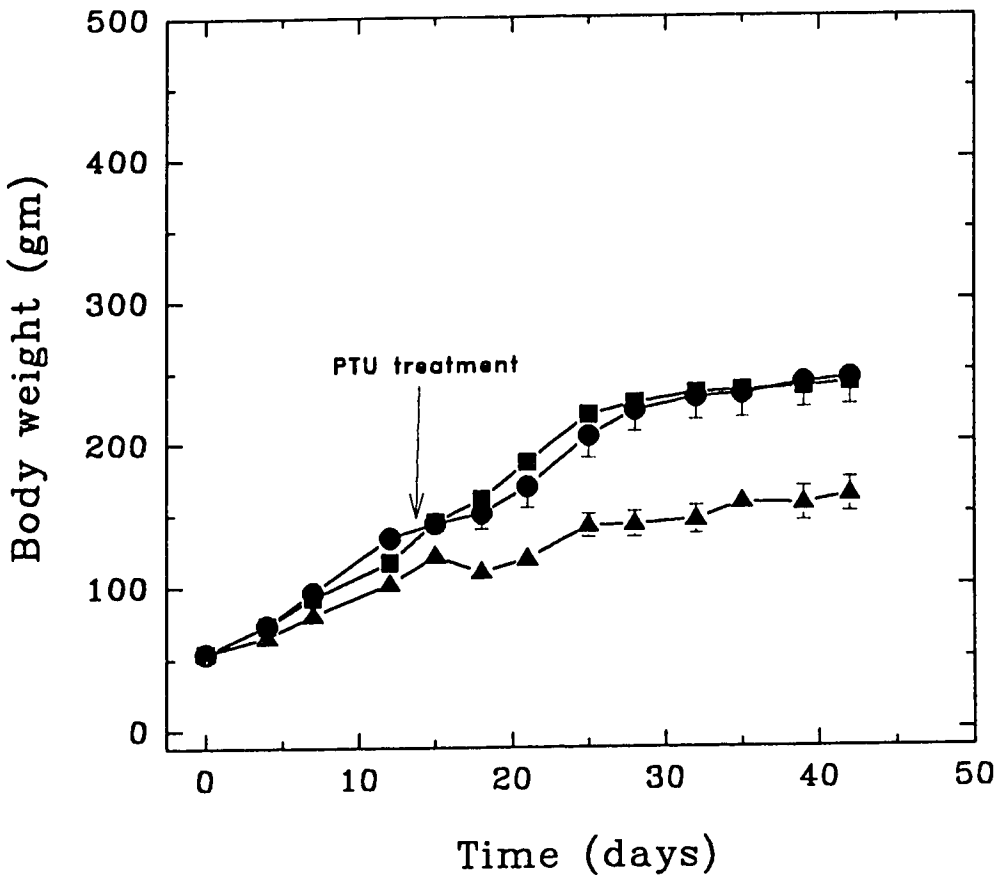

Figure III-2. The growth rate of animals treated with PTU and fed the n-3 diet (●), the n-6 diet (■) or the SAT diet (▲) over six weeks. Data are means (± S.E.); n = 5 animals.

Table III-2. Effect of thyroid status and diet on body weights and heart weights of animals.

Thyroid state	Diet	Body weight (gm)		Heart weight (gm)	HW/BW ($\times 10^{-3}$)
		Initial	Final[14]		
Euthyroid	n-3	52.7 ± 2.3	379.5 ± 23.2	1.41 ± 0.08	3.4 ± 0.4
	n-6	52.7 ± 1.7	352.5 ± 13.4	1.50 ± 0.05	3.7 ± 0.6
	SAT	51.9 ± 0.7	309.2 ± 11.4[a]	1.24 ± 0.02[b]	3.6 ± 0.4
Hypothyroid	n-3	53.5 ± 1.6	230.1 ± 21.2	0.75 ± 0.06	3.2 ± 0.2
	n-6	54.0 ± 0.9	242.0 ± 3.4	0.79 ± 0.03	3.3 ± 0.2
	SAT	54.4 ± 2.1	162.9 ± 12.0[c]	0.57 ± 0.04[c]	3.3 ± 0.1

Values are means (± S.E.) of 6 separate determinations.

[a]*Significantly different from n-3 and n-6 ($p < 0.05$).*

[b]*Significantly different from n-6 ($p < 0.01$).*

[c]*Significantly different from n-3 ($p < 0.05$) and n-6 ($p < 0.01$).*

[14]Body weight at the end of the six weeks feeding period.

hypothyroid animals fed the n-3 diet, the n-6 diet or the SAT diet are presented in Table III-3. The mean tension in tissues from animals fed the n-3 diet was higher than those generated in tissues from animals fed the n-6 diet or the SAT diet but the differences are not significant. The data indicate that thyroid state and dietary fatty acids did not significantly affect basal tension. The Ca^{2+} sensitivity of papillary muscles, from animals in different thyroid states and fed the diet formulations, are illustrated in Figure III-3 and Figure III-4. In euthyroid animals, the increase in tension due to increasing extracellular Ca^{2+} concentration caused by the SAT diet was higher compared to the n-6 diet or the n-3 diet. A comparison between the n-6 diet and the n-3 diet indicate that the latter increased the sensitivity to Ca^{2+} but reduced the maximal increase in tension. Thus, the n-3 diet had a protective effect on Ca^{2+}-induced inotropy. The lower portion of the Ca^{2+} concentration-effect curves for the n-3 diet and the SAT diet are similar while that for the n-6 diet was shifted to the right. The pD_2[15] for Ca^{2+}-stimulated contraction of papillary muscles is presented in Table III-4. In muscles from euthyroid animals, the n-3 diet increased the pD_2 value by 0.58 units while the SAT diet increased it by 0.20 units relative to the n-6 diet. However, in tissues from hypothyroid animals fed the n-6 diet or the SAT diet the pD_2 values are similar. Thus, the calcium sensitivity of papillary muscles was enhanced by n-3 in tissues from both euthyroid and hypothyroid animals. The tension developed in tissues from euthyroid animals fed the SAT diet declined more rapidly, with further increase in Ca^{2+} concentration, after reaching maximum

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[15] — Log EC_{50} for contraction.

Table III-3. Effect of thyroid status and diet on basal tension developed in papillary muscles upon electrical stimulation.

Thyroid state	Diet	Tension (x 10^{-6} N/mg tissue)
Euthyroid	n-3	43.9 ± 8.8
	n-6	30.7 ± 11.0
	SAT	37.3 ± 6.4
Hypothyroid	n-3	42.5 ± 14.3
	n-6	33.0 ± 7.9
	SAT	32.9 ± 2.4

Values are means (± S.E.); n = 5 animals.

Differences between means are not significant (ANOVA).

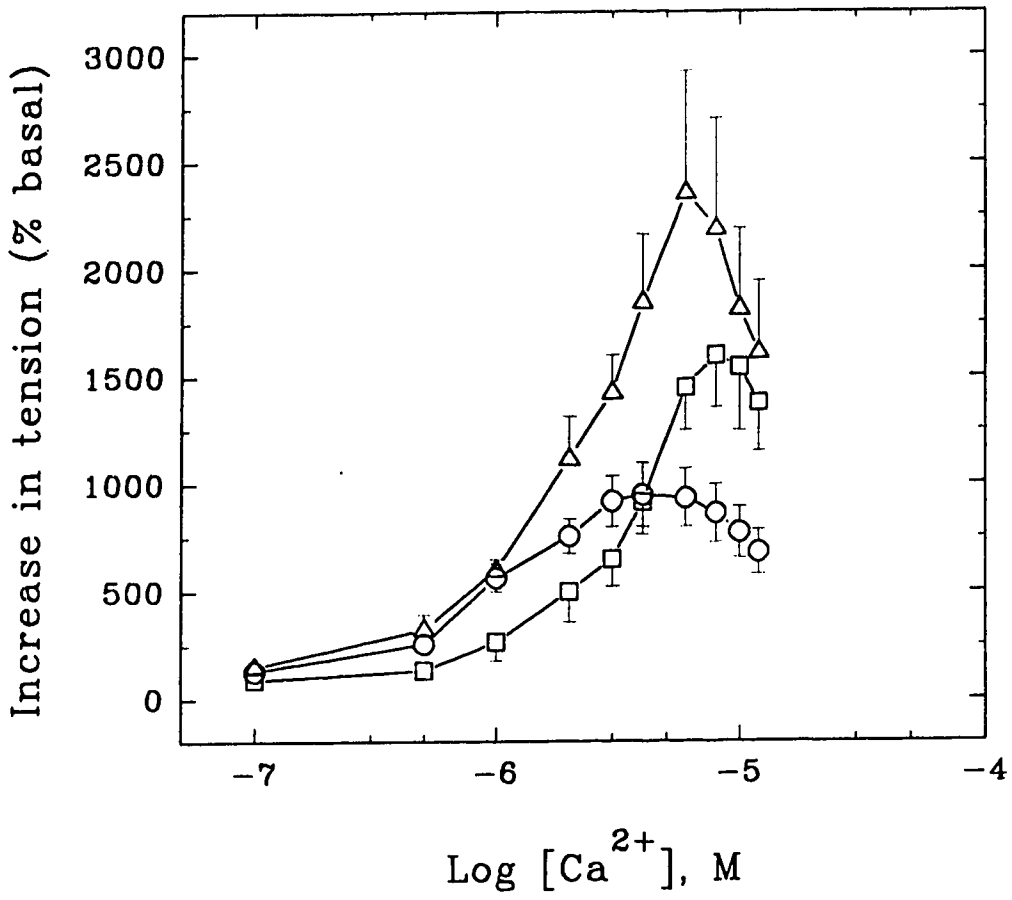

Figure III-3. Calcium sensitivity of papillary muscles from euthyroid animals. Contractile responses to cummulative concentrations of Ca^{2+} in tissues from animals fed the n-3 diet (○), the n-6 diet (□) or the SAT diet (△) are shown. Data are means (± S.E.); n = 5.

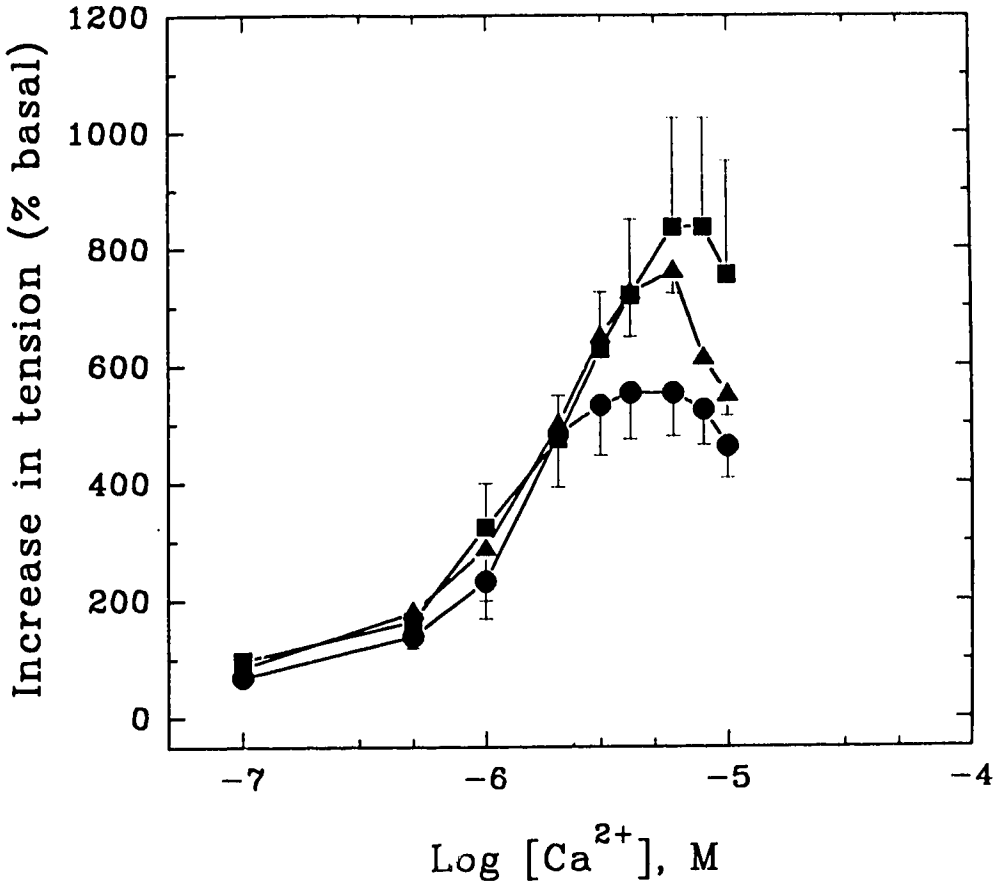

Figure III-4. Calcium sensitivity of papillary muscles from hypothyroid animals. Contractile responses to cummulative concentrations of Ca^{2+} in tissues from animals fed the n-3 diet (●), the n-6 diet (■) or the SAT diet (▲) are shown. Data are means (± S.E.); n = 5.

Table III-4. The effect of thyroid state and diet on pD_2 values for Ca^{2+}-stimulated contraction in papillary muscles.

Thyroid state	Diet	pD_2	C.L.
Euthyroid	n-3	6.03 ± 0.03[a]	6.44 — 5.62
	n-6	5.45 ± 0.06[b]	6.15 — 4.75
	SAT	5.65 ± 0.09	5.93 — 5.36
Hypothyroid	n-3	5.93 ± 0.06	6.17 — 5.42
	n-6	5.83 ± 0.03	6.18 — 5.48
	SAT	5.86 ± 0.05	6.53 — 5.18

C.L. = Confidence limits.

[a]*Significantly different from n-6 and SAT ($p < 0.01$).*

[b]*Significantly different from hypothyroid on same diet ($p < 0.01$).*

compared to those from animals fed the n-3 or the n-6 diets. In hypothyroid tissues, the pattern of decline in maximum force at higher Ca^{2+} concentrations was similar to that in euthyroid tissues. The pD_2 values in all diet groups were similar but the n-3 diet induced a lower maximum tension. Thus, it appears the hypothyroid state had no effect on tissue sensitivity to extracellular calcium.

The data presented in Figure III-5 illustrates the calcium sensitivity of tissues from euthyroid and hypothyroid animals fed standard laboratory chow. The basal tensions were similar (43.9 ± 8.3 and 44.9 ± 9.4 x 10^{-6} N/mg tissue for euthyroid and hypothyroid, respectively; n = 5) as observed in tissues from animals fed the diet formulations. The data indicate that tissues from euthyroid animals exhibited higher Ca^{2+} sensitivities compared to those from hypothyroid animals. The Ca^{2+} concentration-effect curve was shifted to the right and the maximum reduced by propylthiouracil treatment. The calcium sensitivity of chemically skinned muscle preparations from these animals are shown in Figure III-6. There are no differences between the calcium sensitivity of hypothyroid and euthyroid tissues. The maximal increase in tension was similar and about 25% lower compared to those measured in intact muscles. The data suggest that the difference in tension development was not due to myofibrilar Ca^{2+} sensitivity. Table III-5 shows the pD_2 values obtained in intact and skinned papillary muscles. In intact muscles, hypothyroidism reduced the pD_2 by 0.40 units and the maximum contractile response by about 200% compared to euthyroid. These findings imply that contributions from other components of the excitation-contraction coupling system, possibly those involving the sarcolemma or

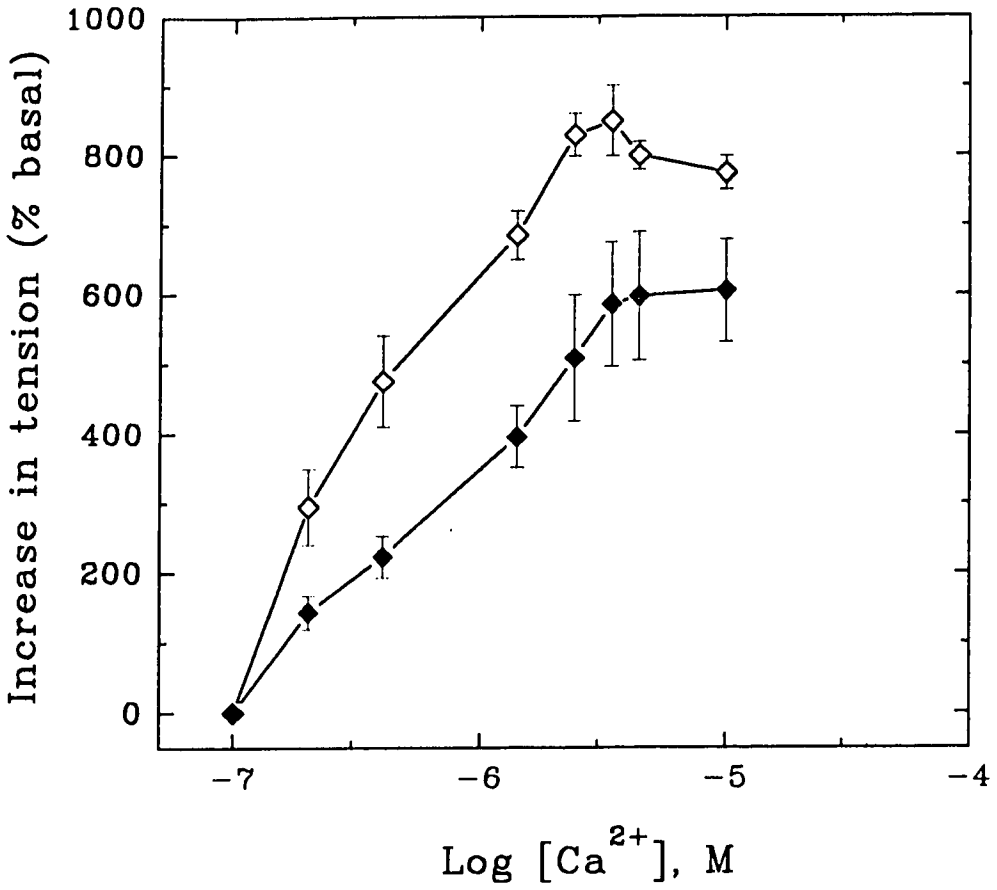

Figure III-5. Calcium sensitivity of papillary muscles from animals fed standard laboratory chow. Contractile responses to cummulative concentrations of Ca^{2+} in tissues from euthyroid (◊) and hypothyroid (♦) aniinals are shown. Data are means (± S.E.); n = 3.

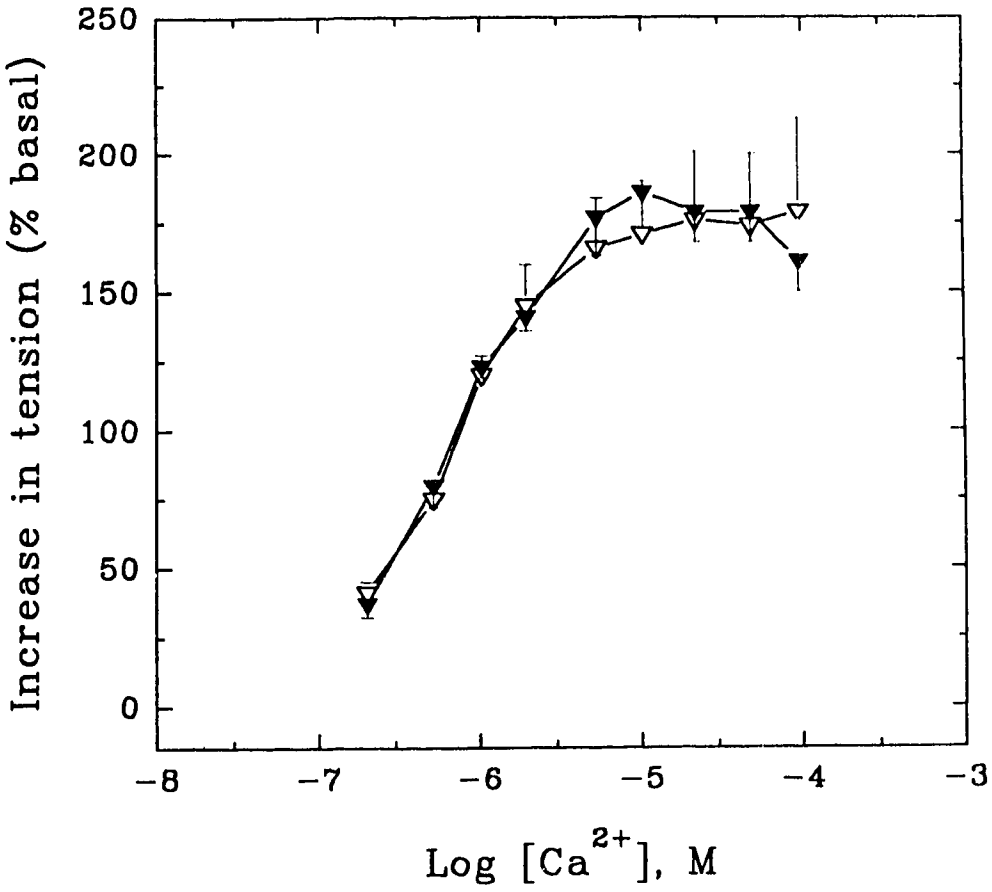

Figure III-6. Calcium sensitivity of skinned papillary muscles from animals fed standard laboratory chow. Contractile responses to accumulative concentrations of Ca^{2+} in tissues from euthyroid (▽) and hypothyroid (▼) animals are shown. Data are means (± S.E.); n = 5.

Table III-5. Effect of thyroid state on pD_2 values for Ca^{2+}-stimulated contraction of intact and chemically-skinned papillary muscles from animals fed standard chow.

Thyroid state	Intact muscles		Skinned muscles	
	pD_2	C.L.	pD_2	C.L.
Euthyroid	6.25 ± 0.07[a]	6.55 — 5.95	6.11 ± 0.04	6.59 — 5.63
Hypothyroid	5.85 ± 0.11	6.29 — 5.40	6.02 ± 0.05	6.76 — 5.28

[a]*Significantly different from hypothyroid ($p < 0.01$).*

the sarcoplasmic reticulum, are responsible for the higher sensitivity to calcium induced by the n-3 diet.

Tissue responsiveness to nifedipine

The effect of the calcium channel blocker, nifedipine, in reducing contractile force in papillary muscles from euthyroid and hypothyroid animals fed the different diet formulations is shown in Figure III-7. In tissues from euthyroid animals, the data indicate that the SAT and the n-6 diets reduced the sensitivity of tissues to nifedipine relative to the n-3 diet. The sensitivity of papillary muscles to nifedipine decreased in animals fed the n-3 diet through those fed the n-6 diet to those fed the SAT diet. Similarly, in tissues from hypothyroid animals, the n-3 diet increased the sensitivity to nifedipine compared to the n-6 diet. The pD_2[16] values for the inhibition of contraction by nifedipine are presented in Table III-6. The sensitivities of tissues from euthyroid animals fed the n-6 diet were similar to those determined for tissues from hypothyroid animals fed the n-3 diet.

Radioligand binding

To determine if thyroid state and dietary fatty acids have any effect on calcium channel density in rat myocardium, specific binding of [^{3}H]nitrendipine to crude plasma membrane preparations from the ventricles of euthyroid and hypothyroid animals fed the various diet formulations was determined. The data presented in

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[16] — Log IC_{50} for inhibition.

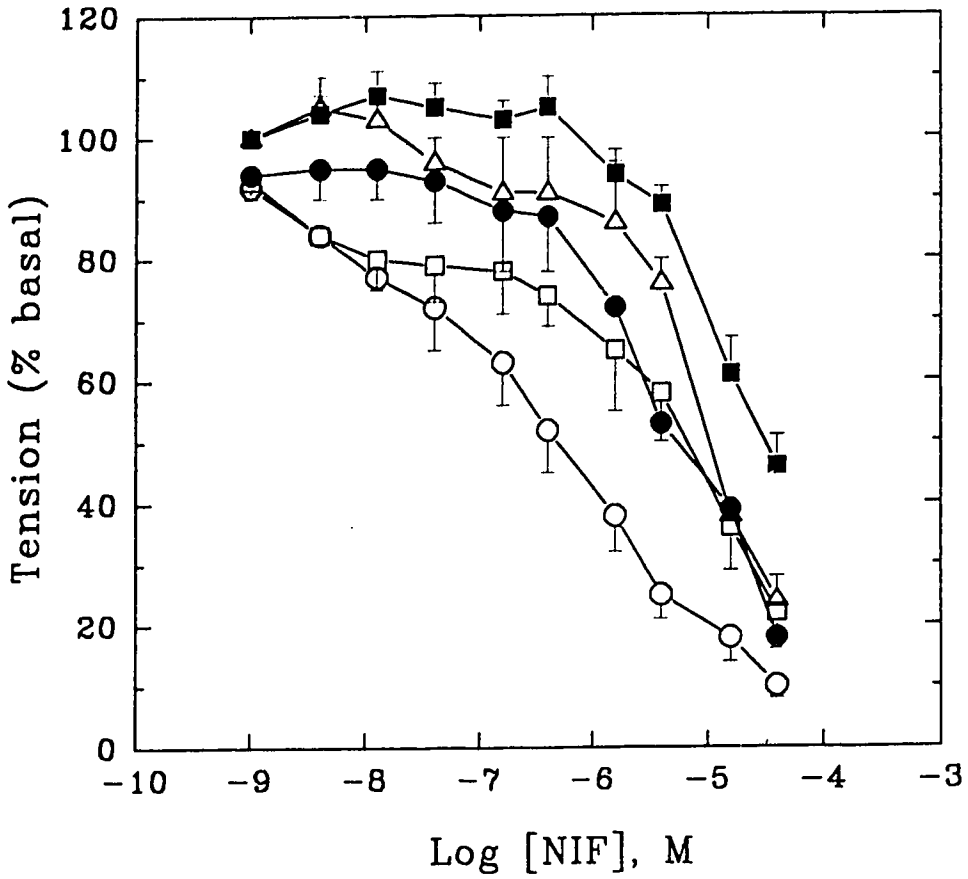

Figure III-7. Negative inotropic effect of nifedipine in papillary muscles from euthyroid (○, □, △) and hypothyroid (●, ■) animals fed the n-3 diet (○, ●), the n-6 diet (□, ■) or the SAT diet (△). Data are means (± S.E.); n = 4-6.

Table III-6. Effect of thyroid state and diet on pD_2 values for the inhibition of contraction of papillary muscles by nifedipine.

Thyroid state	Diet	pD_2	C.L.
Euthyroid	n-3	6.40 ± 0.08	6.62 — 6.18
	n-6	5.48 ± 0.13[a]	5.88 — 5.07
	SAT	4.99 ± 0.06[a]	5.29 — 4.19
Hypothyroid[b]	n-3	5.45 ± 0.02	5.54 — 5.36
	n-6	4.69 ± 0.03[a]	5.02 — 4.36
	SAT	N.D.	-

C.L. = 95% confidence limits; N.D. = Not determined.

Values are means (± S.E.) of at least 5 separate determinations.

[a]*Significantly different from n-3 ($p < 0.01$).*

[b]*Significantly different from euthyroid animals on same diet ($p < 0.01$).*

Figure III-8 show the results from a typical ligand binding experiment obtained in membranes from euthyroid animals fed the various diet formulations. The data demonstrate saturable binding in all cases. There are no apparent differences between the binding curves for nitrendipine. The Scatchard plots obtained in tissues from animals fed the n-3 diet and the n-6 diet are similar. There are no significant differences between the K_D values obtained in membranes from euthyroid animals fed the n-3 diet or the n-6 diet, however, these were higher than those determined in membranes from animals fed the SAT diet. In membranes from animals fed the n-6 diet, the B_{max} values are significantly different ($p < 0.05$) from those obtained in membranes from animals fed the n-3 diet or the SAT diet.

In membranes from hypothyroid animals fed the various diets, binding was again saturable as demonstrated by the binding curves (Figure III-9). The binding of nitrendipine was higher in membranes from animals fed the n-6 diet compared to those fed the SAT diet or the n-3 diet. The Scatchard plots of data obtained in membranes from animals fed the n-6 diet and the n-3 diet are parallel suggesting similar binding affinities but different binding site densities. The mean binding constants, K_D and B_{max} determined by computerized, non-linear, least-squares mass-law analysis of untransformed radioligand binding data, however, are higher in membranes from animals fed the n-6 diet compared to those fed the n-3 diet or the SAT diet. The binding data for euthyroid and hypothyroid animals are summarized in Table III-7.

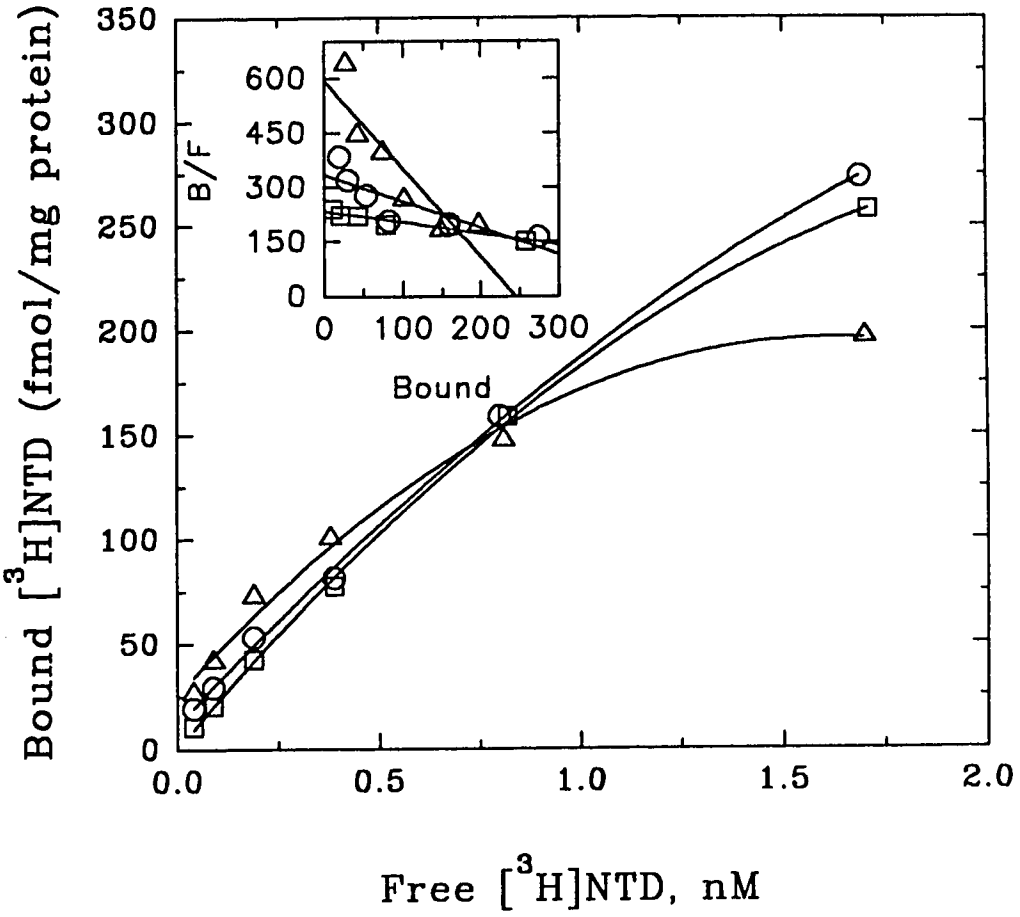

Figure III-8. Site-specific binding of [^{3}H]nitrendipine to crude plasma membranes from euthyroid animals fed the n-3 diet (○), the n-6 diet (□) or the SAT diet (△). Inset are the Scatchard plots of the binding data. Each point is a mean of duplicate determinations; n = 3-6.

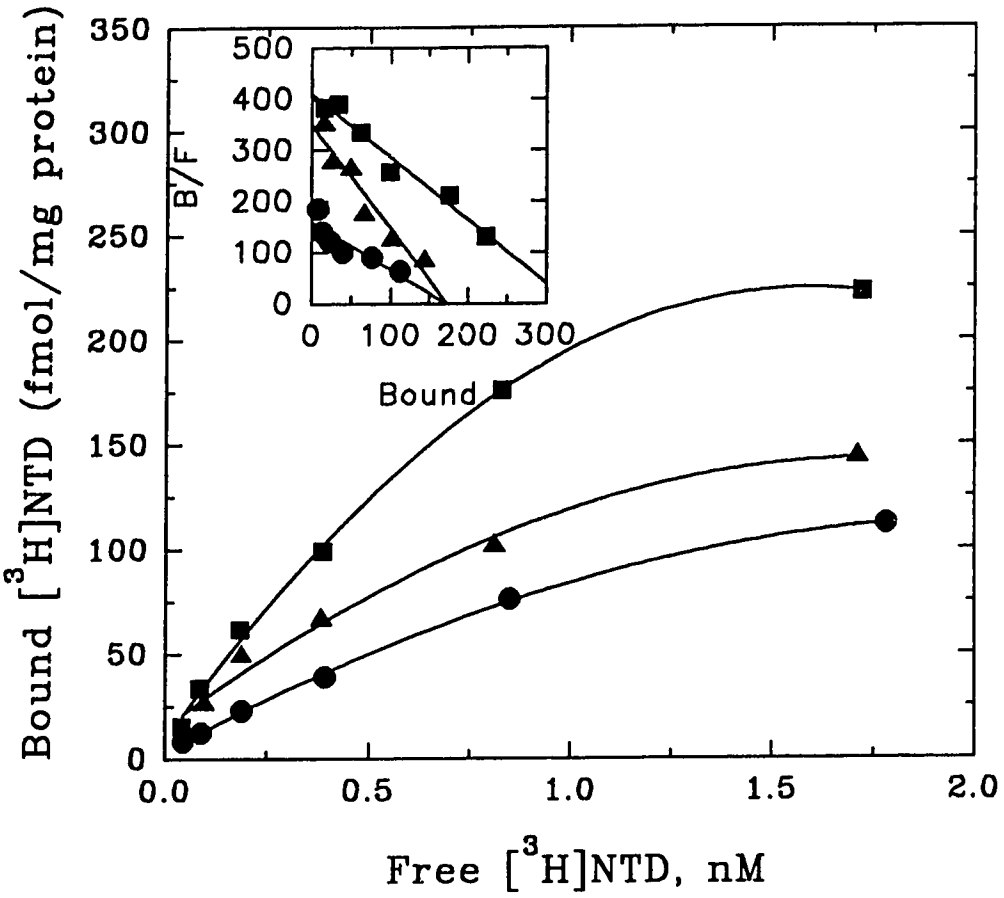

Figure III-9. Site-specific binding of [^{3}H]NTD to crude cardiac plasma membranes from hypothyroid animals fed the n-3 diet (●), the n-6 diet (■) or the SAT diet (▲) Inset are the Scatchard plots of the binding data. Each point is a mean of duplicate determinations; n = 3-6.

Table III-7. Effect of thyroid status and diet on K_D and B_{max} values for the site-specific binding of [^{3}H]nitrendipine to crude cardiac plasma membranes determined by mass-law analysis of untransformed radioligand binding data.

Thyroid state	Diet	K_D (nM)	B_{max} (fmol/mg protein)
Euthyroid	n-3	0.26 ± 0.07	47.60 ± 10.27[a]
	n-6	0.31 ± 0.10	82.60 ± 12.01
	SAT*	0.17 ± 0.02	64.69 ± 15.37
Hypothyroid	n-3	0.18 ± 0.04	42.12 ± 11.93
	n-6	0.33 ± 0.04[b]	114.64 ± 8.74[b]
	SAT*	0.16 ± 0.02	58.64 ± 8.53

*Values are means (± S.E.) of 3-6 separate determinations; *n = 3.*

[a]*Significantly different from n-6 ($p < 0.05$; ANOVA).*

[b]*Significantly different from n-3 and SAT ($p < 0.01$; ANOVA).*

Tissue responsiveness to acetylcholine

The effect of thyroid state and feeding of the n-3 diet or the n-6 diet on the inotropic response of papillary muscles to acetylcholine, a muscarinic receptor agonist is shown in Figure III-10. Acetylcholine elicited biphasic responses in papillary muscles from animals fed the n-3 diet. At low concentrations (1×10^{-8} - 1.4×10^{-6} M), ACh caused reduction in tension development whereas, at high concentrations (4.4×10^{-6} - 4.4×10^{-4} M) contractions were observed. On the other hand, in tissues from animals fed the n-6 diet, ACh elicited only contractile responses. The contractions in tissues from euthyroid animals fed the n-6 diet were more pronounced than those elicited in tissues from hypothyroid animals. The maximal response in euthyroid tissues were significantly larger than the response seen in tissues from hypothyroid animals.

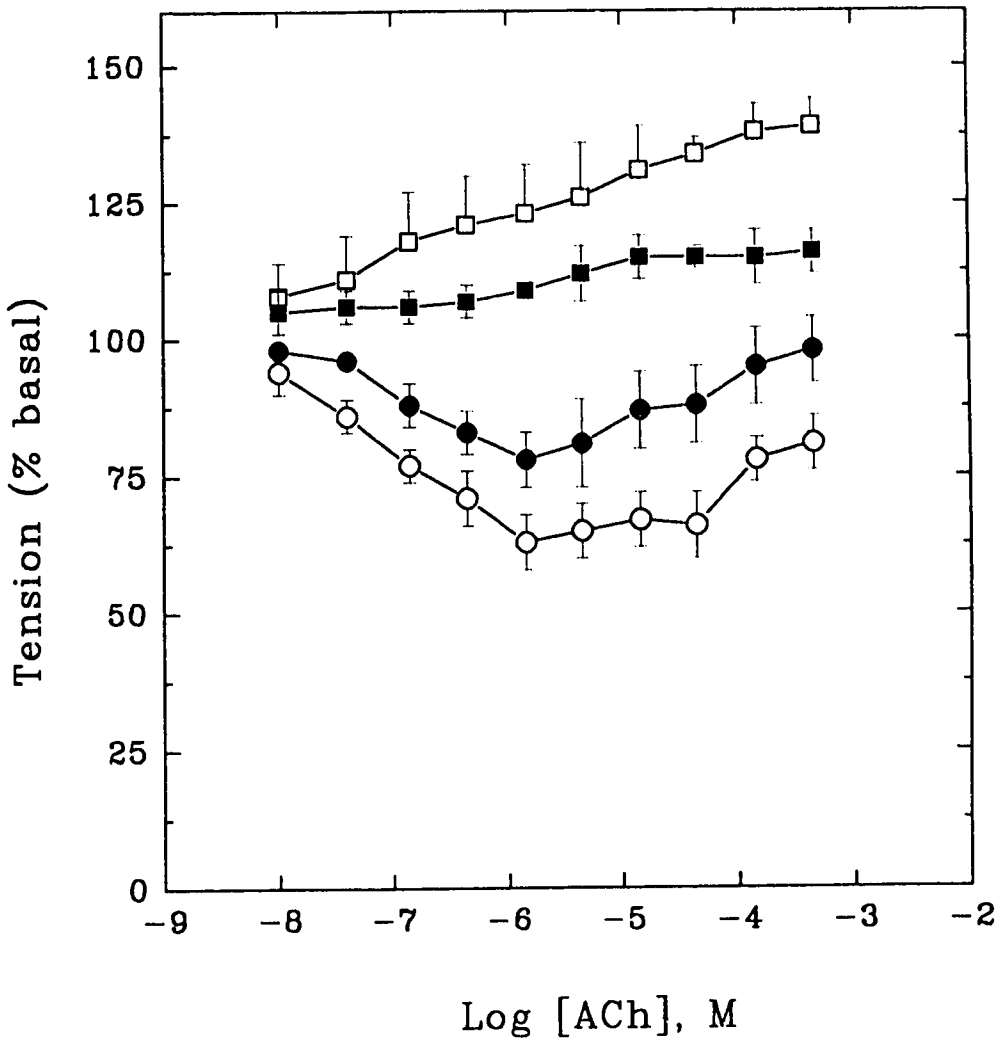

Figure III-10. Negative and positive inotropic effects of acetylcholine in papillary muscles from euthyroid (○, □) and hypothyroid (●, ■) animals fed the n-3 diet (○, ●) or the n-6 diet (□, ■). Data are means (± S.E.); n = 6.

E. Discussion

Changes in thyroid status alters physiological function some of which are very prominent in developing hypothyroid rats which show slower rates of body growth compared to euthyroid controls (Gay *et al.*, 1988; Dieckman and Solaro, 1990; Chin and Pennefather, 1992). The present study has revealed similar effects of hypothyroidism and both n-3 and n-6 fatty acids appear to offset the reduced growth rate when compared with animals fed the saturated fat diet. These results suggest that the polyunsaturated fatty acids at levels higher than 11% in a 20% (w/w) fat diet have beneficial effects on growth and development.

The force of contraction of the heart is controlled by influx of Ca^{2+} into myocardial cells via slow calcium channels. The calcium transient serves to couple sarcolemmal excitation to contraction and is a major determinant of the force of myocardial contraction and ATP consumption (Sperelakis, 1984). The enhancement of the contractile force of cardiac muscle can be achieved by an increase in the availability of Ca^{2+} ions which activate the myofilaments and/or by an increase in the responsiveness of myofilaments to Ca^{2+}. Thus, modulation of contraction can occur at the level of the sarcolemma which controls Ca^{2+} movement into the cell, at the sarcoplasmic reticulum which regulates cytosolic Ca^{2+} concentration or at the myofilaments. Maximum tension development in the myocardium is closely related to the magnitude of the calcium flux across the sarcoplasmic reticulum (Suko, 1973). Thyroid hormone has been shown to increase Ca^{2+} uptake by the sarcoplasmic

reticulum as well as to stimulate Ca^{2+} movement across the sarcolemma (Rudinger *et al.*, 1984; Rodgers *et al.*, 1986). The enhancement of myocardial contractility in response to thyroid hormone may therefore include alterations in the calcium handling properties of both the sarcolemma and the sarcoplasmic reticulum that would directly influence the excitation-contraction coupling response as well as myofilament Ca^{2+} sensitivity. Increase in calcium uptake may result from increased density or open-close probability of voltage-dependent calcium channels. The force of contraction may be limited by decreased Ca^{2+} influx, decreased sensitivity of sarcomeres to Ca^{2+} or stimulation of Ca^{2+} sequestration by the sarcoplasmic reticulum which would reduce the availability of activator Ca^{2+} (Schouten *et al.*, 1990). Calcium channel blockers, such as nifedipine, specifically reduce Ca^{2+} influx through voltage-dependent channels resulting in negative inotropy and chronotropy (Schram and Towart, 1988).

Dietary lipids are important determinants of the fatty acid composition of mammalian tissues and alterations in the saturation/unsaturation levels in cardiac plasma membranes may change the ordered state of the membrane and hence the activities of transport proteins. The side-by-side orientation of phospholipids may increase or decrease steric hindrance and lead to the regulation of ionic movement across the membrane (Steck and Fox, 1972). Dietary polyunsaturated fatty acid supplementation in the rat provides significant protection against isoproterenol-induced dysrhythmia in isolated papillary muscles concomitant to reduction in contractility and in the inotropic responses to Ca^{2+} (McLennan *et al.*, 1987). From

studies with cardiac myocytes, it was suggested that the observed protective effects of n-3 fatty acids were due to modulation of nitrendipine-sensitive L-type calcium channels (Hallaq *et al.*, 1992). The n-3 fatty acids were found to prevent excessive influx of Ca^{2+} into cells as well as enhanced insufficient influx and it was suggested these effects could be due to the presence of different numbers of calcium channels or different kinetics of calcium transport. Elevated intracellular concentrations of Ca^{2+} could be due to increased influx into myocardial cells from the extracellular space via L-type Ca^{2+} channels and Na^{+}/Ca^{2+} exchanger or to increased release from sarcoplasmic reticulum.

The present study demonstrates that the n-3 diet increased the sensitivity of euthyroid tissues to extracellular Ca^{2+} compared to the n-6 diet, however, this diet also exerted a protective effect on intracellular Ca^{2+} accumulation at higher concentrations. The n-6 fatty acids had a larger effect on maximum tension compared to n-3 fatty acids but the latter was more potent in increasing tissue sensitivity to Ca^{2+} at low concentrations. Thus, the effect of n-3 is two-fold: (i) it increases tissue sensitivity to extracellular calcium hence increases contractile force, and (ii) it prevents calcium accumulation by the cell hence limits the force of contraction. This finding agrees with that reported by McLennan *et al.* (1987a) who showed that n-3 fatty acids reduced Ca^{2+}-induced contractility in rat papillary muscles, and offers an explanation for the dual role for n-3 fatty acids suggested by Hallaq *et al.* (1992). The difference between the effects of the n-3 diet and the n-6 diet suggests that these may be due to changes in membrane lipid environment induced by the diets. In

hypothyroid tissues, there are no apparent differences in calcium sensitivity suggesting that thyroid hormone plays a role in modulating Ca^{2+} translocation. Incubating muscle preparations in EGTA-Triton X-100 solution eliminates the influence of the sarcolemma and intracellular organelles, particularly that of the sarcoplasmic reticulum, on contraction. Hence, the results from the Ca^{2+}-sensitivity experiments employing skinned muscle preparations compared to intact muscles suggest that the dietary fatty acid-induced alteration in sensitivity was not due to differences in the Ca^{2+} sensitivities of the myofibrils. This finding implies that changes in sarcolemmal calcium transport or SR calcium release and/or sequestration may be involved.

Studies have shown that thyroid state influences the unsaturation index of rabbit cardiac sarcolemma (Szymanska *et al.*, 1992), as well as the phospholipid deacylation-reacylation cycle (Hoch, 1988). Thus, alteration of the activities of membrane-bound enzymes catalyzing such processes may be involved. Such alterations, in addition to changes in ion transport may result from changes in the phospholipid composition of membranes which provide the structural matrix for enzymes, receptors and transport proteins. Shifts in lipid composition to more unsaturated state may regulate Ca^{2+} movement across the sarcolemma as well as its sequestration in the sarcoplasmic reticulum. A more fluid membrane environment would influence the kinetics of the transport proteins in the lipid bilayer as the mobility of such proteins would be increased. The basal force developed in euthyroid and hypothyroid muscles were about the same suggesting reduced Ca^{2+} uptake by the sarcoplasmic reticulum in hypothyroid tissues.

The higher sensitivity to nifedipine observed in tissues from animals fed the n-3 diet and the K_D and B_{max} values for [^{3}H]nitrendipine binding suggest that fewer calcium channels are present in these tissues compared to those from animals fed the n-6 diet or the SAT diet. The ligand binding affinities in tissues from animals in the latter group were higher although no significant differences were found between the diet groups. These results therefore suggest that animals fed the n-6 diet expressed more calcium channels in their myocardium in order to compensate for the apparent reduction in calcium channel activity, whereas those animals fed the saturated fat diet expressed high affinity channels in their myocardium. An increase in calcium channel density with no change in binding affinity in experimental hypothyroidism compared to the euthyroid sate has been reported in rat cardiac and vascular tissues (Hawthorn *et al.*, 1988). The K_D values obtained in the present study are in agreement with that reported for ventricular membranes from adult rats (Kojima *et al.*, 1990). The expression of fewer channels in cardiac sarcolemma from animals fed the n-3 diet suggests that channel activity is high probably as a result of changes in the membrane environment which allows for a more rapid rate of Ca^{2+} transport into the cell during contraction. However, this process is apparently reversed once enough Ca^{2+} has entered the cell to activate the contractile elements. If this is true then a concomitant mechanism to reduce the cytosolic concentration of Ca^{2+} should operate. Thus, rapid calcium sequestration into the sarcoplasmic reticulum and/or rapid removal via Na^+/Ca^{2+} exchange may serve this purpose. The above mechanism may be important in moving enough Ca^{2+} into the cell to maintain adequate contraction and at the

same time prevent Ca^{2+} overload. Taffet *et al.* (1993) have shown that increasing the ratio of docosahexaenoic acid to arachidonic acid as well as that of total n-3 to n-6 fatty acids decreased ATP-dependent Ca^{2+} uptake and Ca^{2+}-ATPase activity in rat heart sarcoplasmic reticulum. Thus, dietary fatty acids can alter the function of Ca^{2+} transport proteins and other integral membrane transport proteins in the sarcolemma as well as the sarcoplasmic reticular membrane. The involvement of other components of the excitation-contraction coupling system can be determined by examining the effect of calcium on contractile responses in the presence of caffeine or ryanodine as well as calcium uptake by isolated sarcoplasmic reticulum (Shattock and Bers, 1989; Bers, 1991).

The direct inhibitory effect of acetylcholine on the force of contraction of ventricular muscle is thought to involve reduction in adenylate cyclase activity (Loffelholz, 1985). However, at high concentrations, acetylcholine can cause norepinephrine release from nerve endings and, therefore can indirectly cause contraction (Levy *et al.*, 1981). The results obtained with acetylcholine in the present study show that contractions occured in tissues from animals fed the n-6 diet whereas the n-3 diet induced biphasic responses. The biphasic response could be due to activation of separate cell surface receptors, thus each response depends on the occupancy of the receptor and intrinsic agonist efficacy for each receptor, receptor density or coupling efficiency (Kenakin and Boselli, 1991). Another posibility is that a single receptor interacts with different G proteins which mediate two biochemical responses. The negative inotropic effect of acetylcholine could be due to activation

of K^+ channels through a G protein. Carbachol increases K^+ conductance, hyperpolarizes the membrane and decreases action potential duration thereby reducing the influx of Ca^{2+} resulting in negative inotropy (Pfaffinger *et al.*, 1985; Bohm *et al.*, 1986). It has been suggested that two different G proteins regulate K^+ conductance at low and high concentrations of the agonist. In the latter case, the G protein couples the muscarinic cholinoceptor to inositol lipid metabolism. Upon agonist stimulation of the muscarinic receptor, hydrolysis of membrane phosphatidylinositol 4, 5-bisphosphate (PIP_2) to produce myoinositol (IP_3) and diacylgycerol (DAG) occurs.

A number of studies have shown that IP_3 can mobilize Ca^{2+} from sarcoplasmic reticulum in the heart (Nosek *et al.*, 1986; Putney *et al.*, 1989; Kentish *et al.*, 1990) as well as activate contractile proteins and ionic channels (Downes and Michell, 1982). Kohl *et al.* (1990a) have shown that carbachol has a slight positive inotropic effect in papillary muscles, which was preceeded by an increase in IP_3 level, indicating that muscarinic cholinoceptor-mediated increase in IP_3 and force of contraction may be related. Cholinoceptor agonists have also been shown to enhance the formation of inositol phosphates in rat and guinea pig hearts (Brown *et al.*, 1985; Brown and Jones, 1986; Scholz *et al.*, 1988), and inositol metabolism has been demonstrated in human heart (Kohl *et al.*, 1990b). Thus, like carbachol, the positive inotropic effect of acetylcholine in papillary muscles observed in this study could be due to increased IP_3 levels. The results suggest the generation of higher concentrations of IP_3 in cardiac cells from animals fed the n-6 diet compared to those

fed the n-3 diet and probably the mobilization of more Ca^{2+} from the sarcoplasmic reticulum.

In conclusion, this study has shown that thyroid status and dietary fatty acids modulate myocardial contractility through effects on the voltage-sensitive calcium channel and probably through effects on Na^{+}/Ca^{2+} exchange in the sarcolemma. N-3 fatty acids were more effective than n-6 fatty acids probably, because of effects on the kinetics of calcium transport resulting from changes in membrane fatty acids. The results suggest that dietary fatty acids have a greater effect on calcium sensitivity than thyroid hormone.

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IV. CHANGES IN FATTY ACID COMPOSITION OF VENTRICULAR SARCOLEMMAL PHOSPHOLIPIDS INDUCED BY DIFFERENT THYROID STATES AND DIETARY FATTY ACIDS

A. Introduction

Thyroid hormone action on the myocardium produces responses such as changes in protein turnover, increased sensitivity to other hormones (e.g., catecholamines) and increased mechanical performance (Mylotte *et al.*, 1985; Kim *et al.*, 1987; Rohrer and Dillman, 1988; Beekman *et al.*, 1990). Changes in the mechanical activity of the heart in different thyroid states have been related to altered calcium handling properties of the myocardium (Kim and Smith, 1985; Marsh, 1990) as well as changes in the ß-adrenoceptor/adenylate cyclase system (Bahouth, 1991; Birk *et al.*, 1992). In the hypothyroid state, for example, myocardial contractility is depressed partly because of a decreased responsiveness of the ß-adrenergic system. This may be related to changes in sarcolemma phospholipid fatty acid composition (McMurchie *et al.*, 1987). Thyroid hormone alters the fatty acid composition of membrane phospholipids through effects on fatty acid synthesis and desaturation as well as on deacylation-reacylation of phospholipids during which new fatty acids are inserted into the membrane (Faas and Carter, 1981; Faas and Carter, 1982; Hoch, 1988; Dang *et al.*, 1989). The suppressed adrenergic activity observed in hypothyroidism may therefore be due to alterations in the lipid environment of the ß-adrenoceptor/adenylate cyclase complex and/or calcium transport proteins induced

by inadequate levels of thyroid hormone.

Influences of membrane lipids on ion channels may be responsible for the thyroid hormone-dependent changes in Ca^{2+} sensitivity observed in rat myocardium in an earlier study (Chapter III). Hence, thyroid hormone may be important in the regulation of membrane lipid metabolism and the maintenance of normal cardiac function. Desaturation and chain elongation of dietary essential fatty acids play an important role in determining the fatty acid composition of mammalian tissue and the activities of the enzymes involved in these processes, which are dependent on the type of fatty acids in the surrounding membrane, are also affected by thyroid hormone (Hoch *et al.*, 1981; Faas and Carter, 1982). Specific fatty acids are key elements of membrane protein function, as is the type of phospholipid head group and side chain which strongly affect membrane permeability of ions and organic molecules (Rudinger *et al.*, 1984; Kim and Smith, 1985; Marsh, 1990). In rabbit cardiac sarcolemma, however, it was shown that changes in the fatty acid composition of phospholipids observed in different thyroid states was not due to alteration in desaturase enzyme activity but to differences in phospholipase activity (Szymanska *et al.*, 1991).

Membrane fatty acids contribute most to fluidity and are easily altered particularly by dietary manipulation. Dietary fatty acids can profoundly alter the fatty acid composition of membrane phospholipids in mammalian cells (Charnock *et al.*, 1985; Garg *et al.*, 1989; Leaver *et al.*, 1992; Howie *et al.*, 1992). Dietary fatty acids are precursors for the synthesis of a number of long chain saturated and unsaturated fatty

acids and are therefore able to produce changes in the levels of saturated and unsaturated fatty acids as well as the extent to which polyunsaturated fatty acids are available for incorporation into cell membranes. While the major effects of thyroid hormone are mediated through protein synthesis, direct membrane effects independent of protein synthesis have also been observed (Davis *et al.*, 1989). However, the role of the sarcolemma in producing contractile defects associated with chronic hypothyroidism is not clear. The present study therefore examined the influence of thyroid state on the metabolism of dietary fatty acids in ventricular sarcolemmal membrane.

B. Materials

Fatty acid standards for gas-liquid chromatography (GLC) and 6-*n*-propyl-2-thiouracil (PTU) were purchased from Sigma, St. Louis, MO. All other chemicals were of reagent grade available commercially. Precoated HP-K high performance silica gel thin layer Chromatography (TLC) plates (200 μ, 10 x 10 cm) were purchased from Whatman. Basal mix diet was from Tekland Diets, Madison, WI. Safflower oil, linseed oil, olive oil and beef tallow were purchased on the open market, and fish oil (EP-28) was from Nishin Co., Tokyo, Japan.

C. Methods

Animals and diets

Weanling male Sprague-Dawley rats (50-55 gm) were fed diets containing 20% fat (wt/wt) enriched with n-3, n-6 or saturated (SAT) fatty acids for two weeks as previously described (Chapter III). Half of the animals in each diet group were then administered PTU (0.05% solution) in drinking water to render them hypothyroid and the feeding continued for another four weeks. The other half served as euthyroid controls. The basal diet contained the following (in gm/kg of diet): Starch, 408; casein, 270; non-nutritive cellulose, 50; vitamin mix, 10; mineral mix, 50.85; inositol, 6.25; choline chloride, 2.75 and L-methionine, 2.5. The fatty acid composition of diets is presented in Table IV-1. The polyunsaturated/saturated ratio of the n-3 diet reflects the Canadian Heart Association recommendation for fat consumption. Diets

Table IV-1. Fatty acid composition of diets.

Fatty acid	Composition of diet (mol %)		
	n-3	n-6	SAT
14:0	4.5	1.7	5.7
16:0	14.6	15.1	30.9
16:1	5.0	-	-
17:0	2.1	0.7	2.0
18:0	12.6	20.5	48.5
18:1(n-7)	27.0	10.4	2.2
18:1(n-9)	2.1	-	-
18:2(n-6)	11.7	50.5	11.1
18:3(n-3)	2.5	1.2	1.2
20:5(n-3)	13.0	-	-
22:6(n-3)	5.0	-	-
Σ Poyunsaturates	*32.2*	*51.7*	*11.1*
Σ Saturates	*31.7*	*38.0*	*84.7*
Σ n-6	*11.7*	*50.5*	*11.1*
Σ n-3	*20.5*	*1.2*	*1.2*
P/S	*1.0*	*1.4*	*0.1*
n-6/n-3	*0.6*	*42.0*	*9.3*

were stored at 4 °C and feeding cups replenished with fresh diets daily after discarding any uneaten portion.

Preparation of cardiac plasma membranes

Rats were anesthetized by sodium pentobarbital (50 mg/kg) injection and hearts rapidly excised. The ventricles were minced in 10 volumes (w/v) of ice-cold Tris-HCl buffer of the following composition (in mM): Sucrose, 25; $EDTANa_2$, 1; $MgCl_2$, 1 and Tris-HCl, 20 (pH 7.4, 22 °C), and homogenized (polytron; 50% maximum speed, 10 sec). Homogenates were initially centrifuged at 1,000 x g for 3 min and the supernatant filtered through four layers of gauze. The pellet was suspended in 50 ml of Tris-HCl buffer, centrifuged at 800 x g for 10 min and the supernatants from the two procedures combined and centrifuged at 8,700 x g for 15 min. The supernatant was then centrifuged at 48,000 x g for 30 min to obtain a crude plasma membrane which was stored at -70 °C in Tris-HCl buffer and used for lipid extraction.

Extraction and separation of membrane phospholipids

Membrane phospholipids were extracted by a method similar to that described by Hargreaves *et al.* (1989). After pelleting membrane samples, the supernatant was decanted off and the pellets extracted, first in 20 volumes of chloroform/methanol (2:1, v/v) followed by 14 volumes of chloroform/methanol (1:1, v/v) containing 0.1 M KCl, on ice. All solvents contained butylated hydroxytoluene as anti-oxidant.

Membrane phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SPM) were separated by one-dimensional TLC on pre-activated[17] Whatman HP-K high performance silica gel plates using chloroform/methanol/2-propanol/0.25% KCl/ triethylamine (30:9:25:6:18 by volume) as the solvent system (Touchstone *et al.*, 1980). TLC plates were dried in a vacuum dessicator for 5 min, sprayed with 0.05% ANS[18] in water and individual phospholipid spots, visualized under UV light, were scraped off into test tubes for methylation. Fatty acid methyl esters of the phospholipids were prepared directly from the silica gel.

To the scraped samples was added 1 ml of 14% BF_3 in methanol[19]/hexane (1:4, v/v) and the mixture heated at 100 °C for 1 hr (1.5 hr for SPM). After cooling to room temperature, distilled water was added and the mixture vortexed and left to stand for 2 min. The hexane layer was then removed into vials and dried down with N_2, followed by addition of 100 μl of chloroform for storage at -70 °C. Samples were analyzed for fatty acid composition by gas-liquid chromatography .

Gas-liquid chromatography analysis of fatty acid esters

The fatty acid methyl esters prepared as above were identified by GLC (Vista 4600, Varian Instruments, Georgetown, ONT.) with a flame ionization

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[17]TLC plates were activated by heating at 110 °C for 1 hr.

[18]8-Anilino-1-naphthalene-sulphonic acid.

[19]The BF_3 reagent was prepared fresh each week.

detector and System Gold™ data system (Beckman Instruments Inc., San Ramon, CA). Chromatography was performed using a fused silica capillary column (BP-20, 25 mm x 0.25 mm I.D.; S.G.E., Victoria, Australia). Helium was used as the carrier gas at a flow rate of 1.8 ml/min using split injection mode[20], and the stationary phase was polyethylene glycol which is suitable for the separation of fatty acid methyl esters. The oven temperature was programmed to be at 90 °C initially, increased to 170 °C at 20 °C/min and held for 15 min, then increased to 220 °C at 2.5 °C/min for 25 min giving a total analysis time of 40 min. The injection and detector temperatures were 250 °C. Under these conditions, all saturated, mono-, di- and polyunsaturated fatty acids ranging in chain length from C_{12}-C_{24} were separated. Identification of the fatty acids was based on the retention times of standards methylated by the same method.

Statistical Analysis

Comparisons between different diet groups and thyroid states were made using two-way analysis of variance (ANOVA) and the Neumann-Keuls multiple range test at 95% and 99% confidence limits.

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[20]The prefered method for quantitative analysis of major components in a sample. Fraction of sample entering the column is calculated from the ratio of the flow through the column to total flow of carrier gas.

D. Results

Body weights of animals

The final body weights of euthyroid animals fed the n-3, the n-6 or the SAT diets were 379.5 ± 23.2, 352.5 ± 13.4 and 309.2 ± 11.4 gm, respectively. The corresponding final body weights of the hypothyroid animals were 230.1 ± 21.2, 242.0 ± 3.4 and 162.9 ± 12.0, respectively. The increase in body weights of hypothyroid animals in all diet groups during the feeding period was slower compared to euthyroid animals on the same diet ($p < 0.01$). The n-6 and n-3 diets produced higher growth rates compared to the saturated fat diet.

Fatty acid composition of sarcolemmal phospholipids

After six weeks of feeding the experimental diets to euthyroid and hypothyroid animals, the fatty acid compositions of ventricular plasma membrane phospholipids were determined.

Phosphatidylcholine. Table IV-2 illustrates the effect of thyroid status and diet on the fatty acid composition of membrane PC extracted from the membranes. This fraction contained substantial quantities of palmitic (16:0) and stearic (18:0) acids as well as linoleic (18:2n-6) and arachidonic (20:4n-6) acids. Oleic acid (18:1)[21] was also present in high quantities. A comparison of the levels of arachidonic acid in

[21]Both 18:1n-7 and 18:1n-9 were present in a ratio of 1:1 except for membranes from animals fed the SAT diet in which the ratio was 1:2.

Table IV-2. Fatty acid composition (mol% ± S.E.) of phosphatidylcholine extracted from ventricular plasma membranes. Animals were fed diets as described in "Methods".

Fatty acid	Euthyroid			Hypothyroid		
	n-3	n-6	SAT	n-3	n-6	SAT
14:0	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	2.0 ± 0.3	0.5 ± 0.1[a]	1.7 ± 0.1
14:1	0.2 ± .01	n.d	0.1 ± .01	0.2 ± .01	n.d.	0.3 ± .01
16:0	28.7 ± 2.3	22.2 ± 1.8	19.9 ± 0.9[a]	27.8 ± 0.9	19.8 ± 1.9[b]	24.6 ± 3.0
16:1(n-7)	0.3 ± .01	0.2 ± .02	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	n.d.
16:1(n-5)	0.4 ± .04	0.2 ± .02	0.3 ± .03	0.3 ± .01	0.2 ± .03	1.6 ± 0.2[a]
17:0	0.6 ± 0.1	0.6 ± .02	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.2 ± 0.2[a]
18:0	29.1 ± 1.1	31.6 ± 2.4	33.8 ± 1.1	27.2 ± 0.6	33.1 ± 1.6	26.7 ± 2.6
18:1(n-7)	4.1 ± 0.1	2.5 ± 0.1[a]	4.6 ± 0.2	5.2 ± 0.2	3.1 ± 0.4[a]	4.8 ± 0.6
18:1(n-9)	3.3 ± 0.2[b]	2.6 ± 0.2	2.9 ± 0.1	3.6 ± 0.2[a]	2.1 ± 0.1	2.0 ± 0.2
18:2(n-6)	9.1 ± 0.4[b]	12.0 ± 0.4	10.3 ± 1.5	10.1 ± 0.5	14.4 ± 0.6[b]	10.5 ± 1.2
18:3(n-3)	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.1
20:1(n-9)	n.d.	n.d.	n.d.	n.d.	n.d.	1.8 ± 0.3
20:3(n-6)	0.5 ± 0.2	n.d.	n.d.	0.3 ± .03	n.d.	0.6 ± 0.2
20:4(n-6)	13.1 ± 0.9[a]	23.8 ± 1.0	23.1 ± 0.8	11.6 ± 1.0[a]	22.4 ± 0.8	16.4 ± 0.7[c]
20:5(n-3)	1.6 ± 0.2	n.d.	n.d.	2.5 ± 0.2	n.d.	n.d.
22:4(n-6)	n.d.	1.0 ± 0.1	0.6 ± 0.1	n.d.	1.2 ± 0.1	0.8 ± 0.1
22:5(n-6)	n.d.	1.2 ± 0.1	0.6 ± 0.1	n.d.	0.6 ± 0.1	3.1 ± 0.9[a,c]
22:5(n-3)	1.3 ± 0.1	0.4 ± 0.1[a]	0.6 ± 0.1	1.4 ± 0.1[a]	0.5 ± 0.1	0.5 ± 0.2
22:6(n-3)	7.0 ± 0.4[a]	1.3 ± 0.2	1.4 ± 0.1	6.9 ± 0.6[a]	1.3 ± 0.2	1.5 ± 0.4
ΣPolyunsat.	*32.5 ± 2.3*	*39.6 ± 1.8*	*36.5 ± 2.9*	*32.7 ± 2.4*	*40.2 ± 1.7*	*35.8 ± 3.8*
ΣSaturates	*58.8 ± 3.5*	*55.0 ± 4.3*	*55.3 ± 2.0*	*57.6 ± 1.9*	*54.0 ± 3.6*	*54.2 ± 5.9*
Σn-6	*22.6 ± 1.5*	*37.9 ± 1.6*	*34.6 ± 2.7*	*21.9 ± 1.5*	*38.5 ± 1.5*	*20.9 ± 2.9*
Σn-3	*10.1 ± 0.7*	*1.7 ± 0.2*	*2.0 ± 0.2*	*10.8 ± 0.9*	*1.7 ± 0.2*	*2.1 ± 0.5*
Σn-6/n-3	*2.3*	*22.6*	*17.6*	*2.0*	*22.1*	*10.1*
18:2/20:4	*0.7*	*0.5*	*0.5*	*0.9*	*0.7*	*0.7*

(n = 4-6; n.d. = not detected; Differences between means significant within the same thyroid group ([a]p > 0.01; [b]p > 0.05); [c]Significantly different from euthyroid on same diet (p > 0.01).

membranes from euthyroid animals shows that a higher molar quantity of this fatty acid was formed in animals fed the n-6 diet or the SAT diet compared to those formed in animals fed the n-3 diet. In the latter case, the low levels of arachidonic acid were accompanied by high levels of 20:5n-3, 22:5n-3 and 22:6n-3. In comparison to both the n-6 and SAT diets, feeding the n-3 diet resulted in a marked increase in the proportions of 22:5n-3 (300%) and 22:6n-3 (over 500%) in this major fraction of rat cardiac phospholipid. The levels of palmitic, stearic and linoleic acids were not significantly affected by hypothyroidism. The levels of arachidonic acid in hypothyroid animals fed the n-6 diet or the n-3 diet were not different from those found in euthyroid animals, however, a significant reduction ($p < 0.01$) in the level of this fatty acid was observed in hypothyroid animals fed the SAT diet.

To determine if hypothyroidism had any effect on desaturation activity, the ratio of the n-6 fatty acids, 18:2/20:4 were compared. Hypothyroidism caused an increase in this ratio suggesting inhibition of Δ^6-desaturase activity. In animals fed the n-6 diet, the reduction (50%) in the level of the longer chain polyenic fatty acid, 22:5n-6 but not 22:4n-6 suggests inhibition of Δ^4-desaturase enzyme also. Hypothyroidism had little effect on total cardiac saturated and n-3 polyunsaturated fatty acids. Similarly, total n-6 polyunsaturated fatty acid did not change in animals fed the n-6 diet or the n-3 diet, however, in animals fed the SAT diet this was reduced mainly because of the lower levels of arachidonic acid compared to those in euthyroid controls. This decrease was accompanied by the appearance of significant quantities of 20:3n-6 and 22:5n-6.

Phosphatidylethanolamine. The data in Table 1V-3 shows the fatty acid composition of the PE fraction. In membranes from euthyroid animals, the levels of palmitic acid did not change with the feeding of different diets while stearic acid levels were higher in membranes from animals fed the n-3 diet compared to those fed the n-6 diet or the SAT diet. The proportions of linoleic acid and arachidonic acid were also different. Higher levels of palmitoleic (16:1) and oleic[22] acids were found in membranes from animals fed the n-6 diet or the SAT diet compared to those fed the n-3 diet ($p < 0.01$). In addition to these, high levels of 22:4n-6 and 22:5n-6 were also found in the PE fractions from animals fed the n-6 or the SAT diets. The proportions of of 20:5n-3, 22:5n-3 and 22:6n-3 were also high in all diet groups and 22:6n-3 was markedly increased in animals fed the n-3 diet (> 400% increase over those fed the n-6 or the SAT diet).

In hypothyroid animals, the level of palmitic acid was highest in membranes from animals fed the SAT diet compared to those fed the n-6 diet or the n-3 diet. At the same time, the levels of linoleic acid and arachidonic acid increased two- to three-fold, respectively in animals fed the n-6 diet compared to those fed the n-3 or the SAT diets. The levels of n-3 polyunsaturated fatty acids, notably 20:5n-3, 22:5n-3 and 22:6n-3 were similar to those found in euthyroid animals suggesting that hypothyroidism had no apparent effect on the incorporation of these fatty acids into PE, but altered the metabolism of the n-6 fatty acids. This is evident in the increase in the ratio of 18:2/20:4 in hypothyroid animals similar to that observed in

[22]Present as isomers in ratios similar to that found in PC.

Table IV-3. Fatty acid composition (mol% ± S.E.) of phosphatidylethanolamine extracted from ventricular plasma membranes. Animals were fed diets as described in "Methods".

Fatty acid	Euthyroid			Hypothyroid		
	n-3	n-6	SAT	n-3	n-6	SAT
14:0	1.2 ± 0.3	2.7 ± 0.3	1.7 ± 0.2	3.8 ± 0.6	2.7 ± 0.3	1.7 ± 0.2[a]
14:1	0.6 ± .04	0.5 ± .07	0.3 ± .03[b]	0.5 ± .06	0.6 ± .04	1.3 ± 0.1[a]
16:0	18.3 ± 1.2	15.1 ± 0.6[b]	19.5 ± 0.5	22.4 ± 0.9	21.5 ± 1.8	32.5 ± 1.4[a]
16:1(n-7)	0.5 ± .04	2.3 ± 0.5	n.d	4.7 ± 1.0	0.2[b]	n.d.
16:1(n-5)	2.2 ± 0.2[a]	9.7 ± 0.4	11.6 ± 2.0	1.4 ± 0.	0.1	4.5 ± 0.3[a]
17:0	0.6 ± .01	0.5 ± .03	0.6 ± .04	0.5 ± .0	0.1	0.4 ± .04
18:0	32.5 ± 0.7[a]	23.9 ± 1.0	23.5 ± 1.1	23.3 ± 1.	0.7	15.5 ± 0.5[b,c]
18:1(n-7)	3.5 ± 0.1[b]	2.1 ± 0.1	2.9 ± 0.1	5.1 ± 0.7	0.2	4.4 ± 0.3
18:1(n-9)	2.1 ± 0.1	5.4 ± 0.8	8.3 ± 0.6[a]	4.9 ± 1.0	0.3	9.6 ± 0.5[a]
18:2(n-6)	4.3 ± 0.3	6.3 ± 0.7	3.4 ± 0.2[a]	3.7 ± 0.2	0.3 ± 0.2[a]	5.4 ± 0.9[c]
18:3(n-3)	n.d.	0.6 ± .04	n.d.	0.5 ± 0.1	0.7 ± .03	n.d.
20:1(n-9)	n.d.	n.d.	n.d.	2.2 ± 0.1	n.d.	2.3 ± 0.1
20:4(n-6)	10.6 ± 0.2[a]	16.5 ± 1.0	18.7 ± 1.6	6.9 ± 0.	21.1 ± 2.1[a]	7.0 ± 1.1[c]
20:3(n-3)	n.d.	1.8 ± 0.2	n.d.	n.d.	2.2 ± 0.2	n.d.
20:5(n-3)	3.1 ± 0.1	n.d.	n.d.	2.9 ± 0.2	n.d.	n.d.
22:4(n-6)	n.d.	3.9 ± 0.4	1.2 ± 0.1[a]	n.d.	2.3 ± 0.1	2.8 ± 0.4
22:5(n-6)	n.d.	4.7 ± 0.2	2.3 ± 0.1[a]	n.d.	n.d.	3.9 ± 0.5
22:5(n-3)	3.0 ± 0.5[a]	0.8 ± .04	0.7 ± .03	1.9 ± 0.3	1.5 ± 0.1	1.6 ± 0.1
22:6(n-3)	17.5 ± 1.5[a]	4.1 ± 0.2	5.2 ± 0.5	15.2 ± 2.1[a]	3.6 ± 0.4	7.2 ± 0.8[d]
ΣPolyunsat.	*38.5 ± 2.3*	*38.6 ± 2.5*	*31.5 ± 2.5*	*30.6 ± 3.4*	*43.7 ± 3.0*	*27.9 ± 3.8*
ΣSaturates	*52.6 ± 2.2*	*42.1 ± 1.9*	*45.4 ± 2.1*	*50.1 ± 2.9*	*43.7 ± 2.8*	*50.2 ± 2.2*
Σn-6	*14.9 ± 0.4*	*31.4 ± 2.1*	*25.6 ± 2.0*	*10.6 ± 1.0*	*35.7 ± 2.4*	*19.1 ± 2.9*
Σn-3	*23.6 ± 2.1*	*7.3 ± 0.4*	*5.9 ± 0.5*	*20.5 ± 2.6*	*8.0 ± 0.6*	*8.8 ± 0.9*
Σn-6/n-3	*0.6*	*4.3*	*4.3*	*0.5*	*4.5*	*2.2*
18:2/20:4	*0.4*	*0.4*	*0.2*	*0.5*	*0.6*	*0.8*

(n = 4-6); n.d. = not detected; Differences between means significant within the same thyroid group ([a]p < 0.01, [b]p < 0.05); Significantly different from euthyroid on same diet ([c]p < 0.01; [d]p < 0.05).

the PC fraction. The data indicate that Δ^6-deasturase activity is inhibited. Total saturated fatty acid remained relatively the same, irrespective of thyroid status and diet feeding, however, total n-3 fatty acid in membranes from euthyroid and hypothyroid animals were similar and much higher than those measured in the PC fraction.

Phosphatidylserine. The fatty acid composition of cardiac PS is shown in Table IV-4. Membranes from euthyroid animals fed the n-3 diet contained over 50% stearic acid accompanied by a lower proportion of palmitic acid. Linoleic acid levels were similar in all diet groups but higher proportions of oleic acid, particularly 18:1n-7[23] were found. Arachidonic acid was present in similar proportions in membranes from animals fed the n-6 diet or the SAT diet and over 250% more than those from animals fed the n-3 diet. As observed for the PE fraction, the proportions of 22:5n-3 and 22:6n-3 were higher in animals on the n-3 diet compared to those on the n-6 diet or the SAT diet. Total saturated fatty acids were similar in all diet groups mainly because of the significant increase in stearic acid levels. On the other hand, total n-6 fatty acid was lower in membranes from animals fed the n-3 diet compared to those fed the n-6 diet or the SAT diet. A similar pattern of total fatty acids was found in hypothyroid animals, however, total n-6 fatty acid was lower in animals on the SAT diet compared to those on the n-6 diet.

[23]The ratio of 18:1n-7 to 18:1n-9 is about 7:1.

Table IV-4. Fatty acid composition (mol% ± S.E.) of phosphatidylserine extracted from ventricular plasma membranes. Animals were fed diets as described in "Methods".

Fatty acid	Euthyroid			Hypothyroid		
	n-3	n-6	SAT	n-3	n-6	SAT
14:0	6.0 ± 0.7	5.9 ± 1.0	7.2 ± 0.5	4.1 ± 0.6[a]	7.4 ± 0.9	7.4 ± 1.0
14:1	0.3 ± .01	0.2 ± .01	0.3 ± 0.1	0.2 ± .01	0.3 ± .03	0.2 ± .03
16:0	4.3 ± 0.2[a]	17.0 ± 2.3	13.2 ± 1.2	7.0 ± 0.9	18.6 ± 1.9	11.8 ± 0.3[a]
16:1(n-7)	n.d.	1.6 ± 0.2	n.d.	0.6 ± 0.1	0.2 ± .01	0.4 ± 0.1
16:1(n-5)	0.7 ± 0.1	0.9 ± 0.2	0.8 ± .04	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
17:0	0.6 ± 0.1[b]	0.9 ± 0.2	1.3 ± 0.3	0.5 ± .03[a]	1.1 ± 0.2	0.6 ± 0.2[c]
18:0	53.1 ± 0.6[a]	40.2 ± 3.9	39.7 ± 3.8	46.5 ± 2.0[b]	35.5 ± 3.2	39.2 ± 1.5
18:1(n-7)	7.1 ± 0.9	5.9 ± 0.2	6.1 ± 0.4	7.9 ± 0.6	5.9 ± 0.6	5.6 ± 0.9[b]
18:1(n-9)	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	1.4 ± 0.3	0.5 ± .01	1.6 ± 0.2
18:2(n-6)	3.4 ± 0.3	3.5 ± 0.3	2.7 ± 0.5	3.7 ± 0.2	3.7 ± 0.7	2.3 ± 0.4[a]
20:1(n-9)	n.d.	n.d.	4.1 ± 0.7	n.d.	4.5 ± 0.2	n.d.
20:1(n-7)	n.d.	n.d.	n.d.	n.d.	2.3 ± 0.3	0.9 ± 0.2[a]
20:3(n-6)	1.0 ± .04	1.0 ± 0.3	1.0 ± 0.1	2.2 ± 0.1[a]	1.2 ± 0.2	2.1 ± 0.4[c]
20:4(n-6)	2.5 ± 0.4[a]	7.1 ± 0.9	7.2 ± 0.4	2.3 ± 0.2[a]	6.3 ± 0.2	7.1 ± 0.7
20:3(n-3)	n.d.	1.2 ± 0.1	0.8 ± 0.1[a]	1.6 ± 0.2	2.0 ± 0.2	0.8 ± .03
20:5(n-3)	0.3 ± .02	n.d.	0.3 ± .02	0.8 ± .02	n.d.	2.3 ± 0.2
22:3(n-9)	n.d.	n.d.	4.1 ± 0.9	n.d.	4.8 ± 0.4	5.1 ± 0.9
22:4(n-6)	n.d.	4.5 ± 0.3	2.6 ± 0.4[a]	n.d	1.7 ± 0.3	4.4 ± 1.1
22:5(n-3)	2.9 ± 0.4[a]	0.8 ± .02	1.3 ± 0.3	3.0 ± 0.2	n.d.	1.0 ± 0.3[a]
22:6(n-3)	17.0 ± 0.7[a]	4.2 ± 0.8	4.9 ± 1.0	17.6 ± 0.5[a]	2.1 ± 0.3	4.2 ± 0.6
ΣPolyunsat.	*27.1 ± 1.9*	*26.5 ± 3.3*	*26.7 ± 4.0*	*31.3 ± 1.2*	*23.0 ± 2.2*	*31.8 ± 5.6*
ΣSaturates	*64.0 ± 1.6*	*64.0 ± 7.3*	*61.4 ± 5.8*	*58.1 ± 3.4*	*62.6 ± 6.2*	*58.9 ± 3.0*
Σn-6	*6.8 ± 0.7*	*20.3 ± 2.4*	*15.2 ± 1.7*	*8.2 ± 0.4*	*14.2 ± 1.4*	*18.5 ± 1.2*
Σn-3	*20.3 ± 1.2*	*6.2 ± 0.9*	*7.4 ± 1.3*	*23.1 ± 0.8*	*4.1 ± 0.5*	*8.2 ± 1.2*
Σn-6/n-3	*0.3*	*3.3*	*2.1*	*0.3*	*3.4*	*2.3*
18:2/20:4	*1.4*	*0.5*	*0.4*	*1.6*	*0.6*	*0.3*

(n = 4-6); n.d. = not detected; Differenes between means significant within the same thyroid group ([a]p < 0.01, [b]p < 0.05); Significantly different from euthyroid on same diet ([c]p < 0.01; [d]p < 0.05).

Phosphatidylinositol. The PI fraction from membranes prepared from euthyroid animals also contained substantial quantities of palmitic acid and stearic acid (Table IV-5) although the latter was much lower than that determined in the PS fraction. The major component of the n-6 fatty acids was arachidonic acid present at levels comparable to those found in PC and PE and much higher than those in PS. The 18:1n-7 and the 18:1n-9 isomers of oleic acid were present in a ratio of 2:1 in this fraction. The levels of n-3 fatty acids were very low, in comparison to the PE and PS fraction, particularly those in membranes from animals fed the n-6 diet. Total n-3 fatty acids in the PI fraction were similar in membranes from animals fed the n-3 diet or the SAT diet and higher than those from animals fed the n-6 diet.

Hypothyroidism reduced stearic acid as well as arachidonic acid levels in the PI fraction from animals fed the SAT diet when compared to euthyroid controls. This reduction was accompanied by an increase in the level of 22:4n-6 suggesting inhibition of Δ^4-desaturase. The ratio of 18:2/20:4 was increased by the hypothyroid state in all animals.

Sphingomyelin. The saturated fatty acid component of the SPM fraction is also predominantly palmitic and stearic acids (Table IV-6). In membranes from euthyroid animals, the proportions of all other fatty acids were lower than 10%. In addition, significant levels of a number of minor fatty acids such as 18:4n-3, 20:1n-7 and 20:1n-9 were also detected. The proportions of oleic, arachidonic and docosahexaenoic acids were similar in all diet groups. Total saturated fatty acids were

Table IV-5. Fatty acid composition (mol% ± S.E.) of phosphatidylinositol extracted from ventricular plasma membranes. Animals were fed diets as described in "Methods".

Fatty acid	Euthyroid			Hypothyroid		
	n-3	n-6	SAT	n-3	n-6	SAT
14:0	10.9 ± 2.7	5.7 ± 0.7[a]	9.7 ± 0.3	12.9 ± 3.3	7.4 ± 1.8[d]	20.9 ± 0.2[a,c]
14:1	0.3 ± .03	0.5 ± .04	0.3 ± .02	0.2 ± .03	0.5 ± 0.2	0.3 ± .05
16:0	12.0 ± 1.5	11.6 ± 1.7	16.0 ± 3.2	14.3 ± 2.5	15.5 ± 0.8	18.8 ± 1.6
16:1(n-7)	0.5 ± 0.1	0.5 ± .06	0.3 ± .05	0.3 ± 0.1	1.3 ± 0.2	2.3 ± 0.1[a,c]
16:1(n-5)	4.4 ± 0.4	1.4 ± 0.1[a]	5.7 ± 1.3	2.5 ± 0.9[c]	5.3 ± 0.4[c]	3.5 ± 0.2[a,c]
17:0	0.6 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	1.4 ± 0.2	1.4 ± 0.1[a]
18:0	31.5 ± 2.4	33.8 ± 3.5	30.7 ± 4.6	30.5 ± 1.5	29.1 ± 3.1	11.6 ± 0.6[a,c]
18:1(n-7)	4.3 ± 0.5	2.4 ± 0.2[a]	4.0 ± 0.6	5.7 ± 0.3[c]	4.3 ± 0.2[a]	n.d.
18:1(n-9)	3.2 ± 0.2	1.0 ± 0.1[a]	2.7 ± 0.3	2.8 ± 0.3	2.5 ± 0.3	3.8 ± 0.2[a]
18:2(n-6)	6.1 ± 0.7	8.4 ± 0.3	4.6 ± 0.9[a]	6.0 ± 0.9	9.6 ± 0.2[d]	2.9 ± 0.2[a,d]
18:3(n-3)	n.d.	0.4 ± .04	0.3 ± .03	n.d.	1.1 ± 0.1	n.d.
20:1(n-9)	n.d.	0.7 ± 0.1	1.2 ± 0.2[a]	4.0 ± 0.2	1.5 ± 0.8	n.d.
20:1(n-7)	n.d.	6.8 ± 0.4	n.d.	n.d.	3.8 ± 1.1	5.5 ± 0.2
20:3(n-6)	0.7 ± 0.2	0.3 ± .02	n.d.	0.7 ± 0.2	n.d.	2.2 ± 0.2
20:4(n-6)	17.2 ± 0.6	20.5 ± 3.1	11.2 ± 1.6[a]	15.3 ± 0.9[d]	11.3 ± 1.6[c]	4.5 ± 0.7[a,c]
20:3(n-3)	n.d.	0.3 ± 0.1	1.2 ± 0.2[a]	n.d.	n.d.	4.5 ± 0.6[c]
20:5(n-3)	2.1 ± 0.3	0.4 ± 0.1	n.d.	2.4 ± 0.4	n.d.	2.2 ± 0.3
22:3(n-9)	n.d.	2.4 ± 0.2	n.d.	n.d.	4.9 ± 0.3[c]	n.d.
22:4(n-6)	n.d.	1.2 ± 0.1	5.1 ± 0.9[a]	n.d.	n.d.	10.4 ± 0.5[c]
22:5(n-3)	1.3 ± 0.1	0.4 ± .04[a]	1.2 ± 0.6	1.4 ± 0.2	n.d.	n.d.
22:6(n-3)	5.2 ± 0.9	0.7 ± 0.1[a]	4.8 ± 1.3	4.5 ± 1.4	0.4 ± 0.1[d]	1.4 ± 0.4[a,d]
ΣPolyunsat.	*32.4 ± 2.9*	*34.9 ± 4.1*	*28.4 ± 6.1*	*30.2 ± 4.7*	*27.3 ± 1.2*	*28.0 ± 1.7*
ΣSaturates	*55.0 ± 6.7*	*51.9 ± 6.1*	*57.4 ± 8.2*	*58.4 ± 7.4*	*53.4 ± 6.0*	*52.7 ± 2.5*
Σn-6	*23.9 ± 1.5*	*30.4 ± 3.5*	*20.9 ± 3.4*	*22.0 ± 2.2*	*20.9 ± 1.8*	*20.1 ± 1.6*
Σn-3	*8.5 ± 1.4*	*2.2 ± 0.5*	*7.6 ± 2.7*	*8.3 ± 0.5*	*1.5 ± 0.1*	*8.0 ± 1.3*
Σn-6/n-3	*2.8*	*14.1*	*2.8*	*2.7*	*13.8*	*2.5*
18:2/20:4	*0.4*	*0.4*	*0.4*	*0.4*	*0.9*	*0.7*

(n = 4-6); n.d. = not detected; Differences between means significant within the same thyroid group ([a]p < 0.01, [b]p < 0.05); Significantly different from euthyroid on same diet ([c]p < 0.01; [d]p < 0.05).

highest in all diet groups compared to the other phospholipid fractions. The hypothyroid state did not appear to have any significant effect on the fatty acid profile except in membranes from animals fed the SAT diet.

The major fatty acids in membrane phospholipids which were altered by dietary treatment of euthyroid and hypothyroid animals are summarized in Table IV-7 and Table IV-8, respectively. The proportions of palmitic acid in PC, PS, PI and SPM were fairly constant in membranes from euthyroid and hypothyroid animals. Similarly, the levels of 18:1(n-7) and 18:1(n-9) did not change significantly with PTU treatment.

Table IV-6. Fatty acid composition (mol% ± S.E.) of sphingomyelin extracted from ventricular plasma membranes. Animals were diets as described in "Methods".

Fatty acid	Euthyroid			Hypothyroid		
	n-3	n-6	SAT	n-3	n-6	SAT
14:0	6.2 ± 1.3	8.6 ± 1.1	12.7 ± 1.3[a]	4.0 ± 1.2[c]	13.2 ± 2.7[c]	4.2 ± 1.6[a,c]
14:1	0.4 ± .05	0.4 ± .02	0.2 ± .02	0.4 ± 0.1	0.2 ± .04	0.5 ± 0.2[a,d]
16:0	29.4 ± 1.3	27.1 ± 0.7	25.9 ± 1.7[b]	29.8 ± 2.1	24.0 ± 1.7[c]	33.8 ± 2.1[b,c]
16:1(n-7)	4.5 ± 0.5[a]	0.4 ± 0.1	0.4 ± 0.1	4.2 ± 0.8	n.d.	1.0 ± 0.1[a,c]
16:1(n-5)	0.7 ± 0.1	1.0 ± 0.1	1.6 ± 0.3[a]	1.1 ± 0.2[d]	1.1 ± 0.1	2.4 ± 0.3[a,d]
17:0	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	1.8 ± 0.3	1.7 ± 0.2
18:0	25.3 ± 0.8[b]	31.5 ± 2.2	31.8 ± 3.7	20.8 ± 1.5[d]	36.8 ± 2.1[a]	27.3 ± 2.4
18:1(n-7)	4.0 ± 0.2	3.1 ± 0.3	3.2 ± 0.5	4.7 ± 0.4	2.5 ± 0.2[a]	5.5 ± 0.9[c]
18:1(n-9)	2.3 ± 0.1	0.9 ± 0.1[a]	2.1 ± 0.4	2.6 ± 0.2	1.3 ± 0.2	2.3 ± 0.1[a]
18:2(n-6)	4.4 ± 0.4[b]	2.7 ± 0.4	3.4 ± 0.7	6.5 ± 1.3[c]	3.2 ± 0.6[a]	3.0 ± 1.3[c]
18:4(n-3)	6.8 ± 0.9	7.1 ± 0.9	4.6 ± 0.6	8.8 ± 0.2[a]	4.1 ± 0.8	.9 ± 0.8
20:1(n-9)	0.4 ± 0.1	1.5 ± 0.2	2.5 ± 0.3[a]	0.3 ± .03	0.3 ± 0.1	n.d.
20:1(n-7)	0.9 ± 0.2	3.0 ± 0.2[a]	n.d.	1.5 ± 0.1[d]	1.4 ± 0.2[d]	n.d.
20:3(n-6)	n.d.	n.d.	n.d.	0.6 ± 0.2	n.d.	n.d.
20:4(n-6)	2.1 ± 0.2	2.6 ± 0.1	4.6 ± 1.1[b]	2.8 ± 0.2	2.6 ± 0.6	n.d.
20:3(n-3)	n.d.	3.4 ± 0.2	n.d.	n.d.	n.d.	n.d.
20:5(n-3)	5.5 ± 0.6	n.d.	3.8 ± 0.9[a]	5.3 ± 0.7[a]	n.d.	2.3 ± 0.5[d]
22:4(n-6)	1.6 ± 0.2	1.2 ± 0.2	0.2 ± .02[a]	1.5 ± 0.3	4.6 ± 1.8[c]	0.8 ± 0.3[a,c]
22:5(n-3)	2.6 ± 0.3	2.7 ± 0.3	n.d.	2.6 ± 0.5	n.d.	n.d.
22:6(n-3)	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	2.8 ± 0.8[a]	1.5 ± 0.1
ΣPolyunsat.	*24.8 ± 2.8*	*21.5 ± 2.1*	*18.4 ± 3.3*	*29.7 ± 2.4*	*17.3 ± 4.6*	*14.9 ± 2.9*
ΣSaturates	*62.1 ± 3.6*	*68.6 ± 4.0*	*71.8 ± 6.9*	*55.6 ± 5.0*	*75.9 ± 6.8*	*67.0 ± 6.3*
Σn-6	*8.0 ± 0.7*	*6.5 ± 0.6*	*8.2 ± 1.8*	*11.4 ± 1.9*	*10.4 ± 2.9*	*8.8 ± 1.6*
Σn-3	*16.7 ± 2.1*	*15.7 ± 1.5*	*10.3 ± 1.6*	*18.3 ± 1.6*	*6.9 ± 1.6*	*7.6 ± 1.4*
Σn-6/n-3	*0.5*	*0.4*	*0.8*	*0.6*	*1.5*	*1.2*
18:2/20:4	*2.1*	*1.1*	*0.7*	*2.3*	*1.2*	-

(n = 4-6); n.d. = not detected; Differences between means significant within the same thyroid group ([a]p < 0.01, [b]p < 0.05); Significantly different from euthyroid on same diet ([c]p < 0.01; [d]p < 0.05).

Table IV-7. Summary of changes in major fatty acids (mol% ± S.E.) in the phospholipid fractions from ventricular plasma membranes from euthyroid animals. Animals were fed diets as described in "Methods".

Fatty acid	Diet	Phospholipids				
		PC	PE	PS	PI	SPM
16:0	n-3	28.7 ± 2.3[a]	18.0 ± 1.2[a,b]	4.3 ± 0.2[a,b]	12.0 ± 1.5[a,b]	29.4 ± 1.3[b]
	n-6	22.2 ± 1.8[a,b]	15.1 ± 0.6[a]	17.0 ± 2.3[a]	11.6 ± 1.7[a]	27.1 ± 0.7[a,b]
	SAT	19.9 ± 0.9[a,b]	19.5 ± 0.8[a]	13.2 ± 1.2[a]	16.0 ± 3.2	25.9 ± 1.7[a,b]
18:0	n-3	29.1 ± 1.1	32.5 ± 0.7	53.1 ± 0.6[c]	31.5 ± 2.4	25.3 ± 0.8
	n-6	31.6 ± 2.4	23.9 ± 1.0	40.2 ± 3.9[c]	33.8 ± 3.5	31.5 ± 2.2
	SAT	33.8 ± 1.1	23.5 ± 1.1[c]	39.7 ± 3.8	30.7 ± 4.6	31.8 ± 3.7
18:1(n-7)	n-3	4.1 ± 0.1	3.5 ± 0.1	7.1 ± 0.9[c]	4.3 ± 0.5	4.0 ± 0.2
	n-6	2.5 ± 0.1	2.1 ± 0.1	5.9 ± 0.2[c]	2.4 ± 0.2	3.1 ± 0.3
	SAT	4.6 ± 0.2	2.9 ± 0.1	6.1 ± 0.4[c]	4.0 ± 0.6	3.2 ± 0.5
18:1(n-9)	n-3	3.3 ± 0.2	2.1 ± 0.1	0.9 ± 0.1[c]	3.2 ± 0.2	2.3 ± 0.1
	n-6	2.6 ± 0.2	5.4 ± 0.8[c]	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
	SAT	2.9 ± 0.1	8.3 ± 0.6[c]	0.9 ± 0.1	2.7 ± 0.3	2.1 ± 0.4
18:2(n-6)	n-3	9.1 ± 0.4[c]	4.3 ± 0.3	3.4 ± 0.3	6.1 ± 0.7	4.4 ± 0.4
	n-6	12.0 ± 0.4[c]	6.3 ± 0.7	3.5 ± 0.3	8.4 ± 0.3	2.7 ± 0.4
	SAT	10.3 ± 1.5[c]	3.4 ± 0.2	2.7 ± 0.5	4.6 ± 0.9	3.4 ± 0.7
20:4(n-6)	n-3	13.1 ± 0.9	10.6 ± 0.2	2.5 ± 0.4[d]	17.2 ± 0.6	2.1 ± 0.2
	n-6	23.8 ± 1.0	16.5 ± 1.0	7.1 ± 0.9	20.5 ± 3.1	2.6 ± 0.1[c]
	SAT	23.1 ± 0.8	18.7 ± 1.6	7.2 ± 0.4	11.2 ± 1.6	4.6 ± 1.1[c]
22:6(n-3)	n-3	7.0 ± 0.4	17.5 ± 1.5	17.0 ± 0.7	5.2 ± 0.9	1.9 ± 0.2[c]
	n-6	1.3 ± 0.2	4.1 ± 0.2	4.2 ± 0.8	0.7 ± 0.1[c]	1.9 ± 0.1
	SAT	1.4 ± 0.1[d]	5.2 ± 0.5	4.9 ± 1.0	4.8 ± 1.9	1.9 ± 0.1

Values within a row bearing common superscript are significantly different ([a]$p < 0.01$; [b]$p < 0.05$);
[c]Significantly different from others within row ($p < 0.01$);
[d]Significantly different from others within row except SPM ($p < 0.01$).

Table IV-8. Summary of changes in major fatty acids (mol% ± S.E.) in the phospholipid fractions from ventricular plasma membranes from hypothyroid animals. Animals were fed diets as described in "Methods"

Fatty acid	Diet	Phospholipids				
		PC	PE	PS	PI	SPM
16:0	n-3	27.8 ± 0.9	22.[illegible] ± 0.9	7.0 ± 0.9[c]	14.3 ± 2.5	29.8 ± 2.1
	n-6	19.8 ± 1.9	21.5 ± 1.8[a]	18.6 ± 1.9[b]	15.5 ± 0.8[a]	24.0 ± 1.7[c]
	SAT	24.6 ± 3.0	32.5 ± 1.4	11.8 ± 0.3[c]	18.8 ± 1.6	33.8 ± 2.1
18:0	n-3	27.2 ± 0.6	23.3 ± 1.3	46.5 ± 2.0[c]	30.5 ± 1.5	20.8 ± 1.8
	n-6	33.1 ± 1.6	18.9 ± 0.7[c]	35.5 ± 3.2	29.1 ± 3.1	36.8 ± 2.1
	SAT	26.7 ± 2.6	15.5 ± 0.5	39.2 ± 1.5	11.6 ± 0.6[c]	27.3 ± 2.4
18:1(n-7)	n-3	5.2 ± 0.2	5.1 ± 0.7	7.9 ± 0.6[d]	5.7 ± 0.3	4.7 ± 0.4
	n-6	3.1 ± 0.4	3.3 ± 0.2	5.9 ± 0.6	4.3 ± 0.2	2.5 ± 0.2
	SAT	4.8 ± 0.6	4.4 ± 0.3	5.6 ± 0.9	—	5.5 ± 0.9
18:1(n-9)	n-3	3.6 ± 0.2	4.9 ± 1.0[d]	1.4 ± 0.3	2.8 ± 0.3	2.6 ± 0.2
	n-6	2.1 ± 0.1	5.2 ± 0.3	0.5 ± 0.1[c]	2.5 ± 0.3	1.3 ± 0.2
	SAT	2.0 ± 0.2	9.6 ± 0.5[c]	1.6 ± 0.2	3.8 ± 0.2	2.3 ± 0.1
18:2(n-6)	n-3	10.1 ± 0.5[c]	3.7 ± 0.2	3.7 ± 0.2	6.0 ± 0.9	6.5 ± 1.3
	n-6	14.4 ± 0.6[c]	12.3 ± 0.2[d]	3.7 ± 0.7	9.6 ± 0.2	3.2 ± 0.6
	SAT	10.5 ± 1.2[c]	5.4 ± 0.9	2.3 ± 0.4	2.9 ± 0.2	8.0 ± 1.3
20:4(n-6)	n-3	11.6 ± 1.0	6.9 ± 0.5	2.3 ± 0.2[e]	15.3 ± 0.9	2.8 ± 0.2
	n-6	22.4 ± 0.8	21.1 ± 2.1	6.3 ± 0.2	11.3 ± 1.6	2.6 ± 0.6[c]
	SAT	16.4 ± 0.7[c]	7.0 ± 1.1	7.1 ± 0.7	4.5 ± 0.7	—
22:6(n-3)	n-3	6.9 ± 0.6	15.2 ± 2.1	17.6 ± 0.5	4.5 ± 0.2	1.7 ± 0.1[c]
	n-6	1.3 ± 0.2	3.6 ± 0.4	2.1 ± 0.3	0.4 ± 0.1[c]	2.8 ± 0.8
	SAT	1.5 ± 0.4	7.2 ± 0.8[c]	4.2 ± 0.6	1.4 ± 0.4	1.5 ± 0.1

Values within row bearing common superscript are significantly different ([a]$p < 0.01$; [b]$p < 0.05$); Significantly different from others within row ([c]$p < 0.01$; [d]$p < 0.05$;
[e]Significantly different from others within row except SPM ($p < 0.01$).

E. Discussion.

The heart preferentially utilizes fatty acids for energy production (Dhalla *et al.*, 1992), hence the availability of exogenous fatty acids determines not only fatty acid oxidation but also the synthesis of triglycerides and membrane phospholipids in the myocardium. Thus, alterations in the composition of membrane phospholipids can influence cardiac performance through effects on membrane-bound enzymes, receptors and transport proteins. For example, N-methylation of membrane phospholipids has been shown to alter sarcolemmal Ca^{2+}-pump and Na^{+}/Ca^{2+} exchange as well as sarcoplasmic reticular Ca^{2+}-pump ATPase activities (Gangaly *et al.*, 1985; Panagia *et al.*, 1986; 1987). Hence, altered membrane phospholipid fatty acid composition, induced by dietary manipulation can be invoked to explain alterations in myocardial function. Changes in the lipid environment of membrane-bound proteins can potentially alter ionic equilibrium across the the sarcolemma and therefore change contractile response.

Thyroid hormone changes the fatty acyl composition of membrane phospholipds particularly through effects on desaturation (Hoch *et al.*, 1981; Faas and Carter, 1981; Hoch, 1988). Defects in liver desaturase activity have been observed in the hypothyroid state as well as during large intakes of saturated and trans-fatty acids (Brenner, 1989). Diets high in n-3 fatty acids (20:5 and 22:6) have also been shown to depress Δ^{6}-desaturase activity and hence the synthesis of 20:4n-6 and 22:4n-6 from 18:2n-6 (Brenner, 1982). Phospholipids are essential components

of myocardial membranes in which they modulate structural, transport and receptor functions (Dhalla *et al.*, 1992). They are involved in such functions as, determination of fluidity and permeability, calcium storage, anchoring of enzymes and proteins, regulation of membrane enzyme activity as well as serving as precursors for prostaglandins and substrates for methyltransferases (Bers, 1991; Maranesi *et al.*, 1991). Their importance in myocardial function and/or dysfunction are therefore dependent on their fatty acid composition which is partly determined by dietary intake of essential fatty acids. A number of studies have demonstrated thyroid hormone-dependent (Hoch, 1988) and diet-specific (Charnock *et al.*, 1985; 1986; 1992) changes in the fatty acid composition of cardiac membrane phospholipids. Feeding diets enriched with linoleic acid produces reduced levels of this fatty acid in rat ventricular membranes concomitant to increased arachidonic acid levels. (Charnock *et al.*, 1986). In contrast, saturated animal fat diet lowers the level of arachidonic acid in cardiac phospholipids. Incorporation of n-3 fatty acids, particularly docosahexaenoic acid into cardiac membranes occurs when fish oil supplements are fed and this is accompanied by a reduction in the proportions of arachidonic acid. Thus, the most important changes in the fatty acid composition of cardiac phospholipids occur in the polyunsaturated fatty acids as opposed to saturated fatty acids. Generally, the n-6/n-3 ratio of phospholipids is altered in favour of the fatty acids administered.

In the present study, the fatty acid composition of cardiac membrane phospholipids also varied with the type of dietary fatty acid and, to some extent, with

the thyroid status. In the PC fraction, for example, feeding the n-3 diet reduced the Σn-6/n-3 ratio ten-fold in both euthyroid and hypothyroid animals compared to the n-6 diet. Similarly, the ratios in PE, PS and PI were reduced 5- to 10-fold upon feeding the n-3 diet. Feeding the SAT diet produced Σn-6/n-3 ratios in PC, PE and PS that were greater than those obtained after feeding the n-3 diet but lower than those measured after feeding the n-6 diet. The major fatty acids which were altered significantly include 18:1, 18:2n-6, 20:4n-6 and 22:6n-3.

In membranes from euthyroid animals the 18:2 levels in SPM were similar to 20:4 levels suggesting that dietary feeding had no effect on the membrane levels of these fatty acids in this phospholipid fraction. Similarly, 18:1 and 22:6n-3 levels did not change with diet feeding. The relative proportions of 18:2 compared to 20:4 in all phospholipid fractions, except SPM suggests that there was no interference in the conversion of the former into the latter. The 20:4 levels were highest in PC, PE, and PI fractions suggesting that these phospholipids could be more susceptible to alteration in this fatty acid component. Increased 20:4 levels could profoundly increase the biosynthesis of eicosanoids via the cyclooxygenase and lipoxygenase pathways as more substrate will be available. High levels of eicosanoids synthesized from arachidonic acid could therefore render tissues more susceptible to dysrhythmia (McLennan *et al.*, 1987; Abeywardena *et al.*, 1991a; 1991b). As far as the n-3 fatty acids are concerned, the incorporation of 22:6 was higher than 20:5 and the levels of the former were particularly high in PE and PS fractions from animals fed the n-3 diet and this may have inhibitory effects on the metabolism of 20:4 to prostanoids

(Abeywardena *et al.*, 1991a; 1991b; Horrobin, 1991). The levels of 22:6 were high in the PE and PS fractions compared to the other fractions and this may offer some protection against the biosynthesis of eicosanoids in animals fed the n-3 diet since the levels of 20:4 were concomitantly reduced (Needleman *et al.*, 1982; Corey *et al.*, 1983; Bjørneboe, 1988; Charnock *et al.*, 1992). Considering that the n-3 diet contained a higher proportion of 20:5n-3 compared to 22:6n-3, the detection of significant levels of 22:5n-3 and much higher levels of 22:6n-3 in membranes from animals fed this diet suggests that most of the 20:5n-3 was metabolised via elongation and desaturation.

The incorporation of 22:6n3 into cardiac phospholipids was not affected by rendering animals hypothyroid indicating that esterification of this fatty acid is not dependent on thyroid status. The 20:4 levels in PC, PE and PI fractions in membranes from animals fed the SAT diet were significantly reduced suggesting inhibition of desaturase activity. The ratio of 18:2/20:4 was increased in the hypothyroid state when the n-6 diet or the SAT diet were fed but not when the n-3 diet was fed to animals. This finding suggests an apparent reduction in the effect of hypothyroidism on desaturation acitivity. Thus, it appears the increase in the level of 22:6n-3 in membranes from hypothyroid animals offsets any defect in Δ^4-desaturase activity. Of all the phospholipid fractions analysed in this study, the least change in fatty acid composition was observed in sphingomyelin. This suggests that, at least for the major fatty acid species, this phospholipid is relatively resistant to modification by dietary manipulation. However, the detection of substantial amounts of minor fatty

acids, such as 18:4n-3 implies that significant changes in fatty acid profiles of all cardiac phospholipids can be achieved through dietary lipid changes.

An interesting finding in the present study is the variation in the proportions of oleic acid isomers (18:1n-7 and 18:1n-9) in the PS fraction which suggests that the apparent inhibition of desaturase may be partly due to the effect of cis-trans fatty acids. Cis- and trans-vaccenic acids are known to modify membrane fluidity hence the uptake of Ca^{2+} by chick brush border membranes from animals deficient in vitamin D (Bikle, 1990). The cis-isomer increased Ca^{2+} accumulation while the trans-isomer had no effect in hormone deficient animals but reduces Ca^{2+} uptake in hormone-replete animals (Fontaine *et al.*, 1981). These findings were partly confirmed by Bikle *et al.* (1984) who showed that trans-isomers stimulated Ca^{2+} uptake in hormone-replete membranes by altering membrane fluidity. The detection of 20:1(n-7) and 20:1(n-9) in some of the membrane phospholipids in addition to 18:1(n-7) and 18:1(n-9) suggests that cis- and trans-fatty acids are important components of rat cardiac membrane phospholipids. The presence of 20:1, 22:1 and 18:1 have been shown in membrane phospholipids from hearts of rats fed different fats (Ruiz-Gutierrez *et al.*, 1990). Thus, the distribution of these fatty acids observed in the present study can be expected to contribute to changing the lipid environment of cardiac membranes hence the activity of sarcolemma enzymes. Stenson *et al.* (1989) have demonstrated that altered specific activity of enzymes results from changes in local lipid environment rather than changes in the biophysical characteristics of the membrane, and alteration of phospholipase-A activity in rat

myocytes by n-3 fatty acids has been reported (Grynberg *et al.*, 1992). Thus, alteration of the unsaturated fatty acid component of membranes is a likely mechanism by which modulation of Ca^{2+} translocation can be achieved therefore the changes in cardiac membrane phospholipids described in the present study may be responsible for the altered myocardial contractility observed in different thyroid states.

In summary, this study has shown that thyroid status and dietary fatty acids influence the fatty acyl profile of cardiac membrane phospholipids through effects on metabolism of n-6 fatty acids and incorporation of n-3 fatty acids. These changes may affect the activities of intrinsic membrane proteins which mediate contractile responses in the myocardium.

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V. GENERAL DISCUSSION

The influence of thyroid state on cardiac performance was recognized long ago but the actual biochemical mechanisms involved are still elusive. Reduced myocardial contractility in hypothyroid state compared to the euthyroid state (Sharp *et al.*, 1985; Liu and Gerdes, 1990) has been attributed to changes in the ß-adrenergic receptor/adenylate cyclase system, particularly to reduced receptor density (Williams and Lefkowitz, 1983; Stiles *et al.*, 1984). However, this may not be solely responsible for the decreased adrenergic effect since reduced receptor density is observed in hypothyroid, pithed rats as well as in animals treated with 6-hydroxydopamine in which ß-receptors are only slightly activated through the influence of the sympathetic nervous system (Gross and Lues, 1985). It is therefore necessary to consider other factors, such as calcium handling, to explain the cardiovascular changes in hypothyroidism and hyperthyroidism.

The positive inotropic effect and increased force and velocity of contraction induced by thyroid hormone are attributable to modulation of myosin ATPase isoenzymes (V_1, V_2 and V_3) and sarcoplasmic Ca^{2+} pumping activity (Kim and Smith, 1985; Rohrer and Dillman, 1988). However, effects on L-type calcium channels have also been reported (Binah *et al.*, 1987; Rubinstein and Binah, 1989; Mager *et al.*, 1992). The slow inward Ca^{2+} current in guinea pig ventricular myocytes was shown to increase in hyperthyroidism but decreased in hypothyroidism. Furthermore, the antiarrhythmic drug amiodarone, which is similar to thyroid hormone, suppresses

inotropic effect by reducing ß-receptor density as well as directly activating ionic channels (Yin *et al.*, 1992). Thus, cardiovascular changes associated with hyperthyroidism and hypothyroidism result from the complex interaction of several factors and alteration in ß-adrenoceptors represents one molecular mechanism for explaining the cardiac effects of thyroid hormone.

Contractility is a function of muscle size, the muscles' environmental condition (e.g., ionic composition), as well as the sensitivity of myofilaments to calcium (Rupp, 1986). Decreased myocardial contractility may therefore be the consequence of (i) interference with release of Ca^{2+} from the sarcoplasmic reticulum, (ii) reduced sensitivity of contractile proteins to Ca^{2+}, (iii) inhibition of Ca^{2+} influx via voltage-dependent, dihydropyridine-sensitive calcium channels or (iv) interference with signal transduction at several levels in the pathway of excitation-contraction coupling. The intrinsic and extrinsic regulation of myocardial contractility appears to involve alterations in the response of the myofilaments to Ca^{2+} and constitutes an important part of the overall regulation of activity. However, such a mechanism involves alterations in Ca^{2+} delivery to myofilaments via membrane pumps and channels (Honerjager, 1986).

Different local lipid environmental influences can change the characteristics of receptors and channels because events such as coupling of G proteins depend on specific lipids, and binding affinities are altered by changes in membrane fluidity (Steck and Fox, 1972). Fatty acids are known to alter the functional properties of myocardial membranes and changes may contribute to decline in myocardial

contractility (Charnock *et al.*, 1992; Dhalla *et al.*, 1992). In addition to these, lipid intermediates may alter intracellular ion concentrations (Dhalla *et al.*, 1992). Free fatty acids can also increase Na^+ and Ca^{2+} permeability in cardiac sarcolemma (Lamers *et al.*, 1984), therefore regulation of contractility may result from changes in membrane fatty acids. The effect of variations in fatty acid content of membrane phospholipids on Cl^- and K^+ fluxes, and consequently on action potentials in rat cardiac myocytes have also been reported (Hasin *et al.*, 1982). Dietary fatty acids have been shown to modulate the pathway of inositol phosphate generation in platelets (Medini *et al.*, 1990), and the rate of α-adrenoceptor-stimulated hydrolysis of phosphatidylinositol-4,5-bisphosphate was found to be depended on the polyunsaturated fatty acid[24] composition of membrane phospholipids (Lamers *et al.*, 1992).

Stenson *et al.* (1989) have demonstrated the lipid dependence of enzyme activity and attributed this to changes in local lipid environment rather than alterations in the biophysical characteristics of the membrane. Lee and Hamm (1989) have shown that the affinity and density of glucagon receptors are unaffected by diet and suggested that the modulation of adenylate cyclase activity by dietary lipids was due to changes in signal transduction, the G_s protein or the catalytic unit of the enzyme. Thus, the lipid millieu is important for receptor, transporter and enzymatic functions (Clandinin *et al.*, 1991). One can therefore expect that alterations in the

[24]Linoleic acid and eicosapentaenoic acid reduced the rate of inositol phosphate production probably as a result of altered levels of phosphatidylinositol-4,5-bisphosphate.

fatty acid profiles of membrane phospholipids will, presumably alter the functions of enzymes such as adenylate cyclase, Na^+/K^+-ATPase and Ca^{2+}-ATPase in the myocardium. Adenylate cyclase is very susceptible to membrane lipid changes (Ross and Gilman, 1980) hence dietary feeding of essential fatty acids, which have the potential to alter membrane structure (Charnock *et al.*, 1984; 1985; 1986), can affect the efficiency of ß-adrenoceptor coupling to effector systems.

In the study described in this thesis, the hypothesis that depressed myocardial contractility observed in hypothyroidism is due to changes in the lipid environment of the ß-adrenoceptor/adenylate cyclase complex induced by inadequate levels of thyroid hormone was tested. The effect of experimental hypothyroidism and dietary fatty acid feeding on catecholamine-induced inotropic responses of papillary muscles is described in Chapter II. The combined effects of thyroid status and dietary fat did not change ß-adrenoceptor density or affinity suggesting that differences in the coupling mechanism to post-receptor effector systems may account for the variability in tension generation. The n-3 diet and euthyroid state both enhanced contractile responses to isoproterenol but reduced the incidence of dysrhythmia to the same agent. This finding suggests that the development of dysrhythmia may not be due to increased sensitivity of the ß-adrenoceptor/adenylate cyclase system but may be related to intracellular calcium overload (Bers, 1991). Catecholamines are known to promote arrhythmogenesis through the induction of afterdepolarizations leading to triggered activity (Wit, 1984). The mechanism is related to intracellular Ca^{2+} fluctuations. Stimulation of the ß-adrenoceptor affects calcium channels by decreasing

the time that the channel stays closed at a particular voltage (Cachelin *et al.*, 1983; Brum *et al.*, 1984). Isoproterenol can therefore induce arrhythmias by increasing slow inward calcium current (I_{Ca}). However, depolarizing Na^+/Ca^{2+} exchange currents can also produce arrythmogenic currents which are associated with cellular calcium overload. Modification of dietary fatty acid intake can modulate Ca^{2+} handling, presumably through membrane compositional changes (McLennan *et al.*, 1987). Thus the arrhythmia observed in the present study may have resulted from transient inward currents associated with aftercontractions. This current has two components, (i) an intracellular calcium activated non-selective current estimated to be 15% of total current and (ii) a Na^+/Ca^{2+} exchange current which constitutes about 85% (Fedida *et al.*, 1987; Kimura, 1988). It therefore appears that the Na^+/Ca^{2+} exchange current is substantial in tissues from animals fed the n-6 diet compared to those from animals fed the n-3 diet. The n-3 fatty acids apparently offer protection against intracellular calcium overload and by doing so exert beneficial effects on the performance of the heart. The differences in the anti-arrythmogenic effects of n-3 and n-6 fatty acids therefore suggest different mechanisms of action. Bers *et al.* (1988) have shown that calcium entry via Na^+/Ca^{2+} exchange can contribute quantitatively to the direct activation of the myofilaments since contractions and calcium transients can be activated by action potentials and long depolarizing voltage clamp pulses even when both sarcolemmal calcium channels and sarcoplasmic reticulum calcium release are inhibited.

The modulation of Ca^{2+} entry may be an important post-receptor mechanism

that can regulate myocardial contractility. Enhanced Ca^{2+} influx gives rise to increased intracellular Ca^{2+} concentration and the subsequent rise in the amount of Ca^{2+} stored in the sarcoplasmic reticulum. Since stimulation of ß-receptors eventually leads to enhanced calcium influx (Le Peuch and Demaille, 1986; Bers, 1991), the increased Ca^{2+}-sensitivity of tissues and reduced calcium channel number induced by the n-3 diet in euthyroid animals compared to the n-6 diet as described in Chapter III, may be the consequence of increased calcium transport kinetics. Mager *et al.* (1992) have suggested that the Ca^{2+} current contributing to enhanced contractility induced by thyroid hormone in guinea pig myocytes is due to increased adenylate cyclase activity leading to increased phoshorylation of L-type calcium channels. However, in the present study the n-3 diet enhanced contractility in both euthyroid and hypothyroid myocardium without increasing adenylate cyclase activity, suggesting that enhanced contractility may not be due to effects on adenylate cyclase but partly dependent on thyroid hormone-induced influence on membrane lipid environment which in turn modulates L-type Ca^{2+} channel activity, probably the kinetics of Ca^{2+} influx and efflux. Direct evidence for such an effect of dietary fatty acids on calcium and other ion channels can be obtained from patch-clamp studies evaluating the variation in action potential parameters under conditions employed in the current study.

The sensitivity to extracellular calcium was reduced in the hypothyroid state compared to the euthyroid state, but there was no apparent difference in sensitivity to intracellular calcium. This finding suggests that larger changes in intracellular

calcium levels occur after excitation, probably as a result of increased Ca^{2+} release by the sarcoplasmic reticulum or a large influx of extracellular Ca^{2+} and/or reduced uptake by the sarcoplasmic reticulum. Thus, in tissues from animals fed the n-3 diet, intracellular calcium levels may have increased significantly hence the increase in contraction observed in both euthyroid and hypothyroid tissues. At the same time, the sarcolemmal efflux and sarcoplasmic reticulum uptake may be sufficient to return the Ca^{2+} level to equilibrium. The nifedipine and nitrendipine data indicate that enhancement of tension apparently is not exclusively due to calcium channel change and suggest involvement of other components of the excitation-contraction coupling system, such as the sarcoplasmic reticulum (Ca^{2+} uptake and release) or sarcolemmal Na^+/Ca^{2+} exchanger. The reduced B_{max} of nitrendipine binding due to n-3 indicate fewer L-type calcium channels, however, larger contractility was observed. This also suggests that the effect is on increasing intracellular calcium levels, thus the effect of n-3 appears to be larger on sarcoplasmic reticulum calcium release or Na^+/Ca^{2+} exchange compared to n-6.

Fatty acids alter the functional properties of myocardial membranes which results in altered ionic permeability (Lamers *et al.*, 1984). The findings described in Chapter IV indicate that both thyroid state and dietary fatty acids altered the fatty acid profiles of cardiac membrane phospholipids. The fatty acid composition of ventricular membrane phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin depend on thyroid status and the type of dietary fat. This effect is apparently related to desaturase enzyme

activity which is inhibited in the hypothyroid state when the n-6 diet or the SAT diet was fed. Altered fatty acid composition of membrane phospholipids modulates calcium transport in rat myocardium (Taffet *et al.*, 1993), therefore the changes described in this study may be important in regulating Ca^{2+} influx into myocardial cells hence contractility. The myocardial sarcolemma contains a very active Na^+/Ca^{2+} exchange system which may regulate intracellular calcium levels and consequently, contractility (Bers, 1991).

A number of studies have shown that the Na^+/Ca^{2+} exchanger is regulated by ionic components in the membrane lipid environment as well as by fatty acids (Philipson *et al.*, 1985; Philipson and Ward, 1985; 1987). Unsaturated fatty acids were found to be more potent than saturated fatty acids in stimulating Na^+/Ca^{2+} exchange. This effect, which is primarily due to a decrease in the apparent $K_M(Ca^{2+})$, correlates positively with passive Ca^{2+} permeability. Based on these studies, it was concluded that local lipid disorder and anionic charge regulate Na^+/Ca^{2+} exchange. Thus, perturbation of the lipid bilayer hydrophobic region by fatty acids contribute to increased Ca^{2+} influx. The kinetics and amplitude of the calcium current play a critical role in controlling the amount of Ca^{2+} released by the sarcoplasmic reticulum therefore calcium extrusion from the cell during the same cardiac cycle is necessary for the maintenance of steady state. Any uncompensated calcium influx could eventually constitute a calcium overload for the cell, hence compromise relaxation and contraction and may become arrhythmogenic (Bers, 1991).

In summary, the present study has shown that decreased myocardial

contractility associated with the hypothyroid state involves reduced coupling of the ß-adrenergic receptor/adenylate cyclase system to post-receptor effector systems, notably the L-type calcium channel and possibly the Na^+/Ca^{2+} exchanger as well as reduced calcium sensitivity of myocardial tissue. These effects probably result from the influence of inadequate levels of thyroid hormone on the metabolism and incorporation of essential fatty acids into ventricular membrane phospholipids. Although thyroid state was associated with some differences in phospholipid fatty acid composition, these were complex and did not demonstrate any direct link with the contractility studies.

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