EFFECTS OF CHOLESTEROL AND ITS OXIDATION PRODUCTS ON Ca^{2+} TRANSPORT IN CARDIAC AND VASCULAR SMOOTH MUSCLE

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Physiology
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

September, 1991

ISBN 0-315-76831-2

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ACKNOWLEDGEMENTS

I would like to thank the members of the division of Cardiovascular Physiology for their support and scientific contributions throughout the course of this research. I would specifically like to thank Drs. Dhalla, Panagia, Singal and Cole for their continued encouragement. The technical assistance of Thane Maddaford and Bram Ramjiawan was very much appreciated; thanks guys for your friendship.

Most of all, I would like to thank Dr. Grant Pierce, my supervisor, mentor and friend. His enthusiasm and wisdom were greatly appreciated. Completion of this thesis was a very arduous task, and without Grant's encouragement, I doubt I would have endured. I hope someday to repay your kindness Grant.

ABSTRACT

Modification of the cholesterol content and *in situ* oxidative modification of cholesterol in purified sarcolemma (SL) from dog ventricles was performed in order to study the effects of cholesterol and its oxidation products on SL ion transport. Na^+-Ca^{2+} exchange measured in cholesterol enriched SL vesicles was increased 48% over control values. This stimulating effect was specific to the Na^+-Ca^{2+} exchange process, since SL ATP-dependent Ca^{2+} uptake was depressed by 40% with cholesterol enrichment. Na^+,K^+-ATPase activity was depressed in both cholesterol enriched and depleted SL vesicles. *In situ* oxidation of cholesterol to cholestenone by cholesterol oxidase (CO) inhibited Na^+-Ca^{2+} exchange activity and ATP-dependent Ca^{2+} uptake in a concentration dependent manner. ATP-dependent Ca^{2+} uptake was inhibited and conversely, passive Ca^{2+} binding to the membrane was stimulated by CO. In order to determine if low density lipoproteins (LDL) have the capacity to alter Ca^{2+} movements in vascular smooth muscle, isolated rabbit aortic cells were maintained in culture for 24, 48, or 72 h with various concentrations of LDL. The rate of Ca^{2+} exchange in a slowly exchangeable pool was twice as fast in the LDL treated cells as in a control population. A La^{3+}-displaceable, rapidly exchangeable cell membrane associated Ca^{2+} fraction was significantly larger in the LDL treated cells. These data suggest that cholesterol can directly affect ionic interactions in SL vesicles and vascular smooth muscle cells. Measurement of serum oxidized cholesterol derivatives in a population known to be at low risk for the development of coronary heart disease revealed that level of oxidized cholesterol may not be a reliable risk marker for this process.

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A. INTRODUCTION

The regulation of Ca^{2+} movements across the sarcolemmal membrane is of critical importance to cardiac integrity and the excitation-contraction coupling process in the heart[315]. The observations of Reeves and Sutko[201] identified the presence of a highly active ion transport pathway in the isolated cardiac sarcolemmal membrane which exchanged Na^+ for Ca^{2+}. It has been proposed that Na^+-Ca^{2+} exchange is important in the regulation of intracellular Ca^{2+} concentration and thus, force generation in the heart[403]. Thus, any compound which can alter the activity of the Na^+-Ca^{2+} exchanger should significantly modulate contractile activity in the heart.

Cholesterol is a common and relatively prominent component of the lipid bilayer in the cardiac sarcolemmal membrane[404]. Its role as a regulatory factor in myocardial ion transport has been proposed on the basis of muscle function and *in vitro* membrane biochemical studies. For example, mechanical dysfunction was observed in papillary muscle from hypercholesterolemic rabbits which was suggested to be a consequence of a defect in Ca^{2+} transport of the cardiac cell[367]. Manipulation of the cholesterol content of isolated membranes from a variety of tissues has been shown to cause alterations in the activities of Na^+,K^+-ATPase[141,364-366] and Ca^{2+}-Mg^{2+} ATPase[149,150]. Thus precedents exsist for the interaction of cholesterol with cardiac sarcolemmal enzymes.

Several observations support the possibility that membrane cholesterol may influence Na^+-Ca^{2+} exchange. Cholesterol has a known capacity to alter the physical properties of a membrane[146]. Cholesterol has also been shown to directly interact with

membrane-bound proteins[405,406]. Thus, if the Na^+-Ca^{2+} exchange protein is sensitive to the membrane lipid environment, cholesterol may be important in regulating its function. Studies using doxyl group or amphiphile incorporation[319,322], phospholipase[319], or saponin treatment[407] of the cardiac sarcolemmal membrane to alter its physical characteristics and composition have demonstrated a significant dependence of the Na^+-Ca^{2+} exchange mechanism upon the membrane lipid environment. Thus in view of (i) the capacity of cholesterol to alter the function of other sarcolemmal ion transport enzymes and (ii) the known dependence of the Na^+-Ca^{2+} exchanger on the membrane lipid environment, it is likely that cholesterol may modify Na^+-Ca^{2+} exchange. However, the effect of cholesterol on cardiac sarcolemmal Na^+-Ca^{2+} exchange is presently unknown. In view of the proposed role for Na^+-Ca^{2+} exchange in cardiac function, cholesterol modification of its activity has potential significance.

The formation of reduced oxygen intermediates has been demonstrated to have the capacity to cause contractile dysfunction and cellular damage in the heart. These reduced oxygen intermediates, or oxygen free radicals, have been implicated mechanistically in a number of pathophysiological processes including the oxygen paradox, calcium paradox, adriamycin-induced cardiomyopathy, and ischemic/reperfusion injury[375,408,409]. Because the underlying defect in these pathologies is likely to be alterations in ion transport in the heart, research attention has focussed on the effect of oxygen free radicals on ion flux. Exposure of cardiac membranes to free radicals results in increased membrane permeability characteristics[379], depressed Na^+ and Ca^{2+} pump activities[379,410-412], and altered Na^+-Ca^{2+} exchange[235,380].

Several sites of action within the myocardial cell have been proposed to explain the deleterious effects of oxygen free radicals. Oxidation of specific amino acid residues in membrane bound proteins[412,413], and peroxidation of unsaturated fatty acids in the membrane[410,411] are the two sites of attack by oxygen free radicals that are most frequently cited. However, another cellular site of attack for free radicals that may be altering ion flux is membrane cholesterol[340,378]. However it is unknown what effects cholesterol oxidation may have on the myocardium. It is possible that oxidative modification of membrane cholesterol *in situ* may also affect ionic interactions within the membrane.

Hypercholesterolemia and elevated circulating low density lipoprotein (LDL) levels have long been recognized as risk factors in the pathogenisis of atherosclerosis. Excessive cholesterol deposition in the arterial wall is an early process in atherogenesis[28]. The vascular smooth muscle cell is a site for this cholesterol accumulation within the atheromatous plaque[29,30]. Arterial calcification is also a common feature of the atherosclerotic plaque[30], and many reports have documented an increased calcium content of human atherosclerotic arteries[126] and arteries from hypercholesterolemic experimental animals[127,128]. This increased tissue calcium observed in atherosclerotic lesions has been shown to be located, in part, inside the cells[382,383]. However, the direct relationship between high levels of circulating cholesterol (or LDL) and Ca^{2+} flux is less clear.

Several studies showing altered Ca^{2+} transport in arterial tissue segments exposed to elevated cholesterol levels have been reported[131,132]. It was felt that changes in Ca^{2+} movements in in smooth muscle cells were responsible for the observed alterations[131,132].

A number of problems are associated with measuring ion flux in whole tissue preparations and relating these data to the smooth muscle cell. These include the presence in whole tissue of a variety of cell types which all may respond differently to cholesterol enrichment and may possess quite different Ca^{2+} flux characteristics. Kinetic analysis of Ca^{2+} flux in whole tissue is also complicated by Ca^{2+} movements through the interstitial space. This renders it difficult to resolve the movements into separate components and introduces the complication of large amounts of matrix bound Ca^{2+} in the extracellular space[385]. These problems can be avoided by examining ion transport characteristics in isolated cultured smooth muscle cells[352,385].

Oxidatively modified LDL particles have also been suggested to be involved in atherogenesis[85-87], and in particular, oxidation of the cholesterol moiety has been implicated in the atherogenic process[95]. Both animal studies[98] and epidemiological data[417] have shown that diets high in oxidized derivatives of cholesterol results in markedly increased morbidity resulting from coronary heart disease. In addition, a similarity between *in vitro* oxidatively modified LDL particles and LDL from patients suffering from ischemic heart disease has recently been suggested[394]. It has been hypothesized that oxidized cholesterol may be a synergistic risk factor for atherosclerosis[95,98,417].

Epidemiological studies have identified two populations with a higher than expected morbidity and mortality from atherosclerosis without the commonly associated major risk factors[418,419]. It is felt that dietary exposure to cholesterol oxides, resulting in high levels of circulating serum oxidized cholesterol may be responsible for the high frequency of atherosclerotic complications in these populations[417], however this has never

been tested. In contrast, certain indigenous populations, such as the traditionally living Inuit have been spared the current epidemic of ischemic heart disease[420]. It is possible that in this population, serum levels of oxidized derivatives of cholesterol may be lower than in the high risk urbanized western population.

The purpose of the present study was to determine the effects on ion transport of cholesterol enrichment and *in situ* cholesterol oxidation of isolated sarcolemmal membranes. Cholesterol oxidase was used to selectively oxidize membrane cholesterol residues. Ca^{2+} exchange was also examined in isolated cultured vascular smooth muscle cells after cholesterol enrichment using isolated low density and very low density lipoproteins. These results demonstrate that enrichment and oxidation of membrane cholesterol can significantly alter ionic interactions in cardiac sarcolemmal vesicles. In addition, alterations in Ca^{2+} transport can be induced in vascular smooth muscle cells by interaction with LDL.

We also tested the hypothesis that serum levels of oxidized cholesterol, in particular oxidized LDL associated cholesterol, can be used as a risk marker for the development of coronary heart disease by comparing the levels of lipoprotein associated cholesterol and its oxidized derivatives in two populations with differing risks for the development of coronary heart disease. The two populations chosen were the traditionally living Inuit and the urbanized western population. The data presented suggest that oxidized cholesterol may not be a suitable risk marker for the development of CHD.

B. REVIEW OF THE LITERATURE

I. The Pathogenesis of Atherosclerosis

Cardiovascular disease remains the chief cause of death in North America[1]. It is estimated that more than 80,000 people will die as a result of circulatory diseases in Canada in 1989[2]. This accounts for greater than 40% of deaths from all causes[2]. Of these deaths attributable to circulatory causes, more than 49,000 will occur as a result of atherosclerotic coronary heart disease[2]. Despite the fact that atheroscerotic vascular disease is the major cause of mortality in developed countries, its cause remains unknown.

a) Risk Factors

i] *Lipids as risk factors*

Incontrovertible evidence for the central role of cholesterol in the pathogenesis of atherosclerosis and coronary heart disease is derived from experimental animal, epidemiological, genetic and prospective intervention studies. A large body of evidence linking elevated serum cholesterol to coronary heart disease (CHD) was reviewed comprehensively in the recent report on diet and health from the National Research Council - National Academy of Sciences[3]. The most prominent studies which confirm the link between cholesterol and CHD include:

The Framingham Heart Study
The Multiple Risk Intervention Trial (MRFIT)
The "Seven Countries Study"
Brown and Goldstein's LDL receptor research
The Coronary Primary Prevention Trial
The Helsinki Heart Study

The Coronary Drug Project.

The Framingham Heart Study, an ongoing study which began 40 years ago, is perhaps the best known of the clinical trials which provided evidence on the relationship between elevated serum cholesterol and risk of morbidity and mortality from CHD[4]. In their study, Kannel et al[4] reported the cholesterol and coronary histories of 2,282 men and 2,845 women in Framingham, Massachusetts over a period of 14 years. Most of the participants in the study exhibited total serum cholesterol levels between 150 and 300 mg/dL (3.9 and 7.8 mmol/L). The investigators found a positive correlation between serum cholesterol levels and rates of CHD across the range of cholesterol measurements; low levels of serum cholesterol being associated with low rates of CHD while high levels were found to be associated with high rates of CHD.

One of two uniquely powerful studies by virtue of their sample size was the Multiple Risk Factor Intervention Trial (MRFIT)[5]. This was a randomized, primary prevention trial which tested the effects of modifying several coronary risk factors in 12,866 high risk men, aged 37-57, selected from a cohort of more than 360,000 middle-aged men who had no history of hospital admission for myocardial infarction. The serum cholesterol levels of the larger cohort were measured and the CHD death rate over the next six years was observed. This extraordinary epidemiological data showed: 1) the relationship between cholesterol and CHD mortality is curvilinear and continuous over the whole plasma cholesterol distribution (150 - 300 mg/dL, 3.9 - 7.8 mmol/L) without evidence of a threshold effect, 2) the risk for CHD mortality increases steadily, particularily above levels of 200 mg/dL (5.2 mmol/L), and 3) the magnitude of increased

risk is large, fourfold in the top 10% as compared with the bottom 10%. On the basis of studies such as this, the American Health Foundation in 1979 concluded that individual plasma levels of greater than 200 mg/dL (5.2 mmol/L) were not optimal for health. The MRFIT study showed that not only is there a concentration dependent relationship between cholesterol and the risk of CHD, but that this relationship is powerfully modified by the major additional risk factors of smoking and hypertension.

Keys's "seven countries study"[6] was the other uniquely large study which linked cholesterol and CHD. This study established that the countries with the highest CHD mortality rate had the highest consumption of saturated fats and the highest circulating cholesterol levels.

In 1985 the Nobel Prize in Medicine was awarded to Michael Brown and Joseph Goldstein for their investigations on low density lipoprotein (LDL) receptors[7]. These investigators recognized that individuals with a deficiency of LDL receptors were prone to atherosclerosis and premature development of CHD. They observed that a diet rich in cholesterol decreases the number of LDL receptors in the liver, which are involved breakdown of the atherogenic LDL particles. Brown and Goldstein suggested that lifestyle-induced deficiencies of LDL receptors results in an increased LDL concentration and a greater risk of CHD.

The first of the major trials to provide evidence demonstrating that a reduction in plasma cholesterol, particularily LDL cholesterol, decreases the risk of CHD was the Lipid Research Clinics Coronary Primary Prevention Trial (CPPT)[8]. This multicenter, randomized, double-blind study screened more than 300,000 men to identify 3,806 who

met strictly defined criteria. At the time of entry, the men were free of clinical manifestations of coronary disease, were between ages 35 and 59 and had cholesterol levels over 265 mg/dL (6.89 mmol/L). The men were randomized on the basis of cigarette smoking, diabetes and various other factors and assigned either to a treatment or a placebo group. Both groups were placed on cholesterol lowering diets to reduce their serum cholesterol levels by about 4%. The treatment group also received the bile acid sequestrant cholestyramine. The "hard endpoints" were nonfatal myocardial infarction and death due to coronary heart disease. Over the seven year trial period, subjects in the treatment group reported reductions in total cholesterol and LDL cholesterol of 13.4 and 20.3% respectively, which resulted in a 24% decrease in CHD mortality and a 19% decrease in nonfatal myocardial infarction. The CPPT results and those of the Framingham Study indicate that a 1% reduction in an individual's total serum cholesterol translates into an approximately 2% reduction in CHD risk.

Like the CPPT study, the Helsinki Heart Study[9] of 1987 was a randomized, double-blind trial. It involved 4,081 asymptomatic middle-aged men (40-55 years) with primary dyslipidemia (non-HDL [high density lipoprotein] cholesterol $\geq$ 5.2 mmol/L). One group received cholesterol lowering drug therapy, the other placebo. Individuals in the treated group exhibited an average of 8% reduction in total and LDL cholesterol with a concommitant increase of 10% in their levels of HDL cholesterol (there exists a strong negative correlation between HDL cholesterol and CHD, as will be discussed below). After five years there was a 34% reduction in the incidence of CHD in the treatment group, although there was no difference between the groups' total death rate.

The Coronary Drug Project was a double-blind study of more than 8,000 male heart attack survivors which began in the early 60's[10]. The object of this study was to evaluate the lipid lowering agents available at that time. At initial follow-up at six years, the 1,119 treatment subjects had significantly lower levels of total serum cholesterol, LDL, very low density lipoprotein (VLDL), triglycerides, and increased HDL level. In addition, these men had experienced 27% fewer non-fatal myocardial infarctions, however overall mortality was not reduced. Drug treatment was discontinued at this stage, and after a further nine year period, there was a significant 10% reduction in overall mortality in the treatment group.

These combined studies documented that a marked reduction in serum cholesterol, particularily LDL cholesterol was associated with a lesser progression of CHD in addition to a regression of cardiovascular disease in some. The results of these clinical trials created an impetus to develop a comprehensive program for the treatment of hyperlipidemia.

ii] *Non-lipid risk factors*

In addition to hyperlipidemia, several other risk factors for the development of CHD have been identified. Male gender has been recognized as contributing to significant risk for the development of CHD. The rates of CHD are three to four times higher in middle-aged men than in women[11] and men develop CHD, on average, 10-15 years earlier than women[11]. A familial predisposition to premature CHD has been shown. In some instances this may represent clustering of other risk factors within families rather than a unique genetic predispostion. Nevertheless, there are families with

high rates of CHD in which none of the known risk factors appears to operate, but the determinants of such susceptibility are not clear[12].

The association between cigarette smoking and susceptibility to CHD is firmly established. Friedman et al[13] reported smoker/nonsmoker mortality ratios, crude and adjusted, to be 4.7 and 3.6 respectively for coronary heart disease. Several studies have emphasized that reduction in cigarette smoking in those who smoke forty or more cigarettes a day is associated with a marked reduction in the risk of dying from CHD[14,15].

Hypertension has been shown to markedly accelerate atherogenesis and the development of CHD[16]. Data from the Framingham study showed that hypertensive subjects experienced a three-fold rise in coronary heart disease as well as a doubling of peripheral atherosclerosis when compared with normotensive individuals[17]. An exponentially increasing risk was shown to be present over the whole range of blood pressure[17], the risk of CHD in individuals with diastolic pressures greater than 105 mm Hg was four times that of those with pressures 84 mm Hg or less[16]. On the average, for every 10 mm Hg rise in pressure, there appears to be about a 30% increase in cardiovascular risk[17]. The International Atherosclerosis project involving 14 countries and 23,000 autopsied cases has provided possibly the best data on the influence of hypertension on the extent and severity of arterial lesions[18]. The mean extent of fatty streak and raised lesions in the coronary arteries and aortas of hypertensives was significantly greater than in controls, particularly in young individuals. In addition, fibrous plaques and advanced lesions were more frequently seen in hypertensives. Of clinical significance was the fact that the prevalence of significant coronary artery

stenosis was also greater in hypertensive subjects than normotensive individuals.

Diabetes is known to increase the risk of CHD[19]. The acceleration of atherosclerosis with diabetes is poorly understood, and genetic, environmental, metabolic and hormonal factors all seem to be involved. A number of the factors operating in the diabetic are similar to risk factors found in the general population, others are unique to diabetics[19].

Many reports have indicated a connection between obesity and CHD[20,21]. The relationships between obesity and CHD are confounded by the many metabolic effects of obesity (increased blood pressure, increased serum cholesterol, induced glucose intolerance and reduced serum HDL). Data from the Framingham study indicate however, that obesity is an independent risk factor for CHD[22].

Thus, there has been general acceptance of the epidemiological evidence linking atherosclerosis and several independent risk factors, despite the inherent weaknesses of epidemiological studies. Epidemiology has a limited applicability to a chronic, insidious, degenerative disease such as atherosclerosis. It is difficult in epidemiological studies to avoid bias, to have a truly representative sample of the human population, and to study relevant factors of importance to a disease of such chronicity and slow progression. Many of the studies mentioned used the "soft" endpoint of a "clinical" diagnosis of coronary heart disease, which is a reflection of the complication of the disease and not an indication of the disease itself. It is generally accepted that any clinical diagnosis has inherent diagnostic error, the conservative estimate for the error involved in the diagnosis of CHD is $\pm 30\%$[23]. Yudkin[24] revealed in a very practical way that epidemiological

evidence cannot stand alone. He demonstrated a correlation between the sale of radio and television sets in the United Kingdom and the mortality rate for CHD. Epidemiology never proves a cause and effect relationship and requires strong pathologic and experimental supportive evidence for scientific plausibility.

b) The Lesions of Atherosclerosis

Several investigations have clarified the specific cellular constituents of human atheromatous lesions[25]. The earliest lesions of atherosclerosis can usually be found in young children and infants in the form of a lesion called the fatty streak. Classic fatty streaks are raised, narrow, nonobstructive lesions that extend in the direction of blood flow. Grossly, the fatty streaks appear as areas of yellow discoloration due to the large amount of deposited lipid. The streaks are characterized by subendothelial collections of foam cells, smooth muscle cells, T-lymphocytes and an extracellular matrix of lipid, collagen, elastin and proteoglycan[26]. Monoclonal antibody studies have shown that foam cells are primarily monocyte-derived macrophages and occasionally smooth muscle cells that have ingested cholesterol and cholesteryl esters[27]. Fatty streaks were observed by Stary[28] in a series of children and young adults. He demonstrated that by the age of ten years, the fatty streaks consisted primarily of lipid laden macrophages, beneath which accumulated lipid laden smooth muscle cells. Stary[28] also studied the fatty streaks in the coronary arteries in a series of children and found that they were localized at anatomical sites identical to the sites in older individuals that were occupied by advanced fibromuscular lesions, or fibrous plaques, suggesting that fatty streaks are the precursors of more advanced lesions.

The advanced lesion of atherosclerosis is called the fibrous plaque. When the fibrous plaque becomes involved with either thrombosis, hemmorhage or calcification, it is often called a complicated plaque or lesion. Fibrous plaques are white in appearance and are usually elevated and, if sufficiently large, they may compromise the flow of blood. The major cell type identified in these heterogenous lesions has been shown to be lipid laden intimal smooth muscle cell (foam cell)[29], together with numerous macrophages[30]. When these cells contained lipid, the lipid was found to be in the form of cholesterol or cholesteryl esters[30]. The proliferated smooth muscle cells were shown to be surrounded by collagen, elastin fibers, large amounts of proteoglycans and in hypercholesterolemic individuals, varying amounts of deposited lipid. Beneath this cell rich region, there often existed an area of necrotic debris, cholesterol crystals and calcification. It was shown that fibrous plaques were characteristically covered with a fibrous cap composed of multiple layers of a particular form of smooth muscle cell in a lacunar-like arrangement in which the lacunae consisted of alternating layers of basement membrane and proteoglycan[30].

The principal clinical implications of advanced fibrous plaques are their capacity to partially or totally occlude the lumen of the affected artery, or because of developed cracks and fissures lead to thrombosis and embolism, or to aneurysmal dilatation (particularily in large vessels).

c) Hypotheses of Atherogenesis

i] *Response to injury hypothesis*

Historically, the first hypothesis to explain atherogenesis was the thrombogenic

theory put forth by Rokitansky in 1852[31], and subsequently elaborated upon by Duguid[32]. Their belief was that an incrustation of a small mural thrombi existed at the sites of arterial injury and that these thrombi went on to organize by the growth of smooth muscles into them. The second hypothesis, namely the insudative or imbibition hypothesis, was the result of Virchow's pioneering work in 1856[33]. Virchow believed that a form of low grade injury to the artery wall resulted in a type of inflammatory insudate, which in turn caused increased passage and accumulation of plasma constituents in the intima of the artery.

In 1973, these two notions about atherogenesis and accumulated knowledge of the cellular and molecular biology of the artery were combined in a hypothesis termed the response-to-injury hypothesis of atherosclerosis[34]. This has become the most popular theory of the pathogenesis of atherosclerosis as it takes into account the many aspects of the behavior of the arterial wall as well as the numerous risk factors that have been associated with atherogenesis, including hyperlipidemia, altered rheological forces as may occur in hypertension, and alteration of the endothelial barrier by factors associated with cigarette smoking and diabetes.

According to the protagonists of the response-to-injury hypothesis, the key event in the initiation and perpetuation of the fibrous plaque, the pathognomic lesion of atherosclerosis, is the proliferation of the smooth muscle cell in the arterial wall. This event is followed by the deposition of intracellular and extracellular lipid and the accumulation of extracellular matrix components including collagen, elastic fibers and proteoglycans. In this hypothesis, a disruption of the arterial endothelial barrier is

essential for the proliferation of the smooth muscle cells.

Disruption of the endothelial barrier may take many forms, which cover the spectrum from subtle changes in function to frank necrosis and denudation. The vascular endothelium responds to various stimuli by undergoing specific alterations in function, metabolism and structure. Some of these alterations are adjustments in normal constitutive functions, and others are due to inductions of new functions and molecules[35].

An example of a potentially injurious stimuli is that of hypercholesterolemia or hyperlipidemia. In chronic hyperlipidemia, the response to injury hypothesis proposes that an increase in plasma lipoproteins, particularly low density lipoprotein, would result in changes in the surface characteristics in both the endothelial cells and the circulating leukocytes. Evidence for these effects has come from many sources. Jackson and Gotto[36] described the effects of an increase in the number of cholesterol molecules on the plasma membranes of cells such as endothelium. Alterations in the cholesterol:phospholipid ratio of the plasma membranes could lead to increased membrane viscosity with subsequent changes in cell surface enzyme activities. Such changes could decrease the malleability of endothelial cells, particularily at branches or bifurcations of the arterial tree, where they are exposed to altered rheologic forces. This altered plasticity has been proposed to explain the observed endothelial retraction over fatty streaks seen in hypercholesterolemic monkeys which in some instances is so severe that the underlying connective tissue is exposed to the circulation[37,38]. This provides opportunities for platelet adherence, aggregation and mural thrombosis.

One of the earliest cellular interactions that occurs in hypercholesterolemia is the

attachment of monocytes, the precursors of macrophages, to endothelial cells[37,39-41]. When these monocytes adhere, they probe and are chemotactically attracted to migrate between endothelial cells and localize subendothelially, where they are converted to macrophages active as scavenger cells which take up lipid[37,38]. The lipid enters the subendothelium in large quantities in the hypercholesterolemic state resulting in the formation of foam cells and in the development of fatty streaks[37]. The accumulation of macrophages in the intimal space would then establish conditions that could lead to further alterations in the endothelium. Macrophages are well known to be able to synthesize and secrete numerous injurious agents including oxidative metabolites, which in this instance could further injure the overlying endothelial cells. It has been shown in vitro that macrophages are capable of secreting superoxide anion and peroxide which may occur in vivo as well[42].

A further and potentially important reaction of the activated macrophage is related to its capacity to form growth factors. Macrophages have been shown to be capable of synthesizing and secreting at least five potent growth factors These include: [1] platelet derived growth factor (PDGF)[43], a growth factor for mesenchymal cells such as smooth muscle and fibroblasts; [2] interleukin 1[44], which is also somewhat mitogenic for fibroblasts; [3] fibroblast growth factor (FGF)[45], a mitogen for endothelial cells; [4] epidermal growth factor (EGF) and EGF-like molecules[46] (transforming growth factor alpha TGF-a), both of which are capable of stimulating the growth of epithelial cells and; [5] transforming growth factor beta (TGF-b)[47], which acts synergistically with some of the aforementioned growth factors. Thus, appropriately activated macrophages in the

subendothelial space could potentially be involved in the secretion of growth factors that could chemotactically attract smooth muscle cells to migrate from the media into the intima, to proliferate within the intima and form an intimal, fibromuscular, proliferative lesion.

The interaction of a third cell, the platelet, is also proposed by the response-to-injury hypothesis. The hypothesis suggests that with endothelial injury, endothelial cell-cell interaction may be affected and cell dysjunction may occur, leading to retraction of endothelial cells and exposure of underlying cells or connective tissue. This would permit opportunities for platelets to interact, adhere, aggregate, and form mural thrombi. Platelets are capable of little or no protein synthesis, however, they contain within their granules a number of factors that participate in the coagulation cascade and at least three extremely potent growth factors or mitogens; namely, PDGF[48], EGF[49], and TGF-b[50]. With interaction with the subendothelium, the platelet could provide a potent source of growth factors contributing to the proliferation of smooth muscle lesions of atherosclerosis.

Arterial endothelial cells and smooth muscle cells are also capable of synthesizing and secreting at least two mitogens; PDGF and FGF[51-54]. Thus one is able to envision paracrine mechanisms where degranulated platelets, infiltrating macrophages, and damaged or activated endothelial cells release growth factors that, in turn, stimulate smooth muscle cell migration from the media and proliferation in the intima. Alternatively, since smooth muscle cells themselves synthesize PDGF and FGF, autocrine stimulated growth of smooth muscle cells might be, in part, responsible for the

development of atherosclerotic lesions.

The response-to-injury hypothesis suggests that at least two pathways may lead to the formation of initial smooth muscle proliferative lesions[55]. One pathway, demonstrated in hypercholesterolemia, involves monocyte and possibly platelet interactions which may stimulate fibrous plaque formation by growth factor release from the various involved cells, as described above. The second pathway involves direct stimulation of endothelium which may release growth factors that can induce smooth muscle migration and proliferation and possibly autogenous growth factor release by proliferating smooth muscle cells. This pathway may be important in diabetes, hypertension, and cigarette smoking.

ii] *Monoclonal hypothesis*

Another popular hypothesis of atherogenesis is the monoclonal hypothesis proposed by Benditt and Benditt[56]. Their hypothesis suggests that each lesion of atherosclerosis is derived from a single smooth muscle cell that serves as the source of all the smooth muscle cells within the lesion. This theory is based upon the Lyon, or inactive X chromosome, hypothesis which states that each tissue is made up of a small tract of related cells that have either an active maternal or an active paternal X chromosome, but not both. Certain females are heterozygous for the X linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) which exists in two isozymic forms that can be separated by electrophoresis. This finding was taken advantage of by Benditt and Benditt[56] who examined a series of plaques from a small number of females heterozygous for G-6-PD at autopsy. They found that some atherosclerotic plaques of heterozygotes

contained only one of the isoenzymes, which was interpreted to indicate monoclonal proliferation of smooth muscle cells. Atherosclerosis could thus be viewed as similar to a benign neoplastic process in which the cell has been transformed by viruses, chemicals or by other mutagens.

There are many adversaries to this hypothesis. Fialkow[57] has indicated that the presence of only one isoenzyme in a lesion does not necessarily indicate monoclonal origin. He maintains that single enzyme phenotype might instead be a reflection of the smooth muscle mosaic composition or tract size and distribution within the normal intima. In addition, monoclonality has been shown to occur as a consequence of the selection of a subpopulation of cells in several forms of focal hyperplasia[58].

In summary, there is no dearth of mechanisms to account for the smooth muscle proliferation of atherosclerosis. In the case of denuding endothelial injury, as can be induced experimentally, platelets and macrophages can release PDGF and potentially other growth factors. In non-denuding injury, factors from adherent macrophages (as in hypercholesterolemia) or possibly from injured endothelial cells may cause smooth muscle proliferation. Which of these mechanisms is relevant *in vivo* is uncertain. Pharmacologic intervention which inhibits platelet adhesion and platelet function, and antiplatelet antibodies have been shown to inhibit the acute smooth muscle proliferation which occurs after endothelial denudation[59,60]. To date, anti-PDGF antisera have not been shown to supress smooth muscle proliferation. One of the major shortcomings of both of the theories of atherogenesis is the failure to define the genetic basis[61] that underlies the increased susceptibility of some persons to this disease and the relative

resistance of others.

d) Lipoproteins, Cholesterol Metabolism and Atherosclerosis

The major lipids of plasma are, in order of decreasing concentration by weight, cholesteryl esters, phospholipids, triglycerides, free cholesterol, and free fatty acids[62]. None of these is water soluble and they do not circulate in a free form in the blood. Except for free fatty acids (FFA), they are complexed with a specific group of proteins called apolipoproteins. Lipids (except FFA) and apolipoproteins circulate through the bloodstream in macromolecular complexes called lipoproteins, which have been classified according to size, density, and electrophoretic mobility. These differences impart to the lipoproteins the variations in physical and chemical properties which have become the basis of analytical methods for their study. There are six major groups of lipoproteins which may be separated by ultra-centrifugation: the chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), high density lipoproteins (HDL), and very high density lipoproteins (VHDL). The unesterified fatty acids (FFA) of plasma are complexed to albumin by noncovalent forces. This fraction is not identifiable by the usual lipoprotein techniques and must be measured by chemical methods and will not be further discussed. A thorough knowledge of lipoproteins and their functions is essential to the understanding of cholesterol homeostasis and their involvement in atherogenesis.

The typical structure of a plasma lipoprotein is that of a spherical particle in which the water insoluble nonpolar lipids (cholesteryl ester and triglyceride) are shielded from the aqueous environment by a surface monolayer consisting of phospholipid and

apolipoproteins. Cholesterol occupies an intermediate position between the surface layer of apoliporoteins and phospholipid and the core of neutral lipid[63].

i] *Apolipoproteins*

As already mentioned, each of the lipoproteins possesses one or more protein constituents called apolipoproteins. The apolipoproteins exhibit the type of polymorphism and heterogeneity seen with other plasma proteins. An important function of certain apolipoproteins is to mediate the binding of the lipoproteins to specific receptors on the surface of cells.

To date at least eleven different apolipoprotein types have been identified, and are associated with the various lipoproteins. Several functions have been identified for the apoprotein component of the lipoprotein complexes and include structural, enzymatic, coenzymatic and receptor binding. Currently, a great deal of interest is focussed in the use of apoproteins as markers for CHD. In particular, investigators have found that apolipoprotein A-I (apoA-I), apolipoprotein B-100 (apoB-100) or apolipoprotein a (apo(a)) plasma levels are better predictors of CHD than are total plasma lipids or lipoproteins.

ii] *Lipoproteins*

1. Very High Density Lipoproteins (VHDL). In addition to the FFA-albumin complexes, from 8 to 15% of the total serum phospholipids and small amounts of cholesterol and triglyceride are not floated by prolonged ultracentrifugation at a density of 1.21 g/ml[64]. These lipoproteins, which resemble HDL immunologically but have a very small lipid content, are called VHDL. VHDL may function as acceptors of lipids

destined for transport. There is evidence, however, that some of the VHDL molecules are artificially produced from HDL by the stress of ultracentrifugation[65].

2. Chylomicrons. These are the largest of the lipoproteins. The predominant function of the chylomicrons is the transfer of the exogenously derived triglyceride and cholesterol from the intestinal lumen to sites of metabolism or storage. Dietary fats in micelles are broken down to fatty acids and monoglycerides in the intestinal lumen. The lipid components enter the intestinal villi where they are reassembled into triglycerides. In the intestinal cells, cholesterol is esterified to cholesteryl esters through the enzymatic action of acyl cholesterol acyl transferase (ACAT). The triglycerides and esterified cholesterol are then complexed with apoB-48, apoA-I, apoA-II and apoA-IV within the intestinal wall. The chylomicrons enter the sytemic circulation via the lymphatic circulation. Apo-E and apo-C are added outside of the intestine.

As the chylomicrons circulate in the blood, the triglycerides are hydrolyzed by the action of lipoprotein lipase on endothelial surfaces, which results in the production of cholesteryl ester-rich chylomicron remnants. These remnants are recognized by a hepatic receptor specific for apo-E[66,67], which does not seem to be down regulated as chylomicron remnants are taken up[68]. Overall, the two step process delivers dietary triglyceride to adipose tissue and muscle and dietary cholesterol to the liver.

The presence of chylomicrons in fasting plasma is abnormal and may indicate a type of hyperlipoproteinemia. While chylomicronemia per se is not thought to result in premature CHD, the accumulation of remnant particles through prolonged clearance is damaging to the vascular endothelium and is thus thought to predispose to accelerated

atherosclerosis[69].

3. Very Low Density Lipoproteins (VLDL). Once the chylomicron remnant is taken up by the hepatocyte, its cholesteryl esters are metabolized to cholesterol, which together with triglycerides, are packaged for secretion into the circulation within VLDL. They contain triglyceride as the predominant lipid, and function to transport cholesterol and endogenously produced triglyceride from the liver to their sites of utilization. In this way, the metabolism of dietary fat and endogenously synthesized fat is coordinated to supply needed amounts of fuel and cholesterol to body tissues despite fluctuations in dietary intake. VLDL contains two apolipoproteins which can be recognized by the LDL receptor, apoB-100 and apoE, in addition to the lipoprotein lipase regulatory apolipoprotein apoC.

A distinct subclass of VLDL called beta-VLDL has been shown to accumulate in type III hyperlipoproteinemia (characterized by an abnormal apoE) and in animals and humans fed high fat and cholesterol diets[70,71]. The beta-VLDL differ from VLDL in that the former are much higher in cholesterol. The presence of beta-VLDL in plasma has been linked with the development of accelerated atherosclerosis (for review see Mahley and Innerarity[68], Mahley[70], and Mahley et al[71]).

4. Intermediate Density Lipoproteins (IDL). IDL are formed from the metabolism of VLDL triglyceride by lipoprotein lipase in the peripheral tissues. After their formation from VLDL approximately one half of the IDL is cleared within two to six hours by the high affinity apoE receptor on the liver[72]. The IDL not removed by this mechanism remains in the circulation for a prolonged period where the last traces of triglyceride are

hydrolyzed and the apoE and apoC moieties are transferred to HDL, leaving an LDL particle.

5. Low Density Lipoproteins (LDL). LDL are the major cholesterol carrying components of plasma. ApoB-100 is virtually the only apolipoprotein present in LDL. LDL are mainly formed from the breakdown of VLDL, but may be synthesized directly[73]. LDL are cleared from the circulation much more slowly than VLDL or IDL, the half life of LDL in the circulation being approximately sixty hours[72].

LDL is metabolized in liver and in extrahepatic tissues by at least two pathways. One pathway involves specific LDL (apoB/E) receptors first described in cultured fibroblasts by Goldstein and Brown[74], receptors which are located on the surface of hepatic and extrahepatic cells. The receptor recognizes the apoB-100 component of LDL. Binding leads to the uptake of LDL through receptor mediated endocytosis. LDL proceeds through a pathway of endocytic vesicles to lysosomes where the LDL are hydrolyzed, liberating amino acids from apoB and free cholesterol from cholesteryl esters. The free cholesterol migrates into the cytoplasm where it elicits three intracellular regulatory responses that maintain cholesterol homeostasis and protect the cells from the overaccumulation of cholesterol. First, LDL derived sterols suppress the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-controlling enzyme in cholesterol biosynthesis, thereby turning off cellular cholesterol biosynthesis[75]. Second, the cholesterol activates a cholesterol-esterifying enzyme called acyl-CoA:cholesterol acyl transferase (ACAT) which allows the cells to store excess cholesterol in the form of cholesteryl esters[76]. Third, the synthesis of new LDL receptors

is suppressed (down regulation of expression), preventing further cellular entry of LDL and thus cholesterol or cholesteryl ester overloading[77]. This LDL-receptor based regulatory system allows the coordination of intra- and extracellular sources of cholesterol, which all cells require for synthesis of new plasma membranes and which specialized cells need for the synthesis of steroid hormones and bile acids.

In humans, the LDL receptor mediates the degradation of about two thirds of the LDL particles that are metabolized each day[78]. The remainder of LDL is metabolized by LDL receptor independent pathways. Some of this degradation is believed to occur in macrophages and cells of the reticuloendothelial system, which degrade all plasma proteins. Brown and Goldstein have referred to these pathways collectively as "the scavenger pathway"[79]. It is likely that several pathways and mechanisms are involved, some receptor dependent and some nonspecific.

Two lines of evidence strongly suggest that the arterial uptake of LDL, giving rise to foam cells and fatty streaks, are independent of the LDL receptor. First, lesions rich in macrophage foam cells develop even in patients and animals deficient in LDL receptors (homozygous patients with familial hypercholesterolemia and Watanabe heritable hyperlipidemic rabbits)[79-81]. Second, normal monocytes and monocyte-derived macrophages in culture cannot be converted to foam cells by incubation with even high concentrations of native LDL[79].

Goldstein and his coworkers[82] were the first to descibe a modified form of LDL that could be taken up rapidly enough by macrophages to convert them into foam cells. They found that chemical acetylation converted LDL to a form recognized by a saturable

specific receptor on the macrophage which could not recognize native LDL. They designated this receptor as the "acetyl LDL receptor" or the "scavenger receptor". Subsequently, it was found that oxidatively modified LDL is also recognized by the acetyl LDL receptor[83,84] and recent evidence suggests that oxidative modification may play a significant role in atherogenesis in vivo[85-87].

In vitro observations show that three major cells in the arterial wall; endothelial cells[83,88], smooth muscle cells[88-90] and macrophages[84,91,92] are able to modify LDL to a scavenger receptor recognizable form. These cells are able to generate active oxygen species, which can induce the peroxidation of polyunsaturated fatty acids in the LDL lipids[93]. A rapid reaction sequence follows fatty acid lipid peroxidation, that amplifies the number of free radicals and leads to fragmentation of the fatty acid chains. These fragments have been shown to attach covalently to apoprotein B, masking the epsilon-amino group[94], which is recognized by the scavenger receptor. It should be noted that LDL cholesterol is also oxidized during LDL modification, which has been postulated to enhance its atherogenicity[95].

In vitro studies have suggested four mechanisms by which oxidatively modified LDL might contribute to the atherogenic process. First, oxidatively modified LDL is taken up via scavenger receptors which have been shown to be present on macrophages[82-84], endothelial cells[96,97], Kupffer cells[97] and have been postulated to be present on smooth muscle cells[66], which could promote foam cell formation.

The second way oxidatively modified LDL may be atherogenic is by being chemotactic for monocytes. Recent studies[98] have shown that oxidatively modified LDL,

but not native LDL, is a potent chemoattractant for circulating human monocytes, but not for neutrophils. As one of the earliest events observed in experimental atherosclerosis is the binding of monocytes to the arterial endothelium[37,39-41], oxidized LDL may serve to recruit monocytes into the subendothelial space. Neutrophils are very rarely seen in early atherosclerotic lesions. Quinn et al have shown that most of the chemotactic activity of oxidatively modified LDL resided in the lipid component, and much of it was attributable to the lysolecithin generated during the conversion of LDL to its oxidized form[99].

Not only is oxidatively modified LDL chemotactic for the circulating monocyte, but it has also been shown to be a potent inhibitor of the motility of the macrophage[98]. Both basal motility and the increase in motility in response to chemoattractant stimuli have been shown to be strongly inhibited by the oxidized LDL[98]. Thus, the third way in which modified LDL may contribute to atherogenesis is by retaining the macrophage in the arterial wall after its phenotypic conversion from a monocyte.

A fourth means in which oxidatively modified LDL may be atherogenic is by way of its cytotoxicity. Oxidized LDL, in particular the oxidized derivatives of cholesterol, have been shown to be cytotoxic to a variety of cells including endothelium[100,101] and smooth muscle cells[101-103]. Thus, oxidized LDL/cholesterol may contribute to the loss of endothelial cells that has been observed on the surface of established fatty streaks[37]. It has been hypothesized that the cytotoxicity of oxidized LDL may be sufficient to lead to epithelial denudation which would promote further infiltration of lipoproteins, circulating monocytes and platelets, setting the stage for the evolution of the advanced

atherosclerotic plaque[93].

Critical to the importance of oxidized LDL in the atherogenic process is the demonstration of the presence of these modified derivatives *in vivo*, in particular associated with atherosclerotic lesions. Several lines of evidence indicate that oxidized LDL is generated *in vivo*. First, antibodies against *in vitro* oxidized LDL have been shown to recognize material in rabbit aortic atherosclerotic lesions, but not in the normal areas of the rabbit aorta[87]. Second, LDL that is eluted from atherosclerotic plaques from humans and rabbits show cross reactivity with antibodies produced to exogenously oxidized LDL[87,105]. Third, the plasma of Watanabe Heritable Hyperlipidemic rabbits and that of human subjects has been shown to contain autoantibodies that react with various forms of oxidized LDL[87]. Fourth, atherosclerotic lesion LDL showed many of the chemical and physical properties of oxidized LDL[105,106] and lesion LDL (but not normal intimal LDL or plasma LDL) was shown to be chemotactic for monocytes[105], as was oxidized LDL[98]. Finally, oxidated LDL has been isolated from human plasma[107].

6. <u>High Density Lipoproteins (HDL)</u>. These lipoproteins are produced by the liver and the gut and by the peripheral catabolism of chylomicrons and VLDL. They contain by weight approximately 30% cholesterol, 45% protein, 25% phospholipid (predominantly phosphatidylcholine) and a small amount of triglyceride. HDL may be subdivided into several subfractions, $HDL_{1,2,3}$, according to their behavior in the ultracentrifuge. HDL_1 is a minor component and of uncertain physiologic significance. HDL_2 consists of larger particles that are more lipid rich and of lower density. HDL_3 particles are smaller and more protein rich, lipid poor, and denser. There is a very

strong negative correlation between HDL cholesterol and CHD. Some studies suggest that HDL_2 may correlate with the presence of disease better than does HDL_3[108].

The most attractive hypothesis concerning the protective mechanism of HDL is the reverse cholesterol transport hypothesis advanced by Glomset[109]. This hypothesis stemmed from the observation that Tangier disease, which is an inherited deficiency of HDL, is associated with a very low serum cholesterol level and generalized deposition of cholesterol esters in tissues. The reverse cholesterol transport hypothesis proposes that HDL facilitates the removal of cholesterol from tissues, particularly the cells of the reticuloendothelial system by a receptor mediated "reverse endocytosis" process with subsequent delivery to the liver for excretion. There is evidence for the existence of an HDL receptor which seems to interact with apoA-I or apoA-II[110] facilitating the removal of cholesterol from peripheral tissues. Several studies have shown even greater complexity of HDL subclasses exists when the lipoprotein is examined using newer methods. About 5% of the HDL population has been shown to contain apoA-I only[111,112]. This subfraction, which itself is heterogenous, has been suggested to be particularly important in reverse cholesterol transport because it contains most of two proteins believed to be involved in that process; lecithin:cholesterol acyltransferase (LCAT) and the cholesterol ester transport protein[113]. The apoA-I only subfraction is also the most active fraction in vitro in transferring free cholesterol from the surface of cultured cells to plasma[113]. Unfortunately, all of the evidence for the reverse endocytosis hypothesis has come from in vitro studies and direct in vivo quantification and demonstration of reverse cholesterol transport is still lacking.

There are alternative hypotheses on the inverse relationship between levels of HDL and CHD. HDL has been shown to inhibit the binding of LDL to matrix connective tissue[114], subfractions of HDL with apoE can compete with LDL for uptake by way of the LDL receptor[68], HDL inhibits the oxidation of LDL which may protect against its cytotoxic action[90], and HDL inhibits the receptor mediated uptake of oxidized LDL[115]. Finally, the possibility exists that HDL is itself not directly involved in protecting against atherogenesis. Instead, a high HDL level may only be a marker for some metabolic process that somehow interferes with atherogenesis and only incidently leads to a rise in plasma HDL levels.

7. Lipoprotein a. A unique lipoprotein given the label lipoprotein a (lp(a)) was first identified in 1963 and is found mainly in the density range of HDL[116]. It is a relatively minor lipoprotein containing less than 15% of the plasma cholesterol but is ubiquitous in human plasma[117]. Although there are no published prospective studies of lp(a) as an atherogenesis risk factor, numerous retrospective epidemiologic studies have shown that plasma lp(a) levels seem directly related to cardiovascular risk[118-120]. The range of levels, given in units of g/L, is from near zero to more than 1.0 g/L. A threshold level of 0.2 g/L seems to be directly related to atherosclerosis risk[121]. Furthermore, elevated lp(a) levels seem to be an independent risk factor for myocardial infarction unrelated to other differences in cholesterol, lipoprotein subfractions or non-lipid risk factor[79].

Lp(a) is very similar to LDL except that it contains apolipoprotein (a) (apo(a))[122]. Apo(a) is a heterogenous protein which forms disulfide bonds with the apoB-100 apolipoprotein of the LDL molecule[122]. Apo(a) has been shown to exhibit significant

structural similarity to plasminogen. Plasminogen is the precursor of plasmin, a naturally occuring anticoagulant, and can avidly bind fibrin; as can apo(a). One of the theories of the atherogenic nature of lp(a) is that it is this fibrin binding ability which brings the cholesterol laden lp(a) directly to the site of intravascular damage where fibrin has been deposited. Lp(a) with both its cholesterol and protein components, might then be taken up by the macrophages at the site of the intravascular lesion.

Another possible effect of the lp(a) on atherosclerosis may result from the homology between apo(a) and plasminogen. Apo(a) has no intrinsic fibrinolytic activity and cannot be activated by tPA (tissue plasminogen activator). Lp(a) has been shown to compete with plasminogen for the plasminogen binding site on vascular endothelial cells with an equivalent affinity and capacity, but with no effect on circulating plasmin[123-125]. It has been estimated that at plasma concentrations of 0.3 g/L lp(a) reduces cellular plasminogen binding by 20%, thereby supressing endothelial cell fibrinolysis and producing a procoagulant state[123]. In addition, there has been shown a striking accumulation of lp(a) on the endothelium of atherosclerotic coronary arteries of humans, but not in normal blood vessels[124]. Thus, the atherogenic potential of lp(a) may be based on two putative mechanisms; a potential role in plaque formation and a potential role in thrombogenesis.

e) Calcium and Atherosclerosis

Arterial calcification has long been recognized as a universal feature of the atherosclerotic plaque[30]. Many reports have documented an increased calcium content of human atherosclerotic arteries[126] and arteries from hypercholesterolemic experimental

animals[127,128]. Although the calcified deposits in coronary atherosclerosis have been shown to be primarily intimal, medial calcinosis has been reported by several investigators at necropsy of individuals with hypercholesterolemia and concommitant hypercalcemia[129,130]. The increased tissue calcium seen in atherosclerotic lesions of experimental animals has been shown to be a result of, in part, increased cellular calcium uptake[131,132]. Kinetic analysis of altered calcium transport in atherosclerotic rabbit aorta suggested an increase in total intracellular calcium content, consistent with a 4.8-fold elevation in calcium permeability when compared with controls[131]. Enrichment of membrane cholesterol of human erythrocyte has also been shown to increase Ca^{2+} influx[133]. The observed increase in intracellular calcium supports the hypothesis, advanced by several groups of investigators, that atherogenesis is dependent upon increased cytosolic calcium[129,130,134,135].

Two lines of evidence support the hypothesis that increased intracellular calcium is atherogenic. First, it has been demonstrated numerous times that componds which prevent extracellular to intracellular calcium transport can ameliorate or prevent atherosclerosis (for review see Jackson et al[136]). These agents have been effective without reducing arterial blood pressure or blood lipids, and compounds from each of the three major classes of organic calcium channel blockers has been shown to be effective. Second, Phair[137] has reported a 2.1-fold increase in the cholesteryl ester content of thoracic aortic tissue of rabbit that had been artificially loaded with calcium using the calcium ionophore A23187. This preliminary study illustrates that at least one of the many cellular events associated with atherogenesis can be initiated by artificially

increasing cellular calcium.

The precise mechanism whereby cellular calcium participates in atherosclerosis is presently unclear. Regardless of the ntaure of the underlying risk factor, the resulting atherosclerotic lesions all progress in a similar manner. This has led to the suggestion that regardless of the initiating stimulus, atheroma formation is mediated by a single common factor. Ca^{2+} has been implicated as that final common mediator[131].

Increased smooth muscle cell membrane cholesterol has been suggested to cause the intracellular calcium overload seen in tissue from atherosclerotic lesions[137]. There is considerable evidence that the function of integral membrane transport proteins can be regulated by their lipid environment[138]. Cholesterol modulation of membrane ion transport systems has been studied in detail on two ion pumps in particular: Na^+,K^+-ATPase and Ca^{2+}-ATPase.

i] *Na^+,K^+-ATPase*

The Na^+,K^+-ATPase of plasma membranes is an electrogenic enzyme responsible for pumping sodium out of the cell and potassium into the cell against their respective concentration gradients. Inhibition of the ouabain-sensitve ATP hydrolyzing activity (Na^+,K^+-ATPase) by high cholesterol levels (above those found in native membranes) was observed in reconstituted enzyme of human erythrocytes[139], human erythrocyte membranes[140,141], rabbit erythrocyte membranes[142], guinea pig erythrocyte membranes[143], rat liver membranes[144], and kidney basolateral membranes[145]. It has been speculated that the inhibition results from the physical effects of cholesterol on membrane properties[138]. Cholesterol has been shown to increase the anisotropic motional ordering of the bilayer

as a result of the effects of its rigid sterol structure on the lipid components of the membrane[146]. Cholesterol also exhibits a preferential ordered distribution within a membrane characterized by "lines" that are arranged in a rhombic pattern[147]. This general increase in ordering may also lead to constraints in the conformation of the Na^+,K^+-ATPase[138], which may inhibit its function.

When low levels of cholesterol are present in the membrane, cholesterol has been shown to stimulate the Na^+,K^+-ATPase enzyme[145]. Since other sterols did not show similar stimulation, it has been suggested that the stimulatory effect was a result of a direct site specific sterol-protein interaction[145].

ii] *Ca^{2+}-Mg^{2+}-ATPase*

This enzyme has been observed to optimally pump two calcium ions out of the cell per ATP hydrolyzed and can maintain transmembrane calcium gradients of several orders of magnitude in ion concentration. Increasing the cholesterol content of reconstituted membranes has been shown to depress the activity of the Ca^{2+}-Mg^{2+}-ATPase (Ca^{2+} pump) of skeletal muscle sarcoplasmic reticulum[148,149]. Several investigators have proposed the presence of a cholesterol free, phospholipid annulus surrounding the ATPase protein which is essential for full enzyme activity[148,149]. They suggested that the inhibition of enzyme activity that they observed when cholesterol was included in the annulus was a result of the motion-restricted environment produced by cholesterol. Similarly, the Ca^{2+}-Mg^{2+}-ATPase of isolated rabbit cardiac sarcolemma was shown to be depressed with cholesterol enrichment[150]. In reconstitution experiments in which phosphatidylethanolamine was used as a dominant lipid component in the

membrane, cholesterol appeared to stimulate the Ca^{2+} pump[151]. Thus, under special circumstances, cholesterol appears capable of stimulating the function of the calcium pump protein.

f) Coronary Artery Spasm

Coronary artery spasm can be defined as a transient reduction in lumen diameter of an epicardial (or large septal) coronary artery. This abnormal constriction of the conductive arteries is of sufficient degree to produce myocardial ischemia in the absence of any significant increases in heart rate or blood pressure. The ischemia is a transient phenomenon and is promptly reversed by the administration of nitroglycerin.

Coronary spasm had been proposed as an important mechanism of myocardial ischemia over 140 years ago, and the concept remained popular through the early part of this century (for review see Glazier et al[152]). In the 1940's, particularily as a result of a strong rebuttal by Blumgart[153], the coronary artery spasm hypothesis fell into disrepute. Many criticized the theory because there was no direct way to visualize coronary arteries *in vivo* and also because some believed the thickened, fibrotic coronary arteries found at necropsy in nearly all patients with angina were too rigid to have undergone spasm.

The concept of coronary artery spasm began to regain favour once again with the description in 1959 by Prinzmetal and his collegues[154] of variant angina. They postulated that the mechanism of chest pain in patients with this finding was due to coronary artery spasm associated with fixed coronary obstructive disease. Theirs was the first description of angina occurring at rest associated with ST-segment elevation on electrocardiogram,

often associated with arrythmias, and without an obvious increase in myocardial oxygen demand. They proposed that in these patients, the mechanism of ischemia was an increase in coronary vasomotor tone around an eccentric lesion. Concommitant with the observations of Prinzmetal and his colleagues came the development of coronary arteriography. In 1962 the first of many reports documenting coronary artery spasm during coronary arteriography of a patient experiencing angina was published[155].

Coronary artery luminal narrowing is now recognized to be associated with angina pectoris, acute myocardial infarction and sudden death[156]. In addition, when coronary artery spasm occurs, it usually does so at the site of an atherosclerotic but not necessarily stenotic lesion[157]. Several mechanisms have been proposed for the change in smooth muscle tone as seen with coronary artery spasm.

i] *Mechanical injury mechanism*

Focal coronary artery atherosclerosis has been produced in animals subjected to balloon catheter induced endothelial denudation and hypercholesterolemia[158,159]. After development of these lesions, provocative testing with histamine produced localized coronary artery spasm[158], while ergonovine and serotonin produced exaggerated constrictor responses[159]. Thus it seems plausible that, *in vivo*, endothelial injury leads to increased constrictor response of the vascular smooth muscle to vasoactive mediators. The possibility that endothelial injury from causes other than mechanical denudation can result in increased coronary artery smooth muscle sensitivity has not been addressed.

ii] *Endothelial dependent vasoactive mediator mechanism*

Since Furchgott and Zawadzki[160] demonstrated the obligatory role of endothelial

cells in the relaxation of arterial smooth muscle by acetylcholine, numerous findings have indicated that this cell layer modulates the degree of contraction of the underlying vascular smooth muscle[161-163]. It does so by releasing vasoactive substances such as prostacyclin, endothelium-derived relaxing factor (EDRF)[161], endothelium-derived hyperpolarizing factor (EDHF)[164], and endothelium-derived contracting factor (EDCF)[165]. EDRF has recieved much attention, and changes in the release of EDRF are thought to be involved in the pathogenesis of coronary artery spasm[166].

Among the physiologic stimuli that can elicit the release of EDRF are platelet products, thrombin, hormones, neurotransmitters, local autacoids, shear stress and changes in oxygen tension[161-163]. EDRF has been shown to cause relaxation of the underlying smooth muscle by the activation of soluble guanylate cyclase leading to cyclic GMP-dependent protein phosphorylation[167]. Elevation of cyclic GMP evoked by EDRF inhibits Ca^{2+} release from intracellular storage sites and Ca^{2+} influx through receptor-operated channels[168]. Since contraction and tone of vascular smooth muscle depends on an increase in free intracellular calcium available to the contractile proteins (*vide infra*), EDRF may effectively inhibit vascular smooth muscle contraction through its effects on intracellular Ca^{2+} concentrations.

It has been observed that the activity of EDRF appears to be diminished or lost as a consequence of atherosclerosis, and stimuli that cause vasodilation via the EDRF pathway in normal vessels seem to cause vasoconstriction in atherosclerotic arteries[166,169]. It addition chronic hypercholesterolemia causes a reduction in the production of EDRF in endothelial tissue[170]. Oxidized LDL has also been observed to enhance agonist-induced

vasoconstriction by a direct effect on vascular smooth muscle[171]. It is speculated that this occurs through modulation of voltage-gated Ca^{2+} channels through stimulation of phosphatidylinositol metabolism. Thus it is apparent that cholesterol and Ca^{2+} regulation by vascular smooth muscle cells are interrelated, and involved in the pathogenesis of both coronary artery spasm and atherosclerosis. These studies lend credence to the hypothesis that both atherosclerosis and coronary artery spasm are consequences of a single disease process[172,173].

II. Excitation-Contraction Coupling in Heart and Smooth Muscle

Since the pioneering work Ringer[174] on the dependence of cardiac contraction on the presence of calcium ions (Ca^{2+}), Ca^{2+} has been recognized as having a fundamental role in the contraction of both cardiac and vascular smooth muscle as well as in the coupling of the excitatory event of membrane depolarization to the mechanical event of muscle shortening (excitation-contraction coupling). It is the level of intracellular calcium which is now recognized to play the central role in the contraction-relaxation cycles of muscle tissue; however, important functional interactions of Ca^{2+} with other vital cations such as sodium, potassium and magnesium are involved as well. Fundamental differences are known to exist between cardiac and vascular smooth muscle in the excitation-contraction coupling process.

a) Excitation-Contraction (E-C) Coupling in Cardiac Muscle

E-C coupling in the heart can be separated into four steps. First an action potential depolarizes the sarcolemma. Next, the depolarization releases Ca^{2+} from the

subsarcolemmal cisternae of the sarcoplasmic reticulum (SR) and/or allows entry of extracellular calcium. Third, Ca^{2+} binds to the troponin-C protein on the thin filament of the myofibrils, and by a complex sequence of events, permits actin and myosin to interact. Fourth, relaxation occurs when the intracellular Ca^{2+} concentration falls as a result of SR reaccumulation and extrusion of Ca^{2+} through the sarcolemma causing the dissociation of Ca^{2+} from troponin-C.

i] *Depolarization of the myocardial cell*

Depolarization of the myocardial cell leads to a rapid and brief period of inward movement of Na^+ ion through tetrodotoxin sensitive sodium channels into the sarcoplasm from extracellular sources (phase 0). After a brief delay from the onset of membrane depolarization, there is a slower and more prolonged inward movement of Ca^{2+} ion through voltage dependent slow Ca^{2+} channels or via a Na^+-Ca^{2+} exchange mechanism (phase 0,1 and 2)[175].

The Ca^{2+} current through the voltage sensitive membrane channels (I_{Ca}) is composed of an initial fast component, followed by a slow component[176]. In mammalian cardiac cells, which have an extensive SR, most of the inflowing Ca^{2+} seems to be rapidly taken up by SR Ca^{2+} stores. From there it can be released during subsequent depolarizations into the cytoplasm as activator Ca^{2+} for the contractile proteins[177]. Therefore, the amount of Ca^{2+} entering the cell during an action potential determines the degree of filling of SR stores and hence the contractile state of the heart. The fast component of the I_{Ca} triggers a tension transient mediated by Ca^{2+}-induced release of Ca^{2+} from the SR[176]. The slow component does not affect the tension transient caused

by the fast component, but does potentiate subsequent tension transients[176]. Thus the fast initial component of I_{Ca} triggers release of Ca^{2+}, whereas the slow component loads the SR with Ca^{2+} that becomes available for release during subsequent beats.

The Ca^{2+} channels are gated by the electric potential across the sarcolemma membrane. Upon depolarization of the membrane, inward Ca^{2+} currents become apparent at potentials around -60 mV and reach a maximum around 0 mV[178]. At least two types of Ca^{2+} channels have been identified in mammalian sarcolemma, the T (transient) channel and the L (long) channel[178]. Ca^{2+} conductance is low in the T channel (as a result of their rapid inactivation) and high in the L channel (which are more slowly inactivated)[179]. A significant difference between T and L channels is in their sensitivity to blockade: only nickel ion can inhibit Ca^{2+} conductance through T channels, while L channels are sensitve to blockade by the organic calcium channel blockers (diltiazem, verapamil and nifedipine) and to cadmium[179,180].

The cardiac cell membrane contains a number of receptors that, on activation with an appropriate neurotransmitter (catecholamines, acetylcholine, histamine, serotonin, adenosine, and vasopressin), can modulate the function of the voltage dependent Ca^{2+} channel. The current evidence suggests that phosphorylation through the cyclic AMP-dependent protein kinase enhances the entry of Ca^{2+} through the voltage dependent channels[181]. Increases in cyclic AMP produce a rapid increase in the number of slow channels available for voltage activation during membrane depolarization, increase the probability of a slow channel opening at a given voltage and increase the mean time during which the channel remains in an open state[182]. These changes in channel function

are presumed to be associated with phosphorylation of a specific protein site in the slow channel, which leads to a conformational change favouring the passage of Ca^{2+} through the water filled central pore of the channel[182]. Guanine nucleotide-binding proteins may have a role in activating and inactivating these Ca^{2+} channels[183,184].

ii] *Sarcoplasmic reticulum Ca^{2+} release and Na^{+}-Ca^{2+} exchange*

The cardiac cell SR, is a tubular, lipid bilayer membranous structure within the cell which is the analogue of the endoplasmic reticulum in noncontracting cells. The main functions of the SR are to release stored Ca^{2+} to provide the signal for the activation of the contractile elements, and to reaccumulate Ca^{2+} to afford relaxation of the muscle. The SR calcium pump and the Ca^{2+} release channel are two primary protein components of the SR which give rise to the necessary Ca^{2+} exchange. The Ca^{2+} pump is responsible for accumulation of Ca^{2+} to effect relaxation, whereas the Ca^{2+} channel is responsible for the rapid release of Ca^{2+} to provide for contraction.

Several mechanisms operate in cardiac muscle cells to stimulate the release of Ca^{2+} from the SR. It is thought that the most physiologically important mechanism operating in cardiac muscle is Ca^{2+}-induced release of Ca^{2+}. The hypothesis of Ca^{2+}-induced release of Ca^{2+} is that the increase in cytosolic free Ca^{2+} resulting from the transsarcolemmal influx of Ca^{2+} is insufficient to activate the myofilaments directly, but induces a release of Ca^{2+} from the SR which activates the myofilaments. Experiments on skinned cardiac cells suggest that the activation of Ca^{2+} induced release of Ca^{2+} is both Ca^{2+} dependent and time dependent[185]. Thus, a fast increase of free Ca^{2+} will trigger a release of Ca^{2+}, whereas a slower increase to the same final level will load the

SR with an amount of Ca^{2+} that will be available for release during subsequent contractions.

Stimulation of some cell surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers; diacylglycerol (DAG) and inositol 1,4,5-triphosphate [Ins(1,4,5)P_3][186]. These messengers are generated by a membrane transduction process composed of three main components; a receptor, a coupling G protein and phosphoinositidase C. Hirata et al[187], were the first to show that Ins(1,4,5)P_3 can induce Ca^{2+} release from isolated cardiac SR. The amount of Ca^{2+} release induced by Ins(1,4,5)P_3 was only a small fraction of the total intravesicular Ca^{2+}. These results were not reproducible by others[188], and results from studies on isolated skinned myocytes[188] and skinned multicellular muscle preparations[189] have not been able to demonstrate Ca^{2+} release by Ins(1,4,5)P_3. It is accepted, however, that Ins(1,4,5)P_3 can potentiate the caffeine induced release of Ca^{2+} by the SR[189-191]. Although a primary role for Ins(1,4,5)P_3 in E-C coupling seems unlikely, it has been postulated to have a physiological function in receptor mediated cardiac inotropism[192].

The relative importance of SR sequestered Ca^{2+} in E-C coupling in the cardiac myocyte has been the subject of intense investigation (for review see Feher and Fabiato[193]). According to one hypothesis, the SR is the sole source of activator Ca^{2+}, and all of this Ca^{2+} is reaccumulated into the SR[185,194]. An alternative postulate is that some of the Ca^{2+} which activates the myofibrils is derived from transsarcolemmal influx[195-197]. Until recently, most of the studies on the mechanisms of E-C coupling in skeletal and cardiac muscles have been performed on preparations in which the

sarcolemma has been made permeable to Ca^{2+} by chemical treatment[198,199]; the skinned fibre preparation. This preparation allowed control of the chemical environment surrounding the fibre by varying the bathing solution. With the advent of new experimental approaches for the study of SR released Ca^{2+} and refinements in the preparations of isolated cardiac myocytes, the relative importance of the SR in E-C coupling in cardiac myocytes has come under question.

It is accepted that most of the Ca^{2+} crossing the sarcolemma, whether it be for triggering Ca^{2+} release by the SR, sequestration by the SR, or direct activation of the myofilaments, traverses the membrane via Ca^{2+} channels. There exists another pathway that has been postulated to contribute to transsarcolemmal Ca^{2+} influx, namely the sarcolemmal Na^{+}-Ca^{2+} exchanger. The Na^{+}-Ca^{2+} exchange system was first described by Reuter and Seitz[200] more than 20 years ago, and subsequently identified in isolated cardiac sarcolemmal membranes[201]. Since its first description, much effort has been expended in attempts to isolate the exchange protein. Using a novel isolation technique, Barzilia et al have achieved the isolation of a 70 kD protein from brain plasma membrane which they have identified as the exchanger[202,203]. In addition, the Na^{+}-Ca^{2+} exchanger from Bovine rod outer segments has been isolated by two separate groups[204,205], and found to be a glycoprotein with a M_r of about 215 kD. Recently, Nicoll et al[206] have reported the molecular cloning, expression and deduced amino acid sequence of a 108 kD protein from canine cardiac sarcolemma that they have identified as the Na^{+}-Ca^{2+} exchange protein.

The cardiac Na^{+}-Ca^{2+} exchange protein has been extensively investigated using

biochemical, patch clamp and Ca^{2+}-sensitive dye techniques. It is known that the stoichiometry of Na^+-Ca^{2+} exchange in the heart is three Na^+ for each Ca^{2+} transported[207-211], making it electrogenic and sensitive to membrane potential[212-215].

Many kinetic studies have been published reporting the $K_M(Ca^{2+})$ of $[Na^+]_i$-dependent Ca^{2+} uptake measured in various cardiac sarcolemmal vesicle preparations[173,201,213,217,223,228,232,316,322,356,377]. Despite the varied preparation techniques and heterogeneity of the vesicle preparations (inside out vs right side out), most values fall between 15 and 40 μM. Unfortunately, the affinity of the exchanger for external Ca^{2+} $\{K_M(Ca_o^{2+})\}$ reported for cultured myocytes does not correlate well with those obtained using isolated sarcolemma. Values for the $K_M(Ca_o^{2+})$ have been reported as 150 μM[217] and 350 μM[218]. More difficult to measure is the affinity of the exchanger for internal calcium $K_M(Ca_i^{2+})$ in intact cells. Using isolated internally perfused pig ventricular cells, Miura et al[219] have reported a $K_M(Ca_i^{2+})$ of 0.6 μM. This finding suggests that an assymetry may exist in the Ca^{2+} binding sites of the cardiac Na^+-Ca^{2+} exchanger *in situ*. Inclusion of the ion chelator EGTA in the perfusion medium of the study of Miura et al[219] may have confounded their results as EGTA has been shown to increase the apparent affinity of the exchanger several fold[220]. In contrast, the external Na^+ dependence of the Na^+-Ca^{2+} exchanger has been measured by several groups and found to be reproducibly between 12 and 30 μM in both cardiac sarcolemmal vesicles[221-224] and isolated myocytes with similar values measured for $K_M(Na_i^+)$[209,219].

The regulatory control of the Na^+-Ca^{2+} exchanger has been the subject of much interest. It appears that the intacellular surface of the exchanger has a Ca^{2+} binding site

that modulates Na^+-Ca^{2+} exchange activity. Internal Ca^{2+} regulatory sites have been described in cardiac cells[219,225,226], with a $K_M(Ca)$ of near 50 nM[219,226]. Intracellular Ca^{2+} increases the apparent affinity of the exchanger for Na^+ and increases the maximal rate of exchange[216]. The physiological significance of the internal Ca^{2+} regulatory site is still unclear, but it is hypothesized to prevent the Na^+-Ca^{2+} exchanger from extruding too much Ca^{2+} from the cell[227]. It has been suggested that as the exchanger lowers Ca^{2+} below 100nM, the regulatory site loses bound Ca^{2+}, Na^+-Ca^{2+} is inhibited, and further Ca^{2+} efflux stops[227].

Phosphorylation/dephosphorylation reactions have been shown to regulate the Na^+-Ca^{2+} exchanger in cardiac sarcolemmal vesicles[228]. The reactions can be catalyzed by a kinase and a phosphatase endogenous to the sarcolemmal membrane. Both the kinase and the phosphatase require Ca^{2+} and calmodulin. To date no cAMP-dependent regulation has been found. Phosphorylated sarcolemmal vesicles have been shown to have both a higher Ca^{2+} affinity and a V_{max} than dephosphorylated vesicles[230]. It has been speculated that the intracellular Ca^{2+} concentration would determine the phosphorylation state *in vivo*[228], although the physiologic significance of this regulatory mechanism has not yet been determined.

Na^+-Ca^{2+} exchange activity has been shown to be strongly pH dependent, with activity being inhibited at low pH and stimulated at high values[217,229-233]. Ca uptake has been shown to be strikingly inhibited at pH 6 and stimulated at pH 9 which has led to the suggestion that the ionization of a histidine residue may be of importance in Na^+-Ca^{2+} exchange[234]. The mechanism appears to be through competition between H^+ ions

and Ca^{2+} for binding sites on the exchange protein, the effects of pH being much more noticeable at low Ca^{2+} levels than at high levels[229]. Thus, in the intact myocardium, a change in extracellular pH would have little effect on Na^{+}-Ca^{2+} exchange due to the high external Ca^{2+} concentration. In contrast, a fall in intracellular pH could drastically inhibit Na^{+}-Ca^{2+} exchange due to the effective competition of increased proton concentration with the relatively low intracellular Ca^{2+}. Since the cellular pH can change in many pathophysiological situations, effects of pH on Na^{+}-Ca^{2+} may have significant clinical relevance.

A variety of oxidation/reduction reactions have been shown to stimulate the Na^{+}-Ca^{2+} exchanger of cardiac sarcolemmal vesicles[235]. It is suggested that redox modulation of exchange activity is through thiol-disulfide interchange, a process which is thought to alter the conformation of the exchange carrier from a less active to a more active form. It is speculated that this mechanism may protect the myocyte from oxidative stresses, activating Na^{+}-Ca^{2+} exchange and protecting the cells against Ca^{2+} overload[235].

The relative contribution of the Na^{+}-Ca^{2+} exchanger to calcium influx during the cardiac contraction cycle is an area of active investigation. Although sarcolemmal Ca^{2+} channels are thought to provide the principal source of trigger Ca^{2+} for the release of Ca^{2+} from the SR as well as providing Ca^{2+} for the loading of the SR[236], recent studies have supported the hypothesis that the Na^{+}-Ca^{2+} exchanger may contribute to Ca^{2+} entry into cardiac cells and trigger sarcoplasmic calcium release. Using voltage-clamped, isolated cardiac myocytes Leblanc and Hume[237] have shown that in the absence of calcium entry through voltage-dependent calcium channels, membrane depolarization

elicited release of Ca^{2+} from the sarcoplasmic reticulum, supporting a role for the Na^{+}-Ca^{2+} exchanger as provider of trigger Ca^{2+}. It has also been shown that the Na^{+}-Ca^{2+} exchanger is a high capacity system that under certain conditions can supply Ca^{2+} for activation of the contractile apparatus. Bers et al[238] have shown that in the presence of nifedipine to block Ca^{2+} channels, caffeine or ryanodine to inhibit SR function and acetylstrophanthin to raise intracellular Na^{+}, rabbit ventricular muscle demonstrated substantial contractile activity. With the recent identification of a potent inhibitor of the cardiac sarcolemmal Na^{+}-Ca^{2+} exchanger[239], detailed studies on the relative contribution of the exchange mechanism to Ca^{2+} influx through the sarcolemma will now be possible.

iii] *Myofibrillar interaction*

Cytosolic Ca^{2+} in cardiac tissue serves as the activator for myocardial contraction. Although not as well characterized, the proteins that make up the contractile apparatus in cardiac muscle are very similar to those of skeletal muscle. Parallel arrays of thick and thin filaments are interdigitated, which allows an energy dependent sliding motion between the two types of filaments, causing shortening of the muscle fibre during contraction. Upon relaxation, the sliding motion is passively reversed. Connected in series, these parallel arrays consist of individual sarcomeres, which are made of of groups of individual myofilaments. The sarcomeres are grouped to form fibrils, and sheets of fibrils cooperate to form functional whole muscle.

The thick filament consists of specifically aligned myosin molecules. Each myosin molecule is a dimer of two identical subunits; two chains associated as an alpha-helical coiled tail, with each chain terminating at one end in a globular head. The

resulting dimeric myosin molecule is assymetric, with both globular regions present at the same end. In each thick filament, myosin is oriented so that the overall filament is bipolar, with the alpha-helical tails oriented to the center of the filament. The result is that of a filament with globular heads protruding in a staggered pattern at each end of the filament with a narrow midregion devoid of globular "heads". A myosin ATPase present in the globular head of the myosin molecule is activated by a specific interaction of myosin with actin. The association of myosin with actin, coupled with the consequent enzymatic activity is termed actomyosin ATPase. The hydrolysis of adenine triphosphate (ATP) to adenine diphosphate (ADP) and inorganic phosphate (P_i) by the actin activated myosin ATPase provides the energy for contraction.

The actin of actomyosin is found in the thin filament of the contractile complex. Actin is composed of filamentous actin (F-actin) plus tropomyosin and troponin, in a molar ratio of 7:1:1[240]. The F-actin itself is a double-stranded helix composed of globular actin (G-actin) monomers. Tropomyosin is a helical dimer which associates as a head to tail continuum of dimers along the actin polymers in or near the helical groove of the actin double strand. It has been hypothesized that the movement of tropomyosin in and out of the actin helical groove effects the activation and inactivation of the actin-myosin interaction[241].

The movement of tropomyosin is thought to be caused in part by the thin filament associated troponin, a complex of three functionally distinct subunits associated in a 1:1:1 molar stoichiometry. Troponin I (TnI) is the subunit that functions to inhibit actomyosin ATPase activity. Calcium sensitivity is afforded by another subunit, troponin C (TnC).

With Ca^{2+} binding to TnC, the inhibitory action of TnI on ATPase is decreased in the presence of troponin T (TnT). In the absence of TnT, TnC can restrict the inhibitory action of TnI regardless of the presence or absence of calcium. In addition to mediating the effect of TnC upon TnI, the TnT subunit appears to anchor the troponin complex to tropomyosin.

By examining the amino acid sequence of bovine cardiac TnC, van Eerd and Takahashi[242] predicted that the molecule would bind three moles of calcium per mole of TnC. Subsequent studies have shown that whole bovine cardiac troponin does indeed bind three moles of Ca^{2+} per mole of protein as predicted[242]. Three sites with two different affinities for Ca^{2+} are seen. Two of the sites had an affinity binding constant for calcium of 1.47×10^7 M^{-1}, and the third site had a constant of 2.5×10^5 M^{-1}. In the presence of 4 mM magnesium, the affinity for calcium at the higher affinity sites decreased to 3.6×10^6 M^{-1}, whereas calcium binding affinty at the third site remained unchanged[243].

At a suffient level of cytosolic free Ca^{2+}, association of calcium to the calcium specific binding site of TnC takes place. At a threshold point of increasing binding of calcium, the myofilament associated TnC undergoes a still undefined change in its intimate interaction with another troponin subunit, TnI. That association influences the relationship of TnT with TnI and with tropomyosin. It is supposed that the protein-protein interactions in the myofilament induces a shift of tropomyosin relative to actin that also includes a shift of TnI association with actin, thereby removing the TnI inhibition of actin-myosin interaction. The association of myosin with actin activates

myosin ATPase, ATP is hydrolyzed, and biochemical energy is transduced to mechanical force causing contraction. Changes in the amount of cytoplasmic free calcium or changes in the strength of binding at the several sites of troponin C are the means by which the physiologic mechanism can be modulated to provide greater or lesser contractile responsiveness.

iv] *Relaxation*

Relaxation of the cardiac myofibrillar apparatus is a passive process and occurs when the concentration of calcium in the vicinity of the troponin-tropomyosin complex decreases below a critical level. As the intracellular concentration of free Ca^{2+} decreases, Ca^{2+} ion dissociates from its binding site on troponin C, and the regulatory protein resumes its function of modulating the interaction between actin and myosin. The fall in intracellular Ca^{2+} occurs with the sequestration of myoplasmic Ca^{2+} by the sarcoplasmic reticulum and the surface membrane. An ATP-dependent Ca^{2+} pump is responsible for the accumulation of Ca^{2+} into the sarcoplasmic reticulum. The sarcolemma contains two mechanisms that mediate the efflux of Ca^{2+} from the myocyte; an ATP-dependent Ca^{2+} pump, and the sarcolemmal Na^{+}-Ca^{2+} exchanger.

The involvement of a specific ATPase in the pumping of Ca^{2+} out of cells was first suggested by Dunham and Glynn in 1961[244], and first demonstrated in erythrocyte membranes by Schatzmann in 1970[245]. The existence of an ATP-dependent Ca^{2+} transport protein (Ca^{2+}-ATPase) in the myocardial sarcolemma was established by Caroni and Carafoli[246]. Since its identification in cardiac sarcolemma, the pump has been studied in great detail in many laboratories.

The sarcolemmal Ca^{2+}-ATPase has been isolated as a single polypeptide of 140 kD[247]. It has been shown to be an ATPase of the P-type, as it forms a phosphoenzyme during the transport cycle. Phosphorylation of heart sarcolemmal membranes by the cAMP-dependent protein kinase has been shown to stimulate the incorporation of ^{32}P from labeled ATP into the membrane as well as stimulate the activity of the Ca^{2+} pump[248]. In reconstituted liposomes, it has been shown to transport Ca^{2+} with a 1:1 stoichiometry to ATP[249]. The sarcolemmal Ca^{2+}-ATPase is a high affinity enzyme which, in the presence of activating calmodulin interacts with Ca^{2+} at a K_m of 0.4 μM[247]. It has, however, a low capacity, transporting only about 0.5 nmoles of Ca^{2+} per mg of membrane protein per second[247]. The high affinity for Ca^{2+} suggests that the pump operates continuously, extruding Ca^{2+} from the cell during both diastolic and systolic periods.

The dominant Ca^{2+} efflux mechanism of cardiac myocytes appears to be the Na^+-Ca^{2+} exchanger. In comparison to the sarcolemmal Ca^{2+}-ATPase it is a high capacity, low affinity efflux process. Many investigations have supported this idea. Bers and Bridge[250] studied relaxation of rabbit ventricular muscles. In tissue specially treated such that relaxation relied on transsarcolemmal transport, they showed that relaxation was slowed by almost an order of magnitude in the absence of external Na^+. Other studies have concluded that Na^+-Ca^{2+}, but not the sarcolemma Ca^{2+} pump, contributes to beat-to-beat relaxation[251,252].

b) Excitation-Contraction and Pharmacomechanical Coupling in Vascular Smooth Muscle

E-C coupling in vascular smooth muscle can be considered in four separate steps.

First, membrane depolarization occurs. Second, the membrane depolarization causes an increase of myoplasmic calcium from both extracellular sources, as well as from intracellular SR stores. Third, this intracellular calcium causes activation of myosin light chain kinase (MLCK) which leads to muscle contraction. Finally, Ca^{2+} is resequestered into the SR and extruded from the cell allowing relaxation.

Although E-C coupling in skeletal and cardiac muscle involves membrane excitation followed by calcium release into the myoplasm, in vascular smooth muscle, membrane depolarization does not seem to be a prerequisite for contraction. There exsist in smooth muscle nonelectric or pharmacomechanical coupling mechanisms which can operate independently of membrane potential. In both excitation-contraction coupling and pharmacomechanical coupling the final common pathway is an increase in the myoplasmic free calcium concentration.

i] *Depolarization of the smooth muscle cell*

Vascular smooth muscle is usually subdivided into two functional classes, phasic and tonic[253]. Phasic muscle (eg., that in the portal vein) generates action potential spikes, which frequently occur in bursts. In contrast, tonic muscle (eg., that in large arteries) does not generate action potentials, rather it exhibits prolonged low amplitude (5 mV) membrane potential oscillations. The electrochemical gradient of ions in tonic vascular cells supports a membrane potential of -55 to -60 mV in resting cells, while that of the phasic type is less negative and averages around -40[254-256]. Several ion pumps and exchangers which support the electrochemical gradient have been identified in vascular smooth muscle. The sarcolemma has been shown to contain a Na^+,K^+-ATPase[257], as

well as an outwardly directed Ca^{2+} pump[258], a Na^{+}-H^{+} exchanger[259], and a Na^{+}-Ca^{2+} exchanger[260,261].

The sarcolemma of vascular smooth muscle cells has been shown to lack tetrodotoxin sensitive Na^{+} channels, and to possess lower permeability to K^{+} ions compared with cardiac muscle[262]. In response to depolarization, the inward current is carried by Ca^{2+} ions through voltage-dependent Ca^{2+} channels that are functionally analogous to the L-type channels described earlier in cardiac muscle[263,264]. Unlike cardiac sarcolemma, vascular smooth muscle does not contain an L channel that can be phosphorylated by a ß-receptor-mediated activation of adenylate cyclase[265]. Evidence exsists for another type of voltage regulated L-type channel in vascular smooth muscle cells[265,266] in which Ca^{2+} entry is modulated by a receptor operated mechanism. This channel is coupled directly by a G protein to an α_2-adrenergic receptor, stimulation of which increases calcium influx through this channel[265,267]. A transient T-type calcium channel analogous to that in cardiac muscle has also been identified in vascular smooth muscle[180].

ii] *Extracellular Ca^{2+} entry and SR Ca^{2+} release*

Like cardiac muscle, vascular smooth muscle contains a sarcoplasmic reticular network that functions as the sequestration site for contractile calcium. The amount of SR in a particular smooth muscle type varies depending on the type of tissue. For example, large conduit arteries appear to have a much more extensive SR than do smaller muscular arteries[268]. In all vascular smooth muscle, however, contraction can be inhibited by the drug ryanodine, indicating that Ca^{2+} release channels of SR participate

in mobilization of Ca^{2+} during cell activation[269].

Release of Ca^{2+} from vascular smooth muscle SR is thought to be regulated by a calcium-induced calcium release mechanism similar to that operating in cardiac muscle[270,271]. The source of trigger calcium being that traversing the sarcolemma through both voltage and receptor operated L channels, T channels as well as through Na^{+}-Ca^{2+} exchange. In addition to the calcium-induced calcium release from SR, Ca^{2+} can be released from internal sites via an $Ins(1,4,5)P_3$-activated channel[272]. This phosphoinositide pathway and its regulation forms the subcellular basis of "pharmacomechanical coupling" first described by Somlyo and Somlyo[273]. Pharmacomechanical coupling is hormone or drug induced contraction of vascular smooth muscle in the absence of membrane depolarization. α_1-receptor stimulation is coupled to phosphotidylinositol hydrolysis via G-protein[274]. It has been hypothesized that the activated α-subunit of the G-protein complex is phospholipase C[275]. Activated phospholipase C hydrolyzes phosphatidylinositol biphosphate into two active components, diacylglycerol, which can activate protein kinase C, and inositol trisphosphate $Ins(1,4,5)P_3$[186,275,276]. $Ins(1,4,5)P_3$ serves as an intracellular messenger that interacts via a receptor protein[277] with an $Ins(1,4,5)P_3$-responsive Ca^{2+} channel in the SR of vascular smooth muscle to cause Ca^{2+} release from this organelle[278]. It has been suggested that protein kinase C may phosphorylate the Na^{+}-H^{+} and Na^{+}-Ca^{2+} exchangers, stimulating their activities[279,280]. Protein kinase C may also enhance L-type Ca^{2+} channel activity via phosphorylation[281].

Controversy continues to surround the issue of the relative importance of voltage

sensitive versus receptor-activated Ca^{2+} ion release in the control of vascular smooth muscle tone. Recent studies indicate that in peripheral artery smooth muscle, the primary control of intracellular Ca^{2+} resides in the sarcolemmal Ca^{2+} transport systems[267]. It has been proposed that the relatively sparse SR in these vessels functions as a modulator or buffer to help dampen large changes in intracellular Ca^{2+} and not as the primary source of contractile Ca^{2+}. In contrast, in large conduit vessels like the aorta which contains considerably more SR than smaller muscular arteries, it has been suggested that the SR plays an important role in excitation-contraction coupling[269]. Thus in different vascular beds, there may be multiple mechanisms participating in the maintenance of smooth muscle tone.

iii] *Contractile protein activation*

Three types of filaments comprise the contractile apparatus of smooth muscle; thick, thin, and intermediate filaments[282]. The thick filaments (13.5-17.5 nm diameter) are composed of myosin, while the thin filaments (5-8 nm diameter) are composed of actin, tropomyosin and other proteins[282]. Each thick filament is surrounded by thin filaments, and both fibers run mostly parallel to the long axis of the smooth muscle cell. The tone generating capacity of vascular smooth muscle is dependent on the interaction between the thick and thin filaments, analogous to the sliding filament model proposed for cardiac muscle contraction[283].

In smooth muscle, the thin filaments are anchored to the cell membrane via membrane plaques that contain the proteins vinculin, metavinculin, α-actinin, and talin, as well as others[284]. Within the myoplasm, thin filaments insert into fusiform dense

bodies[285], which contain α-actinin as well as other unidentified proteins[286]. The protein filamin crosslinks some of these actin filaments[287]. The dense bodies are held together in a three dimensional network by the intermediate filaments[285] which are composed of vimentin and desmin[288]. The intermediate filaments with their associated dense bodies are thought to form the cytoskeleton of the vascular smooth muscle cell.

The contraction of vascular smooth muscle is regulated by Ca^{2+} in many ways and several mechanisms have been described for the activation of actomyosin ATPase activity. One mechanism involves the phosphorylation of the myosin molecule[289]. Vascular myosin is composed of two heavy chains and two sets of light chains, one of which are the so-called LC_{20} subunits (M_r = 20,000 Da). The LC_{20} subunits are phosphorylated by the enzyme myosin light chain kinase (MLCK), which is activated by Ca^{2+} and the Ca^{2+}-binding protein calmodulin[289]. It is the protein calmodulin which acts as a type of Ca^{2+} receptor, activating MLCK only when it is occupied by four Ca^{2+} ions per molecule[290]. In vitro studies have shown that LC_{20} phosphorylation increases the rate limiting step in the actomyosin ATPase cycle by more than 1,000 fold[291], and hence is the signal that activates the cycling of crossbridges and initiates contraction. Studies on tracheal smooth muscle have shown that MLCK itself can be phosphorylated in vivo which may regulate its activity[292]. Several phosphoprotein phosphatases have been isolated from vascular muscle tissue that dephosphorylate LC_{20}[293], however, none has been shown to exhibit substrate specificity.

Recently, two actin binding proteins with purported regulatory properties have been identified in smooth muscle. Calponin, binds to both actin and tropomyosin and

inhibits actomyosin ATPase activity[294]. This inhibition is reversed by Ca^{2+} and calmodulin, as well as by phosphorylation of calponin by protein kinase C or by Ca^{2+}/calmodulin-dependent protein kinase II[295]. Caldesmon, a much larger protein, also inhibits actomyosin ATPase activity and can be phosphorylated in vitro by several protein kinases[296]. In addition, it is a weak Ca^{2+}/calmodulin binding protein[296]. The amino acid sequence of an avian caldesmon has been determined, and shown to have carboxyl terminus sequence homology to troponin T as well as calmodulin binding proteins[300]. The physiologic importance of these two proteins in the regulation of vascular muscle contractility has not yet been elucidated, but a role for calponin in the modulation of the Ca^{2+} sensitivity of smooth muscle has been suggested[298] while phosphorylated caldesmon is thought to slow crossbridge detachment facilitating tonic contraction[299].

iv] *Relaxation*

As in cardiac muscle, relaxation of vascular smooth muscle occurs when the concentration of myoplasmic Ca^{2+} falls. This fall can be accomplished by either extrusion of cellular Ca^{2+} through the plasmalemma, or uptake into the SR. Sequestration of Ca^{2+} into the SR is under the control of two transport mechanisms. Like cardiac tissue, vascular smooth muscle contains a SR Ca^{2+} pump. It has been suggested that in vascular smooth muscle the affinity of the SR Ca^{2+} pump for Ca^{2+} is enhanced by agents that increase cGMP levels, whereas agents that activate protein kinase C increase the maximal activity of the pump[300]. In addition to the Ca^{2+} pump, vascular smooth muscle SR contains the protein phospholamban[301]. This protein possesses Ca^{2+} channel activity[302], and has been implicated in the cAMP stimulated Ca^{2+}

uptake by SR seen in smooth muscle[303], potentially through a cAMP-dependent protein kinase mediated phosphorylation mechanism.

In addition to its effects on SR Ca^{2+} uptake, activation of cAMP-dependent protein kinase by ß-receptor stimulated increases in cAMP also results in a decrease in the Ca^{2+} sensitivity of the contractile structures[304-306]. This is thought to occur by phosphorylation of MLCK by the cAMP-dependent protein kinase inhibiting its interaction with calcium and calmodulin[307]. The result is relaxation and inhibition of actomyosin ATPase.

An increase in K^+ efflux from the smooth muscle cell also contributes to cell relaxation. Several types of K^+ channels have been identified in vascular myocytes, including Ca^{2+}-activated K^+ channels, delayed rectifier K^+ channels, and ATP-sensitive K^+ channels[308-310]. Activation of any of these channels leads to increased K^+ conductance and results in membrane hyperpolarization with subsequent inactivation of voltage dependent Ca^{2+} channels[311], and enhanced extrusion via Na^+-Ca^{2+} exchange[312] with resulting reduction in smooth muscle tone. The open time and frequency of opening of Ca^{2+}-activated K^+ channels are increased by 5'-GMP, a metabolite of cGMP[313]. cGMP levels are enhanced in smooth muscle cells by the action of EDRF and atrial natriuretic factor (ANF) on cell surface receptors[313]. In addition cGMP activates a cGMP-dependent protein kinase which, in a manner analogous to that of cAMP-dependent protein kinase, may inhibit the action of MLCK inducing relaxation by a direct effect on the contractile apparatus[314].

c) Membrane Lipid Modulation of Cation Transport

Mammalian plasma membrane consists of an assymetric bilayer of phospholipid (PL), cholesterol and protein. It is generally agreed that membrane proteins differ in their association with this lipid matrix. Some proteins are bound to the surface of the bilayer, while others are integrated into the hydrophobic liquid core and some may span the membrane surfaces. It is not suprising that the biochemical properties of these integral membrane proteins depends not only on the physical state of the cell membrane but also on the nature of their dynamic interactions with the other components of the lipid bilayer. Many studies have shown that the composition of the lipid bilayer modulates the activity of the membrane transport proteins.

The regulation of Ca^{2+} movements across the sarcolemmal membrane is of critical importance in maintaining the contractile, metabolic and electrophysiological integrity of the myocardium[315]. It is for this reason that emphasis has been placed on the study of the regulation of sarcolemmal Ca^{2+} transport mechanisms, in particular the Na^{+}-Ca^{2+} exchanger, by the components of the membrane lipid environment.

Regulatory mechanisms affecting Na^{+}-Ca^{2+} calcium exchange are the subject of very active research. In cardiac sarcolemmal vesicles, Na^{+}-Ca^{2+} exchange may be modulated by pretreatment with various enzymes. Based on experiments with phospholipases[316,317], various amphiphiles[319-322], and solubilization/reconstitution techniques[231] it has been shown that the Na^{+}-Ca^{2+} exchanger must interact with specific anionic lipid components for optimal transport activity. Phospholipase C has been used to remove the phosphate containing head group from neutral phospholipids in sarcolemmal vesicles. The resulting diacylglycerol forms droplets outside the membrane

vesicles, thus increasing the percentage of the negatively charged phospholipids, phosphatidylinositol and phosphatidylserine, in the membrane. It has been shown that Na^+-Ca^{2+} exchange activity is stimulated up to 100% over control when 10% to 70% of the sarcolemmal phospholipid is hydrolyzed[317]. This stimulation is thought to be a result of the greater concentration of negatively charged phospholipids in the lipid milieu of the exchange protein.

More dramatic effects on Na^+-Ca^{2+} exchange activity have been shown to occur after treatment of sarcolemmal vesicles with phospholipase D[315], which converts phospholipids to the negatively charged phosphatidic acid. When about 10% of the membrane phospholipid was converted to phosphatidic acid, Na^+-Ca^{2+} exchange activity increased up to 400% over control values in native vesicles[316]. Apparent K_m(Ca) was shown to decrease from 18.2 to 6.3 μM, whereas V_{max} increased 75% after enzyme treatment. The results of these phospholipase treatment studies are consistent with the hypothesis that negatively charged phospholipids exert a regulatory effect on Na^+-Ca^{2+} exchange. An increase in sarcolemmal bound Ca^{2+} after phospholipase D treatment might be related to the observed stimulation of exchange activity[318].

It has been demonstrated that incorporation of anionic or cationic amphiphiles into cardiac sarcolemmal membranes stimulates or inhibits, respectively, Na^+-Ca^{2+} exchange activity[319-322]. The exogenous amphiphiles act as analogues of charged phospholipids, supporting the hypothesis that exchange is modified by anionic phospholipids. These changes could not be attributed to changes in passive Ca^{2+} permeability or membrane surface potential[319]. Exogenous charged amphiphiles also alter the membrane fluidity of

isolated sarcolemma[323] and therefore, part of the modulation of exchange activity may be due to alterations in the physical environment of the protein.

Reconstitution of solubilized Na^+-Ca^{2+} exchange protein into vesicles of defined lipid composition by Vemuri and Philipson[231] revealed that inclusion of both specific anionic phospholipids and cholesterol are required for optimal activity. The mechanism of action of cholesterol on the exchanger was not determined, although the effects of cholesterol on membrane fluidity, phospholipid spacing and membrane thickness have been established[145].

In order to determine if a direct interaction of cholesterol with the Na^+-Ca^{2+} exchange protein was operating, reconstitution studies were performed incorporating the solubilized exchange protein into lipid vesicles containing cholesterol or one of a variety of cholesterol analogues[324]. The sterol requirement of the Na^+-Ca^{2+} was shown to be highly selective for cholesterol, even cholesterol analogues with minor structural changes were unable to support Na^+-Ca^{2+} exchange. Similar results were also observed for sarcolemmal Na^+,K^+-ATPase.

C. MATERIALS AND METHODS

I. Materials

All chemicals and reagents were purchased from Sigma Chemical Co., St. Louis. Cholesterol oxidase (Pseudomonas fluorescens) was purchased from Sigma. The alamethicin was kindly donated by R.L. Keene, The Upjohn Co. Deoxyribonuclase (DNase) was purchased from Cooper Biomedical Inc., Malvern, PA. All chemicals were of standard reagent grade.

II. Vesicular Preparations

Phosphatidylcholine and phosphatidylcholine-cholesterol liposomes were prepared using a modified method of Papahadjopoulos[325]. Since it has been shown that the addition of 1 mol % of alpha-tocopopherol protects against lipid peroxidation[326], chloroform solutions of phosphatidylcholine and mixtures of phosphatidylcholine with cholesterol (1:0.5, 1:1, 1:2) in the presence of 1 mol of alpha-tocopherol/100 mol of phospholipid were dried in the dark under a stream of nitrogen gas. All traces of chloroform were removed by further drying under vacuum for 12 h at room temperature. Buffer A (140 mM NaCl, 20 mM MOPS {4-morpholinepropanesulfonic acid}, pH 7.4) or buffer B (140 mM KCl, 20 mM MOPS, pH 7.4), 0.2 ml/mol of phospholipid, was added, and the mixture was allowed to swell for 10 min. The aqueous dispersions were sonicated for 45 min in a Bransonic 1200 sonicator. After sonication, the liposome suspensions were centrifuged at 104,000 x g at 5°C for 60 min in a Beckman TL-100

tabletop ultracentrifuge to remove large vesicles and undispersed lipid[327]. The supernatant contained liposomes with cholesterol/phospholipid in proportions of 0:1 to a maximum of 1.5:1, which is near the upper limit reported for incorporation of cholesterol into phospholipid vesicles[328]. The liposomes were used immediately. Liposomal cholesterol/ phospholipid ratios indicate the initial proportion of cholesterol to phosphatidylcholine before incorporation of the sterol into phospholipid vesicles.

Sarcolemmal membrane vesicles were isolated from canine left ventricular tissue as described in detail previously[329-331]. The vesicles exhibited relatively high activities of enzymes commonly associated with the sarcolemmal membrane. For example Na^+, K^+ -ATPase activity in the membranes (n=9) was 26.4 $\pm$ 4.1 and 131.5 $\pm$ 17.5 μmol P_i/mg/hr in the absence and presence, respectively, of 12.5 μg alamethicin/ml reaction medium. K^+ -dependent *p*-nitrophenyl phosphatase activity was 26.1 $\pm$ 1.8 μmol phenol/mg/hr, which represented an enrichment of 107 $\pm$ 22-fold over homogenate values. Cross-contamination of this preparation with other subcellular organelles is minimal[330,331].

Cholesterol enrichment was performed by incubation of sarcolemmal vesicles with cholesterol-rich liposomes at 4°C overnight (16-18 h). Cholesterol-depleted sarcolemma was obtained by a similar incubation of the vesicles with phosphatidylcholine liposomes. The liposome-sarcolemma suspensions were spun for 20 min at 104,000 x g in a Beckman TL-100 ultracentrifuge. The pellets were washed once and the final pellet resuspended in the appropriate medium (buffer A or B).

To ensure that fusion, adsorption, or entrapment of the liposomes with

sarcolemma did not confound our results, sarcolemmal vesicles were treated as described with cholesterol-phosphatidylcholine liposomes prepared in the presence of ^{14}C sucrose (2.8 μM, 350 μCi/μmol). To rule out any nonspecific sucrose adsorption, sarcolemmal vesicles were incubated with a liposome-free [^{14}C]sucrose medium. The sarcolemmal vesicles were incubated overnight with identical amounts of [^{14}C]sucrose in the presence or absence of liposomes, centrifuged and washed once as above, and then resuspended and analyzed for the presence of radiolabeled sucrose.

III. Assay Procedures

All assays were carried out at 37°C. K^+ dependent *p*-nitrophenylphosphatase activity was measured in 50 mM Tris, 5 mM $MgCl_2$, 1mM EGTA, 5 mM *p*-nitrophenylphosphate, and 20 mM KCl, pH 7.8, at 37°C. The K^+-independent phosphatase activity, measured in the same reaction medium without KCl, was subtracted. The reaction volume was 1 ml and contained about 8 μg of sarcolemmal protein. The reaction was quenched after 7 min with 2 ml of 1 N NaOH, and the absorbance at 410 nm was used to determine the amount of *p*-nitrophenol formed. Na^+,K^+ adenosine triphosphatase (Na^+,K^+-ATPase) activity was assayed in a medium containing 50 mM Tris, 120 mM NaCl, 3.5 mM $MgCl_2$, 1 mM EGTA, 5 mM NaN_3, 20 mM KCl, 3 mM ATP, pH 7.0, at 37°C. The reaction time was 10 min. ATPase activity measured in a KCl-free mixture was subtracted. ATPase activity in the absence of K^+ was the same as ATPase activity in the presence of K^+ plus 2.5 μM digitoxigenin. Maximally stimulated ATPase activity was measured in the presence of 12.5 μg of

alamethicin/ml. Inorganic phosphate liberated was measured by the method of Fiske and SubbaRow[332].

Na^+-Ca^{2+} exchange was measured as the rate of Na^+_i-dependent Ca^{2+} uptake as described elsewhere[330,333]. Briefly, 0.005 ml of Na^+-loaded (buffer A) sarcolemmal vesicles (1.2-1.5 mg of protein/ml) was rapidly diluted into 0.245 ml of Ca^{2+} uptake medium containing buffer B with 0.4 μM valinomycin , 0.3 μCi of $^{45}CaCl_2$, and various Ca^{2+} concentrations. After the appropriate reaction time, the Ca^{2+} uptake was stopped by the addition of 0.03 ml of 140 mM KCl, 1 mM $LaCl_3$ via a rapid quenching device, as described[333]. Vesicles were retained on Sartorius cellulose nitrate filters (pore size = 0.45μm) and washed with two 3-ml aliquots of 140 mM KCl, 0.1 mM $LaCl_3$. Values were corrected for passive (Na^+-independent) Ca^{2+} uptake and for bound Ca^{2+} by subtraction of blank values obtained by using Ca^{2+} uptake medium which contained 140 mM NaCl instead of KCl. Previous studies on the time course of Na^+-dependent Ca^{2+} uptake have shown the exchange to exhibit linearity to about 5 s[232].

When cholesterol oxidase (cholesterol:oxygen oxidoreductase) was included in the reaction medium, catalase was also present at 75 times (units:units) the cholesterol oxidase concentration. This catalase concentration was in excess of the amount required to quench the H_2O_2 produced by the reaction of cholesterol oxidase with the membrane cholesterol, assuming that 1 mol H_2O_2 was produced for each mole of cholesterol oxidized[334] and all of the membrane cholesterol was oxidized. Catalase itself had no effect on the Na^+-Ca^{2+} exchange reaction. In preliminary experiments, it was discovered that La^{3+} caused large increases in background counts in the presence of Ca^{2+} and the

cholesterol oxidase. Similar problems have been encountered by others[321]. This problem was circumvented by eliminating the use of La^{3+} to stop the reaction and instead using 0.03 ml of 140 mM KCI, 10 mM EGTA, and 20 mM MOPS (pH 7.4) delivered with a rapid quenching device described elsewhere[333]. A 1-ml aliquot of ice-cold 140 mM KCI, 1 mM EGTA, and 20 mM MOPS (pH 7.4) was then immediately added to the reaction tube, and 1 ml was removed for filtration. The filters were then washed with twice with 3 ml of this same solution. This modified quenching and wash solution has been used previously with success[321]. Background counts were reduced to approximately 5% of total counts, and activity was similar in control experiments whether La^{3+} or EGTA was used as the stop/wash solution. These results were also qualitatively similar using another cholesterol oxidase preparation (Nocardia erythropolis).

In order to ensure that the effect of cholesterol oxidase was not due to generated H_2O_2, sarcolemmal vesicles (1-2 mg of protein/ml) in buffer A or B were incubated for 15 min at 37°C with either 4 units of cholesterol oxidase/mg of sarcolemmal protein or with hydrogen peroxide (0.1 or 100mM) in buffer A or B prior to assay for Na^+-Ca^{2+} exchange activity. Catalase (300 units/mg of sarcolemma protein), preincubated with sarcolemma before the addition of H_2O_2 or cholesterol oxidase, was used as a peroxide scavenger.

Passive Ca^{2+} efflux was assayed as previously described[321]. Vesicles were allowed to accumulate ^{45}Ca via Na^+-Ca^{2+} exchange and were then diluted into a medium to allow Ca^{2+} efflux. In this method, 0.005 ml of sarcolemmal vesicles (1.2-1.5 mg of protein/ml) in buffer A was diluted into 0.245 ml of a medium containing 140 mM KCl,

10mM MOPS (pH 7.4, 37°C), 10 μM Ca^{2+}, 0.3 μCi of $^{45}CaCl_2$. After 2 min at 37°C, 0.245 ml of 140 mM KCl, 0.2 mM EGTA solution was added. After various incubation times, the vesicles were filtered on Sartorius filters as described above and washed with two 3-ml aliquots of 140 mM KCl, 0.1 mM $LaCl_3$. Values were corrected for ATP-independent Ca^{2+} binding by subtracting blank values obtained by using uptake medium which contained 140 mM NaCl instead of KCl, and therefore provided no transsarcolemmal Na^+ gradient. Ca^{2+} efflux was also measured in the presence of cholesterol oxidase plus catalase (as above).

ATP-dependent Ca^{2+} pumping activity of the sarcolemma vesicles was determined by the method of Philipson and Nishimoto[232] which involved addition of 0.005 ml of K^+-loaded sarcolemmal vesicles (1.2-1.5 mg of protein/ml) to 0.245 ml of 140 mM KCl, 1.2 mM Tris/ATP, 1.2 mM $MgCl_2$, 5 μM $CaCl_2$, 0.3 μCi of $^{45}CaCl_2$, 20 mM MOPS, pH 7.4, 37°C. The reaction was stopped by the addition of La^{3+} and the vesicles collected on Sartorius membrane filters as described above. Blank tubes did not contain Mg^{2+} or ATP. In experiments employing cholesterol oxidase the stop/wash solution was as described above for the Na^+-Ca^{2+} exchange.

Measurement of ATP-independent (passive) Ca^{2+} binding was made by the method of Bers et al.[335] in which 0.005 ml of sarcolemmal vesicles suspended at a protein concentration of 1.2-1.5 mg/ml in buffer B was diluted into 0.245 ml of a medium containing 140 mM KCl, 20 mM MOPS (pH 7.4, 37°C), $CaCl_2$ (0.05, 0.50, 1.00, or 2.50 mM), 1 μCi of $^{45}CaCl_2$. The reaction mixtures were incubated at 37°C for 1 min, filtered through Sartorius filters as described above, and washed with two 1-ml aliquots

of ice-cold water. The values were corrected for nonspecific binding of $^{45}Ca^{2+}$ to the filters by subtracting blank values obtained by filtering an equal volume of incubation medium without added sarcolemma, as described[335]. In experiments employing cholesterol oxidase treatment, 1 μM A23187 was included in the incubation medium. The A23187 was included to permeabilize the membranes to Ca^{2+} and allow free access of the ion to both sides of the sarcolemmal vesicles. Thus, even if cholesterol oxidase treatment of the sarcolemma increased vesicular permeability, this change in permeability could not account for the alteration in passive Ca^{2+} binding capacity of the membranes. The reaction was carried out for 1 minute at 37°C and was terminated by filtration.

Cholesterol content of the vesicles was determined by the enzymatic method of Sale et al.[336] after enzymatic conversion of esterified derivatives of cholesterol to free cholesterol. Lipid phosphorus measurements were made using the procedure described by Bartlett[337].

Oxidation of membrane cholesterol by cholesterol oxidase was verified using a thin layer chromatographic procedure described by Smith et al.[338], or when necessary, identification of the oxidized species was made using a high-performance liquid chromatographic (HPLC) technique. For HPLC analysis, sarcolemmal vesicles (15 μl, $\approx$30 μg) were suspended on the side of a ground-glass homogenization vessel, and a reaction was initiated by vortexing with 7 units of cholesterol oxidase (+catalase) in 140 mM KCI and 20 mM MOPS, pH 7.4 (total volume was 250 μl). All reactions were carried out at 37°C and were terminated at specified times with the addition of 2 ml of 2:1 (vol/vol) chloroform:methanol. The sample was further disrupted by homogenization

with a glass pestle; then the vessel was rinsed with 2 ml plus 1 ml of the above solution. Lipids were extracted from membranes in a 2:1 (vol/vol) chloroform:methanol solution as described[339] and were then evaporated under a stream of nitrogen and suspended in 100 μl methylene chloride for separation via HPLC. For the separation of oxidized cholesterol species, a modification of the technique of Sevanian and McLeod[340] was used. A 20-50 μl aliquot was injected into an HPLC system (Beckman Instruments, Inc., Fullerton, Calif.) fitted with a 5-μm particle size Beckman Ultrasphere silica column (4.6 X 156 mm). Flow rate was maintained at 1.0 ml/min, and the mobile phase was 95:5 (vol/vol) hexane:isopropanol. Ultraviolet detection of the peaks was carried out at an absorbance of 208 nm using a Beckman model 166 programmable UV-visible detector. Appropriate standards (Sigma; Steraloids Inc., Wilton, N.H.) were run to identify the cholesterol species.

Protein concentration was determined by the method of Lowry et al[341] using bovine serum albumin as a standard.

IV. Cell Isolation and Culture

Adult male albino rabbits weighing 2 to 3 kg were sacrificed with a lethal i.v. dose of sodium pentobarbital via the marginal ear vein. A 6 to 9 cm segment of the thoracic aorta, distal to the subclavian artery and proximal to the diaphragm was removed under sterile conditions. Vascular smooth muscle cells were isolated from this segment as described elsewhere[342,343]. Briefly, the intima, adventitia and outer third of the medial layer were carefully dissected from the remaining medial layer. The luminal surface was

scraped with a sterile scalpel to remove endothelial cells. The medial tissue was minced and incubated for 120 to 180 min at 37°C with gentle shaking in 10 ml of a medium containing Medium 199 (Gibco), 2.5 mg elastase (Sigma Chemical Co., Type III), 7.5 mg soybean trypsin inhibitor (Sigma Chemical Co.), 10 mg bovine serum albumin (Sigma Chemical Co.) and 20 mg Type I collagenase (Worthington Biochemicals Corp.). This mixture was poured through a 100 um Bellco stainless steel sieve and the tissue retained on the sieve was triturated 10 times through a 12 gauge stainless steel cannula. This digest was resieved and the cells in the filtrate were pelleted by centrifugation and plated on 60 X 15 mm Primaria culture dishes (Falcon Plastics) in Medium 199 which contained 20% fetal calf serum, 2.0 mM glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml. Primary cultures grew to confluence within 1 week. Cells were typically plated at a density of 1 X 10^4 cells/cm^2. This procedure yields a homogenous population of smooth muscle cells as identified previously[343] by positive staining with fluorescence labelled mouse anti-smooth muscle contractile protein monoclonal antibody as described by Gown et al[344].

V. Lipoprotein Isolation Procedure

Rabbits fed for six weeks with a 2% cholesterol diet were used to obtain blood for lipoprotein isolation. Blood was drawn via arterial puncture into evacuated containers to which EDTA (4 mM final concentration) was added as a heavy metal sequestrant to prevent oxidative degradation of the lipoprotein species[345]. Plasma was isolated by centrifugation at 1500 x g at 4°C for 15 min. To the separated plasma, Ellman's reagent

[5,5'-dithio-bis-(2-nitrobenzoic acid)] was added to a final concentration of 1.5 mM in order to inhibit LCAT, and phenylmethyl-sulphonyl fluoride (2 mM) was added to inhibit proteolytic enzymes[346]. Thimerosal (0.08 mg/ml) was added both as a bactericide as well as an inhibitor of lipoprotein lipase[347].

VLDL-chylomicra ($\delta < 1.006$) and LDL ($1.085 > \delta > 1.006$) fractions were prepared from plasma by a serial ultra-centrifugation technique described by Lindgren[348]. Briefly, this involved flotation ultracentrifugation of the treated plasma at 37,000 rpm for 24 h in a Beckman 5100 ultra-centrifuge fitted with a fixed angle type 42.1 rotor. The well defined surface layer corresponding to VLDL particles ($\delta < 1.006$) was aspirated off and the density of the remaining infranatant was adjusted to $\delta = 1.085$ g/ml with solid NaCl according to the equation described by Radding and Steinberg[349]:

$$X = V_i(\delta_f - \delta_i)/1 - V\delta_f$$

where X is the weight in gms of solid NaCl to be added, V_i is the initial volume of plasma, δ_f is the final density required, δ_i is the initial density of the solution, and V is the partial specific volume of NaCl, determined by interpolation of data from Baxter and Wallace[350]. In all calculations, the partial solvent volume (= 0.94 times the actual V) was used to calculate density adjustments instead of the true initial volume to account for the colloidal nature of isolated plasma. LDL particles were isolated by flotation ultracentrifugation of the VLDL-chylomicra free density adjusted ($\delta = 1.085$) infranatant at 42,000 rpm for 24 h in a Beckman 5100 ultracentrifuge fitted with a type 42.1 fixed angle rotor, and aspirated off.

The VLDL and LDL enriched subfractions were dialyzed against a solution

containing (in mM): 133 NaCl, 3.6 KCl, 0.3 $MgCl_2$, 1.0 $CaCl_2$, 16 dextrose, 3.0 Hepes (pH 7.4) in order to remove all preservatives. The dialysed preparations were sterilized by passage through an MSI Cameo IIS 0.22 μm filter unit and immediately used to treat smooth muscle cell cultures. Experimental cells were incubated at confluence for varying times with Medium 199 supplemented with varying aliquots of the sterilized lipoprotein. If the incubation time was longer than 24 hours, the entire medium was replaced daily. Control cells were incubated with Medium 199 which was not supplemented with lipoproteins. After the experimental treatment was complete, cells were scraped from the culture dishes and lipid content analyzed. The cholesterol content (both free and esterified) of control and treated cell monolayers and of isolated lipoproteins was determined from chloroform:methanol extracts[339] by the enzymatic method of Sale et al[336] after enzymatic conversion of esterified derivatives of cholesterol to free cholesterol. The free and esterified cholesterol content of the isolated lipoprotein fractions were determined as described after lipid extraction by the method of Folch et al[351].

VI. Ca^{2+} Exchange in Cultured Smooth Muscle Cells

Ca^{2+} exchange in cultured vascular smooth muscle cells was measured by an on-line isotopic method described in detail elsewhere[343,352]. Briefly, this technique involved the growth of isolated muscle cells on Primaria-coated (Falcon Plastics) polystyrene discs which contained a scintillant material (Bicron, Medford, Ohio). After the cells achieved a confluent monolayer, the discs were inserted into a specially designed flow cell so that the discs with their cells formed the sides of the chamber[352] (Figure 1). The flow cell

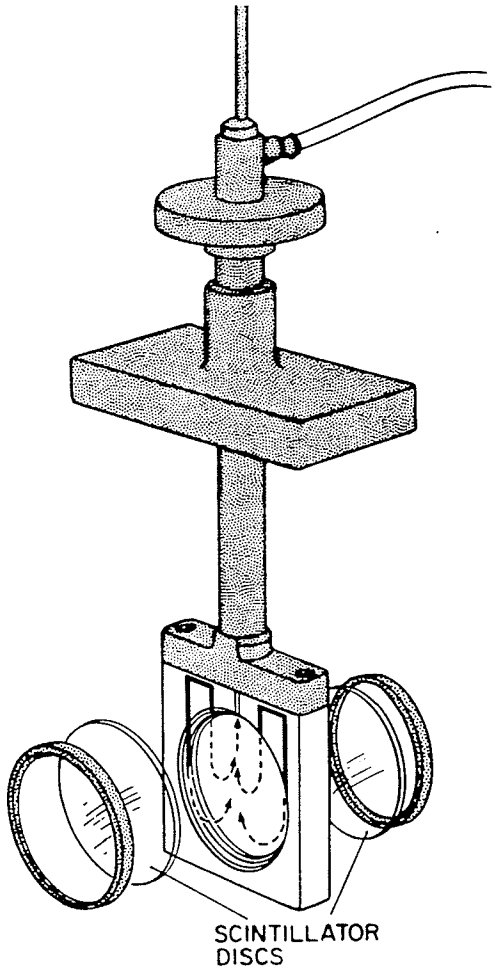


FIGURE 1. *Schematic diagram of specially designed flow cell for on line measurement of ^{45}Ca flux in cultured rabbit aortic smooth muscle cells.* The cells are grown on one side of Primaria-coated plastic discs which contain a scintillant material. The flow cell is assembled such that the discs with the attached cells form the sides of the apparatus. Perfusion medium enters the flow cell via 4 ports, and exits via one larger port. Once assembled, the entire apparatus is inserted into a specially designed spectrometer which has photomultiplier tubes in close apposition to the scintillator discs (4 to 5 mm). See "Materials and Methods" for experimental protocol.

was then inserted into a modified spectrometer (Tennelec, Tennessee) which had photomultiplier tubes (Thorn EMI, Gencom, New Jersey) in close apposition to the flow cell discs. Once the flow cell was inserted into the apparatus, perfusion media of various composition was introduced. The standard perfusate contained (in mM): 133 NaCl, 3.6 KCl, 0.3 $MgCl_2$, 1.0 $CaCl_2$, 16 dextrose, 3.0 Hepes (pH 7.4) and 1.0 μCi ^{45}Ca/ml. In different experiments it was necessary to modify the perfusate composition to include 1 mM $LaCl_3$, or the NaCl concentration was reduced to 35 mM and was replaced with an isosmotic concentration of sucrose.

A typical experiment consisted of perfusing control and treated cell monolayers at 23°C for 30 min with a radioisotope free standard perfusion medium, followed by ^{45}Ca containing perfusate. At an appropriate time, the perfusate was switched to a medium of altered composition, or returned to the radioisotope free standard solution to observe ^{45}Ca washout characteristics. Since the ^{45}Ca associated with the cells on the scintillator disc is counted with a relatively high efficiency (38.8 $\pm$ 1.16%) when compared with the quenched background ^{45}Ca counting efficiency (<5%), on-line monitoring of uptake and washout could be performed continuously[343]. The background count level was high, but stable over many hours of perfusion. Given this stability, and the fact that Primaria-treated discs do not bind Ca^{2+}, changes in cellular Ca^{2+} uptake of $\pm$500 cpm can easily be measured. This represents $\pm 5 \times 10^{-10}$ mol Ca/2 discs. The system was even more sensitive during washout when the background attributable to the chamber falls rapidly to < 200 ct/min. $LaCl_3$ displaceable exchangeable $^{45}Ca^{2+}$ in the smooth muscle cells could be measured by including 1.0 mM $LaCl_3$ in the perfusate.

On completion of Ca^{2+} exchange experiments, the flow cell was drained, disassembled, and the cells scraped onto preweighed filters. These were dried at 100°C for 18 hours, then reweighed to obtain dry tissue weight. The discs, now without the cells, were replaced into the flow cell apparatus and underwent an identical experimental protocol as was completed in the presence of vascular muscle cells. These cell free scintillator disc counts represented background isotopic activity which subsequently could be subtracted from the cell associated ^{45}Ca activity.

^{45}Ca counting efficiency of the cells in the flow cell was determined by comparison to counts made in a liquid scintillation counter and was identical in control and experimental cell preparations. Cells were perfused with normal perfusate which had 4 μCi ^{45}Ca/ml until a near asymptotic value was achieved. The perfusate was then switched to a non-isotopic perfusate and washout continued until it was certain that the flow cell had been cleared of perfusate ^{45}Ca ($\sim$8 min). The final cpm measurement (which was solely cell associated ^{45}Ca) was recorded and the flow cell rapidly drained. The cells were scraped onto filters and placed into a scintillation vial to which 1.0 ml of 0.1 N NaOH was added. This was heated at 80°C for 1 h to solubilize the filter and cell sample, then allowed to cool to room temperature. A 0.1 ml aliquot of 1.0 N HCl was added to neutralize the mixture and then 10 ml of scintillant fluid was added. Radioactivity was measured in a scintillation counter and the cpm obtained was then compared to the final ct/min obtained in the flow cell spectrometer.

VII. Plasma Lipid and Lipoprotein Pattern of Human Subjects

a) Subjects

The study subjects were Inuit males (n=13) of the Keewatin district of the North West Territories greater than 18 years of age who presented to the Churchill Health Centre between May 1, 1989 and June 13, 1989. Control subjects consisted of 12 healthy male Caucasian volunteers aged 18-52 years living in an urban centre.

Examination of the patients included measurements of height and weight. Medical histories were obtained and individuals with documented coronary artery disease or diabetes were excluded from the study.

b) Lipid and Lipoprotein Measurements

All measurements in this study were performed on random draw blood samples which conforms to the guidelines established by the Manitoba Health Services Comission for screening for hypercholesterolemia. All blood was collected at least two hours postprandial to avoid confounding the results with chylomicra. Venous blood samples were collected in the presence and absence of EDTA. Plasma was separated immediately and collected by centrifugation at 1500 x g for 10 min at 4°C, quickly frozen, and maintained at -30°C for later isolation of lipoproteins. Serum was separated from the EDTA-free aliquot by centrifugation at 1500 x g for 10 min at 4°C. Serum samples were maintained at 4°C and sampled for total HDL and LDL cholesterol and triglycerides. None of the samples contained evidence of chylomicrons and all were sampled within 1 week.

Triglyceride concentrations in serum were measured by the enzymatic method of Bucolo and David[353]. Total free and esterified serum cholesterol was assayed by the

enzymatic assay of Sale et al[336] as described. ß-lipoproteins (roughly equivalent to VLDL and LDL fractions) were isolated by precipitation from serum by the addition of phosphotungstate[354]. The remaining supernatant containing lipoproteins (roughly equivalent to the HDL fraction) was assayed for total esterified plus free cholesterol. By using a correction factor, the lipoprotein fraction was corrected for high triglyceride containing particles which correlated with the VLDL fraction.

Lipoprotein isolation from plasma was performed by the method of Lindgren[348], as previosly described. Total apo-protein concentration of the LDL and VLDL solutions was determined by the method of Lowry[341]. HPLC determination of the oxidation products of cholesterol was performed as described above.

VIII. Statistical Analysis

Data were analyzed for statistical significance using a Student's t-test or, where appropriate, a multiple analysis of variance test followed by Duncan's multiple range test for comparison of individual treatment means. A p level less than 0.05 was considered statistically significant.

D. RESULTS

I. Modification of Sterol Content of Sarcolemmal Vesicles

Cardiac sarcolemmal vesicles were incubated at 4°C for various periods of time (0-18 h) in the presence of phosphatidylcholine or cholesterol/phosphatidylcholine liposomes (cholesterol/phospholipid ratio of 2:1 mol/mol). Portions of the mixtures were removed at various times and centifuged at 104,000 x g for 20 min to separate the treated sarcolemmal vesicles from the liposomal vesicles. Previous studies[355] have shown that one centrifugal step is adequate to cleanly separate liposomes from microsomal vesicles. In order to be absolutely sure of this separation, two centrifugal steps were employed in the present study. To futher ensure that any recovered sterol associated with the enriched sarcolemma represented incorporation into the membrane and not entrapment of the liposomes, sarcolemmal vesicles were cholesterol-enriched using [^{14}C]sucrose containing liposomes. Less than 0.5% of the initial [^{14}C]sucrose was associated with sarcolemmal vesicles incubated with either a liposome-free [^{14}C]sucrose medium or a suspension of liposomes containing [^{14}C]sucrose.

Figure 2 shows a typical sterol modulation experiment. After a 16-18-h incubation with pure phosphatidylcholine vesicles, a 17% depletion in sarcolemmal membrane cholesterol content was observed, although this was not statistically significant. The depletion of cholesterol was time-dependent but relatively slow, requiring overnight (ON) treatment. Incubation of sarcolemma with liposomes rich in cholesterol (2:1 mol/mol cholesterol/phospholipid) resulted in a rapid transfer of

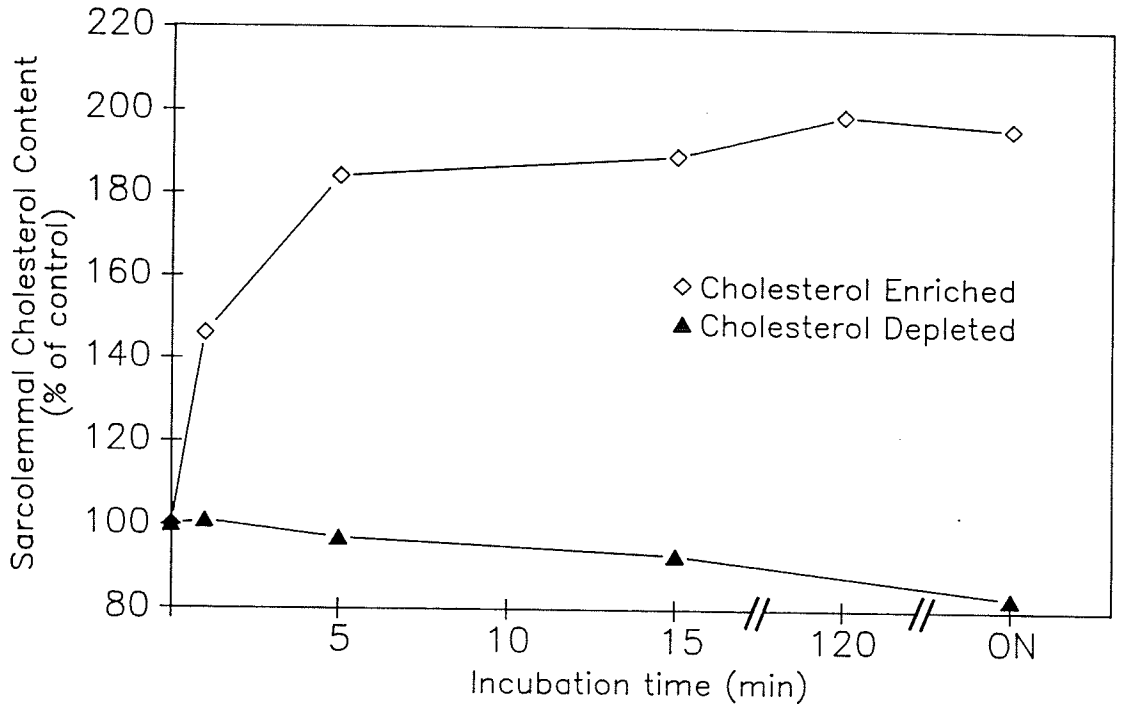


FIGURE 2. *Time dependence of cholesterol incorporation and depletion in sarcolemmal membrane vesicles.* Cholesterol enrichment of membrane vesicles was performed by incubation of sarcolemma with 2:1 cholesterol/phospholipid liposomes at 4°C. Cholesterol-depleted membrane preparations were obtained by incubation of sarcolemmal vesicles with cholesterol-free phosopholipid liposomes at 4°C. See "Materials and Methods" for details. ON = overnight incubation. These data represent the results of a typical experiment.

cholesterol to the membrane vesicles during the first 5 min of exposure. After 18 h, cholesterol enrichiment was 96% over control values.

In a further attempt to control the amount of cholesterol transferred, sarcolemmal vesicles were incubated with liposomes prepared with increasing proportions of cholesterol to phospholipid. As shown in Figure 3, maximal cholesterol enrichment was accomplished using liposomes prepared with an initial cholesterol/phospholipid ratio of 1.5:1 or 2:1. Less cholesterol transfer occurred when liposomes of lower cholesterol/phospholipid ratios were used.

II. Effects of Cholesterol on Ion Transport Activity

The effects of alterations of membrane cholesterol content on Na^+-Ca^{2+} exchange were monitored during the linear phase of the exchange process (1.5 s). Enrichment of sarcolemmal membranes with cholesterol resulted in a stimulation in Na^+-Ca^{2+} exchange activity (Figure 4). With an increase in cholesterol of 64% above control values, there was a corresponding 30% increase in Na^+-Ca^{2+} exchange activity. Modification of sarcolemmal cholesterol content with liposomes of lower cholesterol:phospholipid ratio resulted in no significant change in Na^+-Ca^{2+} exchange.

Na^+-Ca^{2+} exchange in control, cholesterol-depleted, and cholesterol-enriched sarcolemmal membranes was examined as a function of assay reaction time (Figure 5). Similar to the results presented in Figure 4, enrichment of membrane vesicles with cholesterol resulted on a 25% increase in the initial rate of exchange (1.5 s). At longer incubation times, the cholesterol-enriched membrane vesicles exhibited a 48% greater

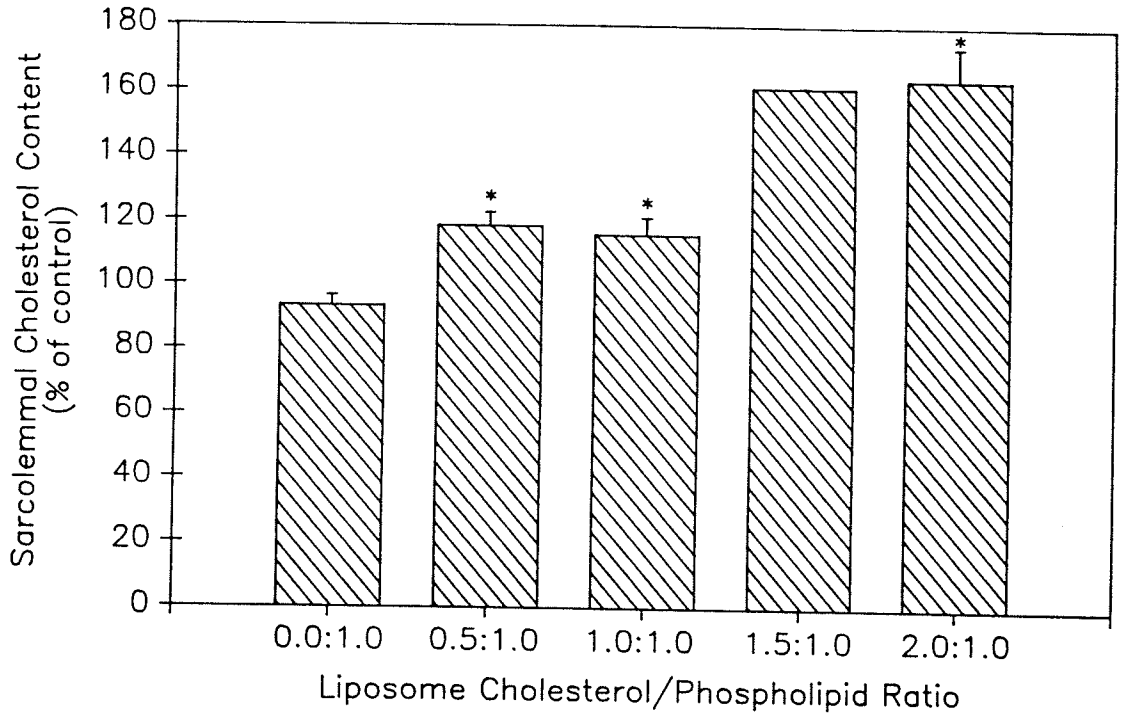


FIGURE 3. *Effect of liposomal cholesterol/phospholipid ratio on cholesterol content of sarcolemma.* Sarcolemmal vesicles were incubated at 4°C for 16-18 h with liposomes prepared with various cholesterol/phospholipid ratios. Ratios indicated represent the mol fraction of initial lipid composition before sonication. See "Materials and Methods" for details. Results are expressed as the average ± S.E. percent increase in cholesterol content over controls (control cholesterol content = 0.49 ± 0.05 μmol/mg of protein). (n = 5-7 except for *bar* at 1.5:1.0 cholesterol/phospholipid which is the mean of two experiments). *, $p<0.05$ *versus* control values.

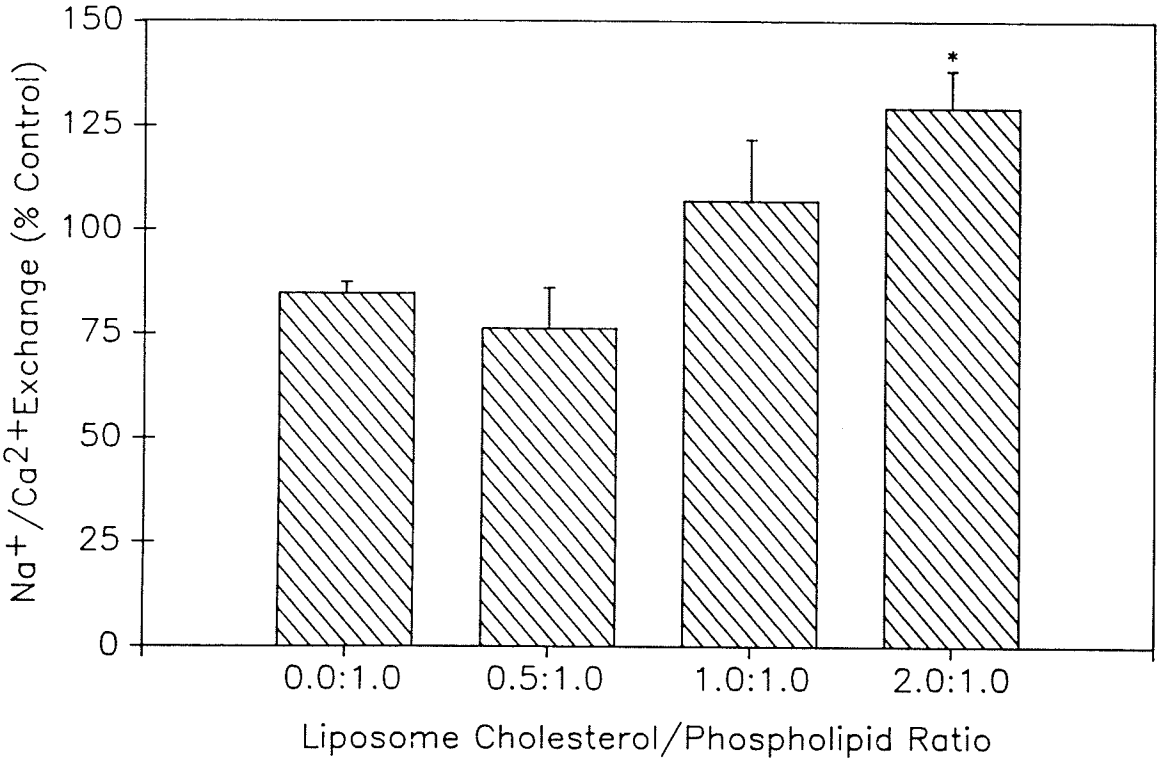


FIGURE 4. *Effect of liposomal cholesterol/phospholipid ratio on Na^+-Ca^{2+} exchange.* Sarcolemmal vesicles were incubated at 4°C for 16-18 h with liposomes prepared with various cholesterol/phospholipid ratios. Ratios indicated represent the mol fraction of initial lipid composition before sonication. The Na^+_i-dependent Ca^{2+} uptake reaction proceeded for 1.5 s at $[Ca^{2+}]$ = 10 μM. Control Na^+-Ca^{2+} exchange was 2.3 $\pm$ 0.5 nmol of Ca^{2+} accumulated/mg/s. See "Materials and Methods" for details. Values represent means $\pm$ S.E. percent of control (n = 5). *, $p<0.05$ *versus* control values.

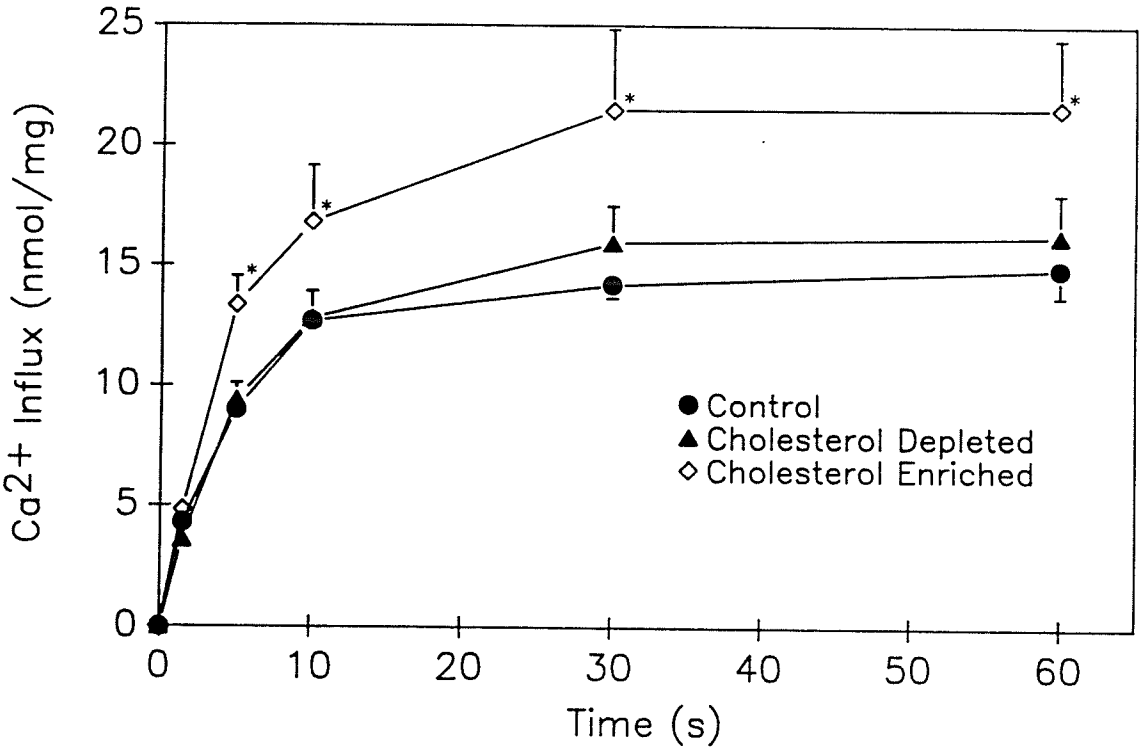


FIGURE 5. *Time dependence of sarcolemmal Na^+-Ca^{2+} exchange in control, cholesterol-depleted, and cholesterol-enriched sarcolemmal membrane preparations.* Cholesterol depletion of sarcolemmal vesicles was performed by incubation of membrane with cholesterol-free phospholipid liposomes for 16-18 h at 4°C. Cholesterol-enriched vesicles were obtained by overnight incubation of sarcolemma with 2:1 cholesterol/phospholipid liposomes at 4°C. The Na^+_i-dependent Ca^{2+} influx reaction proceeded at various times at $[Ca^{2+}] = 10\ \mu M$. See "Materials and Methods" for details. Values represent means ± S.E. (n = 4-6). *, $p<0.05$ *versus* control values.

Ca^{2+} accumulating ability than control vesicles. No significant differences from control were observed in cholesterol-depleted vesicle preparations.

Figure 6 presents the results from several experiments relating Na^+-Ca^{2+} exchange to the sarcolemmal membrane cholesterol content. The exchange shows a positive correlation with cholesterol content ($r = 0.7$).

The dependence of the initial rate of Na^+_i-dependent Ca^{2+} uptake on Ca^{2+} concentrations in control, cholesterol-depeleted, and cholesterol-enriched membranes is shown as a Lineweaver-Burk plot in Figure 7. The K_m for the control preparations was found to be 22.3 ± 3.8 μM which is similar to that reported by others[230,322,356]. Cholesterol enrichment of sarcolemmal vesicles increased the apparent affinity of the exchange mechanism for Ca^{2+} ($K_m = 17.3 \pm 0.9$ μM), while cholesterol depletion had the opposite effect ($K_m = 26.5 \pm 7.2$ μM). The V_{max} of the exchange mechanism for control, cholesterol-depleted, and cholesterol-enriched membrane vesicles was 7.9 ± 0.9, 7.4 ± 1.0 and 8.0 ± 0.8 nmol/mg/s, respectively.

For comparative purposes, the effect of cholesterol enrichment on the sarcolemmal Ca^{2+} pump (Ca^{2+}-Mg^{2+}-ATPase) is shown in Figure 8. The Ca^{2+} accumulating ability of the Ca^{2+} pump is inhibited 40% by cholesterol enrichment at all incubation times.

Since the observed alteration in the Ca^{2+} accumulating abilities of the isolated sarcolemmal vesicles may be due to changes in the Ca^{2+} permeability of these vesicles, it was important to examine passive Ca^{2+} efflux from control, cholesterol-enriched, and cholesterol-depleted membrane preparations after loading the vesicles with $^{45}Ca^{2+}$. No

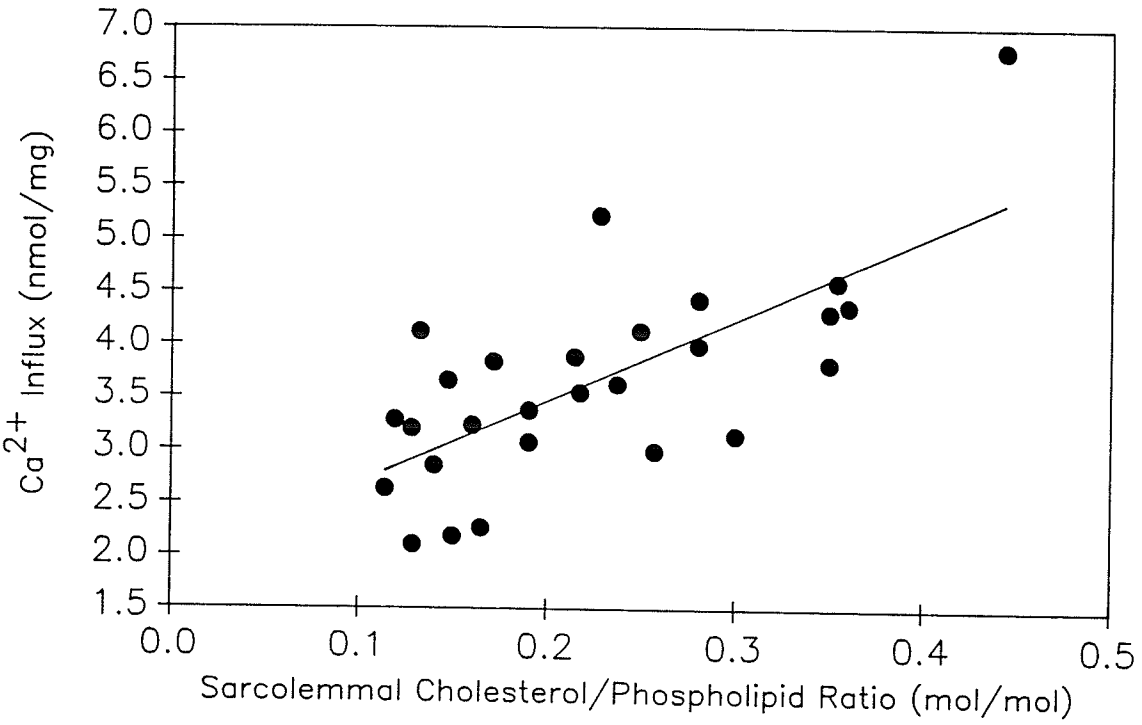


FIGURE 6. *Na^{+}_{i}-dependent Ca^{2+} influx rate as a function of membrane cholesterol/phospholipid ratio.* Sarcolemmal membrane vesicles were incubated overnight with liposomes of various cholesterol/phospholipid ratios. See "Materials and Methods" for details. Ca^{2+} uptake occurred for 1.5 s at $[Ca^{2+}] = 10\ \mu M$.

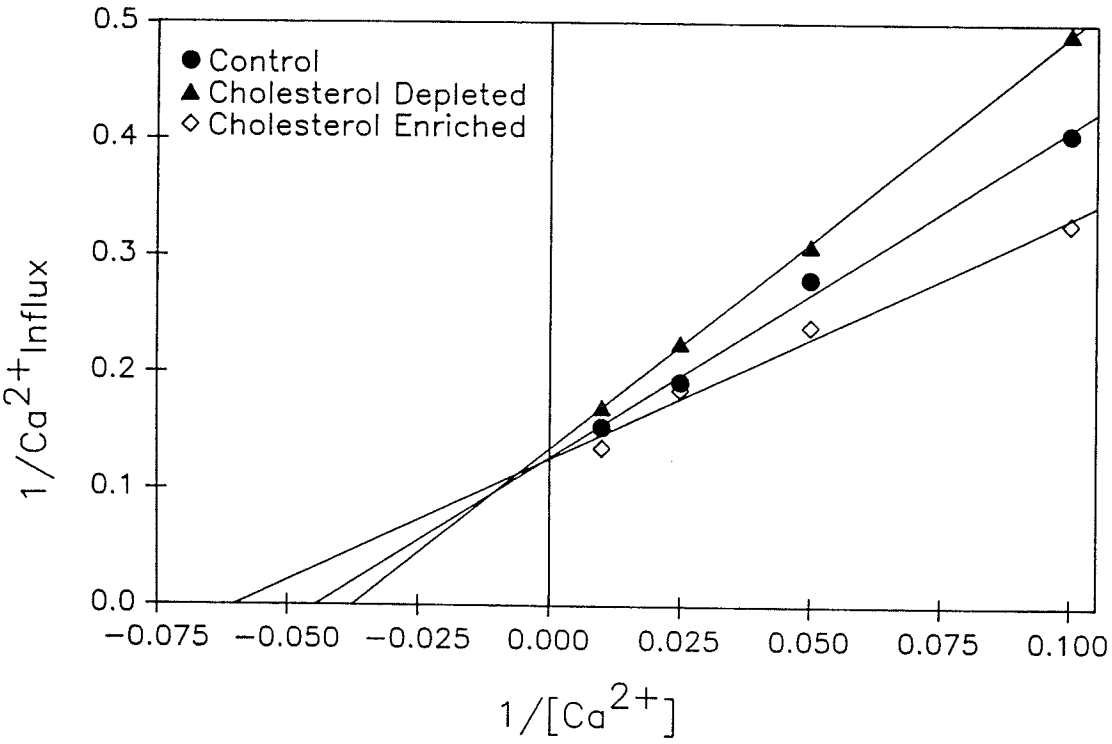


FIGURE 7. *Lineweaver-Burk plot of the Ca^{2+} dependence of Na^{+}_{i}-dependent Ca^{2+} influx in control, cholesterol-depleted, and cholesterol-enriched membrane preparations.* Time for Ca^{2+} uptake was 1.5 s (n = 3).

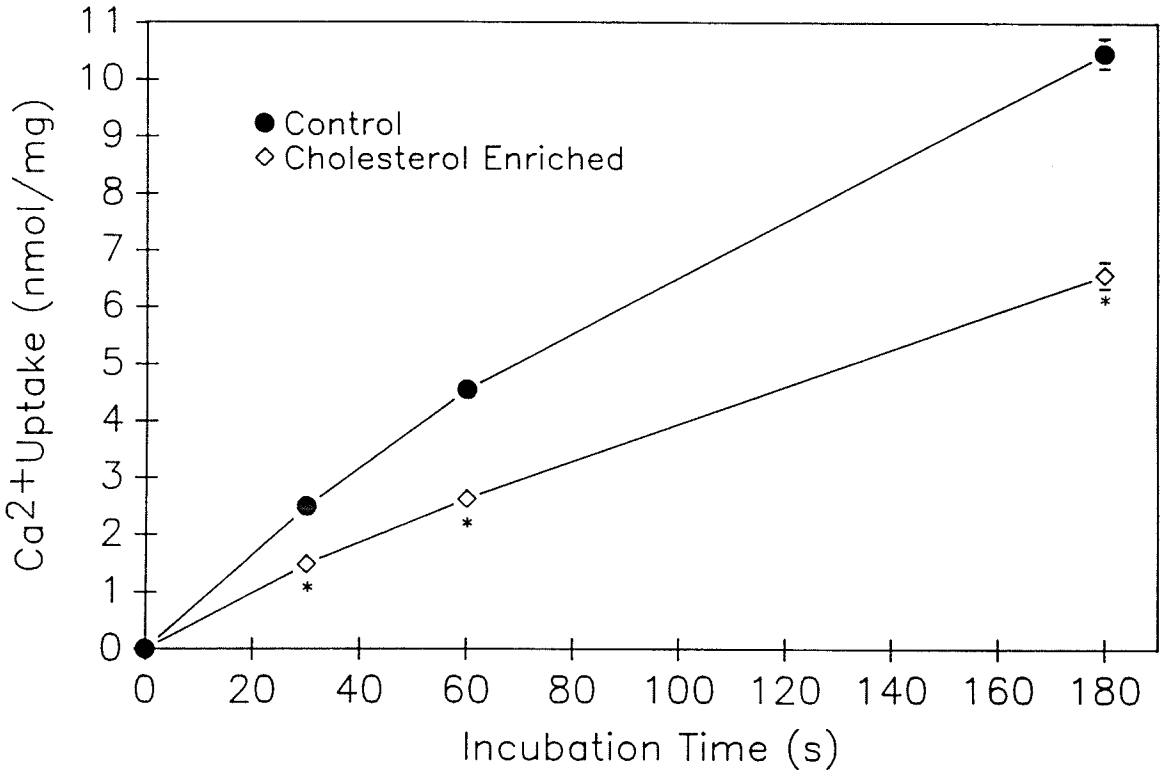


FIGURE 8. *ATP-dependent Ca^{2+} uptake in control and cholesterol-enriched sarcolemma vesicles.* See "Materials and Methods" for details (n =4). *, $p<0.05$ *versus* control values.

differences were observed in passive membrane permeability of treated vesicles compared with control preparations (Figure 9).

It has been suggested that sarcolemmal bound calcium may be involved in the Na^+-Ca^{2+} exchange process[357]. Therefore, it was imperative that the effect of modification of membrane cholesterol content on energy-independent Ca^{2+} binding to sarcolemmal vesicles be investigated. As shown in Table I, no significant differences were observed in the amount of Ca^{2+} bound at any Ca^{2+} concentration examined.

Table II shows the effect of cholesterol enrichment and depletion on Na^+,K^+-ATPase and K^+-*p*-nitrophenylphosphatase activities of control, cholesterol-enriched, and cholesterol-depleted membrane preparations. A reduction in the Na^+,K^+-ATPase activity in cholesterol-depleted membrane preparations was observed. The same qualitative response was seen in cholesterol-enriched membranes although the reduction in Na^+,K^+-ATPase activity was not as marked. This inhibitory trend persisted in the presence or absence of alamethicin.

III. Effects of Oxidation of Cholesterol on Ion Transport

To obtain further information regarding the nature of the cholesterol-modulating effect on transsarcolemmal calcium movements, the effects of cholesterol modification *in situ* were examined. Cardiac sarcolemmal vesicles were treated with cholesterol oxidase which converts cholesterol to several oxidized derivatives, one of which has been identified as cholest-4-en-3-one (cholestenone), as shown in Figure 10. After treatment of sarcolemma with cholesterol oxidase (16 U/ml; $\approx$0.14 U/μg sarcolemmal protein),

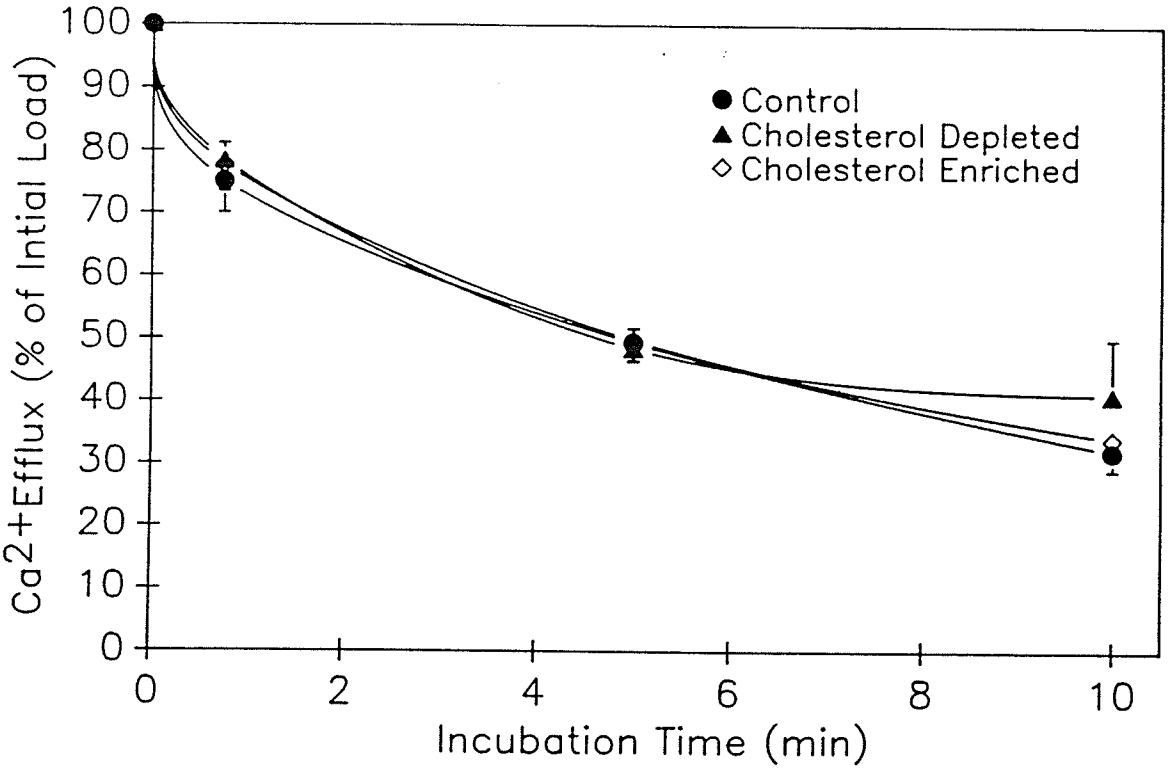


FIGURE 9. *Time dependence of passive Ca^{2+} efflux.* Control, cholesterol-depleted, and cholesterol-enriched membrane vesicles were first loaded with Ca^{2+} by Na^{+}_{i}-dependent Ca^{2+} influx for 2 min in 0.25 ml of uptake medium. After 2 min, 0.25 ml of EGTA solution was added, and Ca^{2+} efflux was allowed to proceed for various times. See "Materials and Methods" for details. The data are presented as the percent of the initial Ca^{2+} load remaining in the membrane vesicles. The initial Ca^{2+} loads were: control, 32 $\pm$ 1 nmol/mg protein; cholesterol-depleted, 34 $\pm$ 1 nmol/mg protein; cholesterol-enriched, 37 $\pm$ 1 nmol/mg protein (n = 3).

TABLE 1

Calcium Concentration	Calcium Bound		
	Control membranes	Cholesterol-depleted membranes	Cholesterol-enriched membranes
mM		nmol/mg protein	
0.05	7.0 ± 0.3	7.0 ± 0.4	7.0 ± 0.2
0.50	38.7 ± 2.0	38.0 ± 2.1	39.1 ± 2.1
1.00	65.4 ± 2.1	69.2 ± 3.7	72.0 ± 2.7
2.50	132.2 ± 4.2	134.6 ± 5.8	136.5 ± 6.2

Effect of cholesterol depletion and enrichment on energy-independent Ca^{2+} binding to isolated sarcolemmal membranes

Cholesterol depletion involved overnight incubation of sarcolemmal vesicles with cholesterol-free phospholipid liposomes, and cholesterol enrichment was performed by incubating membrane vesicles with 2:1 cholesterol/phospholipid liposomes. See "Experimental Procedures" for Ca^{2+} binding protocol. Values are expressed as mean ± S.E. (n = 6).

TABLE 2

Effect of Cholesterol Depletion and Enrichment on K^+-pNPPase and Na^+ K^+ ATPase in Isolated Sarcolemmal Membranes

Cholesterol depletion involved overnight incubation of sarcolemmal vesicles with cholesterol free phospholipid liposomes and cholesterol enrichment was performed by incubating membrane vesicles with 2:1 cholesterol/phospholipid liposomes.

See "Experimental Procedures" for K^+-pNPPase and Na^+ K^+ ATPase assay procedures. Values represent means ± S.E. (n=5).

	Control Membranes	Cholesterol Depleted Membranes	Cholesterol Enriched Membranes
K^+-pNPPase activity (umol phenol/mg/h)	23.0 ± 1.9	22.4 ± 3.5	22.5 ± 1.3
% of control		97.8 ± 14.0	98.8 ± 5.5
Na^+ K^+ ATPase activity (umol Pi/mg/h)	79.1 ± 2.4	58.3 ± 2.3*	65.3 ± 3.1*
% of control		73.8 ± 2.7	82.4 ± 1.7
Na^+ K^+ ATPase activity + alamethicin (umol Pi/mg/h)	213.8 ± 9.5	189.6 ± 12.4	199.0 ± 8.8
% of control		84.5 ± 4.6	92.6 ± 3.6

* $p < 0.05$ vs control values

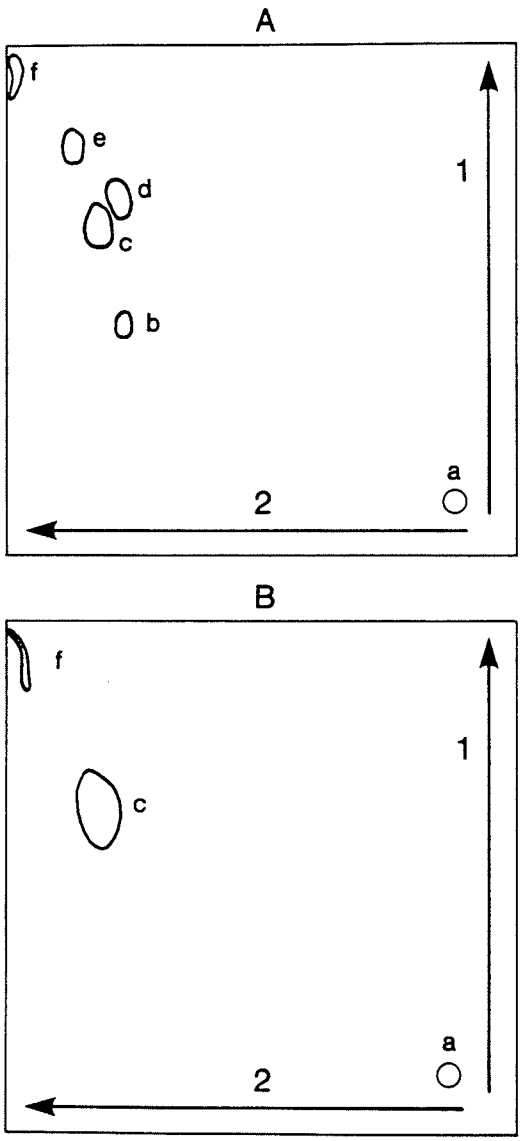


FIGURE 10. *Illustration of two-dimensional thin layer chromatographic plates of cholesterol oxidase-treated and control sarcolemmal vesicles.* **A**, cholesterol oxidase-treated sarcolemma; **B**, control sarcolemma. a, origin; b, unidentified oxidation product; c, native cholesterol; d, cholest-4-en-3-one; e, unidentified oxidation product; f, solvent front materials.

cholest-4-en-3-one was generated (Figure 11) in a rapid, time-dependent manner in the myocardial membrane vesicles (Figure 12). The oxidation product was observed as early as 1.5 seconds after incubation and appeared to exhibit equilibrium after 5-10 minutes of exposure. Generation of cholestenone from cholesterol was also dependent on the cholesterol oxidase concentration (Figure 13).

Na^+-Ca^{2+} exchange was measured as Na^+_i-dependent Ca^{2+} uptake in the isolated cardiac sarcolemmal vesicles. The reaction was linear through the first 2 seconds of the reaction, then exhibited equilibrium by 30-60 seconds. This pattern is similar to results reported previously, as is the absolute activity demonstrated here (Figure 14)[321,330]. Inclusion of 2.0 units/ml cholesterol oxidase in the reaction medium inhibited Na^+-Ca^{2+} exchange. As shown in the inset graph of Figure 14, this inhibition was particularly striking (67%) during the early linear part of the reaction (0.5 seconds), then lessened as the reaction progressed. Approximately 30% inhibition was observed during the nonlinear phase of the reaction (5-60 seconds).

A separate series of experiments was conducted to determine the concentration dependency of the cholesterol oxidase effects. From 1 to 20 units/ml cholesterol oxidase, Na^+-Ca^{2+} exchange (1.5-second reaction time) was inhibited in a concentration dependent manner (Figure 15). Twenty units of cholesterol oxidase almost eliminated exchange activity.

The dependence of the initial rate of Na^+-Ca^{2+} exchange on $[Ca^{2+}]$ in untreated and cholesterol oxidase-treated sarcolemmal vesicles is shown as a double reciprocal plot in Figure 16. Reaction time was 1.0 second, and the cholesterol oxidase concentration

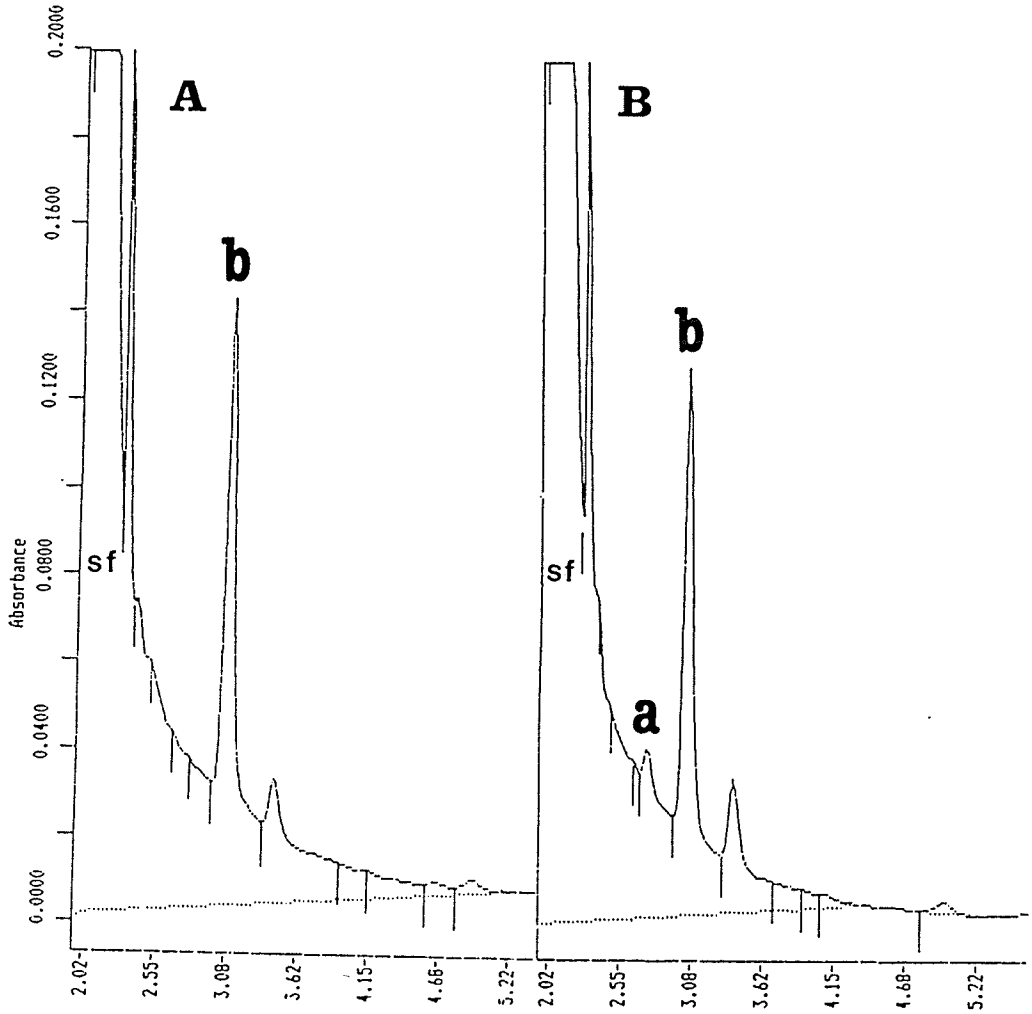


FIGURE 11. *Representative chromatographs obtained from high-performance liquid chromatography analysis of lipids extracted from cardiac sarcolemma before (Panel A) and after (Panel B) exposure to 7 units of cholesterol oxidase for 30 seconds.* Peak a, cholestenone (cholest-4-en-3-one); peak b, cholesterol; peak sf, solvent front.

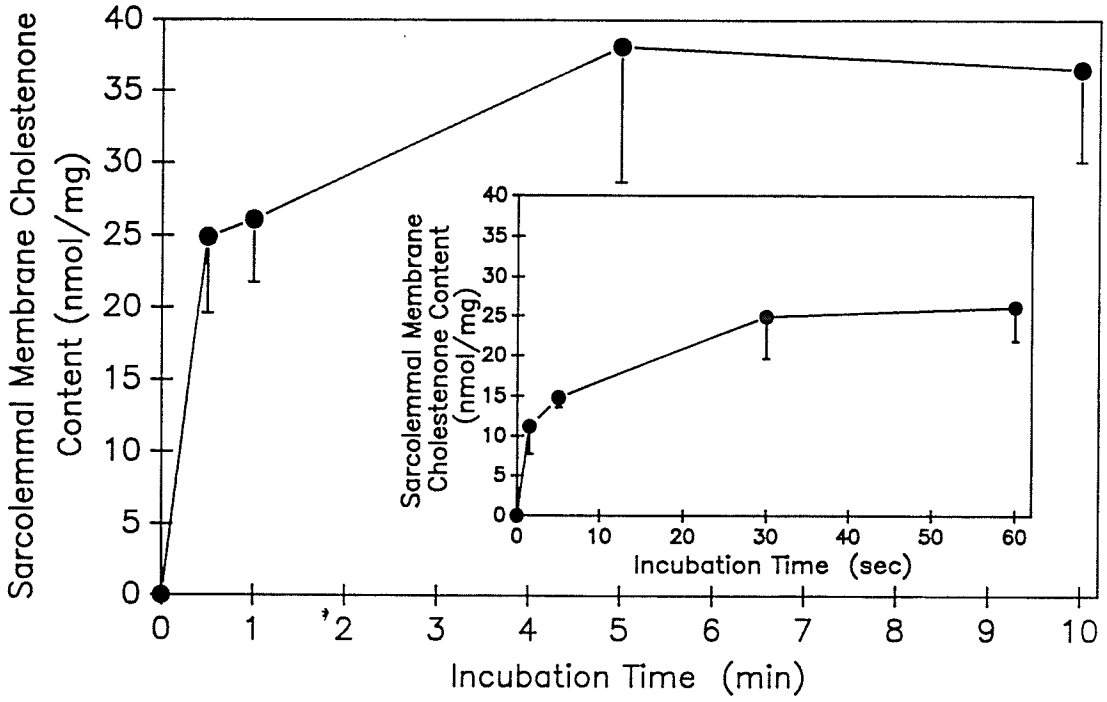


FIGURE 12. *Early (inset) and late time course for the generation of cholestenone (cholest-4-en-3-one) from cholesterol in cardiac sarcolemmal membranes after exposure to cholesterol oxidase.* Values represent mean for five experiments. Cholesterol oxidase concentration = 16 U/ml (= 0.14 U/μg sarcolemmal protein). See "Materials and Methods" for experimental details.

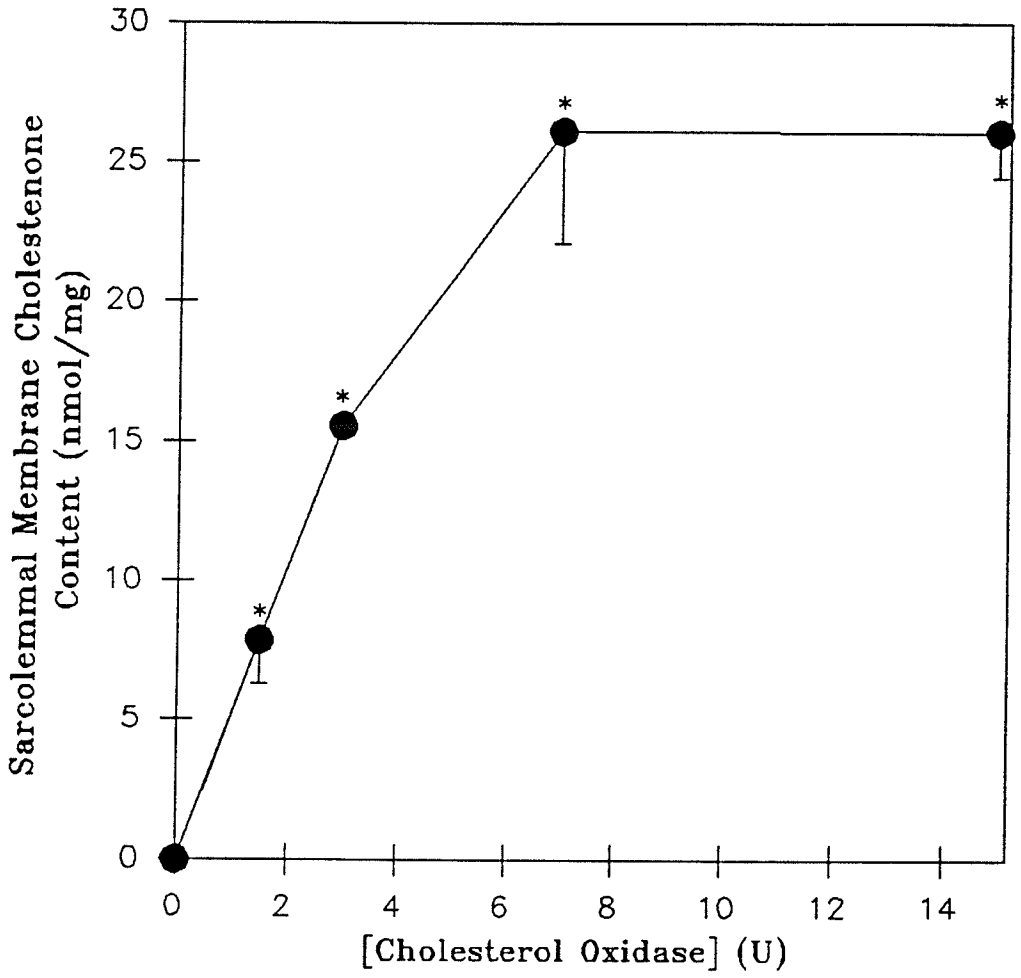


FIGURE 13. *Concentration dependency of cholesterol oxidase for the generation of cholestenone from in situ sarcolemmal membrane cholesterol.* Values represent the mean ± SEM for four to six samples. Reaction time was 1 minute. Cholesterol oxidase concentrations are expressed as U/ml (=U/18 mg sarcolemmal protein). *, $p < 0.05$ *versus* control values.

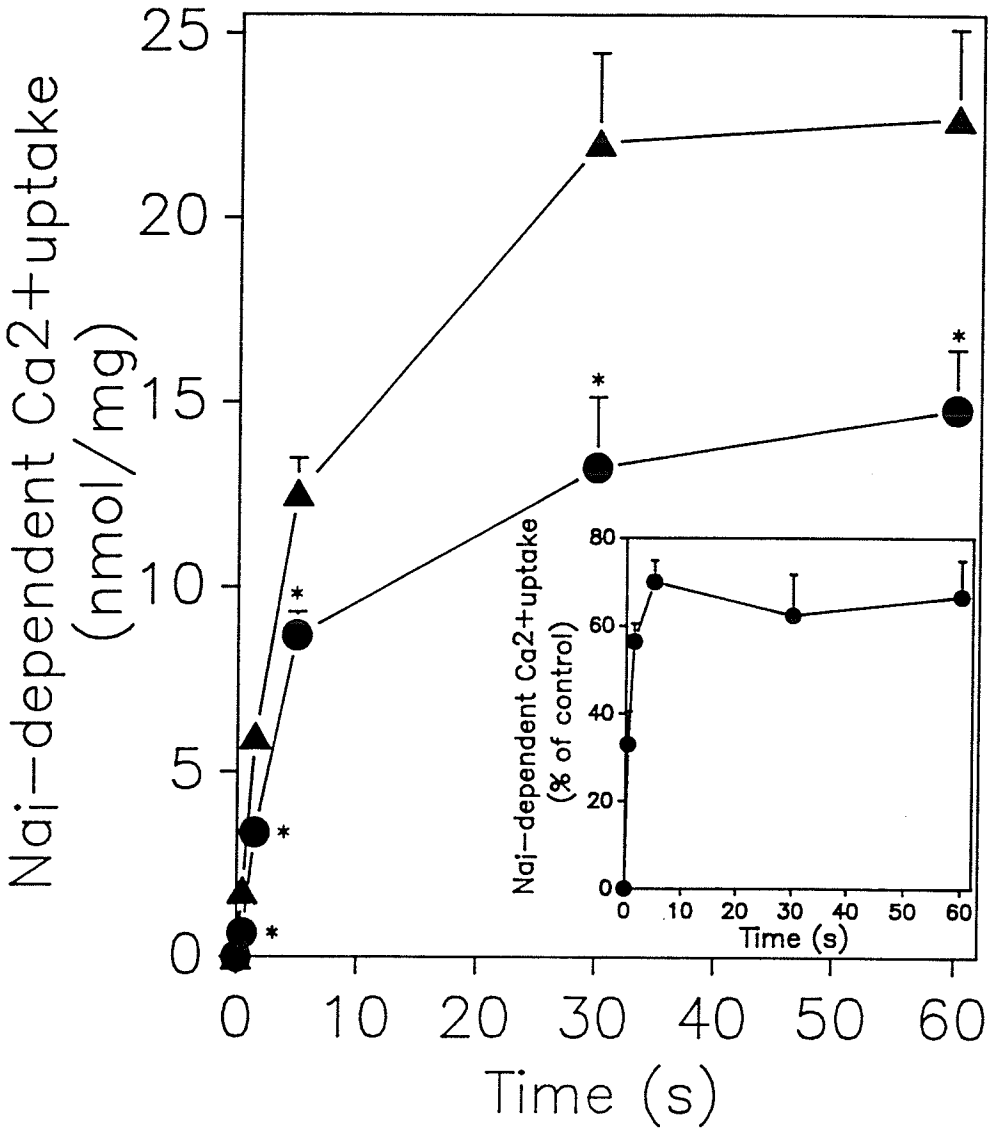


FIGURE 14. *Time dependency of Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles with (●) or without (▲) cholesterol oxidase (2.0 units/ml) treatment.* Values are mean ± SEM (n=5). Insert graph depicts same data presented as a percent of untreated values. Absolute values in the absence of treatment were 1.78 ± 0.16, 5.97 ± 0.19, 12.53 ± 0.85, 22.04 ± 2.17, and 22.70 ± 2.16 nmol Ca^{2+}/mg for 0.5, 1.5, 5, 30, and 60 seconds of reaction time, respectively. *,$p<0.05$ *versus* control values.

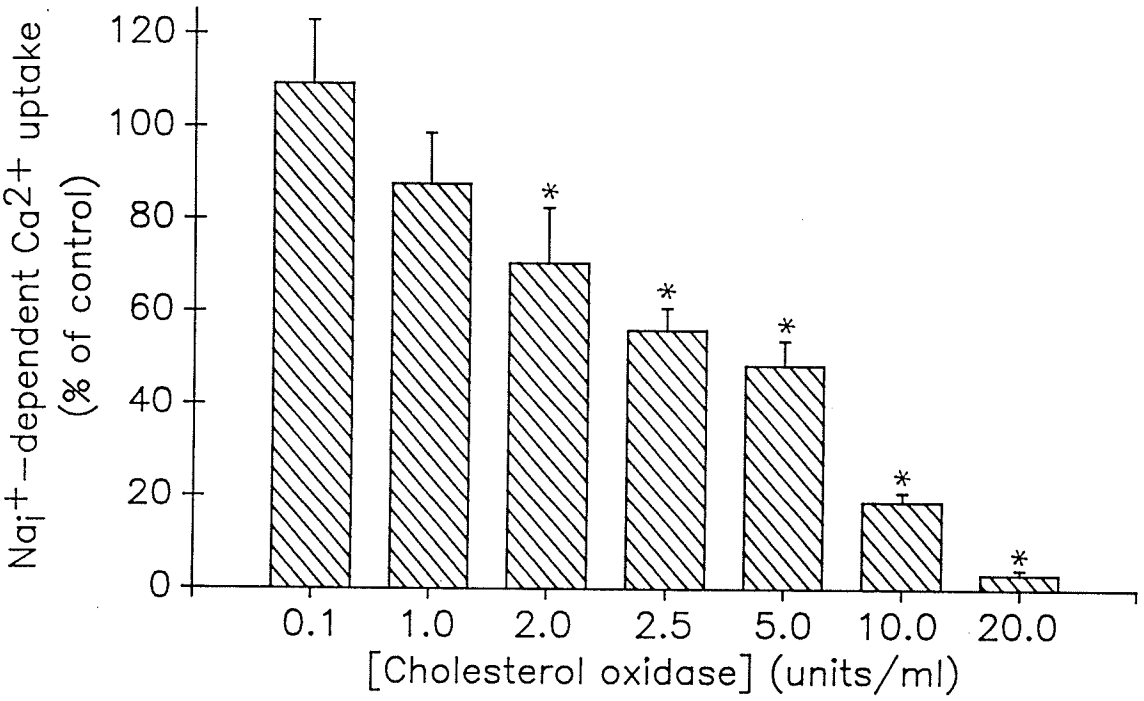


FIGURE 15. *Na^+-Ca^{2+} exchange examined in the presence of varying concentrations of cholesterol oxidase.* Values represent mean ± SEM of three to five experiments. The control value (100%) in the absence of cholesterol oxidase treatment was 4.14 ± 0.78 nmol Ca^{2+}/mg/sec. *,$p < 0.05$ *versus* control values.

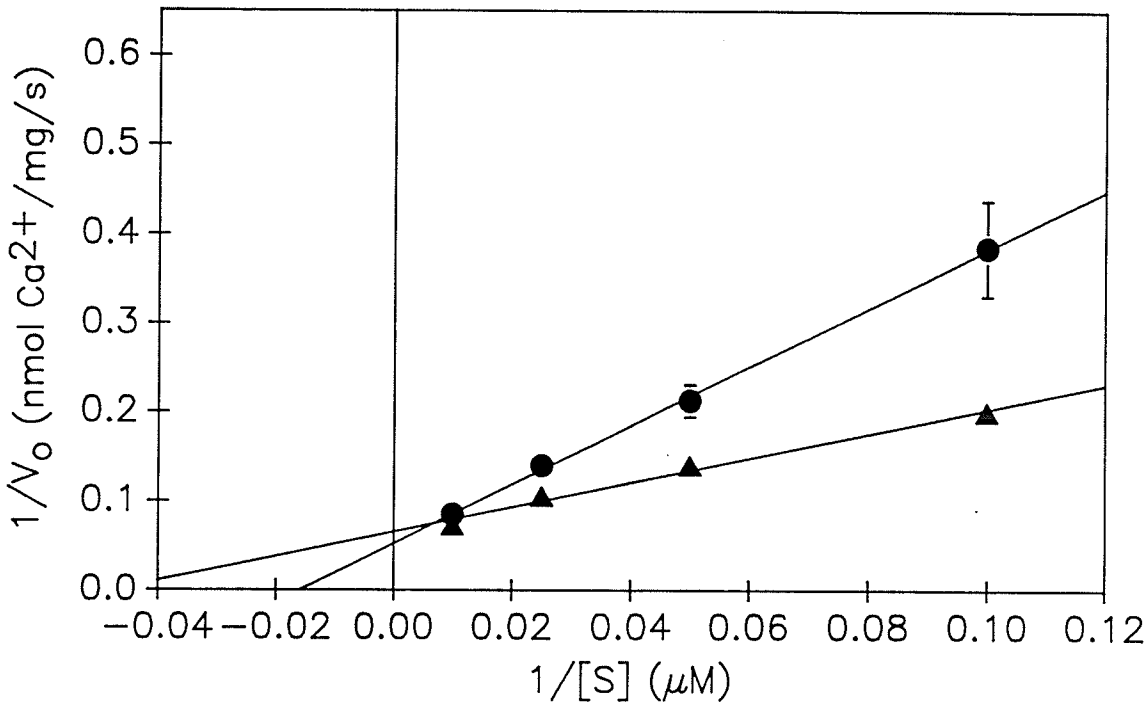


FIGURE 16. *Lineweaver-Burke plot of Na^+-Ca^{2+} exchange in untreated* (▲) *and cholesterol oxidase-treated* (●) *sarcolemmal vesicles.* Absolute values for Na^+-Ca^{2+} exchange in untreated sarcolemma were 5.08 ± 0.33, 7.22 ± 0.39, 9.77 ± 0.66, and 13.87 ± 1.32 nmol Ca^{2+}/mg/sec at 10, 20, 40, and 100 μM Ca^{2+}, respectively. Values represent mean ± SEM of four experiments. If the standard error bar is not present, the data point was larger than the standard error.

was 2.5 units/ml. The K_m for the control preparations (n=4) was 21.73 $\pm$ 1.85 μM, which is similar to values reported previously[321,330]. Cholesterol modification in the membrane vesicles strikingly increased the K_m to 84.03 $\pm$ 10.94 μM (n=4). The V_{max} for the reaction was 15.87 $\pm$ 1.40 and 24.58 $\pm$ 2.72 nmol/mg/sec in the untreated and cholesterol oxidase-treated vesicles, respectively (n=4).

To ensure that the effects of cholesterol oxidase on Na^+-Ca^{2+} exchange activity were due to modified cholesterol effects and not due to the effects of membrane peroxidation, sarcolemmal vesicles were treated with either cholesterol oxidase or H_2O_2 in the presence and absence of catalase. The results of a typical oxidation experiment are shown in Figure 17. On pretreatment of the sarcolemmal vesicles with 4 units cholesterol oxidase per mg membrane protein for 15 min, Na^+-Ca^{2+} exchange activity was virtually eliminated. Since 1 mol of H_2O_2 is generated per mol of cholesterol oxidized, oxidation of all membrane cholesterol with cholesterol oxidase would yield 0.5 μmol of H_2O_2/mg of protein. On pretreatment of the vesicles with increased H_2O_2 (50 μmol/mg of protein) for 15 min, a 30% reduction in enzyme activity was observed. This decrease in Na^+-Ca^{2+} exchange activity caused by 50 μmol of H_2O_2/mg of protein was prevented by the inclusion of catalase in the reaction mixture. Catalase, however, did not protect the membrane vesicles from the inhibitory effects of cholesterol oxidase on the Na^+-Ca^{2+} exchanger.

It seemed possible that the depression in exchange was due to an increase in the permeability properties of the membrane. Thus, experiments were carried out to test the relative permeability characteristics of the untreated and cholesterol-modified membranes

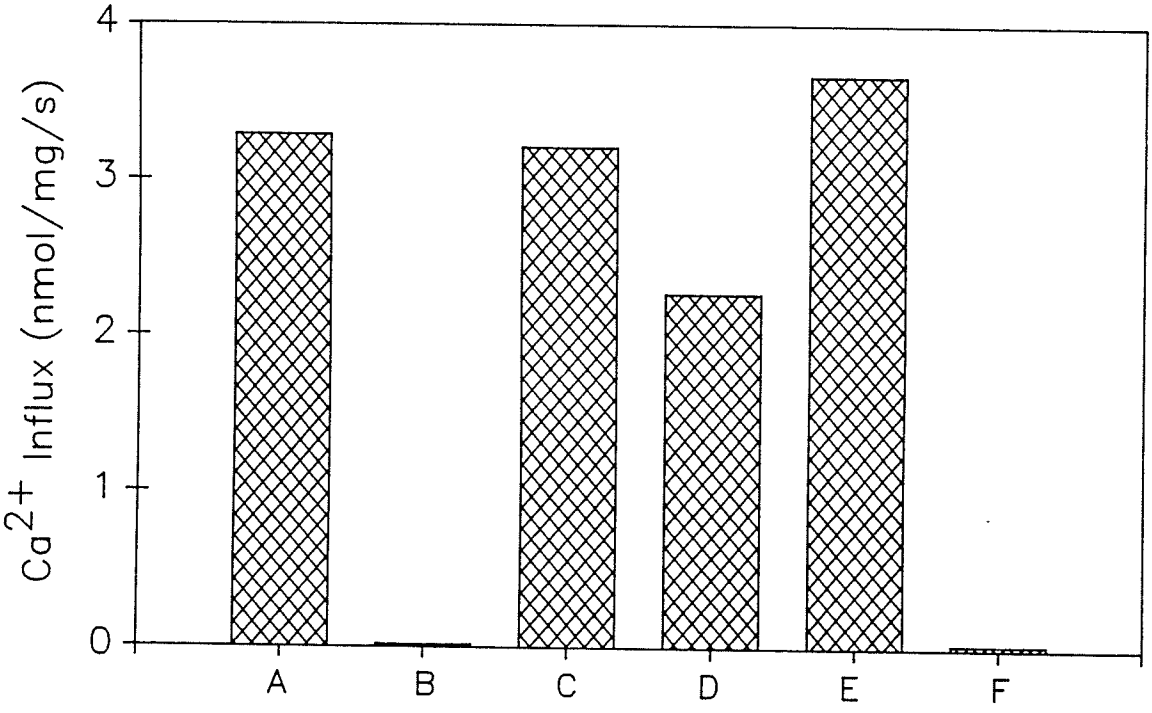


FIGURE 17. *Effect of cholesterol oxidase and H_2O_2 treatment on Na^+-Ca^{2+} exchange.* A, Na^+_i-dependent Ca^{2+} influx in untreated membrane vesicles. B, exchange activity in cholesterol oxidase (4 units/mg protein)-treated sarcolemma. C, membrane treated with 0.05 μmol of H_2O_2/mg of sarcolemmal protein. D, membrane treated with 50 μmol of H_2O_2/mg of protein. E, membrane treated with 50 μmol of H_2O_2/mg of protein in the presence of 300 units of catalase/mg of protein. F, membrane treated with cholesterol oxidase in the presence of 300 units of catalase/mg of protein. See "Materials and Methods" for details. Data are the results of a typical experiment.

to Ca^{2+} flux. Vesicles were loaded with $^{45}Ca^{2+}$ via Na^{+}-Ca^{2+} exchange (initial load was 7.42 $\pm$ 0.67 nmol/mg), then diluted into a medium which was optimal to allow for the passive efflux of $^{45}Ca^{2+}$ from the vesicles, both in the presence and absence of cholesterol oxidase. As shown in Figure 18, after 30 seconds of efflux, vesicular Ca^{2+} content had declined about 10% in the control vesicles. Inclusion of cholesterol oxidase in the efflux medium up to 2.5 units/ml had no significant effects on this efflux rate. However, 10 units/ml did significantly enhance the passive efflux of Ca^{2+} from the sarcolemmal vesicles, indicating that the passive permeability characteristics of the membrane had been compromised. Further study of the time dependency of the efflux reaction (Figure 19) demonstrated that even low cholesterol oxidase concentrations (1.5 U/ml) could increase passive Ca^{2+} efflux from the vesicles if the incubation time was substantially increased up to 5 minutes. This effect was accentuated in the presence of 10 units/ml cholesterol oxidase.

For the purpose of comparison, the effects of *in situ* cholesterol modification on another transsarcolemmal ion transport system, the Ca^{2+} pump, was studied. ATP-dependent Ca^{2+} uptake by the cardiac sarcolemmal vesicles was studied as a function of reaction time in the presence or absence of 4 units/ml cholesterol oxidase (Figure 20). The absolute activity for the control vesicles was 5.48 $\pm$ 0.71 nmol Ca^{2+} taken up per mg/min, which is in the same range as values reported elsewhere[321]. As shown in the inset graph of Figure 20, cholesterol oxidase inhibited ATP-dependent Ca^{2+} uptake by 22%, 36%, and 52% at 15, 30, and 60 seconds of reaction time, respectively.

The concentration dependence of the inhibition of ATP-dependent Ca^{2+} uptake by

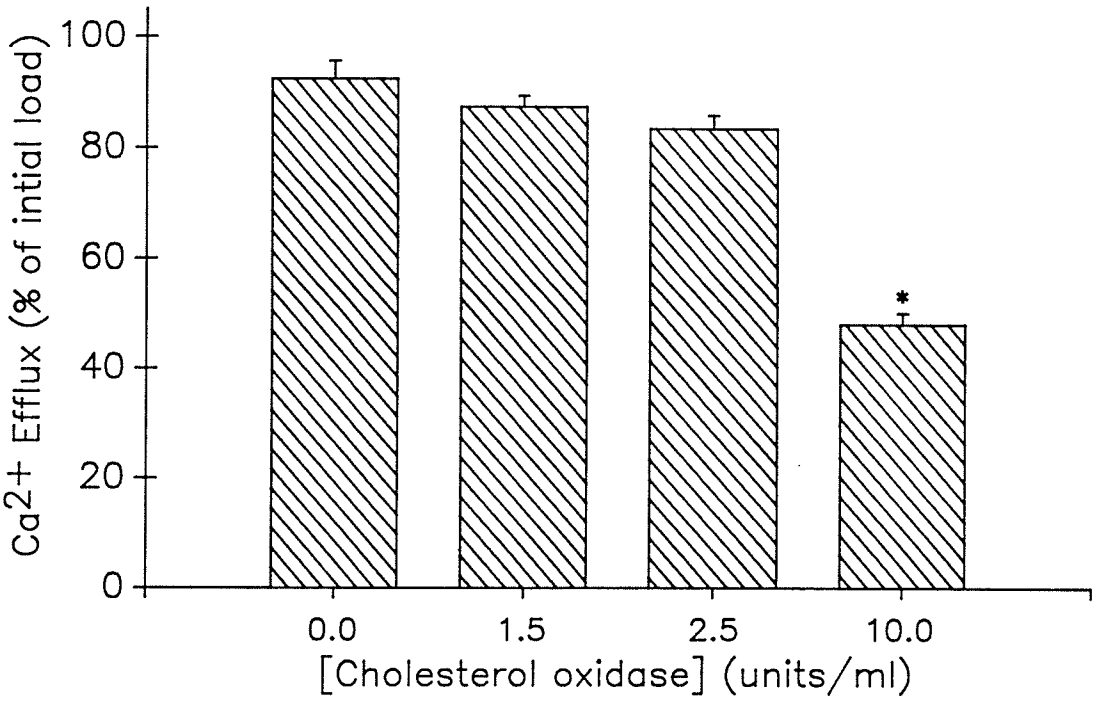


FIGURE 18. *Effects of varying concentrations of cholesterol oxidase on passive efflux of $^{45}Ca^{2+}$ from cardiac sarcolemmal vesicles.* Efflux time was 30 seconds. Results are mean ± SEM of four separate experiments. Values are presented as a percent of total vesicular $^{45}Ca^{2+}$ content at time 0 before initiation of efflux. Cholesterol oxidase, if present, was included only during the efflux period. *, $p < 0.05$ *versus* untreated vesicles.

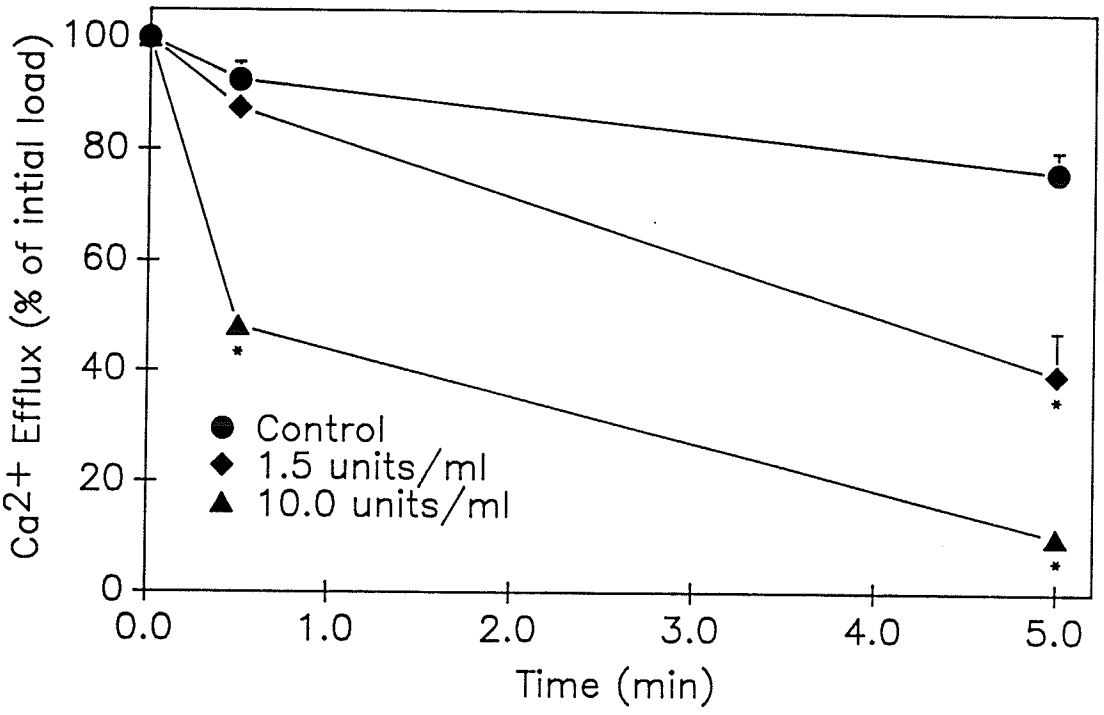


FIGURE 19. *Time dependency of passive Ca^{2+} efflux from sarcolemmal vesicles in the presence of varying concentrations of cholesterol oxidase.* (n=3). Refer to Figure 14 for further experimental details. *,$p<0.05$ *versus* control values.

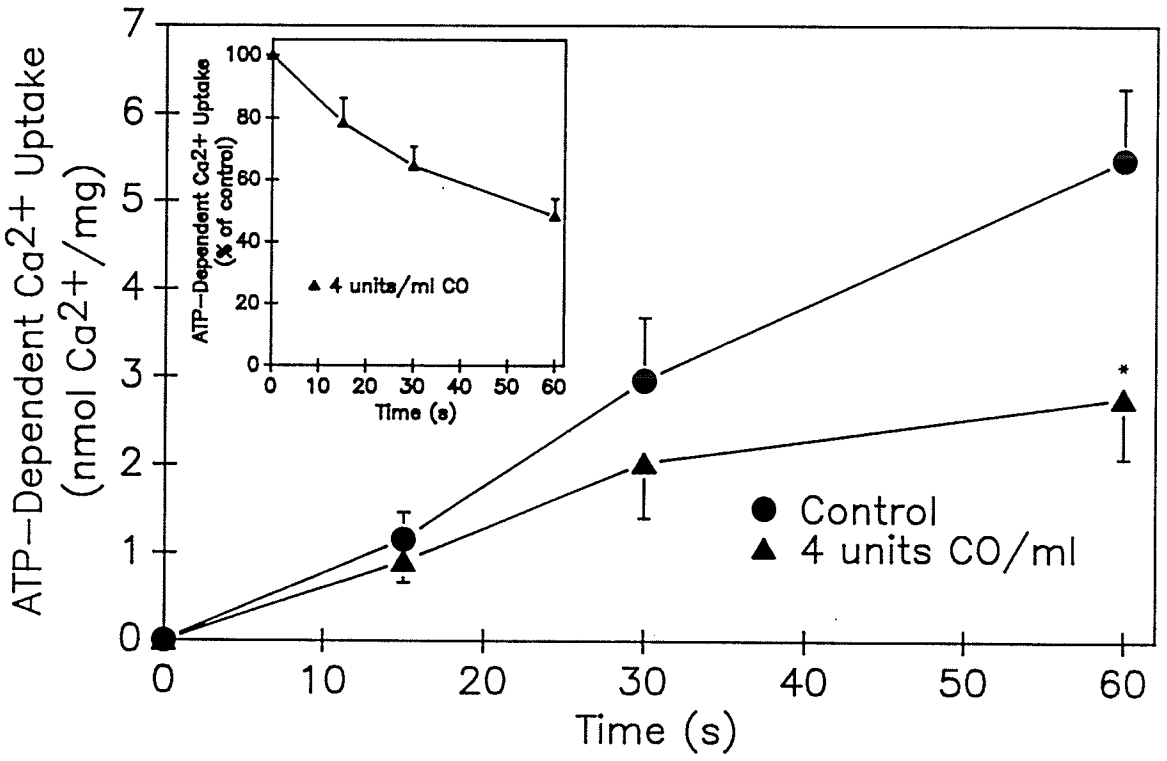


FIGURE 20. *Effect of cholesterol oxidase (CO) on sarcolemmal ATP-dependent Ca^{2+} uptake over varying reaction times.* CO-treated sarcolemmal Ca^{2+} uptake as a percent of control untreated activity is shown (inset). Values are mean ± SEM (n=4). *,$p<0.05$ *versus* control values.

cholesterol oxidase was examined over the 60-second reaction time (Figure 21). Cholesterol oxidase concentrations above 1.0 unit/ml significantly depressed this transport pathway. Cholesterol oxidase (10 units/ml) could reduce uptake almost completely.

Passive (ATP-independent) Ca^{2+} binding to cardiac sarcolemma was examined in the presence of varying concentrations of cholesterol oxidase. Because of the possibility that cholesterol oxidase may make the vesicles leaky and therefore expose more potential Ca^{2+} binding sites on the intravesicular surface of the sarcolemma, 1 μM A23187, a Ca^{2+} ionophore, was included in the reaction medium for both control and oxidase-treated membrane preparations. Thus, artifactual increases in sarcolemmal Ca^{2+} binding produced by the increased membrane permeability to Ca^{2+} were not a factor in the present series of experimental results. As shown in Table 3, cholesterol oxidase concentrations of 1-10 units/ml, produced significant increases in sarcolemmal Ca^{2+} binding capacity. This peaked at 5.0 units/ml, where Ca^{2+} binding capacity almost doubled from control values. Extensive oxidative modification of membrane cholesterol with 20 units/ml resulted in an inhibition of passive binding capacity.

Sarcolemmal passive Ca^{2+} binding was examined in the absence or presence of 2.0 units/ml cholesterol oxidase over a range of Ca^{2+} concentrations (0.01-5 mM). At all [Ca^{2+}] examined, cholesterol oxidase treatment stimulated sarcolemmal Ca^{2+} binding capacity (Figure 22).

IV. Effects of LDL on Ca^{2+} Flux in Cultured Smooth Muscle

a) Cell Appearance

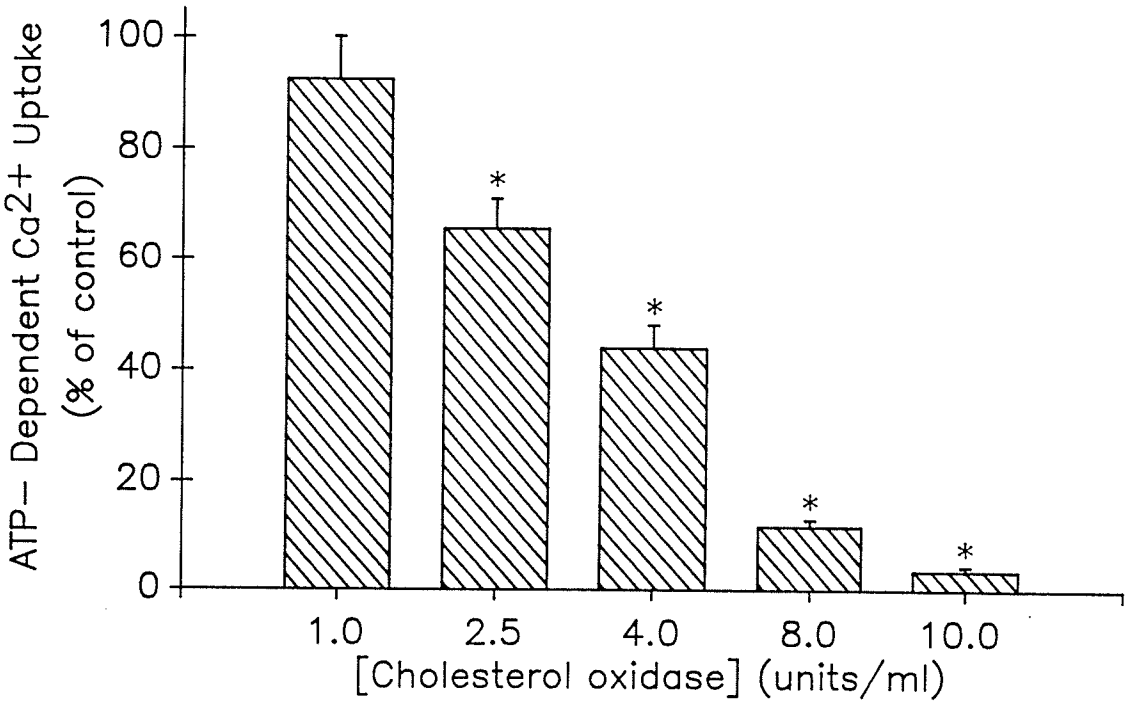


FIGURE 21. *Effect of different concentrations of cholesterol oxidase on ATP-dependent Ca^{2+} uptake.* Absolute values for ATP-dependent Ca^{2+} uptake by cardiac sarcolemmal vesicles was 2.55 $\pm$ 0.44 nmol/mg/min (n=6). Reaction time was 60 seconds at 10 μM [Ca^{2+}]. *,$p<0.05$ *versus* control values.

TABLE 3 Passive calcium binding to isolated sarcolemmal membrane vesicles after treatment with varying concentrations of cholesterol oxidase.

Cholesterol Oxidase (U/ml)	Ca^{2+} Binding (nmol/mg)	% of control
0.0	50.5 ± 3.0	100
0.2	54.5 ± 4.0	108 ± 1
1.0	62.9 ± 4.2	125 ± 2*
2.0	78.8 ± 7.1	156 ± 5*
5.0	89.0 ± 10.9	175 ± 9*
10.0	72.3 ± 13.1	141 ± 15*
20.0	25.7 ± 5.1	50 ± 6*

Values represent mean ± S.E. of 4 separate experiments. Cholesterol oxidase was included in the reaction medium with 1 uM A23187 and 1 mM Ca^{2+} for the entire reaction time (1 min) at 37°C. * $P < 0.05$ vs control (untreated preparation).

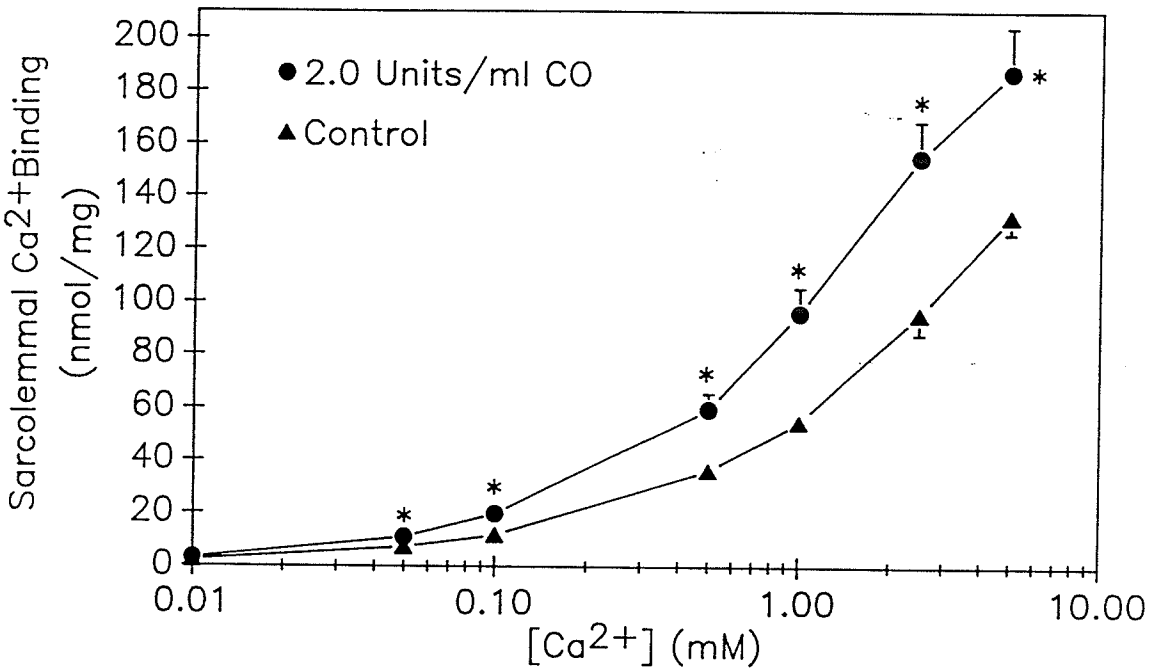


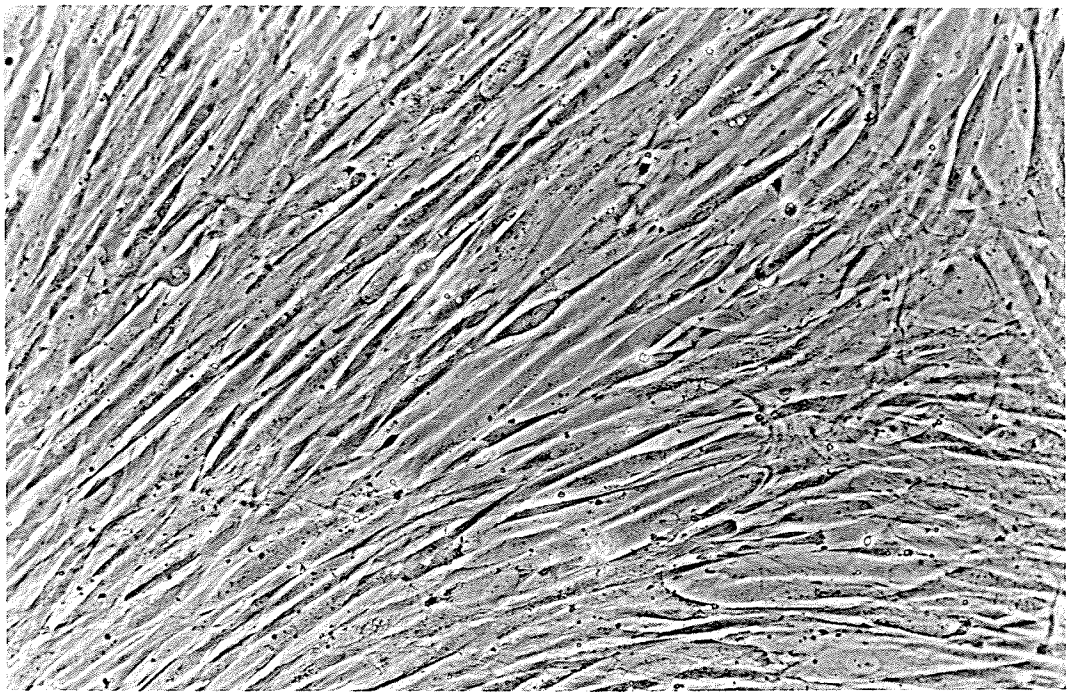
FIGURE 22. *Sarcolemmal Ca^{2+} binding at varying $[Ca^{2+}]$ in the absence* (▲) *or presence* (●) *of cholesterol oxidase (CO).* Values represent mean ± SEM of four separate experiments (if no error bar is present, error was smaller than symbol size). *, $p < 0.05$ *versus* control.

The cells isolated from the rabbit thoracic aorta using the method of Colucci et al[342] represent a homogenous preparation of vascular smooth muscle cells without apparent contamination from other cell types[343]. A typical primary culture of vascular smooth muscle cells at confluence is shown in Figure 23A. These cells show the characteristic spindle shaped morphology of cultured vascular smooth muscle cells with litle cytoplasmic vacuolization. In contrast, cultured vascular smooth muscle cells incubated for 72 h in the presence of sterilized LDL (2.5 μmol LDL cholesterol/ml) showed increased vacuolization with frequent rounded cells, centrally more dense with increased cytoplasmic lucency (Figure 23B).

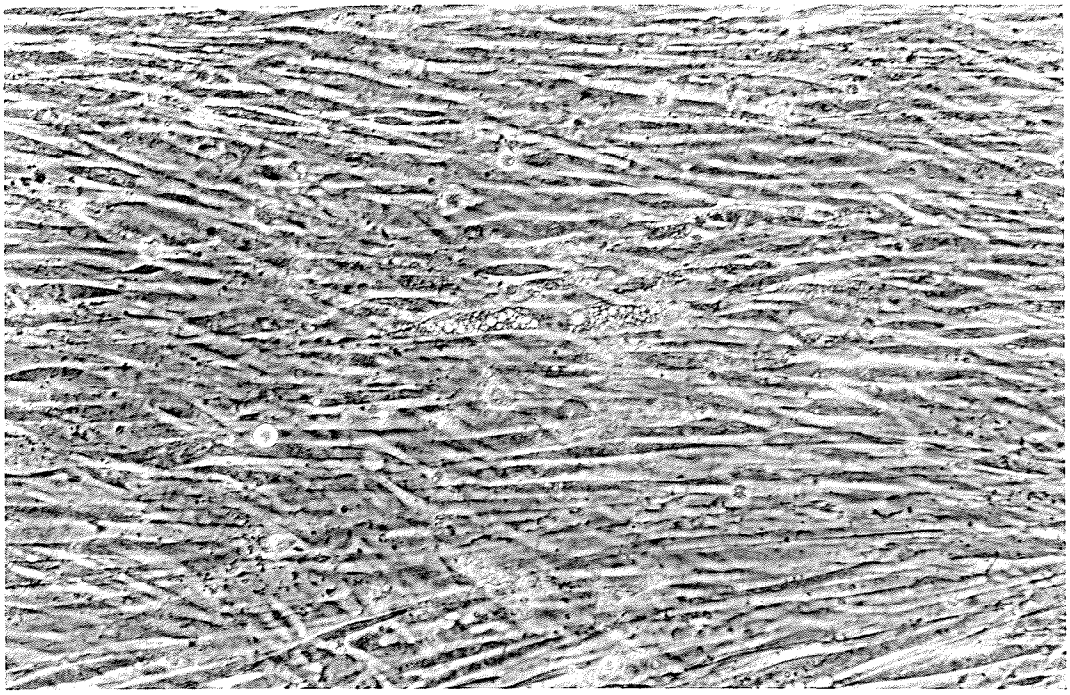
b) Cholesterol Content of Lipoprotein Treated Smooth Muscle

Confluent monolayers of aortic smooth muscle cells were exposed to varying concentrations of LDL for 24, 48, and 72 hours. The cells were then washed briefly, scraped from the culture dishes and the free and esterified cholesterol content of the cells determined. The total cellular cholesterol content (the sum of the free and esterified cholesterol) was also determined. As shown in Figure 24A, free cholesterol content doubled in cells exposed to LDL at a concentration of 2.5 μmol/ml for 24 hours. Free cholesterol accumulation was greater if the incubation period was longer than 24 hours. Furthermore, the cells accumulated more free cholesterol with increasing [LDL], however, an [LDL] of at least 1.25 μmol LDL cholesterol was required to demonstrate statistical significance. For the purpose of comparison, free cholesterol accumulation in cells after exposure to VLDL for the same three time periods was also examined (Figure 24B). Quantitatively, the effects were similar to that observed with LDL, however,

FIGURE 23. *Representative phase contrast micrographs of confluent primary cultures of control and LDL treated vascular smooth muscle cells.* **A**, vascular smooth muscle cells incubated with normal growth medium (Medium 199 containing 20% fetal calf serum, 2.0 mM glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml). **B**, vascular smooth muscle cells incubated for 72 h in normal culture medium supplemented with 2.5 μmol LDL cholesterol/ml.



A



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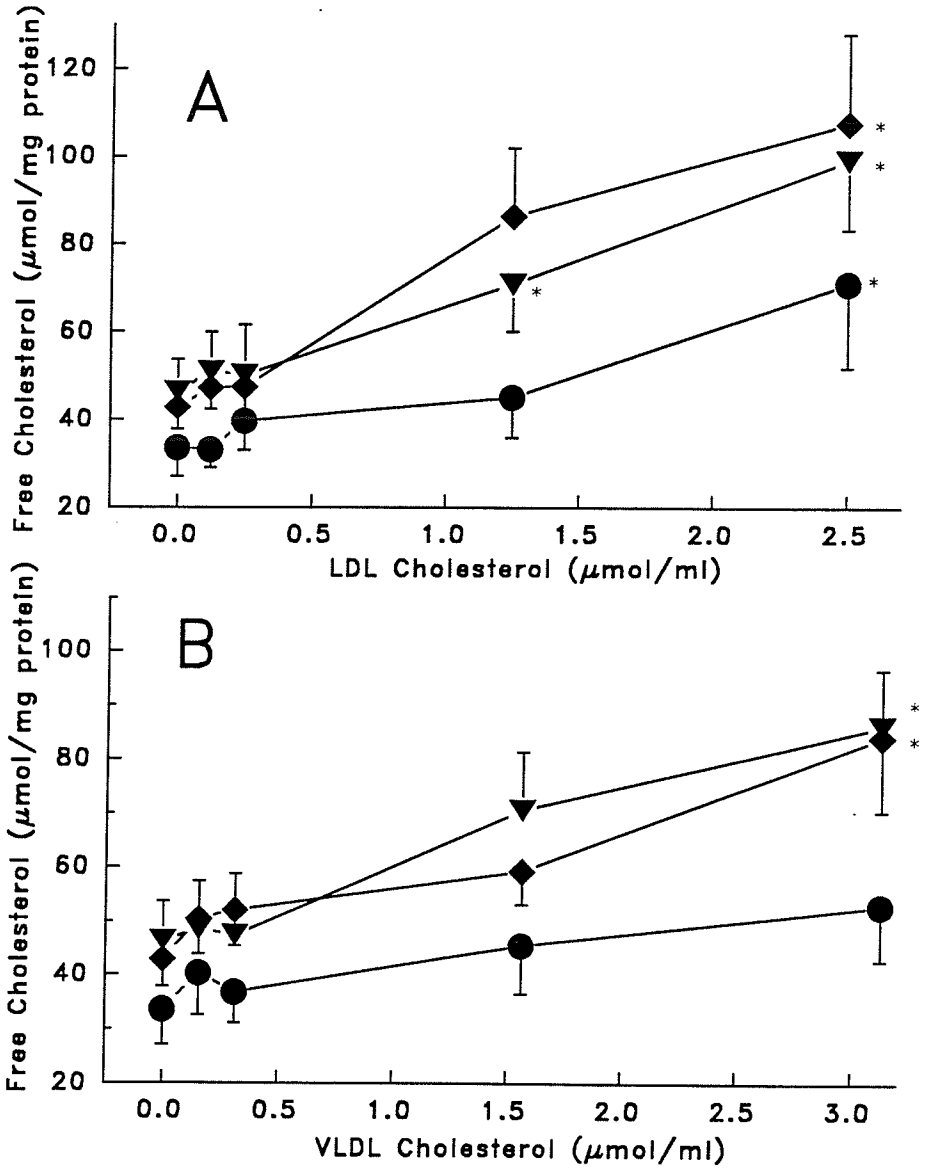

FIGURE 24. *Effect of different concentrations of LDL or VLDL cholesterol and various times of incubation on free cholesterol content of cultured rabbit aortic smooth muscle cells.* **A**, vascular smooth muscle cells treated with 0.0-2.5 μmol LDL cholesterol/ml growth medium for 24 (●), 48 (◆), or 72 (▼) hours. **B**, vascular smooth muscle cells treated with 0.0-3.1 μmol VLDL cholesterol/ml growth medium for 24 (●), 48 (◆), or 72 (▼) hours. After the appropriate incubation time the cells were washed and assayed for free cholesterol. See "Materials and Methods" for details. Values represent mean for six experiments. *, $p<0.05$ *versus* untreated control values.

VLDL was less potent than LDL.

A similar pattern for esterified cholesterol accumulation in cells was observed as for the free cholesterol (Figure 25A). The accumulation of esterified cholesterol was dependent upon both time and [LDL]. Extended exposure of cells to VLDL also resulted in accumulation of esterified cholesterol (Figure 25B). At short exposure times (24 hours), a high [VLDL] was required before a statistically significant increase in cholesterol esters was observed. Since total cellular cholesterol is the sum of free and esterified cholesterol, total cellular cholesterol responded to LDL and VLDL (Figures 26 A and B) in a manner similar to that observed in Figures 24 A and B and 25 A and B. Accumulation of cholesterol in the cells was dependent upon the time of exposure and the concentration of LDL and VLDL. Total cholesterol in the cells was similar at high lipoprotein levels after 48 or 72 hours of incubation which would indicate attainment of equilibrium.

c) Pattern of Ca^{2+} Flux

Figure 27 illustrates a representative ^{45}Ca washout experiment with the cultured vascular smooth muscle cells. After a period of stable ^{45}Ca labelling at 10 ml/min, perfusion medium was switched to non-isotopic medium and ^{45}Ca efflux was monitored at a flow rate of 34 ml/min. The upper curve represents cell specific counts, which are the counts obtained from scintillator discs which had cells attached minus the background counts obtained from washout of blank discs. After 19 min of washout, the flow cell was evacuated, the cells scraped from the discs and the same discs re-inserted into the spectrometer to obtain ^{45}Ca efflux values in the absence of cells (blank values). The

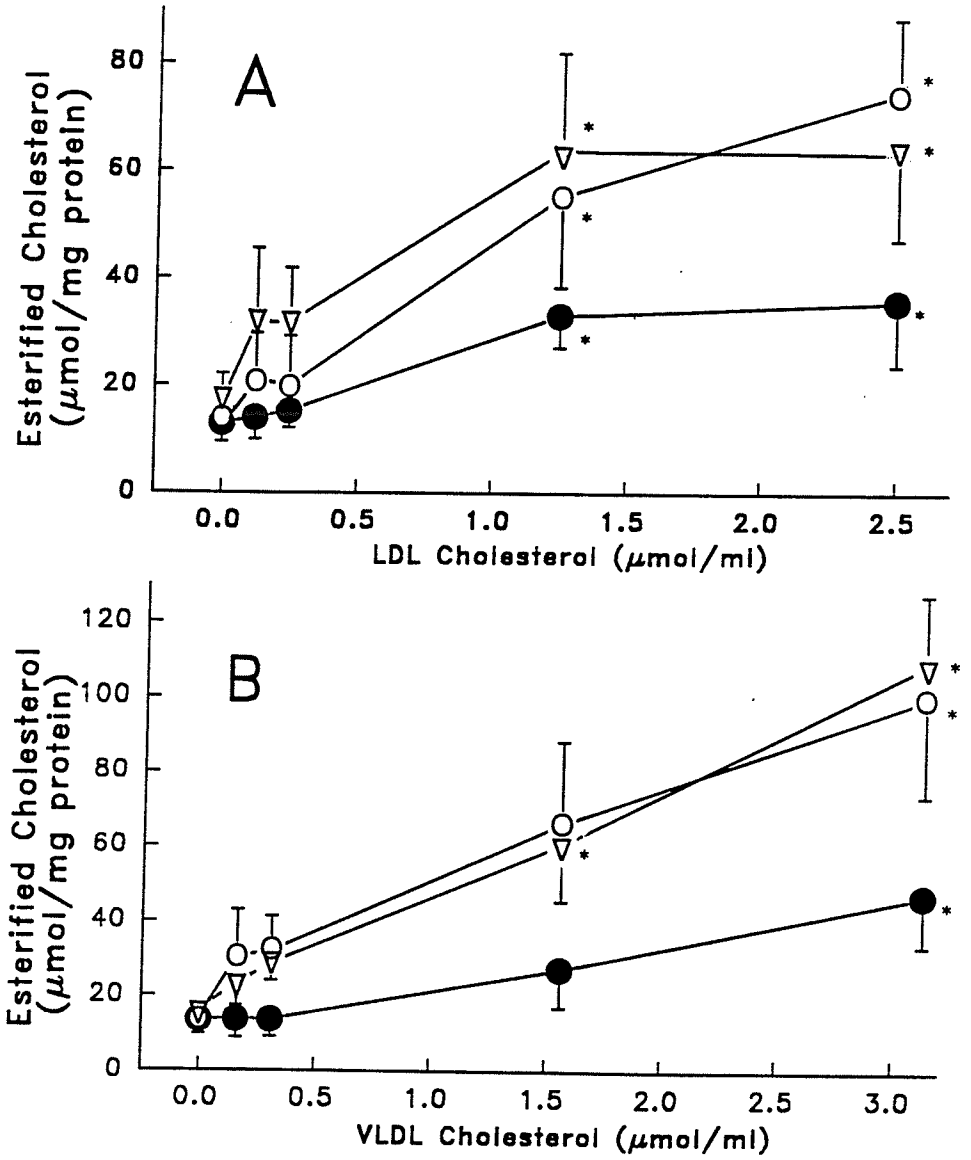

FIGURE 25. *Effect of different concentrations of LDL or VLDL cholesterol and various times of incubation on esterified cholesterol content of cultured rabbit aortic smooth muscle cells.* **A**, vascular smooth muscle cells treated with 0.0-2.5 μmol LDL cholesterol/ml growth medium for 24 (●), 48 (▽), or 72 (o) hours. **B**, vascular smooth muscle cells treated with 0.0-3.1 μmol VLDL cholesterol/ml growth medium for 24 (●), 48 (▽), or 72 (o) hours. After the appropriate incubation time the cells were washed and assayed for esterified cholesterol. See "Materials and Methods" for details. Values represent mean for six experiments. *, $p < 0.05$ *versus* control.

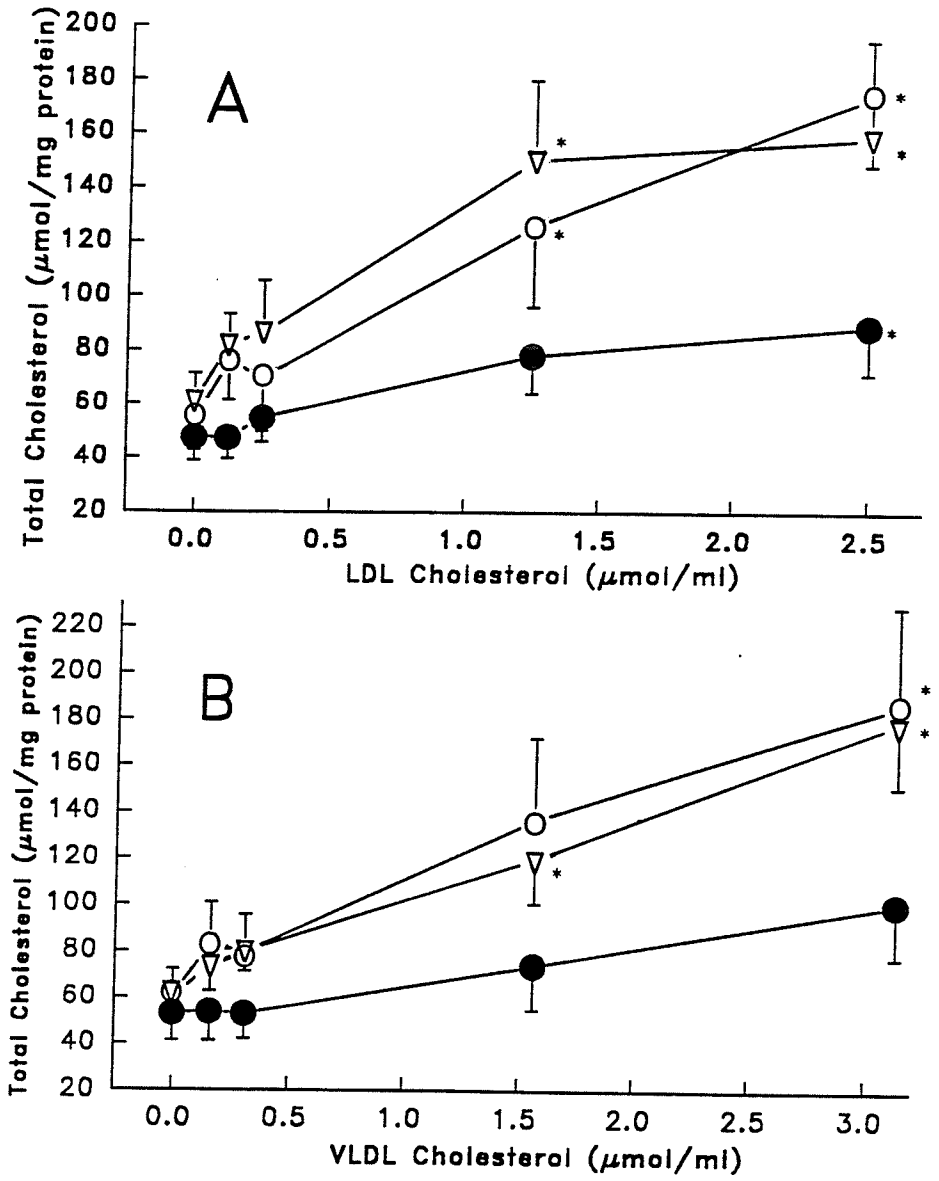

FIGURE 26. *Effect of different concentrations of LDL or VLDL cholesterol and various times of incubation on total (free + esterified) cholesterol content of cultured rabbit aortic smooth muscle cells.* **A**, vascular smooth muscle cells treated with 0.0-2.5 μmol LDL cholesterol/ml growth medium for 24 (●), 48 (▽), or 72 (o) hours. **B**, vascular smooth muscle cells treated with 0.0-3.1 μmol VLDL cholesterol/ml growth medium for 24 (●), 48 (▽), or 72 (o) hours. After the appropriate incubation time the cells were washed and assayed for total (free + esterified) cholesterol. See "Materials and Methods" for details. Values represent mean for six experiments. *, $p < 0.05$ *versus* control.

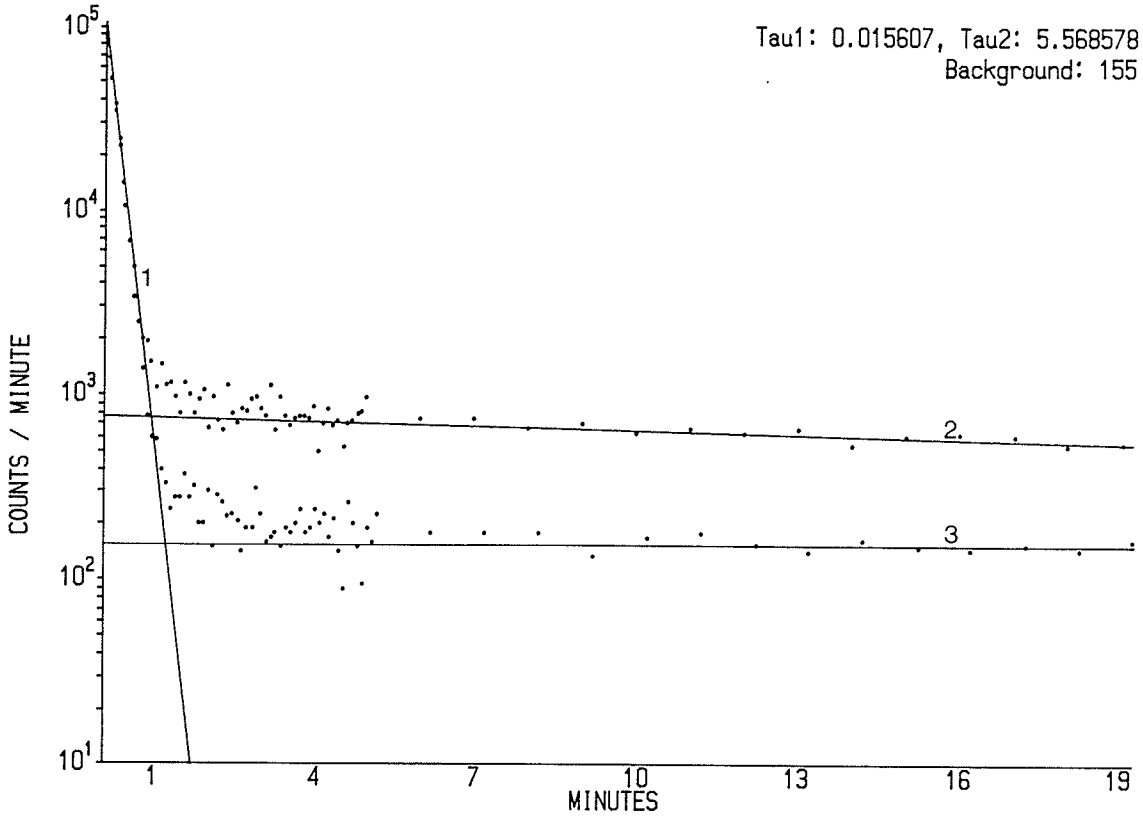

FIGURE 27. *Representative experiment of the ^{45}Ca washout from control smooth muscle cells after equilibration for 30 min in ^{45}Ca-containing perfusate.* The lower curve (3) represent counts obtained from the washout of blank discs. Line no. 2 represents cell specific counts (counts obtained with cells on discs minus line 3). Exchange can be separated into a rapid component (no. 1: rate K = 5.57/min) and a slow component (no. 2: rate K = 0.016/min).

lower curve of Figure 27 was obtained and the counts/min were subtracted from values obtained in the presence of cells to obtain the upper curve which is the cell-specific ^{45}Ca washout curve. In this experiment, the rapid exchange component had a rate constant of 5.57/min (line 1, Tau 2), and the slowly exchangeable component had a rate constant of 1.56×10^{-2}/min (line 2, Tau 1). These values yield a $t_{½}$ of 7.468 s (= 0.124 min) for the rapidly exchangeable Ca^{2+} and $t_{½}$ = 44.40 min for the slowly exchangeable Ca^{2+}. However, the rapidly exchangeable Ca^{2+} remains completely perfusion limited at the 34 ml/min washout rate used. This is because the washout rate of the flow cell and the cell layer are not separable for the first minute of washout. It is clear, however, that the cells wash out at least as rapidly ($t_{½}$ = 7.468) as the flow cell. The zero time intercept of line 2 represents the total ^{45}Ca associated with the slowly exchangeable Ca^{2+} pool at the beginning of washout. In the experiment shown in Figure 27, this amounted to 780 cpm or 1.16 mmol Ca^{2+}/kg dry wt of smooth muscle cells. However, because we know that this pool was not fully equilibrated, (the cells had been exposed to ^{45}Ca for 30 min prior to washout and with a $t_{½}$ for equilibration of 44.40 min, it will be less than 1/2 equilibrated) a correction factor must be introduced. The correction for the phase labelled immediately prior to washout can be determined using the equation:

$$\tau/\tau_o = 1 - e^{-\Phi t}$$

where τ is the activity at any time (t) relative to the asymptotic (τ_o) labelling of the slow phase with a defined exchange rate constant (Φ). In this experiment, therefore, this correction factor is 2.65. Thus the actual capacity of the slowly exchangeable Ca^{2+} pool is 1.16 x 2.65 = 3.07 mmol Ca^{2+}/kg dry weight. This Ca^{2+} was not displaceable by

$LaCl_3$ but $LaCl_3$ could reduce the efflux rate of the slowly exchangeable Ca^{2+} to zero. Similar representative ^{45}Ca washout curves are shown for VLDL and LDL treated vascular smooth muscle cells (Figures 28 and 29 respectively).

The Ca^{2+} exchange kinetics of untreated, VLDL and LDL treated rabbit aortic smooth muscle cells are summarized in Table 4. The rapidly exchangeable Ca^{2+} in control (untreated) vascular smooth muscle preparations had a rate constant of 5.31 ± 0.37/min with an exchange half-time of 0.134 ± 0.009 min. No significant differences were observed in the rapidly exchangeable Ca^{2+} as a function of lipoprotein treatment (72 hours with 3.00 μmol/ml VLDL cholesterol or 2.50 μmol/ml LDL cholesterol). The rapidly exchangeable Ca^{2+} in control smooth muscle cells exhibited kinetics that were over 300 fold faster than the slow component of Ca^{2+} exchange. Its rate constant was 0.015 ± 0.002 with an exchange half-time of over 50 min. The slowly exchangeable Ca^{2+} rate constant was significantly different ($p < 0.05$) in both VLDL and LDL treated aortic smooth muscle cells than control. The exchange half-time ($t_{½}$) was about half or less than control in the VLDL and LDL treated cells.

Calcium exchange compartmentation was examined in control and lipoprotein treated vascular smooth muscle cells. As shown in Table 5, total exchangeable Ca^{2+}, which is the sum of both the rapidly and slowly exchangeable Ca^{2+} components, was 4.27 ± 0.53 mmol/kg dry weight in control cells. Greater than 50% of this exchangeable Ca^{2+} was represented by a La^{3+} displaceable rapidly exchangeable component as has been reported previously[343]. No significant differences were observed in the total exchangeable Ca^{2+} pool in lipoprotein treated vascular smooth muscle cells.

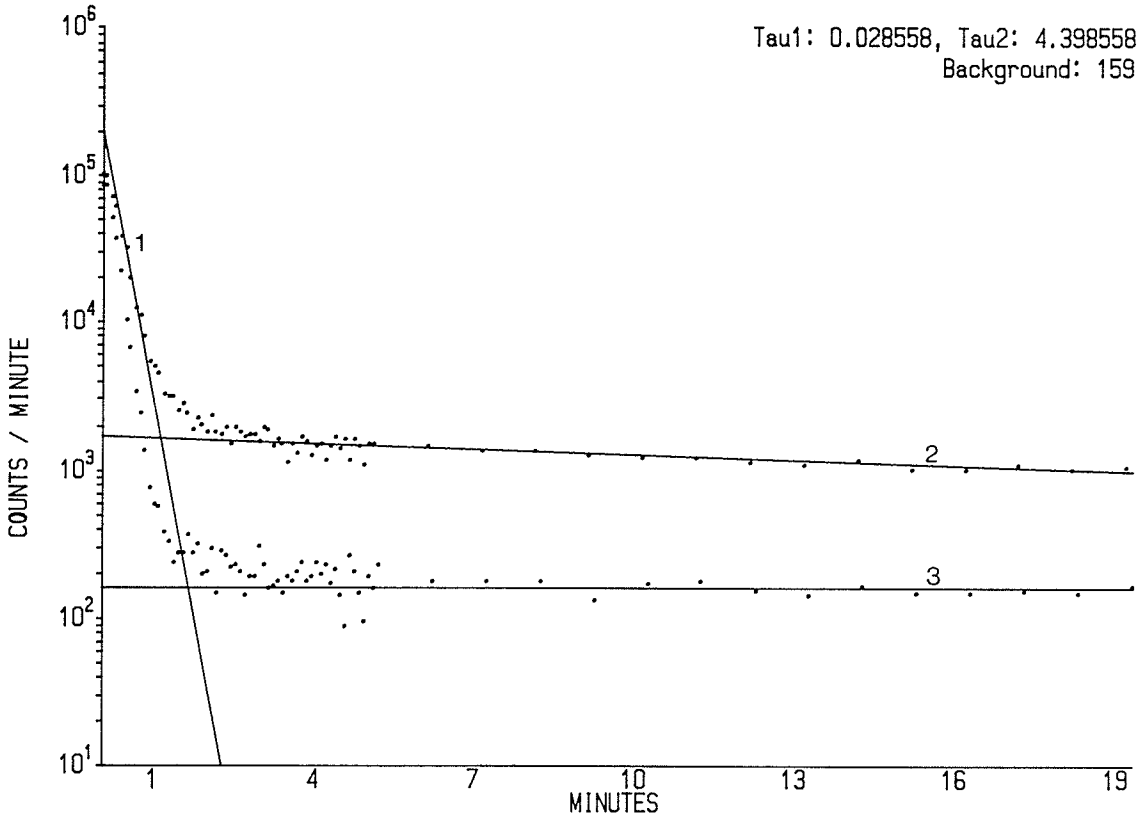

FIGURE 28. *Representative experiment of the ^{45}Ca washout from VLDL treated smooth muscle cells after equilibration for 30 min in ^{45}Ca-containing perfusate.* Cultures were incubated with 3.1 μmol VLDL cholesterol/ml growth medium for 72 h. See "Materials and Methods" for details. The lower curve (3) represent counts obtained from the washout of blank discs. Line no. 2 represents cell specific counts (counts obtained with cells on discs minus line 3). Exchange can be separated into a rapid component (no. 1: rate K = 4.40/min) and a slow component (no. 2: rate K = 0.029/min).

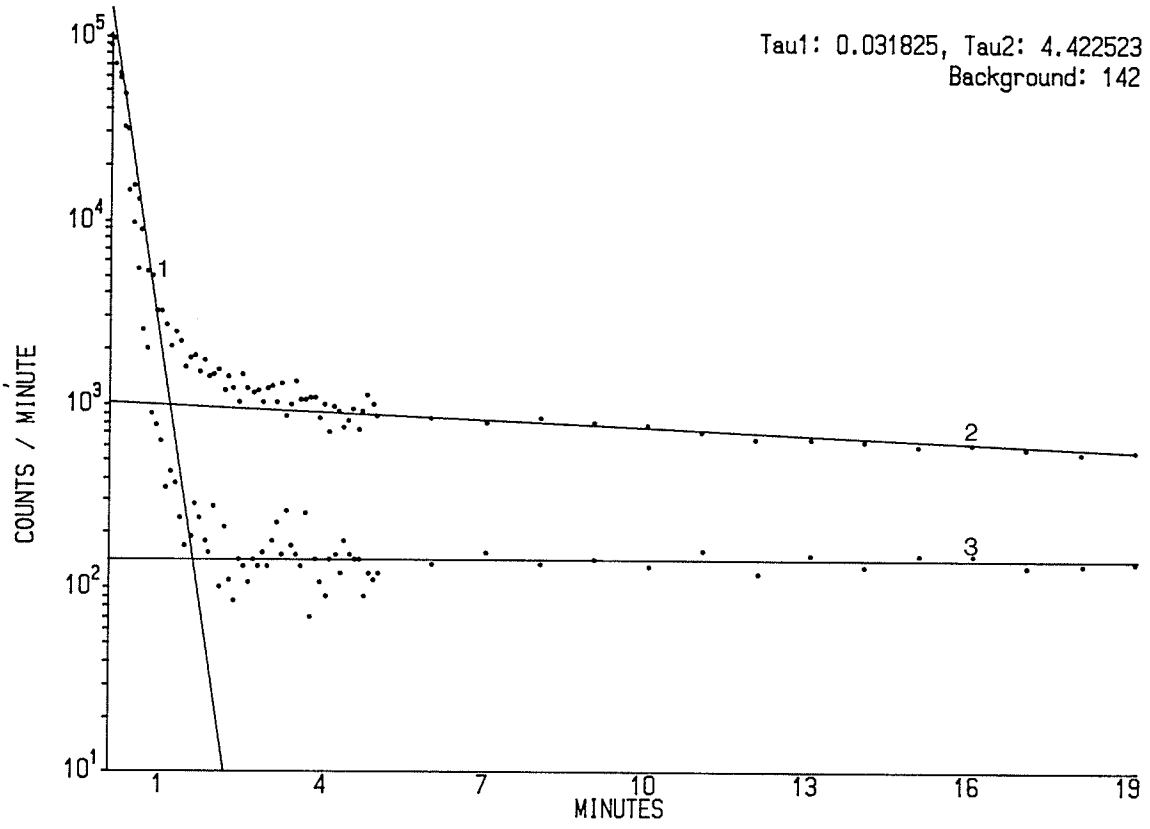

FIGURE 29. *Representative experiment of the ^{45}Ca washout from LDL treated smooth muscle cells after equilibration for 30 min in ^{45}Ca-containing perfusate.* Cultures were incubated with 2.5 μmol LDL cholesterol/ml growth medium for 72 h. See "Materials and Methods" for details. The lower curve (3) represent counts obtained from the washout of blank discs. Line no. 2 represents cell specific counts (counts obtained with cells on discs minus line 3). Exchange can be separated into a rapid component (no. 1: rate K = 4.42/min) and a slow component (no. 2: rate K = 0.032/min).

TABLE 4

Calcium exchange kinetics of vascular smooth muscle cells as a function of treatment with lipoproteins.

Experimental Group	Rate Constant (per min)		Exchange Half Time (min)	
	Rapid Component	Slow Component	Rapid Component	Slow Component
Control	5.31 ± 0.37	0.015 ± 0.002	0.134 ± 0.009	50.4 ± 7
VLDL Treated	4.42 ± 0.28	0.028 ± 0.002*	0.160 ± 0.010	25.3 ± 1*
LDL Treated	4.58 ± 0.33	0.033 ± 0.002*	0.155 ± 0.011	21.6 ± 1*

Values represent mean ± S.E. (n = 4-8). * $P < 0.05$ vs. respective control value.

TABLE 5

Calcium exchange characteristics of vascular smooth muscle cells maintained in the presence or absence of lipoproteins.

Experimental Group	Rapidly Exchangeable La^{3+} displaceable Ca^{2+} (mmol/kg dry wt)	Slowly Exchangeable Ca^{2+} (mmol/kg dry wt)	Total Exchangeable Ca^{2+} (mmol/kg dry wt)
Control	2.23 ± 0.21	2.04 ± 0.34	4.27 ± 0.53
VLDL Treated	3.63 ± 0.59	1.79 ± 0.42	5.42 ± 0.79
LDL Treated	4.72 ± 0.98*	1.42 ± 0.15	6.15 ± 0.92

Values represent mean ± S.E. (n = 4-8). * $P < 0.05$ vs. respective control value.

However, the La^{3+} displaceable Ca^{2+} fraction was significantly increased in the LDL treated cells as compared to control.

d) Modulation of Ca^{2+} Exchange

It has been shown that exposure of cells to a low sodium solution will result in an increase in cell associated Ca^{2+}, and alter Ca^{2+} exchange[358,359]. This intervention was employed in the current study to further understand the nature of the LDL effects on Ca^{2+} exchange in vascular smooth muscle. The results of a typical control experiment are shown in Figure 30. After equilibrium of Ca^{2+} flux had been reached in the smooth muscle cells, perfusate was introduced which contained a lower NaCl concentration (35 mM). ^{45}Ca associated with these cells rose 6100 counts/min or 6.53 mmol Ca^{2+}/kg dry weight during the 55 min low Na^+ perfusion period. On return to the normal perfusate (identical to that used during the first 30 min of perfusion), cell-associated Ca^{2+} declined very quickly for the first 5 minutes, and then at a much slower rate. Extrapolation of this decline back to the zero time intercept (time zero in these experiments being the time of re-introduction of normal perfusate, ie. the 85th min of total perfusion time) represents a slowly exchangeable Ca component. This value was clearly greater than the stable Ca^{2+} pool measured before the introduction of the low Na^+ perfusate. The content of $^{45}Ca^{2+}$ in the slowly exchangeable pool increased 2750 counts/min over the stable values prior to low Na^+ perfusion (131150-128400 counts/min), which represents an increase of 2.64 mmol Ca^{2+}/kg dry weight or 40% of the total increase (6.53 mmol Ca^{2+}). This indicates that the ^{45}Ca associated with the cell increased during low Na^+ perfusion and its slow rate of decline (minutes 90 to 120) suggest that this ^{45}Ca is at least partly

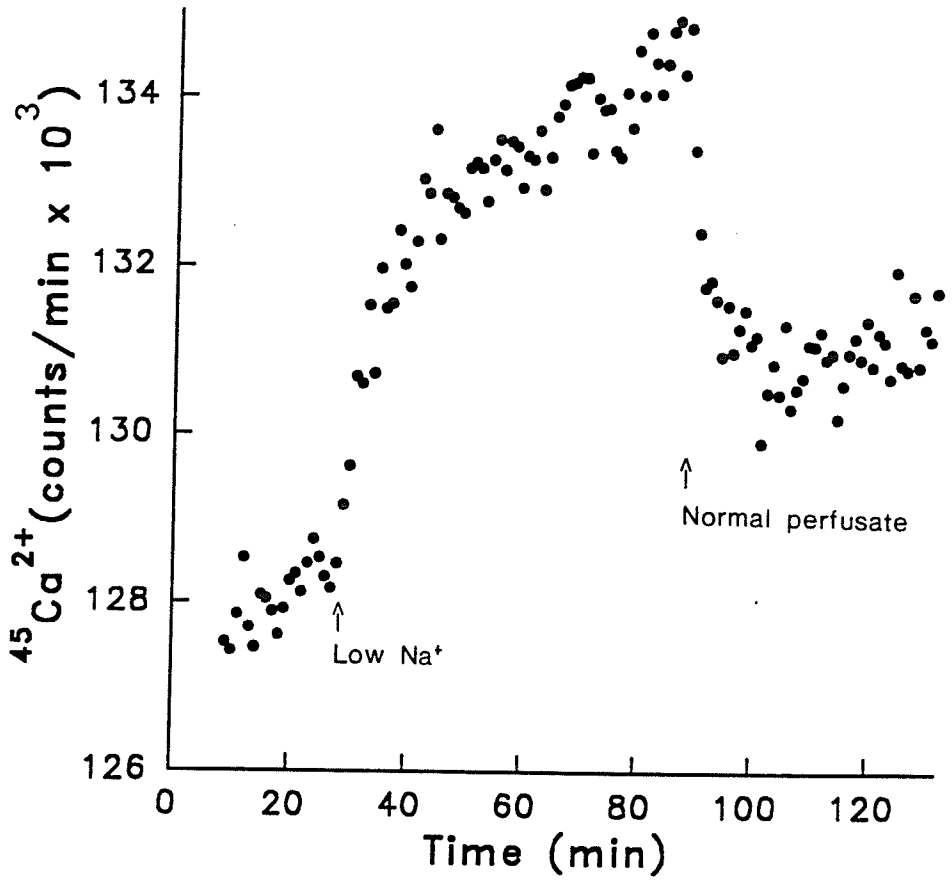

FIGURE 30. *Typical experiment demonstrating the response of vascular smooth muscle cells to perfusate with lowered Na^+ concentration.* After 30 min of equilibration in a normal medium, the perfusate was changed to one which contained 35 mM Na^+ and an isosmotic replacememt of sucrose. This caused an immediate increase in cell associated ^{45}Ca activity which could be partially reversed after 55 min of perfusion in a normal 140 mM Na^+ containing medium. Note the rapid rise in counts from 30 to 40 min., the rapid drop from 85 to 90 min. and the much slower decline in ^{45}Ca activity during the 90 to 125 min. reperfusion period.

intracellular in origin.

Table 6 shows results of a series of experiments involving perfusion of both control and LDL treated smooth muscle cells with low Na^+ containing medium. Perfusion of the cells with low Na^+ perfusion medium resulted in an average increase in cell associated Ca^{2+} of 8.12 $\pm$ 1.21 mmol Ca^{2+}/kg dry weight in control cell cultures. In cells exposed to LDL for 72 hours (2.5 μmol/ml LDL cholesterol) this increase in cell associated Ca^{2+} was significantly greater (13.77 $\pm$ 1.81 mmol) than that of control. The slow exchange Ca^{2+} component immediatly on re-introduction of Ca^{2+} represented 38.57% of the total increase in cell associated Ca^{2+} in control cultures, which was not significantly different fron that seen with LDL treated cells (29.98%).

It has been suggested that the increased cellular Ca^{2+} seen with low sodium perfusion of this type is likely to be associated with the cell surface, as ionic Na^+ is replaced with neutral sucrose in the perfusing solution. Inclusion of La^{3+} in the perfusion solution was used to detect cell surface Ca^{2+}. The results from a typical control experiment are depicted in Figure 31A. Cells at a stable level of Ca^{2+} exchange were exposed to low Na^+ medium at minute 15. This resulted in an increase in cell associated Ca^{2+} of 5.75 mmol/kg dry weight consistent with the results in Figure 30. At minute 35, the cells were exposed to a low Na^+ solution which now included 1 mM $LaCl_3$. This resulted in a reduction of the cell associated Ca^{2+} of 9250 counts/min or 6.63 mmol/kg dry cell weight. Typical results for low Na^+ perfusion of LDL treated vascular smooth muscle cells is shown in Figure 31B. As in control cultures low sodium perfusion resulted in an increase in the cell associated Ca^{2+}. In this experiment, this

TABLE 6

Effect of low Na^+ perfusion on the calcium exchange characteristics of vascular smooth muscle cells maintained in the presence or absence of low density lipoprotein.

Experimental Group	Increase in Cell-Associated Ca^{2+} (mmol/kg dry weight)	La3+ Displaceable Ca^{2+} (mmol/kg dry weight)	Slowly Exchangeable Ca (% of Total Increase)
Control	8.12 ± 1.21	7.40 ± 0.65	38.57 ± 4.61
LDL Treated	13.77 ± 1.81*	14.46 ± 2.49*	29.98 ± 3.55

Values represent mean ± S.E. (n = 5). * $p < 0.05$ vs respective control value.

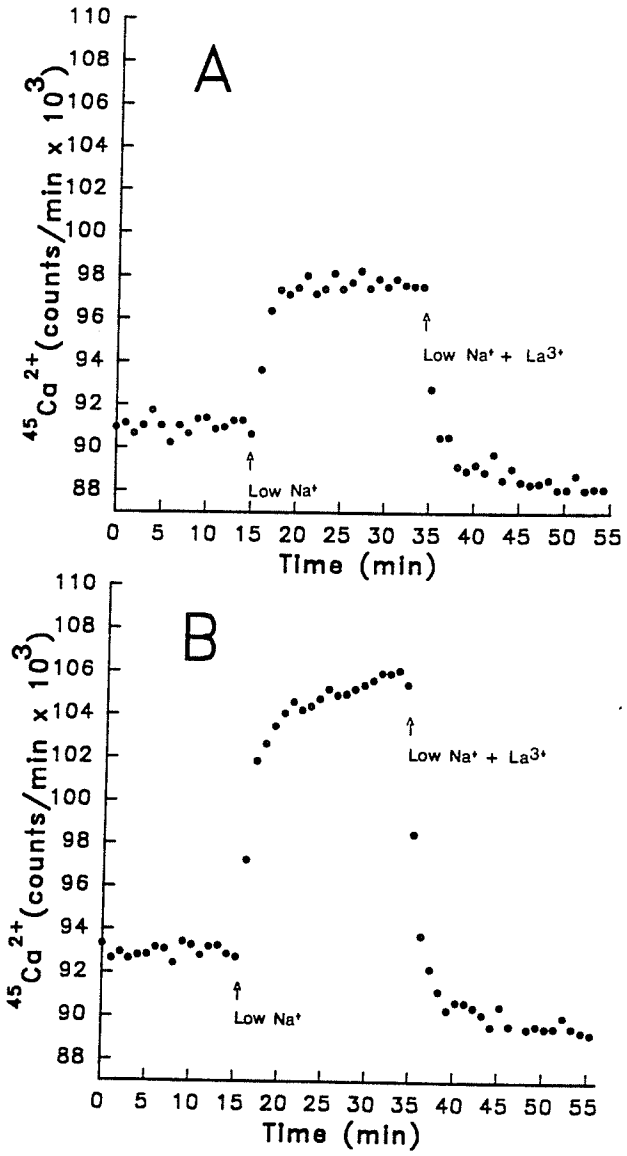

FIGURE 31. *Typical experiments demonstrating the response to La^{3+} of control and LDL treated vascular smooth muscle cells perfused with a low Na^+ medium.* **A**, control cell cultures; after equilibration with a normal medium, the perfusate was changed at minute 15 to one which contained 35 mM Na^+ and an isosmotic replacement of sucrose. This caused an immediate increase in cell associated ^{45}Ca activity. At minute 35, the perfusate was switched to one which contained 1 mM La^{3+}. **B**, cell cultures incubated with 2.5 μmol/ml LDL cholesterol for 72 h prior to experimentation. Cells were perfused as described above for control cultures. Note the difference in the increase in cell associated ^{45}Ca between control and LDL treated cultures on exposure to low Na^+ perfusate and the rapid decline in cell associated ^{45}Ca activity in both control and LDL treated cultures.

increase amounted to 9.55 mmol/kg Ca^{2+} dry weight. On perfusion with a low Na^{+} solution containing 1 mM La^{3+}, cell associated Ca^{2+} was reduced by 10.68 mmol/kg dry weight. In a series of experiments, this La^{3+} displaceable Ca^{2+} amounted to 14.46 $\pm$ 2.49 mmol/kg dry weight in LDL treated cultures, which was significantly different from the control value of 7.40 $\pm$ 0.65 (Table 6). If the normal La^{3+} displaceable Ca^{2+} fraction is 2.23 mmol/kg dry in control cells (Table 5), the extra La^{3+} displaceable Ca^{2+} induced by the low Na^{+} perfusion would be 7.40 - 2.23 = 5.17 mmol/kg dry wt. This extra La^{3+} displaceable Ca^{2+} induced by the low sodium perfusion of 5.17 mmol represents 64% of the total increase of cell Ca^{2+} (8.12 mmol/kg dry wt., Table 6). Similarily for the LDL treated cultures, 14.46 - 4.72 = 9.74 mmol/kg dry wt. which represents 70% of the total increase of cell Ca^{2+} in these treated cultures. Thus, in this series of experiments, low Na^{+} perfusion of the cells for 20 minutes resulted in an increase in Ca^{2+} exchange, of which 64% was La^{3+} displaceable in control cultures, and 70% was La^{3+} displaceable in LDL treated cultures, and the remainder was localized in a slowly exchangeable intracellular compartment.

V. Plasma Lipid and Lipoprotein Pattern of Human Subjects

a) Characteristics of the Study Group

Table 7 shows the age and height/weight relationships of the two groups in this study. The Inuit subjects had a mean age that was significantly greater than the control group. In addition, the Inuit group had a significantly greater ponderal index than controls.

TABLE 7

Age and height/weight characteristics of study population.

	Age	Ponderal Index
Inuit	44 ± 6*	11.8 ± 0.4*
Control	32 ± 3	12.5 ± 0.2

Ponderal index is calculated by dividing height (in inches) by the cube root of weight (in pounds). Values represent means ± S.E. (n = 11 - 12).

* $P < 0.05$ versus control values.

Table 8 shows the lipoprotein profile of serum from both control and Inuit subjects. No significant difference was apparent in the total cholesterol (free plus esterified) or in the triglyceride levels of the serum. The α-lipoprotein fraction (which roughly corresponds to HDL as obtained by floatation ultracentrifugation) was significantly higher in the Inuit population. No difference was observed between the groups in the ß-lipoprotein fraction (roughly corresponding to LDL). The Inuit also exhibited a significantly lower ß-lipoprotein to α-lipoprotein ratio.

In table 9 the VLDL/LDL lipoprotein profile of both control and Inuit plasma as determined by floatation ultracentrifugation is shown. Inuit plasma exhibited significantly lower levels of both VLDL and LDL apolipoproteins. The decreased concentration of LDL apoprotein is reflected in the significantly higher value for LDL native cholesterol (0.92 ± 0.06) and LDL cholesterol esters (1.73 ± 0.13) as determined by enzymatic assay and expressed per mg protein compared with control subjects (0.65 ± 0.03 and 0.88 ± 0.07). No significant differences were observed in the content of VLDL cholesterol or cholesterol esters. For comparison, Table 10 shows the Inuit and control native and oxidized cholesterol content of the isolated LDL and VLDL subfractions of plasma as determined by HPLC. There are no significant differences between the control and the Inuit VLDL cholesterol content whereas the LDL native cholesterol content is significantly higher in the Inuit (0.41 ± 0.17) compared with that of control subjects (0.17 ± 0.02). This is consistent with the data presented in Table 9 obtained using the enzymatic assay. When the oxidized cholesterol species are included, the Inuit VLDL content of total cholesterol (0.97 ± 0.22) is significantly different from that of control

TABLE 8

Serum lipoprotein profile.

	Total Cholesterol (mM/L)	Triglycerides (mM/L)	∝-Lipoprotein Cholesterol (mM/L)	B-Lipoprotein Cholesterol (mM/L)	B/∝
Inuit	5.9 ± 0.7	1.6 ± 0.7	1.8 ± 0.3*	3.4 ± 0.5	2.1 ± 0.4*
Control	5.2 ± 0.3	2.0 ± 0.4	1.2 ± 0.1	3.2 ± 0.2	2.8 ± 0.3

See "Experimental Procedures" for Assay Procedures. ∝-Lipoprotein corresponds roughly to HDL. B-Lipoprotein corresponds roughly to LDL. Values represent means ± S.E. (n = 5-12).

* $P < 0.05$ versus control.

TABLE 9

Plasma lipoprotein profile as determined by enzymatic assay.

	VLDL apo-lipoprotein mg/ml	LDL apo-lipoprotein mg/ml	VLDL Cholesterol umol/mg protein	LDL Cholesterol umol/mg protein	VLDL Cholesterol Esters umol/mg protein	LDL Cholesterol Esters umol/mg protein
Inuit	0.50 ± 0.05*	2.01 ± 0.14*	0.59 ± 0.13	0.92 ± 0.06*	0.63 ± 0.10	1.73 ± 0.13*
Control	0.71 ± 0.10	3.67 ± 0.14	0.79 ± 0.08	0.65 ± 0.03	0.49 ± 0.06	0.88 ± 0.07

See "Experimental Procedures" for assay procedures. Values represent means ± S.E. * $P < 0.05$ versus control.

TABLE 10

Native and oxidized cholesterol content of plasma as determined by HPLC.

	Native cholesterol umol/mg protein		Total cholesterol umol/mg protein	
	VLDL	LDL	VLDL	LDL
Inuit	0.58 ± 0.10	0.41 ± 0.05*	0.97 ± 0.22*	0.43 ± 0.06*
Control	0.42 ± 0.06	0.17 ± 0.02	0.53 ± 0.10	0.18 ± 0.02

Total cholesterol represents the sum of native and the identifiable oxidated derivatives. Values for total cholesterol represent mean ± SE. * $P < 0.05$ versus control.

subjects (0.53 ± 0.10). This information cannot be obtained with the more conventionally employed enzymatic determination of cholesterol as oxidized derivatives of cholesterol are not detected with the enzymatic method.

b) Quantification of Oxidized Derivatives of Cholesterol

In order to determine the content of oxidized derivatrives of cholesterol in the lipoprotein subfractions, aliquots of LDL and VLDL were subjected to HPLC analysis. Table 11 shows the incidence of two identified oxidated derivatives of cholesterol. Cholest-4-en-3-one (4-cholesten-3-one) was observed in 46% of the VLDL isolates from the Inuit plasma. The range of concentrations detected was from 0.004 μmol/mg protein to 0.514 μmol/mg protein. Only 3 of 12 control VLDL isolates (25%) exhibited this derivative (range of 0.108 to 0.199 μmol/mg protein). Only one of the Inuit subjects (8%) demonstrated the 3-keto derivative in the LDL isolate and none of the control LDL isolates exhibited this oxidized form. The more prevalent oxidation product, 25-α-hydroxycholesterol, was present in 46% of the Inuit VLDL isolates (6 of 13), the levels ranging from 0.013 μmol/mg protein to 1.78 μmol/mg protein. Fifty-eight percent (7 of 12) of the control VLDL isolates exhibited the 25-hydroxy derivative, with levels ranging from 0.002 μmol/mg to 0.353 μmol/mg protein. Five of thirteen (38%) of the Inuit subjects showed the 25-hydroxy derivative (0.002 μmol/mg to 0.242 μmol/mg) while only 2 of the control subjects (17%) exhibited this derivative (0.014 and 0.123 μmol/mg).

TABLE 11

Incidence of identified oxidated derivatives of cholesterol in isolated lipoprotein fractions of plasma.

	4-cholesten-3-one		25-∝-hydroxycholesterol	
	VLDL	LDL	VLDL	LDL
Inuit	46	8	46	38
Control	25	0	58	17

Values represent % of total population.

E. DISCUSSION

Incubation of cholesterol-rich liposomes with cardiac sarcolemmal vesicles resulted in a net transfer of cholesterol from liposomes to membrane vesicles. The extent of cholesterol transfer could be controlled by varying the vesicle-liposome incubation time or by altering the liposome cholesterol/phospholipid ratio. Several previous studies have shown that cholesterol moves between vesicles by transfer through the aqueous phase and not via fusion between the donor and acceptor vesicles[360-362]. In this study, experiments involving the incubation of sarcolemma with [^{14}C]sucrose containing liposomes and phospholipid profiles of the cholesterol-enriched membrane preparations (data not presented) indicated that fusion between donor liposomes and sarcolemmal vesicles was not a significant problem. These observations suggest that neither adsorption of liposomes to membrane nor co-sedimentation of liposomes confounded out results.

The present results support an important role for membrane cholesterol in sarcolemmal Na^+-Ca^{2+} exchange. Cholesterol enrichment of sarcolemmal vesicles resulted in a significant increase in the initial rate of Na^+-Ca^{2+} exchange (Figure 4). The stimulation was not due to a decrease in the permeability of the membrane for Ca^{2+} (Figure 9), nor was it due to a change in the amount of Ca^{2+} bound to the membrane (Table 1). Cholesterol enrichment decreased the K_m but did not alter the V_{max} for Ca^{2+} of the Na^+-Ca^{2+} exchange process (Figure 7).

The stimulation of Na^+-Ca^{2+} exchange by cholesterol appears to be a specific

effect. Sarcolemmal Ca^{2+}-Mg^{2+} ATPase activity was inhibited after cholesterol enrichment[150] (Figure 8). Changes in the cholesterol content of sarcolemmal vesicles had no effect on K^+-*p*-nitrophenylphosphatase activity, while enrichment of sarcolemmal cholesterol inhibited Na^+,K^+-ATPase activity, similar to results reported elsewhere[141,364-366]. This enhanced sensitivity of Na^+,K^+-ATPase, as opposed to K^+-*p*-nitrophenylphosphatase, has been observed previously[363]. The present study also showed that cholesterol depletion decreased the activity of Na^+,K^+-ATPase. Using a bovine kidney medulla basolateral membrane preparation, Yeagle et al[145] have also shown inhibition of Na^+,K^+-ATPase activity both when the membrane cholesterol content was enriched, and when the cholesterol content was decreased from that found in native membranes. They reported a linear decrease in ouabain-sensitive ATP hydrolyzing activity with increases in cholesterol content similar in character to the linear increase in motional ordering of the membrane with cholesterol enrichment that has been described elsewhere[146]. They proposed that the correlation between increases in motional order of the lipid hydrocarbon chains, induced by cholesterol, and decreases in enzyme activity, induced by cholesterol, resulted from a decrease in conformational flexibility of the enzyme, limited by the increase in motional order of the lipid hydrocarbon chains in the membrane. They felt the inhibition of Na^+,K^+-ATPase activity with cholesterol depletion suggested an essential role for cholesterol in the activity of the Na^+,K^+-ATPase. A requirement for cholesterol of the cardiac sarcolemmal membrane Na^+,K^+-ATPase and Na^+-Ca^{2+} exchange processes in reconstituted proteoliposomes has recently been shown by Vemuri and Philipson[324]. Using proteoliposomes with varying

phospholipid composition they showed the sterol requirement for both Na^+-Ca^{2+} exchange and Na^+,K^+-ATPase was highly specific for cholesterol. Cholesterol analogues with minor structural changes were unable to support ion transport activity. Their results as suggested a direct interaction of sterols with sarcolemmal ion transporters. The results of the present study support those of Yeagle et al[145] and Vemuri and Philipson[324]. With cholesterol enrichment it is possible that increases in the motional order of the membrane acount for the observed inhibition of Na^+,K^+-ATPase activity. With cholesterol depletion, Na^+,K^+-ATPase activity may be inhibited secondary to removal of this essential sterol from the lipid milieu of the transport protein. In this regard, the cholesterol content found in native bovine kidney basolateral membranes was sufficient to support maximal Na^+,K^+-ATPase activity[145].

The observed inhibition of Na^+,K^+-ATPase with cholesterol enrichment may be of clinical relevance for patients receiving cardiac glycosides, many of whom are hypercholesterolemic. It has recently been shown that not only is the sensitivity to ouabain increased in the myocardium of patients with CHD, (thought to be a reflection of decreased receptor number), but the maximally achievable positive inotropic effect is decreased[104]. The present data may correlate with the latter observation. Inhibition of Na^+,K^+-ATPase with increased membrane cholesterol may produce a positive inotropic effect in the heart, causing the tissue to be relatively refractory to cardiac glycosides with respect to an observed increase in contractility. In the patient with CHD receiving cardiac glycosides, this refractory state with respect to an observed response coupled with a decreased receptor number could contribute to increased toxicity from cardiac

glycosides. A decreased minimum toxic dose of ouabain has been observed in myocardial tissue of individuals with atherosclerosis[104].

The existence of membrane lipid annuli which exclude cholesterol has been proposed for two membrane-bound ion transport proteins (Ca^{2+}-Mg^{2+}-ATPase and Na^+,K^+-ATPase)[149,364]. Conversely our results can best be interpreted considering the presence of a cholesterol-rich annulus surrounding the Na^+-Ca^{2+} exchange protein which modulates its function. In the cholesterol enrichment studies, cholesterol would be preferentially absorbed into membrane regions of lower cholesterol content, away from the cholesterol-rich microdomain. Only with significant cholesterol transfer would the region surrounding the exchanger be affected. In contrast, with cholesterol depletion the driving force for cholesterol desorption would be greatest from the cholesterol-rich annulus, and this region would be depleted earliest. Cholesterol-enriched membrane preparations exhibit decreased fluidity[364], and it has been suggested that the decreased fluidity around membrane transport proteins is one of the mechanisms contributing to the observed depression in the activites of several transport systems[149,364]. The observation of a stimulation of Na^+-Ca^{2+} exchange suggests that cholesterol may be interacting with the exchange protein and modulating its activity. However, further investigation of the mechanism associated with the cholesterol effects is necessary.

Several reports have suggested that hypercholesterolemia may affect the myocardium independent of the atherosclerotic process[414-416]. In view of the present results, it is reasonable to hypothesize that cholesterol may directly alter myocardial function through its effects on sarcolemmal ion transport. Since Na^+,K^+-ATPase is

inhibited by cholesterol enrichment, intracellular Na^+ concentrations may increase in the intact cell, favouring a gain of Ca^{2+} through elevated Na^+-Ca^{2+} exchange. This may be further aggravated by the direct stimulation of Na^+-Ca^{2+} exchange by cholesterol. Coupled with depressed Ca^{2+} pumping activity, intracellular Ca^{2+} overload may develop. In this regard, cholesterol accumulation in the myocardium has been associated with altered Ca^{2+} homeostasis and cardiac dysfunction[367,368]. Derangements in intracellular Ca^{2+} contents secondary to cholesterol enrichment are felt to account for the observed changes in the contractile properties of papillary muscle from hypercholesterolemic rabbits[367], and functional changes in the isolated rat heart perfused with cholesterol containing perfusate[417].

The requirement of Na^+-Ca^{2+} exchange for membrane cholesterol was further demonstrated by oxidative modification of native cholesterol. Cholest-4-en-3-one was the only oxidized species produced in the membrane after incubation with cholesterol oxidase. This resulted in essentially complete inhibition of exchange activity. It was possible that this inhibition of Na^+-Ca^{2+} exchange after cholesterol oxidase was due to the generation of H_2O_2 since hydrogen peroxide inhibited Na^+-Ca^{2+} exchange (Figure 17) and scavenging the H_2O_2 with catalase protected the Na^+-Ca^{2+} exchanger. However, catalase could not protect the Na^+-Ca^{2+} exchanger from the effects of cholesterol oxidase. Further, large concentrations of H_2O_2 (5 mM) are required before an effect (30% inhibition) on Na^+-Ca^{2+} exchange is produced (Figure 17). This [H_2O_2] would not be achieved under the assay conditions in these experiments. These data indicate that the effects of cholesterol oxidase on sarcolemma are not the result of non-specific peroxide

effects on other membrane components. Data from subsequent studies showed that no lipid peroxidation occurred on treatment of LDL particles with cholesterol oxidase in the presence of catalase, as indicated by the absence of malondialdehyde production[421]. These data can be extrapolated to suggest that no lipid peroxidation products result from the treatment of membrane vesicles with cholesterol oxidase.

Other nonspecific effects of including cholesterol oxidase in the assay medium are unlikely. First, pretreatment of sarcolemmal membranes with cholesterol oxidase, and then centrifuging the membranes, resuspending the pellet, and assaying for Na^+-Ca^{2+} exchange (now in the absence of cholesterol oxidase) resulted in a similar inhibition as including the enzyme in the reaction medium directly (data not shown). This would indicate that the effects on Na^+-Ca^{2+} exchange were not due to the presence of the cholesterol oxidase molecule *per se*, but rather, a result of its enzymic properties. Second, the use of a different preparation of cholesterol oxidase elicited similar effects on Na^+-Ca^{2+} exchange (data not shown). These similar effects with two different sources of cholesterol oxidase would, therefore, argue against a contaminant in the enzyme preparation being the cause of the observed depression of Na^+-Ca^{2+} exchange activity. Thus, the effects of cholesterol oxidase on ion flux appear to be due to the generation of oxidized cholesterol *in situ* in the sarcolemmal membrane.

The possibility exists that the depression in ion movements may be due to the increase in membrane permeability. This is highly unlikely with respect to the Na^+-Ca^{2+} exchange reaction for several reasons. First, 2.0 units/ml cholesterol oxidase produced a 35-70% inhibition of the initial rate of Na^+-Ca^{2+} exchange (Figure 14), but 2.5

units/ml did not alter passive permeability characteristics of the membrane (Figure 18). Second, one would expect the inhibitory effect of cholesterol oxidase on Na^+-Ca^{2+} exchange to increase as the reaction progressed in time if membrane permeability were a significant factor. Instead, the most dramatic effects of cholesterol oxidase were at the earliest time points of the reaction (Figure 14, inset). Third, the effects of cholesterol oxidase on exchange were observed after very short reaction times (0.5 second), whereas even after 30 seconds of incubation, passive efflux was unaffected at these lower concentrations of enzyme (Figures 18 and 19). Fourth, because the rate of Ca^{2+} accumulation via exchange is much greater than that lost through passive Ca^{2+} efflux, large increases in membrane leakiness are required before any effect on Na^+Ca^{2+} exchange would be observed[322]. Thus, the data argue for a direct inhibitory effect of oxidized cholesterol on the Na^+-Ca^{2+} exchange pathway.

Oxidation of *in situ* membrane cholesterol also depressed another important transsarcolemmal Ca^{2+} transport pathway; the ATP-dependent Ca^{2+} pump. Changes in membrane permeability may account for the observed depression in this ATP-dependent pathway of Ca^{2+} uptake. Since the concentrations of cholesterol oxidase required to increase membrane permeability and inhibit ATP-dependent Ca^{2+} uptake are similar (Figures 19-21), the incubation times are similar (30-60 seconds), and the rate of ATP-dependent Ca^{2+} uptake is slower than that of Na^+-Ca^{2+} exchange, it is more difficult to separate the effects of cholesterol oxidase on active Ca^{2+} transport from passive Ca^{2+} flux. Certainly, it is clear that Na^+-Ca^{2+} exchange is far more sensitive to oxidized cholesterol in the membrane than is the sarcolemmal Ca^{2+} pump.

The results may provide information on the relationship of membrane cholesterol with these two sarcolemmal ion flux pathways. Oxysterols are less polar than cholesterol, occupy a higher molecular area in the membrane[369,370], and are distributed very differently in the membrane. Cholesterol tends to form cholesterol-rich domains in the membrane and is most effective on the hydrophobic region of the membrane bilayer. Conversely, oxysterols such as cholestenone are randomly distributed across the bilayer plane and do not form defined domains[369]. In addition it has been suggested that oxidation of the 3ß-hydroxy group alters the ability of the molecule to hydrogen bond with membrane phospholipid[371]. It is clear that higher concentrations of membrane oxysterols will cause a disordering of the membrane lipids leading to an increase in membrane fluidity and permeability characteristics[371]. This would account for the observations of enhanced passive Ca^{2+} efflux after extensive treatment of the membrane with cholesterol oxidase. However, membrane fluidity has been shown to be unaltered even after 24% of the membrane cholesterol was oxidized[372]. Thus, although changes in membrane fluidity may explain the increased permeability properties of the membrane, they are unlikely to provide a full explanation for the effects on Na^+-Ca^{2+} exchange. Instead, the disturbance of cholesterol-rich domains in the membrane by the oxidation reaction may better explain the effects demonstrated on the Na^+-Ca^{2+} exchanger. This is consistent with the results of membrane cholesterol enrichment/depletion experiments which are also compatible with a cholesterol rich annulus associated with the Na^+-Ca^{2+} exchange protein.

As indicated earlier, oxidation of cholesterol *in situ* would alter the hydrophobic

region of the membrane[369]. Because cholesterol primarily exerts its effects in the hydrophobic core of the membrane bilayer[369], these results suggest that the Na^+-Ca^{2+} exchanger is more sensitive to this region of the membrane. This interpretation is consistent with the data of Philipson and Ward[322], who used doxyl-stearates and suggested that the hydrophobic region of the membrane may be the most important area for affecting the Na^+-Ca^{2+} exchange reaction. However, this does not detract from the significant effects that modification of the hydrophilic head group can have on Na^+-Ca^{2+} exchange[234,324,330].

Despite a depression in Na^+-Ca^{2+} exchange activity an increase in passive Ca^{2+} binding to the sarcolemma after oxidation of the membrane cholesterol was seen. The observation of a dissociation between the bulk Ca^{2+} binding to the membrane and Na^+-Ca^{2+} exchange (stimulation of binding and inhibition of exchange) is not novel. Other agents have also produced similar qualitative effects[330,373], and the present findings further strengthens the arguement that the two processes are unrelated. Instead, a pool of Ca^{2+} localized in close proximity to the Na^+-Ca^{2+} protein may be far more functionally important to the exchanger than the bulk Ca^{2+} bound to the entire sarcolemmal membrane.

It is important to emphasize that these results should not be interpreted as inferring a role for the enzyme cholesterol oxidase in cardiac pathophysiology. It is found in tissues other than the heart[374]. Instead, the enzyme was used in these experiments to selectively oxidize membrane cholesterol to better understand the effects on ion movements of this oxysterol and of native cholesterol. These observations,

however, may have implications with regard to the mechanism of oxygen free radical action on the myocardium. Oxygen free radicals have been demonstrated to play a role in ischemic/reperfusion damage in the heart[375]. A depression in Na^+-Ca^{2+} exchange in isolated sarcolemmal vesicles[376] and enhanced membrane permeability[377] have been reported during ischemic/reperfusion challenge. Free radicals can oxidize membrane cholesterol[340,378], increase membrane permeability[379] and inhibit Na^+-Ca^{2+} exchange[380], although the effect on Na^+-Ca^{2+} exchange remains controversial[235]. As shown in the present study, oxidized membrane cholesterol can depress sarcolemmal Na^+-Ca^{2+} exchange and increase membrane permeability. The data are consistent, therefore, with the possibility that free radicals may alter ion movements across the sarcolemmal membrane during ischemia/reperfusion challenge through an oxidation of membrane cholesterol residues, as well as other sites on the membrane such as target proteins or fatty acid residues. However, further experiments to determine the presence of oxidatively modified membrane cholesterol in ischemic/reperfused hearts are required.

Oxidatively modified lipids contained in low density lipoproteins have been recently proposed to play an important role in atherogenesis[85-87,93], and oxidation of LDL cholesterol has been postulated to increase the atherogenicity of the LDL particle[95]. Although it is clear that oxidized lipids like cholesterol may enter the cell via a low density lipoprotein receptor-independent scavenger pathway, the role of the oxidatively modified cholesterol once it is deposited within the cell is not known. These data suggest the possibility that this oxidized lipid may alter ionic interactions within the muscle cell.

The importance of increased vascular smooth muscle cell-associated Ca^{2+} in the

process of atherogenesis and in the contractile abnormalities associated with hypercholesterolemia is well documented[127,128,131,132,381-383]. Many reports have identified an increased calcium content of human atherosclerotic arteries[131] and arteries from hypercholesterolemic experimental animals[127,128,381]. This increased tissue calcium seen in atherosclerotic lesions of experimental animals has been shown to be a result of, in part, increased cellular calcium uptake into vascular smooth muscle cells[382,383]. However the direct relationship between high levels of circulating cholesterol and Ca^{2+} flux in arterial smooth muscle cells is less clear. Several studies have shown that arterial tissue segments exhibit altered Ca^{2+} transport when exposed to elevated levels of cholesterol[131,132]. There exists controversy, however, concerning the effects of cholesterol on arterial segments in a perfusing bath. Yokoyama and Henry[384] have suggested that cholesterol may act as a direct vasoconstrictor by altering the intrinsic characteristics of the membrane and thereby altering arterial Ca^{2+} movements and tension development. Others have demonstrated that cholesterol-treated arterial segments exhibit alterations in Ca^{2+} movements and tension development only during stimulation with certain agonists[132]. The results of the present study support the observations of Yokoyama and Henry[384]. In the absence of agonists, significant alterations in the rate of Ca^{2+} exchange in the slowly exchangeable component and in the La^{3+} displaceable Ca^{2+} of vascular smooth muscle cells were observed.

Enrichment with cholesterol and measurement of Ca^{2+} flux in isolated smooth muscle cells as described in the present study has several advantages over the previously reported techniques employing tissue segments from muscular arteries[132,384]. Kinetic

analysis of Ca^{2+} flux in intact tissue preparations is complicated by diffusion delays in the extracellular space, as well as by the presence of large amounts of matrix-bound or free background calcium[385]. The presence of a variety of cell types in intact vascular preparations, with diverse responses to cholesterol and distinct Ca^{2+} flux characteristics renders it impossible to unambiguously relate the exchange characteristics to the muscle cells. These problems can be avoided by examining ion transport characteristics in isolated cultured smooth muscle cells[352,385]. In the present study, it can be conclusively determined that the smooth muscle cell cholesterol content and Ca^{2+} flux are being altered by exposure to lipoproteins.

The scintillator disc flow cell technique for measuring Ca^{2+} exchange in isolated, cultured aortic smooth muscle cells employed in this study has several advantages over other previously described techniques[260,342,385-389]. First, in this method, the cells from each experiment serve as their own controls. Ca^{2+} exchange was measured in the cells, and the same cells were used to to assess sensitivities to low Na^+ or La^{3+}. Second, the measurement of cellular Ca^{2+} movements was on-line and continuously monitored. It was not necessary to remove the cells from their environment, wash and solubilize them to obtain information on Ca^{2+} flux as has been done in other studies. Third, because the system is on-line and the flow cell can accomodate relatively high flow rates, Ca^{2+} exchange kinetics were resolved into very rapid and slow components. These results demonstrate that $> 50\%$ of the exchangeable Ca^{2+} in vascular smooth muscle cells is rapidly exchangeable with a half-time for exchange of < 10 s. Since the more conventional previously employed techniques require more time than this to remove

extracellular Ca^{2+}, much of that Ca^{2+}flux will be missed.

The experimental protocol in this study involved the use of isolated VLDL and LDL to modify the cellular cholesterol levels of isolated cultured vascular smooth muscle. In view of recent arguments regarding the optimal mode of cholesterol enrichment (cholesterol in aqueous suspension versus cholesterol containing liposomes[390]), the present data using isolated lipoproteins must represent an improvement in the methodology for delivering lipid to the cell. This contention is based on two lines of reasoning. First, exposure of smooth muscle cells to lipoproteins represents a more physiological approach to cholesterol enrichment than cholesterol/phosphatidylcholine liposomes or crystalline cholesterol which is only sparingly soluble in aqueous suspension. Second, delivery of lipids via lipoproteins will result in accumulation of esterified as well as native cholesterol. This has not been been possible using only free cholesterol with methods employed in the previous studies.

The rapid and slow Ca^{2+} exchange components are similar to those observed previously[343]. As discussed in detail elsewhere[391], La^{3+} does not displace any Ca^{2+} from the slowly exchangeable fraction and therefore, this component probably represents an intracellular pool of Ca^{2+} localized to the SR. This component is also sensitive to agents like caffeine which are well known to affect Ca^{2+} movements in the SR[392]. The rapidly exchangeable Ca^{2+} component is La^{3+} sensitive. Because La^{3+} does not enter the cell[352,358,359,391], this Ca^{2+} must be in very rapid equilibration with the sarcolemmal membrane of the smooth muscle cell. The bulk of this La^{3+}-displaceable Ca^{2+} is felt to

be associated with the surface of the sarcolemma, however, the possibility exists that an intracellular subsarcolemmal Ca^{2+} pool, if it is in rapid equilibration with plasma membrane Ca^{2+}, may also be affected by La^{3+} perfusion. The presence of such a pool has been advanced for myocardial cell Ca^{2+} exchange[391].

The present investigation demonstrated changes in both the rapidly and slowly exchangeable Ca^{2+} fractions after extended incubation of the cells with isolated LDL. LDL treatment enhanced the rate of exchange in the slowly exchangeable pool and increased the amount of Ca^{2+} associated with the sarcolemma (La^{3+} displaceable Ca^{2+}). Measurement of cell associated calcium in a low Na^{+} environment which is felt to increase cell surface associated Ca^{2+} revealed a significantly greater increase in cell surface Ca^{2+} in LDL treated cells. The pathophysiological consequences of this are unclear, however a plasmalemmal bound Ca^{2+} pool has been suggested to be involved in contractile activity of the rabbit aorta[388]. The concentrations of LDL cholesterol employed in the present study (0.1-2.5 μmol/ml) were within the physiological range for humans[393]. VLDL also increased the rate of exchange in the slowly exchangeable pool and tended to increase the La^{3+} displaceable Ca^{2+} (although the latter did not reach statistical significance). The data are in accord with previous studies which have demonstrated that atherosclerotic conditions or elevated cholesterol levels in aortic tissue result in a substantial increase in tissue associated calcium[126-130]. Although much of the Ca^{2+} found in an atherosclerotic plaque is extracellular in origin, a large portion does appear to be intracellular[131-132]. The data presented here suggest that alterations in the Ca^{2+} handling capabilities of both the SR and the sarcolemma may be responsible for this

change. The data also indicate that LDL is directly capable of affecting these changes in the smooth muscle cell.

The mechanism of action of LDL on the cells is not entirely clear but increased cholesterol deposition within the smooth muscle cell would be the most probable interpretation. Alterations in sarcolemmal membrane cholesterol are capable of affecting specific ion transport pathways in other tissues[148-150]. This would appear to be the case in the present study, although isolation of the plasma membrane would be required for definitive proof of this mechanism. No study to date has examined Ca^{2+} flux in cholesterol-enriched sarcolemmal membranes from vascular smooth muscle. It is clear that the effects observed were not due to a change in the passive permeability properties of the cells as this exchange component is much slower than the 20-50 minute exchange half-time for the slow exchange fraction exhibited by the cells in the present study.

Oxidatively modified LDL particles have been suggested to play a significant role in atherogenesis[85-87], and oxidation of the cholesterol moiety of the LDL complex has been implicated as contributing to this enhanced atherogenicity[95]. Recently, Panasenko et al[394] have shown that incubation of erythrocytes with *in vitro* oxidized LDL particles or LDL particles of patients with known ischemic heart disease resulted in a similar enhanced transport of cholesterol to these cells over that observed using LDL particles from healthy volunteers. They suggested a similarity between oxidatively modified LDL particles and those found in patients with ischemic heart disease. In the present study, blood lipoprotein, lipid, and oxidized cholesterol levels were measured in a group of individuals known to be at decreased risk for ischemic heart disease, namely the

traditionally living Inuit, to ascertain whether serum levels of oxidatively modified derivatives of cholesterol can be used as a risk marker for the development of atherosclerosis.

The Inuit included in this study showed characteristics different from the control population. They exhibited a mean age 12 years greater than controls. In addition, the ponderal index (PI) was significantly lower in the Inuit group. This is consistent with previous studies which have also shown the Inuit to have ponderal indices lower than those of the predominantly caucasian North American population. Insurance statistics have shown significantly higher mortality rates for non-Inuit North Americans with a PI below 12.5[395]. The validity of using PI to assess obesity has come under question[396]. Other assessments of obesity, such as skinfold measurements, have shown the Inuit to be more lean than their North American counterparts, and a lower PI is thought to reflect a constitutive difference in stature in this population[396].

Previous studies have shown that the Inuit population had significantly lower levels of triglycerides, cholesterol and ß-lipoproteins compared with caucasian controls[397,398]. In contrast, the present study shows no significant differences between the Inuit and control subjects with respect to these parameters (Table 8). This discrepancy may in part be due to the acculturization of the Inuit, which has been particularily rapid over the past 15 years[399]. Similar to previous reports[397,398] the data presented in the present study show the Inuit to have significantly higher levels of serum α-lipoprotein (HDL), which has been shown to have a strong negative relationship with coronary artery disease[393]. This may be one explanation for the continued lowered incidence of ischemic

heart disease in the Inuit.

The Inuit showed a decreased concentration of both VLDL and LDL associated apolipoprotein compared with control subjects, despite similar ß-lipoprotein cholesterol levels. Since the apolipoprotein component of LDL particles is thought to consist entirely of apoB[400], this suggests a lower apolipoprotein B level in the Inuit. Elevated apoB has been identified as a predictor for the risk of premature coronary artery disease despite normal serum and LDL cholesterol levels[401,402]. Thus a decreased LDL-associated apoB level might also be contributing to the reduced risk of ischemic heart disease in the Inuit population.

Both the incidence of identifiable species of oxidized cholesterol, and the total amount of oxidized cholesterol expressed per mg of apolipoprotein was found to be higher in the Inuit as compared to the control subjects. Although limited by sample size, the data obtained in this pilot study suggest that in this population, the levels of oxidized cholesterol in the various lipoprotein fractions cannot be used as negative risk markers for the development of coronary artery disease. However, the simultaneous presence of another disease process could also be a confounding factor in the inuit population studied here. In order to conclusively demonstrate the validity of using oxidated derivatives of cholesterol as risk markers, a prospective study involving a large number of subjects needs to be performed.

In summary, the present results demonstrated that incorporation of cholesterol into isolated cardiac sarcolemmal vesicles resulted in a significant stimulation of Na^+-Ca^{2+} exchange. This stimulation was specific to Na^+-Ca^{2+} exchange as the activities of both

Ca^{2+}-Mg^{2+} ATPase (Ca^{2+} pump) and Na^+,K^+ ATPase were inhibited by cholesterol enrichment. Extensive oxidation of membrane cholesterol by cholesterol oxidase significantly increased membrane permeability characteristics. Less extensive oxidation of membrane cholesterol directly inhibited sarcolemmal Na^+-Ca^{2+} exchange and to a lesser extent, Ca^{2+} pump activity. Passive Ca^{2+} binding to the sarcolemma was strikingly stimulated. These data suggest that cholesterol plays an important role in the modulation of cardiac sarcolemma Na^+-Ca^{2+} exchange activity. Oxidation of membrane cholesterol significantly altered ionic interactions in cardiac sarcolemma and may represent an important mechanism whereby free radicals exert their effects.

This study also established that extended incubation of cultured vascular smooth muscle cells with LDL alters Ca^{2+} interactions within the cell. Both the sarcolemmal and SR Ca^{2+} pools appeared to be affected. Since both of these pools have been suggested to be involved in contractile activity of vascular smooth muscle, these results may have implications for abnormal contractile activity of arterial tissue segments under atherosclerotic conditions and may help explain the vascular hyperreactivity observed in hypercholesterolemia and atherosclerosis.

Examination of the serum lipid and lipoprotein profile in a population with decreased risk of developing CHD, namely the Inuit showed levels of triglycerides, cholesterol and ß-lipoprotein similar to a westernized caucasian cohort. Both HDL and oxidized derivatives of cholesterol were found to be higher in the Inuit subjects. These findings suggest that serum levels of oxidized cholesterol cannot be used as a an independent risk marker for the development of CHD. More epidemiological studies are

required to assess the clinical correlates of an increased serum level of oxidized derivatives of cholesterol.

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