ALTERATIONS IN CALCIUM TRANSPORT MECHANISMS OF CARDIAC SARCOLEMMA WITH ADVANCING AGE

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SRISALA NAVARATNAM

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

DEDICATED

TO MY PARENTS

AND

TO MY TEACHER DR.J.C.KHATTER

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A doctoral degree is not achieved through the hard work of one person alone. Many people must help along the way and I wish to thank these people for their invaluable help and encouragement.

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ABSTRACT

We hypothesized that alterations in sarcolemmal Ca^{2+} transport mechanisms may contribute to the characteristic alterations in myocardial function observed with advancing age. Specifically, the possible modifications in Na^{+}-Ca^{2+} exchanger and voltage-gated calcium channel regulation of Ca^{2+} were investigated in the present study.

The activities of the Na^{+}-Ca^{2+} exchanger were studied in relatively pure sarcolemmal vesicles isolated from 3, 6 and 18 month old guinea-pig hearts. The rate and the amount of Na^{+}-dependent $^{45}Ca^{2+}$ uptake by the sarcolemmal vesicles were found to decline progressively with advancing age. This was further characterized by a 70% reduction in the maximum rate of $^{45}Ca^{2+}$ uptake (Vmax) associated with reduced affinity (1/km) for $^{45}Ca^{2+}$ in the vesicles of 18 month old guinea-pig hearts. The initial rates of Na^{+}-dependent $^{45}Ca^{2+}$ extrusion were also lower in the vesicles of older hearts. In addition, there was a greater retention of $^{45}Ca^{2+}$ by these vesicles at the end of 3 minutes of incubation. The decrease in Na^{+}-Ca^{2+} exchange activity was not limited to the aging of guinea-pigs but also observed in rat sarcolemmal vesicles during aging from 2 to 12 months. Since Na^{+}-Ca^{2+} exchange is an important mechanism of Ca^{2+} efflux in the cardiac myocyte, the decreased activity would result in a lower rate and amount of Ca^{2+}

extrusion and this may contribute to the slower myocardial relaxation in older animals.

The myocardial voltage-gated calcium channels were investigated by studying a) myocardial response to calcium channel agonist BAY K 8644 in whole animal and in isolated perfused hearts, (b) properties of dihydropyridine receptor site of voltage-gated calcium channel and (c) phosphorylation of calcium channel subunits. Aging of rats from 2 to 12 months resulted in a substantial increase in positive inotropic and toxic response to BAY K 8644 in whole animal model and in isolated perfused hearts. Similarly, the myocardial sensitivity to BAY K 8644 increased significantly during aging of guinea-pigs to 12 months, which was maintained during senescence to 24 months. The enhanced inotropic response during aging to 12 months, to calcium agonist BAY K 8644, is most likely to be due to the changes in its receptor characteristics of the dihydropyridine site of voltage-gated channel.

The characteristic properties of dihydropyridine receptor sites were studied in relatively pure sarcolemmal vesicles of 2, 12 and 24 month old rat hearts using [^{3}H]BAY K 8644 and [^{3}H]Nitrendipine as radio-ligands. The density of [^{3}H]BAY K 8644 binding sites in 12 month old rat heart sarcolemma was 42% higher than that of 2 month old which may explain the enhanced sensitivity of 12 month old rat hearts to BAY K 8644.

The affinity of the receptors to bind [^{3}H]BAY K 8644 was however lower in the older animal myocardium. Similar to [^{3}H]BAY K 8644, the number of [^{3}H]Nitrendipine binding sites also increased during adult maturation which was then maintained during senescence. Unlike the agonist, the affinity to bind calcium channel antagonist [^{3}H]Nitrendipine remained unaltered in all three age groups of rats. The increase in [^{3}H]Nitrendipine binding in the 12 month old rat heart was seen in all membrane fractions throughout the purification scheme, indicating that the difference in membrane purity was not a contributing factor to the observed increase in dihydropyridine receptor density. Furthermore, the sarcolemmal and the subcellular marker enzyme assays have demonstrated that the extent of purification of the final membrane was similar in all three age-groups. Since dihydropyridine receptor sites are known to represent voltage-gated calcium channels, the increase in dihydropyridine receptor density would indicate that the number of myocardial voltage-gated calcium channels is increased during adult maturation and maintained through senescence.

Photoaffinity labelling of cardiac sarcolemmal membrane using [^{3}H]Nitrendipine has indicated that the protein subunits with the molecular weights of 35 KD, 55 KD and 170 KD represent the calcium channel subunits. The 170 KD protein

subunit appeared as 100 KD and 70 KD under the reducing conditions used in our experiments. The calcium channel subunit of 55 KD molecular weight protein was phosphorylated by an endogenous protein kinase only in the presence of Nitrendipine. The extent of its phosphorylation was almost 72% higher in the membrane of 12 month old than that of 2 month old rat hearts. On the other hand, its phosphorylation by cAMP-dependent protein kinase was lower in 12 month old rat hearts, even in the presence of exogenous protein kinase. These results suggest that during adult maturation and aging there may be an intrinsic modification in channel subunits along with the increase in number, lead to differential alterations in its calcium antagonist and cAMP-induced phosphorylation. The cAMP-dependent protein kinase also phosphorylated other sarcolemmal proteins with the molecular weights of 9 KD, 15 KD, 24 KD and 96 KD. The degree of these protein phosphorylations by endogenous protein kinase were lower in 12 month old rat heart sarcolemma. However, when the exogenous protein kinase was added to the incubation medium, the level of phosphorylations in 2 and 12 month old rat heart membranes were similar. This may indicate a lower activity of cAMP-dependent protein kinase in 12 month old rat heart sarcolemma.

The results from the present study demonstrate that the relative contribution of trans-sarcolemmal Ca^{2+} influx to the

myocardial contraction increases with aging to 12 months, which is then maintained at this level during senescence. This greater trans-sarcolemmal Ca^{2+} influx occurs via voltage-gated calcium channels, which are increased in number. The Ca^{2+} flux through Na^{+}-Ca^{2+} exchanger on the other hand, decreases during aging.

TABLE OF CONTENTS

	PAGE
I. INTRODUCTION	1
MORPHOLOGICAL CHANGES IN AGING	2
AGE-RELATED CHANGES IN MYOCARDIAL FUNCTION DEMONSTRATED IN HUMAN POPULATION	3
AGE-RELATED CHANGES DEMONSTRATED IN IN-VITRO STUDIES	
a) Contraction duration	8
b) Myofilament ATPase	10
c) Myoplasmic Ca^{2+} transient	13
d) Ca^{2+} transport by sarcoplasmic reticulum	14
e) Transmembrane action-potential	16
ALTERED PHARMACOLOGICAL RESPONSE OF AGING MYOCARDIUM	
a) Catecholamines	19
b) Cardiac glycosides	22
CALCIUM TRANSPORT MECHANISMS OF CARDIAC SARCOLEMMA	
a) Voltage-gated calcium channels	24
i. Electrophysiological properties	26
ii. Biochemical properties	28
iii. Identification and purification of calcium channel subunits	30
iv. Regulation of voltage-gated calcium channel	32
b) Na^{+}-Ca^{2+} exchanger	34
c) Sarcolemmal Ca^{2+} pump	39
MYOCARDIAL FUNCTION AND AGING: ESSENCE . . .	40
II. SPECIFIC OBJECTIVES	41
III. METHODOLOGY	
ANIMAL MODEL	42
INVESTIGATION OF Na^{+}-Ca^{2+} EXCHANGE ACTIVITY	
a) Isolation of relatively pure sarcolemmal membrane vesicles	43
b) Na^{+}-dependent Ca^{2+} uptake	44
c) Na^{+}-dependent Ca^{2+} efflux	45
INVESTIGATION OF VOLTAGE-GATED CALCIUM CHANNEL	
a) Cardiovascular response to BAY K 8644 in whole animal model	45

PAGE

b) Myocardial response to BAY K 8644 in isolated perfused hearts.
i. Rat as an animal model 47
ii. Guinea-pig as an animal model 47
c) Investigation of dihydropyridine receptor sites of voltage-gated calcium channel
i. Preparation and characterization of cardiac sarcolemmal membrane 48
ii. Separation of sarcolemmal membrane proteins by SDS-polyacrylamide gel electrophoresis 50
iii. Binding of calcium antagonist [^{3}H] Nitrendipine 51
iv. Binding of calcium agonist [^{3}H]BAY K 8644 52
d) Identification of calcium channel subunits by photoaffinity labelling 53
e) Phosphorylation of cardiac sarcolemmal membrane
i. Nitrendipine-induced phosphorylation 54
ii. cAMP-induced phosphorylation 54

DATA ANALYSIS 55

REAGENTS 56

IV. RESULTS

PHYSICAL CHANGES WITH AGE 57

EFFECT OF AGING ON SARCOLEMMAL Na^+-Ca^{2+} EXCHANGE ACTIVITY
a) Na^+-dependent $^{45}Ca^{2+}$ uptake 57
b) Kinetics of Na^+-dependent $^{45}Ca^{2+}$ uptake 60
c) Na^+-dependent $^{45}Ca^{2+}$ efflux 62
d) Na^+-Ca^{2+} exchange activity in rat heart sarcolemmal membrane 67

EFFECT OF AGING ON MYOCARDIAL VOLTAGE-GATED CALCIUM CHANNELS
a) Cardiovascular response to BAY K 8644 in whole animal model
i. Age-related changes in positive inotropic response to BAY K 8644 69
ii. Age-related changes in pressor response to BAY K 8644 73
iii. Effects of Nifedipine pretreatment on the response of BAY K 8644 73
iv. Arrhythmogenic toxicity of BAY K 8644 75
b) Age-related changes in response to BAY K 8644 in isolated perfused hearts
i. In rat hearts 78
ii. In guinea-pig hearts 80

PAGE

c) Age-related changes in dihydropyridine binding site of voltage-gated calcium channel of cardiac sarcolemma
i. Binding of [^{3}H]BAY K 8644 80
ii. Binding of [^{3}H]Nitrendipine 87
d) Purity of cardiac sarcolemmal membrane of 2, 12 and 24 month old rats 91
e) Identification of calcium channel subunits by photoaffinity labelling 96
f) Phosphorylation of cardiac sarcolemma and its age-related changes
i. Nitrendipine-induced phosphorylation 101
ii. cAMP-induced phosphorylation 105

V. DISCUSSION 110

VI. CONCLUSIONS AND SIGNIFICANCE 133

VII. REFERENCES 135

LIST OF TABLES

TABLE		PAGE
1.	Kinetic constants of Na^{+}-dependent Ca^{2+} uptake in 3 and 18 month old guinea-pig heart sarcolemmal vesicles.	65
2.	Basal cardiovascular parameters of 2 and 12 month old rats.	71
3.	Effect of Ca^{2+} on [^{3}H]BAY K 8644 binding to sarcolemmal membrane of 2 month old rat heart.	85
4.	Binding constants of [^{3}H]BAY K 8644 for 2 and 12 month old rat heart sarcolemmal membranes. . . .	88
5.	Binding constants of [^{3}H]Nitrendipine for 2, 12 and 24 month old rat heart sarcolemmal membranes.	92
6.	Sarcolemmal and subcellular marker enzyme activities in membrane fractions from 2, 12 and 24 month old rat hearts.	95

LIST OF FIGURES

FIGURE		PAGE
1.	Changes in body and heart weight of rats in aging.	58
2.	Changes in body and heart weight of guinea-pigs in aging.	59
3.	Time-course of Na^+-dependent $^{45}Ca^{2+}$ uptake in sarcolemmal vesicles of 3, 6 and 18 month old guinea-pigs.	61
4.	Initial rate of Na^+-dependent $^{45}Ca^{2+}$ uptake as a function of $[Ca^{2+}]$ in guinea-pig sarcolemmal vesicles.	63
5.	Eadie-Hofstee plot of Na^+-dependent $^{45}Ca^{2+}$ uptake.	64
6.	Time-course of Na^+-dependent $^{45}Ca^{2+}$ efflux in sarcolemmal vesicles of 3, 6 and 18 month old guinea-pigs.	66
7.	Na^+-dependent $^{45}Ca^{2+}$ efflux in guinea-pig sarcolemmal vesicles expressed as a percentage of basal value.	68

PAGE

8. Time-course of Na^+-dependent $^{45}Ca^{2+}$ uptake in 2 and 12 month old rat heart sarcolemmal vesicles. 70

9. Positive inotropic response of BAY K 8644 in 2 and 12 month old rats. 72

10. Pressor response to BAY K 8644 in 2 and 12 month old rats. 74

11. Effect of BAY K 8644 on heart rate. 76

12. Arrhythmogenic toxicity of BAY K 8644 in 12 month old rat heart. 77

13. Positive inotropic response of BAY K 8644 in isolated perfused hearts of 2 and 12 month old rats. 79

14. Positive inotropic response of BAY K 8644 in isolated perfused hearts of 2, 12 and 24 month old guinea-pigs. 81

15. Dose-response of BAY K 8644 in 2, 12 and 24 month old isolated guinea-pig hearts. 82

PAGE

16. Specific binding of [^{3}H]BAY K 8644 to the cardiac sarcolemma of 2 month old rat as a function of its concentration. 84

17. Scatchard plots of specific binding of [^{3}H]BAY K 8644 to 2 and 12 month old rat hearts sarcolemma. 86

18. Specific and non-specific binding of [^{3}H] Nitrendipine to sarcolemmal membrane of 2, 12 and 24 month old rat hearts. 89

19. Scatchard plots of specific binding of [^{3}H] Nitrendipine to 2, 12 and 24 month old rat hearts sarcolemma. 90

20. Specific binding of [^{3}H]Nitrendipine to homogenate, microsomes and sarcolemmal membrane of 2 and 12 month old rat hearts. 93

21. Densitometer tracings of electrophrograms of 2 and 12 month old rat heart sarcolemmal membrane. . 97

22. Electrophoretic profile of sarcolemmal membranes of 2 and 12 month old rat heart using 15% SDS-polyacrylamide. 98

PAGE

23. Electrophoretic profile of sarcolemmal membranes of 2 and 12 month old rat heart using 7% polyacrylamide. 99

24. Electrophoretic profile of photoaffinity labelled sarcolemmal membrane of 2 month old rat heart by [^{3}H]Nitrendipine. 100

25. Nitrendipine-induced phosphorylation profile of cardiac sarcolemma of 2 and 12 month old rats. . . 103

26. Time-course of Nitrendipine-induced phosphorylation of 55 KD calcium channel subunit. 104

27. Phosphorylation profile of 2 and 12 month old sarcolemmal membranes by cAMP-dependent endogenous protein kinase. 106

28. Phosphorylation profile of 2 and 12 month old sarcolemmal membranes by cAMP-dependent exogenous protein kinase. 107

29. A proposed model for myocardial voltage-gated calcium channel and its regulation. 131

PAGE

30. Schematic representation of possible alterations in Ca^{2+} transport that may lead to the characteristic alterations in myocardial function in aging. 134

I. INTRODUCTION

In recent years there has been a rapid development of interest in the investigation of pathophysiological changes that occur in man with advancing age. Major impetus has come from the knowledge of demographic characteristics of developed countries. In 1982, those over 65 years of age constituted 11% of the human population in United States of America. The demographers predict that in the year 2035 they will increase to 17-18%. Perhaps more significant is the age distribution of this population. The number of people between 65 and 75 years old will increase from 8 to 18 million and those over 85 will triple from 2.5 to 7.5 million. Similar occurences may also be predicted among the Canadian population. Moreover, it is clear that there is an ever increasing incidence of cardiac diseases with advancing age such that it has become the number one killer in the population over 60 years of age. As such, the knowledge of age-related changes in the cardiovascular system has become an important factor in the practice of current clinical medicine.

The impact of aging on the cardiovascular system has been a subject of considerable research since Master and Oppenheimer demonstrated a decrease in work capacity of older individuals in 1929. Subsequently, many characteristic changes in the cardiovascular system have been described in

humans (Port et al.1980, Rodeheffer et al.1984, Miller et al.1986) as well as in many animal models (Lakatta 1987). The greatest difficulty in this field of research is to separate the disease related changes that are prevalent in the elderly such as ischaemic heart disease and generalized atherosclerosis from the true age-related changes. In attempts to differentiate alterations due to aging from those related to disease, investigators utilize the anatomical and pathological observations in so far as possible to identify the presence of a specific disease state. In addition to the direct demonstration of a disease state, certain other types of evidence may also support the notion that a specific alteration is in fact a true age-related change. The important one of these is the demonstration of similar modifications in more than one species. Several age-related changes have therefore been characterized in many animal species such as rats, guinea-pigs, dogs and rabbits. A second type of evidence is the observation of age-related changes in a longitudinal study of a population rather than in a cross sectional study. This has not always been possible and becomes impossible when it is necessary to sacrifice the animal in order to carry-out in-vitro studies.

MORPHOLOGICAL CHANGES OF THE MYOCARDIUM DURING AGING

Certain morphological changes are commonly observed in

the very elderly heart that usually do not yield any clinical signs of cardiac dysfunction. Autopsy studies of large groups of patients have shown a general increase in heart weight with advancing age, except in the patients aged 90 years and older in whom there is a slight decrease in heart weight (Waller et al. 1984). The increase in weight in aging is also associated with ultra structural changes such as cardiac amyloidosis, basophilic degeneration of the myocardium and brown atrophy (Rosai and Lascano 1970). However, there appears to be no objective morphologic characteristics of the heart which permits an accurate assessment of its age. Even the experienced morphologist can estimate the age of a human heart with only limited success. Apart from the changes in the myocardium, changes in valves such as fibrous thickening, fatty alterations and calcification and changes in coronary vasculature namely increased tortuosity and dilatation, calcific and atherosclerotic plaques are also demonstrated during aging (Waller et al. 1983 & 1984). It is evident however that these morphological changes are not characteristics of aging and are not solely due to advancing age.

AGE-RELATED CHANGES OF MYOCARDIAL FUNCTION DEMONSTRATED IN HUMAN POPULATION

In normal human population, not suffering from ischaemia

or degenerative changes, from the age of 20 years to 80 years, there is a tendency for heart rate to decrease with advancing age, though it is not a significant alteration in all the studies reported (Port et al.1980, Rodeheffer et al.1984). They also display other electrocardiographic changes such as prolongation of P-R interval and QRS complex duration. Earlier studies have indicated that systolic functional parameters such as cardiac output and ejection fraction at rest progressively decreases with advancing age (Granath et al. 1964, Julius et al. 1967). The recent advances in radionucleotide imaging have made it possible to carefully screen the subjects for occult coronary artery disease, measure the cardiac volumes and critically estimate the systolic and diastolic functions of the myocardium (Port et al. 1980, Rodeheffer et al. 1984, Fleg 1986). These recent studies indicate that there is a progressive increase in mean arterial pressure with aging, but as opposed to earlier studies, there were no age-related changes observed in systolic function. Cardiac output and ejection fraction were maintained at the same level throughout the lifespan from 20 years (Port et al.1980, Fleg 1986). The diastolic function determined by the rapid diastolic filling rate on the other hand declines markedly with age (Miller et al.1986, Bryg et al.1987). This would indicate that in spite of increased

afterload due to an increase in mean arterial pressure and impaired relaxation of heart, the older myocardium is still able to maintain its systolic function. This would raise an important question. Are there any other physiological changes that help maintain the systolic function during aging? As early as 1964, Harison et al. studied the duration of contraction, ejection fraction and relaxation of the myocardium of various age groups by utilizing the electrocardiogram and carotid pulse. They described a progressive increase in duration of contraction with advancing age from 20 to 90 years. The prolonged duration of contraction may possibly be a modification that helps the older heart to improve its efficiency and to maintain the systolic function in spite of increased afterload. Although it may appear highly speculative at this point, recent evidence from in-vitro studies in humans and in various animal models suggest that it is in fact a plausible hypothesis.

Myocardial Perfomance of aging population and exercise:

It has been widely observed that the maximum attainable heart rate during exercise decreases with aging (Astrand et al.1959, Port et al.1980, Rodeheffer et al. 1984). The systolic functional parameters such as cardiac output, stroke volume and ejection fraction during exercise were also found to decrease with advancing age (Julius et al.1967, Robinson et al.1943, Port et al.1980). This reduction in myocardial

performance is associated with a decrease in maximum oxygen uptake in older subjects (Dehen et al.1972).

Since evidence obtained in in-vitro studies have indicated relatively little age-related impairment of intrinsic cardiac muscle function (Lakatta 1979, Lakatta and Yin 1982) and since the efficacy of beta adrenergic stimulation (response to catecholamine) decreases with advancing age (reviewed in the following section), it was hypothesized that age-dependent changes in cardiovascular performance during exercise might at least be partly explained by the blunted response to plasma catecholamines. In contrast to these above observations, subsequent cross-sectional studies by Rodeheffer et al. (1984) indicated that there is no reduction in either cardiac output or ejection fraction in older subjects during exercise except that under very heavy workload, the ejection fraction declines in older subjects. It has also been demonstrated that the end diastolic and end systolic volumes of aged myocardium during exercise were greater than that of the younger subjects. Moreover, there is an age-related increase in stroke volume during exercise that was sufficient to prevent the significant decline in cardiac output that otherwise would have been expected because of the decline in heart rate. From these later observations it appears that aging per se does not

limit the cardiac output in subjects either at rest or even during exercise. However, it does alter the mechanism by which cardiac output is maintained during exercise. With advancing age, there is a shift from a catecholamine-mediated increase in heart rate and reduction in end systolic volume as observed in younger subjects to a greater reliance on the Frank-Starling mechanism (increase in end diastolic volume). The reason for the unique observation of this later study (Rodeheffer et al. 1984) is not precisely known. In any event, all the observations made so far agree with the fact that the catecholamine-mediated response during exercise is blunted with advancing age.

The above observations in the aging human population were made by many centres over several years. The common and most important observation is that the alterations in myocardial function are not events at the later stages of life but rather a progressive change that begins as early as in the 3rd and 4th decade of life. This would suggest that in order to characterize an age-related modification, it is necessary to carry out the studies in several age groups of aging animals or human population.

AGE-RELATED CHANGES DEMONSTRATED IN IN-VITRO STUDIES

In order to understand the mechanisms of characteristic alterations in myocardial function in aging, elaborative studies were carried-out by many investigators in various animal models such as rats, guinea-pigs, rabbits and dogs. Since many physiological changes in aging are well characterized in rats and since the age-related disease state namely atherosclerosis is a rare occurence in rat, they are therefore widely used for the investigation of cardiovascular system and aging. The following parameters were specifically investigated and alterations with aging have been documented.

a) Contraction duration:

Trans-sarcolemmal Ca^{2+} influx during depolarization triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) and causes the cytoplasmic $[Ca^{2+}]$ to rise from 10^{-7} M to 10^{-5} M range. The Ca^{2+} binding to troponin results in actomyosin interaction, displacement of actin relative to myosin and force production. As the $[Ca^{2+}]$ in the myoplasm is reduced, mainly by the Ca^{2+} uptake by SR, the Ca^{2+} dissociates from the myofilament and relaxation ensures (Langer 1973).

Lakatta et al. (1975a) have investigated the mechanical properties of aging rat myocardium by using isolated left ventricular trabaculae. During isometric performance, the duration of contraction was significantly prolonged with advancing age from 6 to 24 months. Neither the active tension

nor the maximal rate of tension development changes with advancing age. Subsequently Capasso et al. (1982) indicated that the prolongation of contraction duration occurs as early as during adult maturation from 2 to 10 months of age. The prolonged duration of contraction was contributed by the prolonged time to peak tension. In addition the time for half maximal relaxation is prolonged with advancing age. This could result from changes in myocardial passive visco-elastic properties, active properties or both. To determine whether the age-related difference was from a prolonged active state, Lakatta et al. (1975a) studied the electromechanical dissociation and the overshoot contraction during recovery from hypoxia. They indicated that there was a greater electromechanical dissociation and overshoot in contraction duration following hypoxia in the aged myocardium, suggesting that the prolonged duration of contraction was a result of a change in active state rather than changes in passive properties. These above changes with aging were not limited to rats but appeared to be present in all species in which the cardiac contraction has been measured. eg. Guinea-pigs, dogs, rabbits and humans (Rumbergere et al. 1976, Templeton et al.1978, Frolkis et al.1975, Harison et al.1964).

Prolongation of isometric contraction duration in aging myocardium was also accompanied by an increased myocardial stiffness (Spurgeon et al. 1977). The stiffness during

contraction was increased more than two fold over that measured prior to excitation, whereas the passive component of stiffness did not show any age-related changes. This would indicate that the age-related difference in stiffness was also related to the difference in the activation of myofilament.

A major determinant of contraction duration is the time-course of Ca^{2+}/myofilament interaction. This is determined by 1) the time-course of myoplasmic Ca^{2+} transient, which is determined in part by the duration of sarcolemmal depolarization and in part by the rates of Ca^{2+} release and uptake from SR and 2) the extent and the rate of actin/myosin interaction during contraction. Which itself is determined by the amount of Ca^{2+} bound to troponin before the onset of contraction, the rate of ATP hydrolysis by myofilament (myosin) ATPase and the rate of cross bridge cycling. Any age-related changes in the above activation processes may thus result in the characteristic alterations in mechanical function demonstrated during aging.

b) Myofilament ATPase

Measurement of ATPase activity associated with cardiac myofilament has been a focus of several studies on aging. The enzyme activity has been measured in various preparations ranging from crude myofibrils to relatively pure isolated myosin preparations. Very early studies have employed rather crude myofibrillar preparations and reported that there was a

progressive reduction in the enzyme activity during early maturation, which remained stable from 6 months of age through senescence (Bhatnagar et al. 1984). Subsequently, it was demonstrated that Ca^{2+}-activated ATPase activity in an actomyosin preparation, which contains actin and myosin and is devoid of troponin and tropomyosin, decreases with adult maturation (Capasso et al. 1986). In recent studies, the ATPase activity was determined in purified isolated myosin preparation. These studies have indicated that there is a progressive decline in the activity of myosin ATPase throughout the entire age-range of 24 months (Bhatnagar et al. 1985, Capasso et al. 1986). This led many investigators to believe that the progressive decline in myosin ATPase activity may be responsible for the parallel changes in mechanical function with advancing age. A similar decrease in myosin ATPase activity and parallel decline in shortening velocity have been reported in experimental models in which the haemodynamic loading conditions of the heart were chronically altered (Lompre et al. 1979, Gorza et al. 1981). It has been suggested that the progressive increase in afterload due to an increase in systemic pressure (which was also seen with advancing age) reduces the shortening velocity, and in order to maintain maximum efficiency, the myosin ATPase activity is regulated downwards.

In the myocardium myosin exists in three isoforms V_1, V_2

and V_3. The rate of ATP hydrolysis by V_1 is much greater than V_3 ($V_1 > V_2 > V_3$) and therefore V_1, V_2 and V_3 are named as fast, intermediate and slow isoforms respectively (Lompre et al. 1981). In rats, V_1 isoform predominates during adult life. It progressively decreases and is replaced by V_3 isoform with advancing age and with increase in myocardial loading conditions, which would result in a reduction of myosin ATPase activity (Klotz et al. 1981, Lompre et al. 1981). It has been suggested that the change in myocardial loading condition itself may act as a stimulus for the genetic remodelling of myosin isoforms. Alternatively, a reduction in serum thyroxine, which occurs with advancing age, may be responsible the switch in isomyosin composition. In fact, it has been shown that treatment of older rats with thyroxine can reverse the isomyosin profile to the adult form (Effron et al. 1987). In any event, it is clear that in rats there is a reduction in myosin ATPase activity due to a change in isomyosin composition and which may contribute to the characteristic changes in mechanical function. Alterations in mechanical function is observed not only in rats, but also in humans, rabbits, dogs and guinea-pigs. In many of these species unlike rats, the slow isoform V_3 predominates even during adult life and therefore any major changes in isomyosin composition that would further decrease the myosin ATPase activity during aging is thus highly unlikely (Lompre et al. 1981). Hence, it may not be justified to attribute a

relatively unique observation of switch in isomyosin composition in rats, to a widely observed alteration in mechanical function in other species. Moreover, the activity of enzyme myosin ATPase is determined by the intracellular $[Ca^{2+}]$. This would therefore suggest that changes in Ca^{2+} activation processess and ensuing myoplasmic transient are equally important in the alteration of mechanical function in aging.

c) Myoplasmic Ca^{2+} transient:

The myoplasmic Ca^{2+} transient that follows membrane excitation has been monitored in rats of various age-groups by injecting the chemiluminescent protein aequorine and measuring the light transient that preceeds contraction (Orchard and Lakatta, 1985). The light transient in the cardiac muscle of 24 month old rat was found to be prolonged as compared to that of 7 month old, indicating that there is a prolongation of myoplasmic Ca^{2+} transient in the older myocardium. The changes in myoplasmic Ca^{2+} transient would influence the time-course of Ca^{2+}/myofilament interaction and may be the primary reason for the prolonged contraction duration observed with advancing age. Under physiological conditions, calcium transport mechanisms of the sarcolemma and the SR are the main sites of regulation of myoplasmic Ca^{2+}. Although mitochondria do possess Ca^{2+} transport mechanisms and have larger capacity

for Ca^{2+}, they are not involved in the regulation of myoplasmic Ca^{2+} under physiological conditions but may play an important role during a state of Ca^{2+} overload, such as in ischaemia or digitalis cardiotoxicity (Khatter et al. 1989b). It is therefore conceivable that changes in Ca^{2+} transport mechanisms of sarcolemma and/or SR may be responsible for the prolonged myoplasmic Ca^{2+} transient and contraction duration with advancing age.

d) Calcium transport by sarcoplasmic reticulum

The sarcoplasmic reticulum is an intracellular organelle that plays a vital role in the excitation contraction coupling of myocardium. The Ca^{2+} that enters the cell during the plateau phase of an action-potential (or depolarization itself) triggers a Ca^{2+} release from the SR (Fabiato, 1983). Myocardial relaxation is initiated by the rapid uptake of Ca^{2+} by the SR which is mediated by the energy dependent Ca^{2+} pump that hydrolyses ATP by a Ca^{2+} ATPase. Narayanan (1981 & 1987) and others (Froehlich et al. 1978) have investigated the state of this pump in isolated SR vesicles of various age-groups of rats. It has been demonstrated that there is a progressive reduction in the rate and the amount of ATP-supported, oxalate facilitated Ca^{2+} uptake by SR with advancing age. The decrease in Ca^{2+} uptake is not associated with a decrease in Ca^{2+} ATPase activity of SR vesicles. Therefore, it was speculated that there may be an impaired coupling between ATP

hydrolysis and Ca^{2+} transport. In any event, the reduced rate of Ca^{2+} uptake would lead to a slower relaxation of myofilaments and therefore prolonged contraction duration in aging myocardium. The impaired Ca^{2+} uptake by the SR would reduce the contractile Ca^{2+} reserve within the membrane compartment. The amount of Ca^{2+} released from SR during contraction, therefore would also be lower in aging myocardium. Surprisingly this did not reflect on the tension development of aging myocardium. The maximum developed tension attained in the older myocardium was similar to that of young adults. Furthermore, it has also been demonstrated that the increase in developed tension produced by an increasing concentration of extracellular [Ca^{2+}] was not any different in aging myocardium as compared to the young adults (Gerstenblith et al. 1979, Lakatta et al. 1975b). It is therefore conceivable that there may be a compensatory enhancement of Ca^{2+} influx via an additional Ca^{2+} transport mechanism(s). Since sarcolemmal and SR calcium transports are the primary mechanisms involved in myocardial contraction, the most likely compensatory increase of Ca^{2+} influx would be through sarcolemmal mechanism(s). In fact, the observations with skinned ventricular fragments supported this contention. In a preliminary setting, Fabiato (1982) has shown that the

senescent heart muscle preparation requires a greater Ca^{2+} trigger for the release of SR Ca^{2+} than the preparation from younger animals suggesting that to obtain similar increase in myoplasmic Ca^{2+} the older myocardium may dependent relatively more on the sarcolemmal Ca^{2+} transport mechanisms.

e) Transmembrane action-potential

The age-related changes in transmembrane action-potential have been studied by many investigators in a variety of preparations. In rat atria, there is a prolongation of action-potential duration observed during adult maturation to 12 months (Cavoto et al. 1974). Wei et al. (1984) simultaneously recorded the electrical and mechanical parameters of right ventricular papillary muscle from 7 and 12 month old rats. They observed a prolongation of action-potential duration at all levels of repolarization and a greater amplitude of action-potentials with a parallel prolongation of contraction duration in the muscle of 24 month old versus 7 month old rats. The effect of age on the transmembrane action-potential has also been studied in unloaded rat ventricular muscles and dog Purkinje fibers. These later studies suggested that in unloaded preparations, action-potential increases during adult maturation with no further change during senescence. Regardless of loading conditions, the age-related changes in the action-potential are indicative of age-related differences in current flow

subsequent to depolarization and may therefore relate to the age-related differences noted in contraction.

The duration of transmembrane action-potential is determined by the onset of repolarization, which is initiated by the activation of K^+ outward current and inactivation of Ca^{2+} inward current (Bassingthwaighte et al. 1976, Kass and Tsien, 1976). Since intracellular $[Ca^{2+}]$ has an important role in the activation of K^+ channel, a decrease in the magnitude of Ca^{2+} transient or rapid sequestration of Ca^{2+} by SR leading to lower $[Ca^{2+}]i$ may delay the activation of K^+ channel and thus prolong the transmembrane action-potential duration. In fact, Cavato et al. (1974) proposed the former alternative as a likely explanation. However, evidence presented earlier indicates that neither developed force nor the force/$[Ca^{2+}]$ relation are age-related (Lakatta et al. 1975a, Gerstenblith et al. 1979). This would indicate that the magnitude of the intracellular Ca^{2+} transient with excitation does not decrease with adult aging. Since the onset of repolarization is also dependent on inactivation of voltage-gated calcium channels, prolonged activation of these channels may cause prolonged transmembrane action-potential duration. Prolongation of action-potential duration by a similar mechanism is known to occur in the presence of calcium agonist BAY K 8644, which keeps the Ca^{2+} channel open for a longer duration (Hess et al. 1984). The amplitude of

action-potential is also greater in the aged myocardium. Since the membrane potential above O mv is largely dependent on the Ca^{2+} current, the greater amplitude of this membrane potential would further indicate the possibility of enhanced Ca^{2+} current in aged myocardium. Alternatively, diminution of Na^{+}-Ca^{2+} exchanger activity, that is seen as an outward Ca^{2+} current may also lead to a greater amplitude of action-potential. Studies of Wei et al. (1984) have demonstrated that the changes in action-potential parameters by the elevation of extracellular [Ca^{2+}] were enhanced in senescent versus adult heart muscle, indicating that sarcolemmal Ca^{2+} current may be of greater magnitude in senescent muscle. A significant correlation between the action-potential and contractile parameters with change in extracellular [Ca^{2+}] was also observed in senescent but not in adult heart muscles, further indicating that the transmembrane action-potential is an important factor in the determination of force development of aged myocardium. It is therefore justified to propose that the changes in sarcolemmal Ca^{2+} transport mechanisms may primarily be responsible for the changes in electrical and mechanical function of the aging myocardium.

PHARMACOLOGICAL CHANGES IN AGING MYOCARDIUM

a) Response to catecholamine and related compounds

The myocardial beta-adrenergic stimulation during exercise declines with advancing age (reviewed in the previous section). A most obvious explanation for the apparent age-related difference is that the hormones are not effective at the receptor level of target organs. Alternatively, decreased production of these hormones (catecholamines) upon demand may be responsible for the decreased beta-adrenergic stimulation. However, it has been demonstrated by many investigators that in fact the level of plasma catecholamines and its elevation during exercise are greater with advancing age (Fleg et al. 1985). On the other hand, studies with bolous injections of isoproterenol in indicated that increment in heart rate is lower in the elderly as compared to younger subjects (Lakatta 1979 & 1986). Similar observations have also been made in animal models where full vagal blockade was obtained with atropine prior to isoproterenol infusion (Yin et al. 1979, Scarpace 1986).

In-vitro studies in isolated cardiac muscle and perfused myocardium of rats have also revealed that the catecholamine-induced positive inotropic response decreases with age (Lakatta et al. 1975, Guarneri et al. 1980). The effect of catecholamine on the contraction duration however is not

age-related. These observations in in-vitro studies would suggest that the age-associated defect is intrinsic to the myocardial tissue.

Beta-adrenergic agonist bind to beta-adrenergic receptor and activate the enzyme adenylate cyclase through G-protein mediated processes. The adenylate cyclase in turn catalysis the synthesis of cyclic AMP from ATP on the cytoplasmic side of the plasma membrane. An increase in the level of cAMP results in the activation of cAMP-dependent protein kinases and consequently cAMP-dependent phosphorylation of membrane proteins and troponine. Therefore, beta-adrenergic effects in the myocardium may be explained in terms of cAMP-dependent phosphorylation of membrane (sarcolemma and SR) and contractile proteins (Drummond and Severson 1979, Benson et al. 1985). Changes in any of these above mechanisms of action with advancing age may contribute to a decline in beta-adrenergic response. Studies in human lymphocytes and animal myocardial tissues have indicated that the number of beta-adrenergic receptors does not change with advancing age (Naryanan and Derby 1982, Abrass et al. 1982). However, it has been observed by some investigators that the affinity of the receptor for adrenergic agonist decreases with aging (Narayanan and Derby 1982). O'Connor et al. (1981) indicated that there is a decrease in adenylate-cyclase activity and hence cAMP production in the aging rat myocardium, whereas

Narayanan and Derby (1981) observed no age-related changes in basal adenylate cyclase activity, rather they observed a decrease in guanine nucleotide-dependent stimulation of adenylate cyclase by isoproterenol. Furthermore, when dibutyryl cAMP was used as an agonist, the age-dependent deficit in enhancement of contractility observed with beta-adrenergic agonist persisted (Guarnieri et al. 1980). This would suggest that even though the reduction in adenylate cyclase and reduced production of cAMP may contribute to the blunted beta adrenergic response, mechanisms distal to cAMP production may also be responsible. Which would mean perhaps that the cAMP-dependent activation of protein kinase, that mediates protein phosphorylation may be deficient and/or characteristic changes in target proteins namely voltage-gated calcium channel of sarcolemma, Ca^{2+} uptake mechanism of SR and troponine may occur with aging which may impair the cAMP-dependent phosphorylation. These possibilities have not been tested so far.

b) Enhancement of cardiac glycoside toxicity with aging

Treatment with digitalis is often complicated by the development of cardiac arrhythmias due to its narrow therapeutic index. Manifestation of cardiac toxicity is further increased in the elderly patients resulting in a narrow margin of safety for digitalis (Raisbeck 1952, Irons et al. 1966). The increased sensitivity to digitalis has also

been reported in experimental animals of different species (Katano et al. 1985, Khatter 1985). Variations in the metabolism of digitalis within different age groups does not seem to contribute significantly to the differential digitalis tolerance (Berman and Musselman 1979). Furthermore, the manifestation of arrhythmias in elderly patients does not directly correlate with the plasma digoxin level (Ward and Blatman 1979). This would mean that the increased sensitivity to digitalis is most likely due to the changes in the myocardium with aging. This is further supported by the enhanced toxicity observed in the isolated heart preparation (Khatter 1985). Cardiac glycoside binds and inhibits the sarcolemmal enzyme Na^+, K^+-ATPase and consequently increases the level of intracellular Na^+. The varied intracellular Na^+ exchanges with extracellular Ca^{2+} through electrogenic Na^+-Ca^{2+} exchanger leading to greater Ca^{2+} influx (Langer 1970). Recently it has been suggested that the reduction of Ca^{2+} efflux due to an increased Na^+ may also contribute to the elevation of cytoplasmic Ca^{2+} in cardiac glycoside toxicity (O'Campo and Orrego 1981). Many reports including those from our laboratory have suggested that in the presence of toxic concentrations of cardiac glycosides, the cytoplasmic calcium may rise to a level of "calcium overload" (Khatter et al. 1986, Khatter et al.1990 Kass et al. 1978). The subsequent oscillatory release of calcium from the calcium overloaded SR

would induce an alteration in membrane conduction resulting in after depolarization, which when reaching a sufficent threshold may trigger arrhythmias (Ferrier 1977). Hence it is entirely logical to hypothesize that alterations either in the enzyme Na^+-K^+ATPase and/or in the sarcolemmal Ca^{2+} transport mechanism namely Na^+-Ca^{2+} exchange of the aged heart may be responsible for the increased sensitivity to digitalis-induced arrhythmias. Recently Kennedy et al. (1986) have reported that if the Ca^{2+} influx via voltage-gated calcium channel is increased by a calcium agonist BAY K 8644, the development of digitalis toxicity is potentiated. This indicates the significant role of Ca^{2+} influx via voltage-gated calcium channels in digitalis toxicity.

Our previous studies and studies from other laboratories have demonstrated a significant reduction in cardiac glycoside receptor density and the activity of Na^+, K^+-ATPase with advancing age (Khatter 1985, Katano et al. 1984). In addition, Khatter (1985) has also demonstrated a small increase in affinity of the receptors to bind cardiac glycosides. The possibility of modifications in sarcolemmal calcium transport mechanisms contributing to the enhanced cardiac glycocides toxicity of an aging myocardium has so far not been investigated.

CALCIUM TRANSPORT MECHANISMS OF CARDIAC SARCOLEMMA

Calcium transport across the sarcolemma plays a central role in excitation-contraction coupling of cardiac muscle. Membrane excitation leads to trans-sarcolemmal Ca^{2+} flux, which is generally believed to be relatively small in amount and not be enough to activate myofilament by itself, rather it activates Ca^{2+} release from the SR. The relative contribution of Ca^{2+} through influx and released from SR varies with species and physiological conditions (Bers 1985). The bulk of trans-sarcolemmal Ca^{2+} influx enters via voltage-dependent Ca^{2+} channels. Membrane excitation somehow leads to the opening of voltage-gated Ca^{2+} channels resulting to an inward movement of Ca^{2+} across the membrane. The inactivation of these channels is dependent on membrane voltage and $[Ca^{2+}]i$. The electrogenic Na^{+}-Ca^{2+} exchanger on the other hand is a bidirectional exchanger, involved in both influx and efflux of Ca^{2+} depending on the trans-sarcolemmal electrochemical gradient of Na^{+}. Its contribution to Ca^{2+} influx especially in mammalian myocardium is not well defined (Eisner and Lederer 1985). Recently, $[Ca^{2+}]i$ transients and contractions have been demonstrated at higher positive potentials, which may be dependent on Ca^{2+} entering the cell via Na^{+}-Ca^{2+} exchange (Barcenas-Ruiz et al. 1987, London and Krueger 1986). Therefore, the possibility of Ca^{2+} influx through Na^{+}-Ca^{2+} exchange mechanism contributing to the $[Ca^{2+}]i$ transient must

also be considered. The trans-sarcolemmal Ca^{2+} efflux is mediated through the electrogenic Na^{+}-Ca^{2+} exchanger and by the ATP-dependent Ca^{2+} pump against Ca^{2+} concentration gradient.

Recent evidence indicate that the function of sarcolemma is more than that of a semipermeable membrane with channels and exchanger for ion transport. It has been demonstrated that there is a component of cellular calcium essential for myocardial contraction that could be depleted and repleted in less than a second. A significant portion of this rapidly exchangeable calcium is probably located in the cardiac sarcolemma (Langer 1987). However no definite evidence has so far appeared as to the mode of regulation of myocardial contraction by this sarcolemmally bound Ca^{2+}. It is also not clear whether the well known mechanisms of trans-sarcolemmal calcium influx are regulated by sarcolemmally bound calcium.

a) Voltage-gated calcium channels

Calcium channels are among the most important membrane protein units present in cardiac sarcolemma. Ca^{2+} ion entry through cardiac Ca^{2+} channels is the primary event during excitation-contraction coupling. The inward current carried through the open Ca^{2+} channel helps to determine the action-potential duration and refractory period in myocardial cells, action-potential upstroke and conduction velocity in nodal cells and diastolic depolarization and cardiac rhythm in

pacemaker cells. These voltage-gated calcium channels have been extensively characterized both electrophysiologically and biochemically.

(i) Electrophysiological properties:

In recent years the understanding of Ca^{2+} channel function has been advanced considerably, mainly due to the patch clamp techniques, which has made it possible to record Ca^{2+} channel activities at the level of the individual channels (Hamill et al. 1981, Tsien et al. 1987). Cardiac sarcolemma of most species contain two of the three types of Ca^{2+} channel; L type and T type (Bean 1985, Nilius et al. 1986, Hagiwara et al. 1988). The two channel types differ in the voltage range of their activation and inactivation as well as in the gating kinetics. The L-type channel requires more positive potentials for activation than the T-type channel. The midpoint of the steady state inactivation for L-type channel is 20-30 mV more positive than that of T-type channel. These differences in activation and inactivation properties are advantageous to study these types of channels independently (Bean 1985). Further, the duration of activation is also different between these two types of channels. L-channel current activates and remains relatively well maintained for 100 msec whereas the T-type current activates to a peak and then completely inactivates within few milliseconds. The two channel types also differ in their

pharmacological sensitivities such that the L-type current is increased by calcium agonist BAY K 8644 and beta adrenergic stimulation and decreased by antagonist Nitrendipine, whereas the T-type channel is insensitive to dihydropyridine Ca^{2+} channel agonists and antagonists and fails to respond to beta adrenergic agonists (Reuter 1983, Bean 1985, Nilius et al. 1986, Bonvallet 1987). Furthermore, it has also been demonstrated that the calcium agonist BAY K 8644 increase the influx of Ca^{2+} by keeping the channel open for a longer duration whereas the beta agonist increases the probability of opening of these channels (Tsien et al. 1986). The L-type Ca^{2+} channel is considered to be the "classical cardiac Ca^{2+} channel and investigated by a large number of laboratories. Because of its activation-inactivation kinetics, the L-type channel is one which mainly contributes to the inward current during the plateau phase of the action-potential and this channel alone seems to be able to deliver the Ca^{2+} required for normal excitation-contraction coupling (Tsien 1987). This is further supported by the fact that contractions with normal extracellular Ca^{2+} could be observed with holding potential at which T-type channels are completely inactivated (Weir and Isenberg 1982). On the other hand, the precise role of T-type Ca^{2+} channels in cardiac tissue remains poorly understood. A possible involvement of T-type of Ca^{2+} channels in the generation of the pacemaker potential has been hypothesized (Bean 1985).

ii) Biochemical properties:

Calcium channel antagonists and tritriated congeners of these compounds are the primary tools used in the biochemical characterization of voltage-gated calcium channels. In this form of characterization it is assumed that the calcium antagonist receptors are likely representations of voltage-gated calcium channels. Calcium channel antagonists are heterogenous group of compounds that have diverse chemical structure but share the common mechanism of action of altering the influx of Ca^{2+} via voltage-gated calcium channels (Schwartz and Triggle 1984). They are classified into three groups based on their chemical structure namely phenylalkalamine, dihydropyridine and benzothiazipines (Glossman et al. 1985 a & b). Of the three major structural categories, the greatest number of analogues are available in the dihydropyridine series.

Binding properties of all three major groups of calcium antagonists have been well characterized in many biological tissues of various species. In all, relatively high density of Ca^{2+} channel ligand binding sites were observed in transverse tubules of skeletal muscle and therefore it has been widely used for the isolation and purification of calcium channels. The dihydropyridine Ca^{2+} channel antagonists Nifedipine, Nitrendipine and PN200-110, bind with high affinity to a single class of receptor site in all the tissues

that have been investigated (Vaghy et al. 1984, Glossmann et al. 1985 a & b, Triggle and Janis 1987, Tuana et al. 1987). Two other classes of calcium channel antagonists phenylalkalamine and benzothiazipine similarly bind to independent high affinity receptor sites. The binding of calcium channel ligands are modified by external factors such as membrane potential and extracellular calcium concentration. For Ca^{2+} channel antagonism to occur the drug receptor must be in the correct state, either the inactivated or open state. Repolarization induced deactivation of the channels to the resting state results in rapid drug dissociation (Schilling and Drewe 1986). Calcium has been shown to enhance the binding of dihydropyridines, whereas it decreases the binding of Verapamil (phenylalkalamine) and Diltiazem (benzothiazapine) to their appropriate receptors. Binding of Diltiazem enhances the binding of dihydropyridine and slows the dissociation through allosteric interaction, while Verapamil decreases the binding of dihydropyridine and enhances the dissociation through allosterism. The binding of Verapamil and Diltiazem on the other hand are competitive to each other. These data support a model of calcium antagonist receptor having three allosterically linked binding sites for three groups of calcium channel ligands and a binding site for Ca^{2+}, which interacts with all three receptor sites (Glossmann et al. 1985 a & b).

iii) Identification and purification of calcium channel subunits:

In recent years a great deal of attention has been focused on the purification of the voltage-gated calcium channel. The identification of Ca^{2+} channel antagonist receptor subunits was achieved through specific covalent labelling by reactive derivative of the drugs. Ferry et al. (1984) first succeeded in covalently labelling the dihydropyridine receptor site in transverse tubules of skeletal muscle using [^{3}H]Azidopine. The availability of photoreactive compounds limited these earlier studies only to the dihydropyridine calcium antagonist receptor subunits. Recently photoreactive analogues of Verapamil and Diltiazem have been made available and thus the investigation of other two receptor sites were also made possible (Striessing et al. 1986). Purification of calcium channel receptor subunits was achieved by many investigators using somewhat different procedures. Primarily it involves solubilization with either digitonin or CHAPS and purification by lectin affinity chromatography, ion exchange chromatography and sucrose sediment or SDS-polyacrylamide gel electrophoresis (Glossman and Ferry 1983, Curtis and Catterall 1984, Curtis and Catterall 1986, Borsotto et al. 1985, Campbell et al. 1988, Glossman and Striessing 1988). With the experimental evidence from many laboratories, there exist an agreement that in

skeletal muscle there are three major calcium channel subunits (Catterall 1988, Catterall et al. 1988), a larger molecular weight subunit (175-140 kD) an intermediate (55-50 kD) and a lower molecular weight (35-30 kD) protein in a 1:1:1 ratio (Catterall 1988, Chang and Smilowitz, 1988, Hosey et al. 1986). These three protein subunits are known as alpha, beta and gamma subunit respectively. Further subclassification of these protein subunits to $alpha_1$ and $alpha_2$ have recently been made and additional protein subunits have also been identified (McKenna et al. 1990). The molecular weight of these protein subunits, especially the larger subunit varies depending on the reducing or non-reducing conditions.

As opposed to the knowledge relating to voltage-gated calcium channels of skeletal muscle, the structure of the cardiac calcium channel complex is less clear. Cooper et al. (1987), identified polypeptides of 170 kD and 140 kD before and after reduction respectively, as the calcium antagonist receptor subunits in cardiac muscle. Schmidt et al. (1986) also described similar high molecular weight subunits in cardiac muscle. Rangasamy et al. (1985) reported calcium antagonist receptor subunits with molecular weights of 60 kD, 54 kD and 34 kD, whereas Sarmiento et al. (1986) described 33-35 kD protein subunit as the only calcium antagonist receptor. Recently Tuana et al. (1987) indicated that the protein subunits of 170, 140, 130, 100 and 53 kD as calcium

channel antagonist receptor subunits in cardiac muscle. In addition, antibodies against the 32 kD polypeptide of the skeletal muscle receptor were shown to cross-react against a polypeptide of 170 kD (under reducing condition) and 32 KD in cardiac muscle membranes (Schmidt al. 1986). Taken together, these results suggest that the polypeptide composition of Ca^{2+} antagonist binding sites from cardiac muscle is essentially similar to that of skeletal muscle. Recent isolation of cDNA clones encoding skeletal and cardiac calcium channel subunits however indicate that there may be structural differences between the two subunits (Slish et al. 1989, Mikanu et al. 1989, Lotan et al. 1989).

iv) Regulation of voltage-gated calcium channel:

Many endogenous mediators and exogenous agents namely beta adrenergic agonists, antagonists, calcium channel agonists and antagonists regulate myocardial function by altering sarcolemmal Ca^{2+} flux via voltage-gated calcium channels. Catecholamines and their related compounds bind to beta adrenergic receptors, activate adenylate cyclase and elevate the level of cAMP. Increase in cAMP levels result in the activation of cAMP-dependent protein kinase and therefore its effects on the myocardium may be explained in terms of cAMP dependent phosphorylation of membrane proteins and contractile protein troponine. One of the membrane protein would be the sarcolemmal voltage-gated calcium channel subunit

(Tsien et al. 1986). The involvement of cAMP and cAMP-dependent protein phosphorylation of Ca^{2+} channel in the mechanism of action of catecholamines has earlier been supported by many indirect evidence. Nargeot et al. (1983) and Osterrieder et al. (1982) demonstrated that release of intracellular cAMP by photoactivation of a photolabile derivative produces an increase in Ca^{2+} current. Moreover, the cAMP-dependent phosphorylation of a sarcolemmal protein in an isolated system linerally correlated with voltage-gated Ca^{2+} uptake (Rinaldi et al. 1981 & 1982). Recently Benson et al. (1985) and Hosey et al. (1986) have demonstrated that in skeletal muscle, the beta subunit of the Ca^{2+} antagonist receptor of the voltage-gated calcium channel is phosphorylated by cAMP-dependent protein kinase. Tuana et al. (1987) indicated that in cardiac muscle cAMP-dependent protein kinase phosphorylates 170, 100 and 53 kD molecular weight subunits. These evidences therefore indicate that the beta adrenergic agonist and its related compounds increase the sarcolemmal influx of Ca^{2+} by cAMP dependent phosphorylation of calcium channel. Calcium channel agonist and antagonists on the other hand bind to the appropriate receptor sites of Ca^{2+} channels and alter the transmembrane Ca^{2+} flux through yet unknown mechanisms. It has however been demonstrated that interaction with receptors and subsequent increase in Ca^{2+} flux is mediated neither by cAMP nor by cGMP. Recent studies

in cardiac microsomes have revealed that the calcium channel antagonist Nitrendipine induces phosphorylation of a protein with a molecular weight of 42 kD, which co-migrated with the calcium channel regulatory subunit (Horne et al. 1984). This would indicate that channel phosphorylation by an endogenous protein kinase may be involved in the mechanism of action of Ca^{2+} channel ligands.

b) $\underline{Na^{+} - Ca^{2+}}$ exchanger:

The conceptual origin of the $Na^{+} - Ca^{2+}$ exchange system can be traced to the earlier experiments by Daly and Clark in 1921. The $Na^{+} - Ca^{2+}$ exchange system is a carrier mediated transport process in which transmembrane movements of Ca^{2+} are directly coupled to reciprocal movement of Na^{+}. In the myocardium it is thought to be an important transporter by which cytoplasmic Ca^{2+} concentrations are maintained at submicromolar levels in the face of millimolar concentrations in the extracellular fluid.

Reuter and Seltz (1968) and Glitsch et al. (1970) have suggested that the stoichiometry of the exchange is $2Na^{+}$ per Ca^{2+}. Subsequently it has been indicated that with a 2:1 stoichiometry the Na^{+} gradient might not provide sufficient energy for the Ca^{2+} extrusion and has been suggested that the stoichiometry might be $3Na^{+}$ per Ca^{2+}. On the other hand, a few investigators have indicated (Mullins and Brinley 1975)

that a 4:1 stoichiometry may be necessary. In any event, it has been generally accepted that the stoichiometry is more than 2 Na^+ per Ca^{2+} which makes the Na^+-Ca^{2+} exchanger electrogenic.

The kinetics of the Na^+ - Ca^{2+} exchanger have been studied in isolated sarcolemmal vesicles and in intact tissues. Na^+ - Ca^{2+} exchange activity exhibits Michaelis-Menten kinetics with respect to $[Ca^{2+}]$ in intact tissues as well as in sarcolemmal membrane vesicles (Wakabayashi and Goshima 1982, Reeves and Sutko 1979, Caroni and Carafoli 1983, Khatter et al. 1989). In isolated sarcolemmal vesicles affinity constants ranging from 1.5 to 140 uM have been reported, values between 10 to 40 uM being most widely observed (Reeves and Sutko 1979, 1980 & 1983, Pitts 1979, Khatter et al. 1989). The reasons for these variabilities in Km are obscure and cannot be entirely explained on the basis of differences in experimental techniques among different investigators. The maximum rate of Ca^{2+} transport (Vmax) was in the range of 5-30 nmol/mg protein/sec (Reeves and Sutko 1979, Reeves 1985). The rates of both Ca^{2+} influx and efflux demonstrates a sigmoidal dependence upon $[Na^+]$ (Glitsch et al. 1970). In cardiac tissue a Hill coefficient of 2.3 - 3.2 was obtained for the $[Na]_o$ dependence of Ca^{2+} efflux (Kadonna et al. 1982).

Physiological significance of Na^+-Ca^{2+} exchange:

Na^+ - Ca^{2+} exchange together with influx via slow Ca^{2+} channels and Ca^{2+} ATPase regulate Ca^{2+} fluxes across cardiac sarcolemma and thus the free intracellular Ca^{2+} concentration during myocardial contraction. It is generally accepted that the Na^+ - Ca^{2+} exchanger acts as a system for extruding Ca^{2+} from the cardiac myocyte. Recently it has been shown that influx of Ca^{2+} and contraction could be produced even when the voltage-gated calcium channels are inactivated by holding the membrane potential more positive than equilibrium membrane potential for Ca^{2+}, indicating that Na^+-Ca^{2+} exchange plays an important role in the influx of Ca^{2+} as well (Barcenas-Ruiz et al. 1987). Moreover, its involvement in Ca^{2+}-induced calcium release from the SR has also been demonstrated (Leblanc and Hume 1990). Thus during the plateau-phase of the action potential, Ca^{2+} may enter via both Ca^{2+} channels and Na^+-Ca^{2+} exchanger leading to inward and outward current respectively.

The involvement of Na^+ - Ca^{2+} exchange in the genesis of cardiac arrhythmias has also been proposed. For example, a toxic concentration of cardiac glycosides produces a state of calcium overload which elicit oscillatory after-potentials, which, when they reach a sufficient threshold, may trigger arrhythmias (Kass et al. 1978a, Ferrier 1977, Khatter et al. 1989). Current knowledge suggests that the oscillatory after-potentials are produced by the oscillatory release of calcium from the overloaded intracellular organelle

(sarcoplasmic reticulum) which may then alter the ionic conductance of the sarcolemmal membrane (Kass et al 1978). Recently however, it has been indicated (Lin et al. 1986, Arlock and Katzung 1985) that the Na^+ dependent Ca^{2+} efflux secondary to oscillatory release of calcium from SR, in fact contributes to the generation of after-potentials and hence to the genesis of arrhythmias.

A major experimental limitation in assessing the possible roles of the Na^+ - Ca^{2+} exchange system in cardiac physiology is the absence of specific inhibitors for the exchanger. Nevertheless, certain inhibitory agents have provided useful information. The inorganic ion, La^{3+}, inhibits Na^+-dependent Ca^{2+} uptake and efflux in isolated sarcolemmal vesicles and hence it has been used to terminate the reaction in the isolated system (Reeves and Sutko 1979). The precise mechanism of La^{3+} effect is not clear. It is however, known that La^{3+} inhibits wide varieties of Ca^{2+} dependent biological phenomena (Weiss 1974). This may be due to its similar ionic radius to Ca^{2+}. In addition two kinds of organic inhibitors have been described. The acridine derivative, quinacrine, inhibits both Na^+ dependent Ca^{2+} uptake and efflux. Since it has many fold effects on intact tissue its usefulness in delineating the physiological function of Na^+ - Ca^{2+} exchange system is limited (Sutko et al. 1983). A second class of organic inhibitors is amiloride and its derivatives. These

drugs inhibit Na^+ dependent Ca^{2+} transport in a competitive manner (Schellenberg et al. 1983). However, their action is not specific to Na^+ - Ca^{2+} exchange, as they also inhibit other transport mechanisms eg; Na^+ - H^+ exchange.

Attempts have also been made to isolate the Na^+ - Ca^{2+} exchanger protein(s). However, a major problem in the further development of this important field is the lack of any convincing pharmacology for this exchanger protein. In any event, several groups have reported the solubilization and reconstitution of Na^+ - Ca^{2+} exchanger (Wakabayashi and Goshima 1982, Schellenberg and Swanson 1982). Suggestions have been that the molecular weight of the exchanger is either 70, 82 or 33 kD, and the initial purification is estimated to constitute about 0.1 - 0.2 % of the membrane protein and to have a turn-over at Vmax of approximately 1000 sec^{-1}.

c) Sarcolemmal Ca^{2+} pump

Since the intracellular calcium is ejected out of the cell during myocardial relaxation against an electrochemical gradient, an ATP dependent calcium pump has been proposed as an efflux mechanism in cardiac sarcolemma. The existence of this energy dependent calcium pump (mediated by Ca^{2+}-ATPase) has in fact been conclusively described in recent years by many investigators (Caroni and Carafoli 1980, Narayanan 1981 & 1987) The molecular mechanism governing the calcium pump of sarcolemma has however not been charecterized yet.

Narayanan (1981 & 1987) has investigated the possible age-related changes in calcium pump of SR and sarcolemma in rat myocardium. It has been observed that as opposed to calcium pump activity of SR, the ATP-supported calcium uptake via sarcolemma increases with advancing age. The alteration in pump activity is however not associated with altered Ca^{2+} ATPase activity. The enhanced sarcolemmal pump activity may be a compensatory response to the defective SR Ca^{2+} uptake, but it is not adequate to prevent the relaxation abnormalities of the myocardium seen with advancing age (Lakatta 1987, Miller et al. 1986).

MYOCARDIAL FUNCTION AND AGING : ESSENCE

Progressive decline in diastolic function with unaltered systolic function of myocardium have been demonstrated in humans with advancing age. In-vitro studies in animals have indicated that in older myocardium there is a prolongation of contraction duration, contributed by prolonged time to peak tension. In addition the half maximal relaxation is prolonged with advancing age. The changes in mechanical function could be better explained by the prolonged myoplasmic calcium transient rather than by an alteration in the activity of myosin ATPase. The prolonged myoplasmic Ca2+ transient and subsequent prolongation of contraction duration may be attributed to the defective SR function. However, the maintained developed tension and unaltered response to increase in $[Ca^{2+}]_o$,in spite of defective SR, would suggest that there may be a compensatory increase in calcium flux, possibly through sarcolemmal calcium transport mechanisms, in the aging myocardium. The associated changes in action potential configuration would further support the above contention.

II. SPECIFIC OBJECTIVES

Based on the evidence presented in the previous sections, we hypothesized that modifications of cardiac sarcolemmal calcium transport mechanisms and subsequent alterations in calcium fluxes may contribute to the characteristic alterations in mechanical function with advancing age. Specifically, to test this hypothesis, we propose to study the sarcolemmal Na^+-Ca^{2+} exchanger and the voltage-gated calcium channel in the following manner:

A) Investigate Na^+-Ca^{2+} exchanger activity in isolated cardiac sarcolemmal vesicles of various age-groups of rats and guinea-pigs.

B) Investigate voltage-gated calcium channels by.

(i) Assessment of myocardial sensitivity to calcium agonist BAY K 8644 in whole animal model and in isolated perfused heart of rats and guinea-pigs of various ages.

(ii) Characterization of radioligand binding properties of calcium agonist and antagonist to their receptor sites in relatively pure sarcolemmal membrane of different age group of animals.

(iii) Identification of calcium antagonist receptors by photoaffinity labelling, characterization of their phosphorylation by calcium antagonist and cAMP and their age-related modifications.

III. METHODOLOGY

ANIMAL MODEL

The present investigation of age-related changes in calcium transport mechanisms of cardiac sarcolemma were carried out in male Sprague-Dawley rats and Cavia-porcellus guinea-pigs of various age groups. Sprague-Dawley rats were bred and supplied by Harlen Industries, USA. These rats reach sexual maturity around 2-3 months of age with the life expectancy decreasing after 20 months, such that 50% mortality was seen around 24 months of age. Animals of 2-3 months of age are thus considered as young adults, 10-12 months as mature adults and 24 months and older as senescent or aged rats. The aging process from 2-3 months to 12 months is referred to as adult maturation and aging to 24 months and older as senescence.

Guinea-pig breeders were purchased from Charles River, Quebec, Canada and bred in a controlled environment at the animal centre of the campus. They were kept in a separate room and fed a normal diet without any special treatment. These animals reach sexual maturity at the age of 2-3 months and reach senescence around the age of 24 months, where 50% mortality was seen.

INVESTIGATION OF Na^+-Ca^{2+} EXCHANGE ACTIVITIES IN ISOLATED CARDIAC SARCOLEMMAL VESICLES.

Na^+-Ca^{2+} exchange activities were studied in sarcolemmal vesicles isolated from guinea-pig ventricles of 3, 6, and 18 months of age and rat ventricles of 2 and 12 months of age.

(a) Isolation of relatively pure sarcolemmal membrane vesicles.

Sarcolemmal membrane vesicles of mainly right side out orientation (> 70%) were prepared as described by us earlier (Khatter et al. 1989a). The guinea-pig/rat heart left ventricles from 3-4 animals were homogenized in 0.6 M sucrose, 10 mM imidazole/HCl pH 7.0 (4 vol/g of tissue) using Polytron PT-20 (4 X 15 seconds); the homogenate was centrifuged at 10,000 X g for 20 min. The supernatant was diluted (two fold) with 160 mM NaCl/20 mM MOPS, pH 7.4 and centrifuged at 96,000 X g for 60 min. The pellet was resuspended in NaCl/MOPS, layered over 15 ml of 30% sucrose solution containing 0.3 M NaCl, 50 mM sodium pyrophosphate, and 0.1 M Tris-HCl (pH 8.3) and centrifuged at 95,000 X g for 90 min. The white band at the sample sucrose interface was recovered, diluted with 3 volumes of NaCl/MOPS and centrifuged at 100,000 X g for 30 min. The pellet was resuspended in NaCl/MOPS to a final protein concentration of approximately 1 mg/ml. The Na^+-loaded vesicles were prepared by the above method and the K^+-loaded vesicles were prepared by substituting 160 mM KCl/MOPS for 160 mM NaCl/MOPS.

(b) Na^+-dependent $^{45}Ca^{2+}$ Uptake

Vesicles were preloaded with Na^+ by incubating them in 160 mM NaCl/20 mM MOPS (pH 7.4) for 60 min at 37°C. To initiate Na^+-Ca^{2+} exchange, aliquots of 20 ul (equivalent to 20 ug protein) of Na^+-loaded vesicles were added to a series of tubes containing an incubation mixture (160 mM KCl/20 mM MOPS, pH 7.4 at 37°C) plus 50 uM $^{45}CaCl_2$ (100 cpm/pmol) in a volume of 500 ul. The exchange was terminated by adding the "termination solution" (2 ml ice cold 160 mM KCl/20 mM MOPS/1 mM $LaCl_3$ pH 7.4) at desired times followed by filtration through Millipore filters (0.45 uM) under vacuum. Tubes and filters were rinsed 4 times with 2 ml of "termination solution". The filters were placed into scintillation vials, 10 ml of a scintillation cocktail was added and the radioactivity was counted in a Beckman LS 8100 liquid scintillation counter. In all experiments, non-specific $^{45}Ca^{2+}$ uptake was determined in the vesicles which were loaded with potassium (160 mM KCL/20 mM MOPS pH 7.4) instead of sodium.

In another set of experiment, sarcolemmal vesicles isolated from 3 and 18 month old guinea-pigs were first loaded with sodium and then incubated in the incubation mixture (160 mM KCl/20 mM MOPS, pH 7. at 37°C) with various concentrations of $^{45}Ca^{2+}$ ranging from 20 to 80 uM. The Na^+-dependent Ca^{2+} uptake was terminated after 5 seconds of incubation. The

filtration and counting of $^{45}Ca^{2+}$ on the filter were carried out as described above.

(c) Na^+-Dependent $^{45}Ca^{2+}$ Efflux

Na^+-loaded vesicles were allowed to accumulate $^{45}Ca^{2+}$ for 1 minute in 160 mM KCl/20 mM MOPS (pH 7.4) plus 50 uM $^{45}Ca^{2+}$ in a volume of 500 ul. Calcium efflux was then initiated by increasing the Na^+ concentration of the medium to 90 mM. The exchange was terminated at desired times and filtered through millipore filters as in $^{45}Ca^{2+}$ uptake study and the quantity of $^{45}Ca^{2+}$ was counted by liquid scintillation.

INVESTIGATION OF VOLTAGE-GATED CALCIUM CHANNELS

(a) Cardiovascular response to calcium channel agonist BAY K 8644 in whole animal model

Animal Preparation and Measurements: The animal preparation and recording of cardiovascular parameters were carried out as described by us earlier (Navaratnam and Khatter 1989). Rats of 2 and 12 months of age were anesthetized with -chloralose (60 mg/kg) and urethane (500 mg/kg) intraperitoneally. Tracheostomy was performed and the animals were mechanically ventilated by a Harvard respirator (model 680). The carotid artery was cannulated with a fluid-filled catheter (saline with 2 U/ml heparin) and the arterial pressure was monitored using Statham P23Gb transducer and a Hewlett-Packard recorder (model 1308A). The chest was cut open with minimal bleeding and a fluid-filled (heparinized saline) cannula was inserted

into the left ventricle through the apex and placed in-situ without any interference to the left ventricular function. Left ventricular pressure and the rate max dp/dt were recorded using a Stratham P23Gb transducer and a Hewlett-Packard recorder (model 1308A). The external jugular vein was cannulated for the infusion of drugs during the experiment. Lead II EKG was monitored by the Hewlett-Packard recorder throughout the experiment.

Experimental Protocol: Intravenous dose-response of BAY K 8644 was studied in 2 and 12 month old rats by administering BAY K 8644 as bolus doses. BAY K 8644 was given in doses of 1-30 ug/kg and each dose was tested in a separate animal. The hemodynamic parameters including MAP, LVP, LV dp/dt and EKG were monitored continuously for 15-20 minutes. In one set of experiment (using rats of both 2 and 12 months of age), the Ca^{2+} channel blocker Nifedipine was infused through a second jugular venous cannula at a rate of 3 ug/kg/min. to a total dose of 40 ug/kg prior to the administration of BAY K 8644. A similar protocol was used in another group of rats except that they were given nitroglycerine, a vasodilator at an infusion rate of 8 ug/kg/min for 2 minutes. Nitroglycerine given in this mode has been shown to increase coronary blood flow by 25% (Vatner et al. 1978).

(b) Myocardial response to BAY K 8644 in isolated perfused hearts

(i) Rat as an animal model:

Two and twelve month old rats were sacrificed and the hearts removed quickly. Atria were separated and the hearts cleared of any pericardium or lung tissue. Using a non-circulating Langendorff preparation, hearts were perfused with Krebs-Henseleit solution at 32^{o}C and pH 7.4. The perfusion technique was essentially the same as described by us earlier (Khatter 1985). Hearts were stimulated using pulses of 2 ms duration at 4 Hz and flow rate was adjusted from 8-9 ml/min. based on the weight of the heart. The isometric contractile force was continuously monitored with a Beckman recorder using a force displacement transducer (Grass FT 03) attached to the apex of the heart. All hearts were first perfused with normal Krebs-Henseleit solution (composition in mM: NaCl 118.0; $NaHCO_3$ 27.1; KCl 4.8; KH_2PO_4 1.0; $MgSO_4$ 1.2; $CaCl_2$ 1.27 and Glucose 11.2, arated with 95% O_2 and 5% CO_2) until the tension stabilized (average 20 min.). After this period of stabilization, BAY K 8644 solution was infused into the aortic cannula at a rate of 1% of the total flow.

(ii) Guinea-pig as an animal model:

Hearts from 2, 12 and 24 month old guinea-pigs were isolated and perfused in a manner similar to that described by

us earlier (Khatter 1985). Dose response of BAY K 8644 (10^{-8} to 10^{-6} M) was carried out in all three age groups of guinea-pigs. Drug solutions were infused as described above for the isolated rat heart preparation.

(C) Investigation of dihydropyridine receptor sites of voltage-gated calcium channel.

(i) Preparation and characterization of cardiac sarcolemmal membrane:

Relatively pure sarcolemmal membranes from 2, 12 and 24 month old rat hearts were prepared as described by us earlier (Khatter et al. 1989) A detailed description of the methodology is provided under Na^+-Ca^{2+} exchange studies.

Sarcolemmal and Subcellular enzyme assays:

The relative purity of the final sarcolemmal membrane fractions of 2, 12 and 24 month old rats were judged by various marker enzyme activities in homogenate, microsomes and sarcolemmal membrane. The sarcolemmal enzymes such as patent and ouabain sensitive Na^+, K^+-ATPase and p-nitrophenol phosphatase (pNPPase) were assayed in order to evaluate the distribution of sarcolemmal vesicles.

Sarcolemmal Na^+, K^+-ATPase: The enzyme assay was carried out as described by Khatter and Hoeschen 1982. The sarcolemmal Mg^{2+}-dependent ATPase activity was determined at 37^oC in 1 ml medium containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 4 mM Tris-ATP and 50 ug of protein. Total

ATPase activity was the activity observed in a medium as above with the addition of 100 mM NaCl and 10 mM KCl. The difference between total and Mg^{2+} ATPase activities was taken as Na^+, K^+-ATPase activity. The activity of Na^+, K^+-ATPase was also determined in the presence of 2 mM sodium dodecyl sulphate. Ouabain sensitive Na^+, K^+-ATPase activity was calculated to be the activity of ATPase that was inhibited by 1 mM ouabain. Reactions were terminated after 10 min. by addition of 1 ml of 12% trichloroacetic acid. The released inorganic phosphate was measured in the protein free supernatent as described by Tausky and Shorr (1953).

p-Nitrophenylphosphatase (pNPPase): Basal pNPPase activity was measured in a medium containing 100 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 1 mM Na_2 - EDTA and 3 mM p-Nitrophenylphosphate. Total pNPPase activity was measured in a similar medium with the addition of 15 mM Kcl. K^+-stimulated pNPPase activity was considered to be the difference between the total and basal activities. Ouabain sensitive K^+-pNPPase activity was calculated as the K^+-pNPPase activity inhibited in the presence of 1 mM ouabain. Paranitrophenol concentrations were measured in spectrophotometer at 405 nm wave length (Lamers et al. 1978).

Mitochondrial ATPase:

Since the activity of mitochondrial ATPases are inhibited by sodium azide, 5 mM sodium azide was added to the assay

medium to determine its contribution to the total ATPase activity, which was determined as described earlier.

K^+-EDTA Myosin ATPase:

K^+EDTA ATPase activity is an in-vitro measure of myofibrillar ATPase (Martin et al. 1982). Although it has no physiological significance, it is a sensitive method to determine the presence of myofibrils in in-vitro. The enzyme activity was measured in 450 mM KCl, 45 mM imidazole (pH 7.5), 5 mM $MgCl_2$, 5 mM Tris-ATP, 10 mM NaN_3 and 10 mM EDTA. The released inorganic phosphate was measured as described elsewhere (Tausky and Shorr 1953).

Ca^{2+}-stimulated ATPase:

Ca^{2+}-stimulated ATPase is located in myofibrils and SR and therefore it is used as their marker enzyme (Pierce and Dhalla 1981). The Mg^{2+}-dependent ATPase activity was determined at 30°C in a medium containing 20 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 2 mM Na_2ATP, 10 mM NaN_3 1.6 mM EGTA and 50 mM KCl. Total ATPase activity was determined in a similar medium except that the EGTA was replaced by 1 uM Ca^{2+}. Ca^{2+} stimulated ATPase activity was taken as the difference between the values obtained for total and basal ATPase activities.

(ii) Separation of sarcolemmal membrane proteins by SDS-polyacrylamide gel electrophoresis.

Sarcolemmal membrane proteins of 2 and 12 month old rat hearts were analysed in sodium dodecyl sulphate polyacrylamide

gel electrophoresis (SDS-PAGE) as described by Lammeli (1970). Slab gels were cast with separating gel of 7%, 12% and 15% of acrylamide and stacking gel of 3% acrylamide. The gels were 1.5 mm thick, 16 cm width and 16 cm in length. The separating gel consisted of 0.1% SDS (W/V), 375 mM Tris-HCl (pH 8.8), 0.025% ammonium per sulphate (W/V) and 0.005% TEMED (V/V) with acrylamide: N,N-Methylenebis acrylamide at a ratio of 36.5: 1. The electrode buffer consisted of 0.25M Tris, (pH 8.3) 0.2 M glycine, 0.1% SDS (W/V). Membrane proteins were solubilized by the addition of sample buffer containing 0.3 M Tris HCl (pH 6.8) 5% SDS, 20% glycerol, 2.5 M beta mercaptoethanol (as final concentrations) and 0.006% phenol red as the tracking dye. Samples were immediately heated to 100^{o}C for 1 min. Aliquots of 75-200 ul, depending on the size of the well and standard proteins were subjected to electrophoresis at room temperature. After fixation of gel, it was stained with 0.2% coomassie brilliant blue in acetic acid/methanol/H_2O (1:3:6) and were destained by washing overnight in acetic acid/methanol/H_2O. The molecular weight of the protein peaks were estimated by comparing their mobility with known molecular weight standards run under the identical condition.

(iii) Binding of calcium antagonist [^{3}H]Nitrendipine:

About 80-100 ug of sarcolemmal membrane fraction from all three age groups were incubated in the medium containing 50 mM

Tris-HCl buffer (pH 7.4). After 10 minutes of pre-incubation, the reaction was started by the addition of a given concentration (0.05 - 1.0 nM) of [^{3}H]Nitrendipine (New England Nuclear; specific activity 71 Ci mmol^{-1}) and incubated for 60 min. at 25^{o}C. Non-specific binding was determined in the presence of 0.1 uM non-labelled Nitrendipine in the medium and subtracted form the total [^{3}H]Nitrendipine bound to obtain specific binding. At the end of the incubation period, the samples were immediately filtered through GF/B Whatman filters (Fisher Scientific) using a constant vacuum suction system. The filters were washed three times with 3 ml each of ice-cold, 50 mM Tris-HCl buffer (pH 7.4), dried under vacuum, placed in 10 ml of scintillation cocktail (Fisher Scientific) and counted using Beckman LS8100 liquid scintillation counter. Correction for quenching was performed by the external channel ratio method.

(iv) Binding of Calcium agonist [^{3}H]BAY K 8644:

Sarcolemmal protein of 80 to 100 ug from 2 and 12 month old rat hearts was incubated in a total volume of 5 ml containing 50 mM Tris-HCl (pH 7.4) at 25^{o}C, with varying concentrations (1 - 15 nM) of [^{3}H]BAY K 8644. After 60 minutes of incubation, samples were filtered through Whatman GF/B filters with the aid of a vacuum pump. The filters were washed three times with 3 ml of ice-cold 50 mM Tris-HCl, dried under vacuum, placed into scintillation vials and 10 ml of a

scintillation cocktail (Fisher Scientific) was added. The radioactivity was measured as described for binding of [^{3}H]Nitrendipine. Binding of [^{3}H]BAY K 8644 in the presence of 1 uM non-labelled Nitrendipine was defined as non-specific binding and was subtracted from the total binding to obtain specific binding.

Effect of Ca^{2+} on Specific [^{3}H]BAY k 8644 binding:

To demonstrate that the binding of [^{3}H]BAY K 8644 is to the dihydropyridine site of Ca^{2+} channel, the effect of Ca^{2+} on the specific binding of [^{3}H]BAY K 8644 was tested. Membranes were incubated with 1 mM EDTA for 15 min. and the specific binding in the presence of 1 and 10 nM [^{3}H]BAY K 8644 was carried out as described earlier. In another set of experiments 2 mM Ca^{2+} was added to the membrane (previously incubated with EDTA) and the binding experiments were repeated.

All radioligand experiments were carried-out in duplicate and performed under subdued light to prevent deterioration of light sensitive dihydropyridine derivatives.

(d) Identification of calcium channel subunits by photoaffinity labelling:

Isolated sarcolemmal membrane fractions from a 2 month old rat heart was equilibrated at 25^{o}C for 60 min. with a saturation concentration (10 nM) of [^{3}H]Nitrendipine in 1 ml of 50 mM Tris (pH 7.4) and 0.5 mM PMSF. Non-specific

labelling was determined by the addition to 10^{-6}M cold Nitrendipine. Samples were photoactivated by high intensity UV light (100 watt mercury arc light) at a distance of 15 cm at 4°C for 40 min.

(e) Phosphorylation of cardiac sarcolemmal membrane:

(i) Nitrendipine induced phosphorylation:

Isolated sarcolemmal membranes of 2 and 12 month old rat hearts were suspended in 1 ml of 50 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$ and incubated on ice for 60 min. in the presence and in the absence of 10 nM Nitrendipine. After 60 minutes of incubation the phosphorylation reaction was initiated by the addition of 5 uM [$-^{32}$P]ATP (1 Ci/mmol) and stopped after 30 seconds by addition of NaF and cold ATP to a final concentration of 0.2 M and 2 mM respectively. Time-course of Nitrendipine-induced phosphorylation was studied by stopping the reaction at 30 seconds, 1 min. and 5 min. of incubation.

(ii) cAMP-induced phosphorylation:

cAMP-induced phosphorylation was carried out by endogenous and exogenous protein kinases essentially as described by Lamers et al. (1981). Sarcolemmal membranes from both age gruops were preincubated at 25°C in 45 ul of incubation medium containing 0.05 M Tris-maleate (pH 6.8), 40 mM phosphate, 0.1 M KCl, 5 mM $MgCl_2$, 10 mM theophylline and 5 uM cAMP. Phosphorylation was initiated by addition of 5 ul of

0.1 mM [$-^{32}$]ATP. After 10 min. of incubation, the reaction was stopped by adding the sample buffer that would be used for the SDS-PAGE. To determine the exogenous protein kinase-induced phosphorylation of sarcolemma 2 ug of catalytic subunit of cAMP-dependent protein kinase was added in the incubation mixture instead of cAMP and theophylline.

Photoaffinity labelled and phosphorylated membranes were subjected to gel electrophoresis as described earlier. After destaining, gels were sliced (3 mm), digested overnight with 0.3 ml of 30% H_2O_2 and the radioactivity determined by liquid scintillation counting as described under radioligand binding studies.

DATA ANALYSIS

Statistical analysis in the present investigation was accomplished by student's t-test or when more than two age-groups were compared, the multiple analysis of variance was used and the means were compared by employing Tukey's multiple comparison test. The level of significance was $p < 0.05$.

The kinetic constants of Na^+-Ca^{2+} exchange were obtained from Eadie-Hofstee plots. The best fit line for the plot was determined by linear regression analysis; the Michaelis-Menten constant (Km) was calculated from the slope and the maximum initial velocity was determined from the intercept of the line with the ordinate.

Radioligand binding studies were analyzed by Scatchard analysis and the best fit line was determined by linear regression. Analysis of the data was also carried-out using a computer program (by Munson and Rodbard). The equilibrium dissociation constant (KD) was calculated from the negative reciprocal of the slope and the maximum number of binding sites (Bmax) was determined from the intercept of the line with abscissa. The statistical significance of the difference in binding constants and kinetic constants between various age-groups was determined by student's t-test or analysis of variance as necessary.

REAGENTS

Analytical grade chemicals, dissolved in deionized glass distilled water were used throughout. [^{3}H]BAY K 8644, [^{3}H]Nitrendipine,[^{32}P]ATP and $^{45}Ca^{2+}$ were obtained from New England Nuclear, Quebec, Canada. BAY K 8644 and Nitrendipine were gifts from Dr. Alexander Scriabine, Miles Pharmaceutical. Catalytic subunit of cAMP-dependent protein kinase was obtained from Sigma Chemical Co., USA. Nifedipine capsules and Theophylline were obtained from the Health Sciences Centre pharmacy.

IV. RESULTS

PHYSICAL CHANGES WITH AGE

The changes in body weight and heart weight of rats with advancing age are illustrated in Fig. 1. It can be seen that body weight continues to increase at a rapid pace upto one year of age before levelling off and remaining fairly constant thereafter. Similar changes in heart weight in relation to animal age have been observed, such that the ratio of heart weight to total body weight remains fairly constant in these age range of animals. Figure 2 illustrates the changes in body weight and heart weight of guinea-pigs with advancing age. Similar to the changes observed in rats the body weight and heart weight in guinea-pigs rapidly increase during the first year of life and remain stable thereafter; though they were heavier and their hearts were slightly bigger than those of rats.

EFFECTS OF AGING ON THE SARCOLEMMAL Na^+-Ca^{2+} EXCHANGE ACTIVITY

(a) Na^+-dependent $^{45}Ca^{2+}$ uptake

The Na^+-dependent uptake of $^{45}Ca^{2+}$ was studied using Na^+-loaded vesicles. $^{45}Ca^{2+}$ uptake in K^+-loaded vesicles was taken as non-specific uptake and thus subtracted from the respective values of Na^+-loaded vesicles to obtain net Na^+-dependent calcium uptake. The time-courses of Na^+-dependent $^{45}Ca^{2+}$ uptake in the presence of 50 uM $^{45}Ca^{2+}$ in

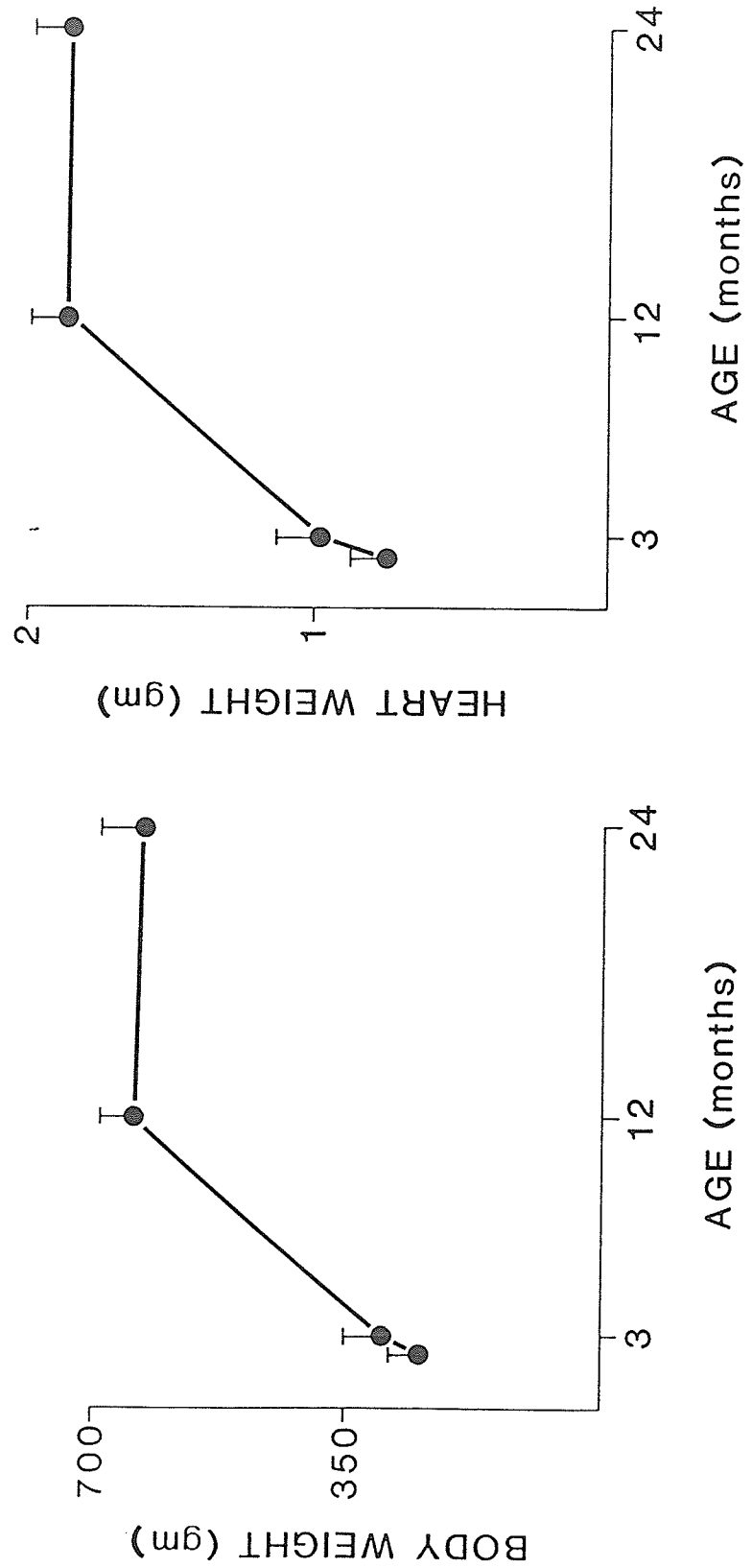


Figure 1. Age-related changes in body and heart weights of rat.

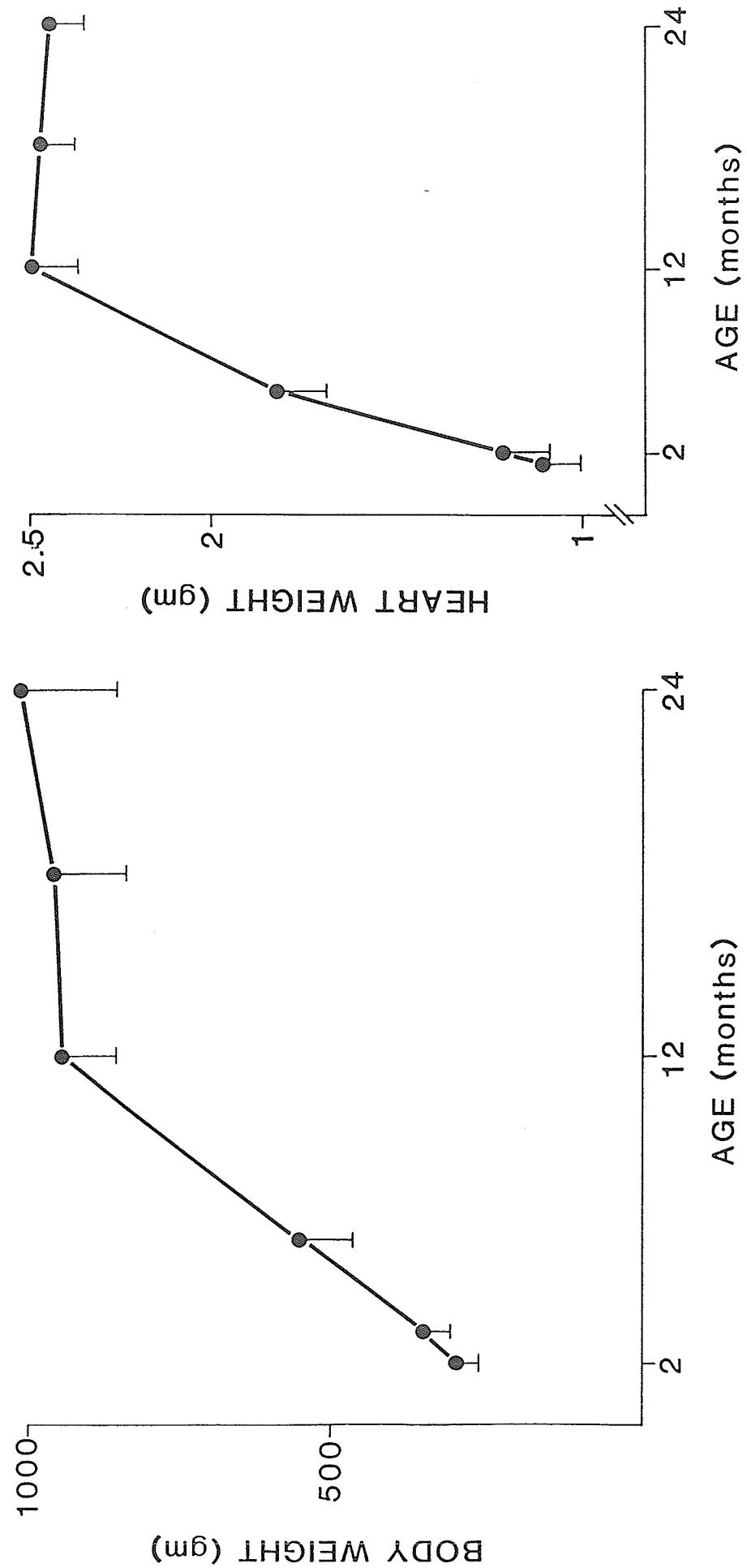


Figure 2. Age-related changed in body and heart weights of guinea-pigs.

vesicles of 3, 6 and 18 month old guinea-pigs are shown in fig. 3. In all three groups of vesicles, there was an initial rapid uptake of $^{45}Ca^{2+}$ followed by a plateau phase. However, substantial differences were seen in the amount of $^{45}Ca^{2+}$ taken up both during the initial period as well as in the plateau phase. The maximum uptake of $^{45}Ca^{2+}$ in vesicles of the control hearts (3 months) was seen within 15-30 seconds and the $^{45}Ca^{2+}$ content of the vesicles at this time was 6.4 $\pm$ 0.34 nmol mg^{-1} protein. In the following 1-1.5 min, calcium content of the vesicles reduced slowly and reached a steady state within 2 min. On the other hand, the vesicles of the older animal hearts (18 months) accumulated calcium at a relatively slower rate with substantially lower maximum accumulated calcium. The calcium content of these vesicles at 45 seconds was 4.63 $\pm$ 0.05 nmol mg-1 protein which was then slowly reduced to 3.13 $\pm$ 0.4 nmol mg^{-1} protein at 5 minutes. The Na^+-dependent $^{45}Ca^{2+}$ uptake in the vesicles of 6 month old guinea-pigs did not show any significant difference in the amount of $^{45}Ca^{2+}$ accumulated either in the initial period or during the plateau phase. The non-specific calcium uptake (taken up in the K^+-loaded vesicles) of 1.55 $\pm$ 0.12 nmol mg^{-1} protein was similar in vesicles of all 3 different age groups.

(b) Kinetics of Na^+-dependent $^{45}Ca^{2+}$ Uptake

The differences observed in Na^+-dependent $^{45}Ca^{2+}$ uptake

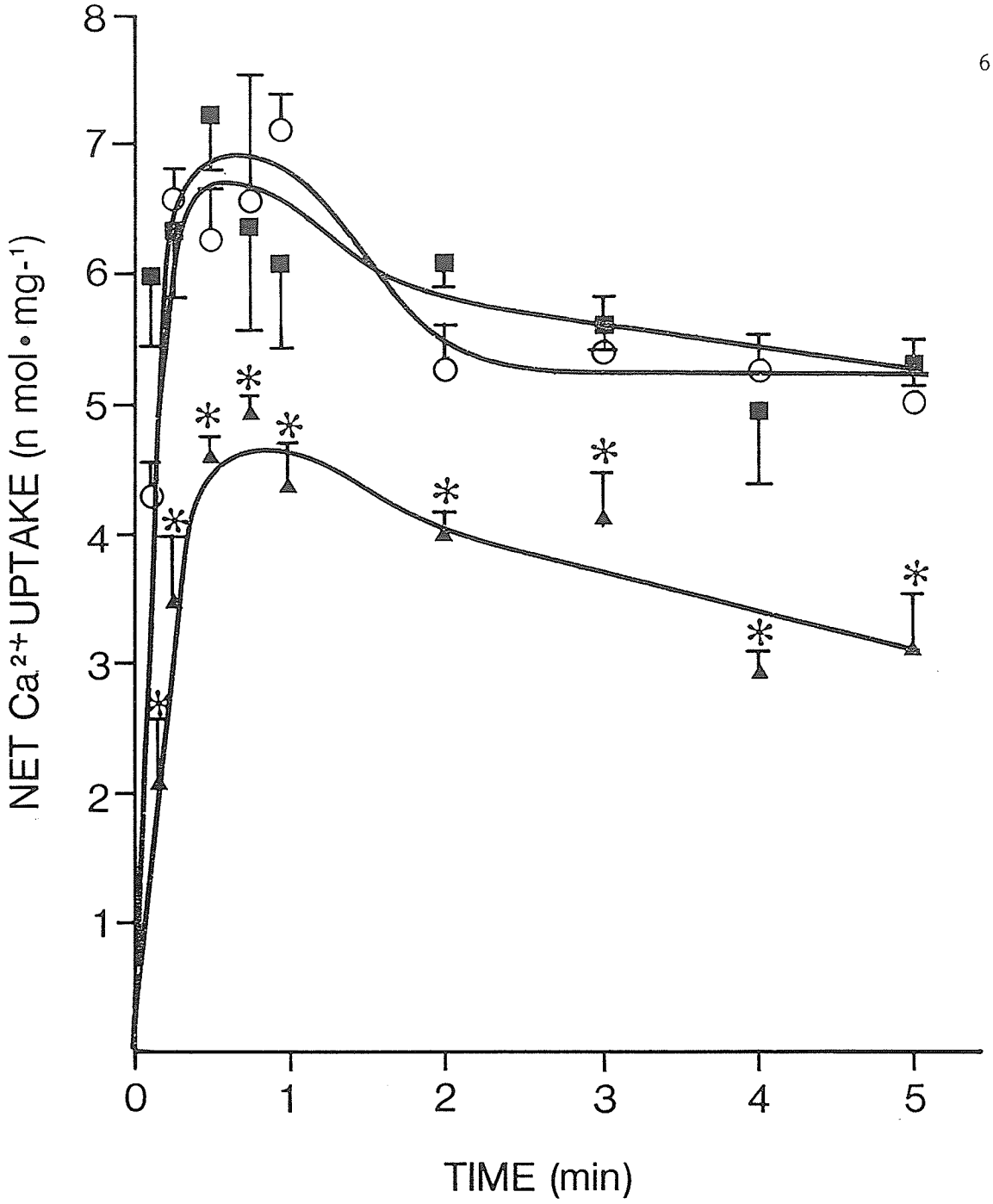


Figure 3. Time-course of Na^{+}-dependent Ca^{2+} uptake in sarcolemmal vesicles of 3(○–○), 6(■–■) and 18 month (▲–▲) old guinea-pigs. Each value represents mean ± S.E. of five experiments carried-out in duplicates in five independent membrane preparations. *Indicates statistically significant difference from the appropriate control values ($P < 0.05$).

the older guinea-pigs (18 month) was further characterized by studying the initial rate of $^{45}Ca^{2+}$ uptake, that was measured in the first five seconds (the uptake of $^{45}Ca^{2+}$ appears to be linear within this period) and the rate of $^{45}Ca^{2+}$ uptake was then calculated. Figure 4 illustrates the initial rates of $^{45}Ca^{2+}$ uptake in the presence of external [$^{45}Ca^{2+}$] ranging from 20 uM to 80 uM. The data shows that there was a substantial reduction in the rate of $^{45}Ca^{2+}$ uptake in the vesicles from the older animal heart (18 months). An Eadie-Hofstee plot of the data shown in Fig. 5 which demonstrates that the apparent maximum initial rate of $^{45}Ca^{2+}$ uptake (Vmax) was reduced by 70% in the vesicles of the older animal hearts. The determined Michaelis-Menten constant (Km) was significantly higher for vesicles of 18 month than 3 month old guinea-pig hearts indicating that the affinity (-1/km) to bind $^{45}Ca^{2+}$ is reduced in the vesicles of aged myocardium. These kinetic constants of Na^+-Ca^{2+} exchange in 3 and 18 month old guinea-pig hearts are given in Table 1.

(c) <u>Na^+-dependent Ca^{2+} Efflux</u>

Calcium efflux was initiated after 1 minute of Na^+-dependent $^{45}Ca^{2+}$ uptake by increasing Na^+ concentration in the incubation medium. Figure 6 illustrates the time course of $^{45}Ca^{2+}$ efflux for vesicles of 3 different age-groups. The initial rate of Ca^{2+} efflux during first 5 to 10 seconds was substantially lower in 18 month old than 3 month old animals

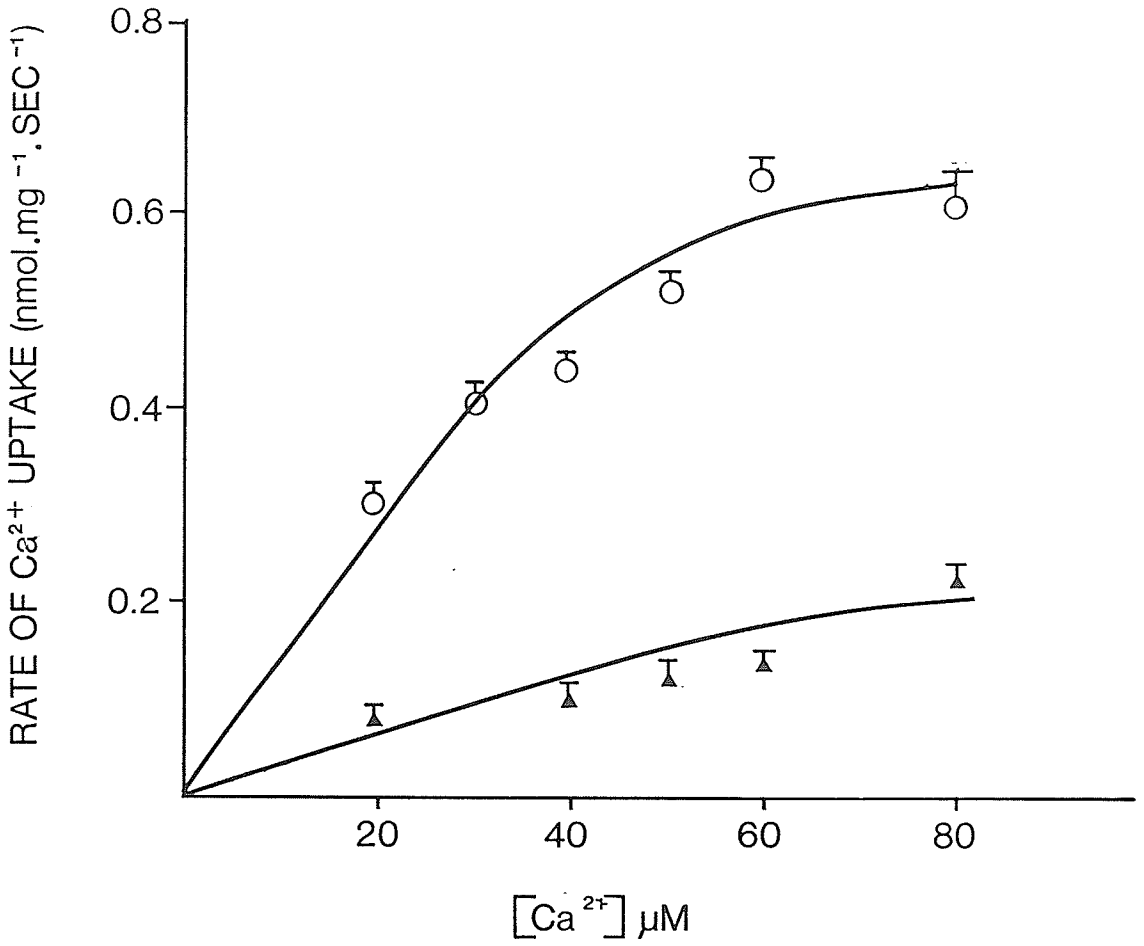


Figure 4. Initial rate of Na^+-dependent $^{45}Ca^{2+}$ uptake as a function of $[Ca^{2+}]o$ in isolated sarcolemmal vesicles of 3(○—○) and 18(▲—▲) month old guinea-pig hearts. (The uptake of $^{45}Ca^{2+}$ measured in 5 sec. of incubation and calculated per second). Each value represents mean ± S.E. of five experiments carried-out in dupicates with independent membrane preparations.

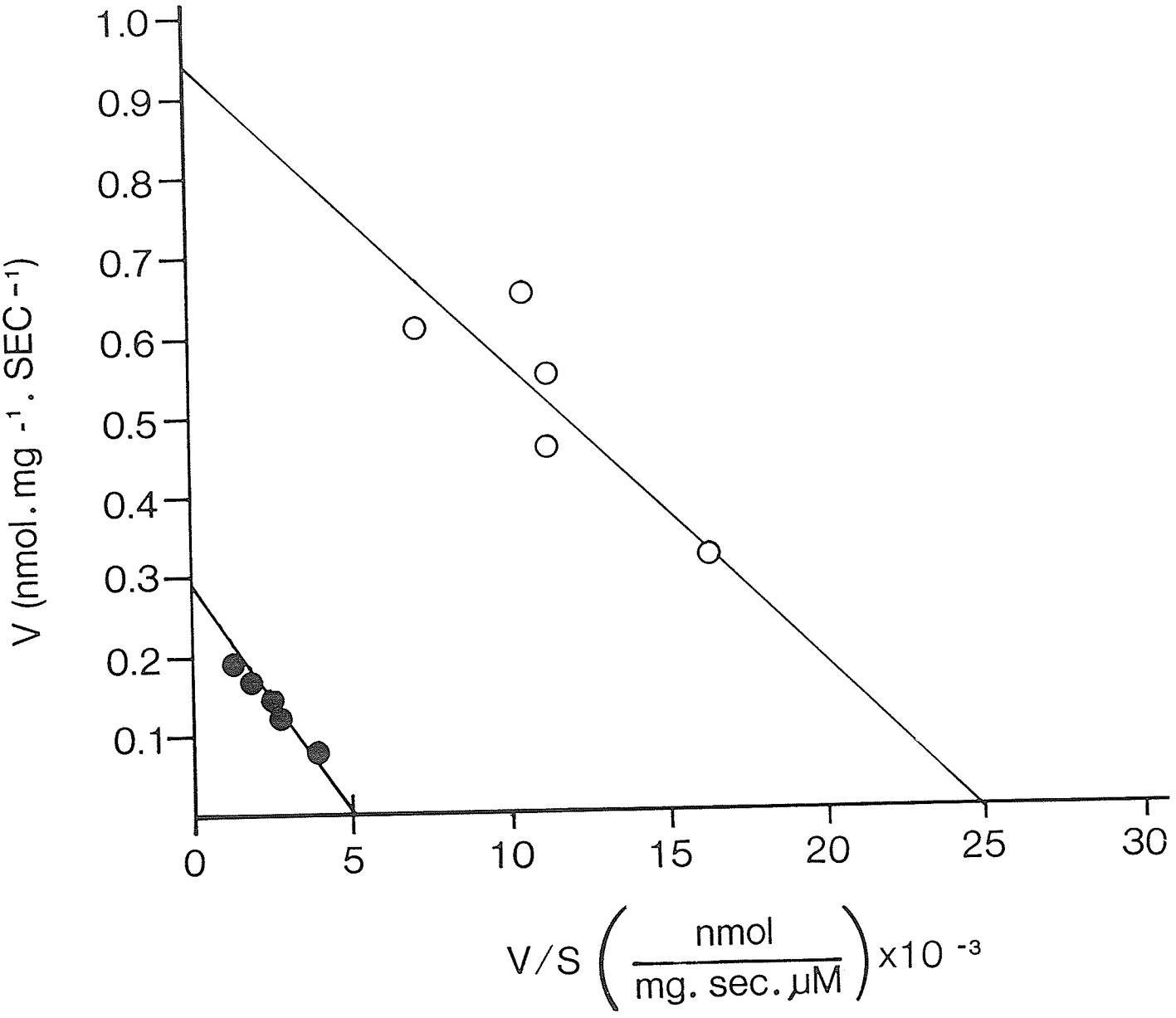


Figure 5. Eadie-Hofstee plot of Na^{2+} dependent $^{45}Ca^{2}$ uptake by the sarcolemmal vesicles of 3 (O—O) and 18(●—●) month old guinea-pigs. V: Rate of $^{45}Ca^{2+}$ uptake. (The uptake of $^{45}Ca^{2+}$ measured in 5 sec. of incubation and calculated per second). V/S: Rate of uptake/concentration of $^{45}Ca^{2+}$. The data represents typical experiment performed in duplicate. The line was determined by linear regression analysis. Experiments were repeated five times with independent membrane preparations.

Table 1. Kinetic constants of Na^+-dependent Ca^{2+} uptake in 3 and 18 month old heart sarcolemmal vesicles.

Age (months)	Vmax (nmol/mg)	Km (uM)
3	54.2 ± 1.7	35.8 ± 1.1
18	20.0 ± 0.8[a]	58.4 ± 6.7[a]

Values represent mean ± standard error of the mean of experiments carried out in five different membrane preparations of 3 and 18 month old guinea-pigs. a - indicates statistical significance from the appropriate value of 3 month old guinea-pig (Student's t-test two tailed, $P < 0.05$)

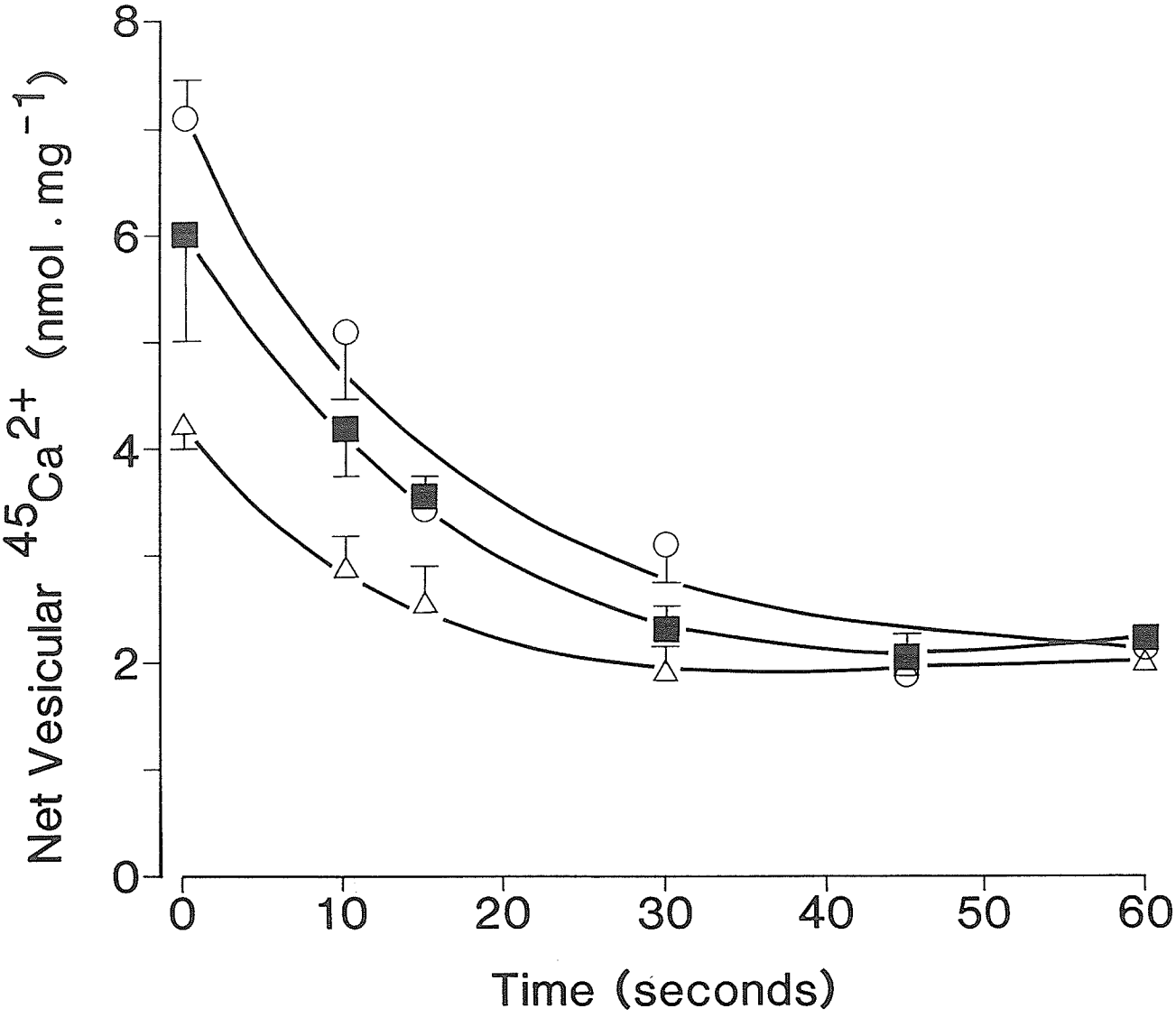


Figure 6. Time-course of Na^+-dependent Ca^{2+} efflux in sarcolemmal vesicles of 3(○–○), 6(■–■), and 18 (△–△) month old guinea-pigs. Each value represents mean ± S.E. of five experiments. The initial rates in older animals determined during 5 seconds, were significantly ($P < 0.05$) lower than that of 3-6 months.

(0.11 ± 0.03 as compared to 0.25 ± 0.02 nmol mg^{-1} protein $second^{-1}$ $p < 0.05$). When the efflux activity was evaluated as a percent $^{45}Ca^{2+}$ retained in proportion to its basal value (Fig. 7), the initial rate of efflux did not seem to differ between 3 age groups. However at the end of 120-180 seconds of incubation, the vesicles from the older animal hearts retained 40% of the basal calcium, which was significantly higher than the vesicles of 3 month old animals, (which retained only 21% of the basal $^{45}Ca^{2+}$) indicating that the Na^+-dependent $^{45}Ca^{2+}$ efflux is reduced in older animals. The $^{45}Ca^{2+}$ efflux in vesicles of 6 month old guinea-pigs was almost similar to the control vesicles (3 months) with slightly greater retention of calcium.

(d) $Na^+ - Ca^{2+}$ exchange activity in rat heart sarcolemmal membrane

The Na^+ dependent $^{45}Ca^{2+}$ uptake was carried out in rats similar to the uptake studies in isolated sarcolemmal vesicles of guinea-pigs. Time-course of Na^+-dependent $^{45}Ca^{2+}$ uptake by the sarcolemmal vesicles of 2 and 12 month old rat hearts are illustrated in Fig. 8. In the vesicles of control hearts (2 month old), there was a rapid initial $^{45}Ca^{2+}$ uptake which reached the maximum level within 30 seconds and remained constant in the following 60 seconds of incubation. The uptake of $^{45}Ca^{2+}$ in 12 month old rat heart vesicles also demonstrated a similar profile in the time-course but the

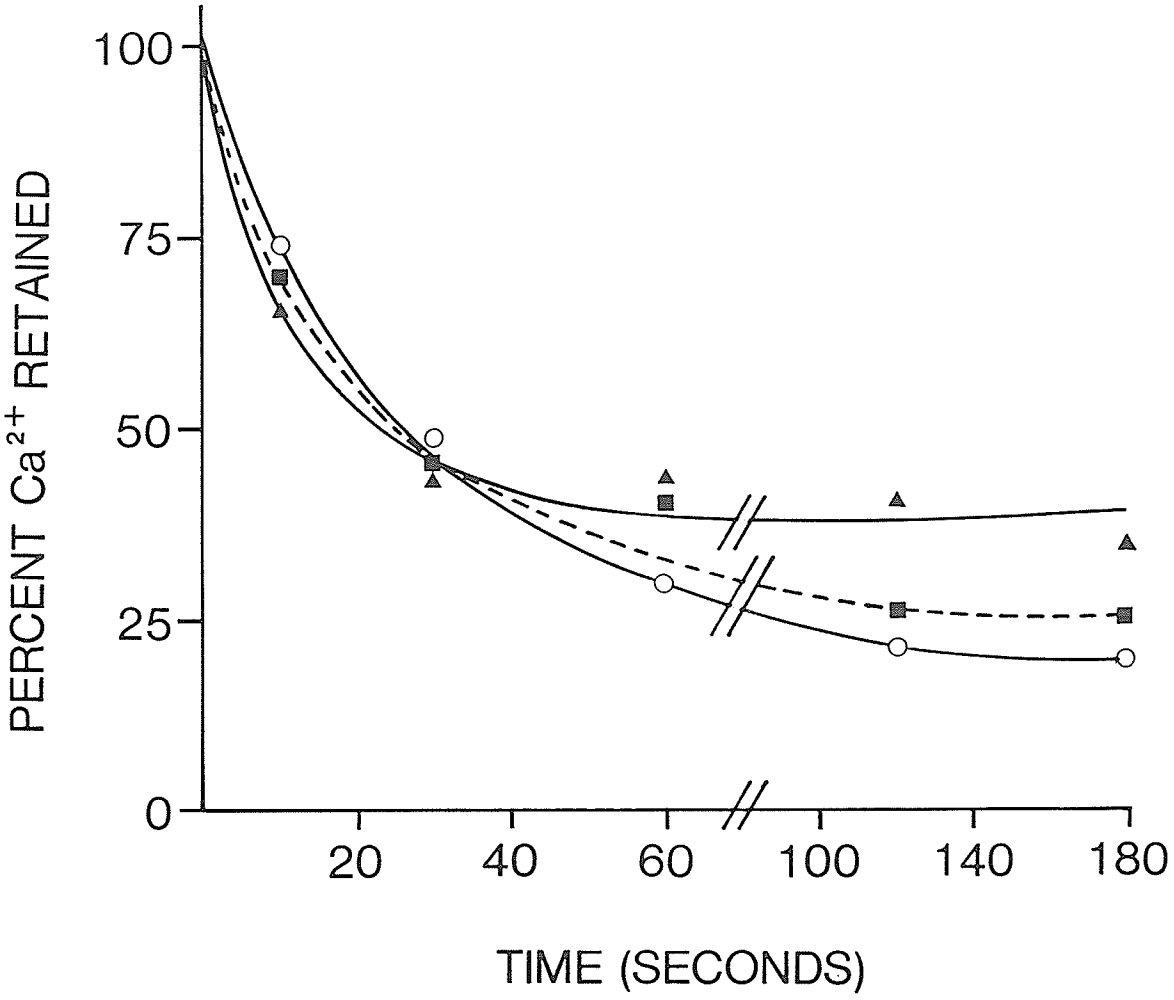


Figure 7. Time-course of percent $^{45}Ca^{2+}$ remaining after incubation of vesicles in high Na^{+} medium. The percent $^{45}Ca^{2+}$ retention indicates the amount of $^{45}Ca^{2+}$ at a particular time in proportion to its initial content. The data represents a typical experiment performed in duplicate. Experiments were repeated five times with independent membrane preparations. Vesicles from 3(○–○), 6(■–■) and 18(▲–▲) month old guinea-pigs.

amount of $^{45}Ca^{2+}$ taken up both during the initial phase (0.8 ± 0.16 vs 1.76 ± 0.28 nmol/mg protein at 5 sec) and during the plateau phase were significantly lower than that of 2 month old rat heart.

EFFECT OF AGING ON MYOCARDIAL VOLTAGE-GATED CALCIUM CHANNEL

a) Cardiovascular response to BAY K 8644 in whole animal model

i) Age-related changes in positive inotropic response to BAY K 8644:

The basal haemodynamic parameters of 2 and 12 month old rats are given in Table 2. The basal MAP, LVP and its dp/dt were not found to be significantly different in 12 and 2 month old rats. Similarly, heart rate, PR interval and QTc (QT interval corrected for heart rate) did not differ significantly between the two age groups.

Figure 9 illustrates the dose-response relationship of BAY K 8644 on percent increase in +dp/dt of LVP. As shown in the figure, the dose-response curve of 12 month old rat heart had shifted to the left and upwards from the curve of 2 month old rat heart. Doses of 1 to 3 ug/Kg of BAY K 8644 produced little or no change in +dp/dt of 2 month old rats. The maximum increase in +dp/dt of 55-60% required almost 20-30 ug/kg of BAY K 8644. Whereas in 12 month old rats, doses as low as 1-3 ug/kg produced almost 15-45% increase in +dp/dt.

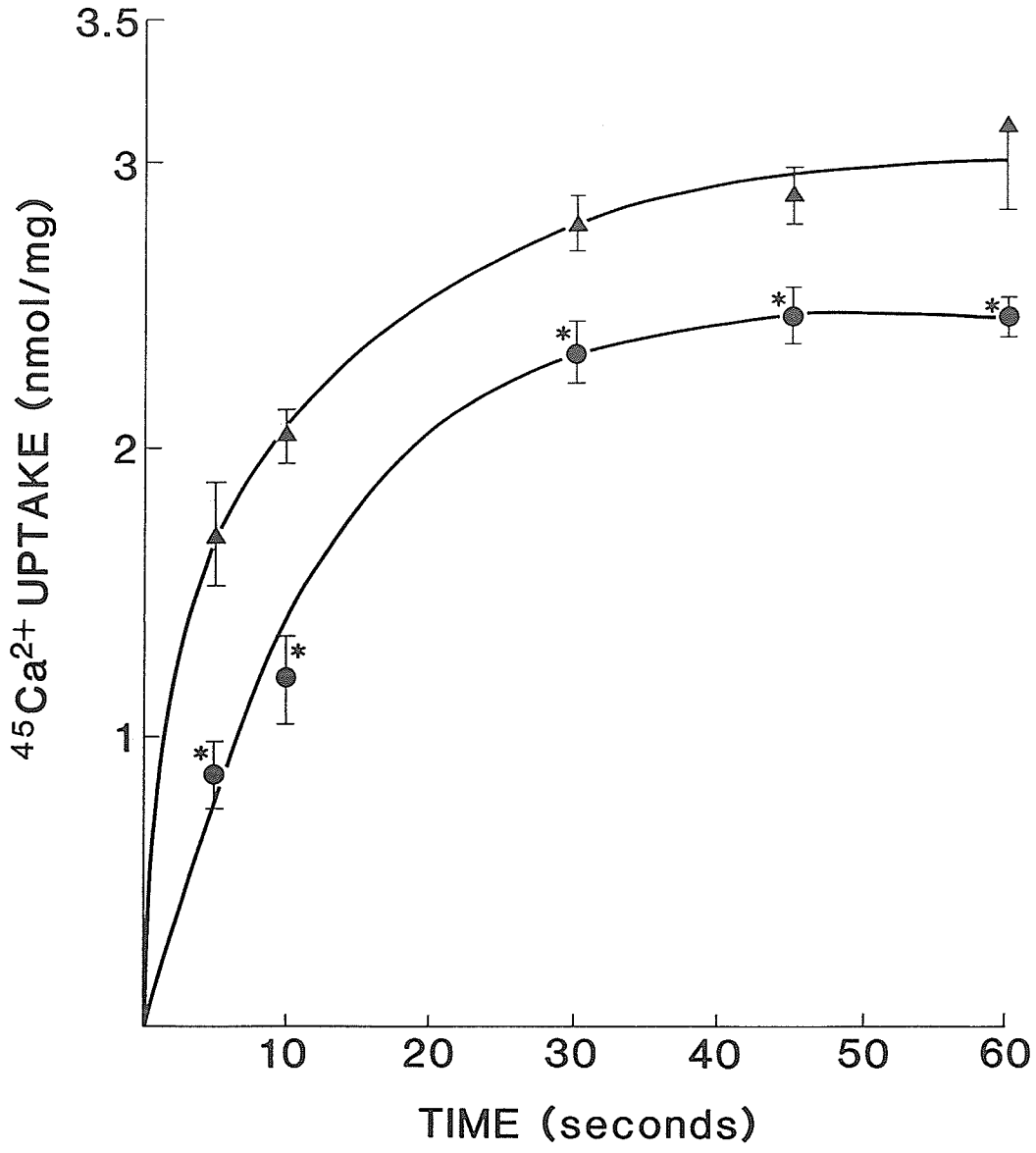


Figure 8. Time-course of Na^{+}-dependent Ca^{2+} uptake in sarcolemmal vesicles of 2(▲—▲) and 12(●—●) old rat hearts. Each value represents mean ± S.E. of five experiments carried-out in duplicates in five independent membrane preparations. *Indicates statistically significant difference from the appropriate values of 2 month old ($P < 0.05$).

Table 2. Basal Cardiovascular Parameters of 2 month and 12 month old rats.

	2 month old rats	12 month old rats
Heart rate (beats/min)	392 ± 12	385 ± 13
PR interval	0.053 ± 0.00	0.053 ± 0.00
QTC (sec)	0.17 ± 0.008	0.17 ± 0.007
Mean Arterial Pressure (mmHg)	83.33 ± 4	86.4 ± 5
Left Ventricular Pressure (mmHg)	120 ± 6	123 ± 8
Left Ventricular +dp/dt (mmHg/sec)	4257 ± 298	4358 ± 208
Left Ventricular -dp/dt	2357 ± 168	2457 ± 180

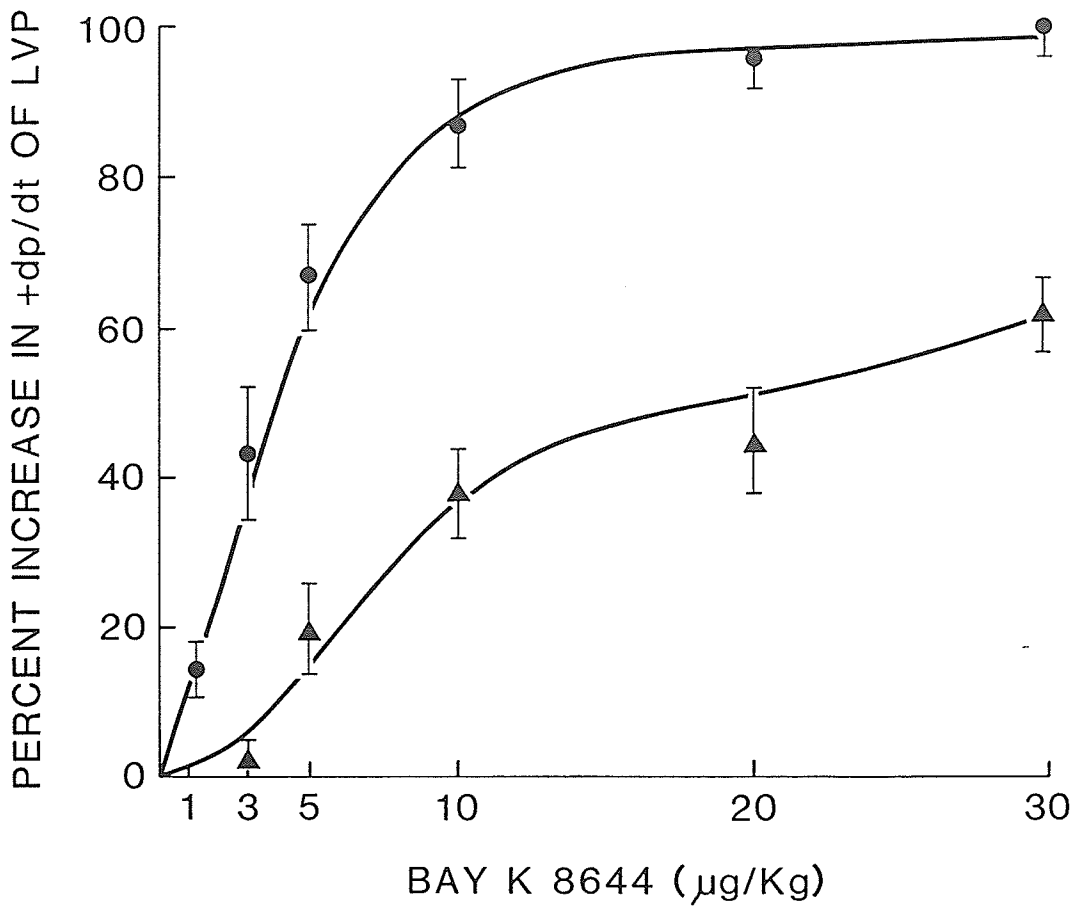


Figure 9. Inotropic response of BAY K 8644 plotted as percent increase in +dp/dt of LV in 2(▲—▲) and 12(●—●) month old rats. Values represent mean ± S.E. of 6 to 8 experiments.

Furthermore, the maximum inotropic response of 85-95% increase in +dp/dt required only 8-10 ug/kg of BAY K 8644. The half maximal increase in +dp/dt (ED50) required 4 ug/kg and 10 ug/kg of BAY K 8644 in 12 and 2 month old rats respectively.

(ii) Age-related changes in pressor response of BAY K 8644:

Dose-response effects of bolus intravenous administration of BAY K 8644 on MAP obtained from 2 and 12 month old rats are illustrated in Fig. 10. Similar to the observations on positive inotropic response, the dose response curve of MAP in 12 month old rat had shifted to the left and upwards from the curve of 2 month old rat. The lower doses (1-3 ug/kg) produced a modest increase in blood pressure of an average of 5 mmHg in 2 month old rats. In 12 month old rat however, the doses of 1-3 ug/kg produced an increase of 10-30 mmHg in MAP. The maximum elevation of MAP (50-57 mmHg) was produced by 10 ug/kg and 20 ug/kg in 12 and 2 month old rats (45-50 mmHg) respectively. The calculated ED 50's for the pressor response were almost similar to the ED 50's for inotropic response in both age groups.

(iii) Effects of Nifedipine Pretreatment on BAY K 8644 response:

Since BAY K 8644 is a putative calcium agonist, we tested the effects of dihydropyridine calcium entry blocker Nifedipine on the inotropic response of BAY K 8644.

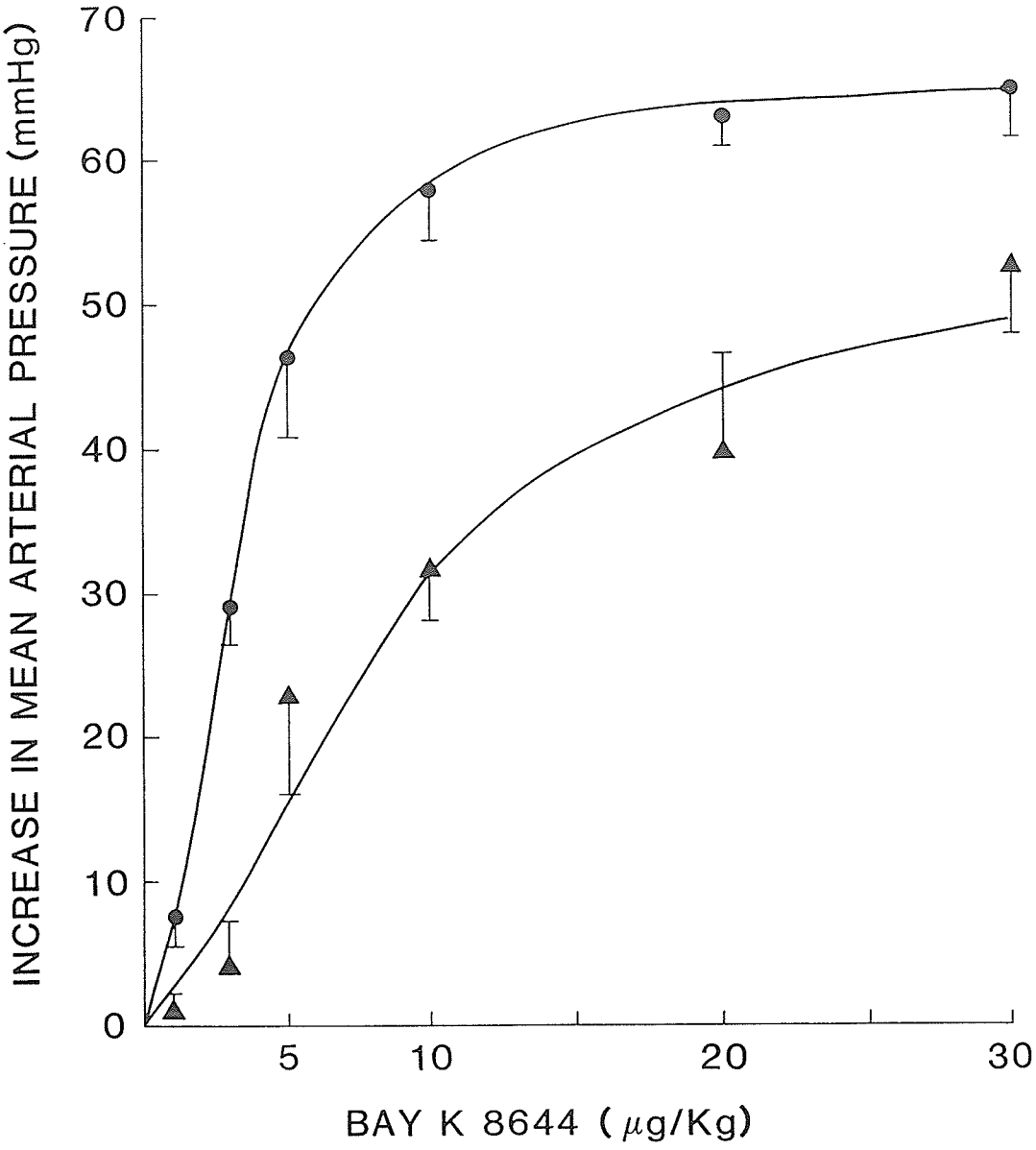


Figure 10. BAY K 8644 induced dose-dependent elevation of mean arterial pressure in 2(▲—▲) and 12(●—●) month old rats. Values represent mean ± S.E. of 6 to 8 experiments.

Intravenous infusion of Nifedipine (3 ug/Kg/min) demonstrated that up to 40 ug/Kg of Nifedipine did not produce any significant changes in either MAP or LVP and its dp/dt in 2 month old rats. The same dose of Nifedipine (40 ug/Kg) however produced a slight decrease of 9 $\pm$ 3% in MAP in 12 month old rats, without any significant ($P > 0.05$) alterations in LVP and its +dp/dt. Since sensitivity to BAY K 8644 was found to be substantially different in 2 and 12 month old rats, in these experiment we used doses of BAY K 8644 which produced half maximal increase in +dp/dt in two age groups. As described earlier (Fig. 9) these doses were 4 ug/Kg for 12 month old rats and 10 ug/kg for 2 month old rat. Pretreatment with Nifedipine (40 ug/kg) reduced the inotropic response to BAY K 8644 by 82 $\pm$ 2% in 12 month old rats and by 65 $\pm$ 3% in 2 month old rats.

(iv) Arrhythmogenic toxicity of BAY K 8644:

As illustrated in Fig. 11 a 5-6% reduction in heart rate was seen with the administration of 5 ug/Kg of BAY K 8644. A further increase in dose of BAY K 8644 caused a further reduction in heart rate such that a 20% reduction was seen with a dose of 20 ug/Kg in 2 and 12 month old animals. A bolus dose of 20 ug/kg of BAY K 8644 however caused ventricular arrhythmias in 12 month old but not in 2 month old rats (Fig. 12). Arrhythmias were of continuous ventricular

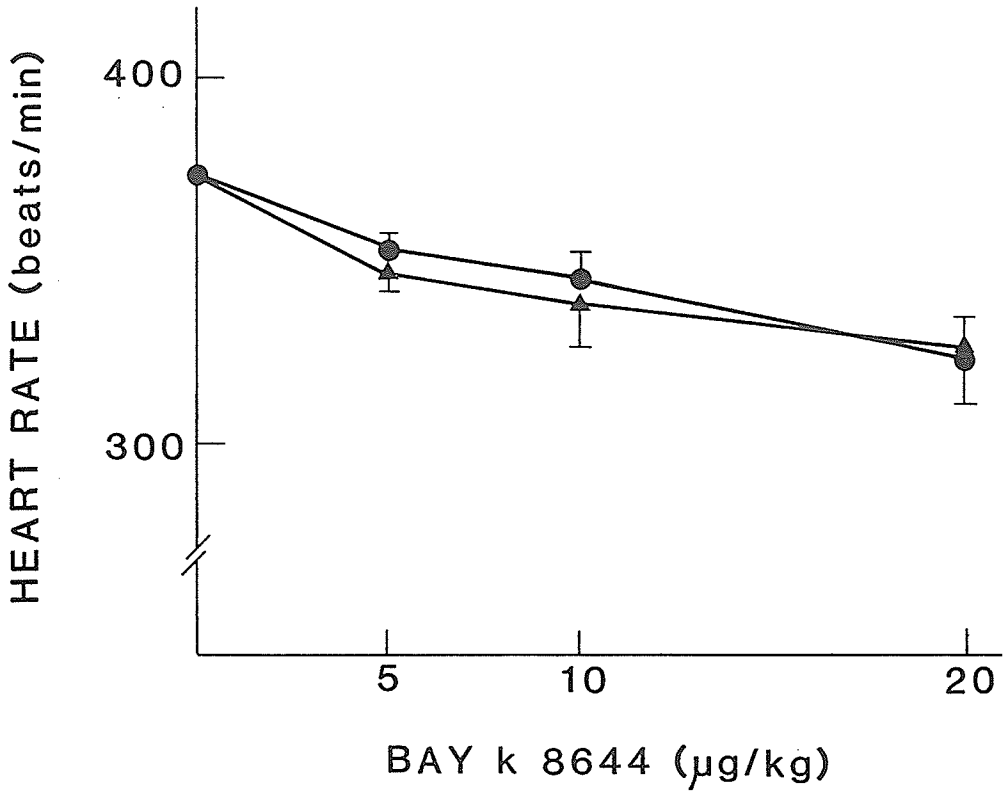


Figure 11. Effect of BAY K 8644 on heart rate of 2(▲—▲) and 12(●—●) month old rats. Values represent mean ± S.E. of 6 to 8 experiments.

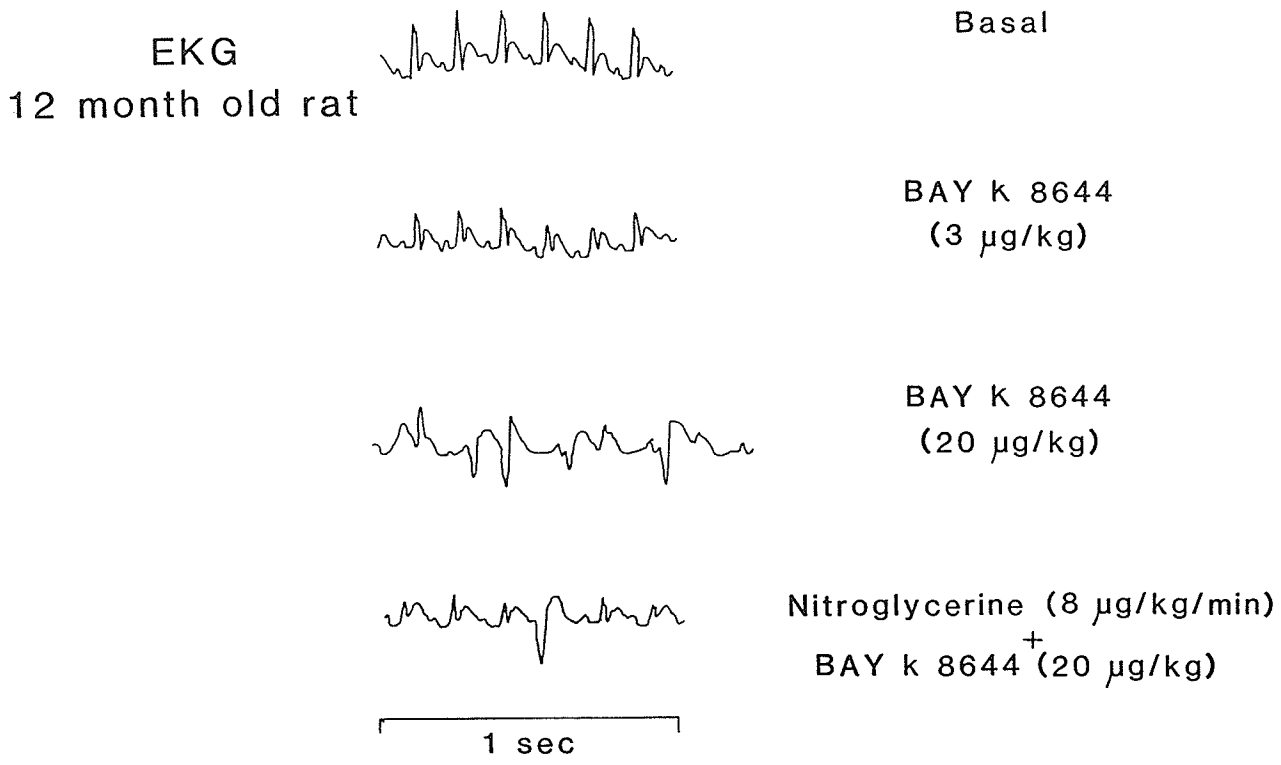


Figure 12. A typical recording of an electrocardiogram of 12 month old rat during administration of 3 and 20 ug/Kg of BAY K 8644 with and without nitroglycerine pretreatment.

ectopics and were seen in the initial few seconds after the administration of BAY K 8644 and a few episodes in the following 10-15 minutes. Upon pre-treatment with 8 ug/kg/min nitroglycerine, a dose which increased coronary flow by 25%, the severity of arrhythmias were reduced from multiple ectopics to a few ectopics, but were not abolished completely.

(b) Age-related changes in response to BAY K 8644 in isolated perfused hearts.

(i) In rat hearts:

After 20 min. of initial equilibration perfusion, when hearts from 2 month old rats were perfused with 10^{-6} M BAY K 8644, there was a steady rise in developed tension to an average maximum of 24% in 2 min (Fig. 13), which then declined at a slow rate to an average of 8.8% after 8 min. of continuous perfusion and maintained at or above the basal level even after perfusion for 45 min. In 12 month old rat hearts however the initial response occurred at much f^{a}ster rate such that a maximum increase in developed tension of 33.3% was produced within 30-40 sec. This initial response was followed by a fast decline in the developed tension. Within 7-8 min. of continuous perfusion, mechanical toxicity developed in 12 month old rat heart such that the developed tension declined below the basal level and was associated with an increase in resting tension.

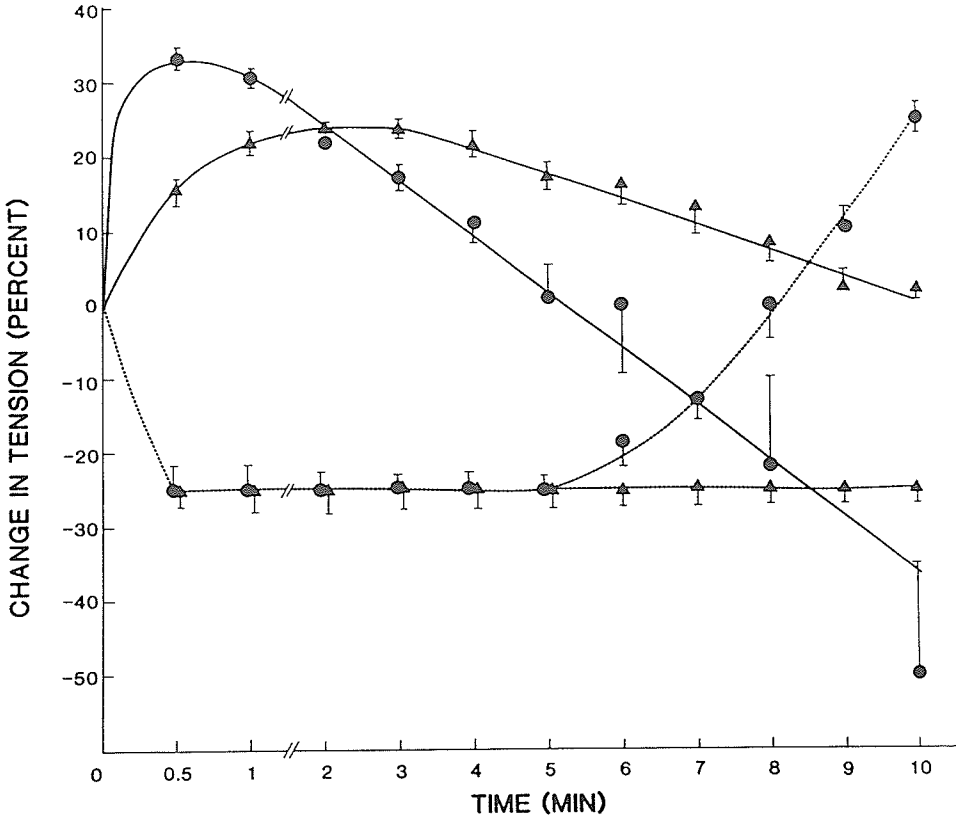


Figure 13. Time-course of developed (——) and resting (– – – –) tensions in isolated hearts of 2(▲) and 12(●) month old rats with perfusion of 10^{-7} M BAY K 8644. Each value represents mean ± S.E. of six experiments.

(ii) In guinea-pig hearts:

Figure 14 illustrates the time course of tension development in 2, 12, and 24 month old isolated guinea-pig hearts during perfusion with BAY K 8644 at a concentration of 10^{-7} M. Just as in rat, the developed tension of 2 month old guinea-pigs increased steadily to a maximum of 35% (on the average) in 5 min., which, unlike the rat, was maintained in the following 30 minutes of continuous perfusion. In 12 month old guinea-pigs on the other hand, the developed tension increased to an average of 82% within 5 min and maintained thereafter. The maximum increase in developed tension and its rate of increase were not significantly different in 12 and 24 month old guinea-pig hearts.

Dose response of BAY K 8644 (10^{-8}-10^{-6}M) was studied in 2, 12 and 24 month old guinea-pig hearts and are illustrated in Fig. 15. It was clearly demonstrated that the dose response curves of 12 and 24 month old guinea-pigs had shifted to the left and upwards from the curve of 2 month old guinea-pigs. The maximum increases in developed tension in 12 and 24 month old guinea-pig hearts were greater and the dose of BAY K 8644 that produced half maximal response was significantly lower (20 nM) than that of 2 month old guinea-pig hearts (65 nM).

C) Age-related changes in dihydropyridine binding site of voltage-gated calcium channel of cardiac sarcolemma

(i) Binding of calcium agonist [^{3}H]BAY K 8644:

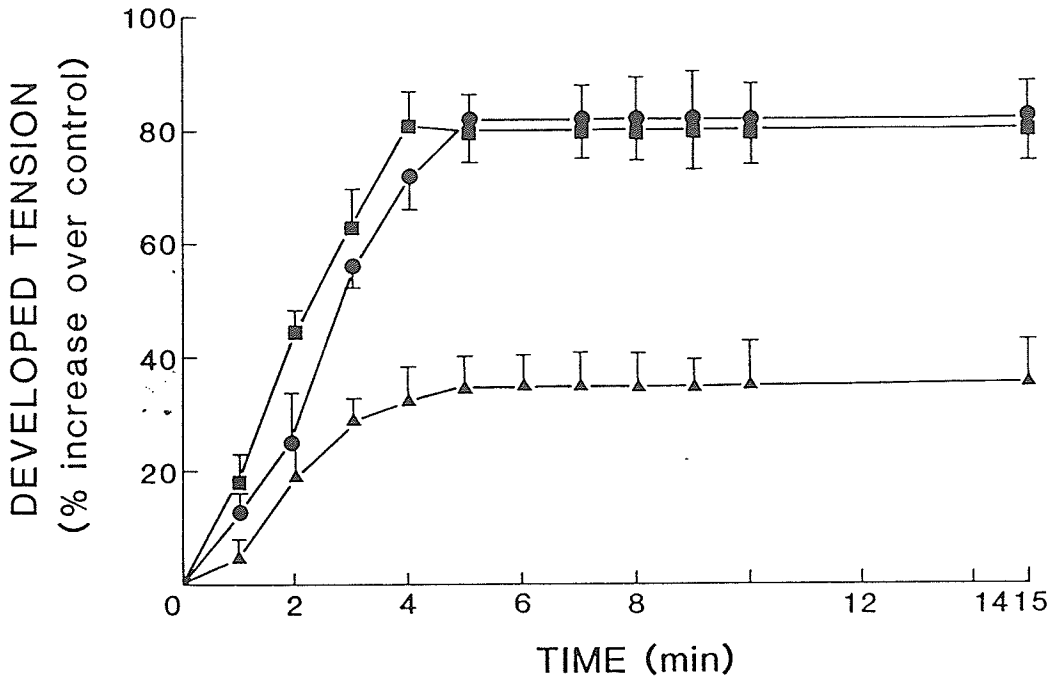


Figure 14. Time-course of tension development in isolated hearts from 2(▲—▲), 12(●—●) and 24(■—■) month old guinea-pigs with perfusion of 10^{-7} M BAY K 8644. Each plot represents a mean ± S.E. of 6 experiments. The maximum responses in 12 and 24 month old guinea-pig hearts were significantly higher than in 2 month old guinea-pigs.

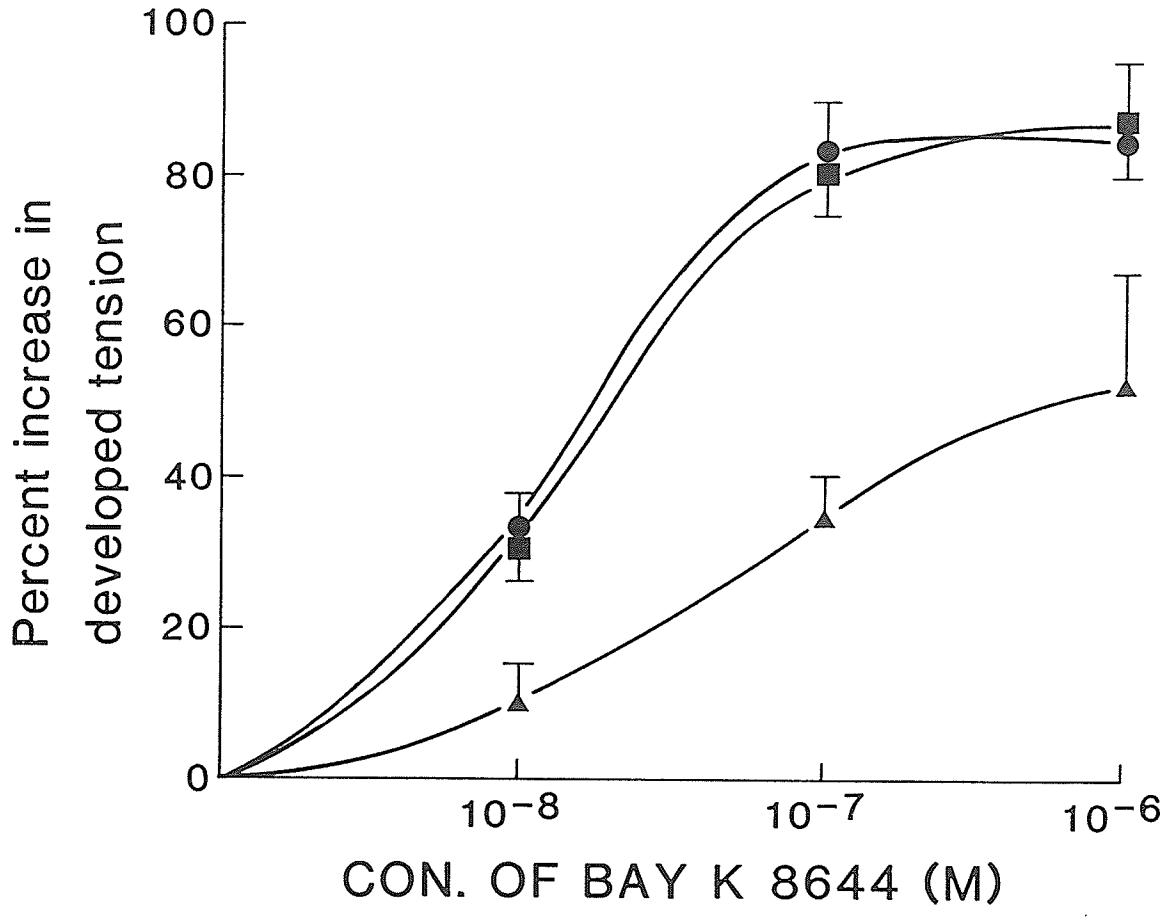


Figure 15. Dose response curves (developed tension) of BAY K 8644 in isolated perfused guinea-pig hearts of 2(▲—▲), 12(●—●) and 24(■—■) month old. Each point is the mean ± S.E. of 6 experiments. The responses of 12 and 24 month old guinea-pig hearts were significantly higher than that of 2 month old at all concentrations of BAY K 8644 tested.

General properties of [^{3}H]BAY K 8644 binding: Figure 16 illustrates the specific binding of [^{3}H]BAY K 8644 to the sarcolemmal membrane of 2 month old rats. Specific binding yielded a monophasic plot over the concentration range tested (1-50 nM), indicating the existence of a single class of specific binding sites for [^{3}H]BAY K 8644. (Since concentrations above 15 nM had very high non-specific binding and provided no further information, they were not included in Fig. 16). Since Ca^{2+} potentiates the ligand binding to dihydropyridine receptor site, its effect was tested on the binding of BAY K 8644 to determine the binding specificity. As shown in Table 3, the specific binding of 1 and 10 nM [^{3}H]BAY K 8644 decreased to 50-55% in the presence of 1 mM Ca^{2+} chelating agent EDTA, which was then restored to control values by an addition of 2 mM Ca^{2+}.

Age-related differences in binding of [^{3}H]BAY K 8644: Although the general binding properties of [^{3}H]BAY K 8644 to the sarcolemmal membrane of 12 month old rat hearts were similar to those of 2 month old rats, significant differences were seen in their binding constants. Scatchard plots of specific binding of [^{3}H]BAY K 8644 in 2 and 12 month old rat hearts are illustrated in Fig. 17. The estimated maximum number of binding sites (Bmax) in 12 month old rat heart was 2.4 $\pm$ 0.1 pmol/mg prot, which was significantly greater than the Bmax in 2 month old rat hearts (1.7 $\pm$ 0.2 pmol/mg prot).

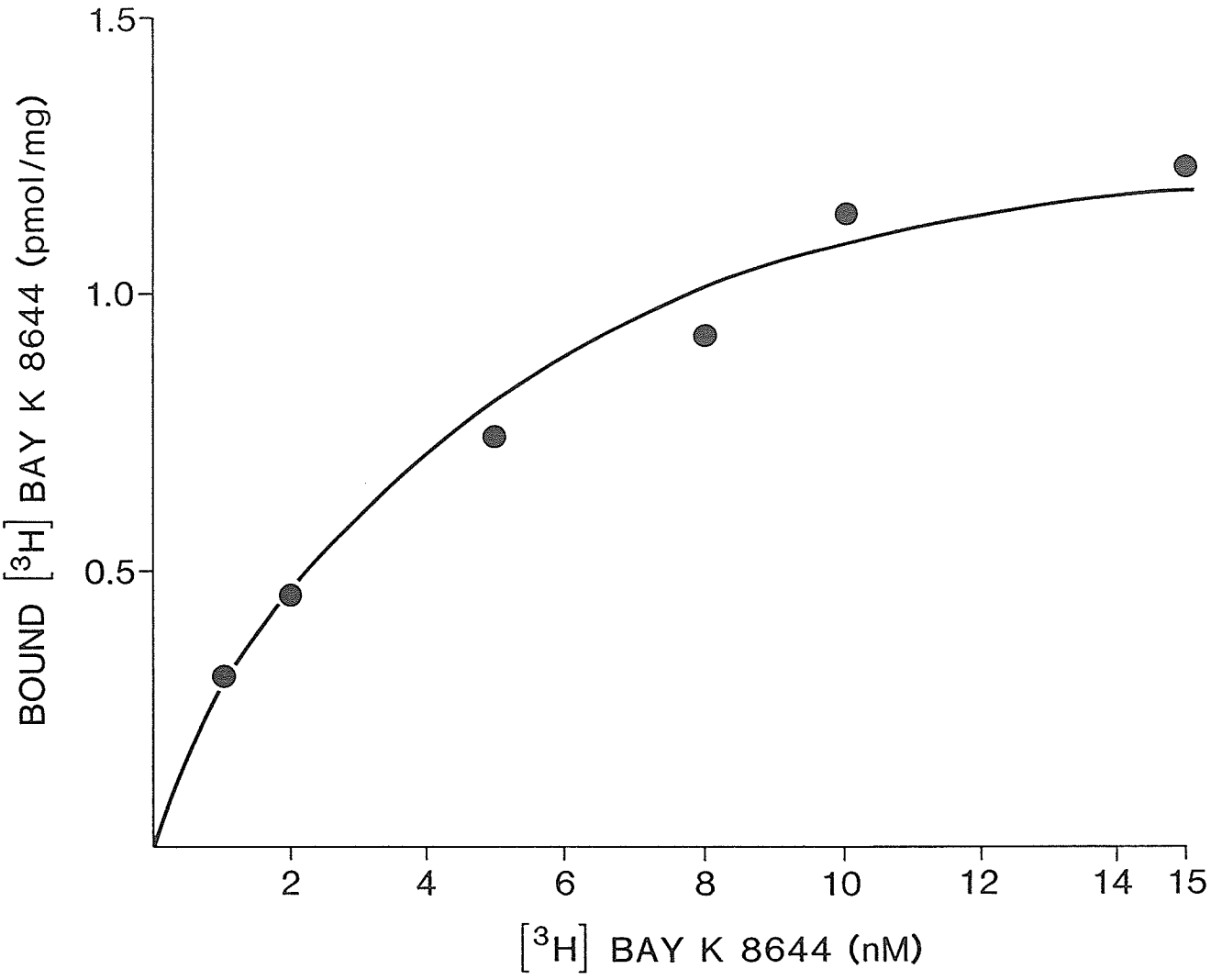


Figure 16. Specific binding of [^{3}H]BAY K 8644 to the sarcolemmal membrane of 2 month old rat heart as a function of increasing concentration. The results depicted in the figure represent the mean of duplicate measurements in a typical experiment. Experiments were carried out in five different membrane preparations.

Table 3. Effects of EDTA and Ca^{2+} on binding of [^{3}H] BAY K 8644 to sarcolemmal membrane of 2 month old rat.

TREATMENT	[^{3}H]BAY K 8644 bound (pmole/mg)	
	1 nM [^{3}H]BAY	10 nM [^{3}H]BAY
Control	0.39 ± 0.007	1.16 ± 0.02
Control + 1mM EDTA	0.14 ± 0.007	0.56 ± 0.02
Control + 1mM EDTA + 2 mM Ca^{2+}	0.38 ± 0.01	1.21 ± 0.01

Mean ± S.E.M. of four to five experiments. Control and plus Ca^{2+} are significantly different from EDTA-treated ($P < 0.05$).

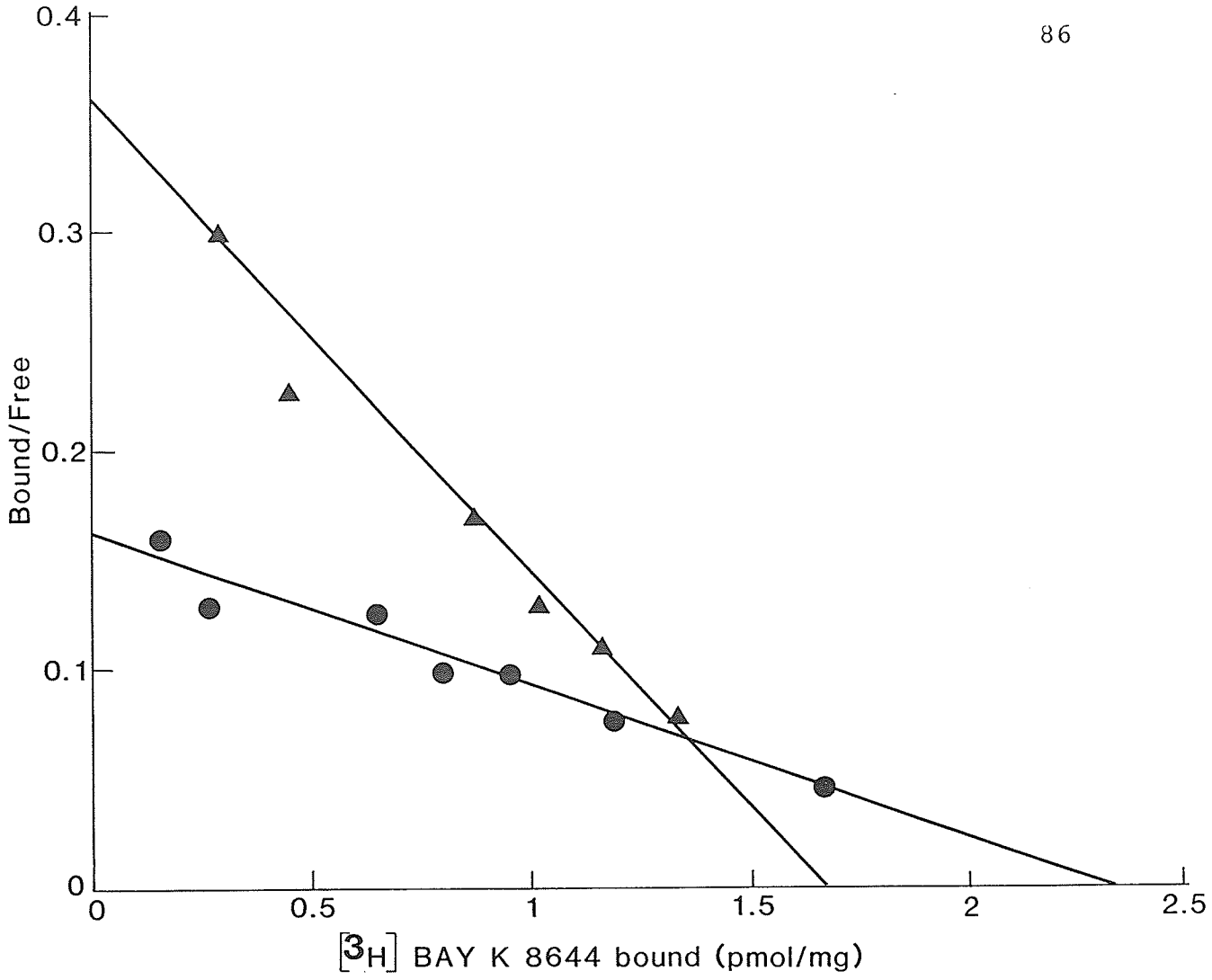


Figure 17. Scatchard plots of specific [^{3}H]BAY K 8644 binding to cardiac sarcolemmal membrane isolated from 2(▲—▲) and 12(●—●) month old rats. The data represent typical experiment performed in duplicate. The line was determined by linear regression analysis (r^2 of 2 month old: 0.95 and 12 month old: 0.88; $P < 0.05$). Experiments were carried out in five independent membrane preparations from both age groups.

The equilibrium dissociation constant (KD) of [^{3}H]BAY K 8644 in 12 month old rat heart was significantly higher than that in 2 month old rat heart (Table 4), indicating that the affinity to bind [^{3}H]BAY K 8644 was lower in older rat hearts.

(ii) Binding of [^{3}H]Nitrendipine:

Figure 18 illustrates the specific and non-specific binding of [^{3}H]Nitrendipine to the sarcolemmal membrane of 2, 12 and 24 month old rat hearts as a function of its concentration. Binding of [^{3}H]Nitrendipine was saturable, which occurred at a concentration of 0.6 nM in 2 month old rat heart membrane. Scatchard plot analysis of the specific binding data yielded a monophasic plot over the concentration range tested (0.05 - 1 nM), indicating the existence of a single class of specific binding sites for [^{3}H]Nitrendipine. Binding of [^{3}H]Nitrendipine to the membrane of 12 month old rat heart was almost 50 to 75 percent greater than that of 2 month old rat heart and there was no further increase in binding observed in 24 month old rat heart membrane (Fig. 18). Scatchard plots of specific binding data of 2, 12 and 24 month old rat hearts are illustrated in fig. 19. The maximum number of binding sites in the membranes of 12 and 24 month old rat hearts were 70% greater than that in 2 month old rat hearts (Table 5). There was virtually no change in equilibrium

Table 4. Binding constants of [^{3}H]BAY K 8644 for 2 and 12 month old rat sarcolemmal membranes.

Age (months)	KD (nM)	Bmax (pmol/mg)
2	4.8 ± 0.3	1.7 ± 0.2
12	14.5 ± 0.8[a]	2.4 ± 0.1[a]

Values represent mean ± S.E.M. of five to six experiments performed in duplicates using independent membrane preparations.
a - indicates statistical significance from the appropriate value of 2 month old rats (Student's t-test, two tailed, $P < 0.05$).

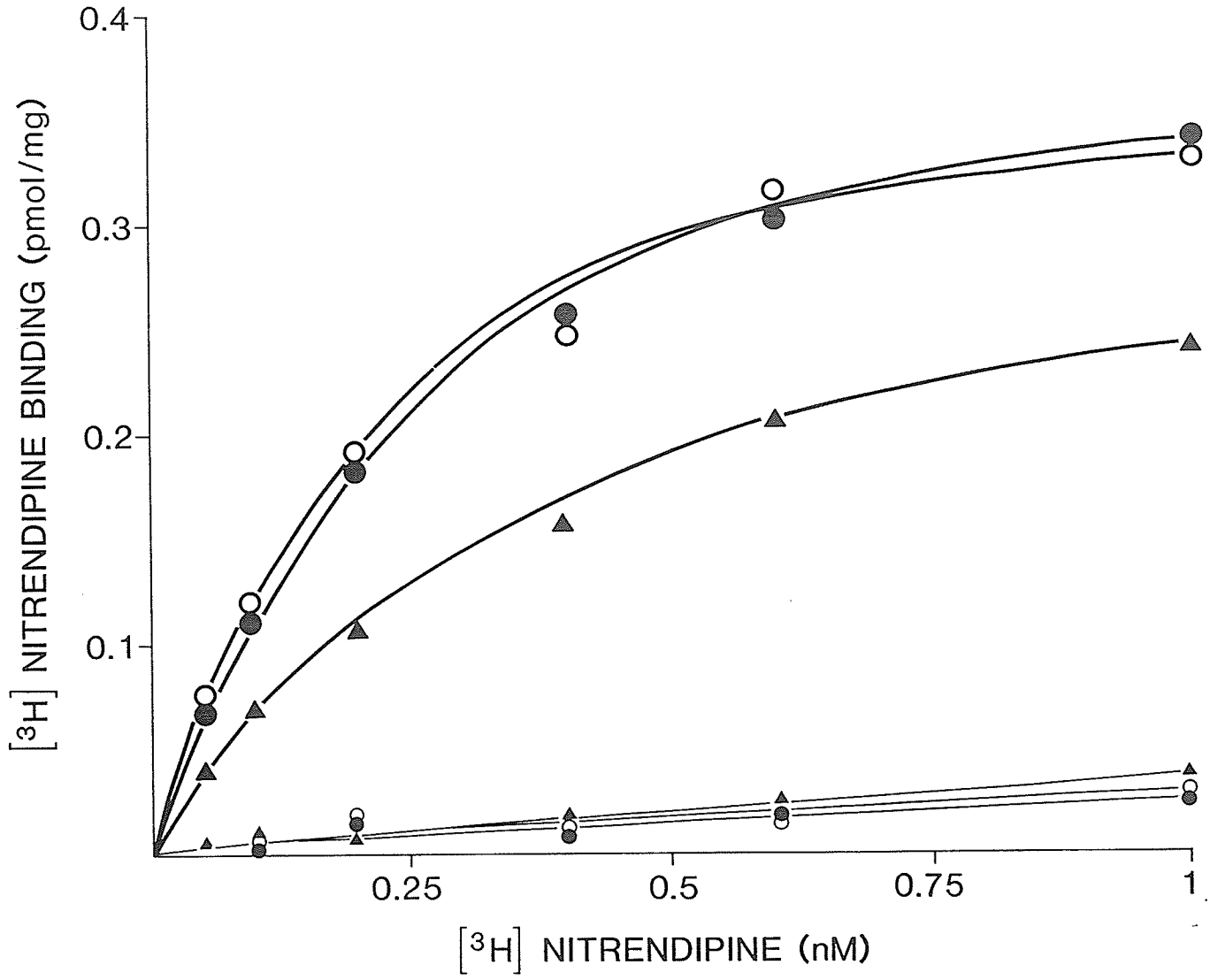


Figure 18. Specific and non-specific [^{3}H]Nitrendipine binding to sarcolemmal membrane of 2(▲—▲), 12(●—●) and 24(0—0) month old rat hearts. The results depicted in the figure represent the mean of duplicate measurements in a typical experiment. Experiments were carried-out in five different membrane preparations from 2, 12 and 24 month old rat hearts.

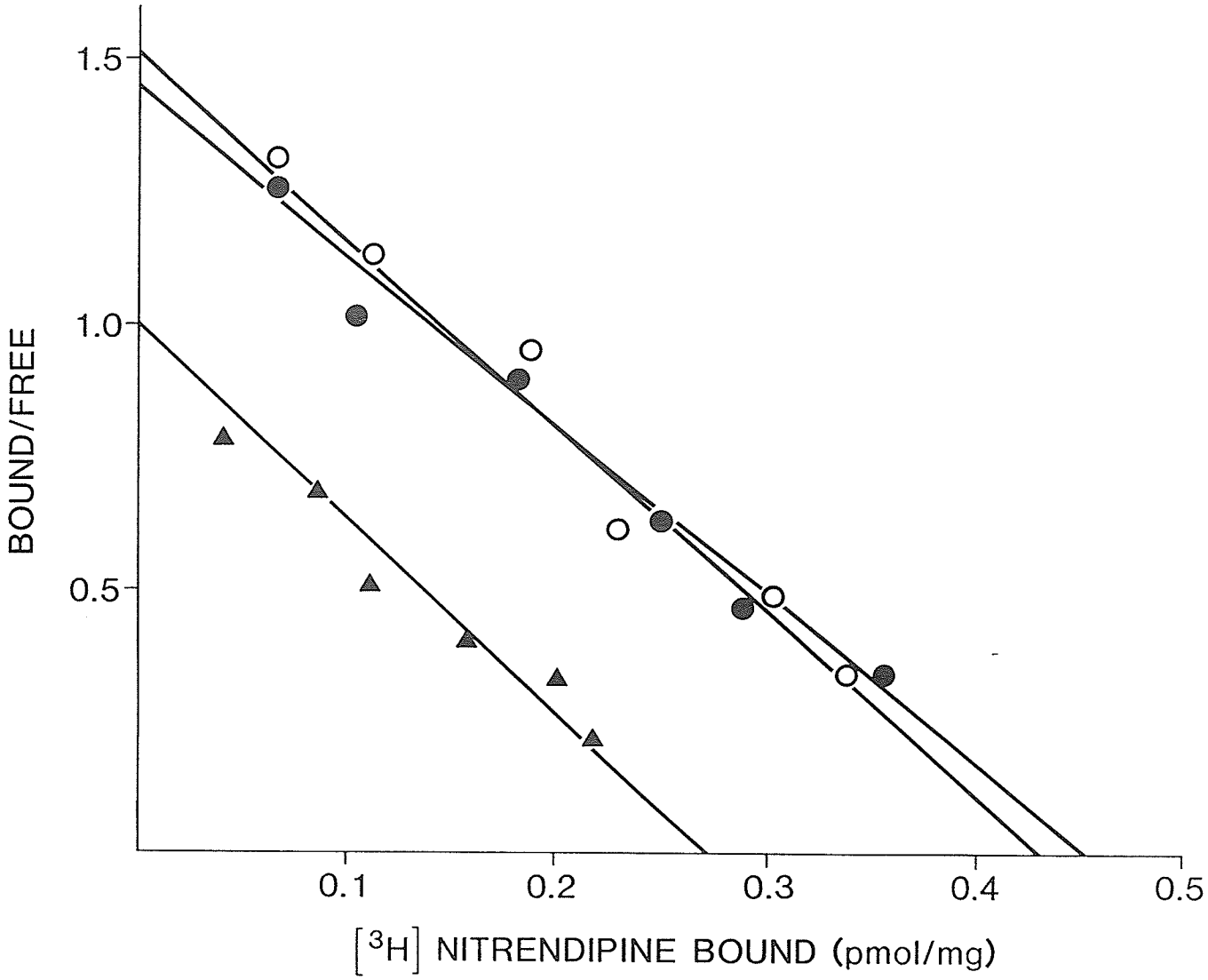


Figure 19. Scatchard plot of specific [^{3}H]Nitrendipine binding to cardiac sarcolemmal membrane of 2(▲-▲), 12(●-●) and 24(0-0) month old rat hearts. The demonstrated values represent the mean of duplicate measurements in a typical experiment. The line was determined by linear regression analysis (r^2 of 2 month: 0.95, 12 month: 0.88 and 24 month: 0.89; $P < 0.05$). Experiments were repeated five times using independent membrane preparations from each age group.

dissociation constant (KD), indicating that the affinity to bind [^{3}H]Nitrendipine was similar in all three age-groups. The maximum number of binding sites in 24 month old rat heart sarcolemmal membrane fraction did not significantly differ from 12 month old rat. The non-specific binding of [^{3}H]Nitrendipine to sarcolemmal membranes of three age groups are illustrated in Fig. 18. As can be seen the non-specific binding was similar in all three age groups and is approximately 15% of the total [^{3}H]Nitrendipine binding.

Figure 20 illustrates the specific [^{3}H]Nitrendipine binding in homogenate, microsomes and sarcolemmal membrane of 2 and 12 month old rat hearts in the presence of 0.6 nM [^{3}H]Nitrendipine, a concentration that provides saturation of their binding sites. The binding of [^{3}H]Nitrendipine to the sarcolemmal membrane fraction in both age groups was 7 fold greater than that of homogenate. Furthermore, the specific binding in the three membrane fractions of 12 month old rat heart was greater by 50% as compared to the respective membrane fractions of 2 month old rat heart.

(d) Purity of cardiac sarcolemmal membranes of 2, 12 and 24 month old rats.

To further ascertain whether the difference in membrane purity was a contributing factor to the above observed age-related changes, cellular marker enzymes were assayed in membranes of 2, 12 and 24 month old rat hearts. The recovery

Table 5. Binding constants of [^{3}H]Nitrendipine for sarcolemmal membrane of 2, 12 and 24 month old rat hearts.

Age (months)	Bmax (pmol/mg)	KD (nM)
2	0.27 ± 0.03	0.27 ± 0.04
12	0.45 ± 0.06[a]	0.31 ± 0.06
24	0.43 ± 0.05[a]	0.29 ± 0.07

Values represent mean ± standard error of the mean of experiments carried out in five different membrane preparations of 2, 12, and 24 month old rats. a - indicates statistical significance from the appropriate value of 2 month old rats (Student's t-test, two tailed, $P < 0.05$). No significant difference was observed between the Bmax values of 24, and 12 month old rats.

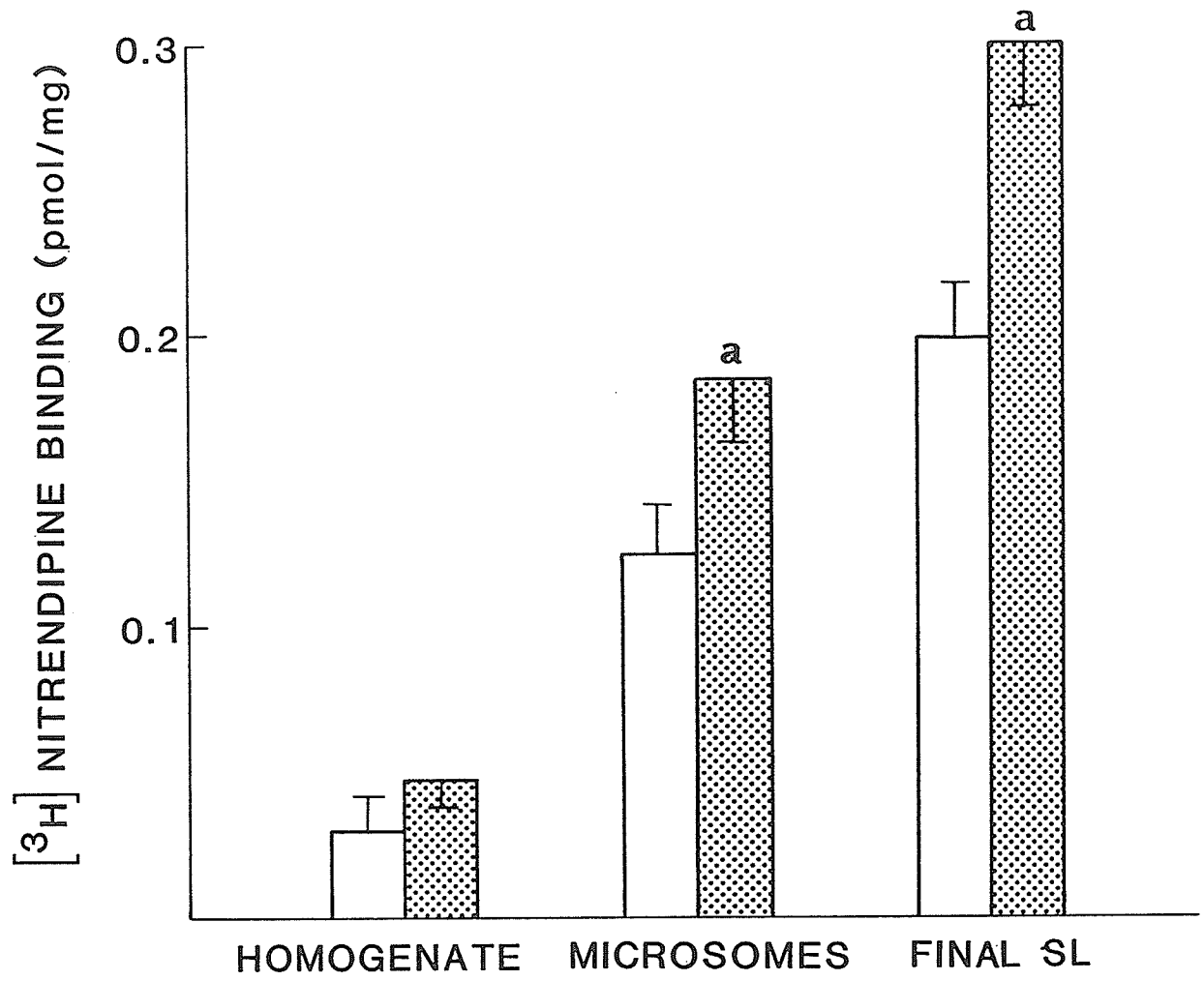


Figure 20. Specific [^{3}H]Nitrendipine binding to homogenate, microsomes and final sarcolemmal (SL) membrane of 2 (□) and 12(⊡) month old rat hearts in the presene of 0.6 nM [^{3}H]Nitrendipine [concentration at which saturation of binding occurred in the final SL membrane]. *Indicates statistically significant difference from the appropriate value of 2 month old rats.

of sarcolemmal membrane protein was virtually similar in all three age groups (0.55 $\pm$ 0.1 mg prot/gm tissue). As given in Table 6, the patent Na^+, K^+-ATPase and ouabain sensitive Na^+, K^+-ATPase in purified sarcolemmal membrane did not differ significantly between 2 and 12 month old rat hearts. The activity of Na^+, K^+-ATPase in 24 month old rat heart however was significantly lower than that of 2 and 12 month old rat hearts. These alterations in Na^+, K^+-ATPase activity have earlier been demonstrated as an age-related phenomenon (Khatter 1985, Katano et al. 1985). The activity of Na^+, K^+-ATPase in the final sarcolemmal membrane was 9 fold greater than that of homogenate and similar fold of purification was seen in all three age groups. The activity of pNPPase was also virtually identical in 2 and 12 month old rat hearts although somewhat lower activity was observed in 24 month old rats. The pNPPase in the final membrane preparation was 6 fold higher than that in the homogenates which again was similar in all three age groups. The final membrane fractions of all age group of animals contained 8-12% of azide sensitive ATPase suggesting a small but equal contamination of mitochondria. Significant activities of Ca^{2+}-stimulated ATPase and K^+-EDTA myosin ATPase were demonstrated in homogenate fractions but they were not detectable in the final sarcolemmal membrane fractions.

Table 6. Sarcolemmal and subcellular marker enzyme activities in membrane functions isolated from 2, 12, and 24 month old rat hearts.

ENZYME	HOMOGENATE			CRUDE MEMBRANE			FINAL SARCOLEMMAL MEMBRANE			PURIFICATION FACTOR		
MONTHS	2	12	24	2	12	24	2	12	24	2	12	24
Na^+,K^+-ATPase (umol/mg/hr)	3.2± 0.4	3.8± 0.6	1.8±[a] 0.6	15.2± 1.7	15.6± 2	7.2±[a] 4	31.6 5	34.7± 4	17.4±[a] 3	9.8	9.1	9.5
Na^+,K^+-ATPase + 1 uM ouabain	NC	NC	NC	6.3± 2.0	5.8± 3.0	2.1± 1.0	18.9± 0.9	21.8± 0.3	9.0± 0.4	-	-	-
PNPPase (nmol/mg/hr)	112± 6	106± 12	93± 4	398.4± 9	390± 9	360± 12	682± 18	664± 17	600± 6	6.1	6.3	6.5
K^+EDTA ATPase (umol/mg/hr)	16.9± 5	18.2± 6	20.0± 6	1.4± 0.8	1.2± 0.7	1.8± 0.8	ND	ND	ND	0	0	0
Ca^{2+}stimulated ATPase (umol/mg/hr)	2.2± 0.8	2.1± 1	3.14± 1	1.3± 0.7	1.6± 0.9	1.8± 0.7	ND	ND	ND	0	0	0

ND: not detected. NC: not carried out.

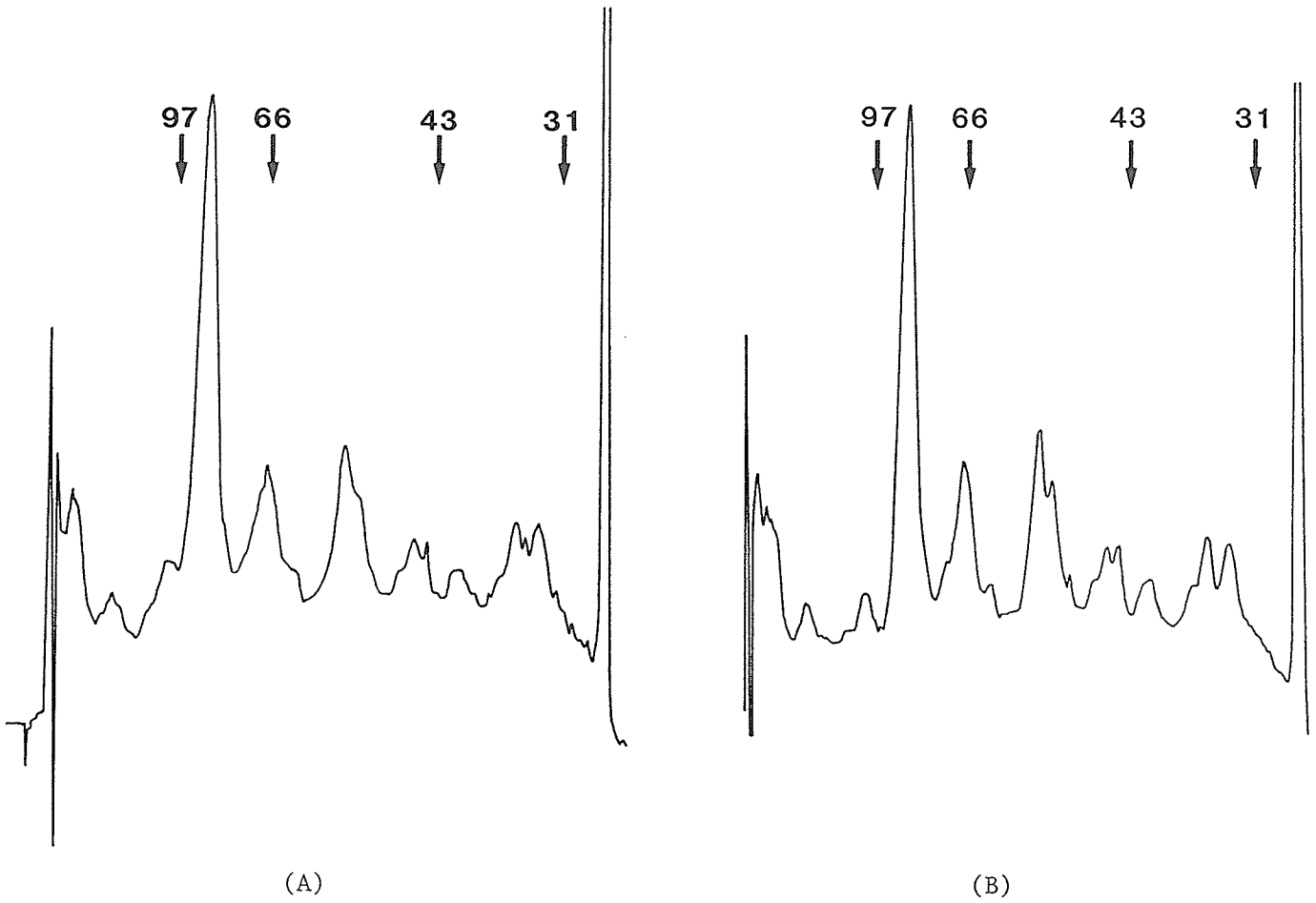
Enzyme activities were assayed in all three membrane fractions (homogenate, crude membrane and final sarcolemmal membrane). The methodological details are provided under methodology. The data represent mean ± S.E.M. of 4-5 experiments carried out in independent membrane preparations. Purification factor indicates the enzyme activity in the final sarcolemmal membrane/homogenate. a-indicates significant difference when compared to the appropriate value of 2 and 12 month old rat hearts (student t test, two tailed, $P < 0.05$).

Protein composition of sarcolemmal membrane:

Polyacrylamide gel electrophoresis of sarcolemmal membranes from 2 and 12 month old rat hearts indicated that the polypeptide composition of the membranes were essentially similar in both age groups. Densitometer scanning and quantitation of electrophorograms did not show any significant changes in the amount of protein in individual polypeptide bands between two age groups (Fig. 21). The densitometer tracings illustrated in Fig. 21 were obtained by analyzing equal amounts of proteins (50 ug) from 2 and 12 month old rat heart sarcolemma in 12% polyacrylamide gel. Studies were also performed in 15% (Fig.22) and 7% (Fig.23) gels, which revealed neither any additional polypeptide bands nor any age related differences.

(e) Identification of calcium channel subunits by photoaffinity labelling

The rat sarcolemmal membrane was labelled with [^{3}H]Nitrendipine in the presence (for non-specific binding) and in the absence (for total binding) of unlabelled Nitrendipine and photoactivated using high intensity UV light. The labelled membranes were analyzed on SDS-PAGE using 7 and 12% acrylamide. Figure 24 illustrates the [^{3}H]Nitrendipine



(A) (B)

Figure 21. Densitometric scans of cardiac sarcolemmal protein separated by SDS-PAGE and stained by coomassie blue. Separation was carried out in a 12% acrylamide gel in the presence of appropriate standard proteins. The numbers indicate molecular weights of standard proteins and their relative mobility. Sarcolemmal membrane protein was isolated from: A. 2 month. B. 12 month old rat hearts.

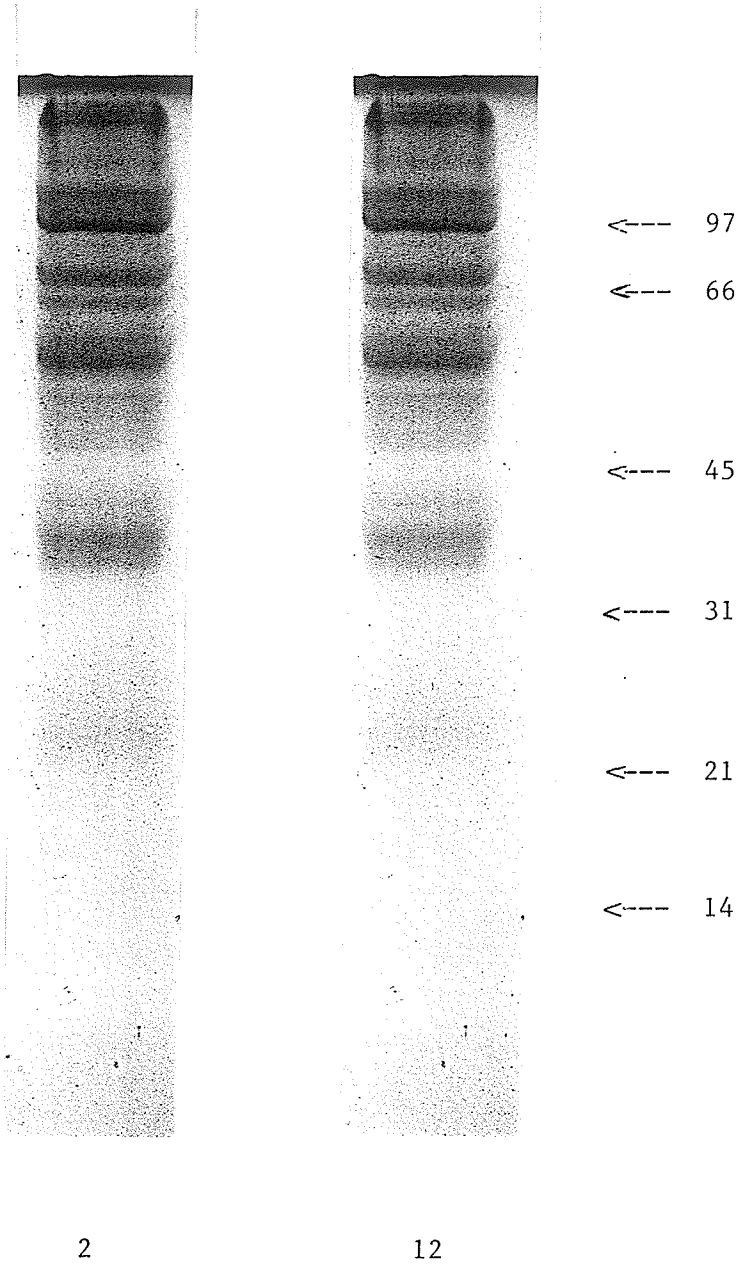


Figure 22. Polyacrylamide gel electrophoresis of sarcolemmal membrane of 2 and 12 month old rat hearts solubilized in SDS. Samples were electrophoresed in slab gel of 15% acrylamide. The numbers indicate molecular weights of standard proteins and their relative mobility.

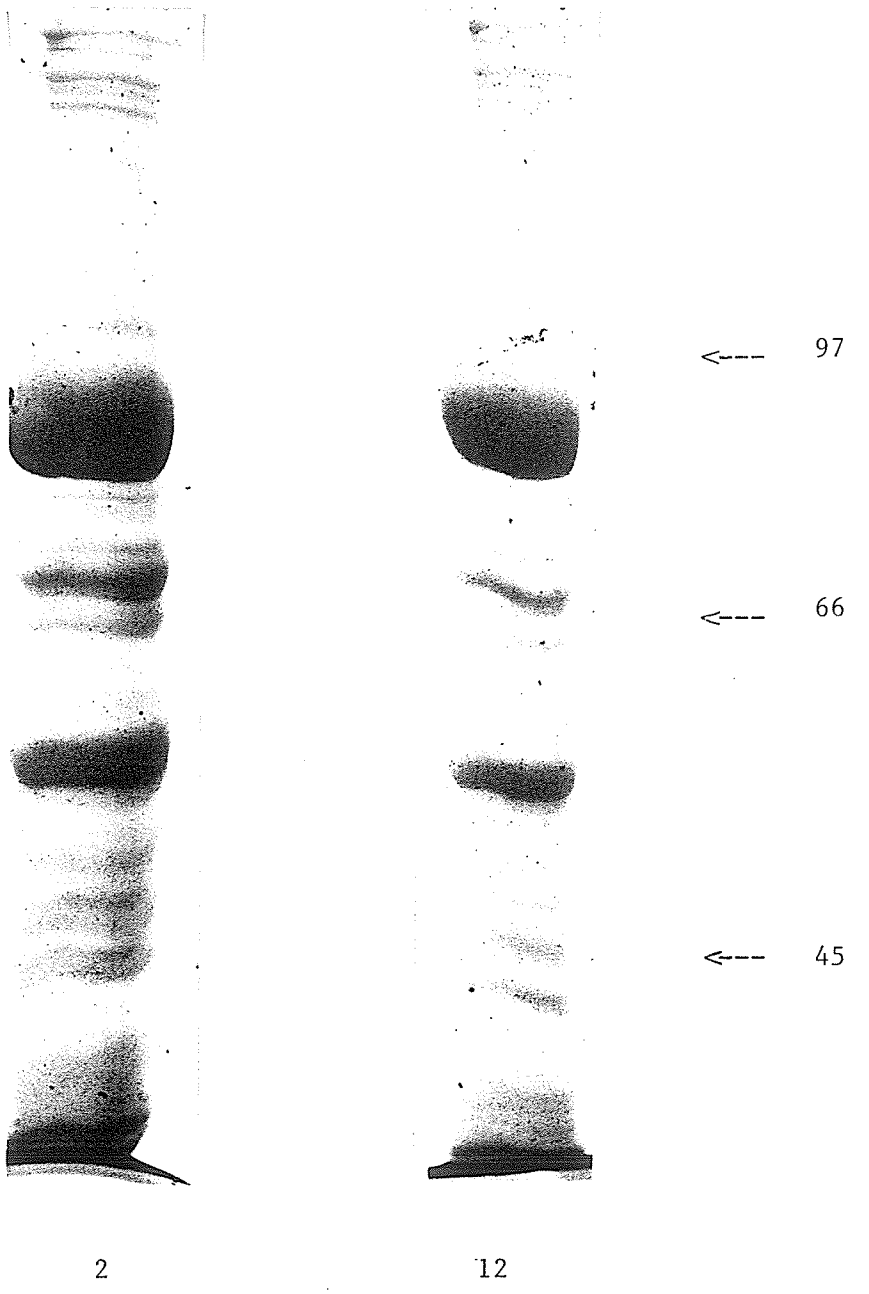


Figure 23. Polyacrylamide gel electrophoresis of sarcolemmal membrane from 2 and 12 month old rat hearts solubilized in SDS. Samples were electrophoresed in slab gel of 7% acrylamide. The numbers indicate molecular weights of standard proteins and their relative mobility.

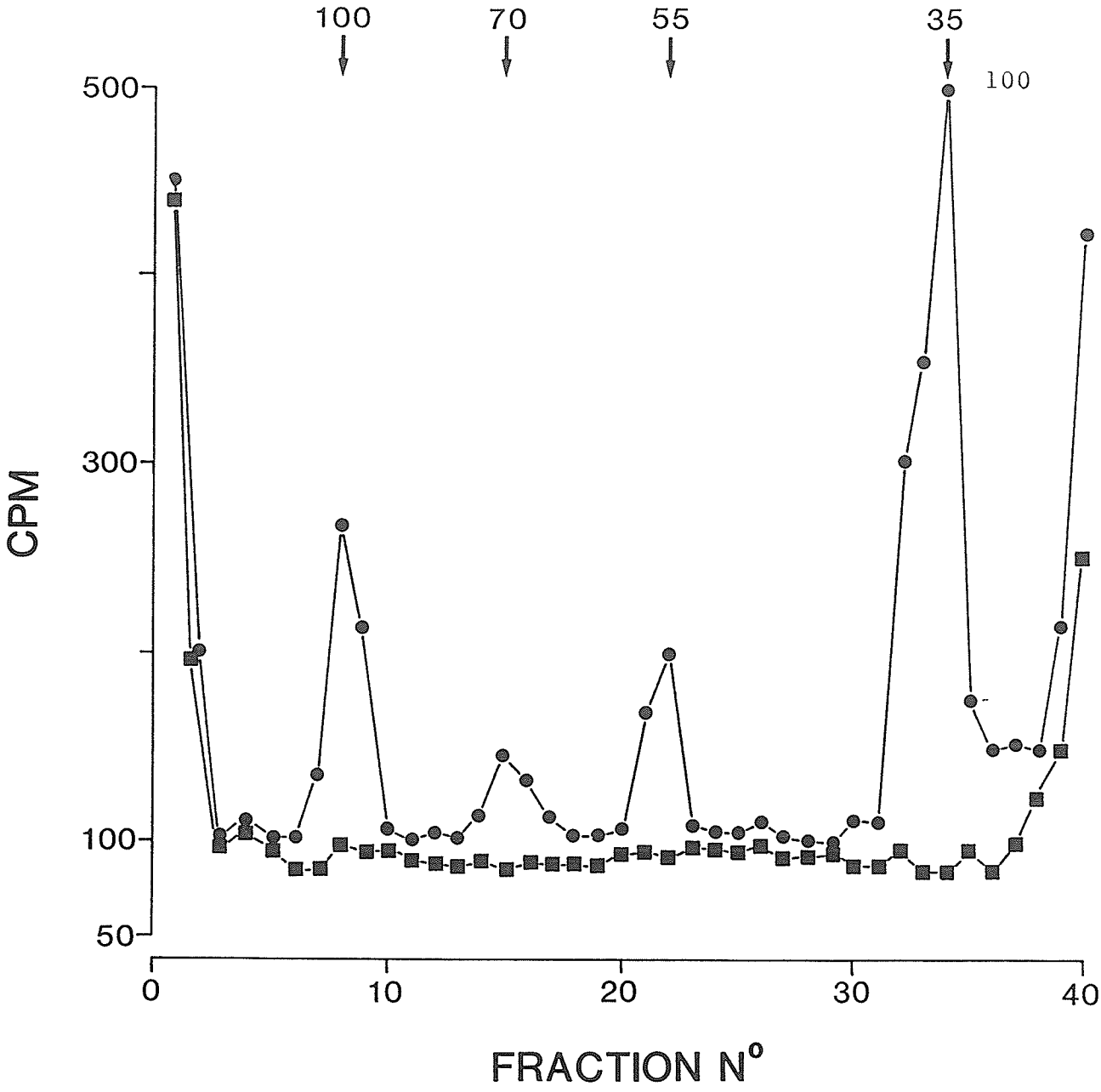


Figure 24. Photoaffinity labelling of cardiac sarcolemmal membrane of 2 month old rat heart with [^{3}H]Nitrendipine and the electrophoretic profile on SDS - 12% polyacrylamide slab gel. Sarcolemmal membrane vesicles were photolabelled in the absence ( ) or presence ( ) of unlabelled Nitrendipine and subjected to SDS-PAGE. Gels were sliced and the radioactivity was determined as described under methodology. Numbers indicate the apparent molecular weights of proteins in kilo-Daltons.

labelling profile analyzed on 12% acrylamide gel. [^{3}H]Nitrendipine was incorporated into four proteins with the molecular weights of 100 KD, 70 KD, 55 KD and 35 KD. The protein with the molecular weight of 35KD had the highest incorporation of [^{3}H]Nitrendipine. Addition of unlabelled Nitrendipine in the incubation medium prevented the incorporation of [^{3}H]Nitrendipine into all four proteins indicating that the incorporation of [^{3}H]Nitrendipine is specific to their receptor subunits. To determine whether there was any [^{3}H]Nitrendipine incorporation to larger molecular weight proteins, labelled membrane was analyzed on 7% acrylamide gel. These studies did not reveal any additional proteins that incorporated [^{3}H]Nitrendipine. Furthermore, none of the above proteins precisely corresponded to the major coomassie blue staining proteins, indicating that either they are glycoproteins that are not stained by coomassie blue or they are relatively minor components of cardiac membrane.

(f) Phosphorylation of cardiac sarcolemma and its age-related changes.

(i) Nitrendipine-induced phosphorylation:

Phosphorylation of cardiac sarcolemmal proteins by an endogenous protein kinase was investigated in the presence and absence of Nitrendipine at 0^{o}C. We repeatedly observed that 10 nM Nitrendipine induces phosphorylation of a 55 KD protein within 30 seconds of incubation with $-^{32}$P[ATP]. In the

absence of Nitrendipine on the other hand, there was no phosphorylation of sarcolemmal protein seen under the same experimental conditions (Fig. 25). To further characterize the nature of Nitrendipine-induced phosphorylation, its time-course was also studied. As illustrated in Fig. 26, the extent of phosphorylation was highest at 30 seconds, which further decreased during 1 and 5 min of incubation. As discussed above, during photoactivation, the [^{3}H]Nitrendipine was incorporated to the 55 KD sarcolemmal protein indicating that it in fact represents a calcium antagonist receptor subunit. When samples of phosphorylated and [^{3}H]Nitrendipine labelled membrane proteins were mixed and subjected to SDS-PAGE, it was further observed that the [^{3}H]labelled protein corresponding to 55 KD subunit co-migrated with the phosphorylated protein. The above phosphoprotein did not correspond to any major coomasie blue staining protein of those that are depicted in Fig. 21.

Age-related changes in nitrendipine-induced phosphorylation of 55 KD sarcolemmal protein:

The Nitrendipine-induced phosphorylation was studied in cardiac sarcolemmal membrane from 2 and 12 month old rats. Incubation with Nitrendipine and phosphorylation of 2 and 12 month old rat heart membranes which were prepared side by side on the same day were carried-out simultaneously. Equal amounts of phosphorylated membrane proteins from both age groups were subjected to SDS-PAGE as duplicates. As illustrated in Fig. 25 the extent of ^{32}P incorporation to

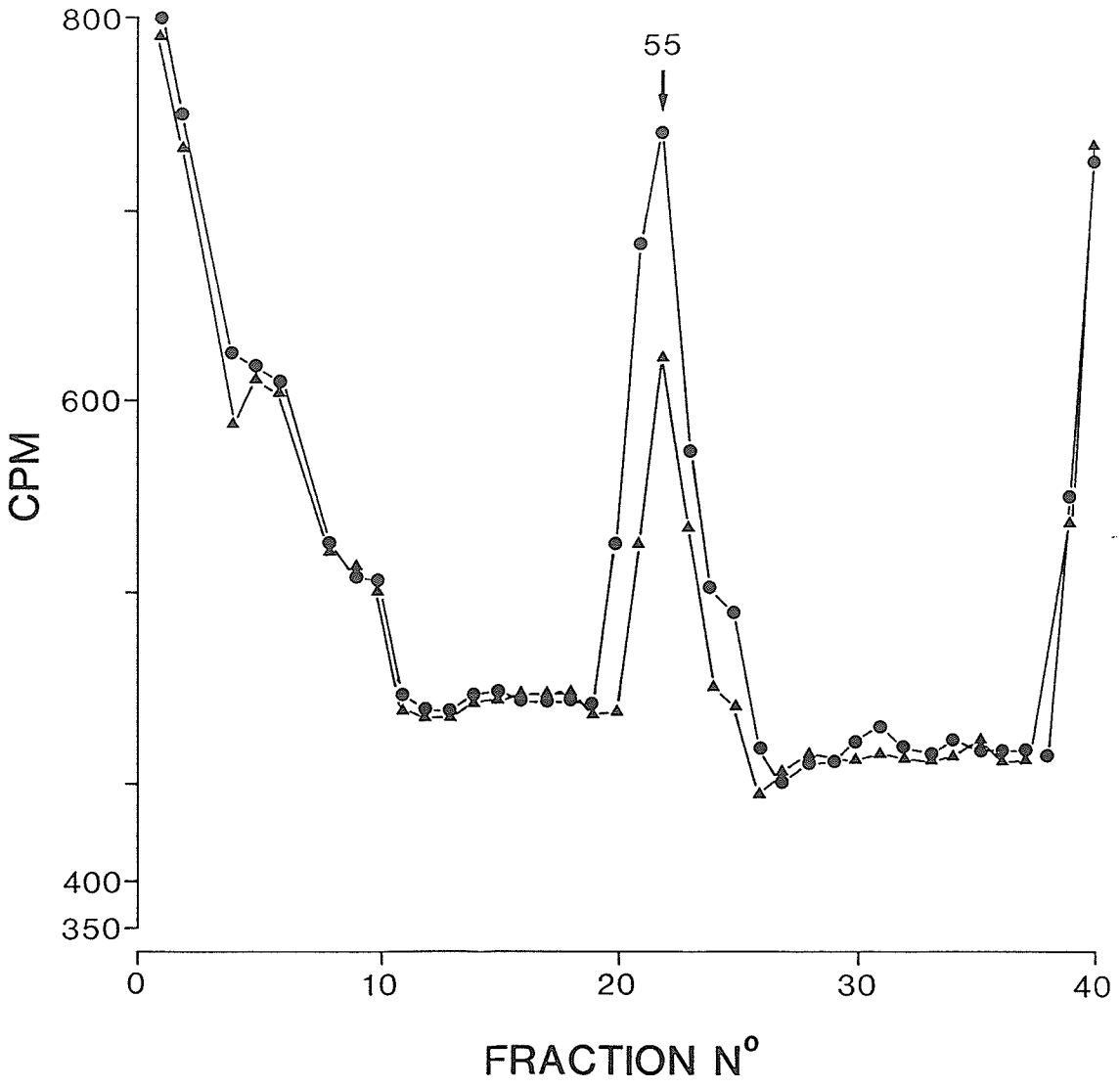


Figure 25. Nitrendipine-induced phosphorylation profile of cardiac sarcolemma of 2(▲—▲) and 12(●—●) month old rats on SDS-12% PAGE. Equal amounts of protein (100-150 ug) from both groups were phosphorylated in the presence of 10 nM Nitendipine and subjected to SDS-PAGE as described under methodology. The apparent molecular weight of the phospho-protein was 55 kilo-Daltons. Four experiments were carried-out using four individual membrane preparations from both age groups.

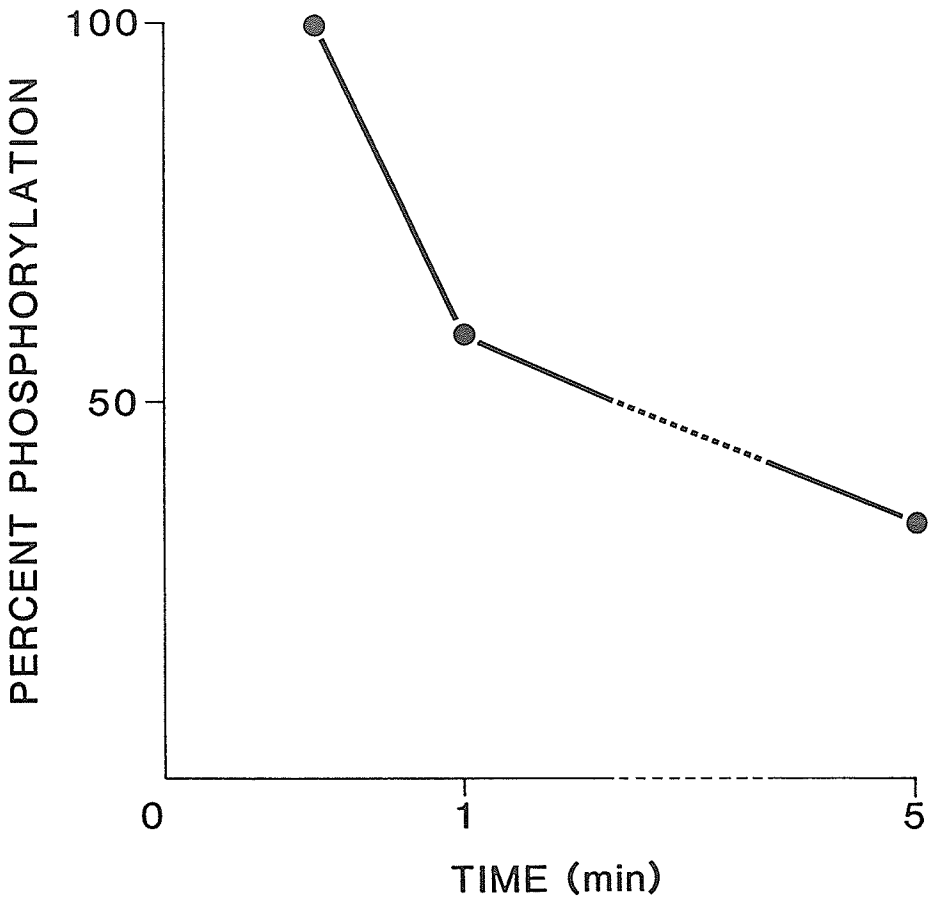


Figure 26. Time-course of Nitrendipine-induced phosphorylation of calcium channel subunit (55KD) in 2 month old rat heart membrane. All points represent the value from a typical experiment. The experiment was repeated with two additional membrane preparations and similar results were obtained.

the 55 KD molecular weight protein was substantially higher in heart membranes from 12 month old than in 2 month old rat. The sarcolemmal phosphorylation was repeated in three independent membrane preparations of both age groups and in all three preparations similar observations were made. When the areas of the peaks of ^{32}P incorporation were calculated, it was apparent that the extent of phosphorylation of 55 KD molecular weight protein in 12 month old was 72 $\pm$ 19% greater than that of 2 month old rat heart membrane.

(ii) cAMP-induced phosphorylation by endogenous and exogenous protein kinases:

Phosphorylation of cardiac sarcolemma membrane by cAMP-dependent endogenous protein kinase was tested in the presence of 5 uM cAMP and 10 mM theophylline. Under the above experimental conditions ^{32}P was incorporated in to five sarcolemmal proteins with the molecular weights of 74, 55, 24, 15 and 9 KD (Fig. 27). The extent of phosphorylation was relatively higher in 74 and 9 KD sarcolemmal proteins whereas 55, 24 and 15 KD proteins incorporated almost equal amount of ^{32}P. When phosphorylation was induced by exogenous protein kinase (by adding the catalytic subunit of cAMP-dependent protein kinase), identical proteins were phosphorylated, but the extent of phosphorylation was substantially greater (Fig. 28). This increased phosphorylation was more marked in 55 and

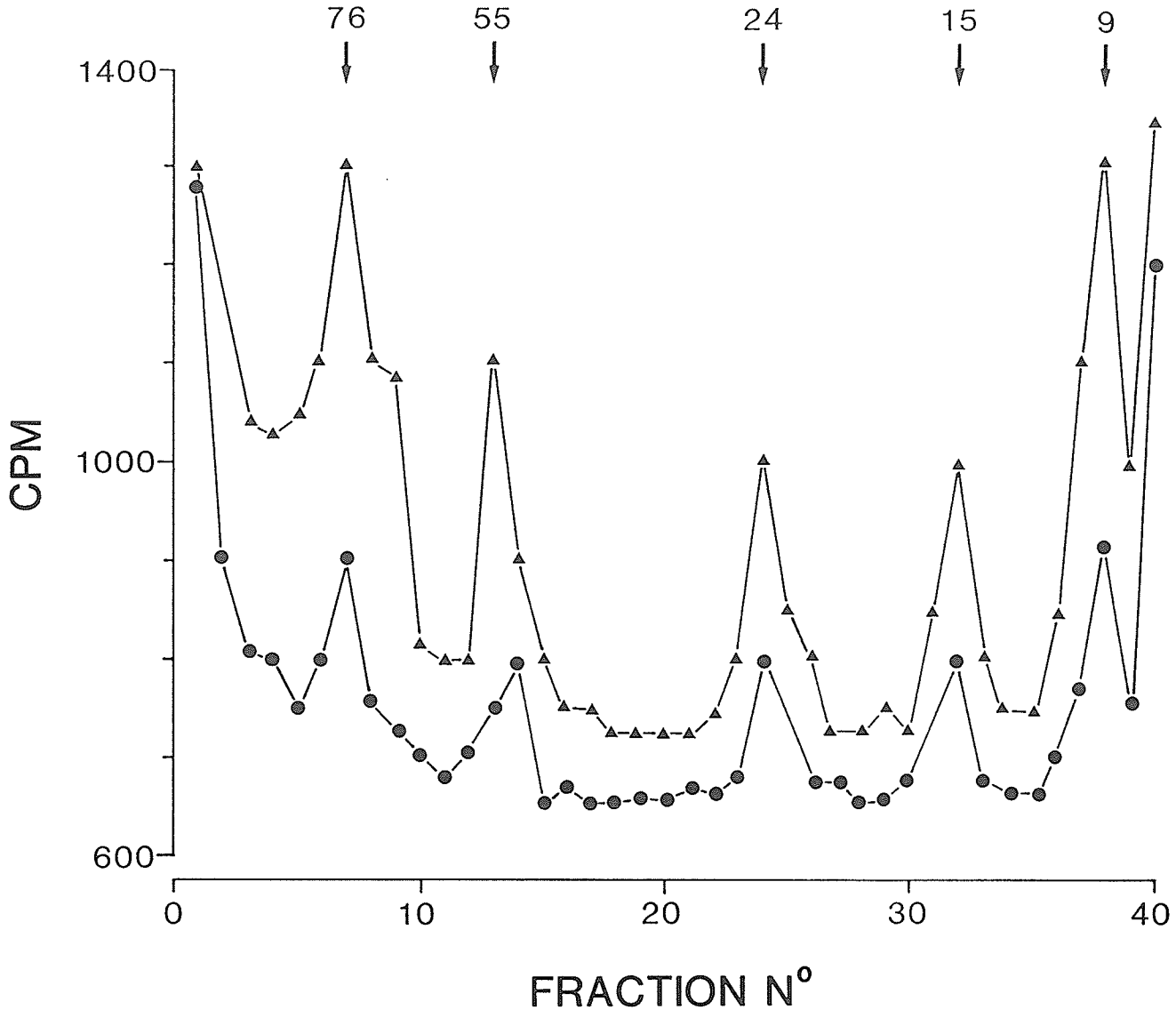


Figure 27. Cyclic-AMP induced phosphorylation profile of cardiac sarcolemma from 2(▲—▲) and 12(●—●) month old rats on SDS-15% PAGE. Equal amounts of sarcolemmal proteins (25 ug) were phosphorylated by cAMP-dependent endogenous protein kinase and subjected to SDS-PAGE. Experiments were repeated with three independent membrane preparations from both age-groups and similar profiles were obtained. Numbers indicate the apparent molecular weights of protein in kilo-Daltons.

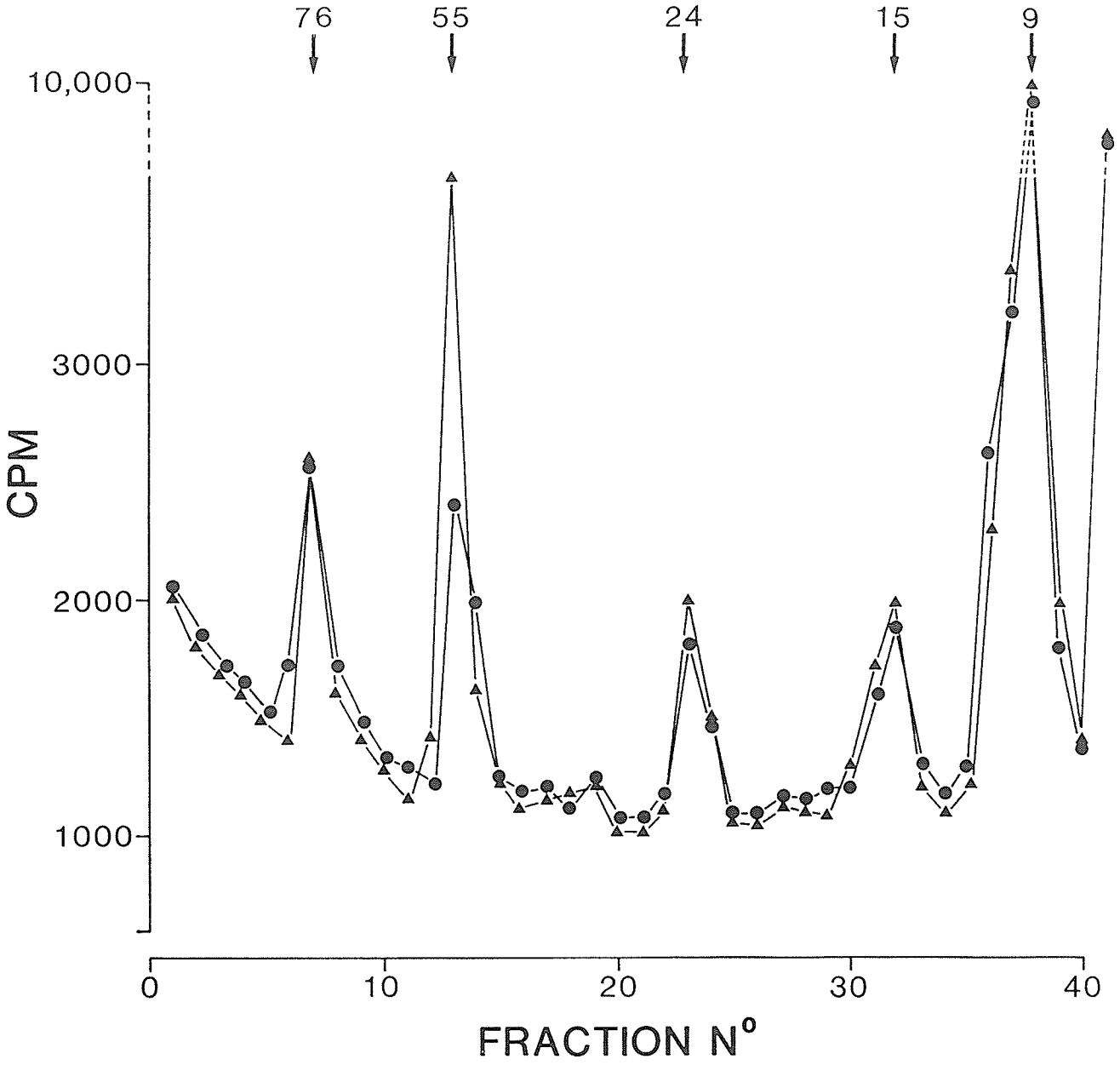


Figure 28. Cyclic AMP-induced phosphorylation profile of cardiac sarcolemma from 2(▲—▲) and 12(●—●) month old rats in the presence of catalytic subunit of cAMP-dependent protein kinase. 25ug of sarcolemmal protein from both age groups were phosphorylated and subjected to SDS-PAGE using 15% acrylamide. Experiments were repeated with three independent membrane preparations from both age groups and similar profiles were obtained. Numbers indicated the apparent molecular weights of proteins in kilo-Daltons.

9 KD molecular weight proteins such that in the presence of exogenous protein kinase almost 8 fold increase in phosphorylation of 9 KD protein and 2.5 fold increase of 55 KD protein were seen. It should be pointed out that in the presence of exogenous protein kinase the baseline radioactivity or non-specific incorporation of ^{32}P to sarcolemmal proteins was also higher than in the absence.

Age-dependent changes in cAMP induced phosphorylation: Figure 27 illustrates the phosphorylation profile by endogenous protein kinase in 2 and 12 month old rat heart sarcolemmal membrane. A generalized reduction in ^{32}P incorporation to the membrane of 12 month old rat heart was repeatedly observed such that the baseline radioactivity is relatively lower in these membranes. Moreover, the extent of phosphorylation of all five proteins by endogenous protein kinase in 12 month old heart membranes was significantly lower as compared to the membrane of 2 month old rat hearts. On the other hand when the phosphorylation was induced by an exogenous protein kinase, the degree of phosphorylation of all five proteins, except 55 KD, in 12 month olds reached the level of phosphorylation obtained in 2 month old rat heart membrane. The amount of ^{32}P incorporation to the 55 KD molecular weight protein was almost 50% lower in 12 month old rat heart even in the presence of exogenous protein kinase (Fig. 28). Experiments were repeated in 2 other

independent membrane preparations of both age-groups and similar results were observed.

V. DISCUSSION

An impairment of diastolic function with no significant changes in systolic performance have been indicated in the aging myocardium (Fleg 1986, Miller et al. 1986). In-vitro studies have demonstrated a prolonged contraction duration, associated with prolonged time to peak tension and half maximal relaxation with no significant alteration in developed tension (Lakatta 1987). These changes may partly be explained on the basis of the defective SR Ca^{2+} uptake and reduced activity of myosin ATPase demonstrated in aging myocardium (Narayanan 1981 & 1987, Capasso et al. 1986). However, in order to maintain systolic performance there should be additional compensatory changes in the Ca^{2+} transport processes.

In the present study the Na^{+}-dependent Ca^{2+} uptake progressively decreased with advancing age in guinea-pigs, which was further characterized by a significant reduction in the rate of $^{45}Ca^{2+}$ uptake (Vmax) and a reduced affinity (1/km) for Ca^{2+}. The passive diffusion of $^{45}Ca^{2+}$ from the vesicles were not determined in different age-groups. However, it is very unlikely that the substantial changes observed in kinetic constants of Na^{+}-dependent $^{45}Ca^{2+}$ uptake are due to the differences in passive diffusion. The initial rates of Na^{+}-dependent Ca^{2+} extrusion also progressively decreases with advancing age. Since the basal $^{45}Ca^{2+}$ content (before the

initiation of extrusion) was different in various age groups, it may be argued that the observed differences in initial rate is a reflection of the lower vesicular $^{45}Ca^{2+}$ content. In spite of the lower $^{45}Ca^{2+}$ content at the end of 3 min. of incubation the vesicles of older animal hearts retained relatively more $^{45}Ca^{2+}$ than the vesicles of 3 month old indicating that the reduction in the initial rates of $^{45}Ca^{2+}$ extrusion is in fact a representation of decreased exchanger activity. In addition to these observations made in guinea-pigs, the Na^{+}-dependent Ca^{2+} transport was also found to decrease during aging of rats from 2 to 12 months, suggesting that the decrease in Na^{+}-Ca^{2+} exchanger activity is not species specific. In the present study the $^{45}Ca^{2+}$ transport activities were studied in isolated sarcolemmal vesicles, the majority (> 70%) of which were right side out vesicles (Pitts 1979). The decrease in Na^{+}-dependent $^{45}Ca^{2+}$ uptake and extrusion should therefore reflect a decline in Na^{+}-dependent Ca^{2+} influx and efflux in intact cardiac myocyte of older animals. Moreover, since the Na^{+}-Ca^{2+} exchanger is a bi-directional and symmetrical exchanger (Reeves 1985), its reduced activity in the aging myocardium would involve both influx and efflux of Ca^{2+} via this mechanism.

The Na^{+}-Ca^{2+} exchanger is an electrogenic exchanger in which transmembrane movements of Ca^{2+} are directly coupled with reciprocal movement of Na^{+} (Reeves 1985). In the myocardium it is thought to be an important transporter by

which Ca^{2+} is extruded to maintain the cytoplasmic Ca^{2+} at micromolar levels (Baker 1986). The present observation of decreased activity of the Na^{+}-Ca^{2+} exchanger would therefore suggest that the rate and the amount of Ca^{2+} extrusion from the myocyte are lower in the older myocardium. Myocardial relaxation is initiated by rapid uptake of Ca^{2+} by the SR. A significant portion is however extruded through sarcolemmal transport mechanisms as well. It has been earlier demonstrated that there is a progressive reduction in SR Ca^{2+} uptake with advancing age (Narayanan 1981 & 1987). Taken together with our present observations it is apparent that the Ca^{2+} transport mechanisms involved in myocardial relaxation are defective in the older myocardium. This would explain the prolonged time to half maximal relaxation and thus the prolonged contraction duration observed in in-vitro studies, and decreased rapid diastolic filling rate observed by echocardiogram in an aging human population (Lakatta 1987, Miller et al. 1986). In spite of slow relaxation of the aging myocardium, neither the resting tension in isolated tissues nor the end diastolic pressure/volume at rest are age-related (Rodeheffer et al. 1984). This would mean that there might be a compensatory increase in Ca^{2+} efflux which occurs through an additional mechanism. In fact, Narayanan (1981 & 1987) has demonstrated that the reduction of SR Ca^{2+} uptake is associated with an enhanced Ca^{2+} pump activity of sarcolemma

with advancing age. However, it appears that the rate of efflux via this latter mechanism is not sufficient to prevent the lower rate of relaxation observed in older myocardium. Recently, evidence has appeared in the literature suggesting that the Na^+-Ca^{2+} exchanger played an important role not only in the efflux of Ca^{2+} but also in Ca^{2+} influx during membrane excitation (Barcensd-Ruiz et al. 1987). If this is true, then the impairment of Na^+-Ca^{2+} exchanger activity observed in the older animal myocardium would mean that there is a reduced contribution of Ca^{2+} from this mechanism to the trans-sarcolemmal Ca^{2+} influx. Since the activity of Na^+-Ca^{2+} exchanger declines during aging, the Ca^{2+} influx via voltage-gated calcium channels should be greater in the aging myocardium in order to maintain the developed tension.

In addition to the physiological role of Na^+-Ca^{2+} exchanger, it is also an intermediate step in the mechanism of action of cardiac glycosides (Kass et al. 1978, Khatter et al. 1990). Cardiac glycosides inhibit the sarcolemmal Na^+ pump whereby the elevation the $[Na^+]i$ results in a reduction of Na^+ linked Ca^{2+} extrusion and elevation of $[Ca^{2+}]i$ to a level of Ca^{2+} overload, which has been demonstrated to be the primary mechanism involved in its cardiac toxicity (Khatter et al. 1986, 1989 & 1990). The decline in Na^+-Ca^{2+} exchanger activity of the older myocardium would therefore make it much more

suceptible to cellular Ca^{2+} overload and toxicity in the presence of cardiac glycosides than of younger animals (Katano et al., 1984, Khatter et al. 1985 & 1990).

In the present study, the investigation of the voltage-gated calcium channel was approached by studying (a) the myocardial response to calcium channel agonist, (b) the receptor sites of voltage-gated calcium channels and (c) the phosphorylation of calcium channel subunits.

Aging of rats from 2 to 12 months caused a substantial enhancement of cardiovascular response to BAY K 8644 such as increases in MAP, LVP and LV +dp/dt. Increase in +dp/dt of LVP has been shown to be a reliable parameter of the inotropic response in normal heart (Wallace et al. 1963), though it may depend to some extent on changes of heart rate, aortic pressure and end-diastolic filling. Since the basic haemodynamic parameters were not altered with aging to 12 months, these were unlikely to be contributory factors to the observed difference in the elevation of +dp/dt. In addition, the dose-dependent effects of BAY K 8644 on heart rate were also similar in the two age groups . The observed greater increase in +dp/dt is thus a reflection of enhanced positive inotropic response to BAY K 8644 in 12 month old rat. Furthermore the maximum developed tension and its rate of development with the perfusion of BAY K 8644 were found

greater in the isolated heart of 12 month old than 2 month old rats.

Myocardial sensitivity of BAY K 8644 was also studied in isolated guinea-pig hearts. The positive inotropic response to identical dose of BAY K 8644 was greater in guinea-pig than in rat myocardium. This may be due to the fact that the guinea-pig myocardium is more dependent on the sarcolemmal calcium transport mechanisms than rats and therefore would be more sensitive to the agents that act through sarcolemmal mechanisms (Langer 1978, Fabiato 1982). In spite of these differences in Ca^{2+} metabolism, it is important to note that an enhancement of inotropic response to BAY K 8644 was demonstrated during adult maturation of both rats and guinea-pigs. The similar myocardial response of 24 month old guinea-pigs to BAY K 8644 indicates that the enhanced inotropic response during adult maturation to 12 months is maintained through senescence to 24 months. In addition to the enhanced inotropic response, the toxic and the pressor response of BAY K 8644 also increased during adult maturation. Higher doses of BAY K 8644 when administered intravenously produced arrhythmias only in 12 month old rats which may be attributed either to its direct myocardial effect and/or secondary to coronary constriction. However, the arrhythmias were not totally abolished by the pre-treatment of coronary vaso-dilator Nitroglycerine. Furthermore, Preuss et al.

(1985) have indicated that with similar doses of BAY K 8644, as used in the present study, there is in fact an increase in coronary blood flow secondary to increased metabolism. It is therefore conceivable that the arrhythmias in 12 month old rats were largely contributed by the enhanced myocardial sensitivity.

The pressor response of BAY K 8644 may be due to a direct action on vascular smooth muscle and/or is secondary to its positive inotropic response (Schramm et al. 1983 & 1985, Gross et al. 1985, Lefer et al. 1986). The enhanced pressor response in 12 month old rats could therefore be attributed to their enhanced inotropic response. The recent observation of greater blood pressure lowering effect of calcium antagonist in the elderly (Buhler et al. 1985) may however suggest the possibility that the vascular sensitivity is also increased with aging. In any event, it is important to point-out that in spite of greater pressor response (or afterload), which could have a negative influence, the positive inotropic response to BAY K 8644 is substantially greater in older rats.

Myocardial response to inotropic agents namely cardiac glycosides and isoproterenol has been studied during adult maturation and aging by many laboratories. The inotropic response to ouabain does not change during adult maturation but declines during aging (Katano et al. 1984). The inotropic and chronotropic responses to isoproterenol on the other hand progressively decline during adult maturation and aging

(Lakatta et al. 1975b, Abrass et al. 1982, Fleish 1981). Furthermore, the inotropic response to increase in extracellular [Ca^{2+}] does not change either during maturation or senescence (Wei et al. 1984). This would indicate that the enhanced inotropic response during adult maturation is specific only to calcium agonist BAY K 8644. Hence, any changes in intracellular Ca^{2+} metabolism such as a greater Ca^{2+} release from SR or greater Ca^{2+} sensitivity of myofibrils are unlikely to be the contributing factors, but rather it may be due to the changes in its receptor characteristics. Since BAY K 8644 binds to the dihydropyridine receptor site of the voltage-gated calcium channel, it is conceivable that a greater density and/or greater affinity of these dihydropyridine receptor sites in older animal myocardium that may contribute to the enhanced sensitivity of the older animal myocardium.

In the present study, the dihydropyridine receptor site of voltage-gated calcium channel was therefore investigated in relatively pure sarcolemmal membrane using dihydropyridine calcium channel ligands [^{3}H]BAY K 8644 and [^{3}H]Nitrendipine. [^{3}H]BAY K 8644 binds to a single class of high affinity binding sites and the binding is calcium dependent, which is a characteristic binding to a dihydropyridine site of the calcium channel (Sarmiento et al. 1987). Although a low

affinity binding site has also been reported in rabbit myocardium, these studies were carried out in a microsomal preparation (Sarmiento et al. 1987) which in addition to sarcolemmal vesicles also consisted of membrane vesicles of intracellular organelles that have been shown to possess low affinity receptor sites for calcium channel ligands (Campbell 1984, William and Jones 1983, Tuana et al. 1987).

The density of [^{3}H]BAY K 8644 receptor sites in purified sarcolemmal membrane increased significantly in mature adult rats of 12 months of age. The affinity of these receptors to bind [^{3}H]BAY K 8644 on the other hand decreased with adult maturation. The increase in receptor density may explain our earlier observation of enhanced myocardial sensitivity to BAY K 8644 during adult maturation. However, the significance of reduced affinity, in the wake of increased receptor density, is not exactly clear to us. We therefore characterized the dihydropyridine receptor site also using an antagonist [^{3}H]Nitrendipine.

Binding of [^{3}H]Nitrendipine to a purified sarcolemmal membrane fraction demonstrated an interaction to a single class of saturable binding sites, which is in agreement with earlier observations (Wagner et al. 1986, Depover et al. 1982, Vaghy et al. 1984). The KD value of 0.27 nM obtained in this study for [^{3}H]Nitrendipine binding to the membrane of 2 month old rat heart is similar to the KD values reported earlier

(Vaghy et al. 1984, Wagner et al. 1986). In general the maximum number of binding sites (Bmax) observed in studies reported earlier (Wagner et al. 1986, Depover et al.), with the exception of the study by Vaghy et al. 1984 in dog cardiac sarcolemma, were lower (0.062-0.187 pmol/mg) than what we observed in the present study. These differences in Bmax values are probably due to the differences in the experimental conditions and/or different degree of membrane purity. The differences in animal species may also be a contributing factor.

Similar to [^{3}H]BAY K 8644, the density of [^{3}H]Nitrendipine binding sites was also found to increase significantly during adult maturation to 12 months and maintained at this level during aging to 24 months. However, unlike [^{3}H]BAY K 8644, the affinity of the receptor site to bind [^{3}H]Nitrendipine was same in mature adult and aging rat heart. This would suggest that observed age-related decrease in affinity for [^{3}H]BAY K 8644 is rather specific to calcium channel agonist. [^{3}H] BAY K 8644 and [^{3}H]Nitrendipine bind to the dihydropyridine site of voltage-gated calcium channels. The binding of [^{3}H]Nitrendipine is competitively inhibited by BAY K 8644 and vice versa (Vaghy et al. 1984), which further suggests that both ligands bind to the same receptor site. As such, one would expect to find an equal density of their binding sites with both the ligands in a given biological

membrane. However, the densities of these binding sites in cardiac sarcolemma determined with [^{3}H]BAY K 8644 and [^{3}H]Nitrendipine were not similar in the present study. The dissimilarity in receptor density was also observed by others (Janis et al. 1984) in rabbit heart microsomes. In any event, it is important to note that in spite of the differences in the binding characteristics of ligands used, the data clearly indicate that the density of dihydropyridine receptor site significantly increases during adult maturation and is maintained through senescence.

The binding of [^{3}H]Nitrendipine was also carried-out in membrane fractions separated during the purification scheme. All of these membrane fractions demonstrated a similar fold increase in binding, indicating that differences in the purity of the final sarcolemmal membrane was not a contributing factor to the greater dihydropyridine receptor density in 12 and 24 month old rat heart. Moreover, it was apparent from the sarcolemmal marker enzyme assays that the extent of purification of the final membrane preparation was in fact similar in all three age-groups. The purification factor of pNPPase was lower than that of Na^+, K^+-ATPase in membranes of all three age groups. It should be pointed out that, exact parallelism is not always observed during enrichment of various sarcolemmal marker enzymes (Kidwai et al. 1971). Our final membrane fraction had minor contamination from

mitochondria, which was equal in all three age groups and had no contamination either from SR or from myofibrils. Furthermore, a differential alteration in the dihydropyridine receptor density (an increase) and Na^{+}-Ca^{2+} exchange activity (a decline) was observed in the same purified sarcolemmal membrane preparation with advancing age. Therefore, it is highly unlikely that either lesser or greater distribution of the sarcolemmal vesicles in the final membrane fraction is a significant contributing factor to the decreased Na^{+}-Ca^{2+} exchange activity and increased dihydropyridine receptor density respectively.

The myocardial voltage-gated calcium channel possesses three receptor sites for calcium channel antagonist namely dihydropyridine, phenylalkalamine and benzothiazipine (Glossman et al. 1985a & b). The properties of these receptor sites in fact reflect voltage-gated calcium channels and are widely used in the biochemical characterization of these channels. Therefore, the present observation of greater density of dihydropyridine receptor sites would also mean that the number of voltage-gated calcium channels increases during adult maturation and is maintained through senescence.

Influx of Ca^{2+} through the myocardial calcium channel is the primary event in excitation and contraction coupling. During the plateau phase of membrane action-potential each voltage-gated calcium channel undergoes brief cycles of

opening and closing. Inactivation of these channels along with the activation of K^+ outward current would initiate membrane repolarization. When the duration of opening of individual channel is prolonged, as for example in the presence of calcium agonist BAY K 8644 (Hess et al. 1984, Nowycky et al. 1985), their inactivation is also delayed. This leads to a prolongation of action-potential duration by BAY K 8644 (January et al. 1989, Hryshko et al. 1989). Similarly, if the calcium channel number is increased, as observed in the aging myocardium, the total duration in which the channels remain activated would be prolonged. This would result in a delayed inactivation of the channels and prolongation of action-potential duration in aging myocardium.

Prolonged action-potential duration as a result of changes in Ca^{2+} channel would also reflect on the myoplasmic Ca^{2+} transient and ensuing mechanical activity. The increase in number of sarcolemmal calcium channels may thus be the primary alteration responsible for the prolonged contraction duration with advancing age (Capasso et al. 1982 & 1983, Lakatta et al. 1975a, Lakatta 1987). It has been demonstrated that the prolonged contraction duration is progressive beyond the age of 12 months (Lakatta 1987). We however observed no further change in the number of voltage-gated calcium channel during aging from 12 to 24 months. Additional alterations that progress beyond the age of 12 months such as defective SR

function or Na^{+}-Ca^{2+} exchange activity may be responsible for these progressive changes in mechanical function (Narayanan 1981 & 1987).

We earlier hypothesized that in order to maintain the developed tension, in the wake of defective SR·Ca^{2+} metabolism there may be a compensatory influx of Ca^{2+} through sarcolemmal Ca^{2+} transport mechanisms with advancing age. We further demonstrated in the present report that the greater influx of Ca^{2+} does not occur through Na^{+}-Ca^{2+} exchanger, in fact it seems to decrease with advancing age. We proposed that the greater Ca^{2+} influx could therefore occur through voltage-gated calcium channel. In support of this contention, we have now demonstrated that there occurs an increase in the density of voltage gated calcium channels with advancing age. This would result in a greater trans-sarcolemmal influx of Ca^{2+} in the older myocardium. The greater influx of Ca^{2+} and greater inward current along with decreased outward current due to declined Na^{+}-Ca^{2+} exchanger activity may also contribute to the greater amplitude of action-potential in the aged myocardium (Lakatta 1987).

Activity of the myocardial voltage-gated calcium channel is not only an integral step in excitation and contraction coupling but the channel is also a primary site through which many of the endogenous and exogenous agents regulate myocardial function. With an increase in number of these

channels one would also anticipate to find an altered response to these agents. In fact, in the present report we have clearly shown that the myocardial response to calcium agonist BAY K 8644 substantially increases during adult maturation. Furthermore, Rosen et al. (1978) have reported that the sensitivity of action-potential parameters of purkinje fibers to calcium antagonist AH 2666 significantly increases during aging of dogs to 5 years and is maintained to 9 years of age. This is in agreement with our observations of altered sensitivity to BAY K 8644 during adult maturation and stabilization thereafter. The enhancement in sensitivity would also point to the fact that the increase in number of Ca^{2+} channel is associated with its greater function. However, electrophysiological studies are necessary to directly demonstrate its enhanced function which are beyond the scope of this present report.

We have so far demonstrated that there is an increase in number of myocardial voltage-gated calcium channels during adult maturation which results in an enhanced myocardial sensitivity of the older animals to calcium channel ligands. Even though the precise mechanism of modification of channel function by calcium channel ligands is not completely defined, it has been indicated that the phosphorylation of calcium channel subunits or related proteins are likely possibilities (Horne et al. 1984). Therefore, it may be anticipated that the increase in number of calcium channels and the enhanced

sensitivity to calcium channel ligands of the older animal myocardium would also be associated with a greater phosphorylation of channel proteins.

Another major group of agents namely catecholamines and related compounds also regulate myocardial function by modifying the Ca^{2+} influx via voltage-gated calcium channels. The myocardial sensitivity to these compounds progressively decreases with advancing age, which cannot be attributed to the changes in beta-adrenergic receptor sites (Narayanan and Derby 1981, Abrass et al. 1982). It has earlier been proposed that the changes distal to the activation of cAMP-dependent protein kinase are the most likely possibilities. This could mean that the changes in cAMP-induced phosphorylation of calcium channel (and other membrane and contractile) proteins may be at fault. In the present study we therefore investigated the calcium channel antagonist and cAMP-induced phosphorylation of cardiac sarcolemma during adult maturation of rat.

In order to translate the significance of sarcolemmal phosphorylation to calcium channel function, calcium channel subunits were first identified by photoaffinity labelling using [^{3}H]Nitrendipine, which has been successfully used earlier by others (Campbell et al. 1984). In cardiac sarcolemmal membrane it has been suggested, although no general agreement exist as in skeletal muscle, that there are

three major groups of calcium channel protein subunits namely a larger molecular weight subunit (170 KD and 140 KD before and after reduction), an intermediate (55-50 KD) and a lower (35-30 KD) molecular weight subunit (Tuana et al. 1987, Catterall et al. 1988). In the present study we observed an intermediate molecular weight 55 KD and a lower molecular weight 35 KD proteins as calcium channel subunits. We did not see any larger molecular weight subunits comparable to either 170 KD or 140 KD. However, we did observe the incorporation of [^{3}H]nitrendipine to 100 KD and 70 KD molecular weight proteins, which may be components of 170 KD complex that are released under the reducing condition used in the present experiment.

The calcium channel subunit with the molecular weight of 55 KD was phosphorylated by an endogenous protein kinase only in the presence of calcium antagonist Nitrendipine. It's time-course revealed that the maximum phosphorylation occurred within 30 seconds, indicating it's physiological significance. Future studies are necessary to further elucidate the characteristics of this protein kinase. It is however, important to point out that an intrinsic protein kinase that is neither cAMP nor calmodulin dependent has also been identified in skeletal muscle triads that was involved in the phosphorylation of calcium channel subunits (Imagawa et al. 1987). Horne et al. (1984) also observed the calcium

antagonist Nitrendipine induced the phosphorylation of cardiac microsomal proteins. However they observed more than one protein subunit with the molecular weights of 42 KD, 45 KD and 52 KD as phosphoproteins. In addition, the phosphorylation was seen even in the absence of Nitrendipine, which was increased by two fold in the presence of Nitrendipine. These observed differences in their study with regards to the number of phosphoproteins and phosphorylation even in the absence of Nitrendipine may be explained by the fact that they used a microsomal membrane preparation, which in addition to sarcolemmal proteins could also have subcellular membrane proteins and protein kinases. In any event, one of the phosphoproteins (52 KD) was comparable to the phosphorylated calcium channel subunit in our study (55 KD). However, the authors failed to obtain any evidence indicating that it was a calcium antagonist receptor rather they indicated that 42 KD molecular weight protein as the possible calcium channel subunit, which is a unique observation made only by these investigators so far.

In general, receptor antagonists such as alpha and beta adrenergic antagonists bind to their appropriate receptors and produce an antagonistic response by displacing the endogenous agonist. As such, they do not cause any modification either in the receptor or in the subsequent cascades of events. The above contention however cannot be applied to calcium channel antagonists because, so far no endogenous calcium channel

ligand has been identified, even if there is one in existence, it cannot interact with all three receptor sites for the major groups of calcium channel ligands. This would mean that both calcium channel agonist and antagonist initiate biological response by inducing changes in their receptor sites. This has been proven to be true by the present demonstration that the phosphorylation of calcium channel subunit is induced by a calcium channel antagonist. In the present study, an antagonist was chosen over an agonist BAY K 8644 to induce phosphorylation because of its higher affinity to bind to the receptor site. When the extent of Nitrendipine-induced phosphorylation of 55 KD calcium channel subunit was compared in 2 and 12 month old rat hearts, there was almost 70% increase in phosphorylation observed in 12 month old rats. It was however not possible to precisely state whether the ^{32}P incorporation per unit of calcium channel subunit was different between the two age groups, unless a sensitive method such as immunolabelling was used to quantitate the calcium channel subunits in the two different age groups. The quantitation by photoaffinity labelling is not precise, since only a fraction of calcium channel subunits were labelled by this method (Campbell et al. 1984).

As opposed to enhanced Nitrendipine-induced phosphorylation, the degree of cAMP-dependent phosphorylation of 55 KD calcium channel degree of subunit was significantly lower in

12 month old rat hearts even in the presence of exogenous protein kinase. This would suggest that, associated with the increase in number of voltage-gated calcium channels there may be an intrinsic alteration of the channel characteristic that results in impaired cAMP-dependent phosphorylation during adult maturation. This may contribute to the decreased beta-adrenergic response in spite of increase in number of calcium channels with aging.

The calcium antagonist and cAMP phosphorylated the same calcium channel subunit (55 KD), despite the fact that both have opposing influence on the Ca^{2+} influx via voltage-gated calcium channel. This would mean that both are likely to phosphorylate different residues, causing a different confirmational change of the protein subunit. Furthermore, the present observation of phosphorylation of only 55 KD protein subunit and the significant modification in the aging myocardium indicates its significant role in the Ca^{2+} channel function and regulation. The calicum antagonist was also incorporated to the other two major protein subunits which were not, however, phosphorylated in the presence of Nitrendipine. It is not clear whether the binding of calcium antagonist to these two subunits was necessary to induce the phosphorylation of 55 KD protein or were they also phosphorylated in the presence of calcium antagonist and the phosphorylation was not being detected in the present

experimental condition. Based on these present experimental findings we propose a model for the function of the myocardial voltage-gated calcium channel and its regulation (Fig. 29). The myocardial voltage-gated calcium channel possesses three protein subunits with molecular weights of 170 KD, 55 KD and 35 KD. The binding of calcium antagonist to these protein subunits induces an endogenous protein kinase, which phosphorylates the 55 KD molecular weight protein. The 55 KD protein is also phosphorylated by cAMP-dependent protein kinase. As such, it plays a central role in the regulation of channel function. The differential alteration in calcium antagonist and cAMP-induced phosphorylation of 55 KD protein is reflected in the differential myocardial sensitivity to calcium antagonist and catecholamines with advancing age which would further signify its important role in channel function.

The endogenous cAMP-dependent protein kinase also phosphorylated other cardiac sarcolemmal proteins with the molecular weights of 9 KD, 15 KD, 24 KD, and 76 KD. The 9 KD molecular weight protein was the major protein phosphorylated and which has been demonstrated to be a component of Calciductin; 24 KD (Lamers and Stinis 1980). Identical proteins were phosphorylated also by exogenous protein kinase but the extent of phosphorylation was substantially greater. The degree of phosphorylation of cardiac sarcolemmal proteins (including that of 55 KD) of 12 month old rat by

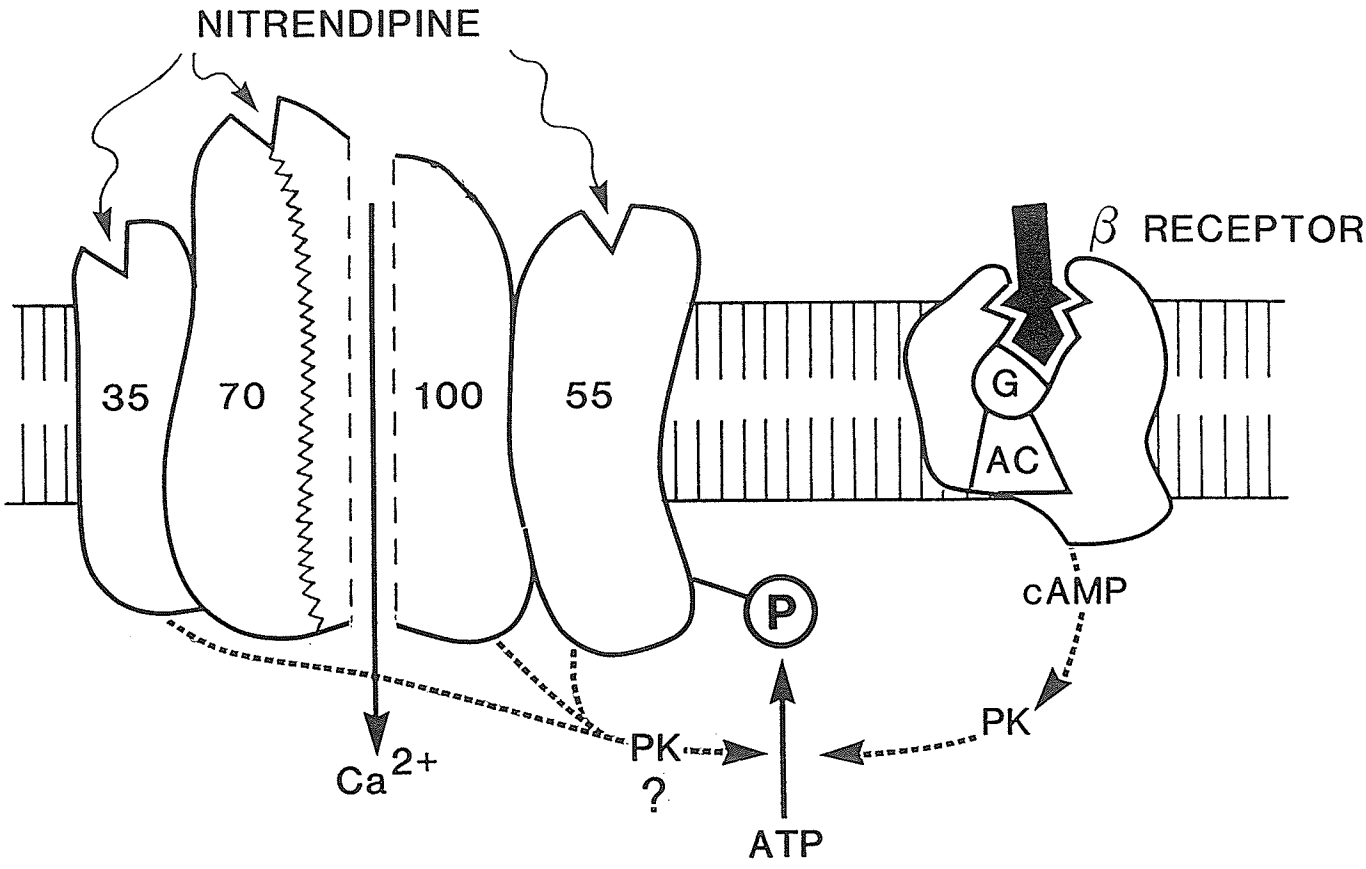


Figure 29. A proposed model of myocardial voltage-gated calcium channel and its regulation. Three calcium channel subunits with molecular weights of 35 KD, 55 KD and 170 KD were identified. The latter appeared as 100 KD and 70 KD subunits in the present experiment. The 55 KD subunit was phosphorylated by an endogenous protein kinase only in the presence of Nitrendipine and also by cAMP-dependent protein kinase.

cAMP-dependent endogenous protein kinase was generally lower than that of 2 month old. This may suggest that the activity of cAMP-dependent protein kinase was lower in the membrane of the 12 month old rat. Alternatively, increased dephosphorylation of phosphoproteins by the enzyme phosphatase may lead to a generalized reduction of ^{32}P incorporation. However, when exogenous protein kinase was added to the incubation medium, with the exception of calcium antagonist subunit 55 KD molecular weight protein, the extent of phosphorylation of all identified proteins were identical to that of 2 month old rat. This would indicate that reduction in phosphorylation is due to the lower activity of cAMP-dependent protein kinase in the 12 month old rat heart.

Recently, Sakai et al. (1990) have demonstrated a reduction in Norepinephrine-induced phosphorylation of myofilament protein of aging myocardium. Unlike our present study, the phosphorylation was tested in cardiac myocyte in which cAMP was produced within the cell. Therefore, they suggested that the observed reduction in phosphorylation of myofilament may be due to a decrease in cAMP production in the aging myocardium which has also been observed earlier by O'Connor et al. (1981). From the present study it is however apparent that not only cAMP production is reduced but also the activity of cAMP-dependent protein kinase and cAMP-induced phosphorylation of Ca^{2+} channel subunits are decreased witn advancing age.

VI. CONCLUSIONS AND SIGNIFICANCE

The activity of the cardiac sarcolemmal Na^{+}-Ca^{2+} exchanger declines with advancing age, which may contribute to impaired relaxation of the aging myocardium . The number of myocardial voltage-gated calcium channels and their sensitivity to calcium channel ligands on the other hand increase during adult maturation and are maintained through senescence. Thus it appears that, in order to compensate for the defective SR function, the contribution of trans-sarcolemmal Ca^{2+} influx to the myocardial contraction progressively increases with advancing age. Moreover, the greater trans-sarcolemmal Ca^{2+} flux occurs via voltage-gated calcium channels whereas the contribution of the Na^{+}-Ca^{2+} exchanger decreases during aging (Fig. 30).

The calcium antagonist Nitrendipine may regulate Ca^{2+} channel function by inducing phosphorylation of the Ca^{2+} channel subunit of 55 KD molecular weight . This subunit is also involved in Ca^{2+} channel regulation by cAMP mediated processes. Differential alterations in its phosphorylation induced by calcium antagonist and cAMP, along with a decrease in cAMP-dependent protein kinase activity may contribute to the differential sensitivity of the aging myocardium to calcium channel ligands and beta-adrenergic stimulation.

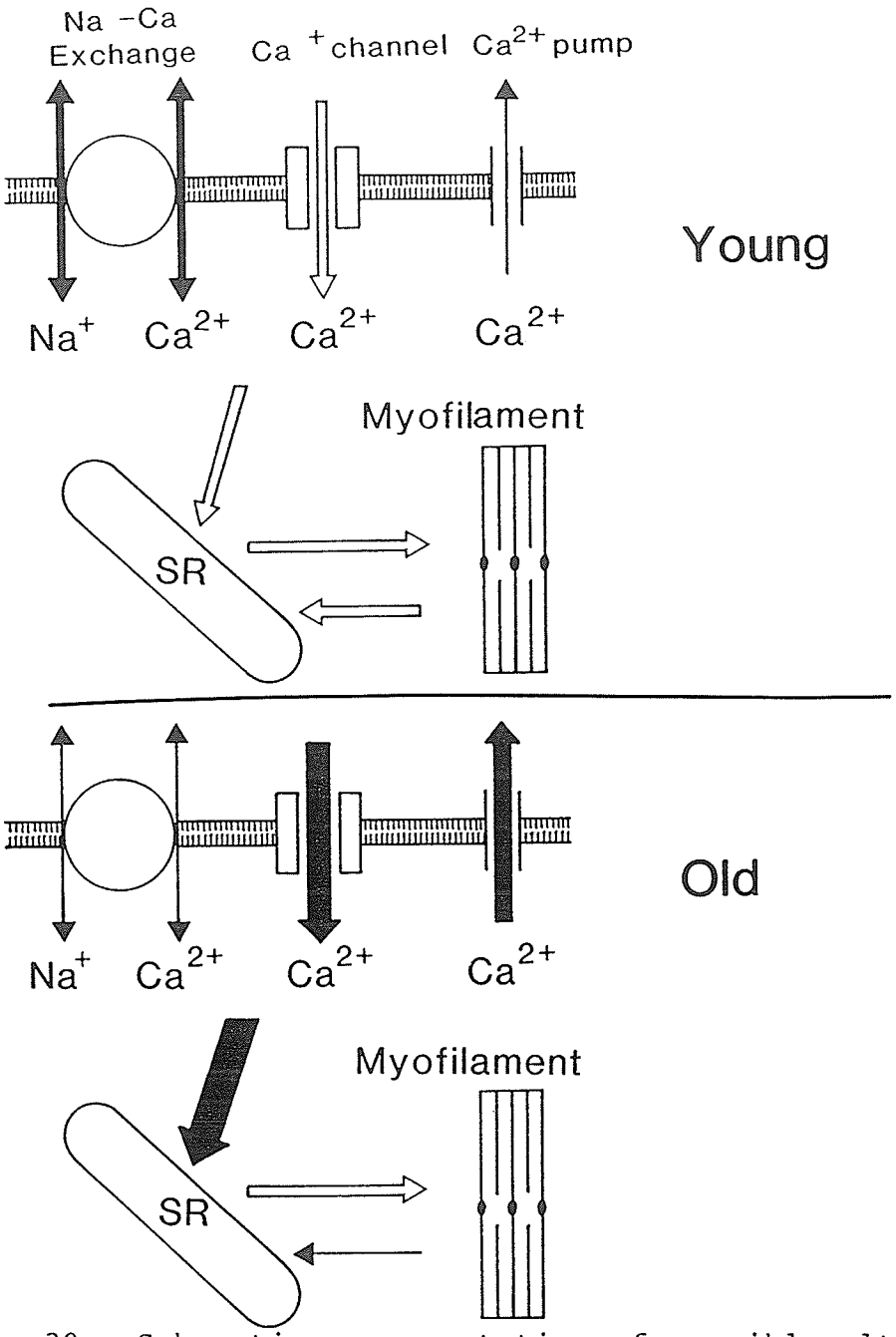


Figure 30. Schematic representation of possible alterations in calcium transport that may lead to the characteristic alterations in myocardial function in aging. There was a reduction in sarcolemmal Na^{+}-Ca^{2+} exchanger activity and Ca^{2+} uptake by the SR with advancing age. The reduced Ca^{2+} uptake would lead to a reduction in the subsequent release of Ca^{2+} from the SR. These changes were compensated by a greater transarcolemmal Ca^{2+} flux due to an increase in number of voltage-gated calcium channels with advancing age.

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