AGE RELATED DIFFERENCES IN THE FUNCTIONAL RESPONSE OF CARDIOMYOCYTES TO SUB-OPTIMAL CONCENTRATION OF MAGNESIUM

A THESIS PRESENTED

BY

PREETHA NAIR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM

JUNE 1999

Dedicated to my parents

CERTIFICATE

I , PREETHA NAIR hereby certify that I had personally carried out the work depicted in the thesis entitled "Age related differences in the functional response of cardiomyocytes to sub-optimal concentration of magnesium" , except where external help was sought and is acknowledged .

Preetha Nair

PREETHA NAIR

Date : 14-6-99

Dr . R . RENUKA NAIR

Division of Cellular and Molecular Cardiology
Sree Chitra Tirunal Institute for
Medical Sciences and Technology
Thiruvananthapuram

DECLARATION

This is to certify that Ms . PREETHA NAIR in the Division of Cellular and Molecular Cardiology of this Institute , has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and technology , Thiruvananthapuram. The work relating to her thesis entitled "AGE RELATED DIFFERENCES IN THE FUNCTIONAL RESPONSE OF CARDIOMYOCYTES TO SUB-OPTIMAL CONCENTRATION OF MAGNESIUM" was carried out under my direct supervision .

R . RENUKA NAIR
(Guide)

The thesis
entitled

AGE RELATED DIFFERENCES IN THE FUNCTIONAL RESPONSE OF CARDIOMYOCYTES TO SUB-OPTIMAL CONCENTRATION OF MAGNESIUM

submitted

BY

PREETHA NAIR
(DIVISION OF CELLULAR AND MOLECULAR CARDIOLOGY)

for
DOCTOR OF PHILOSOPHY

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM

evaluated and approved
by

18/2/2000

Name of the Guide
(Dr. R . Renuka Nair)

18/2/2000

Names of thesis examiners

ACKNOWLEDGEMENT

I take this opportunity to express my sincere gratitude to Dr . R . Renuka Nair for her sustained interest , sound counsel and encouragement during the course of this study .

I express my sincere thanks to Prof . C . C . Kartha , Head of the Division and member of the doctoral advisory committee for his advises . I also acknowledge my appreciation to the other members of doctoral advisory committee; Prof . K . Radhakrishnan , Dr . P . S . Appukkuttan and Dr . Annie John for their valuable suggestions .

I am thankful to Dr . P . S . Sharma for the help extended by him in carrying out the statistical analysis of this work ; and to Dr . K . Rathinam and Dr . Arthur Vijayan Lal for the maintenance and supply of experimental animals .

I take this opportunity to thank Dr . K . Mohandas , Director of SCTIMST , for providing the opportunity and required facilities to carry out this work , and I also wish to thank Dr . K . Shivakumar and Dr . John . T . Eapen for their opinions .

The completion of this work could not have been accomplished without the sincere help and encouragement of my colleagues K . Remani , Preeta . R , Kumar . C and Rajasree . S .

I am thankful to the University Grants Commission , New Delhi for JRF and SRF and also to the Roussel Scientific Institute for the financial assistance .

I wish to express my gratitude to my parents , husband , relatives and friends for their the unfailing support and sincere encouragement .

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYNOPSIS

Introduction :

The adult stage after completion of development, and before aging sets in, is considered to be a more or less stable period in the life-span of an animal. A point worthy of mention is that even during this period, there is physiologic hypertrophy of the myocardium. This could be associated with changes in the frequency and function of the channels and receptors at the level of sarcolemma (SL) and sarcoplasmic reticulum (SR), leading to variation in myocardial function .

The extracellular milieu also has a significant influence on the function of the cardiomyocytes and in turn the cardiac performance. Calcium (Ca) is the major ion controlling cardiac contractility. The importance of magnesium (Mg) as an ion influencing myocardial performance is a recent finding. The incidence of cardiovascular diseases is high in regions with soft water,and tissue Mg-levels are low. As Kerala is a region with soft water, assessment of functional response of cardiomyocytes to suboptimal levels of extracellular Mg^{2+} ($[Mg^{2+}]_o$) gains importance. The type and degree of inotropic response to variation in $[Mg^{2+}]_o$ is envisaged to be age dependent , if the capacity to regulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) during stress varies with age.

This scientific inquiry has been conducted with the following objectives:

1) Set up an experimental model to assess myocardial mechanics
2) Delineate age-related variation in cardiomyocyte mechanics
3) Assess the mechanical response to variation in $[Mg^{2+}]_o$
4) Identify the SL and SR - ion channels that could be influenced by Mg-insufficiency using ion-channel modulators.

Experimental design :

Isolated cardiac myocytes formed the experimental model for recording the cardiomyocyte mechanics. Freshly isolated myocytes from mature adult male rats of three different age groups - 2 -,6 -and 12 - months; belonging to Sprague-Dawley strains were used. Inotropic response was quantified as % change in cell length using a video based edge detection device. The contribution of the channels affecting cardiac inotropy in response to the variables under consideration was assessed using ion-channel modulators. Intracellular diastolic cation levels were measured spectrofluorimetrically using ion - sensitive fluorochromes.

Methodology :

Difficulties in the isolation of viable cardiac myocytes from adult heart has restricted its use as an experimental model. Modifications to existing methods were introduced to optimise the yield. The crucial stages in the isolation procedure were found to be the initial step of calcium depletion for isolation of relaxed myocytes and the final stage of calcium repletion for isolation of calcium tolerant cells. Inclusion of EGTA and taurine in the perfusate for Ca^{2+}-depletion, and of trypsin in the medium during Ca^{2+}-repletion has led to four fold increase in cell yield.

Briefly , the method consisted of retrograde perfusion of the isolated heart with HEPES modified , Ca-free Kreb's Ringer Hensleit (KRH) buffer containing EGTA and taurine to relax the cells , followed by perfusion with Ca-free buffer containing collagenase for the dissolution of the extracellular matrix to dissociate the cells. To the cells so dissociated , Ca^{2+} was introduced step by step to isolate Ca-tolerant cells. The cells were plated in Medium-199 supplemented with 4% fetal bovine serum and incubated for 2 hours in a humidified atmosphere with 95% air and 5% CO_2 at 37°C, before any experimental manipulation.

To elicit contractions the cells were field stimulated at 0.5 Hz using bipolar constant current pulses. The extent of contraction measured as a function of change in cell length was monitored by an edge detection device connected to a video camera and output to a strip chart recorder. The extent of contraction is expressed as percentage change in diastolic cell length . The inotropic response to a variable was characterised as the change from the baseline value .

Statistical analyses : The data are presented as mean ± SEM values for each set. Each experimental observation was based on 10 - 20 cells from atleast 3 separate adult ventricular myocyte preparations . Difference in selected means were evaluated via unpaired Student's t-test or Scheff's test with a significant difference said to exist at $p < 0.05$. The group means were first compared by ANOVA and if a difference was found then the experimental means between two groups were compared .

Results :

Good yield of viable cells could be obtained from animals of all the 3 age groups.

Age related variation in cardiomyocyte mechanics : Advancing age was associated with increase in sarcomere length. Contractile amplitude normalised to cell length was also found to increase with age. As Ca^{2+} is the major ion governing mechanical performance, the cells were exposed to various ion-channel modulators which directly or indirectly affects Ca^{2+} transients . $[Mg^{2+}]_o$ was maintained at 0.8 mM. The negative inotropic response to antagonists of Ca - influx and efflux channels increased with age. Negative inotropic effect with verapamil, the L-type channel antagonist was 20-24% in 2 and 6 month old myocytes and 56% in 12 month old myocytes. The response to T-type channel antagonist $NiCl_2$ and Na-Ca exchange antagonist $MnCl_2$ were not significanly different from the base line in 2 month old

myocytes, while 20-25% reduction with $NiCl_2$ and 50-60% reduction with $MnCl_2$ were observed in 6 and 12 month old cardiomyocytes. Age related variation in SR-functional capacity as assessed with ryanodine (50-60% reduction) and caffeine (20-45% increase) was not significant , but its functional contribution to myocyte contractility was significant ($p<0.05$). Na,K-ATPase activity varied with age, the increase in inotropy being 10% at 2 months, 30% at 12 months and 80% in 6 month old cardiomyocytes, with the inhibitory cardiac glycoside ouabain. Diastolic $[Ca^{2+}]_i$ - level was found to be elevated with ouabain and caffeine in all ages, while a reduction was observed with $MnCl_2$, verapamil and ryanodine .

Inotropic response to variation in $[Mg^{2+}]_o$: The contractile response to variation in $[Mg^{2+}]_o$ was assessed by exposing the myocardial cells to different levels of $[Mg^{2+}]_o$, ranging from 1.8 mM to nominally Mg-free conditions. The mechanical response to variation in $[Mg^{2+}]_o$ was found to follow the same pattern in the 3 age groups. A positive inotropy at 0.48 mM compared to the amplitude at 0.8 mM Mg^{2+} (30-50% - $p<0.05$) and a reduction in inotropy (15-25%) in nominally Mg-free conditions were observed. At 0.48 mM $[Mg^{2+}]_o$, an increase in diastolic Ca^{2+}-level and decrease in $[Na^+]_i$-level was observed suggesting the possibility of Na - dependent Ca - transport , leading to positive inotropy at 0.48 mM $[Mg^{2+}]_o$. Variations observed in contractility at the lower levels of Mg were found to be reversible on changing to Mg-sufficient medium .

In animal experiments conducted in our laboratory, serum Mg-levels of animals on Mg-sufficient and Mg-deficient diet were found to be 0.88 and 0.45 mM respectively.

Effect of extracellular Mg^{2+} on the response to ion-channel modulators : Inotropic response of the myocytes at 0.48 mM concentration of $[Mg^{2+}]_o$ in the presence of ion-channel modulators was assessed and compared with the positive inotropy observed at

0.48 mM $[Mg^{2+}]_o$. Decrease in $[Mg^{2+}]_o$ was found to be associated with relatively enhanced effect of the antagonists. The extent of reduction in the SL-L-type channel activity with verapamil was found to be in the range of 60-65% in the 3 age groups. As in sufficient Mg (0.8 mM) , age dependent alteration was observed with $NiCl_2$, the SL-T-type channel blocker , but the negative response was significantly higher at 0.48 mM $[Mg^{2+}]_o$ (30-60% reduction . $p<0.05$) . The response to Na-Ca exchanger blocker was found to vary with $[Mg^{2+}]_o$ depending upon age ($p<0.05$). Complete cessation of contractility was observed in myocytes from 2 month old rats and ~55% reduction in 6 and 12 month old myocytes. Inhibition of Na,K-ATPase with the cardiac glycoside ouabain induced a negative inotropic response in 2 month old (20% reduction) compared to the contractile amplitude at 0.48 mM ; no significant change in 6 month old animals and positive inotropy in the 12 month old . This suggests that the positive inotropic response expected on treatment with cardiac glycosides may not be effected in the younger age groups , if the extracellular Mg-levels are low. The expected positive inotropic response to caffeine was also not observed , when the $[Mg^{2+}]o$ was low, and the negative effect of ryanodine was higher (65-70%), without significant variation between different ages. Intracellular diastolic levels of Ca was found to be lower in the presence of caffeine , ryanodine and $MnCl_2$ The value remained unaffected with ouabain . Reduction in $[Ca^{2+}]_i$-level with $NiCl_2$ was noticed in 12 month old myocytes. As the diastolic Ca^{2+} levels are not directly related to inotropic variation, Ca^{2+} transients may be responsible for the variable response.

These observations suggest that the function of ion-channels and receptors and their response to channel modulators and inotropic agents can be significantly affected by the level of extracellular Mg, depending on the age of the animal.

Conclusion :

An experimental model has been standardised, which can be used for studying cardiomyocyte mechanics. The experimental data suggest that significant differences exist, in the sensitivity of the cardiomyocytes to alterations in extracellular Mg^{2+} and to inotropic agents, depending on the age of the adult animal. The study has also shown that the negative inotropic effects of Ca-channel antagonists are accentuated and the positive inotropic effects of cardiac glycosides and caffeine are attenuated at suboptimal levels of $[Mg^{2+}]_o$. The extent of response was found to vary with age.

The inotropic effects may be due to Ca^{2+}-transients effected by variation in the distribution and/or function of SL-ion channels.

CHAPTER - I

INTRODUCTION

The contractile function of the heart is regulated by intrinsic factors and extrinsic variables . Age is one of the factors that can influence cardiac function . Ontogenic differences in cardiac contractile performance have been documented from observations on developmental stages such as fetal , newborn and adult . The other stage that has received much attention during the recent times is adult aging. The developmental stages are marked by the morphological and physiological changes leading to maturity and then gradually slowing down to the fully grown adult . The adult phase is considered a more or less stable phase in the life span of an individual . The functional capacity is said to be at its peak at this stage (Srivastava and Snehlata , 1993) . It is infact a period of slow transition from maturation to aging , the latter being the result of progressive accumulation of changes , which beyond a threshold manifests as the complications accompanying senescence .

In the mature adult , age associated variation in cardiac contractility and morphological characteristics have been recorded (Qi and Rouleau , 1997 ; Walsh and Dorn , 1998) . A point worthy of mention is that the heart is continuously enlarging in size during the life span due to hypertrophy (Walsh and Dorn , 1998) . Physiological hypertrophy of the adult myocardium being the result of cellular hypertrophy , can be associated with changes in the localisation and function of the membrane channels and receptors of the cardiac myocyte . Such changes can be accompanied by profound modifications in excitation - contraction coupling . The response to changes in cytosolic composition and extracellular components can also vary .

The maintenance of normal cardiac function is dependent mainly on thecontrol and modulation of Ca^{2+} . The cytosolic Ca^{2+} - levels and Ca^{2+} - transients vary in response to physiological changes and in pathological conditions . Many of

the pharmacological interventions used in the treatment of cardiovascular diseases exert their effects on Ca^{2+} - regulation by modulating the mechanisms controlling the intracellular Ca^{2+} - transients at the level of the sarcolemma and / or sarcoplasmic reticulum . Calcium homeostasis in cardiac myocytes results from the integrated functioning of trans-sarcolemmal Ca^{2+} - influx and efflux pathways, modulated by membrane potential and from intracellular Ca^{2+} - uptake and release caused predominantly by the sarcoplasmic reticulum . Alterations in these processes accompany physiological remodelling . It can also occur in different disease states as well as in pharmacological interventions . Changes in systolic and diastolic $[Ca^{2+}]_i$ cause significant alterations in the contractile function of the cardiac myocyte .

In addition to K^+ and Na^+ that are known to affect Ca^{2+} - homeostasis , an ion that has gained importance in recent times is Mg^{2+} . Magnesium acts as a cofactor in phosphorylation reactions and also as a Ca^{2+} - ion channel blocker , both of which can influence Ca^{2+} - homeostasis and in turn cardiac contractility . High extracellular Mg^{2+} is found to have a protective effect (Jyng and Falck , 1995) and hypomagnesemia has been identified as the cause for the prevalance of cardiovascular disorders in regions with soft water (Durlach *et al* , 1989) . Studies on the effect of lower levels of extracellular Mg^{2+} on cardiac function are comparatively less compared to reports on the protective effects of Mg - supplementation .

Investigation of the effects of Mg - deficiency on cardiac function gains importance because of the presence of soft water in Kerala . Though water Mg - content upto 10 mg / L is common in many places (WHO , 1984) , the Central Ground Water Board , Trivandrum observed that the Mg - content of potable water of Kerala is less than 5 mg / L in most of the regions (Personal communication) . In

a survey conducted in the region , serum and red cell Mg^{2+} was found to be low , significantly in children from the low socio - economic group (Nair *et al* , 1995) . Drinking water being an important source Mg^{2+} , dietary insufficiencies can be compensated in regions with hard water , but can lead to subclinical deficiency in regions with soft water . *In vivo* studies have established that dietary deficiency of Mg leads to hypomagnesemia without change in the Mg - content of the cardiac tissue (Chang and Bloom , 1985 ; Kumar *et al* , 1996) . In addition to dietary deficiency , hypomagnesemia can ensue pharmacological interventions , in pathological conditions , alcoholism , pregnancy and stress (Durlach et al , 1993 ; Seelig , 1994) .

The contents of the extracellular space have a direct participation in cardiac function . Hence , variation in the concentration of Mg^{2+} in the extracellular milieu can have a significant influence on cardiomyocyte contractility . Thirty six percent of the cell circumference at its widest part is within 200 nm of capillary (Frank and Langer , 1974) . This close proximity to capillaries emphasizes the fact that in vascularly perfused ventricular muscle , the cell has rapid access to the vascular contents . Hypomagnesemia can therefore have a direct influence on cardiac function.

This scientific inquiry has been taken up with the view of obtaining a better understanding of the contractile function of the heart , choosing from among the innumerable variables that regulate cardiac function , the influence of the age of mature adults and the effect of variation in extracellular Mg^{2+} on cardiomyocyte mechanics .

It has been hypothesised that : ***Cardiac contractility can be affected in response to decrease in extracellular Mg^{2+}; and, the inotropic response of the myocardium to suboptimal levels of Mg^{2+} can be dependent on age .***

The hypothesis is based on the **assumption** that there is an age dependent variation in cardiac mechanics associated with changes in the function and localisation of the ion - channels that influence Ca^{2+} - transients .

It has also been assumed that all the processes responsible for the exchange of ions with the environment and for excitation - contraction coupling are preserved at the level of individual cardiomyocytes , making the isolated cardiomyocytes a good experimental model for measurement of cardiac mechanics .

Due to practical difficulties in procuring human tissue with the required specifications , the use of animal tissue is imperative . In spite of significant interspecies differences , it seems likely that the age dependent changes involving the regulation of contractile function may exist in all mammals and the basic mechanism modulating cardiac contractility may be conserved during evolution . Rat is a commonly used experimental animal . Size of rat cardiomyocyte being very close to that of man compared to rabbit and guinea pig , the use of rats as the experimental species seems justifiable .

This study has been designed with the following **objectives** :

(i) Set up the adult rat myocardial cell culture as an experimental model for assessment of cardiac mechanics

(ii) Delineate the age related variation in cardiomyocyte mechanics using cells isolated from adult animals of different age groups

(iii) Record the variation in cardiomyocyte mechanics in relation to change in concentration of extracellular Mg

(iv) Assess the influence of $[Mg^{2+}]_o$ on the mechanical response following exposure to sarcolemmal and sarcoplasmic reticular ion channel modulators .

Magnesium being a weak Ca^{2+} - channel blocker , it is presumed that the mechanical response to Ca^{2+} - channel blockers can vary in Mg - insufficiency .

As with all experimental studies , experimental design of this study is also subject to certain **limitations** . The use of ion - channel modulators for identification of the ion channels that vary with age and those that are influenced by Mg - insufficiency , suffers a drawback . Most of the channel specific modulators cross react with other ion - channels . Antagonists with minimum cross - reaction have been chosen for the study . Another fact is that , when one of the channels is blocked , the other channels show a compensatory response . So the mechanical variation observed in response to an antagonist cannot be regarded as the consequence of blocking the channel , but a large fraction would be the contribution of the channel of interest with minor contribution from related channels . This is also true of pharmacological interventions used in the treatment of cardiovascular diseases that exert their effects by modulating the function of the ion - channels . Therefore *in vitro* assessment of the inotropic changes to specific channel modulating agent is useful as it helps to interpret the sensitivity of cardiac contractile function to the inotropic agent in response to intrinsic or extrinsic variables .

In an attempt to correlate the mechanical variation with changes in intracellular free Ca^{2+} , the diastolic levels of Ca^{2+} was assessed with the help of Ca^{2+} - sensitive fluorochromes , the fluorescence intensity being measured by the fluorescence - ratio method , using a dual wavelength excitation spectrofluorometer . Basal Ca^{2+} - level is important because the response to additional $[Ca^{2+}]_i$ is related to

the calmodulin kinase II already activated and is reflected in the mechanical response . A better predictor of the role of intracellular Ca^{2+} would be the Ca^{2+} - transients , where the level of both systolic and diastolic $[Ca^{2+}]_i$ are measured . This has not been feasible due to lack of facilities . As the correlation of mechanical variation with change in intracellular calcium was not part of the objectives of this study , it can be taken up as a future project .

Single cell studies cannot replace whole heart experiments and *in vivo* studies. The advantage of the system is that , they help to distinguish between inherent contractile properties that are intact at every level of organisation and those that are a consequence of the complex geometry of the heart , the connective tissue and the neuro-humoral factors . The chemical messages in the environment are sensed and translated into meaningful physiological action by the cardiac myocytes . In spite of difficulties in defining the absolute level of myocardial contraction , the cardiomyocyte model helps to document the changes in contractility and inotropic changes can be regarded as the response to the variable under investigation , either physiological , pharmacological or pathological . Nevertheless the use of the isolated cardiomyocyte model for assessment of the mechanical function is advantageous , as the inotropic changes in the heart are mediated through mechanisms intrinsic to the cardiac cell .

DEFINITION OF TERMS

Action potential : A change in the electrical potential of an active cell or tissue .

Arrhythmia : Loss of rhythm , denoting especially an irregularity of the heart beat .

Acidosis : A state characterised by relative decrease of alkali in body fluids , in relation to acid content due to accumulation of acid metabolites , so that pH is decreased .

Ca - tolerant myocytes : Isolated cardiac myocytes that remain viable and functional in medium containing calcium .

Cardiomyocyte mechanics : Study of motion and force of cardiomyocyte contraction.

Diastole : Normal post systolic dilatation of the heart cavities during which they fill with the blood .

Depolarisation : The abolition or disappearance of a difference in electrical charge between inside and outside of the cell wall .

Excitation - contraction coupling : The translation of the bioelectrical events at the surface of the cell to mechanical events .

Exchanger : Proteins that promote the movement of one type of ion into or out of the cell in exchange for another ion .

Homeostasis : Physiologic arrangements which serve to restore the normal state , once it has been disturbed .

Ion - channels : Structures on the membrane , which when activated permit the passage of ions into or out of the cell .

Inotropy : Influencing the contractility of muscular tissue . Negative inotropy ie., weakening of muscular action and positive inotropy ie., strengthening of muscular action .

Polarisation : Development of potential differences between inside and outside of the cell wall in living tissues .

Pumps : Proteins that actively transport ions across the membrane against a concentration gradient , driven by hydrolysis of ATP by the membrane bound enzyme ATPase .

Receptor: Molecular structure within the cell or on the cell surface characterised by selective binding of a specific substance and accompanied by specific physiologic changes in the cell .

Resting Membrane potential : Difference in the density of electrical charge at two sides of the sarcolemma in a resting cell , in which inner surface of the cell membrane is negative relative to the fluid outside .

Sarcolemma : The membrane that surrounds a muscle cell .

Sarcoplasmic reticulum : Net work of tubules surrounding the myofibrils . They are responsible for regulation of $[Ca^{2+}]_i$ inside the heart cell .

Sarcomere : The fundamental structural and functional unit of contraction , delineated by Z - bands and made up of thick and thin filaments .

Systole : Contraction of the heart , especially of the ventricles by which the blood is driven through the aorta and pulmonary artery to traverse the systemic and pulmonary circulations .

Voltage gated channels : Ion channels that are gated by alterations in membrane potential .

ABBREVIATIONS

ATP : Adenosine tri phosphate

ATPase : Adenosine tri phosphatase

BSA : Bovine serum albumin

Ca : Calcium

$[Ca^{2+}]_i$: Intracellular calcium ion concentration

$[Ca^{2+}]_o$: Extracellular calcium ion concentration

CICR : Calcium induced calcium release

EGTA : Ethylene glycol - bis (β - amino ethyl ether) N,N,N',N' - tetra acetic acid .

FBS : Fetal bovine serum

HEPES : N-2 - Hydroxy ethyl piperazine - N - 2 ethane sulphonic acid

K : potassium

$[K^+]_i$: intracellular potassium ion concentration

KRB : Kreb's Ringer Buffer

Mg : Magnesium

$[Mg^{2+}]_i$: Intracellular magnesium ion concentration

$[Mg^{2+}]_o$: Extracellular magnesium ion concentration

$MnCl_2$: Manganese chloride

Na : Sodium

$[Na^+]_i$: Intracellular sodium ion concentration

Na- pump : Sodium pump

Na - Ca exchanger : Sodium - calcium exchanger

Na,K - ATPase : Sodium , potassium adenosine tri phosphatase

$NiCl_2$: Nickel chloride

PBFI : Potassium salt of benzofural isothiocynate

SL : Sarcolemma

SR : Sarcoplasmic reticulum

SEM : Standard error of mean

SBFI : Sodium salt of benzofural isothiocynate

T - tubules : Transverse tubules

Tn : Troponin

Tm : Tropomyosin

CHAPTER - II

REVIEW OF RELATED LITERATURE

The history of cardiac and circulatory physiology can be traced back to the second century A.D (Williams and Dry , 1948) . It was Galen (200 AD) who first gathered and catalogued concepts regarding cardiac function and suggested that the heart set the blood in motion. The basis of modern concepts of circulation was laid down by Harvey (1578 – 1657), who described that the pumping action of the heart was responsible for the circulation of blood. Harvey began a series of direct experimental observations of circulatory dynamics both in animal and human cadavers; and his theories were published in 1628 in 'De Motu Cordis'. Harvey's description of the circulation of blood was the single most revolutionary and significant advance in the history of medical science and majority of his principles are still relevant and accepted . By suggesting that " the blood is driven around a circuit with an increasing sort of movement, as an activity or function of the heart which it carries out by virtue of its own pulsation" , Harvey not only was describing circulation , but was also drawing attention to the heart's ability to provide the mechanical force necessary to sustain the circulatory flow. Subsequent discoveries by Richard Lower and Stephen Hales further advanced the discipline of hemodynamics. It was Carl Ludwig , who in the middle of 19th century established ' experimental cardiac physiology ' by the development of kymograph pressure recording device . This primitive technology evolved over the next 50 years into more precise recording systems. Ludwig and his students ; Adolph Fick, Julius Cohnheim , Henry Bowditch and Otto Frank established most of the important principles of contemporary cardiac physiology ; including length – dependancy of myocardial function . Contemporaneously with the work of Ludwig school ; Francois –

Frank and Mary from France , and several other workers from Britain and US made significant contributions to measure changes in ventricular volume indirectly .

It was on this back ground , Ernest Starling began his experiments at University college, London in the early 1900's (Starling , 1918). Working with an isolated mammalian heart - lung preparation, he showed that the heart adapts to increasing arterial pressure by a compensatory rise in filling pressure which then tends to maintain cardiac output. He stated that the law of the heart is same as the law of muscular tissue in general , and that the energy of contraction however measured is a function of the length of the muscular fibre . He had also stated that chemical changes occuring in a muscle as a result of excitation affect surfaces arranged longitudinally in the muscle.

A better understanding of the mechanical properties of the heart was attained by the description of the ultrastructure of the muscle cells and the ' sliding filament hypothesis' of muscle contraction put forward by Huxley & Hanson in 1950's based on the structure - function relationship (Huxley and Hanson , 1954) . Frank had established another important principle that the ventricular muscle could contract isovolumically without any change in the heart volume (Frank , 1958).

The introduction of the patch - clamp technique and the discovery of ion specific fluorescent indicators in the last decade has provided an understanding of the molecular basis of excitation & contraction . The ion - channels play a particularly prominent role in the cardiovascular system . The ion channels in a nodal cell enable it to pace whereas those in a myocyte enable it to contract . In addition to mediating ion fluxes across cell membranes , ion channels are the primary receptors of

a variety of clinically important drugs including antiarrhythmic agents and calcium (Ca) channel antagonists.

The technological advances made recently have shown that the heart cannot be considered as a mechanical pump alone . It is also a sensitive organ responding to the metabolic needs of the body during rest and exercise , health and disease by carefully regulating the rate and force of contraction.

The Heart - Anatomical and Physiological Overview :

The mammalian heart is a compact organ consisting of four main chambers ; the right and left atria, and right and left ventricles . The left and right sides of the heart pump blood to the systemic and pulmonary circuits of the vascular system. Venous blood enters the right atrium through the superior and inferior venacavae , passes into the right ventricle via the tricuspid orifice and is then pumped through the pulmonary arteries to the lungs where gaseous exchange occurs. This oxygenated blood returns to the left atrium via the pulmonary veins , and passes to the left ventricle through the mitral orifice, for distribution through out the body by the aorta and its branches . All these functions are based on the contractile activity of the cardiac wall.

The wall of the heart consists of three layers ; an inner layer – the endocardium , a middle layer – the myocardium and an outer layer – the epicardium. The heart also has a fibrous skeleton which serves as support and firm anchorage for the origin and insertion of the atrial and ventricular musculature as well as the valvular tissue. The endocardium is the inner lining of atrial and ventricular cavities and covers

all the structures that project into the lumen of the chambers. The endocardial lining is continuous with the endothelium of the blood vessels entering and leaving the heart. These endothelial cells rest on a thin subendothelial layer of delicate connective tissue . The underlying myocardium is composed largely of cardiac muscle cells. They comprise more than 70% of the volume of the heart wall , but make up less than 30% of the total cell number. The epicardium covers the heart externally and constitutes a layer of mesothelial cells supported by a thin layer of contractile tissue.

The heart muscle tissue possesses a system of specialised cells, whose function is to co ordinate the heart beat by regulating the contraction of atria and ventricles. Contraction of the atria is followed by contraction of ventricles. The initiating impulse begins in the cells of sino-atrial node, passes through the atrium to the atrio-ventricular node, and is collected by the atrio-ventricular bundle , which branches and passes into the ventricular tissue via the Purkinje fibres and transitional cells. All areas of the specialised conduction system of the heart as well as the cardiac muscle cells are capable of spontaneous discharge (Canale *et al* , 1986).

Thus , the three major properties of cardiac muscle are ;

a) automaticity - the ability to initiate an electrical impulse,

b) conductivity - the ability to conduct or propagate the electrical impulse ,

c) contractility - the ability to do the mechanical function of contraction.

Functionally , the heart constitutes two different systems ; the conduction system and the working myocardium. The latter constitutes the major mass of the

heart. Heart works as a homogeneous unit benefitting from the heterogeneity of its different parts or subunits (Zimmer , 1994).

During its functional performance as a pump, the heart regulates its output according to the changing requirements of the organism . The force and rate of contraction influences the cardiac output. Though the pacemaker cells are responsible for regulating the heart rate and the working myocardial cells for maintaining the force of contraction; modulation of these functional parameters are brought about by changes in the biochemical environment , changes in the number and localisation of carrier systems that mediate the ion – fluxes and changes in the receptor - effector systems .

Cardiac muscle : Cardiac muscle is made up of cross – striated fibres , quasi – cylindrical in shape which bifurcate and connect with adjacent fibres to form a 3-dimensional network. Each fibre is a linear unit composed of several cardiac musclecells joined end- to-end or end-to-side by specialised junctional complexes called intercalated discs (Sommer , 1982). In certain places the apposed membranes are fused across the disc at the so called tight - junctions . It has been suggested that these junctions are regions of low electrical resistance through which current can pass from one cell to another , thus permitting the muscle to contract as a single unit (Kelman, 1977). Each myofibre is composed of a group of myocytes held together by the surrounding collagen weave. Eventhough made up of distinct myofibres , myocardium is functionally a syncytium.

Most of the muscular tissue of the heart is made up of the working myocardial cells with contractile capacity. The rest consists of pacemaker and

conducting tissue which are concerned with the generation and propagation of electrical activity.

The contractile muscle cells (cardiomyocytes) account for more than half of the heart's weight. The myocytes are roughly cylindrical in shape. Those in the atrium are smaller being less than 10μm in diameter and about 20μm in length . Ventricular muscle cells are larger measuring up to 10 – 25 μm in diameter and 50 – 100 μm in length as seen in rat heart (Opie , 1991a). Under the light microscope both atrial and ventricular myocytes show cross – striations and are branched . Except for variation in size , the other features of the muscle cells from different species of mammals are comparable . Harding *et al* (1990) compared the cell lengths and sarcomere lengths of individual cardiomyocytes isolated from hearts of different species. Among them cardiomyocytes from rat heart showed more similarity with human cardiomyocytes. Moody *et al* (1990) have observed that lengths of isolated cardiomyocytes and sarcomere are similar in man and rat.

Cardiomyocyte - (ultrastructure) : Individual cardiac muscle cells are bound by a complex cell membrane the sarcolemma (SL) . Sarcolemma invaginates at intervals to form an extensive tubular network, the T – tubules. It extends the extracellular space into the interior of the cell. Coupled to it interiorly is the sarcoplasmic reticulum (SR), which forms a cylindrical mesh of anastamosing tubules. The nucleus contains almost all of the cell's genetic information . Its ploidy level varies from species to species. Beneath the SL and surrounded by SR – tubules , are the myofibrils . Interspersed between the myofibrils and immediately beneath the SL are many mitochondria.

Innumerable different molecules and assemblies such as the channels and receptors are characteristic of the SL – membrane . The heart cell membrane has the ability to maintain a vast gradient of ions between intracellular and extracellular environments through carefully regulated channels , pumps or exchangers . It also has the ability to respond to the wave of depolarisation by the opening and closing of highly specific ion – channels . The consequence of this ion flux is the critical triggering of the contractile apparatus (McNutt , 1975) .

The other striking feature of cardiomyocytes is the SR which is a completely internal reticulum of membraneous tubules. In muscle it plays a major role in regulating the concentration of calcium in the sarcoplasm and hence the contractility of the cell . The SR forms a 'cylindrical ' mesh of anastamosing tubules intimately surrounding the myofibrils , acting as a link in the coupling of electrical depolarisation of the cell membrane and mechanical contraction of the muscle proteins. Sarcoplasmic reticulum acts as a Ca – storage organelle . The sequential release and accumulation of calcium ions (Ca^{2+}) by SR triggers contraction and relaxation of myofibrils. The myoplasmic Ca^{2+} determines the degree of interaction between the regulatory proteins of the contractile machinery and thereby the force of contraction. Evidence suggests that Ca – binding protein calsequestrin found largely in the SR, plays a major role in sequestering Ca^{2+} during relaxation (Jorgensen *et al* , 1988) .

The nucleus of myocyte is usually cigar - shaped and fusiform and is oriented along the long axis of the cell. Its location is usually central , although it may occasionally be eccentric in position and close to SL . Myocytes with more than one nucleus are a common finding in the adult animals , and their presence appears to be

age related (Bishop & Drummond , 1979) . The nuclear chromatin is usually dispersed with scattered clumping . Under the electron microscope , nucleus is seen to be surrounded by a nuclear envelope consisting of two membranes separated by narrow perinuclear cisterna. The nuclear envelope is interrupted by nuclear pores and is continuous with SR. The cytoplasmic side of the nuclear envelope may be associated with ribosomes . The perinuclear region at each pole often contain prominent golgi complex , glycogen granules , lipid droplets , lysosomes and with advancing age deposits of lipofuscin granules.

Cardiac muscle cells contain more mitochondria compared to other cell types. Mitochondria are the energy producers of the cell. Located beneath the SL , they are wedged in between the myofibrils. Thus the major source of energy supply is close to the main site of energy use. Much of the mitochondrial machinery is involved in the production of adenosine tri phosphate (ATP), which the cells need for survival and proper functioning. Besides generation of ATP , cardiac mitochondria have another important role - the assimilation of Ca^{2+}. This Ca – uptake helps to maintain the level of Ca^{2+} in the cytosol and prevent Ca – overload.

The contractile proteins are regularly arranged in a way which gives the myocardial cell its typical striated appearance under the microscope. The basic contractile unit is the myofibre which is approximately 0.5 μm in diameter and consists of interdigitating filaments of the proteins actin and myosin.

Electron microscopic studies reveal the presence of a series of light and dark bands . The dark anisotropic band (A – band) is formed of thick filaments of myosin and light isotropic band (I – band) is formed of thinner filaments of actin. In the centre

of each light band there is a thick dark line , the Z – line . The functional unit of myofibrils is the sarcomere which is defined as the area between two adjacent Z – lines. The length of the sarcomere determines the degree of overlap between actin and myosin and is inversely related to the state of contraction of the myocyte (Katz , 1992a). The contractile apparatus can be differentiated into the thick and thin filaments.

The thick filament , myosin is a large molecule . Its filamentous tail maintains the structural rigidity of thick filament , and the globular head performs the biological activities . It has two important biological activities ; the ATPase activity (the ability to hydrolyze the terminal PO_4 of ATP thereby releasing the chemical energy of the nucleotide) and the ability to bind actin (the interaction with the thin filaments) .

The thin filament , actin is much smaller than myosin and it is globular . Activation of myosin ATPase and the physicochemical interaction with myosin are the two important properties of actin . These properties allow the two proteins actin and myosin to liberate chemical energy from ATP and undergo physicochemical changes during the process of contraction . Tropomyosin (Tm) and troponin (Tn) –complex are the two regulatory proteins . Tropomyosin is a relatively inflexible elongated molecule that regulates interaction between actin and myosin. Along with troponin , tropomyosin mediates the regulation of muscular contraction. Tropomyosin binds to actin in the thin filament and adds structural rigidity to the thin filament. The Troponin – complex is made up of three discrete proteins. Troponin – I , together with tropomyosin regulates the interaction between actin and myosin . The most important regulatory effect being inhibition of actin – myosin binding. Troponin – T binds the troponin – complex to

tropomyosin . Troponin – C contains the Ca – binding sites that regulate the muscular contraction . The signal for muscular contraction is initiated by an increase in cytosolic Ca^{2+} . The three troponin – components interact in a co operative manner with each other, with tropomyosin and with actin and myosin (Katz , 1983) to regulate the force and rate of contraction and relaxation.

Contractile elements in mammalian cardiac muscle is estimated to make up 50 – 70% of the cell volume and are arranged as a more or less continuous mass (Canale *et al* , 1986) . Mechanical function of the cardiomyocytes , 'contractility' is effected by excitation - contraction (E-C) coupling. The primary factor responsible for the initiation and also variation in contractility is the concentration of activator Ca^{2+} . It was Ringer (1883) who showed the requirement of Ca for normal contractile activity. His work led to the modern concept that Ca^{2+} – fluxes into and out of the myocardial cell and its intracellular regulation explain most aspects of the contractile behaviour of the heart. Cytosolic - Ca^{2+} regulates contractility by indirectly controlling the interaction between myosin heads with actin filaments , which in turn is regulated by Tn – C and Ca^{2+} binding .

Entry of Ca into the myocardial cell is controlled by the electrical state of the cell . Sarcolemmal – depolarisation plays a major role in Ca – influx. Thus electrical excitation triggers the mechanical function of contractility.

Electrical excitation : Under physiological conditions , the interior of the myocardial cell is electrically negative with respect to the surrounding extracellular fluid. The transmembrane electrical potential difference depends on the differences in ionic

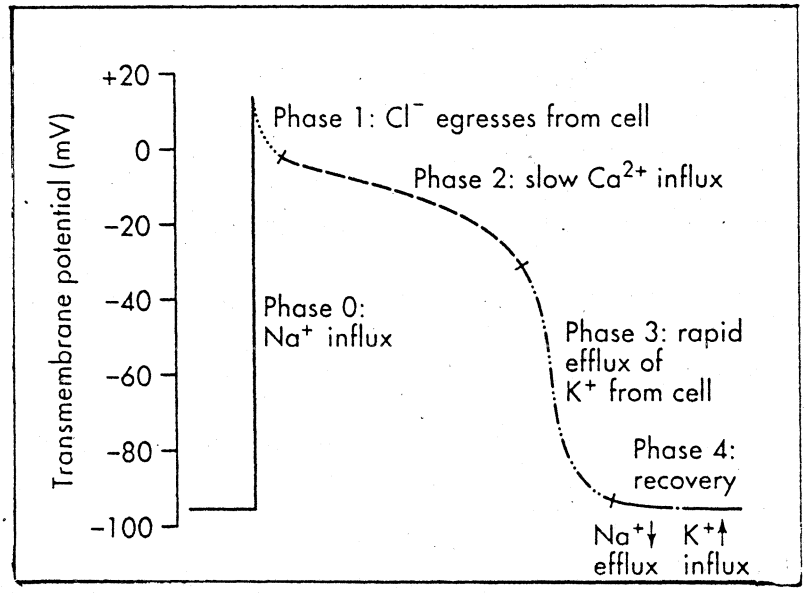
composition between the intracellular and extracellular fluids and the differential permeability of the cell membrane to various ions.

The processes that signal the myocardium to contract begin when an action – potential depolarises the SL. The electrical impulse for this is generated in the sino – atrial node, conducted through the atria, atrioventricular node , His – bundle and its branches and finally reaches the ventricles. As the wave of electrical excitation arrives , it initiates depolarisation and the flow of current opens the voltage activated gates of the sodium (Na) – channel to allow the rapid entry of Na^+ - ions which constitute the rapid upstroke or 'phase – 0' . As the Na - current fades away , and the cell loses its polarity, the Ca – channel which is activated by a less negative voltage , opens . From the peak of depolarisation the overshoot is lost (phase – 1). Inflow of Ca^{2+} -ions and outflow of K^+ -ions forms the the 'phase – 2' , the action potential plateau. By this time the K – current starts to flow again into the cell causing repolarisation (phase – 3). This rectifying current helps to terminate the action – potential plateau. Finally the resting potential (phase – 4) is regained . (Fig: 1) The shape of the action potential is determined by the current flowing across the SL

The SL calcium channels which respond to the changes in voltage . The voltage activated mechanisms brings the first inward Ca – current . The second inward Ca - current is slower and lasts longer . The small amount of Ca that enters the myocardial cell , triggers the release of much larger amount of Ca – ions from SR – stores. This initiates contraction of the cell (Barry & Bridge ,1993) . Release of Ca^{2+} - ions from SR induces contraction and uptake of Ca^{2+} -ions by the SR causes relaxation

FIGURE - 2.1

INTRACELLULAR ACTION POTENTIAL



Ruegg ,1990) . The Ca – flux links the wave of electrical excitation to contraction by the process of E – C coupling .

Myocardial contraction : The essential components of the heart's contractile machinery are those proteins concerned with contraction (actin and myosin) and those which are regulatory (Tn and Tm) .

A comprehensive dynamic model of E – C coupling for a single cardiac cell can be extended to the myocardium as a whole . This model is used to simulate the basic mechanical characteristics of the cardiac muscle and the inotropic and lusitropic changes associated with variation in extracellular environment .

The physical nature of actin – myosin interaction was proposed as early as 1954 by Huxley and Hanson , popularly known as the sliding – filament hypothesis of muscular contraction . According to this hypothesis, actin and myosin filaments are linked by chemical bonds and activation of the contractile proteins produces forces which cause the thick and thin filaments to slide past one another , thus decreasing the inter – Z – distance and causes the shortening of the muscle. The attached heads of the thick filaments pull the thin filaments a very small distance towards the centre of the sarcomere , before detaching and reattaching to repeat the cross – bridge cycle.Cross - bridge cycle is better explained by the Eisenberg – Greene model (1980). It proposes that the myosin heads go through an oar-like motion from the 90° configuration to a 45° angle . The simplest model to explain these changes was proposed by Eisenberg and Hill in 1985. According to their proposal the myosin head alternates between two major configurations. When ATP is bound to the head , there is

a weak binding conformation and when inorganic phosphate is released there is a strong binding conformation. The model depends on different types of binding between actin and myosin in response to the molecular configurations of the myosin head and the ATP hydrolysis - resynthesis cycle.

The stages of the contractility involves two important cross – bridge states , namely actin – attached and actin – detached states. It is the attached state that is force generating. The rate at which cross – bridges attach and detach in the cyclic manner may be quite different under various conditions.

Brenner in 1988 proposed that the cross bridge cycling rate is dependent on two rate constants , the ' f - constant ' controlling the rate at which cross bridges enter the attached state and the ' g - constant ' controlling the rate of cross bridge detachment . According to Brenner it is predominantly the cross bridge attachment constant that is affected by Ca^{2+} . Thus any increase in free Ca^{2+} - ion concentration in the myoplasm would enhance the Ca^{2+} - occupancy of the Tn-C , which in turn would increase the probability of cross bridge attachment . The first effect of Ca^{2+} - ions would be to increase the probability of cross bridge attachment . This effect of Ca^{2+} is because , the rate constant of force (or pressure) increase is maintained proportionally to the sum of the rate constants (f + g) (Brenner , 1988) .

The first effect of Ca – ions is to increase steady – state force development. Additionally , indices of contractility, such as the rate of pressure change in the intact heart (dp/dt) also would be altered by Ca^{2+} (Ruegg , 1990).

Landsberg and Sideman (1994) has presented a dynamic model describing the mechanical regulation of cardiac muscle by coupling Ca – kinetics with cross – bridge

cycling. Their study described the regulation of mechanical activity in the intact cardiac muscle , the effects of free calcium – transients and the mechanical constraints ; and emphasized the central role of Tn – complex in regulating muscle activity. Their model includes two feed back mechanisms : a positive feed back or co operativity , in which the cycling cross – bridges affect the affinity of Tn for Ca and a negative mechanical feed back , where the filament – sliding velocity affects cross – bridge cycling .

Summarizing the process of excitation - contraction coupling (Figure - 2.2) ; it comprises ,

!) Arrival of the wave of depolarisation to ventricular myocardial cells ,

2) Entry of Na^+ through the fast inward Na^+ – channels into the cell reducing the negative potential ,

3) Entry of Ca^{2+} through the voltage gated Ca^{2+} – channels into the cell ,

4) Ca^{2+} - triggered Ca^{2+} - release from SR through the SR - Ca^{2+} - release channel ,

5) Activation of contractile proteins , following Ca^{2+} - binding to Tn – C ,

6) Removal of Ca^{2+} , predominantly by SR – Ca – ATPase and to a lesser extent by SL Na – Ca exchanger and SL Ca – ATPase ,

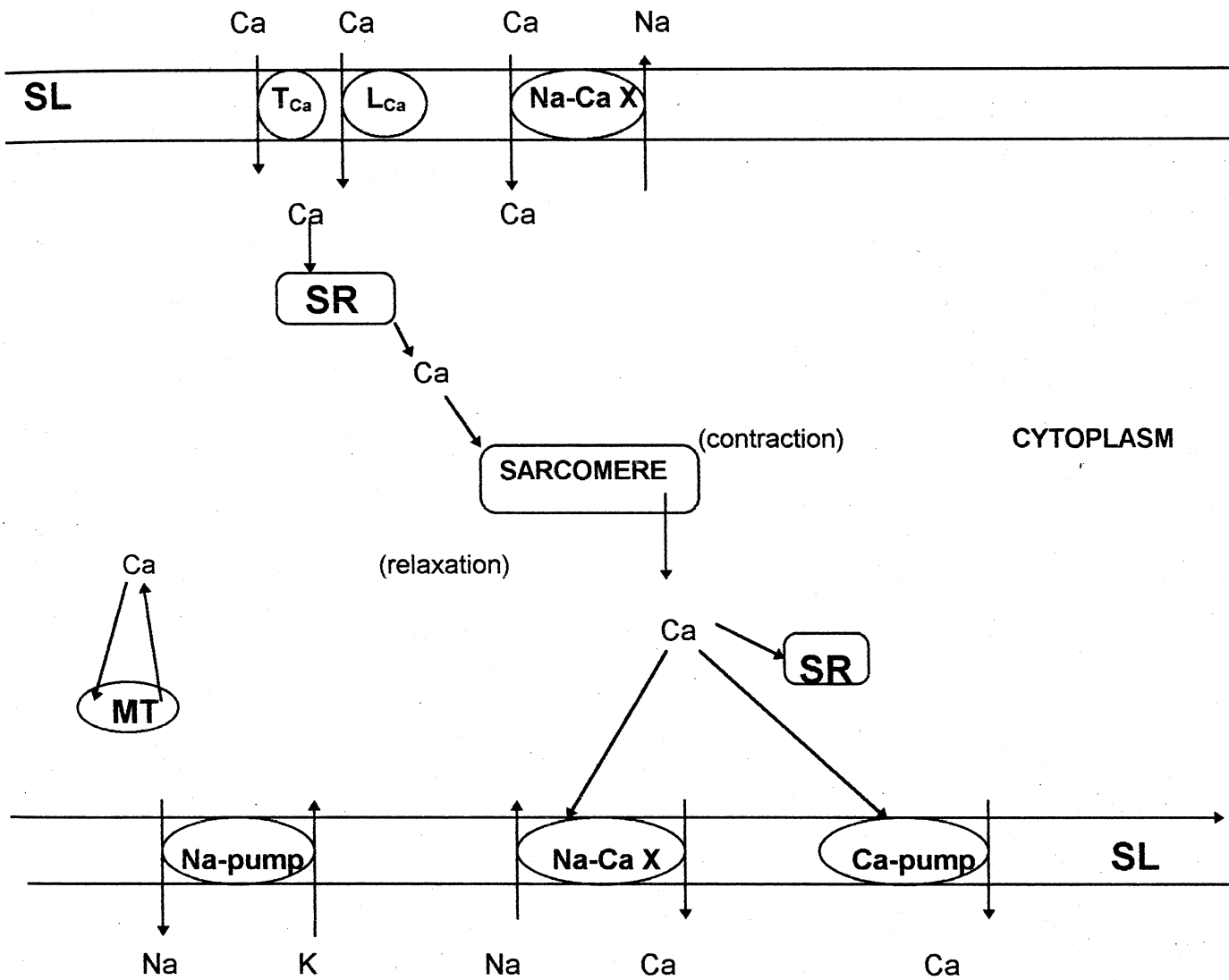
7) Repolarisation and attainment of resting state .

Thus Ca^{2+} is found to be an essential factor for myocardial contractile function. In most instances myocardial contractility is regulated by changing Ca –fluxes across the SL and it is modulated by either alteration in the number of Ca – channels or the extent of channel opening in response to SL – depolarisation. The extent of channel opening is influenced by in trinsic and extrinsic factors .

Besides physiological regulators of contractility , pathological factors can also influence myocardial mechanics .

FIGURE - 2.2

SCHEME OF CALCIUM - CYCLE IN A CARDIOMYOCYTE



SL - Sarcolemma

SR - Sarcoplasmic reticulum

Ca - Calcium ion

MT - Mitochondria

Na-Ca X - Na - Ca - exchanger

Na - Sodium ion

L_{Ca} - L - type Ca - channel

T_{Ca} - T - type Ca - channel

K - Potassium ion

Na-pump - Na, K - ATPase

Ca-pump - Ca - ATPase

Regulators of myocardial contractility :

Myocardial contractile function is regulated by physiological factors that influence membrane – ion – transport , adrenergic and cholinergic systems , neuro transmitters , etc. Besides these , pathological factors , pharmacological agents , toxic substances from external environment , etc can also modulate myocardial contractility.

Membrane transport systems : Contractile function of the cardiomyocyte is known to be regulated by both intra and extra cellular ionic concentration. The inflow and outflow of ions are mediated by some type of carrier such as channels , exchangers or pumps located on the SL - membrane.

The past decade has witnessed tremendous advancement in the knowledge of the structure and function of membrane channels in cardiac cells . Technical advances including studies on isolated cardiomyocytes , voltage – clamp recordings and molecular biological techniques have contributed tremendously to this progress. Characterisation of the nature of cardiac ion – fluxes has allowed a detailed examination of the origin of the cardiac action – potential and the relation between cardiac membrane potential and contractile function. The movement of ions is facilitated by carriers or proteins in the membrane , mainly the channels , pumps and exchangers.

Channels : Channels may be held closed in the absence of an effector molecule or at a particular membrane potential, and in such a state there is no ion – transport . A gated channel can be held open or closed by a chemical or at a specific voltage. The

latter are called the voltage - gated channels , which are functional at a specific voltage .

Ion – channels are classified broadly by the principal ion they carry – Na^+ , K^+ , Ca^{2+} , Mg^{2+} or Cl^- and the mechanism by which they are opened or closed . Changes in the membrane voltage or concentrations of intracellular ions and molecules such as ATP regulate the opening and closing of ion – channels (Laniado *et al* , 1990).

Guarding each channel there are two or more gates that control its opening. Ions can pass through the channel only when both the gates are opened . Movement of ions across the voltage gated - channels depend upon the voltage and time after the onset of depolarisation . So they are considered to be voltage gated and time dependent. In fact only a small percentage of the potentially active channels are functional at any one time . It is called the 'probability of channel opening'. The reason for this is that channels are not simply opened or closed. Open state is the last of a sequence of many molecular states , varying from fully closed to fully opened configurations. The voltage signal is passed along each of these configurations and the channel is functional only when it reaches the fully opened state. Besides, there are selectivity filters which determine the type of the ion to pass through any particular channel (Colquhoun and Hawkes , 1982) . In addition to voltage – gated channels there are ligand operated channels. Ligands bind to cell – membrane at receptor sites and they convey the signal to the channel gate via G – proteins or by some other yet unidentified signal (ligand gated ·G-protein dependent or ligand gated G-independent channels) . The third category responds to mechanical stretch (mechanoreceptors).

The resting potential of ~ -90 mV in the myocardium is largely the result of unequal distribution of K – ions across the SL . As the wave of depolarisation reaches the ventricular myocardial cell , the potential falls . When it reaches -70 to -60 mV , the threshold of activation of Na – channels , there is rapid inward Na – current. At the resting membrane potential in the case of Na and Ca – channels , the activation gate is shut and the inactivation gate is open. With the fall of the resting membrane potential during depolarisation of the myocardium , first the activation gate of the Na – channel opens , followed by that of Ca – channel ; in response to specific voltages . At this stage both activation and inactivation gates are open and the inward flow of Na and Ca - ions through their respective channels produces Na and Ca inward currents. As depolarisation continues and higher voltage is reached , the inactivation gate is closed so that the currents are switched off in the order Na first and then Ca.

Besides the time and voltage factors , another inactivation signal is an increase in intracellular level of calcium, which is essential to avoid harmful effects of Ca – overload (Lee *et al* , 1985).

There are two major sub populations of Ca – channels relevant to cardiovascular system ; L – channels and T – channels . 'L' or long lasting channels open at a less negative voltage, – 35 to -25 mV and accounts for the later phase of Ca – influx , while 'T' or transient channels opens at a more negative voltage -60 to -50 mV . They have short bursts of activity and account for the early phase of Ca – influx (Nilius *et al* , 1985)

It is generally accepted that under normal physiological conditions two transmembrane inward currents ; the fast influx of Na and the subsequent slow

inward Ca - flux operate during excitation of the mammalian myocardial cell. There is also the evidence of a preferentially Mg – carrying transport system besides these two in the excited cell membrane. This was inferred by Spah and Fleckenstein (1979) by increasing the extracellular K^+ concentration to depolarising values , so that both Na and Ca channels remain in the resting state and the cell does not contract. But the myocardial cell still exhibited contractility and the action – potential was propagated . It appeared to be exclusively carried out by a slow channel. This slow channel was found to be responsible for the non specific transfer of Mg – ions. Under normal conditions when the resting membrane potential is high and extracellular Na^+ , K^+ , Ca^{2+} and Mg^{2+} concentrations are within the physiological range , the contribution of this 'Mg - channel' to the electrogenesis of action – potential is negligible and its role may be in metabolic processes.

The routes of Mg – entry and exit remains controversial . Romani *et al* (1993) proposed that there is a slow inward Mg – leak which gets balanced by an equivalent efflux to maintain Mg – homeostasis . Handy *et al* (1996) later suggested that Mg – uptake occurs in exchange for intracellular Na^+ by reversal of Na – Mg antiporter , which is regarded as a mechanism for Mg – extrusion. Mg leaves the Mg - loaded cell primarily through this Na – dependent mechanism. In addition to this , physiologic conditions such as hormonal stimulation was also found to result in Mg – extrusion (Romani *et al* , 1993) . β - adrenergic stimulation has also been reported to produce Mg – efflux , which is mediated through a rise in the cytosolic cAMP – level (Romani *et al* , 1993) .

The resting membrane potential is regained by the potassium – currents. K – channels are both voltage gated and ligand operated. The major voltage - gated K – currents are the back ground outward K – current and delayed rectifier K – current. The first one flows more strongly during the resting diastolic phase of cardiac cycle , contributing more to resting membrane potential. While the second K - current has rectification properties, it is also outward in direction but induced after the onset of depolarisation at a specific voltage. Early transient K – current contributing to very early repolarization is considered as the third voltage – gated K – current (Heidbuchel *et al* , 1990).

There are other K – currents that flow in response to definite molecular stimuli for example , when certain effector molecules bind to receptors called ligands. They are of two types ; those that need G – proteins and others that are non – G – gated . An example of ligand operated G – dependent K – channel is that operated by acetylcholine (Ach) the parasympathetic messenger. It plays a major role in pacemaker cells and is absent in ventricular cells. Another example is the adenosine sensitive channel which is similar to Ach – sensitive channels and requires ATP breakdown product , adenosine. In these K – channels , the G – protein regulates the gating mechanism directly. Another K – current , which is ATP – sensitive responds to its ligand ATP and is G – independent. The increase in ATP levels lead to closing of this channel (Ho *et al*,1993).

Exchangers : To keep the system of ions under homeostatic control , there are also regulators called exchangers, which are under feed - back control .

Once the wave of depolarisation has left , there will be a gain of Na^+ and Ca^{2+} - ions and a loss of K^+ - ions . With time , these ionic imbalances will gradually build up. The cytosolic Ca – level should be guarded within strict limits to avoid Ca – overload. The chief mode of exit of Ca – ions from cardiac cell is by Na – Ca exchanger, which bring in Na – ions as Ca – ions leave. Usually three Na^+ - ions are exchanged for one Ca^{2+} -ion . Its activity depends on ion – concentration as well as membrane potential. At rest , when the cell is normally polarised , the Na^+ - ions flow inward along their electrochemical gradient and the Ca^{2+} - ions flow outward against the electrochemical gradient. The exchanger responds to relatively small increases in the internal Ca – concentration (Leblanc and Hume , 1990).

The exchanger is electrogenic and is sensitive to the prevailing membrane potential. During depolarization , membrane potential is such that the Na – Ca exchanger operates to allow inward flow of Ca^{2+} - ions , contributing to the action – potential plateau and increasing the action – potential duration. During repolarisation Ca^{2+} - ions flow out in exchange for the Na^+ - ion .

Another exchanger , Na^+ - H^+ exchanger transports protons out of the cell after vigorous working of the heart inorder to reduce acidosis. Counter transport of Na^+ - ions into the cell takes place simultaneously.

Pumps : Consequent to the counter transport of Ca^{2+} through Na – Ca exchanger , there is a Na^+ - influx and the intracellular Na^+ - level rises . The Na^+– ions that have accumulated in the cell must be returned to the extracellular space, to prevent Na^+ overload , which could lead to absorption of water by osmosis . This is corrected by

Na – K pump , which is an active transport system. It extrudes Na^+ out of the cell and permits entry of K^+ into the cell , against the electrochemical gradient. It is also called Na, K – ATPase, as it is activated by internal Na^+ or external K^+ and uses energy in the form of ATP complexed with Mg. One ATP molecule is used for each transport cycle. For three Na - ions exported , only two K – ions enter the cell as a result of which the cell will be negatively charged (Skou , 1990).

Other active transport sytems which require ATP are energy utilising Ca – pumps in SR and SL – membranes which extrude Ca – ions accumulated in the cytosol along with the Na – Ca exchanger inorder to avoid Ca – overload (Sasaki *et al* , 1992).

Calcium- ions that enter during the plateau phase of action potential is not sufficient for tension - development. Thus it was speculated that there may be an internal source of Ca^{2+} , and consequently led to the discovery of Ca - induced Ca - release from SR (Fabiato , 1983). Moravec and Bond (1991) are of the opinion that not all the Ca^{2+} within the SR is released with each beat and that the SR Ca^{2+}-release can be graded by the amount of trigger - Ca^{2+} entering the cell . Calcium spontaneously released from SR may induce adjacent SR to release Ca^{2+} , thereby propagating a 'wave' of increasing $[Ca^{2+}]_i$ resulting in contraction (Berlin *et al* , 1989) . The Ca^{2+} that is released from SR initiates contraction by binding to the contractile proteins. Decline in the myoplasmic Ca^{2+} - level occurs by the reuptake of Ca^{2+} into the SR and also by extrusion of Ca^{2+} from the myoplasm through Na - Ca exchanger and SL Ca - pump (Barry and Bridge , 1993) . SR – membrane contains a Ca^{2+} - specific ATPase which transports Ca^{2+} from sarcoplasm into the vesicles of SR with high velocity and affinity. Transition from systole to diastole is mediated by Ca – accumulating activity of this SR

Ca – ATPase (Barry and Bridge , 1993) . These pumps are switched on by a membrane protein called phospholamban, which requires a PO_4 group for its maximal activity (Fujii *et al* , 1987) . Phospholamban when unphosphorylated , binds and inhibits the activity of SR - Ca - ATPase. On phosphorylation by cAMP - dependent protein kinase, inhibitory effect of phospholamban will be removed (Sasaki *et al* , 1992) , resulting in Ca - transport into the SR . Dephosphorylation of phospholamban occurs via type - I phosphatase (Steenaart *et al* , 1992). After uptake by SR - Ca - ATPase , Ca^{2+} is bound to calsequestrin which is located primarily in the SR. Calsequestrin is the major Ca^{2+} - binding protein in cardiac muscle . Binding and release of Ca^{2+} by calsequestrin is important in E - C coupling (Barry and Bridge , 1993) . Thus Ca - uptake and Ca - release mechanisms located in the SR - membrane regulates myoplasmic Ca - concentration , and in turn myocardial contractility.

Adrenergic and cholinergic systems : The contractile activity of myocyte is also influenced by the autonomic nervous system. The extent of channel opening is influenced by β - adrenergic stimulation and by changes in the configuration of the action – potential by adrenergic and cholinergic stimulation (Winegrad , 1984). Both adrenergic and cholinergic discharge involves a system of cellular signals starting with binding of the primary messenger to the receptors on the SL, and the production of a second messenger. The second messenger acts through a series of intracellular signals to increase cytosolic Ca – levels . The resulting rise in intracellular Ca – concentration results in a positive inotropic effect.

There are receptors for various adrenergic agonists on the SL. Occupancy of the β - receptor by a primary messenger like epinephrine stimulates the reaction between the G – protein and GTP. GTP stimulates the adenylate cyclase into activity which converts ATP to cAMP , the second messenger. cAMP in turn activates protein kinases , which phosphorylates certain crucial cellular proteins to alter their properties. For example , a subunit of SR Ca – channel is phosphorylated to enhance the uptake of Ca by the SR (Gasser *et al* , 1988).

α - adrenergic system also modulates intracellular Ca^{2+} , though its action is mainly on vascular smooth muscle cells. Stimulation of α - receptor leads to hydrolysis of phosphatidyl inositol with formation of two intracellular messengers namely IP_3 which stimulates release of Ca^{2+} from SR and protein kinase - C which phosphorylates some of the contractile proteins (Berridge and Irvine , 1989).

The parasympathetic nervous system decreases the force of contraction and heart - rate , by decreasing the rate of release of norepinephrine from terminal neurons by the process of neuromodulation ; and also by stimulating an enzyme which breaks down GTP , so that the crucial step in the activation of adenylate cyclase is inhibited. It acts through the muscarinic receptor system with acetyl choline (Ach) as the extracellular first messenger , and G – protein system as the intracellular signalling system (Flemming and Watanabe , 1988) . Acetyl choline the mediator of parasympathetic system influences cells of atria and sino atrial and atrio ventricular node. In ventricular myocardium they have less physiological significance. Ach produces the pacemaker activity as the resting potential is increased due to slowing of the rate of closure of Ach – sensitive K – channels. In the pacemaker cells the diastolic

depolarization will be slow. Only at extremely high concentrations Ach reduces acceleration of repolarization in His – Purkinje system and ventricular myocardium. Norepinephrine released from sympathetic nerve terminals in all regions of the heart , and epinephrine which is released into the blood stream along with norepinephrine from adrenal glands enhances contractility , accelerates conduction , increases heart rate and shortens the action – potential duration. The general mechanism by which these effects could be mediated is by cAMP – protein kinase system (Katz , 1992b).

Intracellular calcium cycling mechanisms *:* Calcium signals modulate a large number of reactions in heart cells , most importantly the contraction and relaxation of myofibrils. The signalling function demands a very low (μM or sub μM) concentration of ionic Ca^{2+} inside the cells . Heart cells are normally bathed in fluids where the ionic concentration of Ca^{2+} reaches the mM range. The extracellular pool is the source of Ca^{2+} , that crosses the plasma membrane to initiate the contractile events. This is a carefully regulated phenomenon . SL Ca – transport mechanisms are the only means available for the extracellular Ca^{2+} to gain access to the intracellular myoplasm . Heart cells modulate their Ca – sensitive processes by mobilising the Ca^{2+} present in the cells (Carafoli , 1985).

In cardiomyocytes the major Ca – transporting systems are in the SL , SR and mitochondria. The multiplicity of transporting systems emphasizes the importance of a messenger functioning for maintaining the concentration of Ca^{2+} in heart cells. Ca must be controlled vigorously and the efficiency of control is based on concerted operation

of transport mechanisms located on the membranes responding to difference in the Ca – concentration in the surrounding space.

Important Ca^{2+} – transporters that control the intracellular Ca – homeostasis in heart cells are ;

1) Voltage – dependent Ca – channels in the SL ,
2) Na – Ca antiporter at the SL ,
3) Ca , Mg - dependent Ca – pump at the SL ,
4) Mitochondrial Ca – uniporter ,
5) SR Ca - release channel and SR Ca , Mg –ATPase or Ca – pump .

Voltage dependent Ca - influx channels , as mentioned earlier are the major influx channels . The Ca – channel mediated Ca – influx is increased by β - adrenergic activation by increasing the duration of channel opening by the cAMP – directed phosphorylation of a protein component at the inner side of the membrane (Reuter, 1983).Sarcoplasmic reticulum is the structure on which the heart cells depend for the regulation of Ca^{2+} in the ambient surrounding the myofibrils.Ca - induced Ca - release (CICR) from SR during systole satisfies the demand for Ca^{2+} during contraction. Transition from systole to diastole involves Ca – accumulating activity of Ca – pump located in the SR (Chamberlain *et al* , 1983). Thus SR has a prominent role in Ca – regulation during contraction/relaxation cycle .

The importance of mitochondria in Ca – homeostasis is in its role as a long– term Ca – buffer which makes it possible to accumulate large amounts of Ca^{2+} .

Enzymatic activities in the mitochondrial matrix are also regulated by Ca^{2+} . Activity of pyruvate dehydrogenase , isocitrate dehydrogenase , α - keto glutarate dehydrogenase , etc are modulated by Ca. Functioning of respiratory chain and in turn production of ATP are under the control of Ca^{2+} (McCormack and Denton , 1984). The dynamic balance of Ca between mitochondria and sarcoplasm (myoplasm) is the result of the operation of an electrophoretic pathway , which is used exclusively for the uptake of Ca^{2+} and it is a Na^{+} triggered route. This constitutes the ' mitochondrial Ca – cycle ' (Carafoli , 1985).

In order to prevent calcification of the cell , [illegible] Ca in the myoplasm has to be kept sufficiently low . Considerable amount of Ca is sequestered by the SR. Two SL – systems involved in removing Ca from the myoplasm are Ca – ATPase and Na – Ca exchange mechanism. Ca – ATPase or Ca – pump is a high affinity , low capacity system expelling Ca^{2+} with the same efficiency through out the entire functional cycle of the heart cells (Caroni and Carafoli , 1980) . Na - Ca exchanger is electrogenic and its activity is dependent on ionic concentration and membrane potential . At rest when the cell is normally polarized , the Na^{+} - ions flow into the cell along their electrochemical gradient , and the Ca^{2+} ions flow outward against an electrochemical gradient . The exchanger responds to relatively small increases in the $[Ca^{2+}]_i$. During depolarisation the membrane potential is such that the exchange system could operate to allow the inward flow of Ca^{2+} - ions , thus contributing to action potential plateau and increases the action potential duration .

Other endogeneous Ca – modifiers are calmodulin , cAMP , cytosolic pH etc. Ca – ions are also seen bound to Tn – C , parvalbumin or calsequestrin (Snowdone *et al* , 1985).

All these elements function concomitantly and influence each other , thus controlling the cytosolic concentration of Ca , which in turn is responsible for the initiation and regulation of contractility.

In addition to the basic regulatory mechanisms , myocardial contractility and the ion - transients can be affected in pathologic conditions ; consequent to pharmacologic interventions and also due to physiological variations .

Pharmacological agents influencing myocardial contractility :

Pharmacological interventions can influence myocardial contractility . Positive inotropic agents such as digitalis compounds , β - receptor agonists , etc can mediate their effects by increasing either intracellular calcium concentration or the sensitivity of the myofilaments for calcium. Elevation of intracellular calcium concentrations can be achieved by several mechanisms : Most importantly β - receptor mediated cAMP increasing agents increase trans sarcolemmal Ca - influx via the L - type Ca - channel and diastolic Ca - reuptake of the SR via phosphorylation of phospholamban and decrease the sensitivity of the myofilaments for calcium . Agents that increase cAMP by coupling to the stimulatory G - protein include agonists of β - receptors (eg: adrenaline , noradrenaline , dobutamine) . Other cAMP increasing agents include direct activators of the adenyl cyclase (eg: forskolin) , and inhibitors of the phosphodiesterase III that hydrolyze cAMP (eg: mirinone). The positive inotropic effect of these agents is

in contrast to cAMP - independent agents , accomplished by shortening of the contraction and relaxation time. An increase in intracellular calcium can also be achieved directly by Ca - channel agonists . It has been shown experimentally that elevation of extracellular Ca - concentrations increase Ca - influx through the L - type Ca - channel . Higher intracellular Na levels enhance Na^+ - efflux and Ca^{2+} - influx via the Na -Ca exchanger . This can be mediated by inhibitors of the Na , K - ATPase (digitalis glycosides) or by agents that prolong the open states of the Na - channel (eg : DPI 201-106 , BDF 9148) (Mittmann *et al* , 1998).

The mechanism of action of α - receptor agonists or of endothelin and angiotensin II (at the atrium) , which mediate their positive inotropic effects via an activation of the phospholipase C , is unclear in human myocardium . It has been questioned whether they increase intracellular calcium concentrations sufficiently . It has also been proposed that an increase in action potential duration , inositol triphosphate content , diacyl glycerol , pH or in the sensitivity of the myofilaments to Ca^{2+} may play a role (Terzic *et al* , 1993) .

Negative inotropic agents like Ca - antagonists , β - receptor antagonists , etc can depress myocardial contractility . Antagonists to voltage - operated Ca - channels are effective antihypertensive and antianginal agents , but they depress myocardial contractility (Sarsero *et al* , 1998) . The Ca - antagonists are a class of drugs which block the inward movement of calcium into cells through ' slow channels ' from extracellular sites. By inhibiting phase - 0 depolarisation in cardiac pacemaker cells and phase - 2 plateau in myocardium , and by depressing Ca - ion flux in smooth muscle cells of blood vessels , these agents exert profound effects on the cardiovascular

system . These agents induce sinus node depression , impaired atrio - ventricular conduction , depressed myocardial contractility and peripheral vasodilatation . Verapamil is the most potent inhibitor of cardiac conduction and contractility . Diltiazem also behaves similarly . Nifedepine is the most potent vasodilator . Significant pharmacodynamic effects were obtained by combination therapy with Ca - antagonists , especially with verapamil and β - blockers (Pearigen and Benowitz , 1998) . Verapamil , diltiazem and nifedepine act by blocking Ca - flux through L - type channels . T - channel currents are also involved in vasoconstriction and cardiac pacemaker activity . T - channel blockers (eg: mibefradil) lowers blood pressure in hypertensive patients and dilates coronary arteries in patients with angina , without triggering a reflex activation of pressor systems. The noteworthy point is that heart rate decreases rather than increase in response to this new Ca - antagonist . Another important feature of it is the lack of negative inotropic effect (Hermsmayer , 1998) . β - blockers have been employed with increasing success in congestive heart failure. Reduction in heart rate may also play a major role in the therapeutic efficacy of β - blockade (Just , 1996) . In hypertensive patients with left ventricular hypertrophy , the pure β - blocker propanolol was more effective in regressing left ventricular hyperterophy (Szlach ic *et al*,1990). β - adrenergic blocking agents act by inhibiting the interaction between β - agonists and the β - adrenergic receptor , so that the stimulation of adenylate cyclase reduces with consequent decrease in the formation of cAMP . The consequence is that the slow inward Ca - current is inhibited with resultant negative inotropy of the myocardium .

Pathological factors modulating myocardial contractility :

Pathologic conditions such as cardiac arrhythmias , ischemia and reperfusion injury , cardiomyopathy , congestive heart failure and myocardial infarction affect contractility of the myocardium (Mittmann *et al* , 1998).

Arrhythmias are common in heart failure and is considered as a marker of the disorder rather than a predictor of sudden cardiac death (Mitamura , 1996 ; Ponikowski *et al* , 1996) . The three major mechanisms for the development of ventricular arrhythmias are ; the development of automaticity , reentry circuits and abnormalities of repolarization . Arrhythmias may be primary or secondary . Primary arrhythmias are those occuring as a result of an electrophysiological disturbance . They are not associated with significant changes in hemodynamic function . In contrast , when a disease process causes a hemodynamic abnormality , it initiates electrical disturbances leading to arrhythmia . It is referred to as secondary arrhythmias. Depressed conduction due to atrio ventricular nodal block results in slowing of the conduction of the impulse , and the consequent block between atria and ventricles leads to ventricular asystole . This condition resulting in decrease in the rate of ventricular beating is called bradycardia . Extreme bradycardia causes the blood pressure to fall. Tachycardias (increase in the rate of ventricular beating) can arise from a number of mechanisms which cause premature systoles. These include both accelerated pacemaker depolarisations and the various phases of Ca – reentry mechanisms (Katz , 1992c). Ca – overload can also lead to sustained contracture . Excess Ca – cycling in and out of the SR may explain certain arrhythmias (Opie,1991b).

Acute myocardial infarction is the acute or sudden development of a localized or circumscribed area of myocardial necrosis due to severe ischemia from inadequate blood flow and / or oxygenation. After an infarction myocardial ischemia develops and the myocardium is at risk for further necrosis (Chiche , 1974). Myocardial ischemia occurs when the reduction of coronary flow is so severe that the supply of oxygen to the myocardium is inadequate in relation to the O_2 - demands of the tissue . It is initially reversible . Prolonged ischemia causes irreversible changes , with the development of cell death and necrosis or myocardial infarction . The two major mechanisms of ischemia - reperfusion injury are formation of free radicals and Ca - overload . Both induce membrane damage and impaired contractility (Opie , 1989) . Observations of different workers provide substantial evidence that cytosolic calcium is increased during ischemia (Lee *et al* , 1987 : Kihara *et al* , 1989 ; Steenbergen *et al* , 1990 ; Marban *et al* , 1990) . The consequence of a rise in Ca - level is increased depolarization and ischemic contracture . In addition to this Ca^{2+} may not return rapidly to the static diastolic level at the end of relaxation phase . This results in contractile dysfunction . A compensatory cytosolic Ca - overload via SR Ca - release channel during ischemia in rat has been reported and SR inhibitory agents like ryanodine is found to prevent such arrhythmias (Thandroyen *et al* , 1988 ; Mubagawa *et al* , 1997) . There are reports indicating the beneficial effect of Ca - antagonists inhibiting trans SL - Ca- influx preventing Ca - overload, during ischemia (Finelli *et al* , 1993). Yoshimo (1991) reported that preserving the activity of Na,K - ATPase prior to ischemia may be beneficial in preventing ischemia induced injury . This observation suggests that Na^+_i - rises due to impaired Na,K - pump function and Na - Ca exchanger mediated Ca - overload ensues .

Myocardial failure may also occur following primary myocardial disease for example , cardiomyopathy. In cardiomyopathy , for a given end - diastolic volume the tension generation is inadequate. In primary cardiomyopathy the causative factor is seldom known . In hypertrophic cardiomyopathy , the muscle cells may undergo excessive growth in size , which is associated with high systolic ejection and diastolic dysfunction . This may be due to the small size of the left ventricular cavity . In familial hypertrophic cardiomyopathy , mutations in genes encoding myofibrillar proteins leads to functional defect in the sarcomere (Bonne *et al* , 1998). Dilated cardiomyopathy develops whenever a large mass of myocardium is damaged as in myocarditis ,in severe coronary artery disease or after a large myocardial infarct . In dilated cardiomyopathy , the initial event is myocardial failure with poor pressure generation marked by an decrease in wall stress . Loss of myofibril organization is a common feature of chronic dilated cardiomyopathy (Sussman *et al* , 1998) . Myocarditis is thought to be commonly caused by various viruses and accumulating evidence links viral myocarditis with the eventual development of dilated cardiomyopathy . Cytokines are being increasingly recognized as an important factor in the pathogenesis and pathophysiology of myocarditis and cardiomyopathy . Elevated levels of cytokines have been reported in heart failure and various cytokines have been found to inhibit myocardial contractility *invitro* and *invivo* (Matsumori , 1997) . Excess Ca - entry in myocarditis and dilated cardiomyopathy is reported to occur as a result of activation of Ca^{2+} permeable cationic channels by the autoantibodies (Tominaga *et al* , 1993) .

An important concept in cardiac pathology is that of Ca – overload, first emphasized by Fleckenstein (1971). Cytosolic Ca – overload can occur in response to myocardial ischemia , reperfusion and excess catecholamine stimulation.

Ischemia induced acidosis can lead to cardiac reperfusion injury , including arrhythmias and stunning . An accelerated $Na^+ - H^+$ exchange on reperfusion might be arrhythmogenic . Secondary efflux of Na^+_i in exchange for Ca^{2+}_o is the reason (Dennis *et al* , 1990). Acidosis has been known to depress myocardial contractility for more than 100 years (Gaskell ,1880) and is an important factor to be considered because acidosis is a major consequence of myocardial ischemia and may contribute to the ischemic decline in force. The situation is potentially complicated by the fact that changing pH can modify virtually every cellular system involved in Ca - regulation and force development . Increase in intracellular $[H^+]$ could increase diastolic $[Ca^{2+}]_i$ by competing with Ca - ions for intracellular Ca- binding sites (Bers and Ellis , 1982) . But acidosis decreases the myofilament Ca - sensitivity and decreases the force regulation (Blanchard and Solaro , 1984).

Physiological determinants of myocardial contractility :

Physiological determinants of myocardial contractility include exercise and rest , stress , age of the animal , extracellular ionic levels , etc . The influence of age and extracellular Mg have been reviewed in detail as these are the variables taken up for the study .

During exercise the cardiac output must increase manyfold , hence either stroke volume or heart rate or both must increase . The major adaptation to exercise

generally is increased heart rate . The expected increase in end - diastolic volume as a result of the increased venous return may in part be annulled by increased contractility brought about by increased adrenergic discharge , which in turn allows the heart to increase the stroke volume without any change in the diastolic fibre length (Iskandrian *et al* , 1983) . Force - frequency effects of the intact left ventricle are markedly enhanced by exercise . Exercise increases the efficiency of conversion of metabolic energy to external work by the left ventricle resulting in increased production of mechanical energy and enhanced contractility (Nozawa *et al* , 1994 ; Mima *et al* , 1998) . The plasma norepinephrine concentration has been reported to be increased during moderate and severe exercise above resting values (Peronnet *et al* , 1981) . Cardiac inotropic function during exercise is suggested to be largely β1 - adrenoreceptor mediated with little or no β2 - adrenoreceptor involvement (Nyako - Adomfeh , 1991). Verapamil impairs left ventricular function during exercise in hypertensive patients (Ashmore *et al* , 1990) indicating the probable involvement of SL - L - type Ca - channel in enhancing Ca - influx during exercise . After exercise the initial rate and maximal capacity of Ca - uptake of isolated SR were reported to be siginificantly depressed (Byrd *et al* , 1989) . This depression was paralleled by decreased activity of Ca - ATPase .

Myocardial contractility is influenced by various kinds of stress . Wall stress has been used as one of the parameters determining myocardial mechanics . Wall stress is an essential determinant of myocardial O_2 - consumption and is also an important determinant of the myocardial contractile state and diastolic function and it also regulates electrical properties (Sugushita et al , 1994). If wall stress deviates from the

normal range , inspite of compensatory mechanisms , severe cardiac damage occurs .

Certain cardiovascular diseases are associated with free radical generation . Free radicals are highly reactive chemical species with unpaired electrons in their outer orbital. Superoxide anion , hydroxyl radical , H_2O_2 , etc are included in this category. Free radicals are found to have a role in arrhythmias (Opie , 1991c). Free radicals can induce peroxidation of poly unsaturated lipids of the membrane . Membrane damage due to lipid peroxidation , leads to increased membrane permeability . Owen *et al* (1990) stressed the point that free radical induced membrane damage can lead to Ca – overload . This may lead to abnormal contractile function (Vandeplassche *et al* , 1990) .

Emotional stress is a potent source of catecholamine discharge . It can lead to enhanced activity of the adrenergic system especially with increase in circulating epinephrine , so that β - adrenergic activity is enhanced . The increased β - adrenergic discharge also leads to a series of events that enhance myocardial O_2 - uptake , tachycardia , increased contractility and increased cardiac output (Ruddel *et al* , 1988) .

Influence of age on myocardial contractility : Aging is associated with selective changes in the cardiovascular system . Independent of diseases , age - associated changes in cardiovascular function result , consequent to anatomical , biochemical and haemodynamic remodelling without pathological consequence. Growth of the vertebrate heart during embryonic and fetal life is characterised by hyperplasia of myocardial cells . Shortly after birth , myocardial cells lose the capability of division and further

growth of the heart is due to cardiomyocyte - hypertrophy and nonmuscle cell - hyperplasia . This process which is referred to as hypertrophic growth results in 30 - 40 fold increase in volume of individual myocardial cells during normal postnatal growth and maturation . The transition from hyperplasic to hypertrophic growth is associated with the formation of binucleated myocardial cells , as a result of karyokinesis without cytokinesis . The molecular mechanism of this transition is uncertain. The response of the heart to increased metabolic demands or to an increased work load depends on the age of the animal . In addition to cellular enlargement , structural remodelling of the myocardial cells , including alterations in the relative proportions of cellular organelles and in the ultrastructure of individual organelles are reported to occur during physiological hypertrophy of the adult heart (Oparil *et al* , 1984) . The expansion of the ventricular myocardium during maturation shows a remarkable degree of well balanced compensatory response , as the capillary microvasculature , parenchymal cells and subcellular components of myocytes also grow in proportion to the increase in cardiac mass . While in induced cardiac hypertrophy , the capillary luminal volume and surface ; and mitochondrial to myofibrillar volume ratio are affected . This indicates that physiological hypertrophy varies from pathological hypertrophy where an inadequate growth adaptation of the component structures responsible for tissue oxygenation occur and the hypertrophied myocardium may exhibit structural abnormalities that can be expected to increase its vulnerability to diseases (Anversa *et al* , 1986) . Several physiological , functional and biochemical modifications occur progressively in the myocardium during the course of life . Cardiovascular system is the first functionally regulated organ system, recognised in the vertebrate ontogeny. From a straight tubular

heart to a complex four chambered pump is a rapid transformation and occurs early in the mammalian life (Artman *et al* , 1996). Ontogenic differences have been observed in cardiac contractile performance (Kato *et al* , 1996) .

Morphometric changes include basically an increase in cardiac mass which may be due to hypertrophic or hyperplasic growth of myocytes or a combination of both the processes (Rakussan , 1993). This characteristic chain of developments is probably common to all mammalian hearts . First hyperplasia takes place ; which is then replaced by hypertrophy (Claycomb , 1992) . It is not clear when or why the proliferation stops.

Cardiac development can be divided into three phases . First phase comprises the entire embryonic and fetal period until birth with growth by the proliferation of myocytes (hyperplasia). The second phase is of short duration and occurs during early postnatal life , when the myocytes become bigger , concomittent with cell proliferation. Soon after that the cells cease to divide and the myocytes grow by hypertrophy (vander Hoff *et al* , 1997). As the cardiomyocytes terminally withdraw from the cell – cycle , genes which encode proteins characteristic of the mature muscle cell will be activated (vander Hoff *et al* , 1997) .

A large number of genes are newly transcribed during terminal differentiation including those that encode the sarcomeric proteins , the specialised channels and receptors and the muscle's metabolic enzymes . Rakussan (1993) proposed that in rat heart the 'cut – off' point has been somewhere between third and sixth week of postnatal life . Li and his coworkers (1996) also

supported this rapid transition of cardiomyocytes from hyperplasia to hypertrophy during postnatal development.

Canale *et al* (1986) revealed that measurements of isolated rat cardiomyocytes exhibited increase in both width and length during postnatal growth , the average cell being 15.7 X 88.2 μm at 60 days and 21.5 X 110.3 μm at 250 days. The postnatal changes of the rat heart cells has been worked out in detail. According to a study conducted by David *et al* (1979) , several structural changes take place in the rat heart. They have examined the myocadium of male Wistar rats at birth and at ages 1 , 2, 3 , 4 , 5 , 6 , 7, 14 , 21 days and 1 , 2 , 3 , 4 , 5 , 6 months. Their observations can be summarised as follows : volume density of myofibrils significantly decreased from 0.52 at birth to 0.33 on fifth day, increased again and reached a peak in the third month . Then it remained at 0.55. The proportion of sarcoplasm decreased continually from 0.327 at birth to 0.018 in the sixth month . The volume density of mitochondria rose from 0.16 at birth to 0.18 - 0.20 on the seventh day .

Aging is associated with variation in myocardial performance as observed in senescent animals (Muscari *et al* , 1992) . The adult stage , after completion of development and before aging sets in is considered as a more or less stable period in the life span of an animal . Even during this period , there is physiological hypertrophy of myocardium (Walsh and Dorn ,1998) . Lakatta (1990) has pointed out that the heart wall thickness increases modestly due to an increase in myocyte size . It could be associated with changes in structure and function and distribution of channels and receptors at the level of SL and SR leading to variation in myocardial function .

Mechanical response of cardiomyocytes to physiological disturbance or pharmacological interventions can therefore vary with age .

According to Canale *et al* (1986) , T – tubules in the SL develop postnatally and is initially confined to the periphery of the cell, but later localised varicosities appear , which usually form a coupling with SR. They comprise 27 – 36% of the total SL – area. Profiles of SR are too infrequent in new born rats . Even on the tenth day of age , cells rarely show SR – network . Intercalated discs which joins the cells end – to - end also show marked changes during development . Immature discs are short , usually less than 1–2 μm wide. Further development which is presumably related to mechanical function is associated with complexity of interdigitation and appearance of end – to – end junctions.

The subcellular mechanisms responsible for developmental changes in action potential configuration and excitation - contraction coupling have been elucidated recently . This task was made more difficult by significant age and species differences in cardiac electrophysiology . Mature myocytes utilize Ca^{2+} - entry via SL - voltage gated Ca - channels to trigger Ca - induced Ca - release (CICR) from SR , while in neonatal rat SL - mediated Ca - entry is more prominent than intracellular Ca - cycling .

The ontogeny of cardiac Ca - channels is highly species dependent . In chick heart a progressive decrease in Ca^{2+} - influx via $I_{Ca,L}$ during early development was noted (Wetzel and Klitzner , 1996) . In similar studies in rat heart , Ca - current density in cultured neonatal myocytes was much greater than in isolated adult myocytes (Cohen and Lederer , 1988) . But , during aging the magnitude of L - type Ca - channel current increased significantly in parallel with cell enlargement , eventhough $I_{Ca,L}$ density

remained unaltered (Zhou *et al* , 1998) . In contrast , Ca - channel expression in rabbit heart was found to increase with development (Osaka and Joyner , 1991). T - type Ca - channels were reported to be present in 91% of adult rabbit heart cells but were evident only in 39% of neonatal cells ; voltage gated Ca - current amplitude , current density and T - type Ca - channel prevalance - all increase with maturation (Wetzel et al , 1991). T-type Ca - currents have been reported to have a rather unclear prominence in mature and immature chick heart (Tohse *et al* , 1992) , while in rat heart 60% decrease in $I_{Ca,T}$ density was observed on maturation from 4 1/2 weeks to 14 weeks of age (Xu and Best , 1992) . Reverse Na - Ca exchange activity has been described in mature myocardium also , but it serves as an important Ca - influx channel in immature myocardium (Frank *et al* , 1992) . Steady state mRNA levels of the SL Na - Ca exchanger peak near birth in developing rabbit and rat hearts (Boerth *et al* , 1994) . In chick heart the exchanger density in adult was only one half of the level than that at 10 days of life ; and in rabbit heart , a decrease in Na - Ca exchange activity from the late fetal and neonatal period to adulthood was noted (Wetzel and Klitzner , 1996) . Significantly greater cardiac Na - Ca exchanger expression was reported in newborn rabbit hearts compared with adults (Haddock *et al* , 1998) . Hanson *et al* (1993) have reported that Na , K - ATPase activity and ouabain binding is higher in adult compared to neonate and in contrast Na - Ca exchange activity remained the same in the two age groups . Earlier studies also had revealed that in congestive heart failure patients , sarcolemmal Na,K - ATPase which act as digitalis receptors were fewer in young patients than in the adult (Legato , 1979). While Kennedy *et al* (1996) observed that in rats aging is associated with a decline in the Na^+ - pump capacity of the myocardium ,

Abete *et al* (1996) are of the opinion that Na - Ca exchange activity is not modified by the aging process , while an age related reduction in Na,K - ATPase activity was observed . Ellingsen *et al* (1994) has reported the down regulation of Na, K - pump in rat myocardial hypertrophy , and in his opinion reduction in the activity may not be due to reduced synthesis of pump protein , but can be ascribed to increased cell volume and increased content of other cell proteins such as contractile proteins . Kjeldsen and Gron (1990) also ascribed the age dependent change in myocardial Na,K - ATPase concentration to variation in the ratio between the amount of the enzyme and muscle mass .

From studies on the right ventricular papillary muscle isolated from senescent rat ventricle , Wei *et al* (1984) have concluded that the prolonged and greater extent of depolarization compared to the young may be related to the prolonged contractile duration and may also be a determinant of the peak force developed in response to excitation under the same conditions . Abete *et al* (1992) evaluated the effects of Platelet Activating Factor (PAF) on contractile characteristics of adult and senescent rat hearts . Their data suggested that age influences the effect of PAF on contractile parameters , coronary flow and conduction arrhythmias by acting on receptors whose function is unaffected by age .

The ontogeny of Na - channel has not been extensively studied . But in neonatal rabbit , rat and mouse heart ; their voltage dependence is similar to that in adult preparations . A direct comparison of neonatal and adult rat myocytes has suggested that voltage - dependence of steady - state inactivation and the time constant of inactivation - both shift towards more negative potentials with development (Xu *et al* ,

1991). Depression of Na - channel activity due to aging process was reported in ventricular myocytes (Wu *et al* , 1997) . K - currents have been described in mature as well as immature cardiac myocytes . Similar to Ca - currents the ontogeny of these channels is also species - specific . In rabbit heart increase in the inward rectifier K - current between neonatal and adult myocytes was associated with more negative resting membrane potential and an increased sensitivity of the resting membrane potential to extracellular K^+ concentration ; this developmental increase in I_K has also been reported in chick and rat heart (Josephson and Sperelakis , 1990 ; Huynh *et al* , 1992 ; Wahler , 1992) . Transient outward K - current has been described in adult myocytes but not in neonatal myocytes in canine myocardium (Varro *et al* , 1993) , while this current was not found in adult guinea pig myocytes. ATP - sensitive and Ach - sensitive K - channels were also found to exhibit single channel conductance and channel density with development (Wetzel and Klitzner , 1996). Overall there appears to be a general increase in K - conductance with development (Wahler *et al* , 1994 ; Wang *et al* , 1996) .

Maturation of the heart is associated with dramatic changes in the structure and function of the SR . The content of SR is low and the SR is less organized in the immmature heart (Nassar *et al* , 1987). Expression of SL voltage gated Ca - channels and SR Ca - release channels is reported to be coordinately regulated during maturation of the rabbit heart (Brilliantes *et al* , 1994) . Developmental changes in SR Ca - transport have been studied in SR vesicles . Ca - uptake , activity of the Ca - pump , number of Ca - pumps and efficiency of the Ca - pump were found to be slower in vesicles from immature hearts compared with that measured in vesicles from mature

heart in rabbits , guinea pigs and sheep ; and the content of Ca - pump mRNA paralleled the increase in pump protein in adult rabbits . Thus transcriptional and / or post - transcriptional regulation of the Ca - pump gene was supposed to be the factor contributing to age related changes in SR Ca - transport (Mahouny , 1996) . Age associated slowing of cardiac relaxation was assumed to be due to diminished Ca - sequestering activity of SR . Western blot analysis showed no significant age related difference in the relative amounts of ryanodine receptor sensitive (RyR) Ca - release channel , Ca - storage protein calsequestrin , Ca - pumping ATPase and Ca - ATPase regulatory protein phospholamban in SR . On the other hand , relative amount of calmodulin dependent protein kinase (CaM kinase) was ~ 50% lower in aged heart . CaM kinase mediated phosphorylation of RyR Ca - release channel , Ca - ATPase and phospholamban were also reduced (~25 - 40%) in the aged heart . Ca - uptake was also significantly reduced with aging . These findings indicate that changes in the intrinsic functional properties of SR contributed to maturational changes in myocardial SR function (Xu and Narayanan , 1998 ; Tanaka *et al* , 1998). Thus the processes responsible for regulating $[Ca^{2+}]_i$ within the myocytes either directly or indirectly were subjected to important changes in structure and function during maturation of the mammalian heart . These changes result in developmental differences in myocardial functional performance (Vornanen , 1996) .

Cardiac mitochondrial phosphorylation system of aged rats was found to be poorly sensitive to variations in external free calcium (Guarineri *et al* , 1993) .

The autonomic nervous system also has an important role in the regulation of cardiac function . β - adrenergic and muscarinic cholinergic agonists in the mature heart

influence the $I_{Ca,L}$ activity . Studies in rabbit heart have demonstrated a decreased responsiveness of $I_{Ca,L}$ to β - adrenergic receptor stimulation with isoproterenol in neonatal myocytes as compared to adult cardiac muscle cells (Osaka and Joyner , 1992) . Similarly , Charpentier *et al* (1996) reported that response to isoproterenol the β - receptor agonist is higher in adult dogs compared to young ones . Age associated reduction in cardiac β-1 and β-2 adrenergic responses without changes in inhibitory G-proteins or receptor kinase was reported by Xiao *et al* (1998) and Ferrara *et al* (1997) . Prominent inhibitory effects of muscarinic agonists in neonatal rabbit myocytes (Osaka *et al* , 1993) suggest a negative influence of these receptors on Ca - currents in immature myocardium . Su *et al* (1995) demonstrated that the negative chronotropic and inotropic responses of the heart to cholinergic muscarinic receptor stimulation are strongly enhanced with aging in the rat model .

In otherwise healthy individuals , the effectiveness of β - adrenergic modulation of cardiovascular performance has been found to decline with age (Xiao and Lakatta , 1992) .

Age dependent variation in contractile characteristics of the adult myocardium has not received as much attention as the changes associated with developmental maturation and aging . Limited number of studies have been carried out in the adult rats between the ages 2 months and 1 year . The heart weight and body weight was found to increase with age , but the heart weight to body weight ratio decreased from 3.2 in 2 month old rats to 2.6 in 12 month old rats , where as the ratio increased in spontaneously hypertensive rats (Tschudi and Luscher , 1995) . Age related decrease in cardiac tolerance to oxidative stress has been observed . H_2O_2 infusion resulted in a

significantly larger increase in end diastolic pressure in hearts of 6 and 12 month old rats than 3 months (Abete *et al* , 1999) . In addition , developed pressure decreased and incidence of arrhythmias showed a higher score . Biochemical changes were also observed with increase in age following maturation . A profound reduction was observed in the percent of V_1 myosin isoenzyme from 75% at 2 months to 50% at 8 months and 14% in the 24 month old rats , paralleled by a decline in Ca^{2+} - myosin ATPase (Effron *et al* , 1987) . Talesara and Arora (1994) , have observed age related decrease in succinic dehydrogenase and myofibrillar ATPase in heart muscle. Endocardial endothelium has been shown to modulate the performance of the adjacent myocardium (Qi and Rouleau , 1997) . Myocardial contractile characteristics were found to vary in rats of different age groups : 7 - weeks , 4 - months and 9 - months . At a low extracellular calcium concentration (0.7 mM) , total tension , maximum rate of tension development (+ dT / dt), time to peak tension and time to half tension decline from maximum tension were increased with increasing age . Increasing the extracellular calcium concentration eliminated age - related differences in total tension and time to half tension decline , but did not affect time to peak tension and caused +dT/ dt to increase to greater levels in 7 week old myocardium . Endocardial endothelium removal had a greater effect on contractile indices in young rats . Increasing extracellular calcium concentration eliminated endocardial endothelium mediated changes in contractile indices , regardless of age . These observations indicate that the contractile effects of endocardial endothelium are both age- and calcium - dependent , with responsiveness to endocardial endothelium decreasing with age (Qi and Rouleau , 1997) .

Influence of extracellular ionic composition on myocardial contractility : Myocardium needs a suitable biochemical environment in order to contract , in addition to an adequate supply of oxygen and nutrients . Blood plasma contains (in mM) : Na^+ - 160 , K^+ - 10 , Ca^{2+} - 2 , Mg^{2+} - 1 and Cl^- - 100 . The free ion - concentration of these elements are kept constant in the circulatory fluid and the extracellular milieu of the cardiomyocyte . Even small deviations from normal levels in man are recognized as symptoms of malnutrition or disease (da Silva and Williams , 1991) . Elevation or reduction in the plasma levels of these ions causes disturbances in cardiac rhythm as is reflected by the abnormalities in the action potential of the myocardial cells . Prolongation of the action potential results in reduced amplitude of contraction and reduction in the duration of action potential leads to rise in the amplitude of contraction. For example hyperkalemia or hypocalcemia has been associated with shortening of the amplitude of action potential , while hypokalemia or hypercalcemia prolongs the action potential (Katz , 1992d) .

Many of the cardiac diseases were found to be associated with hyponatremia, hypokalemia and / or hypomagnesemia (Schwinger and Erdmann , 1992) . Electrolyte disturbances can affect Ca - transport mechanisms and in turn cardiac mechanical function . Applying recirculation fraction of activator Ca (RF) , as an index of myocardial contractility and myocardial force - interval relationships , RF under the influence of Na^+_o and Ca^{2+}_o were calculated . Hyponatremia was found to increase the RF . Similarly, increasing $[Ca^{2+}]_o$ also was found to enhance RF (Morner and Wohlfart , 1992). Low

extracellular K^+ and high extracellular Na^+ increased the heart rate and restored the ability to pace (Kerns *et al* , 1996) , thereby reversing the chronotropic toxicity of β - blockers like propanolol . Lowering $[Na^+]_o$ is , theoretically expected to slow electrical conduction velocity leading to increase in cardiac contractility via suppression of Na - Ca exchange mechanism , thereby increasing the $[Ca^{2+}]_i$. But it has been observed that , in patients with 'water intoxication syndrome' , negative inotropism was caused by low serum Na - concentration (Yanagi *et al* , 1998). Myocardial ischemia and early perfusion is associated with heterogeneity in $[K^+]_o$ and electrophysiologic changes which in turn may play an important role in the genesis of arrhythmias (Hariman *et al* , 1993) . Extracellular K^+ exerts beneficial effects on the frequency dependent force generation in human myocardium (Schwinger *et al* , 1997) . The ion activates Na , K - ATPase and changes the membrane potential , thereby influencing the mode of action of Na - Ca exchanger to produce an inotropic effect . Magnesium and potassium deficiencies play an important role in the development of cardiac arrhythmias (Schwinger and Erdmann , 1992).

Last two decades were associated with the acquisition of a greater understanding of cations in cardiac physiology . The major discoveries have centered on Ca^{2+} , K^+ and Na^+ , but relatively little attention has been paid to the importance of Mg^{2+} . Lack of suitable techniques for measurement of Mg has been one of the reasons .

Role of Mg^{2+} in cardiac physiology : Magnesium (Mg) is an important element both in health and disease . Approximately half of the total Mg in the body is present in the

soft tissue and the other half in bone. Less than 1% of the total body Mg is present in blood. Majority of the experimental information comes from determination of Mg in serum and red blood cells (Elin , 1988). At present we have little information about equillibrium - state of Mg within the body pools. Mg^{2+} - ions are pivotal in the transfer , storage and utilization of energy. Mg regulates and catalyzes around 300 enzyme systems in mammals . The intracellular level of free Mg^{2+} regulates intermediary metabolism , DNA and RNA synthesis and maintenance of cell structure and cell growth. Mg has numerous physiological roles among which the important ones are control of neuronal activity , cardiac excitability , neuromuscular transmission , muscular contraction , vasomotor tone , blood pressure and peripheral blood flow. Mg modulates and controls Ca^{2+} - entry and release (Altura and Altura , 1996) . The Mg – content of adult human body is 21 – 28 gm or ~2000mEq (Wacker and Parisi , 1968). It is the fourth most abundant cation in the body and its intracellular level gives it the second position Apart from the half of body Mg – content in bone , the rest is equally distributed between muscular and non muscular soft tissues. Of the non – osteous tissues ; liver and striated muscle have the highest concentrations - ~15 to 20 mEq / kg.

The average daily requirement of Mg for a normal human adult is in the order 25 mEq. The largest source is nuts , green vegetables etc . Drinking water is a very important source of Mg . Intake of 0.30 - 0.35 mEq / kg of body weight per day is required to maintain the proper Mg - balance in normal human beings (Jones *et al* , 1967) , and 12 - 18 % of Mg can be derived from water (Seelig , 1980) . Hence , the level of Mg in the drinking water can have a significant influence on the intake of Mg, especially when dietary intake from other sources is low .

The physiological roles for Mg^{2+} - ions in cardiac and vascular muscle are limited to regulation of contractile proteins , SR - membrane transport of Ca^{2+} - ions , co factor in ATPase activities and metabolic regulation of energy dependent cytoplasmic and mitochondrial pathways . Small changes in free external $[Mg^{2+}]$ or cytoplasmic Mg^{2+} could exert significant effects on cardiac or vascular smooth muscle contractility (Altura and Altura , 1985). Dietary , metabolic or drug - induced changes in Mg^{2+} - levels appear to play important roles in the etiology of cardiac and vascular disorders.

It has been observed quite early that the content of Mg in mammalian ventricular muscle is very high . Polimeni and Page (1973) determined the total Mg – content in rat ventricle as 17.3 ± 0.2 mM / kg of cell water and Polimeni (1973) reported that, Mg – content of rat ventricular muscle is 43.4 ± 0.4 mM / kg dry wt .

Lack of suitable methods for quantitation of Mg had been the major obstacle in the past. Discovery of sensitive analytical and chemical methods like nuclear magnetic resonance spectroscopy (NMR) , atomic absorption spectrometry (AAS) and fluorescent spectrofluorimetric methods made possible the accurate determination of tissue and cellular Mg – content .

A better understanding of Mg – metabolism requires the knowledge about the state of Mg i.e. free or bound , because free Mg only is physiologically active. Accurate determination of free Mg – concentration was a challenge and various methods have been developed . Use of metallochrome dyes is one method. Metallochrome dyes selectively binds Mg, resulting in a change in the absorbance of the dye. Exchrome blue had been found to be a successful dye to detect free Mg (Casillas *et al* , 1981) . Ion selective micro electrodes can also be used for determining

the Mg - concentration (Blatter , 1986) . Mg - Isotopes have been used as biologic tracer in following the absorption , distribution in the body and the excretion of this ic (Aikawa *et al* , 1960 ; Romani *et al* , 1993) , but ^{28}Mg has a very short half life (~2 hrs) and is not readily available (White and Hartzell , 1989) . NMR employs isotopes lik ^{31}P and ^{25}Mg (Elin , 1988 ; London , 1991). Ultrafiltration / equillibrium dialysis helps separating free and complex Mg - fractions . Fluorescent indicators and their ce permeant probes are also found to be reliable in measuring the cytosolic fre Mg - concentrations (Murphy *et al* , 1989).

According to Polimeni and Page (1973) approximately 12% of cardiac Mg seen in mitochondria and 23% in the myofibrils. A large portion of the intracellular M is complexed with ATP , ADP and AMP . Still smaller amount is bound to enzyme coenzyme complexes.

In the cardiac muscle , Mg^{2+} influences tension development (Shine , 1979). M has been found to impart negative inotropic effect in mammalian ventricular musc (Hall *et al* , 1990) . This effect is due to competitive antagonism against Ca , probably SL – binding sites. Bara *et al,* (1993) have reported that Mg plays an important role a large number of cellular processes by acting as a co-factor in enzymatic reaction and transmembrane ion movements. According to them $[Mg^{2+}]_o$ influences variou processes in the cellular ion - transport through SL - K - channels , Na - channels , Na , K pump and Na - Ca exchanger . Intracellular Mg blocks the outward current by interferin with the passage of K – ions and induces rectification of the channel current – voltag relationship , and acts as a fast blocker of Na - channels . $[Mg^{2+}]_o$ increases the K channel permeability and Na – transport at high concentrations by acting on Na

pump. Both intracellular and extracellular Mg stimulate the Na , K – pump at high concentrations and inhibit at low concentrations . The activity of the Na - Ca antiporter is inhibited by extracellular Mg . The inhibition by Mg is competetive with Ca (Bara *et al* , 1993).

The above mentioned observations indicate that variation of extracellular Mg concentration can alter the functioning of most of the SL ion channels . Digitalis induced arrhythmias is also found to be more frequent in Mg - deficiency (Singh *et al* , 1975) .

Patch – clamp studies showed that Mg – ion acts on both L and T – type Ca – channels and Mg has been dubbed "nature's calcium antagonist" (Millane and Camm , 1992). Schwinger *et al* (1997) are of the opinion that Mg reduces the intracellular Ca – availability through several mechanisms ; such as its inhibitory action on SL , Na – Ca exchanger etc . Howarth and Levi (1998) demonstrated in rabbit myocytes that free $[Mg^{2+}]_i$ might partially inhibit the activity of the Na - Ca exchanger or might limit the ability to trigger Ca - release . It was suggested that SR Ca – pumps are influenced by Mg^{2+} and it inhibits SR Ca – release channel (Meissner and Henderson , 1987 ; Schwinger *et al* ,1997) . Fabiato and Fabiato (1975) noticed that CICR from SR occurs at higher intracellular Ca – concentration, in the presence of high Mg – concentration. Their opinion was that increase in free intracellular Mg – concentration could enhance the capacity and rate of binding of Ca^{2+} by SR. While Meissner and Henderson (1987) reported that Ca – release from cardiac SR is modulated by $[Mg^{2+}]_i$. This channel is inhibited by mM concentrations of Mg^{2+} .

Reduction in Ca^{2+} - inward current in the presence of Mg^{2+} is mediated by its competition with Ca^{2+} binding sites of the SL. This competetion is facilitated by the smaller ioniuc radius of Mg^{2+} (0.6A°) , than that of Ca^{2+} (0.95A°) (da Salva *et al* , 1991) . Enzyme reactions that are known to be catalyzed by ATP require Mg as a cofactor. Magnesium acts as an activator . Magnesium is divalent and does not form intermediates like transitional metals . Perhaps this quality enables it to act as a bridge between a large number of chermical reactions . The effect of Mg - chelation in such reactions helps to lower the free energy of activation eg : Na, K - ATPase , Ca^{2+} - ATPase etc (Aikawa , 1989) .

Magnesium also has a prominent role in tension - development in myofibres , by influencing the activity of contractile proteins . Potter and Gergely (1975) reported that out of the 6 cation binding sites of troponin ; two are Mg^{2+} - specific , two are Ca^{2+} - specific and the remaining two sites can bind either Mg^{2+} or Ca^{2+} . Calcium specific sites have low affinity for Ca^{2+} , and Ca^{2+} has high affinity for Mg - Ca competetive sites . If $[Mg^{2+}]_i$ is higher Ca - Mg sites would be occupied by Mg^{2+}, as during relaxation . Then Ca - binding will be only at low affinity Ca - specific sites. If $[Mg^{2+}]_i$ is less , Ca^{2+} can bind to both high affinity Ca - Mg sites and low affinity Ca - specific sites , in which case tension development will be stronger . Binding of TnC with Ca^{2+} displaces Tm from Tn - complex which otherwise blocks the cross - bridge formation between actin and myosin . Thus tension development in muscle fibres can be strongly influenced by Ca^{2+} / Mg^{2+} ratio. Schwinger *et al* (1997) noticed that Mg^{2+} reduces Ca - binding to Tn - C. Significance of Mg^{2+} - affinity in cardiac TnC - Ca^{2+} exchange was also specified by Wang *et al* , (1998).

The actin and myosin filaments of cardiac muscle attach through cross - bridges . The myosin heads contain myofibrillar ATPase activity. Mg ATP is the substrate for this enzyme and by hydrolyzing MgATP , energy for cross - bridge attachment is obtained . At low $[Ca^{2+}]$ (10^{-8} to 10^{-9} M) interaction of MgATP with myofibrillar ATPase of myosin takes place, leading to cross - bridge interaction. Increasing $[Mg^{2+}]_i$ from 1 mM to 10 mM and decreasing from 1 mM to 0.4 mM , both depress the ATPase activity. Substrate inhibition may be the reason for the observed decrease at higher $[Mg^{2+}]_o$. Thus there is an optimum $[Mg^{2+}]_i$ at which Ca - binding to myofibrils is maximum (Shine , 1979).

Metabolic and functional responses to $[Mg^{2+}]_o$ in perfused rat heart revealed that elevation of $[Mg^{2+}]_o$, dose dependently reduced the contractile function (Headrick *et al* , 1998) . By regulating Ca - transport and also by influencing contractile protein interaction Mg^{2+} affects cardiac contractility.

Magnesium homeostasis : Intracellular free $[Mg^{2+}]$ is critical to cardiac cell function , as it regulates many processes . The concentration of total Mg^{2+} in cardiac ventricular myocytes ranges between 17 and 11 mM and most of the cellular Mg^{2+} is present as a complex with ATP , bound to cytosolic proteins or internalised within intracellular structures . Only a small fraction of total Mg^{2+} is 'free' within the cell (Romani *et al* , 1995) . The values of cytosolic free Mg^{2+} present in cardiac ventricular myocytes under physiological conditions shows variability depending upon the techniques used to measure it . Although the basal value of serum Mg^{2+} broadly ranges between 0.75 - 1.5 mM (Lauler , 1989) , it is unanimously accepted that the free $[Mg^{2+}]_i$ does not change at

all , or if it changes only minimally from the usual 1 mM (Romani *et al*, 1995). A large redistribution of total Mg^{2+} to or from cellular compartments has been identified and characterised in perfused rat heart and in isolated cardiac ventricular myocytes (Romani *et al* , 1993) . Their observations suggests that the amount of Mg^{2+} transported across the cell membrane is large but changes in the cytosolic free Mg^{2+} - content were negligible . The final conclusion made by them was that Mg^{2+} distributes among intracellular organelles. In contrast to this , Handy *et al* (1996) are of the opinion that most of the changes in $[Mg^{2+}]_i$ appear to arise from variation in Mg -transport across the SL , rather than from alterations in intracellular Mg - buffering or Mg - sequestering capacity of sub cellular organelles.

Very little knowledge had been acquired regarding cellular mechanisms that regulate intracellular Mg. Eventhough Romani *et al* (1993) argued that animal cells are well protected from Mg - influx down the electrochemical gradient by the low permeability of cell membrane , they also pointed out the possibility of a slow inward Mg - leak which must be balanced by an equivalent efflux to maintain Mg - homeostasis. Earlier in 1979 , Spah and Fleckenstein had reported the existence of a third transmembrane transport system besides the fast Na and slow Ca - channels , which preferentially carries an inflow of Mg^{2+} - ions. Magnesium uptake also occurs in exchange for Na^+_i by reversal of Na - Mg antiporter (Handy *et al* , 1996) , which is regarded as the mechanism of Mg - extrusion.

Certain conditions such as hormonal stimulation were found to induce Mg - extrusion . Romani *et al* (1993) observed that addition of norepinephrine or isoproterenol like β - adrenergic agonists elicits large Mg - efflux from rat heart.

Mechanisms that control $[Mg^{2+}]_i$ are different in Mg - depleted and Mg - loaded conditions. Handy *et al* (1996) suggested a decrease in $[Mg^{2+}]_i$ below normal in cells kept in Mg - free medium. But loss of membrane integrity or cell death was not reported. On reperfusion with basic medium cells recover their normal $[Mg^{2+}]_i$. In their opinion this recovery is due to reversal of Na - Mg exchange activity.

Magnesium as a therapeutic agent : Intravenous Mg supplementation to treat ventricular arrhythmia associated with digoxin toxicity was practised as early as 1935 by Zwillinger (Millane and Camm , 1992) . Since then there have been numerous case reports of the efficacy of Mg in both supraventricular and ventricular tachycardia . Since the Cardiac Arrhythmia Suppression trial conducted in US (Denes *et al* , 1991), the benefits of this safe and cheap "natural " drug was more readily appreciated and the use of Mg as an antiarrhythmic agent became popular (Woods *et al* , 1992). According to them , administration of 5 - 10 mM $MgSO_4$ over a few minutes will reduce digoxin associated arrhythmia and terminate many sustained ventricular tachycardias . Mg - therapy by oral physiological administration and also through Mg - infusions was reported by Durlach *et al* (1994) . When given at physiological doses , therapy with Mg , corrects the alterations in cellular function resulting from Mg - deficiency , where as at higher dosages which induces hypermagnesemia , Mg possesses pharmacological effects such as inhibition of Ca - influx , which may alter the electrophysiological properties of heart cells . A ubiquitous Ca - channel blockade mechanism is the main and well established way of action of Mg at pharmacological levels (Weiss and Lasserre ,

1994). This suggests that in humans in addition to its physiological role , Mg can also acts as a pharmacological agent .

Effects of magnesium - insufficiency on cardiac contractility : In a classic description of Mg - deficiency , Whang and Welt (1963) described cerebral irritability with muscle tetany and ataxia in dogs . Rapidly progressive hypomagnesemia was associated with severe hypokalemia that was resistant to treatment with K - supplementation . Histologically there was calcification of the myocardium with pathological arterial fibrinoid changes . A progressive cardiomyopathy was seen with a rise in intracellular calcium and mitochondrial calcium deposition (Heggtveit , 1965).

Several experimental observations suggest that subclinical hypomagnesemia can be due to either chronic dietary deficiency , pharmacological interventions or consequent to pathologic situations (Khalil , 1999) . In human cardiac transplant recipients receiving cyclosporin , Mg - deficiency was observed and this myocardial Mg - depletion was associated with Ca - overload . In postmortem studies of ischemic heart diseases , myocardial Mg - content was upto 20% lower and Ca - content upto 6 times higher than in non - ischemic controls (Johnson *et al* , 1979) . Reduced levels of Mg in serum or red blood cells correlated with the occurence of hypertension (Dyckner and Wester, 1983).

Alteration in Mg - concentration could lead to increased permeability of the cell membrane . Mg - deficiency can also increase the membrane fluidity . Both types of changes could be caused by looser packing among the molecules in the bilayer (Heaton *et al* , 1984 ; Heaton and Rayssiguier , 1987).

Increased intracellular Na and Ca - levels were found to be associated with hypomagnesemia . Cardiotoxicity of Mg - deficiency is augmented by inhibition of Na,K-ATPase , which leads to Na - accumulation . High level of intracellular Na^+ potentiates reversal of Na - Ca exchange resulting in an increased intracellular Ca - level (Bloom , 1988) . It must be emphasized that the degree of Ca - accumulation can be high enough to cause morphologically obvious cardiac myocyte calcification usually associated with cell death .

Hypermagnesemia : Clinically significant states of Mg - excess are rare . The most common cause of hypermagnesemia is renal failure . There is evidence that total body stores of Mg are increased in chronic renal failure (Cantigulia *et al* , 1972) . In contrast to the planned therapeutic hypermagnesemia , elevated serum levels can occur when Mg - containing drugs usually antacids and cathartics are ingested chronically by individuals with renal insufficiency (Mordes and Wacker , 1978) , and extreme hypermagnesemia has toxic effects on the heart that are primarily confined to arrhythmias and conduction disturbances . In acute extreme hypermagnesemia it is the neuromuscular blocking action of Mg , resulting in respiratory muscle paralysis that causes death .Mg - containing medications in patients with significant renal disease should be avoided , otherwise close monitoring is necessary.

Reduced tolerance to stress in magnesium insufficiency : Seelig (1994) has pointed out that stress conditions if associated with Mg - deficiency paradoxically increases the risk of cardiovascular damage including arrhythmias . Gunther (1991) suggested

that in Mg - deficiency , intracellular Ca and Fe - contents were increased and more catecholamines was released , particularly when Mg - deficient animals are additionally stressed . Cyran *et al* (1992) suggested that capacity of immature myocardium to regulate intracellular Ca^{2+} is diminished during stress . Kramer *et al* (1997) demonstrated that progressive development of cardiovascular lesion formation due to Mg - deficiency amplified myocardial vulnerability to toxic agents and induced stress conditions including ischemia . Ising et al (1981) observed that chronic exposure to noise stress can enhance the chance of cardiac hypertrophy in Mg - deficient rats. Cardiotoxicity to calcium was higher in Mg - deficient rats , cardiac output was low and supplementation of cadmium to Mg - deficiencient diet was also alleviated myocardial necrosis and Ca - overload , compared to hearts of rats on Mg - sufficient diet (Nishiyama *et al* , 1990) .

Age related changes in Mg - homeostasis : Biological aging is associated with changes that affect the functional capacity of the heart . Tonitou *et al* (1987) conducted a study which underscores the high prevalance of Mg - deficiency in an unselected elderly population . Even in the absence of disease conditions , low concentrations of the cation was noticed in the aged . Reduction in the dietary intake and decreased intestinal absorption may explain atleast in part this deficiency (Seelig , 1981) . Aging constitutes a risk factor for Mg - deficit . Primary Mg - deficit originates from two etiological mechanisms : deficiency and depletion . It may be due to nutritional insufficiency , which is more pronounced in institutionalized than in free living aging groups (Durlach *et al* , 1993) . Magnesium deficit may participate in the clinical pattern of aging : mainly neuromuscular , cardiovascular and renal symptomatologies , as reported by

Durlach *et al* (1998). Villano *et al* (1997) reported that as age progresses, several alterations in electrolyte balance occur including modifications of Mg - homeostasis. These changes can be induced by reduced Mg - intake, reduced intestinal absorption and increased renal excretion of the ion. The age associated changes in Mg - metabolism can lead to a fall in intracellular concentration of the ion. Hypomagnesemia is now known to be a common problem with potentially dangerous heart rhythm abnormalities. Elderly persons are reported to be at greater risk for hypomagnesemia. Since hypertension and other cardiovascular disorders become more common with aging, many elderly persons take diuretics. The tendency to absorb Mg^{2+} from food become less due to diuretic treatment (Harward heart letters, 1991). If their diet tends to be low in Mg - content, then the Mg - intake will be significantly less.

The influence of $[Mg^{2+}]$ on the basal or stimulated activity of adenylate cyclase from the hearts of young (1 month old) and aged (24 month old) rats has been investigated *invitro* (Pignatti *et al*, 1993). The basal activity of cardiac adenylate cyclase, and its responsiveness to stimulatory or inhibitory effects, declined with age, probably due to alterations at the catalytic moeity of the transduction system. Pignatti *et al* (1993) suggested that aging leads to a higher requirement for Mg^{2+} at the allosteric site on the catalytic moeity, whose occupancy is essential for the full expression of stimulated activity. Gunther (1991) is of the opinion that severe chronic Mg - deficiency, particularly when combined with stress, may increase the formation of oxygen free radicals and aging.

Durlach in 1989 reported that in developed countries, the recommended dietary amounts of magnesium have been set at 6 mg / kg / day. The magnesium

requirements for optimal health in the adult population depend on constitutional conditioning factors . They may intervene at every stage of Mg - metabolism : absorption , circulation , storage and excretion . Among the multiple interactions , it is important to emphasize that the maintenance of a Ca / Mg ratio close to 2 in the intake is necessary . According to Durlach , magnesium - deficit and stress reinforce each other in a pathogenic vicious circle . Further studies are necessary to assess the accurate place of Mg - deficit in normal physiology and physiopathology of aging .

CHAPTER - III

DESIGN OF THE STUDY

The inotropic variation in myocardial response has been investigated using the isolated cell - culture model .

Ventricular cardiac myocytes were isolated from adult male Sprague Dawley strains of rats of three different age groups. The mechanical response has been recorded as a function of change in cell length by optical methods . Experiments were designed in keeping with the objectives of the enquiry .

The main objective of this study was to assess the age - related variation in functional response of cardiomyocytes to sub optimal levels of extracellular magnesium ($[Mg^{2+}]_o$).

The following is the methodology employed for the study ;

Setting up of experimental model of isolated cardiac muscle cells to assess myocardial mechanics : The procedure for isolation and culture of cardiomyocytes from adult rat had to be established in the laboratory. This required modification of the existing procedures to obtain satisfactory yield of viable cells for experimental studies.

Morphometric and mechanical characteristics of cardiomyocytes isolated from the adult animals of three different age groups : The age - dependent differences in cell geometry and contractile amplitude were recorded by optical methods . A video based edge detection device was used to measure the cardiomyocyte mechanics non - invasively.

Inotropic response to varying levels of $[Mg^{2+}]_o$: Changes in extracellular milieu can lead to variations in contractility . As the response to Mg - insufficiency may vary with age , the cardiomyocyte mechanics were recorded separately for the three different age groups . In an attempt to correlate the variation in contractility with changes in ion concentration , the cytosolic levels of the ions Ca^{2+} , Mg^{2+} , Na^{+} and K^{+} were measured spectrofluorimetrically using their respective fluorescent probes .

Inotropic reponse to ion - channel modulators : Variation in contractility associated with age or level of $[Mg^{2+}]_o$ may be consequent to variation in the distribution or function of ion - channels that modulate Ca - flux . The influence of intrinsic changes (age - related) and extrinsic factors (extracellular Mg - concentration) on the function of channels was assessed by recording the inotropic response consequent to exposure to channel specific antagonists .

MATERIALS

Chemicals and their source :

HEPES modified Medium - 199 , Taurine , HEPES, Collagenase type - I , Trypsin , EGTA , Ryanodine , Caffeine , Manganese chloride , Ouabain , Verapamil, Caffeine , Acetoxy methyl esters of fura - 2 , Mag fura - 2 , SBFI , PBFI and Fetal Bovine Serum were purchased from Sigma chemical company , St.Louis , USA. Ca , Mg - free Medium - 199 was purchased from HiMedia , India .

Sodium chloride , Potassium chloride , Sodium dihydrogen phosphate , Magnesium chloride , Glucose , Cadmium chloride , Potassium dihydrogen phosphate , Fatty acid free fraction V BSA , Nickel chloride , Sodium dodecyl sulphate , Sodium hydroxide , Concentrated HCl , HNO_3 and Ether were obtained from Sisco Research Laboratories , India.

Heparin , Gentamycin and penicillin were purchased from Gland Pharma Ltd, India . Culture dishes were purchased from Nunc , Denmark .

Instruments used :

CO_2 Incubator (Forma scientific , UK) , High speed refrigerated centrifuge (Hitachi , Japan) , Weighing balance (Sartorius , Germany) , pH meter (Elico , India) , Spectrofluorometer (Shimadzu , Japan), Cell length monitor (HVS Image Analysing , Syrray , UK) , Inverted microscope with phase contrast optics (Diaphot TMD , Nikon , Japan), High resolution CCD camera (Nikon , JVC - TK - 1280E , Japan). Strip chart recorder (L & T Gould electronics , Mysore) , VCR , TV

METHODOLOGY

Isolation and culture of cardiomyocytes :

Cardiac myocytes were isolated following the technical details available from published papers (Young , 1976 ; Berg *et al* , 1989 ; Bouron *et al* , 1992 ; Harding *et al* , 1990). As the cell yield was not satisfactory various modifications to the existing methods were carried out with the objective of maximizing the yield of viable Ca - tolerant myocytes.

Sprague Dawley strains of male rats belonging to three different age groups ; 2 - months , 6 - months , 12 - months were used as the source of cells. Developmental maturation is complete at 1 month of age (Siebrits and Barnes , 1989) , and symptoms of senescence appear only by 15 - 17 months of age (Quigley *et al* , 1990) . So the span of 2 months to 1 year is regarded as mature adults . To avoid the variation due to sex only male animals were taken up for the study .

Isolation of the heart : The rats were subjected to deep ether anesthesia and the heart was quickly excised through a thoracic incision and placed in Kreb's Ringer buffer containing 1 mM calcium and heparin (25 IU / ml). HEPES modified Kreb's Ringer buffer (KRH) of the following composition was used (in mM) : NaCl - 117 , KCl - 5.7 , $NaHCO_3$ - 4.4 , NaH_2PO_4 - 1.5 , $MgCl_2$ - 1.7 , HEPES - 21.1 and Glucose - 11.7 . The pH was adjusted to 7.4 . All the steps were carried out at 37°C and the solutions continuously gassed with 100% oxygen.

Isolation of myocytes : The chunk method and perfusion method are the two methods commonly employed for isolation of myocytes . Both the methods were tried out .

Chunk method : The procedure followed by Harding *et al* (1990) was adopted. The isolated heart was rinsed in KRH with 1 mM Ca . The ventricular portion was chopped into 2 - 3 mm^3 chunks with scalpel blades and incubated in Ca - free buffer with 0.05% w / v collagenase type - I and 0.1% w / v BSA (fraction V fatty acid free) for 30 minutes. The suspension was decanted out leaving behind chunks of undigested tissue. The supernatant was discarded , as it usually contains damaged cells from the cut surface and red blood cells. The chunks were reincubated in the same buffer containing 1% BSA and collagenase (1mg / ml) for 45 minutes with intermittent shaking. At the end of 45 minutes , the suspension was strained through 300 μm gauze , to remove undigested tissue bits and centrifuged at 20 g for 2 minutes. Cells were resuspended in the same medium with 1% BSA and Ca - concentration was increased gradually to 0.5 mM . The centrifugation was repeated and the cells were pelleted.

Langendorff - perfusion method :

Langendorff had devised in 1895 the isolated heart system for experimental studies , which is still widely used . In the Langendorff heart system , the perfusion fluid is provided to the heart through the aorta , as a result of which the valves separating the aorta and the left ventricle , the aortic valves will be closed and the perfusion fluid by retrograde perfusion is forced along the arteries running along the surface of the heart , the coronary arteries . Thus the perfusion fluid enters into

the myocardium through the coronary arteries and then leaves by the coronary veins to drop out through the bottom . The provision of adequate oxygen is critical . Equally important is the maintenance of the correct ionic and pH conditions of the perfusion fluid - a condition not adequately met until 1932 , when Kreb's and Hensleit described in detail the ionic composition of the mammalian blood . Their bicarbonate buffer system resembles that of human blood in ionic composition and is now considered as a standard for heart perfusion system . Most established vertebrate cells tolerate osmolality in the range 260 - 320 mOsm / kg (Waymouth , 1970) .

For isolation of myocytes the principle of Berg et al (1989) was adopted . A photograph of the set up used for the perfusion of heart is given in Figure-3.1 . Isolated heart was placed in KRH buffer containing 1 mM $CaCl_2$ and heparin (25 IU / ml). Then the heart was cannulated via the aorta and perfused retrogradely with the same solution at a flow rate of 10 ml / minute for 5 minutes , inorder to flush out the blood and to ensure proper perfusion. Calcium free buffer containing 0.1mM EGTA and 20 mM taurine was perfused subsequently for 5 minutes inorder to relax the myocardium by depletion of calcium . This was followed by perfusion with Ca free , EGTA and taurine free buffer for 5 minutes , the main purpose being , to wash out the EGTA as it may damage the heart cells . The same Ca-free solution containing 0.06%(w/v) collagenase type - I and 0.1% (w/v) fatty acid free fraction V BSA was perfused by recirculation for 25-30 minutes . By this time , the heart became flaccid losing its toughness because of the dissolution of the extracellular matrix . The ventricular portion was minced and incubated in the buffer with 0.05% collagenase (type - I) and 0.8% BSA for 10 minutes with gentle trituration . The cell suspension was strained through 300μm gauze to remove undissociated

pieces of tissue. The suspension was centrifuged at 20g for 2 minutes at room temperature. Centrifugation at 20g for 2 minutes selectively isolated the healthy myocardial cells separating them from nonmyocardial cells. Cells so isolated were resuspended in the Ca-free buffer containing 0.005% (w/v) trypsin and 1% BSA . Calcium concentration was steadily increased to 0.5mM in 10 steps at 5 minute intervals. The centrifugation was repeated and the cells were plated into polystyrene dishes in Medium 199 (Sigma) containing 1 mM Ca , 0.8 mM Mg , 20mM $NaHCO_3$, 5 mM HEPES and gentamycin (50μg/ml) supplemented with 4% Fetal Bovine Serum (Sigma). Cultures were incubated for 2 hours in a humidified atmosphere (99% humidity) with 5% CO_2 at 37°C .The culture dishes were preplated overnight with serum containing medium to promote cell adhesion.

Cell count and cell viability of the isolated cells were recorded and this served as an index for comparison of cell yields by the different methods. A cell was considered viable and healthy if it had the following features ;

a) rod shaped morphology with clearly visible cross striations,

b) quiescent when unstimulated or has spontaneous contractile waves at a rate of not more than one or two per minute and uniform response to electrical stimulation.

c) absence of blebs and presence of well defined cell edges .

Dead and damaged cells usually have blebs and are hypercontracted . Cardiomyocytes so isolated are suitable for physiological , biochemical or pharmacological studies as the functional characteristics are well preserved despite the fact that they have been physically uncoupled from their usual syncytial state.

The two crucial stages being the initial perfusion for depletion of Ca^{2+}, and the final step of isolation of Ca-tolerant myocytes; the effect of following alternatives were assessed with the objective of improving the cell yield:

I Initial perfusion for relaxation of the myocardium

a) inclusion of 20 mM taurine along with 0.1 mM EGTA ;

b) inclusion of 0.1 mM EGTA in the perfusate ;

c) inclusion of 0.005 % (w/v) trypsin ;

d) inclusion of 25 μM calcium in the perfusate ;

e) use of nominally Ca-free buffer.

The last two alternatives are popularly used by the other workers .

II Enhancement of calcium for isolation of Ca-tolerant myocytes

a) inclusion of 0.005 % (w/v) trypsin ;

b) inclusion of 0.5 mM EGTA ;

c) inclusion of 0.005 % trypsin with 0.5 mM EGTA ;

d) calcium free Kreb's Ringer buffer - as commonly used.

Measurement of cell geometry :

The cell geometry of myocytes was measured on a calibrated video screen. The culture dish containing relaxed cardiomyocytes were placed on the incubated stage of an inverted phase contrast microscope. Measurements were made by video imaging the cells with the help of a high resolution CCD camera attached to the microscope. The video screen was calibrated using a stage micrometer . Cell length and sarcomere length were measured. For determination of sarcomere length , the span of atleast 10 consecutive sarcomeres was measured and the mean was taken as the sarcomere length .

Measurement of cardiomyocyte mechanics :

Myocardial contractility is a parameter extremely difficult to define . However , it is possible to record the change in contractility in response to a variable . A variety of optical techniques have been developed to measure length changes during the contractile cycle of cardiac myocytes. These methods can be used to assess both the whole cell and sarcomere shortening dynamics , to evaluate the "contractility" of myocytes in response to various inotropic or electrophysiological alterations (Delbridge and Roos , 1997) . Based on this principle , the contractile amplitude of cardiomyocytes were measured by the method of Harding *et al* (1988) ; where the force of contraction is computed as a function of change in cell length .

Healthy myocytes adhere to the culture surface within 1-2 hours of plating . The cells were incubated in fresh , O_2 equilibrated medium containing 1 mM calcium . Figures 3.2(a) and 3.2(b) show the set up used for recording the contractile amplitude . The dish with myocytes was mounted on the stage of an inverted microscope and maintained at 37°C using a stage incubator . The cells were viewed with phase contrast optics . To elicit contractions , cells were field stimulated at 0.5 Hz using bipolar constant current pulses. Stimuli were delivered to cells via platinum electrodes positioned 1 cm apart. The cells were preincubated for a minimum of 3 minutes before recording the contractions . A video digitizer edge detection device (SP144) supplied by HVS Image Analyzing (Kingston , Surray) was connected between the camera and the video monitor . The image was sent by the video camera to the edge detector . The image was transmitted from the edge detector to the television screen through the video cassette recorder . The camera was rotated so that the selected cell was aligned with its long axis vertical

to the screen . The magnification was so adjusted that the cell occupied 50-90 % of the height of the video screen.

A rectangular window of adjustible height and width projected by the cell length monitor was superimposed over the selected cell. A variable control sets a cut-off grey level. Tones lighter than this appears as white and dark as black. The grey level cut off was adjusted so that the cell edge appeared white on a black background (Figure - 3.3) .

SP144 digitizes and displays 512 of the possible 625 lines generated , by the camera scan. Cell length is the primary measurement which is got by counting all the lines between the top and bottom of the cell. The extent of contraction as given by the length is output to a strip chart recorder every 20 ms.

The video screen was calibrated using a stage micrometer . The cell length was recorded and the extent of shortening was calculated as percent of cell length. The extent of shortening was determined from a mean of atleast 20 consecutive contractions.

Measurement of intracellular ionic levels :

The four important cations having a regulatory role in cardiomyocyte contractility are Ca^{2+} , Na^{+} , K^{+} , Mg^{2+} . In an attempt to correlate the mechanical changes with the variation in ion concentration , the cytosolic levels of the ions were measured by dual - excitation fluorescent method , using ion sensitive cell permeant fluorescent probes . The ratio of the fluorescence intensity elicited at two different wavelengths was determined and taken as the measure of the cytoplasmic level of the ions . The ratiometric method has the advantage that the measurements

are not influenced greatly by changes in dye concentration or cell volume . The ionic levels were measured at different levels of $[Mg^{2+}]_o$.

After exposing the cells to the desired treatment along with electrical stimulation the cells were incubated in KRH buffer of the same composition , along with the acetoxy methyl ester of the respective fluorescent probes for 60 minutes at room temperature . Loaded cells were then washed with the same buffer without the probe by mild centrifugation at 20g for 2 minutes to remove extracellular and bound dye . The pellet was then suspended in KRH buffer with normal levels of ion under study and placed into quartz cuvette . The fluorescence was measured in the spectrofluorometer at the emission and excitation wavelengths given in the Table - 3.1.

The fluorescent probes alter the excitation spectrum on binding with the cation , but not its emission spectrum. The fluorescence intensity at the peak of the emission spectrum was measured by switching back and forth between the two excitation wavelengths specific to the probe .

Intracellular fluorescence measurements were recorded using a dual wavelength spectrofluorometer . Changes in the 330 / 360 for Na^+ and K^+ and 340 / 380 for Ca^{2+} and Mg^{2+} were considered representative of changes in the intracellular ion - concentration .

Influence of extracellular Mg - concentration on cardiomyocyte contractility :

After stabilisation in the standard culture conditions the cardiomyocytes were exposed to the following levels of $[Mg^{2+}]_o$: (in mM) 1.8 , 1.4 , 0.8 , 0.64 , 0.48 , 0.32 , 0.16 , and nominally Mg free conditions. Cardiomyocytes isolated from the 3

age groups were exposed to different levels of Mg for atleast 10 minutes and inotropic response to variation in $[Mg^{2+}]_o$ was recorded .

Inotropic response to Ca - channel modulators :

Ion channel antagonists are commonly used to assess the functional alteration of different channels to varying physiological and experimental conditions . The influence of age and variation in $[Mg^{2+}]_o$ on inotropic response were assessed in the presence of selected ion channel modulators .

Cardiomyocytes possess specific pathways for Ca-transport across the SL and SR . Changes in the distribution or function of the channels can affect the contractile function . The impact of individual channels can be assessed using competitive channel modulators .

For this purpose , cells that stabilized in culture were exposed to the channel antagonists specified in the Table - 3.2 for 15 minutes and the contractile amplitude was recorded . The change from the base line value was calculated . The contractile amplitude in the Mg - sufficient medium was recorded and taken as the basal value .

Only those cells that attained a steady state contraction was used . The change in contractile response induced by the antagonist was taken as the contribution of the channel to the inotropy of the myocyte .

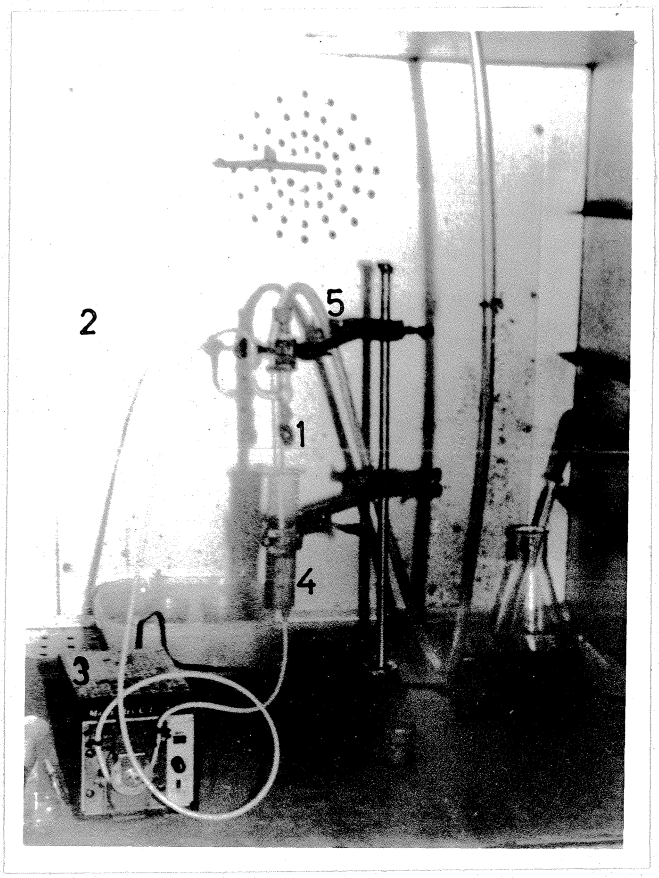
The inotropic response to the antagonist was recorded in the presence of sufficient Mg (0.8 mM) and at suboptimal level (0.48 mM) in all the three age groups.

Statistical analyses :

The data are presented as mean (± SEM) values for each set. Each experimental observation was based on 10 - 20 cells from atleast 3 separate adult ventricular myocyte preparations . Difference in selected means were evaluated via unpaired Student's t-test or Scheff's test with a significant difference said to exist with $p < 0.05$. The group means were first compared by ANOVA and if a difference was found , then pairs of experimental means were compared .

FIGURE - 3.1

SET UP OF LANGENDORFF - PERFUSION SYSTEM



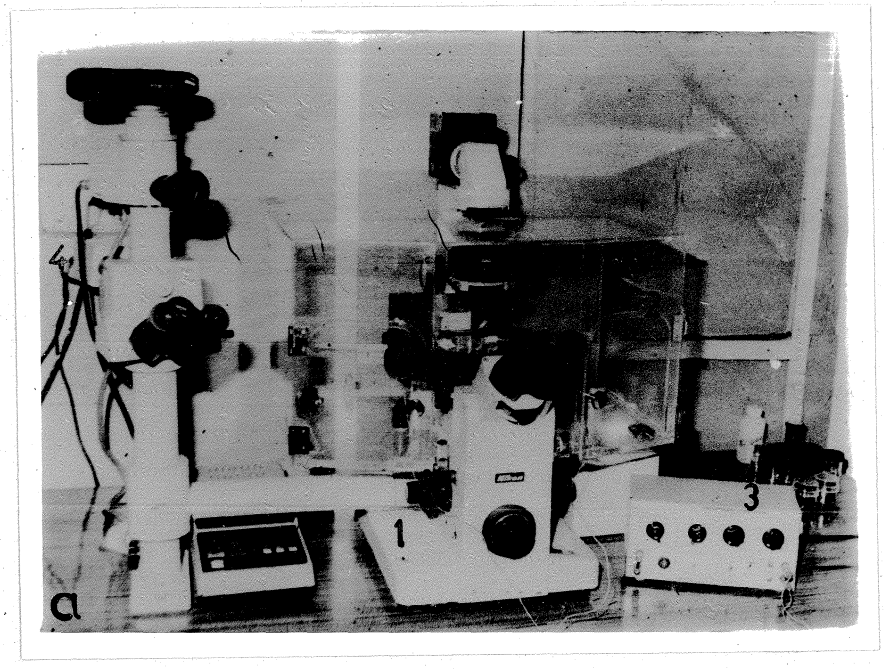
(1) Isolated rat heart (2)Incubator

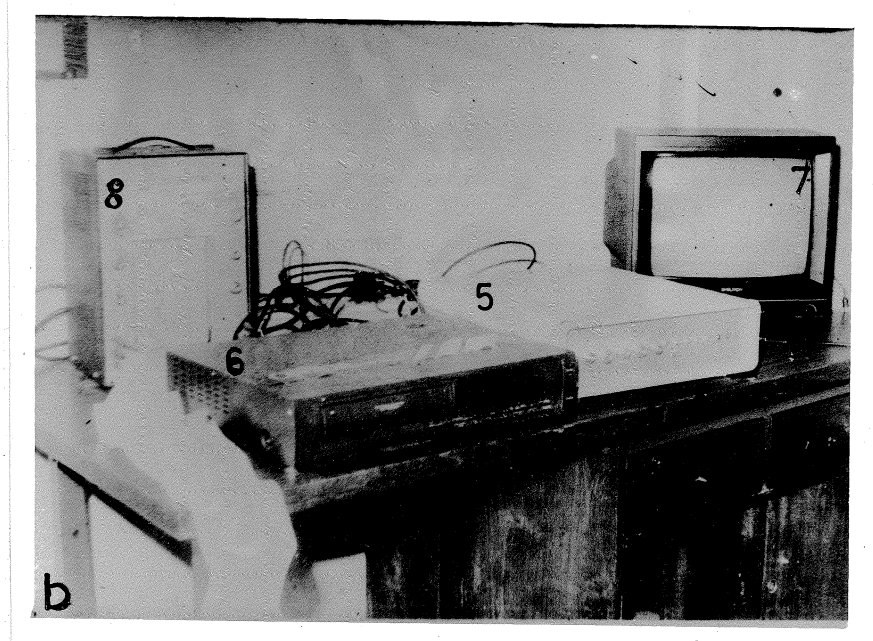
(3) Perfusion pump (4) perfusate

(5)Oxygen

FIGURE - 3.2

SET UP USED FOR RECORDING CONTRACTILE AMPLITUDE





(1) Phase contrast microscope (2) Incubator (3) Stimulator

(4) Video camera (5) Cell length monitor (6) Video casette recorder

(7) Video screen (8) Strip chart recorder

FIGURE - 3.3

CARDIOMYOCYTE WITH THE WINDOW SUPERIMPOSED

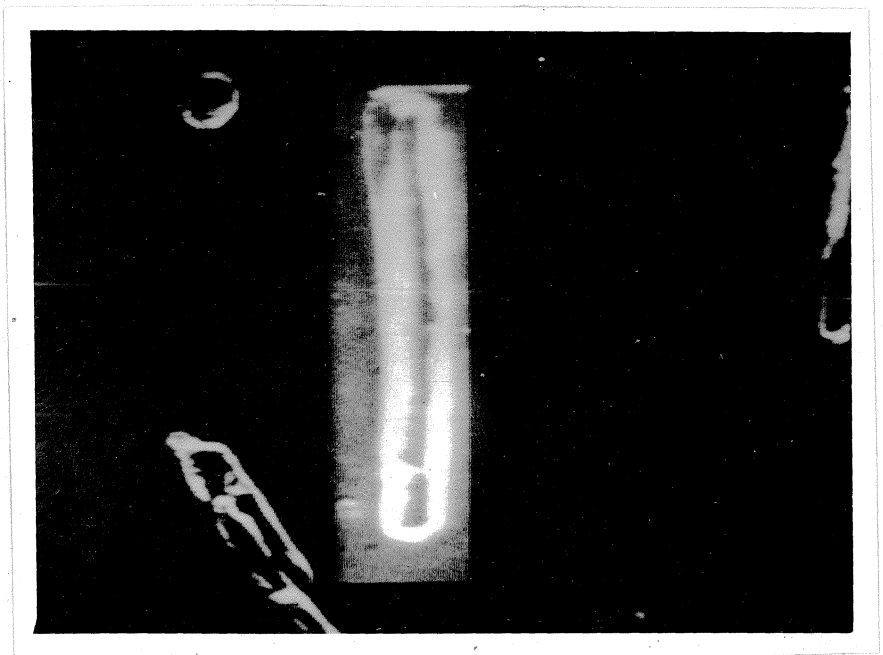


TABLE - 3.1

Ion sensitive fluorochromes for spectrofluorimetric measurement of intracellular ion levels :

Ion sensitivity	Fluorescent probe	Concentration (μM)	Excitation (λ) I	Excitation (λ) II	Emission (λ)	References
Ca^{2+}	Fura - 2	5	340	380	510	Cobbold and Rink , 1987
Mg^{2+}	Mag fura -2	5	340	380	505	Lattanzio and Bartschat , 1991
Na^{+}	SBFI	7	330	360	505	Minta and Tsien , 1989
K^{+}	PBFI	7	330	360	505	Jezek *et al* , 1990

TABLE - 3.2

Ion channel modulators - their specificity and concentrations used :

Channel specificity	Modulator	Concentration	References
SL - L - type Ca - channel	Verapamil	1 μM	Tada *et al* , 1982 ; Yang *et al* , 1996
SL - T - type Ca - channel	$NiCl_2$	40 μM	Hagiwara *et al* , 1988; Marban and O'Rourke , 1995
Na - Ca exchanger	$MnCl_2$	0.2 mM	Coetzee and Opie , 1992
Na , K - ATPase	Ouabain	0.3 mM	Kennedy *et al* , 1986
SR Ca - release channel	Ryanodine	1 μM	Seguchi *et al* , 1986; Su and Chang , 1993
SR Ca - pump	Caffeine	10 mM	O'Neill and Eisner , 1990

CHAPTER - IV

ANALYSIS OF DATA

RESULTS

The experimental results are presented under the following headings :

I . Cardiomyocyte culture and measurement of heart muscle cell mechanics

II . Age dependent variation in cardiomyocyte mechanics

III . Mechanical response of cardiomyoctes to variation in $[Mg^{2+}]_o$

IV . Influence of suboptimal levels of extracellular Mg^{2+} on the functional response of cardiomyocytes to ion - channel modulators in the different age groups

I . Cardiomyocyte culture and measurement of heart - muscle cell mechanics :

Cardiac myocytes from adult rat heart were isolated following protocols available in literature . The two methods widely used are chunk method and perfusion method . The chunk method was tried out first (Harding *et al* , 1990) . The cell yield was found to be very low (< 10% of viable cells) . So Langendorff enzymatic perfusion method (Berg *et al* , 1989) was adopted . Cells were isolated following standard procedures as reported in literature , but the yield was not satisfactory . Certain modifications were introduced to optimise the cell yield.

Adult male rats of the Sprague - Dawley strain were used for isolating ventricular myocardial cells . Six month old rats were used for the standardisation of the technique . Large variations in cell yield and viability were observed depending on the composition of the medium used during the initial perfusion for Ca - depletion and relaxation of the myocardium . Incorporation of 0.1 mM EGTA in the perfusate was found to be beneficial compared to trypsin containing buffer or the buffer alone . The presence of taurine in association with EGTA led to further improvement in yield . The modification resulted in more than seven fold increase in cell yield at

this stage of the isolation procedure (11 . 92 ± 0.77 x 10^6 cells / heart) (Table - 4.1) . Inclusion of calcium in the perfusate , levels as low as 25 μM damaged the cells considerably.

During the final step of reintroduction of Ca^{2+} , trypsin in the medium was found to have a significant effect on cell viability . With the inclusion of 0.005% trypsin in the medium , the yield of viable cells was two and a half times more than that in the absence of trypsin (Table -4.2) . Cell viability and adhesion were found to be affected with higher concentrations (0.1 %) of trypsin .

Therefore , the optimum method for cell isolation is one where Ca - depletion is carried out with EGTA (0.1 mM) and taurine (20 mM) included in the perfusate , followed by collagenase digestion for cell dissociation ; and Ca - tolerant cells are isolated with trypsin (0.005%) included in the medium at the time of Ca - repletion . The cell yield with the modifications introduced was comparatively higher than the reported values (Table - 4.3)

The cells so isolated had rod shaped morphology (Figure - 4.1) with clearly visible cross striations and no large blebs or areas of hypercontracture (Figure - 4.2) ; and were characterised by the absence of spontaneous contractions when unstimulated in Ca - containing medium . The variation in viability observed with the use of Ca - chelators highlights the importance of the maintenance of Ca^{2+} - homeostasis , both in extracellular and intracellular milieu .

The contractile parameters were measured using a video based edge detection device . Figure - 4.3 shows a typical recording of the contractile measurements . Contractile amplitude was found to be 4.44 ± 0.12 % . These cells displayed steady contraction amplitude and diastolic cell length at basal stimulation .

The extent of cell contraction was measured as percentage change in diastolic cell length .

The Ca - tolerant cells were viable even after 10 hours of incubation in the culture medium .

The same procedure could be adopted for isolation of cells from 2 month and 12 month old rats .

II . Age dependent variation in cardiomyocyte mechanics :

Adult rats of three different age groups were used for the experimental studies : 2 months , 6 months and 12 months . Their body weight and heart weight was found to increase with age (Table - 4.4) , but the heart weight / body weight ratio showed a decreasing trend . The increase in body weight is ~ 190 % at 12 months compared to 2 months , while the increase in the heart weight is ~ 100 % .

Age dependent variation in cell geometry :

The cell geometry of isolated cardiomyocytes from three age groups were found to be significantly different (Table - 4.4) . Cell length and sarcomere length increased with age . Increase in cell length (49 %) was associated with an increase in sarcomere length (39%) at 12 months compared to 2 months .This age dependent variation in cell geometry can be associated with cellular remodelling resulting in functional variation .

Age dependent variation in contractile amplitude :

Contractile amplitude normalized to cell length was found to increase with age (Figure - 4.4) the value in 12 month old animals showed 25 % increase

compared to that in the 2 month old . Differences in contractile performance observed may be due to a number of factors . Basal level of intracellular [Ca^{2+}] being an important variable , diastolic Ca^{2+}_i - level was measured spectrofluorimetrically . The $[Ca^{2+}]_i$ - levels were not significantly different in the three age groups . The experimental variation is found to be high . Age associated variation in contractile amplitude may be related to age - dependent variation in Ca^{2+} - flux , rather than differences in the basal levels. This can be brought about by differences in distribution or functioning of ion - channels . Age related variation in ion channels was therefore assessed using selective modulators .

Age associated variation in the mechanical response to sarcolemmal and sarcoplasmic reticular ion - channel modulators :

Cardiomyocytes were exposed to different channel modulators which selectively inhibit a particular channel with minimum effect on other channels .

SL - L - type Ca - channel blocker (Verapamil - 1μM) : Verapamil is an antihypertensive and antiarrhythmic agent known to depress cardiac contractility by blocking the voltage gated slow Ca - channel (Tada *et al* , 1982 ; Yang *et al* ,1996). A negative inotropic effect was observed with verapamil in the three age groups (19.5% , 23.87% and 56.43% reduction in contractile amplitude in 2 , 6 and 12 months respectively) . The response was found to vary significantly between groups (F , $p < 0.05$) . The negative inotropic effect was significantly higher in 12 month old cardiomyocytes (Figure - 4.5) . Exposure to verapamil was associated with a relative decrease in Ca^{2+}_i - level in all the three age groups (Figure - 4.5)

SL - T - type Ca - channel blocker ($NiCl_2$ - 40 μM) : SL - T - type Ca - channel function was examined by using $NiCl_2$ (Hagiwara et al , 1988 ; Marban and O'Rourke , 1995). Significant negative inotropy was observed in 6 month and 12 month old rats (17.15% and 24.85% of reduction in contractile amplitude respectively) .The difference between groups was significant (F , $p < 0.05$) . In older animals (12 month old) , a significant reduction of diastolic Ca^{2+} was also observed . In 2 month old cardiomyocytes diastolic Ca^{2+}_i - level and contractile amplitude were higher than the base line value , but the increase was not statistically significant (Figure - 4.6) .

SL Na - Ca exchanger blocker ($MnCl_2$ - 0.2 mM) : $MnCl_2$ is accepted as a Na - Ca exchanger blocker (Coetzee and Opie , 1992) . As observed with T - type channel , no significant change was observed in 2 month old animals . The response was different in older animals (F, $P < 0.05$) . In 6 and 12 month old cardiomyocytes , significant negative inotropy was observed (Figure - 4.7) . The reduction in contractile amplitude was found to be 46.14 % and 57.54 % respectively in 6 and 12 month old cardiomyocytes . Diastolic Ca^{2+}_i - level was found to decrease in all the three age groups irrespective of age (Figure - 4.7) .

SL Na , K - ATPase inhibitor (Ouabain - 0.3 mM) : The cardiac inhibitory glycoside ouabain that blocks the Na , K - pump (Kennedy *et al* , 1986) produced significant positive inotropy in cardiomyocytes of higher age groups (83. 51% and 32.91% increase in contractile amplitude in 6 and 12 month old respectively). The inotropic response in 2 month old myocytes was lower than that in older animals (F, $P < 0.05$) Maximum contractile amplitude was observed in 6 month old

cardiomyocytes (Figure - 4.8). The diastolic Ca^{2+}_i - level was found to be enhanced compared to control in all the three age groups . The increase in diastolic Ca^{2+}_i - level was inversely related to age , but the difference was not statistically significant . Though the rise in basal calcium was maximum in 2 month old cardiomyocytes , the inotropic response was comparatively less in this group (Figure - 4.8).

SR Ca - release channel modulator (Ryanodine - 1 μM) : Ryanodine inhibits release of Ca^{2+} from the SR (Seguchi *et al* , 1986 ; Su and Chang , 1993) . In the presence of ryanodine , negative inotropy was induced in all the three age groups . The reduction in contractile amplitude (39.84% , 32.49% and 49.89% respectively at 2 , 6 and 12 months) was more pronounced in 12 month old cardiomyocytes , but the difference was not statistically significant . Diastolic Ca^{2+}_i - level was low and showed the same pattern as that of the mechanical function (Figure - 4.9) .

Effect of SR Ca - pump modulator (Caffeine - 10 mM) : Caffeine is known to prevent uptake of Ca^{2+} by the SR , thereby increasing the basal Ca - level (O'Neill and Eisner , 1990) . Exposure to caffeine induced positive inotropy in all the three age groups (45.86% , 24.53% and 21.39% increase in contractile amplitude at 2 , 6 and 12 months respectively) . The increase in contractile amplitude and the diastolic Ca^{2+}_i was inversely related to age , but the difference was not statistically significant (Figure - 4.10) .

Observations and Inference :

1) Cell length and sarcomere length increases with age .

2) Contractile amplitude normalised to cell length shows age dependent increase .

3) Response to voltage gated Ca - channel blockers (both L - type and T - type channel) were more pronounced in older animals .

4) Older animals were found to be more sensitive to blockers of Na - Ca exchanger activity.

5) Response to Na^+ , K^+ - ATPase inhibitor was found to be maximum in 6 month old cardiomyocytes .

6) The response to SR Ca - pump antagonist decreases with age and that to SR Ca - release channel inhibitor increases with age , but the variation between groups is not statistically significant .

Based on the observations , it is inferred that the contractile amplitude increases with age . Age dependent variation suggests that the distribution / function of Ca - influx channels increase with age . Inotropic response to ion channel modulators depend on age .

III . Mechanical response of cardiomyocytes to variation in $[Mg^{2+}]_o$:

The contractile response to variation in $[Mg^{2+}]_o$ - levels were studied by exposing the myocardial cells to different levels of $[Mg^{2+}]_o$ from 1.8 mM to nominally Mg - free conditions . Osmolality of the medium with various concentrations of Mg^{2+}_o when assessed were in the range 265 - 270 mOsm / kg , which falls within the accepted range (260 - 320 mOsm / kg) for cell - culture studies (Waymouth , 1970) .

The mechanical response to different levels of $[Mg^{2+}]_o$ was found to follow the same pattern in the three age groups . A positive inotropy was observed with decrease in $[Mg^{2+}]_o$, with a peak at 0.48 mM followed by reduction in inotropy

with further decrease in Mg (Figure - 4.11) . The extent of increase in contractility at 0.48 mM $[Mg^{2+}]_o$ was of the order 2 months ≥ 6 months > 12 months compared to the amplitude in 0.8 mM $[Mg^{2+}]_o$. The extent of increase in contractile amplitude at 2 (50.31 %) and 6 (51.69 %) months was statistically significant when compared to 12 months (32.04 %) .

Variations observed in contractile amplitude with variation in $[Mg^{2+}]_o$ were found to be reversible on changing back to Mg - sufficient medium .

Variation in intracellular diastolic cation - levels followed the same pattern as that of contractile amplitude . Positive inotropy was associated with enhanced Ca_i - level at 0.48 mM . The Ca - levels showed a decrease in nominally Mg - free conditions (Table - 4.5) . Na_i - level was maximum at 0.8 mM Mg . $[Mg^{2+}]_o$ above and below this value was associated with decrease in $[Na^+]_i$, but the variation was not statistically significant (Table - 4.6). Intracellular levels of Mg^{2+} and K^+ were unaffected by change in extracellular Mg (Tables - 4.7 and 4.8) .

Observations and Inference :

Extracellular Mg^{2+} level has a significant influence on contractile amplitude . The amplitude increases with decrease in $[Mg^{2+}]_o$; the younger animals being more sensitive to the change in Mg^{2+} .

IV . Influence of suboptimal levels of $[Mg^{2+}]_o$ on the functional response of cardiomyocytes to ion channel modulators in the different age groups :

Serum Mg - levels of animals kept on Mg - sufficient diet was found to be ~0.8 mM and that on Mg - deficient diet was in the range of 0.4 - 0.5 mM Mg (Kumar *et al* , 1996) . So further experiments to assess the effect of suboptimal

levels of $[Mg^{2+}]_o$ on cardiac contractility were carried out at 0.48 mM $[Mg^{2+}]_o$, the level at which maximum inotropic response was observed . The control was maintained at 0.8 mM [Mg^{2+}] . As $[Mg^{2+}]_o$ can influence intracellular Ca^{2+} - levels by affecting Ca - flux , the effect of $[Mg^{2+}]_o$ on the response to ion channel modulators was examined . Due to age - dependent variation in the response to ion channel modulators , the effect of $[Mg^{2+}]_o$ was examined separately for the three different age groups .

SL - L - type Ca - channel blocker (Verapamil - 1 μM) : Exposure to verapamil induced significant reduction in contractile amplitude in the three age groups both in Mg - sufficient and Mg - deficient media (Figure - 4.12) . The age related difference in the negative inotropy observed in control conditions was neutralised to some extent by Mg^{2+}_o - reduction (F , $p < 0.05$) . The reduction in contractile amplitude being 32.16% , 32.38% and 46.06% respectively at 2 , 6 , 12 months compared to the amplitude in 0.8 mM Mg. Intracellular diastolic Ca - level was decreased in the presence of verapamil , but compared to control conditions (0.8 mM + verapamil) , no change was observed (Figure - 4.12) .

SL - T - type Ca - channel blocker ($NiCl_2$ - 40 μM) : SL - T - type channel blocker $NiCl_2$ produced negative inotropy in the three ages in Mg - deficient conditions . Age dependent variation was significant (F , $p < 0.05$) . The response was greater than that in Mg - sufficient medium . The effect was maximum in 12 month old cardiomyocytes (48.61% of reduction compared to 0.8 mM Mg) (Figure - 4.13) . Decrease in contractile amplitude was not associated with a corresponding decrease in diastolic Ca^{2+}_i .

SL Na - Ca exchanger blocker ($MnCl_2$ - 0.2 mM) : Variation in extracellular Mg had a significant influence on the response to Na - Ca exchanger blocker $MnCl_2$. The response was age dependent (F , $p < 0.05$). Cessation of contraction was observed in 2 month old cardiomyocytes at 0.48 mM Mg^{2+}_o , in the presence of $MnCl_2$ (Figure - 4.14) . Contractile amplitude in the presence of $MnCl_2$ was slightly higher in Mg - deficiency than in sufficient Mg in the 6 and 12 month old animals (~ 30 - 50% reduction in contractile amplitude) , but the difference was not statistically significant . In nominally Mg^{2+} - free medium , cessation of contraction was observed in all the three age groups . Contractility regained the basal values on changing back to Mg - sufficient medium . Diastolic level of Ca^{2+}_i was found to be less on comparison with 0.48 mM $[Mg^{2+}]_o$, while comparable to control conditions (0.8 mM Mg + $MnCl_2$) (Figure - 4.14) .

SL Na , K - ATPase inhibitor (Ouabain - 0.3 mM) : Inhibition of the Na^+ - pump with cardiac glycoside ouabain induced negative inotropic response compared to the amplitude at 0.48 mM Mg^{2+} in younger age groups . The response varied with age (F , $p < 0.05$) . In 2 month old cardiomyocytes , Mg did not influence the inotropic response to ouabain (16 - 18% of increase in contractile amplitude at 0.8 and 0.48 mM Mg) ; but in the 6 month old ouabain induced an inotropic response ; the effect was less than that in sufficient Mg (increase in contractile amplitude being 83.51% at 0.8 mM Mg and 59.58% at 0.48 mM Mg) and in 12 month old , the effect of ouabain was more in Mg - insufficiency (increase in contractile amplitude from the base line being 32.91% at 0.8 mM Mg and 47.93% at 0.48 mM Mg) .Diastolic Ca^{2+}_i - levels were elevated in the presence of ouabain (Figure - 4.15) .

SR Ca - release channel inhibitor (Ryanodine - 1μM) : Extracellular Mg had no effect when SR Ca - release channel was blocked with ryanodine (40 - 50 % of reduction in the three age groups). The amplitude of contraction was comparable at both levels of Mg . Diastolic Ca^{2+}_i - levels were not affected by Mg - deficiency , though the levels were lower in older animals (Figure - 4.16) .

SR Ca - pump inhibitor (Caffeine - 10 mM) : The inotropic response to caffeine at 0.48 mM extracellular Mg was not to the same extent as in Mg - sufficient medium especially in 2 and 12 month old animals (32.52 % , 29.71% and 10.61% of increase in contractile amplitude from the baseline at 0.48 mM Mg compared to 45.86 % , 24.53 % and 21.39 % at 0.8 mM Mg in 2, 6 and 12 month old cardiomyocytes respectively) . In Mg - deficient medium , diastolic Ca^{2+}_i - level also showed a corresponding decrease , the levels being lower than the basal values . The difference was not statistically significant (Figure - 4.17) .

Observations and Inference :

1. The negative inotropic response to verapamil , the L - type channel blocker was not significantly affected in Mg - deficiency and the response was comparable in the three age groups .

2. Mg - deficiency augmented the negative inotropic effect of SL - T - type channel antagonist ($NiCl_2$) , older animals being more sensitive to the blocker .

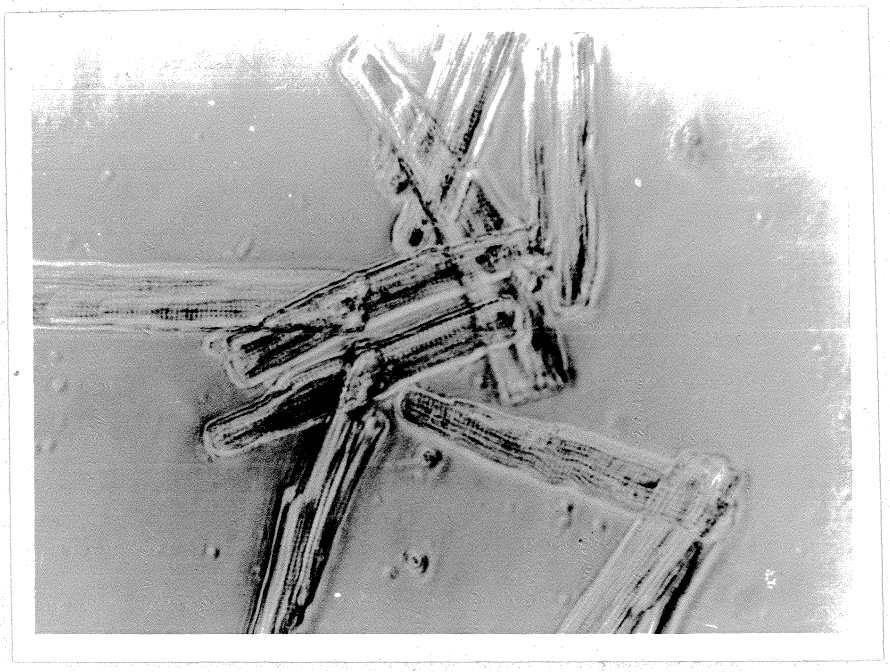
3. The response to Na - Ca exchanger blocker $MnCl_2$ was influenced by extracellular Mg - concentration in 2 month old animals .

4. The extent of positive inotropy with Na^+, K^+ - ATPase inhibitor ouabain, in Mg - deficiency was not as much as in sufficient Mg in 6 month old cardiomyocytes
5. The positive inotropic response to Ca - pump inhibitor was lower, the effect being more pronounced in 2 and 12 month old cardiomyocytes and the negative inotropic response to Ca - release inhibitor was accentuated in Mg - deficiency.

Extracellular Mg has a significant influence on the functional response to ion - channel modulators. An interaction between age and Mg is observed to influence the response to T- type channel blocker, Na - Ca exchanger antagonist and Na, K - ATPase inhibitor.

FIGURE - 4.1

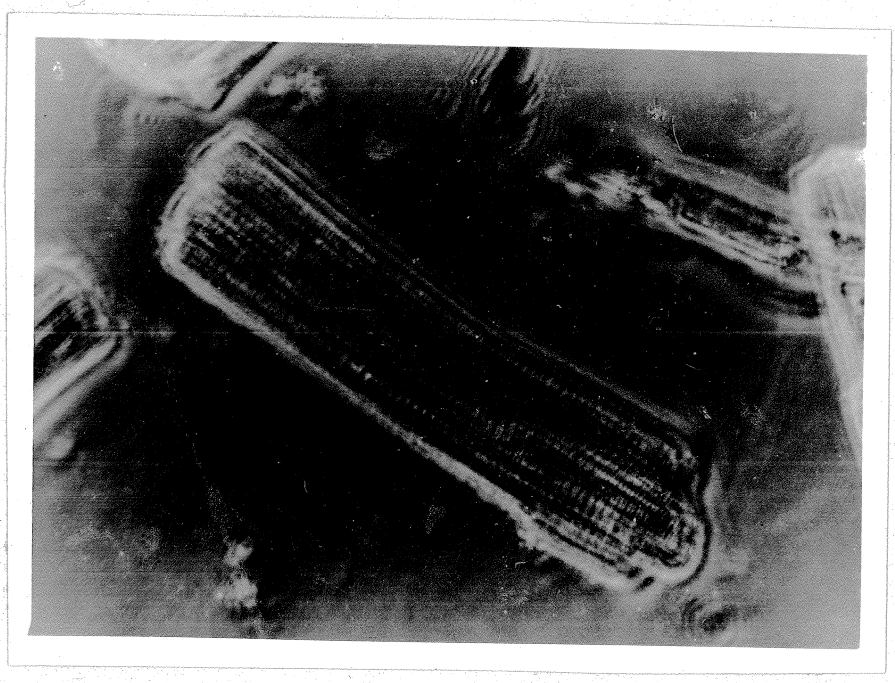
PHASE CONTRAST MICROGRAPH OF ISOLATED RAT VENTRICULAR MYOCYTES



Magnification (280X)

FIGURE - 4.2

PHOTOMICROGRAPH OF A SINGLE VENTRICULAR MYOCYTE



Magnification (560X)

FIGURE - 4.3

TYPICAL RECORDING OF THE CONTRACTILE AMPLITUDE OF CARDIOMYOCYTES

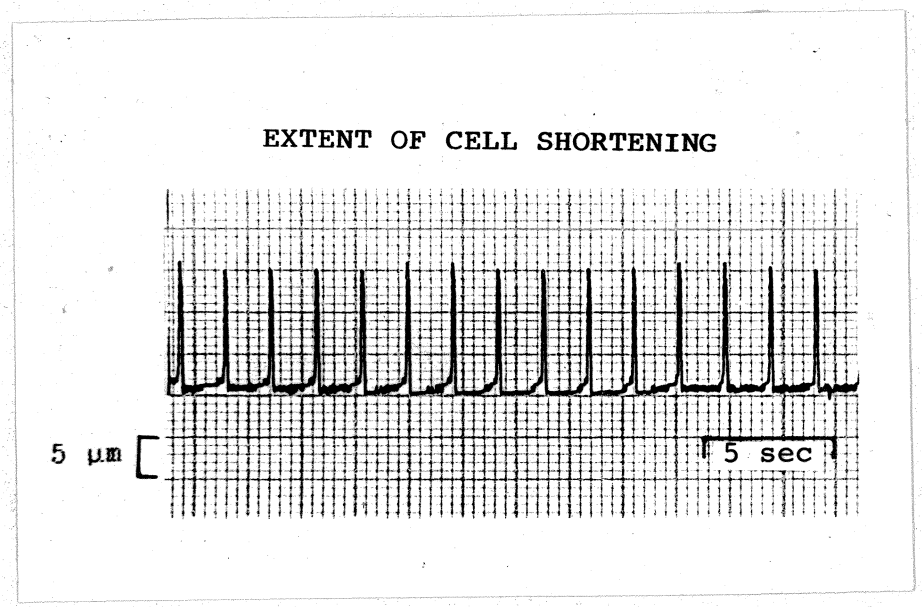
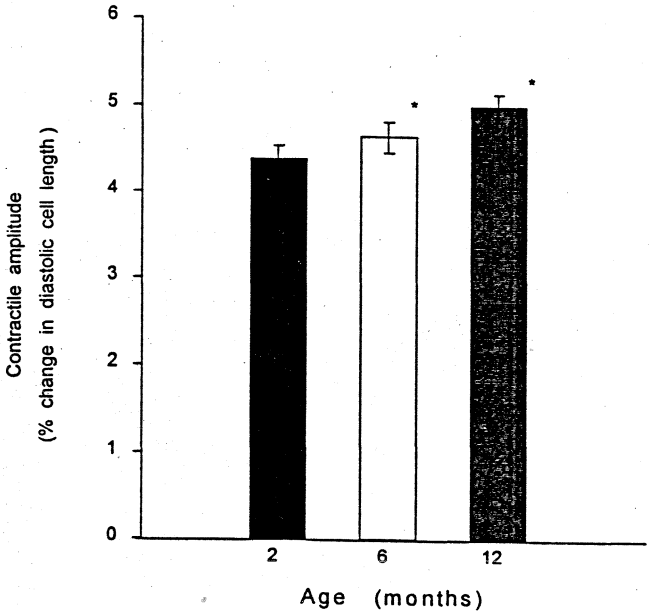


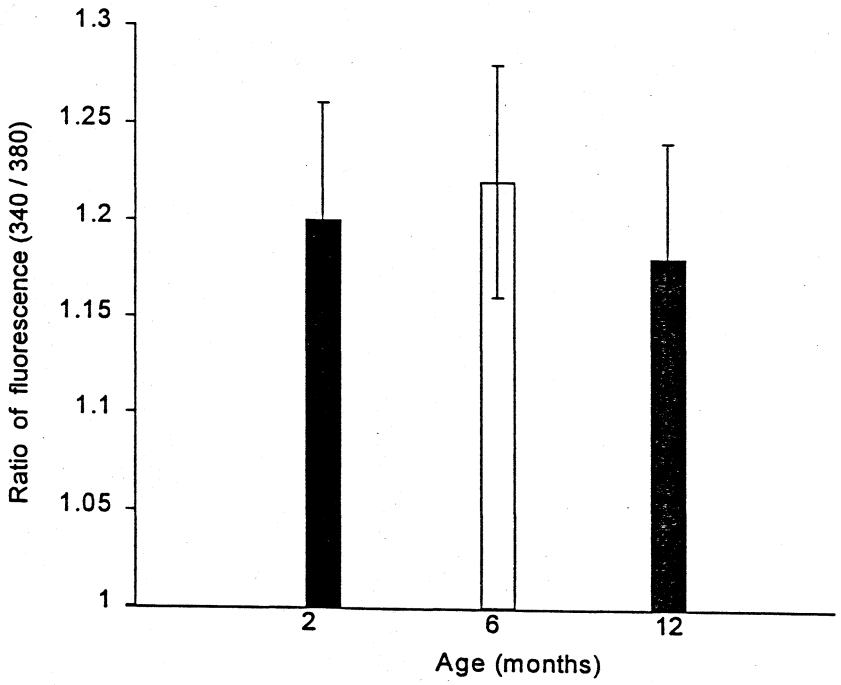
FIGURE - 4.4

Age dependent variation in contractile amplitude of cardiomyocytes

Contractile amplitude :



Diastolic Ca^{2+}_i - level :



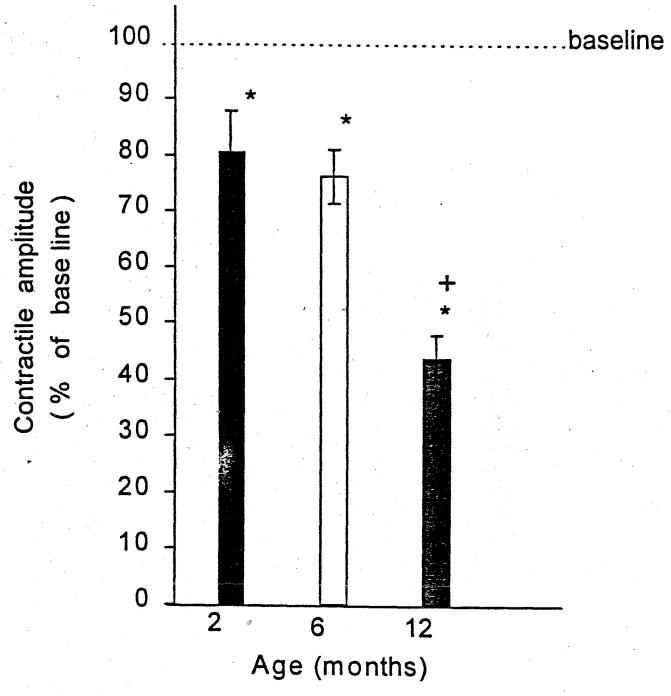
Values are mean ± SEM of n = 10 - 25 cells for measurement of contractile response and n = 3-8 preparations for Cai - level measurement .

* $p < 0.05$ v 2 months .

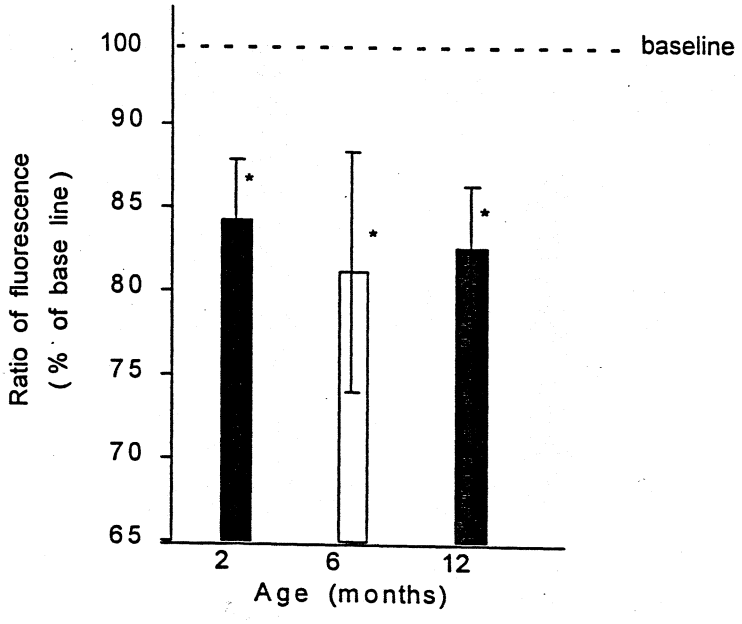
FIGURE - 4.5

Age dependent variation in response to SL - L- type Ca - channel antagonist (Verapamil - 1 μM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :

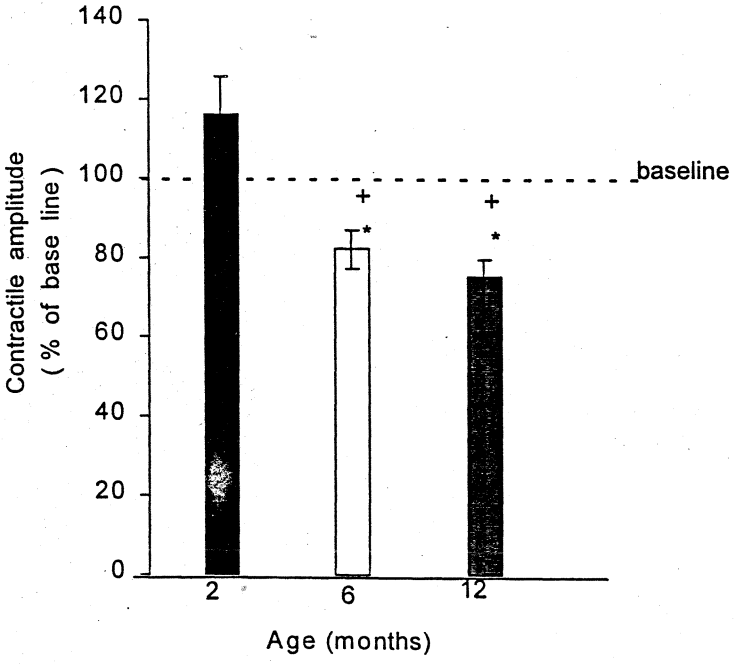


Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Ca_i - level measurement. * $p< 0.05$ v baseline . + $p< 0.05$ *v* 2 & 6 months.

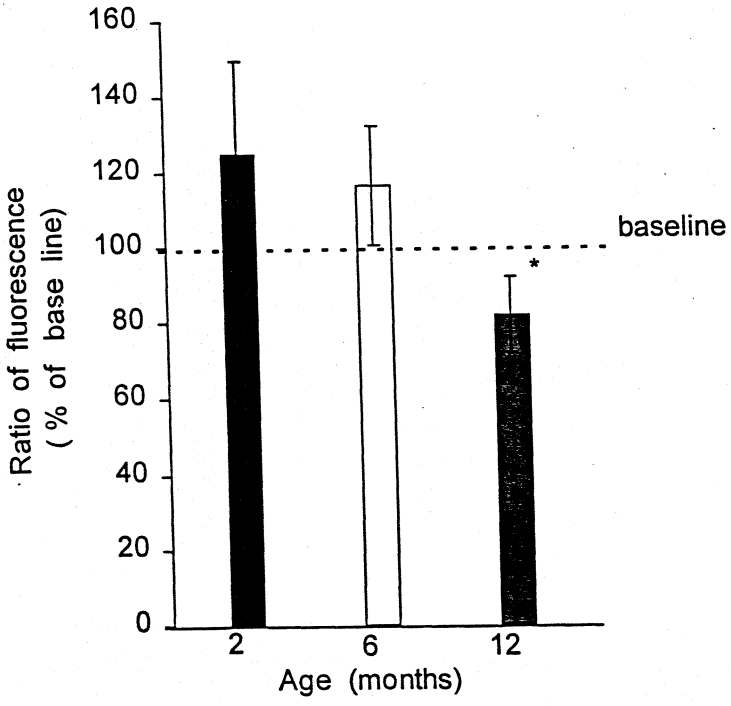
FIGURE - 4.6

Age dependent variation in response to SL - T-type Ca - channel antagonist ($NiCl_2$ - 40 µM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :

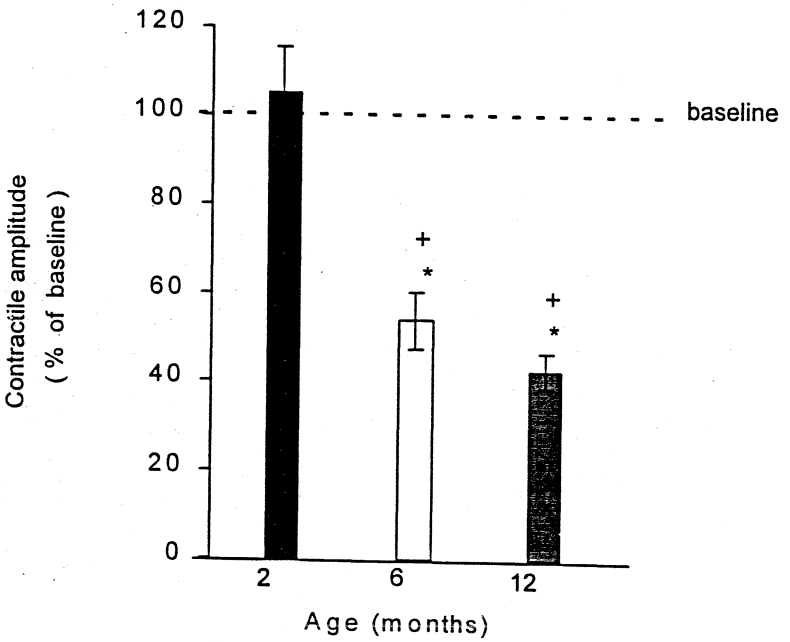


Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Ca_i - level measurement . * $p < 0.05$ *v* baseline , + $p < 0.05$ *v* 2 months.

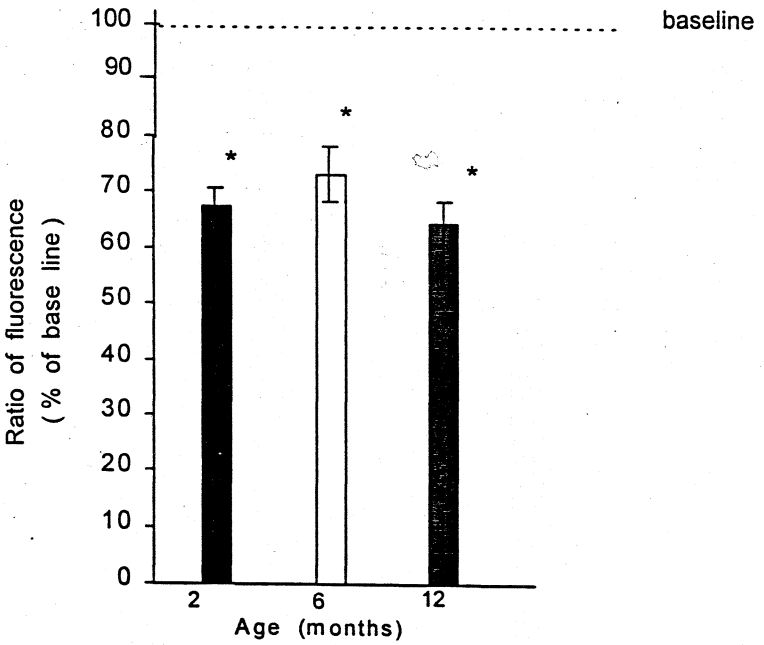
FIGURE -4.7

Age dependent variation in response to SL Na - Ca exchanger antagonist ($MnCl_2$ - 0.2 mM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :

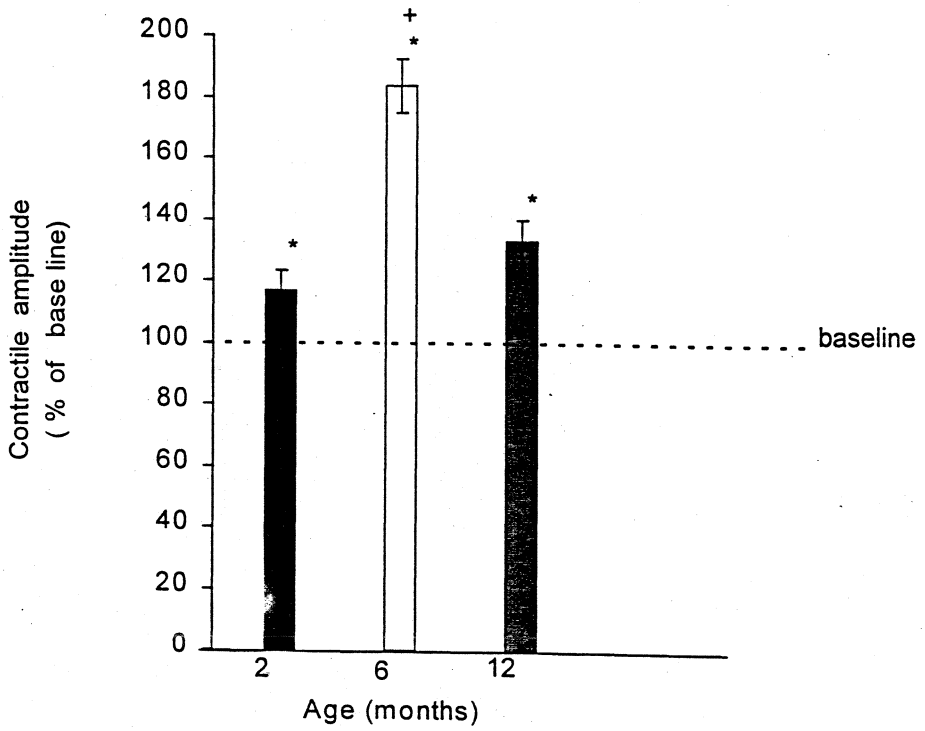


Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Ca_i - level measurement . * $p < 0.05$ v baseline . + $p < 0.05$ *v* 2 months .

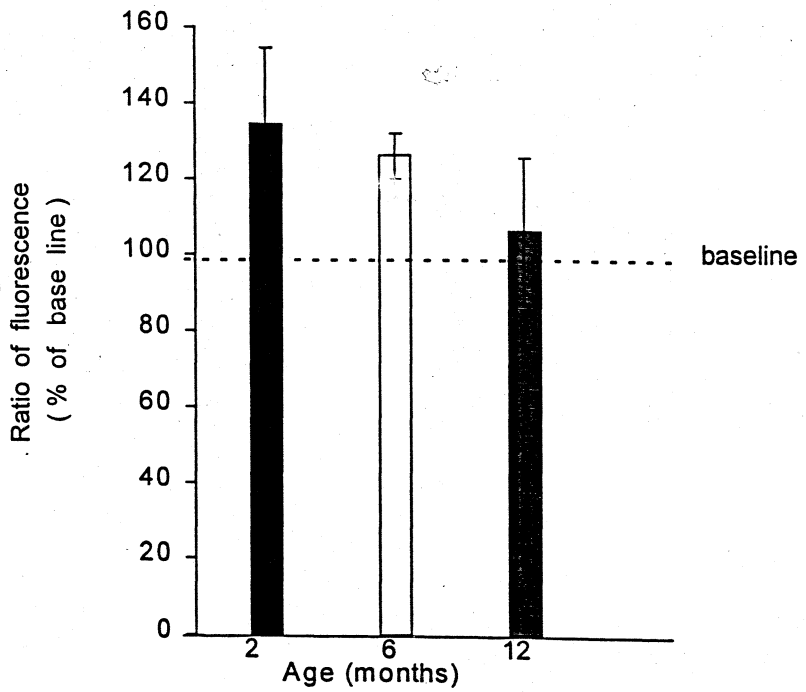
FIGURE -4.8

Age dependent variation in response to Na, K - ATPase inhibitor (Ouabain - 0.3 mM)

Contractile ampltude :



Diastolic Ca^{2+}_i - level :

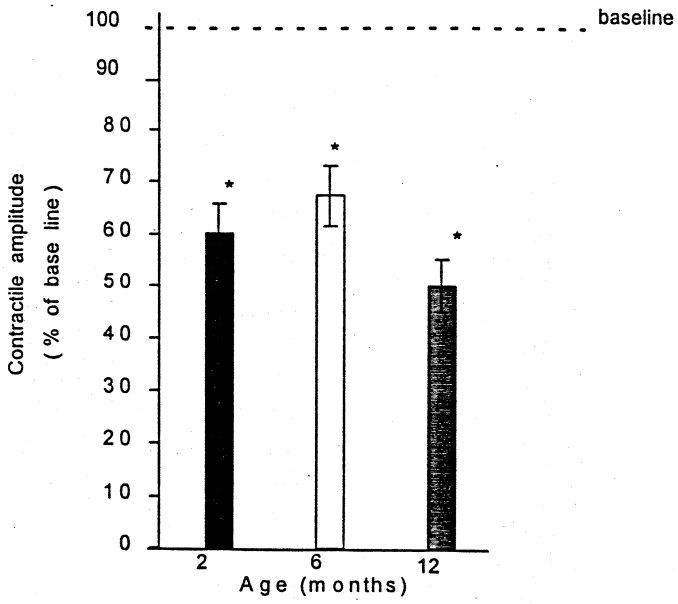


Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and n = 3-8 preparations for Ca_i - level measurement . * $p < 0.05$ v baseline . + $p < 0.05$ v 2 and 12 months .

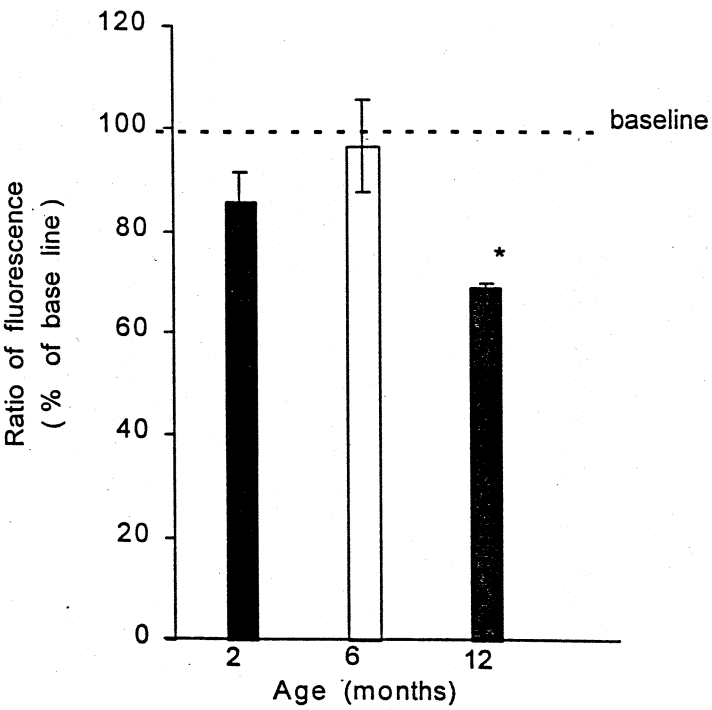
FIGURE - 4.9

Age dependent variation in response to SR inhibitor (Ryanodine - 1μM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :

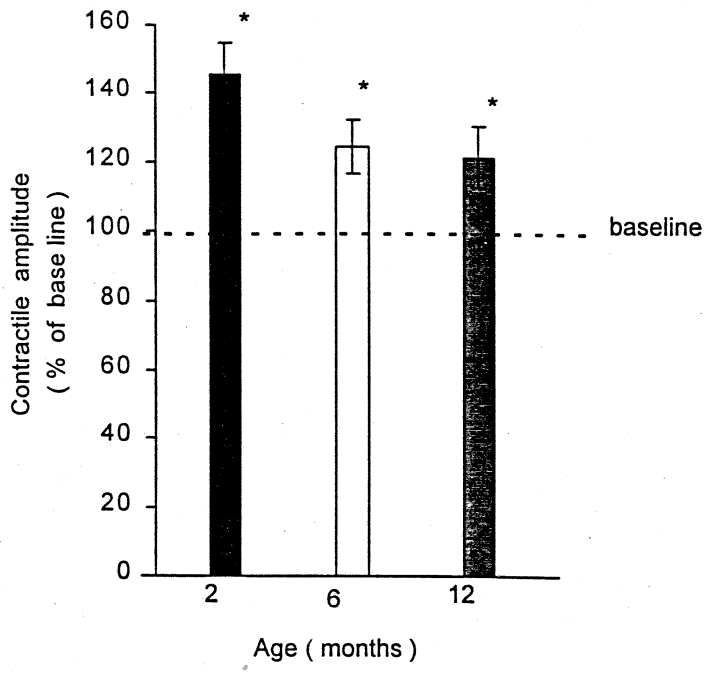


Values are mean ± SEM of n = 10 - 25 cells for measurement of contractile response and n = 3 -8 preparations for Ca_i - level measurement . * $p < 0.05$ v baseline .

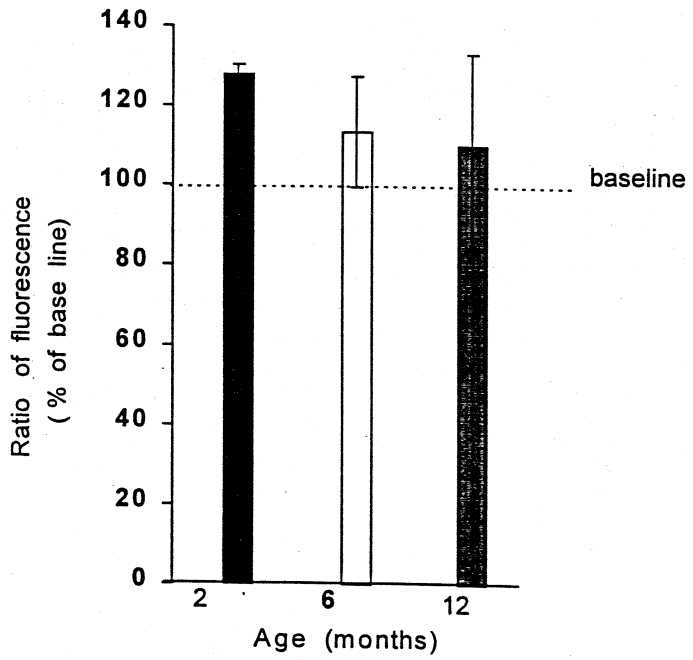
FIGURE - 4.10

Age dependent variation in response to SR - Ca - pump inhibitor (Caffeine - 10 mM)

Contractile amplitude :



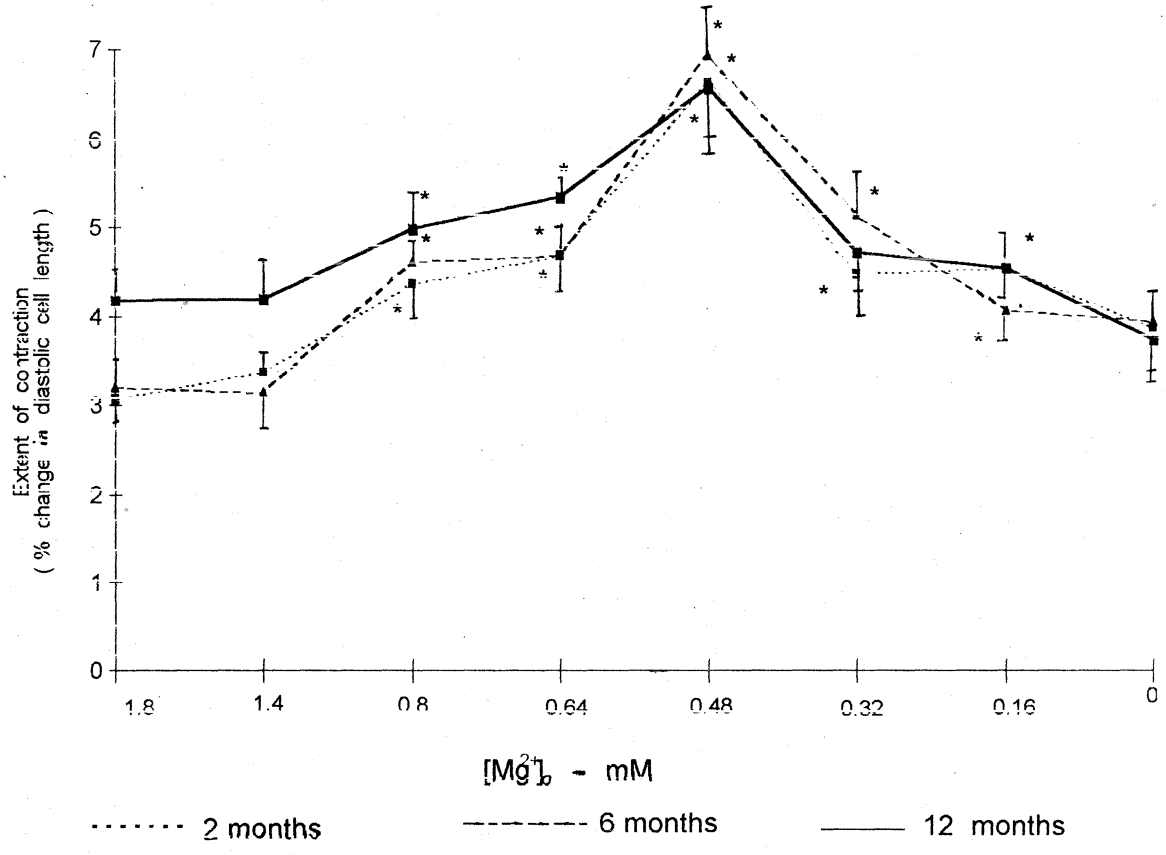
Diastolic Ca^{2+}_i - level :



Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Ca_i - level measurement . * $p < 0.05$ v baseline .

FIGURE - 4.11

EFFECT OF EXTRACELLULAR MAGNESIUM ON THE FUNCTIONAL RESPONSE OF CARDIOMYOCYTES

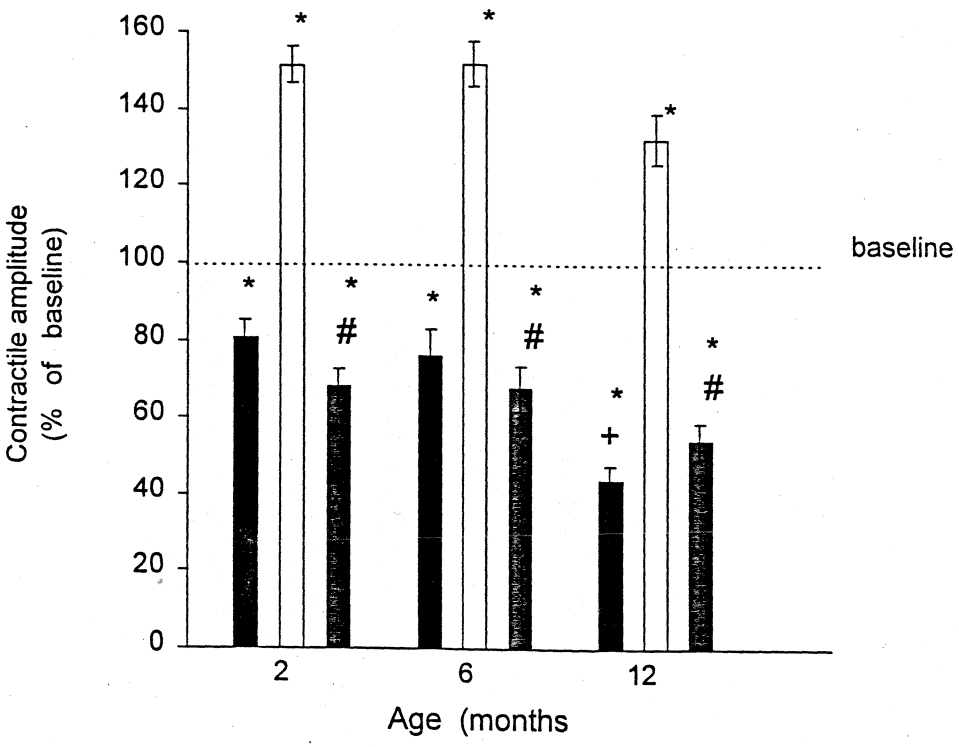


Values are expressed as mean ± SEM of n = 15 - 25 cells . * $p < 0.05$ compared to 1.8 mM Mg.

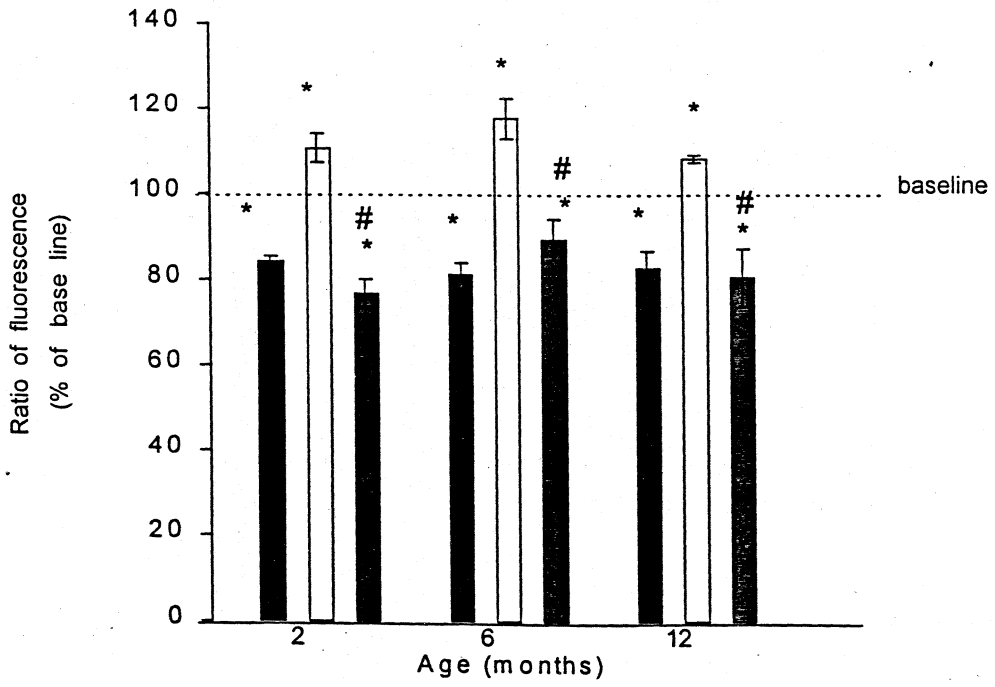
FIGURE - 4.12

Effect of extracellular Mg on the response to SL - L - type Ca - channel antagonist (Verapamil - 1 µM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :



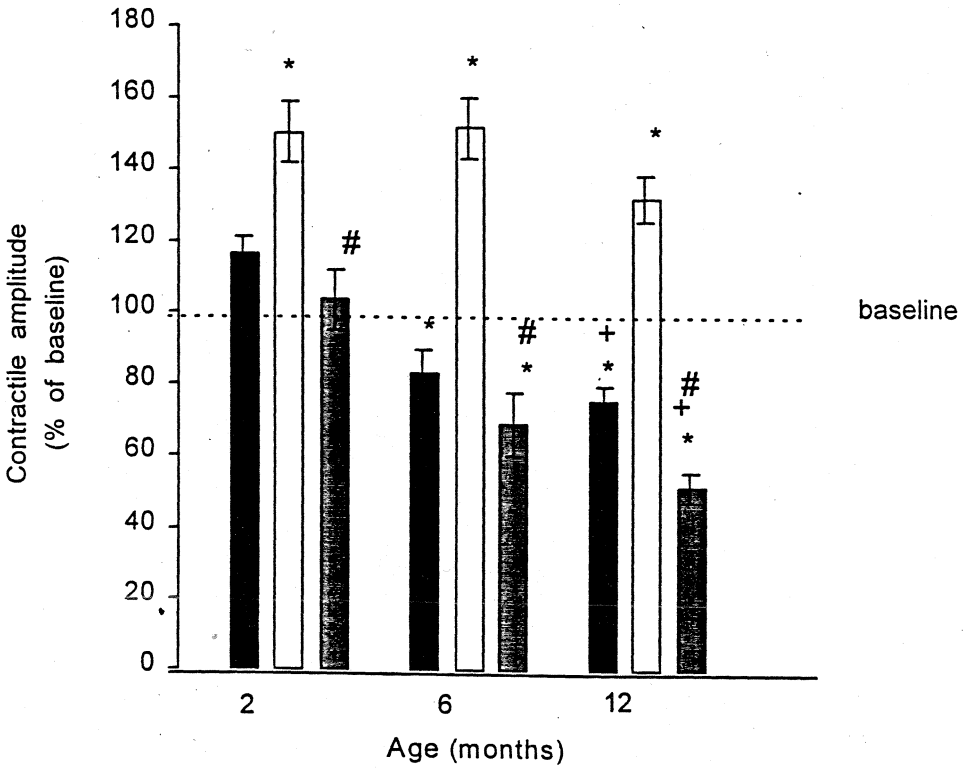
control + Ver Mg deficient Mg deficient + Ver

Values are mean ± SEM of n= 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for intracellular Ca_i - level measurement. * $p \leq 0.05$ v

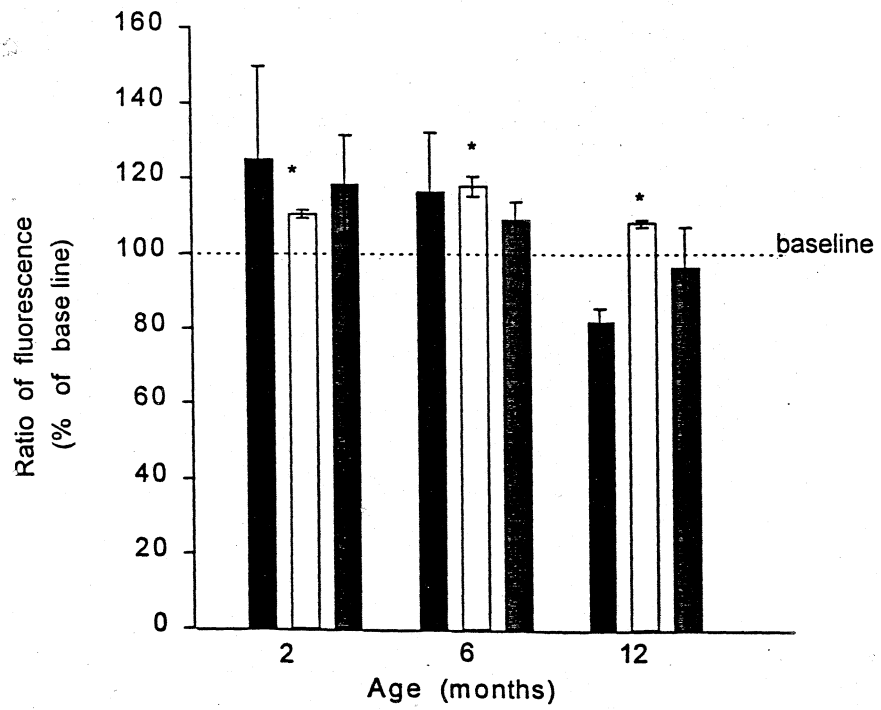
FIGURE - 4.13

Effect of extracellular Mg on the response to SL - T - type Ca - channel antagonist ($NiCl_2$ - 40 μM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :



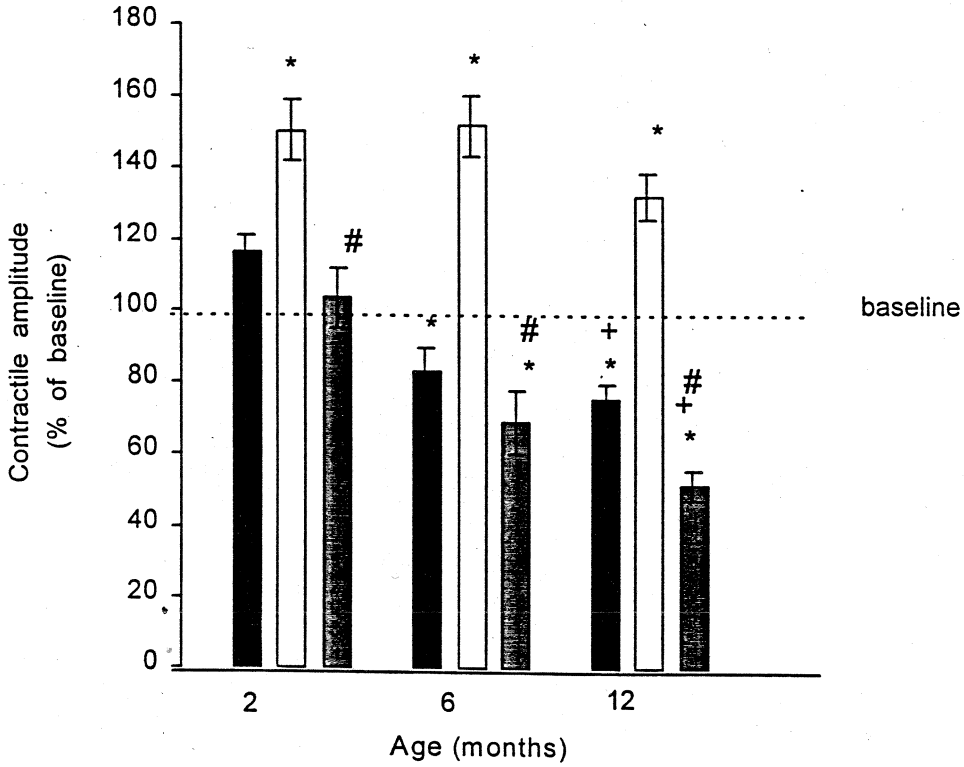
control + $NiCl_2$ ▭ Mg- deficient ▭ Mg- deficient + $NiCl_2$

Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and 3 - 8 preparations for Cai level . * $p < 0.05$ v baseline . + $p < 0.05$ v 2 & 6 months . # $p < 0.05$ v Mg - deficient .

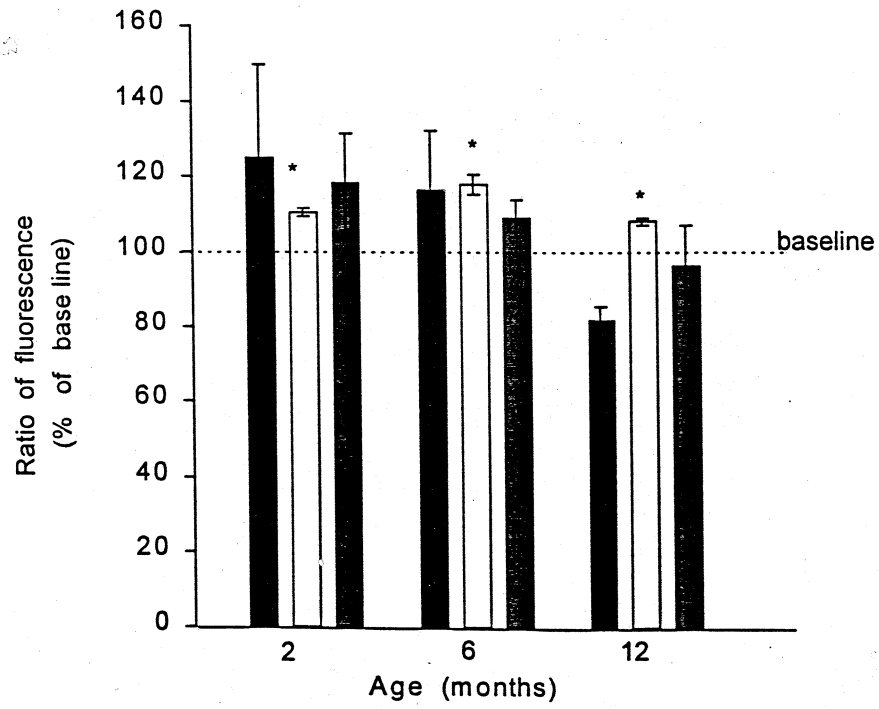
FIGURE - 4.13

Effect of extracellular Mg on the response to SL - T - type Ca - channel antagonist ($NiCl_2$ - 40 µM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :



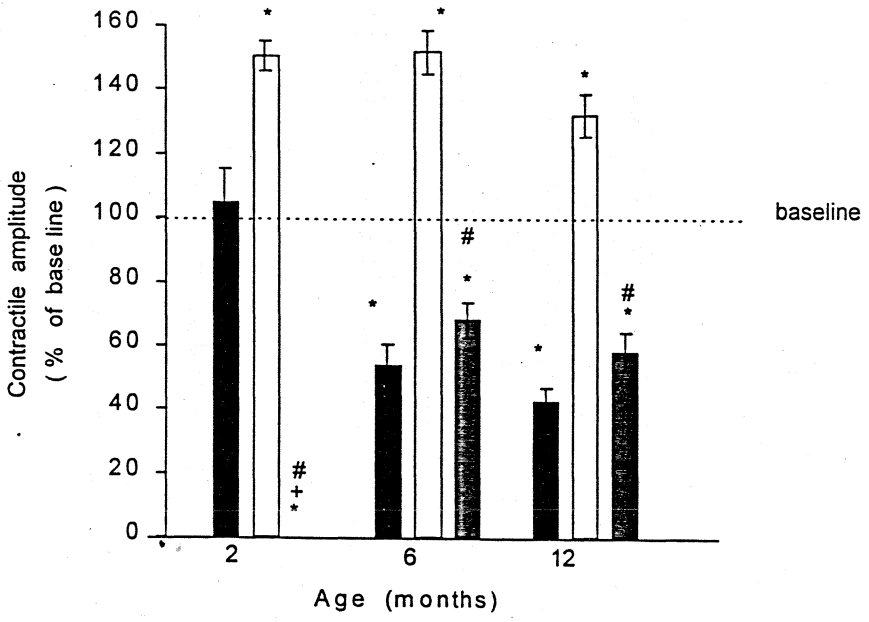
control + $NiCl_2$ Mg- deficient Mg- deficient + $NiCl_2$

Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and 3 - 8 preparations for Cai level . * $p < 0.05$ v baseline . + $p < 0.05$ v 2 & 6 months . # $p < 0.05$ v Mg - deficient .

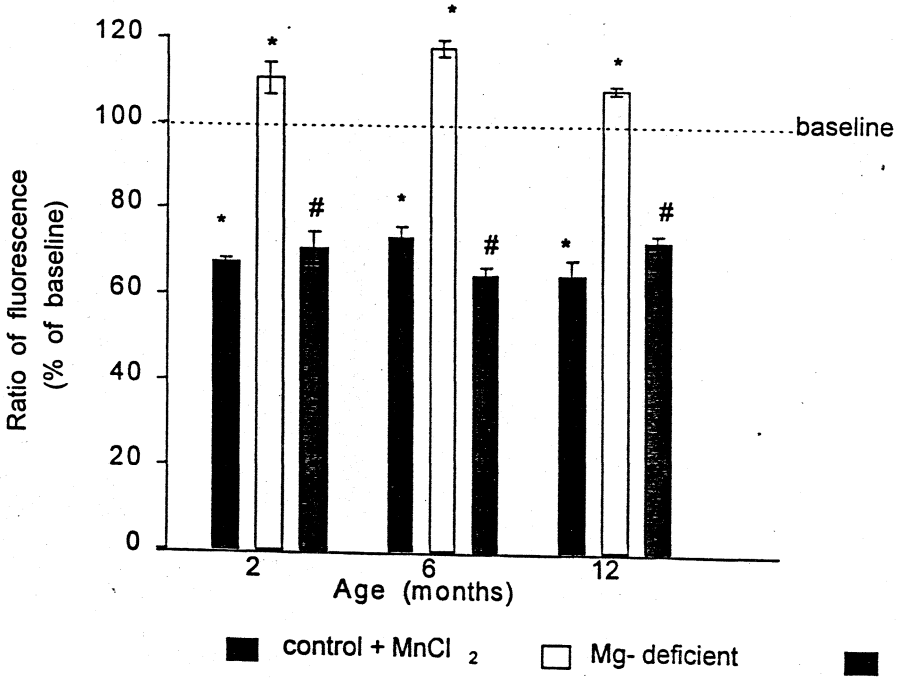
FIGURE - 4.14

Effect of extracellular Mg on the response to SL Na - Ca exchanger blocker ($MnCl_2$ - 0.2 mM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :

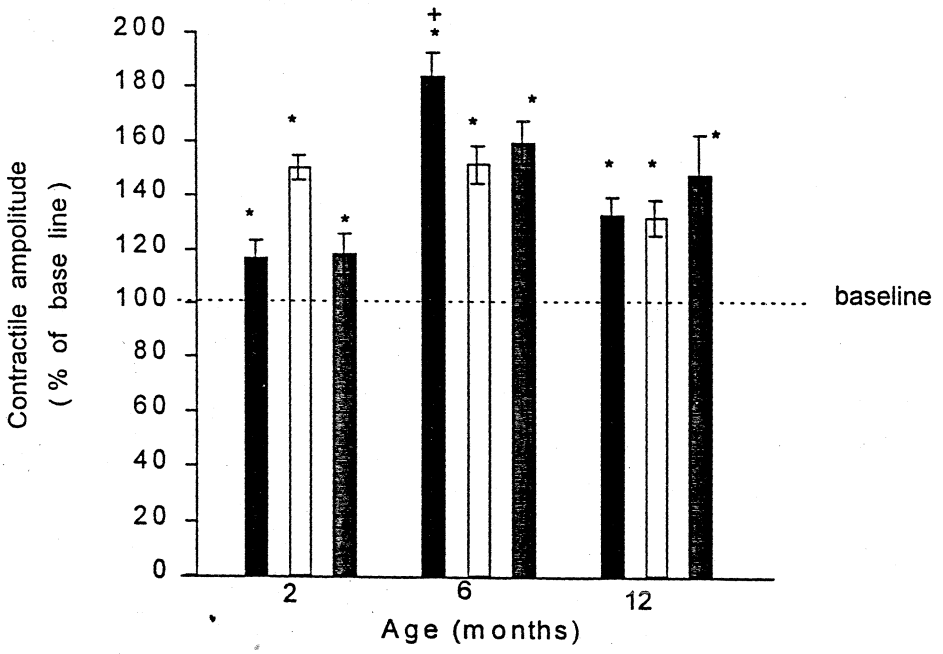


Values are mean ± SEM of 10 - 25 cells for measurement of contractile response and 3 - 8 preparations for Cai - level . * $p < 0.05$ v control / baseline. + $p < 0.05$ v 6 & 12 months . # $p < 0.05$ v Mg - deficient .

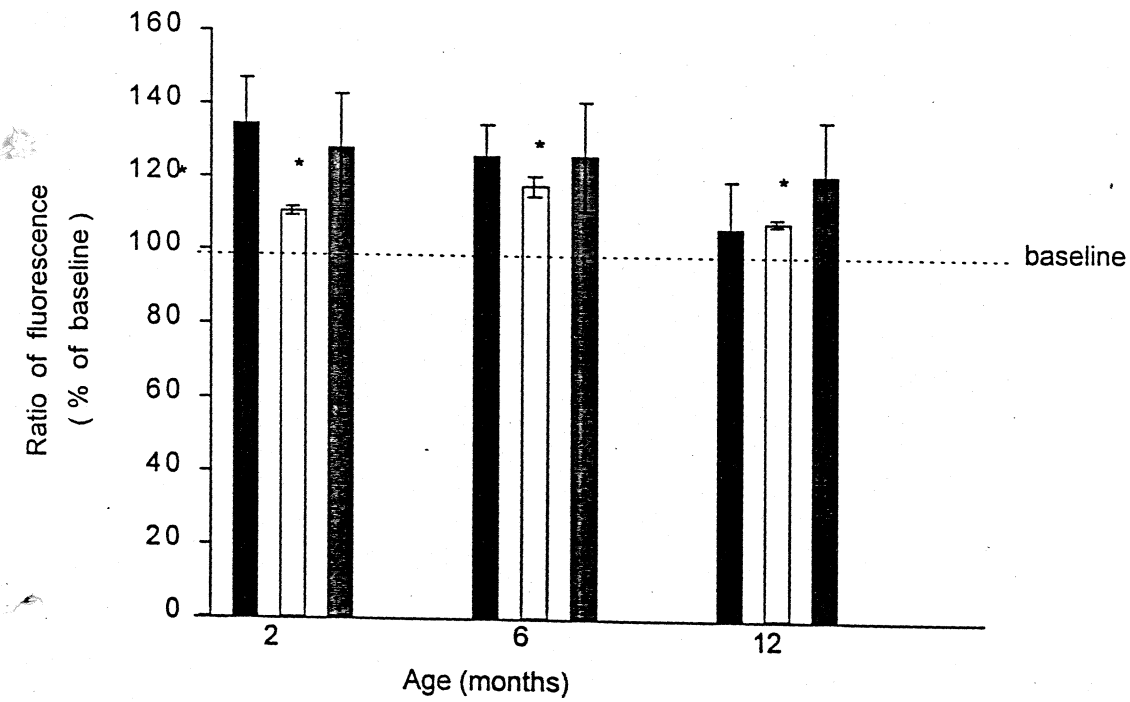
FIGURE - 4.15

Effect of extracellular Mg on the response to Na,K-ATPase inhibitor (Ouabain - 0.3 mM)

Contractile amplitude :



Diastolic Ca^{2+}_i - level :



control + Oub

Mg - deficient

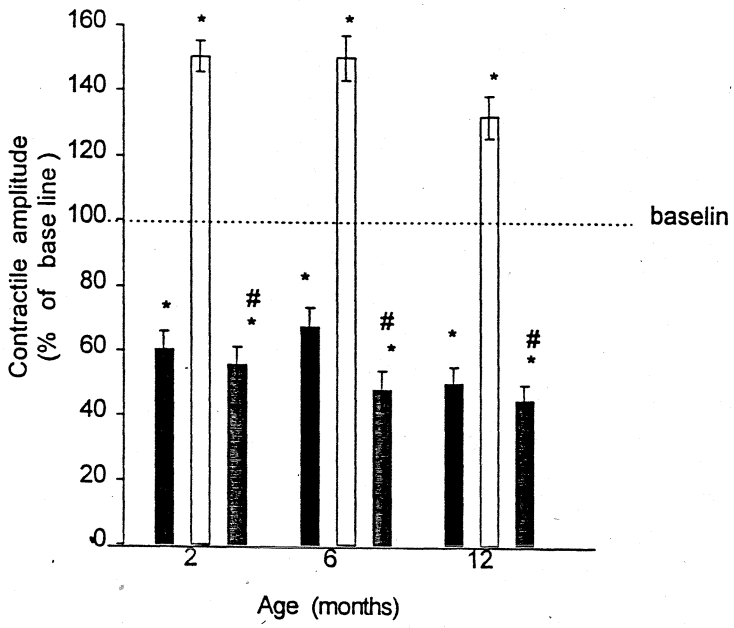
Mg - deficient + Oub

Values are mean ± SEM of n = 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Cai - level easurement . * $p < 0.05$ *v* baseline .

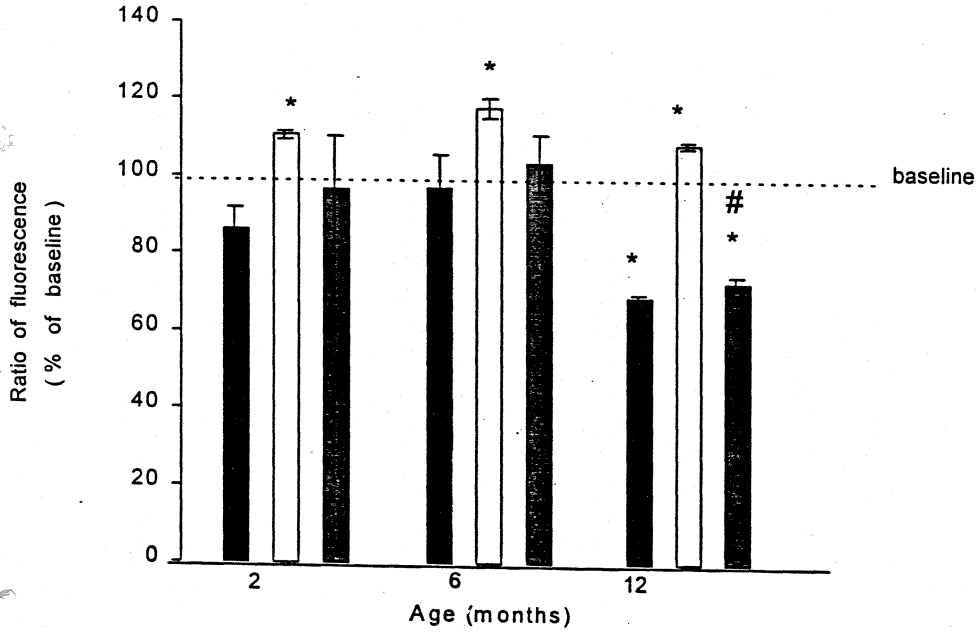
FIGURE - 4.16

Effect of extracellular Mg on the response to SR - Ca - release channel inhibitor (Ryanodine - 1 μM)

Contractile amplitude



Diastolic Ca^{2+}_i - level :



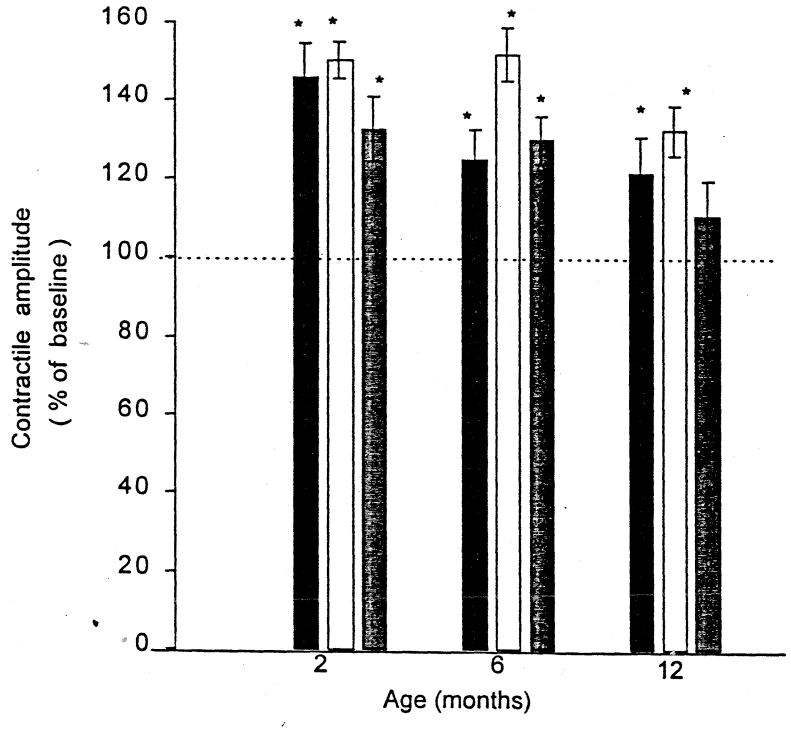
control + Ry Mg- deficient Mg deficient + Ry

Values are mean ± SEM of n = 10 - 25 cells for measurement of contractile response and n = 3 - 8 preparations for Cai - level measurement. * $p < 0.05$ *v* base line . # $p < 0.05$ *v* Mg - deficient .

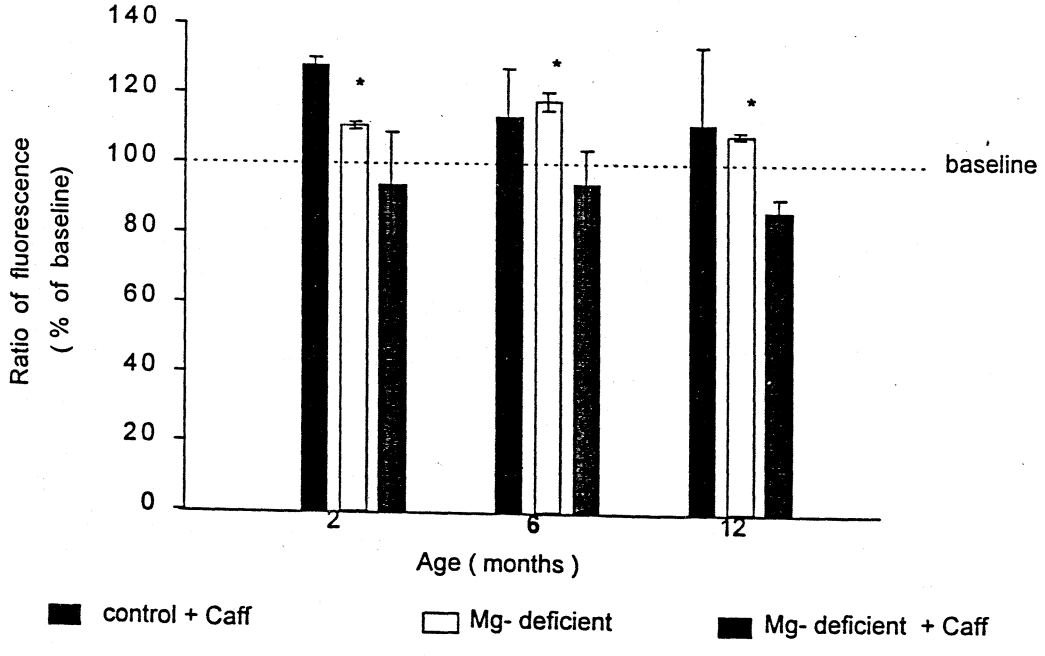
FIGURE - 4.17

Effect of extracellular Mg on response to SR - Ca - pump inhibitor (Caffeine - 10 mM)

Contractile ampltude :



Diastolic Ca^{2+}_i - level :



Values are expressed as mean ± SEM of 10 - 20 cells for measurement of contractile amplitude and 3 - 8 preparations for Ca_i - level measurement .* $p < 0.05$ v baseline .

TABLE - 4.1

Yield / heart of cells isolated in Ca - free medium with different types of perfusates used for Ca - depletion

Number of replicates	Composition of perfusate	Cell count (X 10^6)	Cell viability (%)	# Net Yield of viable cells (X 10^6)
3	KRH - buffer (control)	4.66 ± 0.33	32.53 ± 1.59	1.52 ± 0.16
3	KRH + Trypsin (0.005 %)	7.00 ± 0.58	42.7 ± 2.12	2.96 ± 0.39 *
6	KRH + EGTA (0.1 mM)	12.13 ± 1.53	71.57 ±4.16	8.15 ± 0.59 **
3	KRH + EGTA (0.1 mM) + Taurine (20 mM)	15.00 ± 0.58	79.31 ± 2.73	11.92 ± 0.77 **

KRH - HEPES modified Kreb's Ringer Henseleit Buffer .
represents the cell yield before Ca - repletion .
Values are expressed as mean ± SEM.
*$p < 0.05$ v control , ** $p < 0.01$ v control .

TABLE - 4. 2

Cell yield / heart with different types of media used during Ca - repletion

Composition of medium	Number of replicates	Cell count (X 10^6)	Cell viability (%)	Net yield of viable cells (X 106)
KRH - Buffer (control)	4	3.60 ± 0.80	29.72 ± 4.26	1.60 ± 0.18
KRH + EGTA (0.1 mM)	3	5.60 ± 0.95	33.87 ± 4.51	1.98 ± 0.61
KRH + EGTA (0.1 mM) + Trypsin (0.005 %)	8	5.39 ± 0.82	56.87 ± 4.41	2.98 ± 0.38 *
KRH + Trypsin (0.005 %)	7	5.65 ± 1.07	73.06 ± 3.81	4.23 ± 0.96 *
KRH + Trypsin (0.01 %)	3	6.28 ± 1.80	66.94 ± 2.77	4.17 ± 1.16 *
KRH + Trypsin (0.05 %)	3	5.26 ± 0.36	66.22 ± 4.73	3.26 ± 0.22 *
KRH + Trypsin (0.1 %)	3	6.66 ± 0.66	63.69 ± 5.60	4.21 ± 0.59 *

KRH - HEPES modified Kreb's Ringer Henseleit Buffer with 1% BSA.
Values are expressed as mean ± SEM.
* $p < 0.01$ *v* control.

TABLE - 4.3

Cell yield as per the method standardised in comparison with earlier reports

Reference	Cell count / gm wet wt tissue (X 10^6)	Cell viability	Net yield of viable cells (X 10^6)
* Haworth *et al* (1980)	6.30 ± 1.50	67.50 %	4.25 ± 1.01
# Lundgren *et al* (1984)	4 - 6	60 - 75 %	3 - 4
* standardised method	8.56 ± 1.62	73.06 %	6.25 ± 1.45

* Values represented as mean ± SEM .
# - Values represented in range .

Haworth P A , Hunter D R , Berkoff H A The isolation of Ca2+ resistant myocytes f adult rat *J. Mol . Cell . Cardiol .* 1980 ; 12 : 715 - 723 .

Lundgren E L , Borg T , Mardh S Isolation , characterisation and adhesion of Ca - tolerant myocytes from adult rat heart *J . Mol . Cell . Cardiol .* 1984 ; 16 : 355 - 362.

TABLE - 4.4

Cardiomyocyte geometry , contractile amplitude , heart weight and body weight of male Sprague Dawley rats of different age groups .

Variable	2 months	6 months	12 months
Heart weight (mg)	381.0 ± 11.4	545.6 ± 23.9 *	766.6 ± 20.3 * +
Body weight (g)	101.2 ± 1.2	196.5 ± 3.9 *	294.3 ± 6.2 * +
Ratio (H.wt / B.wt)	3.78 ± 0.27	2.78 ± 0.11 *	2.61 ± 0.06 *
Cell length (μM)	86.4 ± 3.4	116.4 ± 5.5 *	128.8 ± 5.9 * +
Sarcomere length (μM)	1.39 ± 0.05	1.54 ± 0.05 *	1.93 ± 0.04 * +

Values represent mean ± SEM of 10 - 20 estimations .
* $p < 0.05$ *v* 2 month old animals . + $p < 0.05$ *v* 6 month old animals .

TABLE - 4.5

Effect of extracellular Mg on intracellular Ca - level .

$[Mg^{2+}]_o$ in mM	2 months (F340 / F 380)	6 months (F340 / F 380)	12 months (F340 / F 380)
1.8	1.03 ± 0.08 *	1.04 ±0.09 *	1.11 ± 0.08
1.4	1.20 ± 0.10	1.08 ± 0.12	1.14 ± 0.05
0.8	1.20 ± 0.07	1.22 ± 0.06	1.18 ± 0.03
0.48	1.30 ± 0.09 *	1.33 ± 0.10 *	1.24 ± 0.03 *
0	0.78 ± 0.17 *	1.06 ± 0.13 *	0.92 ±0.06 *

Values are expressed as mean ± SEM of 3 - 8 preparations from each age group . Ratio of fluorescence intensities at wave lengths 340 and 380 represent the intracellular diastolic Ca - level .

* $p < 0.05$ compared to 0.8 mM Mg .

TABLE - 4.6

Effect of extracellular Mg on intracellular Na - level

$[Mg^{2+}]_o$ in mM	2 months (F330 / F360)	6 months (F330 / F360)	12 months (F330 / F360)
1.8	1.33 ± 0.05	1.08 ± 0.04	1.13 ± 0.03
1.4	1.33 ± 0.03	1.14 ± 0.03	1.11 ± 0.03
0.8	1.46 ± 0.07	1.25 ± 0.07	1.25 ± 0.03
0.48	1.38 ± 0.09	1.22 ± 0.03	1.11 ± 0.05
0	1.23 ± 0.17	1.08 ± 0.04	1.02 ± 0.06

Values are expressed as mean ± SEM of 3 - 8 preparations .
Ratio of fluorescence intensities at excitation wavelengths 330 and 360 represents intracellular diastolic Na - level .

TABLE - 4.7

Effect of extracellular Mg on intracellular Mg - level

$[Mg^{2+}]_o$ in mM	2 months	6 months	12 months
1.8	2.30 ± 0.17	2.21 ± 0.14	2.35 ± 0.20
1.4	2.32 ± 0.12	2.18 ± 0.15	2.32 ± 0.25
0.8	2.34 ± 0.18	2.23 ± 0.23	2.32 ± 0.28
0.48	2.37 ± 0.22	2.11 ± 0.17	2.35 ± 0.17
0	2.21 ± 0.25	2.03 ± 0.15	2.30 ± 0.24

Values are expressed as mean ± SEM of 3 - 8 preparations .
Ratio of fluorescence intensities at two excitation wavelengths , 340 and 380 nm represents the diastolic intracellular magnesium levels .

TABLE - 4.8

Effect of extracellular Mg on intracellular K - level

$[Mg^{2+}]_o$ in mM	2 months	6 months	12 months
1.8	1.29 ± 0.10	1.05 ± 0.09	1.12 ± 0.09
1.4	1.30 ± 0.11	1.08 ± 0.05	1.10 ± 0.05
0.8	1.33 ± 0.12	1.10 ± 0.04	1.14 ± 0.09
0.48	1.34 ± 0.14	1.11± 0.07	1.13 ± 0.07
0	1.33 ± 0.13	1.12 ± 0.04	1.11 ± 0.11

Values are expressed as mean ± SEM of 3 - 8 preparations .
Ratio of fluorescence intensities at two excitation wavelengths , 330 and 360 nm represents the diastolic intracellular potassium levels.

DISCUSSION

Ontogenic differences have been observed in cardiac performance (Kato *et al* , 1996) and senescence has also been found to be associated with variation in myocardial performance (Muscari *et al* , 1992) . Age associated alteration of the mature myocardium has received comparatively very little attention . Even during this period , there is physiological hypertrophy of the myocardium (Walsh and Dorn , 1998) . This study was taken up based on the surmise that the age associated increase in heart size can be accompanied by variation at the level of muscle cell , which in turn could affect cardiac performance . Such an intrinsic variation with age can also influence the response to physiological and pathological changes in the extracellular milieu .

Contractile performance of the mammalian heart is influenced by intrinsic and extrinsic factors. Importance of Mg^{2+} as an ion influencing myocardial contractile function has received much attention recently and Mg^{2+} is considered as "nature's Ca - antagonist" (Millane and Camm , 1992). Hypomagnesemia has been correlated with the prevalance of cardiovascular diseases in soft water areas (Durlach *et al* , 1989) . Kerala being a region with soft water , this study on functional response of the cardiac muscle cells to variation in extracellular $[Mg^{2+}]$ was taken up based on the assumption that 'sensitivity to sub optimal levels of $[Mg^{2+}]_o$ may be dependent on age'.

Magnesium is the mineral ion of chlorophyll . Hence green vegetables are an important source of magnesium . Drinking water is another important source of Mg^{2+} and slight deficiencies in food is compensated by the Mg in water in hard

water regions . An inverse correlation between water hardness and incidence of cardiac fatalities have been reported and it has been shown that Mg^{2+} present in hard water has a cardioprotective effect (Marier , 1978) . Following this finding several studies have reinforced that Mg - content in water plays a very crucial role in the prevention of cardiac ailments (Rabenowitz *et al* , 1996 ; Yang *et al* , 1997 ; Yang , 1998 ; Rabenowitz *et al* , 1999) . Hypomagnesemia has been observed in regions with soft water (Durlach *et al* , 1989) and in a study conducted in the local population , serum and red cell Mg were found to be low in children from the low socio - economic group (Nair *et al* , 1995) .

Considering these factors , assessment of functional response of cardiomyocytes to sub optimal levels of $[Mg^{2+}]_o$ gains importance . Cardiac mechanical and electrophysiological performance depends critically on the functioning of the cardiomyocytes . The chemical messages in the environment are sensed and translated into meaningful physiological actions by these cells . Cardiac myocytes were therefore chosen as the model for assessment of the functional response of the myocardium to variation in extracellular magnesium .

The experimental studies were carried out in cardiac myocytes from adult male rats of Sprague - Dawley strain of different age groups - 2- , 6- and 12 - months.

Isolated cardiac myocytes as the experimental model for assessment of cardiac mechanics:

Isolated cardiomyocytes offers a viable preparation for the study of cardiac mechanics , as all the processes responsible for the exchange of ions with the environment and excitation - contraction coupling are believed to be preserved in isolated myocytes (Bishop and Drummond , 1979) . Though an excellent model ,

difficulties in isolation and culture of viable cells has eluded the use of the model for experimental studies .

Successful isolation of viable cells is dependent on a delicate interplay of a number of variables . In line with the observation of earlier workers (Glick et al , 1974 , Haworth et al , 1980 ; Berg et al , 1989; Harding et al , 1990) , the enzymatic perfusion method was found to be superior to chunk method .

Introduction of technical modifications to the existing protocols enabled successful isolation of cardiomyocytes with improved yield of viable cells . Existing methods when tried out did not provide satisfactory yield of Ca - tolerant cells . Various factors were found to influence the efficacy of cell - isolation . The two crucial stages in the isolation procedure were found to be the first step of Ca - depletion for relaxation of the myocardium and the final step of Ca - repletion for isolating Ca - tolerant myocytes .

Viable cells can be isolated only if the myocardium is totally relaxed . For that tissue - Ca should be kept as low as possible (Glick *et al* ,1974). During the initial Ca - depletion stage , depending upon the composition of the medium used, large variations in the cell yield and viability was observed (Table - 4.1) . Relaxation of the myocardium is attained by perfusion of Ca^{2+} - free buffer . Incorporation of 0.1 mM EGTA in association with 20 mM taurine in the perfusate had a positive effect on cell yield (Table - 4.1) . This modification resulted in more than seven fold increase in cell yield ($p < 0.05$) , at this stage of the isolation procedure . The beneficial effect of taurine in the isolation procedure has been controversial . Lundgren *et al* (1984) stated that taurine does not confer any specific advantage in cardiomyocyte - isolation , though it has been used in the isolation of ferret myocytes with success (Bouron *et al* ,1992). Taurine is known to modulate the intracellular

Ca^{2+} - accumulation and is expected to diminish the Ca - overload . Taurine retards the accumulation of Ca^{2+} associated with defective SL . It has been demonstrated that taurine increases the affinity for Ca^{2+} of a Ca - binding site on SL , thus bringing about a lowering of free Ca - level within the cell (Chovan *et al* , 1979). It has also been reported that the protective effect of taurine is due to removal of Na^{+} from cells , which may help to resist the Ca^{2+} - overload caused by influx of Ca^{2+} through the Na - Ca exchanger (Caroni et al , 1981) . Taurine is also reported to possess antioxidant properties (Arnoma et al , 1988). The beneficial effect of taurine in the isolation of cardiomyocytes could be due to any one of these factors or the additive effect of two or more factors .

EGTA being a Ca - chelator , lowers the concentration of free Ca^{2+} in the extracellular medium by sequestering Ca - ions released from the cells . EGTA also modulates Na - Ca exchanger activity to avoid Ca - overload (Trosper and Philipson , 1984) .

The use of low $[Ca^{2+}]$ in the perfusion medium remains controversial . Inclusion of 25 μM Ca , as per the procedure of earlier workers (Berg et al , 1989) was found to reduce the cell yield considerably , when compared to the use of Ca-free medium .

The next crucial step in isolating cardiomyocytes is the stage of Ca-repletion for isolation of Ca^{2+} - tolerant cells . Inclusion of trypsin (0.005% w/v) during Ca - repletion was found to confer a significant beneficial effect . The yield of viable cells was two and a half times ($p < 0.05$) more than that in the absence of trypsin (Table - 4.2) . Higher concentrations of trypsin (0.1 %) helped to improve

the yield , but cell viability and cell adhesion were affected . Bers (1993) reported that trypsin can also modulate Na - Ca exchanger activity to prevent Ca - overload . During enzymatic isolation of cardiac cells , removal of basement membrane , tearing of cell membrane or disruption of gap - junction can occur as a result of perfusion pressure . Most of them reseal rapidly , but some remain open . Such disruptions promote Ca - influx . Haworth *et al* (1980) have shown that the gap - junctions of Ca^{2+} - susceptible cells often retain shreds of junctional regions of cells which were formerly contiguous . They suggested that these unseparated gap - junctions which are exposed to external medium could be the site of Ca - entry which stimulate spontaneous beating and promotes cell damage . The inclusion of trypsin was found to prevent the damage . The most straightforward explanation suggested by them is that trypsin removes the membrane shreds allowing gap - junction channels in the intact cells to close , which would prevent Ca - overload and spontaneous beating , thereby improving the yield of viable cells . The commonly encountered Ca - paradox in isolated cell- preparation can therefore be considerably diminished by the use of trypsin .

Thus the two major modifications suggested to optimise the yield of viable and functional cardiac myocytes are ;

1) Inclusion in the initial perfusate , the Ca - chelator EGTA and taurine an aminoacid found in very high concentrations in the myocardium .
2) Introduction of trypsin , a Ca - binding agent in the medium at the time of Ca - repletion for the isolation of Ca - tolerant myocytes .

Improvement of cell yield , apart from providing a larger sample for experimental studies , ensures that the cells obtained are healthier . With the current restrictions on the use of animals for experimental studies and the promotion of

use of alternatives to animal experimentation ; the cell culture model offers the facility for carrying out more number of experiments than would be possible on a single animal.

Different experimental models such as whole heart or intact muscle strips can be used for measurement of cardiac mechanics . These models help to record the contractile mechanics in relation to the connective tissue and other non contractile cell types . The viscoelastic properties of connective tissue matrix along with other non contractile cell types will be manifested in the force - length response of the muscle and obscure the mechanical properties of contractile machinery when muscle strips are used (Parikh et al , 1993) . The isolated cell model is useful when the purpose of the experiment is to assess the age related variation of myocardial cell mechanics and its sensitivity to externally induced stress conditions , without the influence of other extrinsic factors .Obviously there is a difference in the contractile environment between an isolated cardiomyocyte freely settled onto a glass slide and a myocyte in the syncytium of the heart . In the heart , the cell normally experiences varying external load , where as the freely isolated cell is not externally loaded . Despite this and many unknown and undeterminable internal forces , there is remarkable consistency in data from unloaded cell - contraction experiments , which suggests that qualitative assessments can be reasonably made over limited ranges of contractility (Delbridge and Roos , 1997) .

Traditionally , the contractile activity of striated muscle preparations has been evaluated with respect to both isometric force development and isotonic shortening behaviour. Effective techniques for recording force development using isolated

mammalian cardiac myocytes have also been devised. The relationship betwee muscle length or sarcomere length and developed tension was explained by Alle and Kentish (1985) , and the cellular mechanisms such as Ca^{2+} - sensitivity , whic underlie the relation between muscle length and developed tension in muscle ha also been reviewed . Fuchs and Wang (1996) are of the opinion that , both Ca^{2+} sensitivity and Ca^{2+} - binding correlated more closely with change in interfilamer spacing . This suggests that length dependent force generation in cardiac muscle i based primarily on length dependent changes in the separation between myosin an actin filaments .

A variety of optical techniques have been developed to measure length dependent changes during contractile cycle of mammalian cardiac myocytes no invasively (Delbridge and Roos , 1997) , which include two general categories - thos employing light diffraction techniques and those directly monitoring microscopic ce images . These methods have been used with various detection and analysi schemes to provide whole cell , sub-cellular and sarcomere indices of myocyt contractile function . Like most methods , each approach has its inherent advantage and limitations , but the overriding concern is the attainment of adequate spatial an temporal resolution to assess the cell's function unambiguously . The light diffractio method employs monochromatic light and a series of diffracted layer of lines ar observed , whose spacing is inversely proportional to the striation pattern periodicit as observed in an optical microscope . Diffraction patterns arise from the interferenc of light scattered by the A-I band cross - striations ; and it provides an index c average striation spacing of the muscle . This method can monitor sarcomer dynamics from muscle preparations if the striations are relatively well ordered (Roo and Leung , 1987) .

Cell edge detection technique was developed to evaluate relative changes in the contractile magnitude . Both single edge detection and double edge detection techniques have been used . In single edge detection ; the motion of one end of the contracting myocyte has been measured by using a photodiode line array , either directly coupled to the microscope or monitored from the video - generated image (Rich *et al* , 1988) . In the two edge detection imaging , the microscopic image of the whole cell is focussed directly , and cell shortening can be measured with good temporal resolution . A digital imaging technique of a quite different design , based on the output of a 512 - element line scan camera has been developed by Harris *et al* , (1987). In this system one complete scan of cell length occurs each millisecond during a triggered contractile cycle and the camera output of successive scans is digitised to consecutive horizontal lines of a video frame display . This is then analysed to obtain cell length data . It is assumed that all these optical monitoring approaches ultimately provide a measure of the same basic parameter - the time course of myofilament overlap during contraction . The degree of applicability depends on the specific experimental goals and the methods being utilised .

The video - based edge - detection system supplied by HVS Image Analysing (UK) is a device that records the contractile amplitude as a function of change in cell length . This double-edge detection system has been adopted for this study and has been found to be suitable for assessment of the inherent variation in cardiomyocyte mechanics and the response to variation in the extracellular milieu .

Age dependent variation in cardiomyocyte mechanics :

Even after attaining developmental maturity , the heart continues to enlarge in size . Hypertrophy takes the place of hyperplasia during postnatal growth of the heart . In addition to cellular enlargement , structural remodelling of the myocardial cells including alterations in the relative proportions of cellular organelles and also in the ultrastructure of individual organelles occur during hypertrophy of the adult heart (Oparil *et al* , 1984) . In the rat , the switch from cardiac myocyte hyperplasia to hypertrophy occur between days 3 and 4 of postnatal development (Li *et al* , 1996) . Developmental maturation is completed by 25 - 30 days (Siebrits and Barnes , 1989) and according to Liebowitz et al (1991) in male Sprague - Dawley strains of rats puberty is attained by the age of 42 - 49 days . Aging associated changes are said to be initiated approximately at 18 months (Adams and Jones,1982 ; Quigley *et al* , 1990 ; Zakaria et al , 1998) . Hence animals between 2 and 12 months of age were considered suitable for the study .

Very few reports are available on age associated changes in the mature myocardium . Age dependent alteration in contractile characteristics and biochemical features have been reported in rats between the age of 2 months and 12 months (Bhatnagar et al , 1984 ; Qi and Rouleau , 1997) . Cardiac tolearance to oxidative stress was found to decrease with age (Abete et al , 1999) . This study has shown that age dependent alteration in cell geometry is associated with variation in contractile amplitude and differential response to ion - channel modulators .

Age dependent alteration in cell geometry :

Increase in heart weight accompanies enhanced body weight , but the heart weight to body weight ratio shows a decreasing trend (Table - 4.4) . The increase in body weight and heart weight was associated with increase in cell length and

sarcomere length . The results indicate the occurence of physiological hypertrophy as age advances . The heart weight to body weight ratio has been reported to decrease with age in normotensive Fischer rats; the ratio being 3.2 at 3 months and 2.6 at 18 months (Tschudi and Luscher , 1998) , which is comparable with our observation (Table - 4.4) . Enhancement of cell length associated with increase in sarcomere length , suggests that the hypertrophy is at the level of the sarcomere . This alteration in myocyte geometry can be associated with cellular remodelling leading to variation in mechanical characteristics .

Age associated variation in contractile amplitude :

Contractile amplitude was found to increase with age (Figure - 4.4) . As the contractile amplitude is normalised to cell length , the increase in amplitude is not related to the age dependent change in cell length . It is to be inferred that the age associated functional variation is consequent to alteration in other intrinsic factors . Differences in contractile performance could be due to physical factors and activation factors (Allen and Kentish , 1985) . Physical factors which influence contractility include changes in maximum number of active cross - bridges , lateral spacing of thick and thin filaments and the restoring forces associated with sarcomere geometry . There are certain activation factors which can also influence contractility . They are the action - potential and underlying ionic currents , Ca^{2+} - movements which may lead to very rapid rise and fall of myoplasmic Ca^{2+} - concentration and binding of Ca^{2+} to troponin .

The amount of cytosolic Ca^{2+} being an important variable , diastolic $[Ca^{2+}]_i$ - levels were determined . The level of $[Ca^{2+}]_i$ was found to be comparable in the three age groups (Figure - 4.4) . This finding is supported by an earlier report ,

where the diastolic levels of cytoplasmic Ca^{2+} were comparable in myocytes from Fischer 344 rats at 3 months , 8 months and 12 months of age (Nitahara *et al* , 1998) .

As the observed variation in contractile amplitude was not due to variation in myoplasmic [Ca^{2+}] , it may be due to other factors such as distribution or functional capacity of ion - channels or differences in the sensitivity of myofibrillar proteins to Ca^{2+} which can vary with sarcomere length .

Possibility of alteration in the structural and functional features of SL and SR - membrane systems with cell enlargement was suggested by Gwathmey and Morgan (1985) . Since membrane transport systems have a significant role in regulating myoplasmic Ca^{2+} - concentration , the possible role of SL and SR - transporting systems which directly or indirectly regulate Ca_i - levels were examined using selective channel antagonists .

Effect of age on sarcolemmal and sarcoplasmic reticular ion - channels :

The contribution of a channel to the mechanical function was inferred by the conventional method of blocking the channel with a suitable blocker and assessing the consequent mechanical response. The channel modulators were selected in such a way that a particular blocker selectively blocks one particular transport mechanism with little effect on other tranport systems . Ostadal *et al* (1993) and Muscari *et al* (1992) have suggested that along with changes in the expression of myosin isoforms , postnatal development and aging are associated with alterations in the mechanisms controlling the intracellular Ca^{2+} - transport at the level of SL and SR . The mammalian myocardium is known to depend on both transsarcolemmal Ca^{2+} - influx and Ca^{2+} release from the SR . The relative

contribution of the two mechanisms varies significantly during development. The SR of the newborn rat is not fully developed and Ca^{2+} - release from SR is thus much less expressed as compared with the adult animals . The contractility of neonatal myocardium depends therefore to a large extent on the entry of Ca^{2+} across the SL via the Ca^{2+} channels . During the course of development the ability of SR to accumulate Ca^{2+} increases and there is a progressive maturation of the Ca^{2+} - release from the SR (Ostadal *et al* , 1993). In senescent animals contraction and relaxation times are prolonged . This in part is explained by an alteration in excitation - contraction coupling due to an increased duration of action potential due to slower inactivation of SL - L - type Ca^{2+} - channels and prolonged Ca^{2+} - efflux via Na^{+} - Ca^{2+} exchange , reduced biosynthesis of the Ca^{2+} - stimulated ATPase pump of SR and prevalence of the V3 isoform of myosin with slow ATPase activity (Muscari *et al* , 1992) .

Age dependent variation in response to Ca - channel modulators have been observed in the mature adult life span , and the results are discussed below .

Effect of SL - L - type Ca - channel blocker (Verapamil) : Calcium influx through the L - type Ca - channel in the cardiomyocytes is the initiating event in the excitation - contraction coupling process . The L - type Ca - channel plays an integral role in Ca_i - transient mechanisms and in turn in the contractile performance of the cardiomyocyte .

Verapamil , the voltage operated Ca - channel antagonist is a very effective antihypertensive and antianginal agent and depresses myocardial contractility by blocking SL - L - type Ca - channel (Hess *et al* , 1984 ; Bell and McDermott , 1995) .Negative inotropic effect to verapamil reflects the functional contribution of SL - L -

type Ca - channel to myocyte contractility in all the three age groups (Figure -4.5). The activity of this voltage activated Ca - channel is found to be maximum in 12 month old cardiomyocytes , as denoted by the significant depression ($p < 0.05$) in the contractile amplitude in the presence of verapamil (Figure - 4.5).

Diastolic Ca^{2+} - levels were reduced in all the three age groups , without significant variation between groups (Figure - 4.5) .

Zhou *et al* (1998) , have reported that during aging the magnitude of L - type Ca - channel current is significantly increased , even when the $I_{Ca,L}$ density remains unaltered . Increased sensitivity to verapamil in older animals may be the precedence of the aging process . It is inferred that the contribution of the L - type channel in myocardial performance increases as a function of age .

Effect of SL - T - type Ca - channel blocker ($NiCl_2$) : T - type Ca - channels are activated at relatively negative trans-membrane potentials and produces currents of shorter duration (Nilius *et al* , 1985) . T - type Ca^{2+} - currents have been speculated to play a role in pacemaker activity (Hagiwara *et al* , 1988) .Exact physiological significance of the T- type Ca^{2+} - current in ventricular muscle cells remain unclear (Bers , 1993) . T - type Ca - channels have recently been reported to be impressively associated with growth and remodelling (Nuss and Houser , 1993 ; Sen and Smith , 1994) . Though there is a high density of the T - type Ca - channels in cardiac myocytes of embryonic and neonatal animals ; in adults the channels are said to be found only when there is hypertrophy (Hart , 1994) . It has been reported that Ca - influx through these channels can be abolished in the presence of relatively low concentration of nickel (Hagiwara *et al* , 1988 ; Marban and O'Rourke , 1995) .

In this study the sensitivity of the T - type Ca - channel antagonist was found to increase with age as assessed by the use of the blocker $NiCl_2$ (Figure - 4.6) . The diastolic Ca_i - level and the contractile amplitude were higher in 2 month old animals though not significantly different from the baseline (Figure - 4.6). It is possible that in younger animals T - type channels are not functional , but may become functional with increasing age . It has also been reported that the T - type channel activity may be negligible, and that T- type channels are not functional at certain phases of the life span (Xu and Best , 1992) .

Antihypertensive agents which modulates T - type channels have been recently introduced . The Ca^{2+} - antagonist , mibefradil is a selective T - type Ca - channel blocker (Hermsmeyer , 1998 ; van Zwieten , 1998) . Its beneficial effect lies in the absence of negative cardiac inotropy. Though interspecies variation cannot be excluded , the observation of age dependent response to T - type antagonists in experimental animals envisages further studies in man . In addition to the L - type channel , the T - type channel can also account for the age related increase in contractile amplitude .

Effect of Na - Ca exchanger blocker ($MnCl_2$) : Cardiac muscle is one of the richest sources of Na - Ca exchanger activity compared to other tissues (Bers , 1993). Na - Ca exchanger is the major Ca - efflux channel in cardiac myocytes . Leblanc and Hume (1990) are of the opinion that active Na^+ - influx through Na - channel during the phase - 1 (rapid upstroke) of action potential , would reverse the Na - Ca exchanger mode to expel Na^+ out and permit Ca^{2+} into the cell . Thus the Na - Ca exchanger also contributes to the plateau phase of action potential . Na - Ca

exchanger mediated Ca - influx can also trigger Ca - induced Ca - release from SR (Levesque *et al* , 1991).

$MnCl_2$, the Na - Ca exchange modulator did not produce significant change in contractile amplitude in 2 month old cardiomyocytes . But in 6 and 12 month old cardiomyocytes , significant negative inotropy was observed (Figure - 4.7) . Contrary to an expected increase in contractile amplitude and rise in diastolic $[Ca^{2+}]$ due to prevention of Ca - efflux , a decrease in diastolic Ca_i - level was observed in all the three age groups .

The observed reduction in contractility could be due to reduction in Ca - influx . It is known that Na - Ca exchanger is functional as a Ca - influx channel at a very early age (Haddock *et al* , 1998) . But in the 2 month old cardiomyocytes no significant change was observed . One possibility is that there is some other mechanism which brings about functional compensation in the young animals . It has been observed that if Na - Ca exchanger is nullified by decreasing the Na - gradient or by depolarisation ; a large fraction of the Ca^{2+} from the SR will be resequestered and rest decay is slowed . Such a response should be associated with increase in diastolic Ca^{2+} . However the same has not been observed in this study . Depending on the transsarcolemmal $[Na^+]_i$ gradient , rest can either deplete the SR or fill the SR with Ca^{2+} . Clearly a dynamic yet delicate balance exists in the intracellular Ca^{2+} in the heart and changes in these systems can lead to inotropic and lusitropic changes (Bers *et al* , 1993).

Effect of Na , K - ATPase inhibitor (Ouabain) : Na , K - ATPase maintains the high K^+ and low concentration of Na^+ in the intracellular milieu (Skou, 1990) . The inotropy induced by cardioactive steroids can be attributed primarily to Na^+ -

pump inhibition and consequent shifts in Na - Ca exchanger making Ca - influx more favourable and Ca - efflux less favourable (Barry *et al* , 1985 ; Kim *et al* , 1987) . Cardiac glycosides such as ouabain causes augmentation of Na^+_i , as a result of inhibition of Na , K - ATPase .

In the presence of the inhibitory glycoside ouabain , significant positive inotropic response ($p < 0.05$) was observed , the maximum being in 6 month old rats (Figure - 4.8) . The diastolic Ca_i - levels were inversely related to age , but the difference was not statistically significant . The rise in diastolic Ca^{2+} in young animals without a proportionate increase in contractile amplitude , could be due to inefficiency in Ca^{2+} - handling .

Aging has been reported to be associated with reduction in Na^+ - pump activity (Kennedy *et al* , 1996) . Ellingsen (1994) is of the opinion that reduction in Na^+ - pump activity may not be due to reduced synthesis of pump protein and it can be due to increased cell volume and increased content of other cellular proteins such as contractile proteins . But in a study conducted in congestive heart failure patients , age dependent increase in its activity has been reported (Legato , 1979) , as digitalis receptors are fewer in young patients than in the adult. The inhibition of the Na^+ - pump by ouabain and consequent shift in Na - Ca exchanger can increase contractility by increasing diastolic $[Ca^{2+}]_i$, increasing SR Ca - content and its subsequent release and increase in the Ca - influx early in contraction . These are of course all interrelated and it is difficult to determine unequivocally the fractional contribution of each effect (Bers , 1993) . It is possible that the higher $[Ca^{2+}]_i$ in the presence of Na^+ - pump inhibition contributes to the increase of I_{Ca} as suggested by Marban and Tsien (1982) . When the cell gains too much Ca^{2+} due to shift in Na - Ca exchanger activity , negative inotropic and

arrhythmogenic effects occur (Bers , 1993) . This could explain the comparatively lower contractile amplitude in 2 month old cardiomyocytes inspite of higher diastolic calcium .

Effect of SR Ca - channel modulators (Ryanodine and Caffeine) *:* The maintenance of Ca - gradient between the heart cells and extracellular milieu is the result of differential activity of SL Ca - transport systems . At the same time , SL - Ca - fluxes have a quantitatively minor role compared to the total amount of Ca^{2+} needed during a single contractile cycle in cardiac myocytes . The Ca^{2+} crossing the SL triggers release of Ca^{2+} from intracellular Ca - storage sites and derives most of the Ca^{2+} needed for contractile activity . SR is the most important Ca - storage structure responsible for the fine regulation of Ca^{2+} in the cytosol .

The functional role of SR was evaluated using ryanodine and caffeine which influences the inotropic state of cardiac system by regulating release and uptake of Ca^{2+} by the SR (Weber , 1968 ; Rasmussen *et al* , 1987 ; Xu *et al* , 1996) .

Ryanodine , the SR Ca - release channel inhibitor induced negative inotropy in the three ages . The negative effect was maximum in 12 month old animals , but the age related variation was not statistically significant (Figure - 4.9) ; the diastolic Ca_i - level followed the same pattern as that of contractile amplitude , but the difference from the baseline was significant only in 12 month old .

Caffeine has the property of emptying SR of its Ca - content as it increases the Ca - permeability of SR (O'Neill and Eisner , 1990) . In the presence of caffeine , positive inotropy was noted in all the three age groups (Figure - 4.10) , the inotropic response being inversely related to age , though the difference was not statistically significant . The increase in amplitude is found to be in tune with the diastolic Ca_i -

levels . SR uptake of Ca^{2+} slows with increasing age (Walker *et al* , 1993) . This could explain the age dependent decrease in response to caffeine .

The response to SR Ca - release and uptake inhibitors indicates that the SR is functionally active in animals by the age of 2 months .

Conclusion :

The experimental observations lead to the conclusion that physiological hypertrophy is initiated at the level of sarcomere and is associated with changes in the frequency and / or function of membrane bound ion - channels . Differential response to channel modulators indicate that sarcolemmal ion - transport systems exhibit variation in their functional characteristics as the myocardium transcends the different stages of adult life span . Age related increase in contractile amplitude can be attributed to an increase in the functional capacity of voltage activated Ca^{2+} - influx channels . The inotropic response to Na^{+} - pump inhibition was found to be more in 6 month old compared to younger and older animals . These observations also assume importance because of the increasing clinical use of positive and negative inotropic drugs .

Inotropic response to variation in extracellular Mg - concentration :

Magnesium is a potent antiarrhythmic and cardioprotective agent which is likely to exert its beneficial effects via its antagonistic effect to Ca^{2+} . The contractile response to variation in $[Mg^{2+}]_o$ was studied by exposing the myocardial cells to different levels of Mg ranging from 1.8 mM to nominally Mg - free conditions .

A positive inotropy with decrease in $[Mg^{2+}]_o$ attaining a peak at 0.48 mM $[Mg^{2+}]$, followed by a decline in amplitude on further reduction in $[Mg^{2+}]_o$ was observed ; the pattern being similar in all the three age groups (Figure - 4.11) . At

0.48 mM $[Mg^{2+}]_o$, the diastolic Ca^{2+}_i - level was high (Table - 4.5). Na^{+}_i - level was low but not significant (Table-4.6). K^{+}_i and Mg^{2+}_i levels remained unaltered with decreasing Mg^{2+}_o - concentration (Tables - 4.7 and 4.8). The positive association of diastolic Ca - level and contractile amplitude indicates that the inotropic response is mediated by a rise in the basal Ca - level. As observed in this study the cytosolic Ca^{2+} -levels from mature New Zealand rabbits was found to increase at 0.5 mM $[Mg^{2+}]_o$ and the levels were lower at 0 mM $[Mg^{2+}]_o$. But in juvenile rabbits, the peak was at 2.0 mM $[Mg^{2+}]_o$ (Cyran *et al*, 1992).

Mg^{2+} is said to exert its inhibitory effect on Ca - influx, due to its smaller ionic radii (Mg^{2+} - 0.6 A° and Ca^{2+} - 0.95 A°) and it is known to compete for the same site on SL with Ca^{2+} (Fransto da Salva and Williams, 1991). Mg^{2+} is considered as the physiological Ca - antagonist (Iseri and French, 1984) and this may explain the negative inotropy at higher $[Mg^{2+}]_o$ - 1.8 and 1.4 mM. The relaxation of this inhibitory effect at 0.48 mM $[Mg^{2+}]_o$ may be responsible for the rise in Ca_i - level and positive inotropy. Reduction in inotropy observed in the nominally Mg - free medium may be due to Ca - loss through a relatively more permeable membrane. Total depletion of Mg can lead to changes in membrane fluidity and permeability (Heaton *et al*, 1989), and impair myocardial carbohydrate and lipid metabolism along with cardiac bioenergetics (Altura *et a l*, 1996)

Katholi et al (1979) studied the dual dependency of heart cells on both Ca^{2+} and Mg^{2+} for electrical stability. According to them, Ca^{2+} participates in the generation of action - potential, promoting electrical stability, initiating myocardial contraction, and Mg^{2+} has importance as an activator of cation - transport through the SL.

Progressive decrease in $[Na^+]_i$ - level could be due to compensatory changes in the $[Ca^{2+}]_i$ - level . Tillisch *et al* (1979) stated that myocardial contractile response can be affected by the relative concentrations of Ca^{2+} and Na^+ . Compensatory changes in Na^+_i affect Ca^{2+}_i - level by influencing Ca - influx which is largely determined by $[Na^+]_i$. Na^+_i - level is also influenced by $[Mg^{2+}]_o$.

The reversibility of $[Mg^{2+}]_o$ - induced changes on transferring to Mg - sufficient medium indicates that altered contractility observed during Mg_o - reduction is not a permanent change . This is supported by the report that Mg^{2+} ions contribute to the integrity and stability of biomembranes along with Ca^{2+} (Gunther *et al* , 1984 ; Beaven *et al* , 1990 ; Antonenkov *et al* , 1976) , and their absence can lead to functional variations without causing permanent damage to membrane structure .

Inotropic response to variation in $[Mg^{2+}]_o$ followed the same pattern in animals of all age groups , but the younger animals were found to be more sensitive to the changes . The capacity of cardiac cells to respond to external stimuli , either hormonal or nonhormonal declines during senescence (Fleg , 1986 ; Guarineri *et al* , 1980 ; Amerini *et al* , 1985) . The influence of Mg^{2+} on adenylate cyclase activity was investigated from hearts of young (1 month old) and aged (24 months old) rats . The basal activity of adenylate cyclase and its responsiveness to stimulatory or inhibitory effects declined with age . Compared to the enzyme from the heart of aged rats , unstimulated adenylate cyclase from the heart of young rats was more sensitive to an increase in $[Mg^{2+}]$ in the incubation medium . The authors suggest that aging leads to a higher requirement for Mg^{2+} at the allosteric site on the catalytic moiety whose occupancy is essential for the full expression of stimulatory activity (Pignatti *et al* , 1993) . The observed decrease in sensitivity could be the initiation of the aging related change.

Effects of Mg - insufficiency on age related functional response to sarcolemmal and sarcoplasmic reticular ion - channel modulators :

In rats on a Mg - deficient diet the serum Mg - level was found to be 0.45 mM compared to 0.8mM in animals on Mg - sufficient diet (Kumar *et al* , 1996) . In dogs treated with diuretics , 42% decrease in serum Mg was observed (Singh *et al* , 1975) . The *invitro* experiments of this study has shown that , the peak in the inotropic response was at 0.48 mM $[Mg^{2+}]_o$; the decrease from the control Mg - concentration (0.8 mM) being 40% . Interestingly the sensitive concentration in *invitro* experiments corresponds to the reduction in serum Mg in pharmacological interventions as well as nutritional deficiencies . Reports available suggest that Mg^{2+} - concentration can decline to levels as low as 0.3 mM , but that can occur only , in drastic Mg - deficiency (Zhang *et al* , 1992) . Considering these facts , the inotropic response to ion - channel modulators in Mg - insufficiency was examined at 0.48 mM $[Mg^{2+}]_o$ and compared with the control (0.8 mM Mg_o).

Magnesium modulates the functioning of ion - channels located both in the SL and SR either by acting as an antagonist to Ca^{2+} or as an activator of the enzyme systems which use ATP (White and Hartzell , 1989 ; Agus *et al* , 1989) . Hence inotropic response of cardiomyocytes at 0.8 and 0.48 mM concentrations of Mg_o was assessed in the presence of ion channel modulators . The intracellular Ca^{2+} - level was also measured following exposure to ion - channel modulators .

Effect of SL - L - type Ca - channel blocker (Verapamil) : Negative inotropic response to verapamil was comparable at both the levels of Mg_o (Figure - 4.12) . This suggests that Mg^{2+} could be an L - type blocker as the positive inotropic effect

in Mg - deficient medium was neutralised . Extent of reduction in contractile amplitude and diastolic Ca^{2+}_i was similar in all the three age groups and comparable with the control (Figure - 4.12) .

These observations suggest that in all the age groups the positive inotropic effect at 0.48 mM $[Mg^{2+}]_o$ could be mediated by the activation of the SL - L - type Ca - channel . At sub optimal levels of Mg , the extent of reduction in amplitude compared to the value prior to addition of the drug is high ; but Mg has no influence on the action of the blocker .

Effect of SL - T - type Ca - channel blocker ($NiCl_2$) : At sub optimal levels of $[Mg^{2+}]_o$, the negative inotropic effect induced by Ni^{2+} was greater compared to that in sufficient Mg . In the younger animals , $NiCl_2$ did not induce a negative inotropic effect in sufficient Mg , but in Mg - insufficiency the contractile amplitude was significantly reduced (Figure - 4.13) . This in conjunction with the exaggerated response to Mg - insufficiency in older animals suggest that the T- type channel may be activated on extracellular Mg - reduction (Figure - 4.13) .

The diastolic levels of $[Ca^{2+}]_i$ do not correspond to the change in contractile amplitude (Figure - 4.13) . According to observations by Hess (1988) , the T - type channels do not exhibit differences in inactivation depending upon the ion that carries the charge , while L - type Ca - channels displays prominent Ca^{2+} - mediated inactivation .

These observations suggests that age and $[Mg^{2+}]_o$ influences the response to T - type Ca - channel antagonists . This finding gains importance as new T - type Ca - channel modulating antihypertensive agents like mibefradil have been recently introduced (Hermsmeyer , 1998) .

Effect of Na - Ca exchanger blocker ($MnCl_2$) : Na - Ca exchanger function was found to vary with alterations in $[Mg^{2+}]_o$ depending upon age (Figure - 4.14) . The observations suggest the possibility of Ca - influx via Na - Ca exchanger during Mg - insufficiency ; which in the presence of $Mg^{2+}{}_o$ will be inhibited to a certain extent through Ca- antagonistic effect of Mg^{2+} . Similar explanation was given by Dichtl and Vierling (1991) for the inhibition of Ca- inward current in heart ventricular muscle on enhancing extracellular Mg - concentration . Studies on isolated cardiac SL - vesicles also support the existence of such an influence of Mg^{2+} (Trosper and Philipson , 1983) .

In 2 month old cardiomyocytes $MnCl_2$ inhibited contraction . In the absence of Mg , $MnCl_2$ induced cessation of contraction in all the three age groups . The negative effect is found to be reversible suggesting that the cessation of contraction in Mg - deficiency is not due to permanent damage . Mg - deficiency is known to promote Na - Ca exchange activity (Bara *et al* , 1993) . This could be the possibility for the drastic changes observed in Mg - deficiency , associated with the presence of the antagonist .

Effect of Na , K - ATPase inhibitor (Ouabain) : The use of ouabain as an inotropic agent has been in vogue for decades . In 6 and 12 month old rats , ouabain augmented the positive inotropy induced by Mg - deficiency . In 2 month old animals , as observed in Mg - sufficient medium inspite of the high diastolic Ca - levels , the contractile amplitude did not register a compensatory increase and was lower than the amplitude in Mg - deficient medium in the absence of the blocker (Figure - 4.16) . As mentioned earlier and as suggested by Li and Vasalle (1984),

the mechanical function may be affected by Ca - overload . It has been suggested that Mg - deficiency might lead to Ca - overload following an increase in myocardial Na due to inhibition of Na,K - ATPase and reversal of Na - Ca exchange (Ahmad and Bloom , 1989) . Low levels of Mg has been found to inhibit Na , K - ATPase activity (Bara *et al* , 1993) , so exposure to ouabain in Mg - deficiency can have an additive effect . It has also been reported that digitalis toxicity is reduced in the presence of Mg (Seller , 1971) .

Tackett and Holl (1981) have reported that Mg - deficiency lowered both the threshold for sustained ventricular arrhythmia and the inotropic threshold , for ouabain treated dogs . They found that the dose of ouabain producing 50% of the maximum contractile force increases in normomagnesemic animals was equal to the dose , producing sustained arrhythmia in the hypomagnesemic animals . Earlier , Singh et al (1975) observed in mongreal dogs that hypomagnesemia induced by diuretics , resulting in 42% decrease in serum Mg facilitated ouabain induced arrhythmia which could be controlled by intravenous administration of $MgSO_4$. White and Hartzell (1989) have also suggested that hypomagnesemia can predispose to cardiac glycoside toxicity .

The data also indicate that in addition to extracellular Mg , age is another variable influencing the response to inotropic agents that act by modulating the Na^+ - pump .

Effect of SR Ca - channel modulators (Ryanodine and Caffeine) : Even though alterations in $[Mg^{2+}]_o$ have no effect on $[Mg^{2+}]_i$ - levels ; it has been speculated that altered $[Mg^{2+}]_o$ - levels could affect the intracellular Mg - buffering capacity (Koss and Grubbs , 1994 ; Howarth and Levi , 1998) . Both SR Ca - ATPase mediated Ca -

uptake and ryanodine sensitive SR - Ca - release channel functions are reported to be sensitve to Mg^{2+} (Meissner and Henderson , 1987) . Meissner and Henderson (1987) reported that inclusion of 3 mM free Mg shifted the Ca - dependence of Ca - efflux rate in cardiac SR vesicles to higher $[Ca^{2+}]$, and intracellular Mg when lower than 3 mM in cardiac muscle would increase the Ca - sensitivity of the release channel . They also suggested that Ca - uptake in cardiac SR vesicles can be dramatically increased by inclusion of agents like Mg^{2+} and ruthenium red which block the Ca - release channel . Since Mg^{2+} acts as a cofactor in enzymatic reactions which require ATP , it is assumed that Ca - uptake by SR Ca - ATPase also requires an optimal level of Mg^{2+}_i (Yamadi *et al* , 1986) . In red cell membrane vesicle , ATP - dependent Ca - transport was reported to be activated by divalent metal ions (Enyedi *et al* , 1982) . The authors suggested that in such reactions , metal ions from ATP - complexes can serve as an energy donor substrate for the Ca - pump . They are of the opinion that ATP - dependent Ca - transport requires the presence of Ca and Mg at the membrane surface . Thus the molecular basis of this transport is a Ca^{2+} , Mg^{2+} - activated ATPase . Since the Ca- pump activity is dependent on $[Ca^{2+}]$ along with Mg^{2+} and ATP , and the situation is further complicated by Ca - release channel which will alter net Ca - transport by the Ca - pump . Fabiato and Fabiato (1978) speculated that variation in $[Ca^{2+}]_i$ due to $[Mg^{2+}]_o$ - depletion can also affect Ca - uptake and Ca - release by the SR .

Contractile amplitude and diastolic Ca - levels were found to be unaffected by the extracellular Mg - level when exposed to ryanodine (Figure - 4.16) . Inspite of high initiator Ca^{2+} , when Ca - release was blocked as can be expected , the amplitude was not significantly different from that in sufficient Mg , though it was slightly lower in all the three age groups .

Inotropic response induced by sub optimal levels of $[Mg^{2+}]_o$ was attenuated by caffeine. Diastolic Ca - levels were also decreased at 0.48 mM $[Mg^{2+}]_o$, when caffeine was present (Figure - 4.17) . Rasmussen et al (1987) are of the opinion that Ca^{2+} abruptly released by caffeine , from SR may be extruded by an electrogenic Na - Ca exchanger or by a slower Na_o - dependent mechanism presumably a SL Ca - ATPase . O'Neill *et al* (1990) have also suggested that in the presence of caffeine $[Ca^{2+}]_i$ rise is transient and the decline may represent Ca - extrusion from the cell via Na - Ca exchange . It is therefore possible that at reduced $[Mg^{2+}]_o$, Ca^{2+} is extruded through SL Ca - ATPase and / or Na - Ca exchanger , thereby accounting for the lower inotropic response associated with decrease in diastolic Ca_i - levels .

Conclusion :

This inquiry into the effects of suboptimal levels of $[Mg^{2+}]_o$ on cardiomyocyte mechanics leads to the conclusion that variation in the level of extracellular Mg^{2+} has a significant influence on the functioning of ion - channels and the response to ion - channel modulators . Magnesium appears to be an L - type channel blocker and the inotropic response in Mg^{2+} insufficiency is probably induced by an increase in the Ca^{2+} - induced Ca^{2+} - release mediated by greater influx of initiator Ca^{2+} through the SL - L - type Ca - channel . This conclusion is based on the observation of neutralisation of the inotropic response by the L - type channel antagonist , verapamil and the SR - Ca - release channel inhibitor , ryanodine ; without much variation between the groups . The inotropic response to caffeine is attenuated in Mg - insufficiency and the response to T - type Ca - channel blocker , Na - Ca exchange antagonist and Na , K - ATPase inhibitor manifests an age related variation suggestive

of an interaction between age and the $[Mg^{2+}]_o$, influencing the mechanical response Though there may be some ambiguity in the use of ion - channel blockers as predictors of the function of specific channels ; the data shows that the response to ion - channel modulators in pharmacological interventions can be affected by variation in extracellular Mg . The effect can also be dependent on age .

CHAPTER - V

SUMMARY AND CONCLUSIONS

Among the factors that regulate cardiac contractility , age of the mature adult and the influence of extracellular Mg^{2+} are two variables that have not drawn much attention of researchers in the field of cardiac biology . Magnesium has recently been recognised as an ion with significant influence on cardiac contractility (Fry and Procter , 1993) . Hypomagnesemia has been positively correlated with the prevalence of cardiovascular disorders in regions with soft water (Durlach *et al* , 1989) . The assessment of the effects of suboptimal levels of Mg on cardiac function is relevant in Kerala , where the Mg - content of drinking water is low (< 5 mg / L) . Hypomagnesemia can also occur as chronic or acute manifestations of pathological conditions or pharmacological interventions .

The focus of the investigation has been on the effects of Mg - insufficiency on cardiac contractility and assessment of age - related variation in sensitivity; assuming that the contractile properties of the adult myocardium varies with age .

Cardiac myocytes isolated from adult male Sprague - Dawley strains of rats of three different age groups - 2 months , 6 months and 12 months were chosen as the experimental model . The ages were selected on the basis that developmental maturation of rats is completed by 1 month and symptoms of aging are evident after 18 months .

Though a good model for assessment of myocardial contractility , difficulties in the isolation of viable cells has evaded its use for experimental studies . The first step of this investigation was therefore standardization of the technique for isolation of cardiac myocytes . Cells were isolated by the enzymatic perfusion method using collagenase for dissociation of cells . Relaxed myocytes were isolated in Ca - free

medium and the Ca - level was enhanced for obtaining Ca^{2+} - tolerant cells . Preparations with more than 60 - 70 % viable cells were used for experimental studies .

Contractile amplitude was measured as a function of change in cell length using a cell - length monitor . Contraction was quantified as percent change in cell length . Cell length and sarcomere length were determined from a calibrated video screen .

The major objectives of the study were ;

1) Delineation of age - dependent changes in cardiomyocyte mechanics .
2) Assessment of inotropic response to variation in extracellular Mg^{2+} .
3) Assessment of the influence of $[Mg^{2+}]_o$ on mechanical response , following exposure to ion channel modulators .

Description of Procedures :

For characterisation of age dependent changes , cell geometry and contractile amplitude of cells isolated from animals of the three age groups was recorded . The inotropic response was examined following exposure to antagonists of the different SL and SR - ion channels , assuming that the age dependent change in contractile amplitude is due to variation in the channels influencing ion - transients .

Inotropic response to variation in the levels of extracellular Mg^{2+} was assessed . The cells were exposed to ion channel modulators and the effect of lower levels of Mg on contractile amplitude was recorded .

In an attempt to correlate the contractile changes with change in intracellular Ca^{2+} , the diastolic levels of Ca^{2+} was assessed by the dual wavelength excitation method with the help of the ion - sensitive fluorochrome , fura - 2AM .

The response to a variable was recorded as percent change from basal value and presented as mean ± SEM . Variation within group was assessed by F - test and the significance of difference between means was determined by student's t - test . $p < 0.05$ was considered significant .

Major findings :

Cardiac myocytes were isolated successfully in viable condition and the cells were found to be suitable for assessment of cardiomyocyte mechanics . Modification of existing techniques were carried out for optimisation of yield . The use of Ca^{2+} - chelators brought about four fold increase in cell yield compared to that obtained by commonly reported procedures . The cells remained viable for more than 10 hours and responded to electrical stimulation .

The contractile amplitude of cardiomyocytes increased with age . The shortening fraction of cells were 4.37 % ± 0.56 at 2 months , 4.62 % ± 0.85 at 6 months and 4.98 % ± 0.81 at 12 months of age . The sarcomere length and cell length also exhibited an age dependent increase . As the contractile amplitude has been normalised to cell length , the age dependent increase could be associated with changes other than the cell geometry .

Assuming that the change in amplitude could be due to age - dependent variation in the distribution and / or function of ion channels that regulate Ca^{2+} - transients , the inotropic response to channel specific blockers was assessed and age associated variation was observed . The sensitivity to Ca^{2+} - influx channel

antagonists (L- type and T - type) increased with age . The response to Na^+ , K^+ - ATPase inhibitor ouabain was maximum at 6 months of age .

Variation in extracellular Mg within physiological limits was associated with change in contractile amplitude . An inverse relationship between Mg - content and contractile amplitude was observed with the younger animals being more sensitive . In all the three age groups , contractile amplitude showed a peak at 0.48 mM Mg . At 0.8 mM $[Mg^{2+}]_o$, the extent of contraction was of the order 12 months > 6 months > 2 months ; but the shortening fraction in 0.48 mM $[Mg^{2+}]_o$ was 6.63 % ± 0.74 , 6.92 % ± 1.54 and 6.57 % ± 1.55 respectively for 2 - , 6 - and 12 - month old . The percentage increase in contractile amplitude at 0.48 mM $[Mg^{2+}]_o$ was of the order 2 months > 6 months > 12 months . Change in $[Mg^{2+}]_o$ was associated with change in $[Ca^{2+}]_i$ but not of Na^+ , K^+ or Mg^{2+} .

Inotropic response to Mg - deficiency is likely to be mediated by modulation of the channels that regulate Ca^{2+} - flux . Hence , the inotropic response to Ca^{2+} - channel antagonists was examined in association with Mg - deficiency .

The inotropic response at 0.48 mM Mg was neutralised in the presence of the L - type channel antagonist . In 2 month old animals , the T - type channel blocker did not have a significant influence on contractility at 0.8 mM $[Mg^{2+}]_o$, but reduced the contractile amplitude by 33 % at 0.48 mM $[Mg^{2+}]_o$. Even in older animals , the negative inotropic response was greater than that in sufficient medium .

Inotropic response to the Na - Ca exchange blocker $MnCl_2$ was unaffected by extracellular Mg in cardiomyocytes from 6 and 12 month old animals but caused cessation of cardiomyocyte contraction in Mg deficient medium in the 2 month old .

On exposure to the Na,K - ATPase inhibitor ouabain , an interaction between age and $[Mg^{2+}]_o$ was observed . Ouabain induced an additive inotropic response in Mg - deficiency in the 12 month old cardiomyocytes . In 2 month old animals the inotropic response of Mg - insufficiency was neutralised by ouabain and in the 6 month old , the contractile amplitude was comparable at both levels of $[Mg^{2+}]_o$.

The response to SR Ca - release channel inhibitor was unaffected by $[Mg^{2+}]_o$. The inotropic response to caffeine , the SR Ca - pump inhibitor was attenuated in Mg- deficiency .

Conclusions :

Cardiac myocytes suitable for experimental manipulation have been successfully isolated from adult rats . It has been observed that maintenance of Ca^{2+}- homeostasis is vital to the isolation of healthy cells . The inotropic response to experimental manipulation has shown that the processes responsible for the exchange of ions with the environment and for excitation - contraction coupling are preserved , making it a viable preparation for the study of cardiac mechanics . It offers the potential to probe into the molecular mechanisms underlying myocardial behaviour .

The L - type Ca - channel is known to play an integral role in the determination of cardiac performance . From the observed age related increase in sensitivity to SL - Ca - influx channel antagonists , it is reasonable to speculate that age dependent rise in contractile amplitude is associated with enhanced function / distribution of L - type and T - type channels .

Calcium antagonists which reduce rather specifically the Ca^{2+} - influx through the voltage dependent Ca^{2+} - channel in the cardiac and vascular smooth muscle have many clinical applications . The observed age dependent variation in response to calcium antagonists envisages further studies in patients .

This inquiry into the effects of suboptimal levels of Mg on cardiomyocyte mechanics leads to the conclusion that variation in the level of extracellular Mg can influence the functioning of the membrane bound channels , and the inotropic response to ion channel modulators . Magnesium appears to be a blocker of Ca - influx channels . The inotropic response in Mg - insufficiency may be mediated by higher initiator Ca^{2+} and the consequent rise in Ca - induced Ca - release . This conclusion is based on the observation of neutralisation of the inotropic response in Mg - deficiency by L - type channel antagonist and also by ryanodine , the SR Ca - release channel blocker . Augmentation of response to T - type channel blocker in Mg - deficiency suggests that the channel is activated in Mg - deficiency which can also add to the initial Ca - influx . Inotropic response to Na - Ca exchange blocker and the Na,K - ATPase inhibitor indicates an age related variation suggestive of an interaction between age and Mg - level . Conceivably , differences in responsiveness to the cardiac glycoside may be related to changes in Na - Ca exchange activity . Magnesium ion acts as a cofactor in Na , K - ATPase activity of cardiac muscle (Skou *et al* , 1971) and the functioning of the SR Ca - pump also requires MgATP . Though caffeine is known to disable the SR (Barry and Bridge , 1993) , the response to caffeine was attenuated in Mg - insufficiency . Reduction in the activity of the channel in Mg - deficiency can account for such a response , but the possibility of caffeine cross - reacting with other channels has to be excluded .

The data indicates that the response to inotropic agents can be attenuated in Mg - insufficiency .

Recommendations for Further Investigations :

The quest for a solution promotes science not only by the answer , but the lead it gives to an array of newer problems that need attention . As envisaged, this study has also lent scope for further investigations .

1) The choice of an appropriate experimental model is vital for any experiment . The model to be used depends largely on the question at hand . The isolated cell model is appropriate if the primary focus is on assessment of intrinsic cellular mechanisms independent of compensatory mechanisms . Inotropic changes in the heart are mediated through mechanisms intrinsic to the cardiac cell , establishing the suitability of cardiomyocytes for assessment of changes in myocardial contractility . It has been recently reported that cardiac endothelium modulates myocardial contraction (de Keulenaer *et al* , 1995) . Though most of the work is on response to reactive - oxygen species , it may be worth extending this study to examine whether endocardial cells modify the contractile performance of myocardial cells in Mg - insufficiency , by using co-culture of endocardial and myocardial cells .

2) The study has shown that the age dependent inotropic variation is mediated probably by the variation in the ion - channels that regulate Ca - flux . Mg - deficiency also appears to modulate the functioning of the ion - channels . An attempt was made to correlate the inotropic changes with changes in diastolic Ca^{2+} - levels using ion sensitive fluorochromes . A better understanding of the molecular mechanisms underlying the inotropic changes can be obtained if the Ca^{2+} - transients are

studied. Inspite of the limitations on the quantitative details with which we can currently understand Ca^{2+} - regulation , the measurement of Ca^{2+} - flux by microspectrofluorimetry is expected to provide valuable information.

3) The inotropic changes in response to the variables studied have been related to the variation in ion - channels on the basis of the mechanical response to specific channel antagonists . Most of the channel blockers suffer from the disadvantage that the functioning of other channels are also affected . The use of patch - clamp technique for electrophysiological assessment will provide information specific to a channel . This may be studied in relation to the mechanical performance .

4) Apart from the changes in the ion - channels , biochemical variation and alteration in sensitivity of adrenergic and cholinergic receptors can influence the inotropic response . Investigation on these lines is expected to provide additional information on the mechanical performance . Methods such as those reported by Velden *et al* (1998) , where simultaneous measurements of isometric force development in cardiomyocytes along with determination of protein composition can also be applied

5) Experimental studies are carried out on the premiss that the mechanisms underlying the functional changes may be extendible to man . Despite interspecies differences age dependent changes may exist in all mammals and the ionic influence in the regulation of contractile function may be comparable . The ion channels play a prominent role in the function of the cardiovascular system and in addition to mediating ion - fluxes across cell membrane , they are primary receptors for a variety of clinically important drugs including Ca^{2+} - channe

antagonists and anti arrhythmic agents . Ca^{2+} - antagonists which reduce rather specifically the Ca^{2+} - influx through the voltage dependent Ca^{2+} - channel in the cardiac and vascular smooth muscle have been found to have many clinical applications . The inhibitory glycoside ouabain is also commonly used as an inotropic agent . The study has shown that the sensitivity to Ca^{2+} - antagonists increase with age and that the response to ouabain also is age dependent . Prospective or retrospective studies may be carried out on patients to validate the findings .

BIBLIOGRAPHY

Abete P , Ferrara N , Cioppa A *et al* The role of aging on the control of contractile force by Na - Ca exchange in rat papillary muscle ***J . Gerontol . A . Biol . Sci . Med . Sci .*** 1996; 51 : M251 - M259 .

Abete P , Ferrara N , Leosco D *et al* Age ralated effects of Platelet Activation Factor (PAF) in the isolated perfused rat heart ***J . Mol . Cell . Cardiol .*** 1992 ; 24 : 1399 - 1407.

Abete P , Napoli C , Santoro G et al Age related decrease in cardiac tolerence to oxidative stress ***J . Mol . Cell . Cardiol .*** 1999 ; 31 : 227 - 237 .

Adams I , Jones D G Quantitative ultrastructural changes in rat cortical synapses during early -, mid- and late adulthood ***Brain . Res .*** 1982 ; 239 : 349 - 363 .

Agus Z S , Kelepouris E , Duke I , Morad M Cytosolic magnesium modulates calcium channel activity in mammalian ventricular cells ***Am . J . Physiol .*** 1989 ; 256 : C452 - C455 .

Ahmad A , Bloom S Sodium pump and calcium channel modulation of Mg - deficiency cardiomyopathy *Am . J . Cardiovas . Pathol .* 1989 ; 2 : 277 - 283 .

Aikawa J K The role of magnesium in cellular processes **In *Magnesium : its biological significance*** (CRC Press, Florida) 1981 pp 21 - 29 .

Aikawa J K , Gordon S , Rhoades E L Magnesium metabolism in human beings . Studies with ^{28}Mg ***J . Appl . Physiol .*** 1960 ; 15 : 503 .

Allen D G , Kentish J C The cellular basis of the length - tension relation in cardiac muscle ***J. Mol. Cell. Cardiol.*** 1985 : 17 , 821 - 840 .

Altura B M , Altura B T New perspectives on the role of Mg in the pathophysiology of the cardiovascular system : clinical aspects ***Magnesium*** 1985 ; 4 : 226 - 244 .

Altura B M , Altura B T Role of Mg in pathophysiological processes and the clinical utility of Mg - ion selective electrodes ***Scand . J . Clin . Lab . Invest . Suppl .*** 1996 ; 56 : 211 - 234 .

Altura B M , Gebrewold A , Altura B T , Brandbar N Magnesium depletion impairs myocardial carbohydrate and lipid metabolism and cardiac bioenergetics and raises myocardial calcium content invivo : relation to etiology of cardiac diseases ***Biochem . Mol . Biol . Int.*** 1996 ; 6 : 1183 - 1190 .

Amerini S , Fusi F , Plazzesi G , Mantelli L ,Ledda F , Mungelli A Influence of age on the positive inotropic effect mediated by α and β - adrenoreceptors in rat ventricular strips ***Dev . Pharmacol . Ther .*** 1985 ; 8 : 34 - 42 .

Anderson P A W The heart and development ***Semin . Perinatol .*** 1996 ; 20 : 482 - 509 .

Antonenkov V D , Gerasimov A M , Panchenko L F Role of divalent cations in the structural organization of the peroxisomal membrane ***Vopr . Med . Klum .*** 1976 ; 22 : 522 - 528.

Anversa P , Ricci R , Olivetti G Quantitative structural analysis of the myocardium during physiological growth and induced cardiac hypertrophy : a review ***J . Am . Coll . Cardiol .*** 1986 ; 7 : 1140 - 1149 .

Arnoma O I , Halliwell B , Hoey B M , Butler J The antioxidant action of taurine , hypotaurine and their metabolic precursors ***Biochem . J .*** 1988 ; 256 : 251 - 255 .

Artman M , Nakanishi T , Rosin M Developmental cardiology enters adolescence ***Cardiovas . Res .*** 1996 ; 31 : E1 .

Ashmore R C , Corkadel L K , Green C L , Horwitz L D Verapamil not nifedepine impairs left ventricular function during exercise in hypertensive patients ***Am . Heart . J*** . 1990 ; 119 : 636 - 641 .

Bara M , Guiet - Bara A , Durlach J Regulation of sodium and potassium pathways by magnesium in cell membranes ***Magnes . Res .*** 1993 ; 6 : 167 - 177 .

Barry W H , Bridge J H B Intracellular calcium homeostasis in cardiac myocytes ***Circulation*** 1993 ; 87 : 1806 - 1815 .

Barry W H , Hasin Y , Smith T W Sodium - pump inhibition enhanced Ca - influx via Na - Ca exchanger and positive inotropic response in cultured heart cells ***Circ . Res .*** 1985 : 56 ; 231 - 241.

Beaven G H , Parmar J , Nash G B , Bennett B M , Gratzer W B Effect of magnesium ions on red cell membrane stability ***J . Membr . Biol .*** 1990 ; 118 : 251 - 257 .

Bell D , McDermott B J Inhibition by verapamil and diltiazem of agonist - stimulated contractile response in mammalian ventricular cardiomyocytes ***J . Mol . Cell . Cardiol*** . 1995 ; 27 : 1977 - 1987 .

Berg I , Guse A H , Gercken G Carbamoyl choline induced accumulation of inositol mono - , bis - , tris - and tetrakis phosphates in isolated cardiac myocytes from adult rats ***Biochim . Biophys . Acta*** 1989 ; 1010 ; 100 - 107 .

Berlin J R , Cannell M B , Lederer W J Cellular origins of the transient inward current in cardiac myocytes . Role of fluctuations and waves of elevated intracellular calcium ***Circ . Res .*** 1989 ; 65 : 115 - 126 .

Berridge M J , Irvine R F Inositol phosphates and lower cell signalling ***Nature*** 1989 : 341 ; 197 - 205 .

Bers D M , Bassani J W , Bassani R A Competition and redistribution among Ca - transport systems in rabbit cardiac myocytes ***Cardiovas . Res .*** 1993 ; 27 : 1772 - 1777 .

Bers D M , Bridge J H B Relaxation of rabbit ventricular muscle by Na - Ca exchange and SR Ca - pump . Ryanodine and voltage sensitivity ***Circ . Res .*** 1989 ; 65 : 334 - 342 .

Bers D M , Ellis D Intracellular Ca and Na activity in sheep heart purkinje fibres : Effect of changes of external Na and intracellular pH ***Pflugers . Arch .*** 1982 ; 393 : 171 - 178 .

Bers D M Calcium regulation in cardiac muscle *Med . Sci . Sports . Exerc .* 1991 ; 23 : 1157 - 1162 .

Bers D M Sarcolemmal Ca - channels **In** *Excitation - contraction coupling and cardiac contractile force* (Kluwer Academic Publishers , Netherlands) 1993 pp 49 - 69.

Bhatnagar G M , Walford G D , Beard E S , Humphreys S , Lakatta G ATPase activity and force production in myofibrils and twitch characterisation in intact muscle from neonatal , adult and senescent rat myocardium ***J . Mol . Cell . Cardiol .*** 1984 ; 16 : 203 - 218 .

Bishop S , Drummond J L. Surface morphology and cell size measurements of isolated rat cardiac myocytes ***J . Mol . Cell . Cardiol*** . 1979 : 11 ; 423 - 433 .

Blanchard E M , Solaro R J Inhibition of the activation and Tn - Ca binding of dog cardiac myofibrils by acidic pH ***Circ . Res .*** 1984 ; 55 : 382 - 391 .

Blatter L A , McGuigan J A S Free intracellular magnesium ions in ferret ventricular muscle measured with ion selective microelectrodes ***Quart . J . Exp . Physiol*** . 1986 ; 71 : 467 - 473 .

Bloom S Magnesium deficiency cardiomyopathy ***Am . J . Cardiovas . Path .*** 1988 ; 2 : 7 - 17 .

Boerth S R , Zimmer D B , Artman M Steady - state mRNA levels of the sarcolemmal Na - Ca exchanger peak near birth in developing rat and rabbit hearts *Circ . Res* . 1994 ; 74 ; 354 -359.

Bonne G , Carrier L , Richard P , Hainque B , Schwartz K Familial hypertrophic cardiomyopathy : from mutations to functional defects ***Circ . Res .*** 1998 ; 83 : 580 - 593 .

Bouron A , Portreau D , Raymond G The L- type Ca - current in single hypertrophied cardiomyocytes from the right ventricle of the ferret heart ***Cardiovas . Res .*** 1992 ; 26 : 662 - 670 .

Brenner B Effect of calcium - cross bridge turnover kinetics in skinned single rabbit psaos fibres ***Proc . Natl . Acad . USA*** . 1988 ; 85 : 3265 - 3269 .

Brilliantes A B , Bezprovannaya S , Marks A R Developmental and tissue specific regulation of rabbit skeletal and cardiac muscle Ca - channels involved in excitation - contraction coupling *Circ . Res* . 1994 : 75 ; 503 - 510.

Byrd S K , Bode A K , Klug G A Effects of exercise of varying duration on SR - function ***J . Appl . Physiol*** . 1989 ; 66 : 1383 - 1389 .

Canale J R , Campbell G R , Smolich J J . Campbell J H Development of cardiac muscle **In: *Cardiac muscle*** A Okasche and L Vollrath Eds. (Springer - Verlag , Berlin) 1986 pp 1 -10.

Cantigulia S R , Alfrey A C , Miller N *et al* Total body Mg excess in chronic renal failure ***Lancet*** 1972 ; 1 : 1300 - 1302 .

Carafoli E The homeostasis of calcium in heart cells ***J . Mol . Cell . Cardiol .*** 1985 ; 17 : 203 - 212 .

Caroni P , Carafoli E An ATP - dependent Ca - pumping system in dog heart sarcolemma ***Nature*** 1980 ; 283 : 765 - 767 .

Caroni P , Villani F , Carafoli E The cardiotoxic antibiotic doxorubicin inhibits the Na - Ca exchange in dog heart SL - vesicles ***FEBS Lett .*** 1981 ; 130 : 184 - 186 .

Casillas E , Harry D J , Kenny M Measurement of ionized magnesium in biological fluids ***Anal . Biochem*** 1981 ; 116 : 319 - 324 .

Chamberlain B K , Berenski C J , Jung C Y , Fleischer S Determination of the oligomeric structure of the Ca^{2+} - pump protein in canine cardiac SR - membranes using radiation inactivation analysis ***J . Biol . Chem .*** 1983 ; 258 : 11997 - 12001 .

Chang C , Bloom S Interrelationship of dietary magnesium intake and electrolyte homeostasis in hamsters -I . severe Mg - deficiency , elecctrolyte homeostasis and myocardial necrosis ***J . Am . Coll . Nutr .*** 1985 ; 4 : 173 - 185 .

Charpentier F , Liu Q Y , Rosen M R , Robinson R B Age related differences in β - adrenergic regulation of repolarization in canine epicardial myocytes ***Am . J . Physiol .*** 1996 ; 271 : H1174 - H1181 .

Chiche P Myocardial infarction , necrosis and ischemia ***Coeur . Med . Interne .*** 1974 ; 13 : 3 - 9 .

Chovan J P , Kulakowski E C , Benson B W , Schaffer S W Taurine enhancement of Ca - binding to rat heart sarcolemma ***Biochim . Biophys . Acta*** 1979 ; 551 : 129 - 135 .

Claycomb W C Control of cardiac muscle cell division ***Trends . Cardiovas . Med .*** 1992 ; 2 : 231 - 236 .

Claycomb W C , Palazo M C Culture of the terminally differentiated adult cardiac muscle cell : a light and scanning electron microscopic study ***Dev . Biol .*** 1980 ; 80 : 466 - 482.

Cobbold P H , Rink T J Fluorescence and biolumniscence measurement of cytoplasmic free calcium *Biochem . J* 1987 ; 248 : 313 - 323 .

Coetzee W A , Opie L H Effects Of oxygen free radicals on isolated cardiac myocytes from guinea pig ventricle : Electrophysiological studies ***J . Mol . Cell . Cardiol .*** 1992 ; 24 : 651 - 663 .

Cohen . N . M , Lederer . W . J . " Changes in the calcium current of rat heart ventricular myocytes during development " J . Physiol . 1988 ; 406 : 115 - 146 .

Colquhoun D , Hawkes A G On the stochastic properties of bursts of single ion channel openings and of clusters of bursts ***Phil . Trans . R . Soc . Lond .*** 1982 ; 300 : 1 - 59.

Crocker J W , Walmsley R N Routine plasma Mg - estimation : a useful test ? ***Med . J . Austria*** 1986 ; 21 ; 74 - 76 .

Cyran S E , Ditty S E , Baylen B G , Cheung J , La None K F Developmental differences in the response of cytosolic Ca to K - depolarization and cardioplegia in cardiac myocytes ***J . Mol . Cell . Cardiol .*** 1992 ; 24 : 1167 - 1177 .

Da Silva J J R , Williams R J P **In:** ***The biological chemistry of the elements - The inorganic chemistry of life*** (Oxford University Press , London) 1991 pp 243 - 250 .

David H , Mayer R , Marx I , Gusk H , Wenzelides K Morphometric characterization of left ventricular myocardial cells of male rats during post natal development ***J . Mol . Cell . Cardiol*** . 1979 ; 11 : 631 - 638 .

De Keulenaer G W , Andries L J , Sys S U , Brutsaert D L Endothelin - mediated positive inotropic effect induced by reactive oxygen species in isolated cardiac muscle Circ . Res . 1995 ; 76 : 878 - 884 .

Delbridge L M D , Roos K P Optical methods to evaluate the contractile function of unloaded isolated cardiac myocytes ***J . Mol . Cell . Cardiol .*** 1997 ; 29 : 11 - 25 .

Denes P , Gillis A M , Pawitan V , Kammerling J M , Wilhemsen L , Salerno D M Prevalence , characteristics and significance of ventricular premature complexes and ventricular tachycardia detected by 24 - hour continuous electrocardiographic recording in cardiac arrhythmia suppression trial ***Am . J . Cardiol .*** 1991 ; 68 : 887 - 896 .

Dennis S C , Coetzee W A , Crago E J Jr. , Opie L H Effects of proton buffering and of amiloride derivations on reperfusion arrhythmias in isolated rat hearts . Possible evidence for an arrhythmogenic role of Na^+ / H^+ exchange ***Circ . Res .*** 1990 ; 66 : 1156 - 1159.

Dichtl A , Vierling W Inhibition by magnesium of calcium inward current in heart ventriculart muscle ***Eur . J . pharmacol .*** 1991 ; 204 : 243 - 248 .

Dunnett J , Nayler W G Calcium efflux from cardiac sarcoplasmic reticulum : Effects of calcium and Magnesium ***J . Mol . Cell . Cardiol .*** 1978 ; 10 : 487 - 498 .

Durlach J , Bac P , Durlach V , Rassiguier Y , Bara M , Guiet - Bara A Magnesium status and aging : an update ***Magnes . Res .*** 1998 ; 11 : 25 - 42 .

Durlach J , Durlach V , Bac P , Bara M , Guiet - Bara A Magnesium and therapeutics ***Magnes . Res*** . 1994 ; 7 : 312 - 328 .

Durlach J Recommended dietary amounts of magnesium ***Magnes . Res .*** 1989 ; 2 : 195 - 203.

Durlach J , Bara M , Guiet - Bara A Magnesium level in drinking water : its importance in cardiovascular risk **In :** ***Magnesium in health and disease*** Y Itokawa and J Durlach Eds. (John Libbey and Co . Ltd , Great Britain) 1989 pp. 173 - 182 .

Durlach J , Durlach V , Bac P , Rayssiguier Y , Bara M , Guiet - Bara A Magnesium and aging II . Clinical data : Aetiological mechanisms and pathophysiological consequences of Mg - deficit in the elderly ***Magnes . Res .*** 1993 ; 6 : 379 - 394 .

Dyckner T , Wester P O Effect of Mg on blood pressure ***Br . Med . J .*** 1983 ; 286 : 1847 - 1849 .

Effron M B , Bhatnagar G M , Spurgeon H A , Ruano - Arroyoa , Lakatta E G Changes in myosin isoenzymes , ATPase activity and contraction duration in rat cardiac muscle with aging can be modulated by thyroxine ***Circ . Res .*** 1987 ; 60 : 238 - 245 .

Eisenberg L , Hill T L Muscle contraction and free energy transduction in biological ***systems Science*** 1985 ; 227 : 999-1006 .

Eisenberg L , Greene L E The relation of muscle biochemistry to muscle physiology ***Ann . Rev . Physiol .*** 1980 ; 42 : 293 - 309 .

Elin R Magnesium metabolism in health and disease ***J . Dis . Mon .*** 1988 ; 34 : 166 - 218 .

Ellingsen Q , Holthe M R , Svindland A , Aksenr G , Sejersted O M , Ilebekk A Na , K - pump concentration in hypertrophied human hearts ***Eur . Heart . J .*** 1994 ; 15 : 1184 - 1190 .

Enyedi A , Sarkadi B , Nyers A , Gardos G Effects of divalent metal ions on the Ca - pump and membrane phos phorylation in human red cells ***Biochim . Biophys . Acta*** 1982 ; 690: 41 - 49 .

Fabiato A Calcium induced release of calcium from cardiac SR ***Am . J . Physiol*** .1983 ; 245 : C1 - C14

Fabiato A , Fabiato F Effects of magnesium on contractile . activation of skinned cardiac cells ***J . Physiol .*** 1975 ; 249 ; 497 - 517.

Fabiato A , Fabiato F Effects of pH on the myofilaments and the Sr of skinned cells from cardiac and skeletal muscles ***J . Physiol .*** 1978 ; 276 : 233 - 255 .

Fermini B , Nathan R D Removal of sialic acid alters both T - type and L- type Ca – current in cardiac myocytes ***Am . J . Physiol .*** 1991 ; 260 : H735 - H743

Ferrara N , Davia K , Abete P , Rengo F , Harding S E Alterations in β - adrenoreceptor mechanisms in the aging heart . Relationship with heart failure ***Aging*** 1997 ; 9 : 391 - 403.

Finelli C , Aussedat J , Ray A , Lovtet S , Lavandry N , Guarnieri C , Caldarera C M , Rossi A Effect of age on phosphorylated compounds and mechanical activity of isolated rat heart ***Cardiovas . Res .*** 1993 ; 27 : 1978 - 1982 .

Fleckenstein A Specific inhibitors and promoters of calcium - action in the excitation - contraction coupling of heart muscle and their role in the prevention or production of myocardial lesions **In : *Calcium and the heart*** P Harris and L H Opie Eds. (Academic Press , New York) 1971 , pp . 135 - 188 .

Fleg J L Alterations in cardiovascular structure and function with advancing age ***Am . J . Cardiol .*** 1986 ; 57 : 33C - 44C .

Fleming J W , Watanabe A M Muscarinic cholinergic receptor stimulation of specific GTP hydrolysis related to adenyl cyclase activity in canine cardiac sarcolemma ***Circ . Res*** . 1988 ;64 : 340 - 350 .

Frank J S , Langer G A The myocardial interstitium : its structure and its role in ionic exchange ***J . Cell . Biol .*** 1974 ; 60 : 586 - 601 .

Frank K J S , Mottino G , Molday R S , Philipson K D Distribution of Na - Ca exchanger protein in . mammalian cardiac myocytes : an immunofluorescent and immunocolloidal gold labelling study ***J . Cell . Biol .*** 1992 ; 117 : 337 - 345 .

Frank O Zur Dynamik des Herzmuskels ***Z .Biol .*** 1935 ; 32: 370 - 447 . Translated in ***Am . J . Heart .*** 1958 ; 58 : 282 - 317

Fry C H , Proctar A V The effects of magnesium on excitable tissues **In : *Magnesium and cell*** N J Birch Ed . (Academic Press , New York) 1993 pp. 215 - 234 .

Fuchs F , Wang Y P Sarcomere length versus interfilament spacing as determinants of cardiac myofilament Ca^{2+} - sensitivity and Ca^{2+} - binding ***J . Mol . Cell . Cardiol .*** 1996 ; 28 : 1375 - 1383 .

Fujii J , Ueno A , Kitono K , Tanaka S *et al* Complete complementary DNA - derived amino acid sequence of canine cardiac phospholamban ***J . Clin . Invest .*** 1987 ; 79 : 301 - 304 .

Gaskel W H On the tonicity of the heart and blood vessels ***J . Physiol .*** 1880 ; 3 : 48 - 75.

Gasser J , Paganetti P , Carafoli E , Chiesi M Heterogeneous distribution of calmodulin and cAMP dependent regulation of Ca^{2+} - uptake in cardiac SR - subfractions ***Eur . J . Biochem*** . 1988 ; 176 : 535 - 541 .

Gasser R , Eber B , Schwarz T , Schafhalter E , Klein W Calcium antagonists and coronary heart disease - An outline ***Wien . Med . Wochenshen*** . 1993 ; 143 : 500 - 509 .

Glick M R , Burns A H , Reddy W J Dispersion and isolation of beating cells from adult rat heart ***Anal . Biochem .*** 1974 ; 61 : 32 - 42 .

Guarnieri T , Filburn C R , Zitnik G , Roth G S , Lakatta E G Contractile and biochemical correlates of β - adrenergic stimulation of the aged heart ***Am . J . Physiol*** . 1980 ; 239 : H501 - H508 .

Gunther T Magnesium deficiency , oxygen radicals and aging ***Mag . Bull*** . 1991 ; 13 : 78 - 81 .

Gunther T , Merker II J , Averdunk R , Peter H W , Wonigetit K Membrane alterations in Mg - deficiency induced in malignant T - cell lymphoma ***Magnesium*** 1984 ; 3 : 29 - 37 .

Gupta R K , Gupta P , Moore R D NMR studies of intracellular metal ions in intact cells and tissues ***Annu . Rev . Biophys . Bioenerg*** . 1984 ; 13 : 221 - 246 .

Gwathmey J K , Morgan J P Altered Ca - handling in experimental pressure - overload hypertrophy in the ferret heart ***Circ . Res .*** 1985 ; 57 : 836 - 843 .

Haddock P S , Artman M , Coetzee W A Cardiac Na - Ca exchanger expression is significantly greater in newborn rabbit hearts compared with adults ***Pflugers . Arch .*** 1998 ; 435 : 789 - 795 .

Hagiwara N , Irisawa H , Kameyama M Contribution of the two types of calcium currents to the pacemaker potentials of rabbit sino - atrial node cells ***J . Physiol .*** 1988 ; 395 : 233 - 253.

Hall S K , Fry C H , Mc Leod K T The effect of extracellular Mg on the contraction of isolated ventricular myocardium from several mammalian species ***J . Physiol .*** 1990 ; 429 : 23P.

Handy R D , Gow I F , Ellis D , Flatman P W Na - dependent regulation of intracellular free Mg - concentration in isolated rat ventricular myocytes ***J. Mol . Cell . Cardiol .*** 1996 ; 28 : 1641 - 1651 .

Hansen D .E , Craig C S , Hondegham L M Stretch - induced arrhythmias in the isolated canine ventricle . Evidence for the importance of mechano electrical feedback ***Circulation*** 1990 ; 81 : 1094 - 1095 .

Hanson G L , Schilling W P , Michael L H Sodium - Potassium pump and sodium - calcium exchange in adult and neonatal canine cardiac sarcolemma ***Am . J . Physiol .*** 1993 ; 264 : H320 - H326 .

Harding S E , O'Gara P , Jones S M , Brown L M , Vescovo G , Poole - Wison P A Species dependence of contraction velocity in single isolated cardiac myocytes ***Cardioscience*** 1990 ; 1 : 49 - 53 .

Harding S E , Vescovo G , Kirby M , Jones S M , Gurden J , Poole - Wilson P A Contractile responses of isolated adult rat and rabbit cardiac myocytes to isoproterenol and calcium ***J . Mol . Cell . Cardiol .*** 1988 ; 20 : 635 - 647 .

Hariman R J , Louie E K , Krauser R L *et al* Regional changes in blood flow : extracellular potassium and conduction during myocardial ischemia and reperfusion ***J . Am . Coll . Cardiol .*** 1993 ; 21 : 798 - 808 .

Harris P J , Stewart D , Cullinan MC , Delbridge C M , Dally L T , Grinwald P Rapid measurement of isolated cardiac muscle length using a line - scan camera ***IEEE Trans . Biomed . Eng .*** 1987 ; 34 : 463 - 467 .

Hart G Cellular electrophysiology in cardiac hypertrophy and failure ***Cardiovas . Res .*** 1994 ; 28 : 933 - 946 .

Hartzell H C , White R E Effects of magnesium on inactivation of the voltage gated Ca - current in cardiac myocytes ***J . Gen . Physiol .*** 1989 ; 94 : 745 - 767 .

Harward Heart Letters Magnesium : coming of age 1991 ; 6 - 7

Haworth P A , Hunter D R , Berkoff H A The isolation of Ca^{2+} resistant myocytes from adult rat ***J . Mol . Cell . Cardiol .*** 1980 ; 12 : 715 - 723 .

Headrick J P , McKirdy J C , Willis R J Functional and metabolic effects of Mg^{2+}_o in normoxic and ischemic myocardium ***Am . J . Physiol*** . 1998 ; 275 : H917 - H929 .

Heaton F W , Tongyai S , Rayssiguier Y Membrane function in Mg - deficiency **In : *Magnesium in health and diseases*** Y Itokawa and J Durlach Eds. (John Libbey and Co. , London) 1988 pp.27 - 34 .

Heaton F W , Rayssiguier Y Magnesium - deficiency and membrane properties **In :** ***Magnesium in Cellular processes and Medicine*** Altura B . M , Durlach . J , Seelig . M. S . Eds . (Karger, Basel) 1987 pp . 121 - 130 .

Heaton F W , Elie J R Metabolic activity of liver mitochondria from Mg - deficiencient rats ***Magnesium*** 1984 ; 3 : 21 - 28 .

Heidbuchel H , Vercecke J , Carmeleit E Three different K - channels in human atrium ***Circ . Res .*** 1990 ; 66 : 1277 - 1286 .

Hermsmeyer K Role of T - channels in cardiovascular function ***Cardiology*** 1998 ; 89 : 2 - 9.

Hess P Elementary properties of cardiac calcium channels : A brief review *Can . J . Physiol . Pharmacol .* 1988 ; 66 : 233 - 253 .

Hess P , Lansman J B , Tsien R W Different modes of calcium channel gating behaviour favoured by dihydropyridine calcium agonists and antagonists ***Nature*** 1984 ; 311 : 538 - 544 .

Ho H K , Nichlos C G , Ledrer W J *et al* Cloning and expression of an inwardly rectifying ATP - regulated K - channel ***Nature*** 1993 ; 362 : 127 - 133 .

Holroyde M J , Robertson S P , Johnson J D , Solaro R J , Potter J D The calcium and magnesium binding sites on cardiac troponin and their role in the regulation of myofibrillar ATPase ***J . Biol . Chem .*** 1980 ; 255 : 11688 - 11693 .

Howarth F C , Levi A J Internal free magnesium modulates the voltage dependence of contraction and calcium transient in rabbit ventricular myocytes ***Pflugers . Arch .*** 1998 ; 435 : 687 - 698 .

Huxley H E , Hanson J Changes in the cross - striations of muscle stretch and their structural interpretation ***Nature*** 1954 ; 173 : 973 .

Huynh T V , Chen F , Wetzel G T , Friedman W F , Klitzner T S Developmental changes in membrane Ca^{2+} and K^{+} currents in fetal , neonatal and adult rabbit ventricular myocytes ***Circ . Res .*** 1992 ; 70 : 508 - 515

Iseri L T , French J H Magnesium : Nature's physiological calcium blocker ***Am . Heart . J.*** 1984 ; 108 : 188 - 193 .

Ising H , Babisch W , Gunther T , Kruppa B Increased risk of myocardial infarction due to chronic noise stress ***Z . Larinbe . Kampf .*** 1997 ; 44 : 1 - 7 .

Iskandrian A S , Hakki A H , DePace N L *et al* Evaluation of left ventricular function by radionuclide angiography during exercise in normal subjects and patients with chronic coronary artery disease ***J . Am . Coll . Cardiol .*** 1983 ; 1 : 1518 - 1529 .

James J E , Manalo R , Flink E B Magnesium requirements in adults ***Am . J . Clin . Nutr .*** 1967 ; 20 : 632 - 635 .

Jeck C D , Boyden P A Age related appearance of outward currents may contract to develpomental differences in ventricular repolarization ***Circ . Res*** . 1992 ; 71 : 1390 - 1403.

Jezek P , Malidi F , Garlid K D Reconstitution of the beef heart and rat liver K^+ / H^+ (Na^+ / H^+) antiporter ***J . Biol . Chem .*** 1990 ; 265 : 10522 - 10533 .

Jiang M T , Moffat M P , Narayanan N Age related alterations in the phosphorylation of SR and myofibrillar proteins and diminished contractile response to isoproterenol in intact rat ventricle ***Circ . Res .*** 1993 ; 72 : 102 - 111 .

Johnson C J , Peterson D R , Smith E K Myocardial tissue concentrations of Mg and K in men dying suddenly from ischemic heart disease ***Am . J . Clin . Nutr .*** 1979 ; 32 : 967 - 970 .

Jones J E , Manalo R , Flink E B Magnesium requirements in adults ***Am . J . Clin . Nutr .*** 1967 ; 20 : 632 - 635 .

Jorgenson A O , Broderick R , Somylo A P , Somylo A V Two structurally distinct Ca - storage sites in rat cardiac SR . an electron microscopic analysis study ***Circ . Res .*** 1988 ; 63 : 1060-1069 .

Josephson T R , Sperelakis N Developmental increases in the inwardly rectifying K+ - current of embryonic chick ventricular myocytes *Biochim . Biophys . Acta* 1990 ; 1052 ; 123 - 127 .

Just H Pathophysiological targets for β - blocker therapy in CHF ***Eur . Heart . J .*** 1996 ; 17 : 2 - 7

Jynge P , Falck G High Mg improves the post ischemic recovery of cardiac function ***Cardiovas . Res .*** 1995 ; 29 : 439 .

Katholi R H , Woods W T , Kawamura K , Urthales F , James T N Dual dependence on both Ca^{2+} and Mg^{2+} for electrical stability in cells of canine false tendon ***J . Mol . Cell . Cardiol*** . 1979 ; 11: 435 - 445 .

Kato Y , Masumiya H , Agata N , Tanaka H , Shigenobu K Developmental changes in action potential and membrane currents in fetal , neonatal and adult guinea pig ventricular myocytes ***J . Mol . Cell . Cardiol .*** 1996 ; 28 : 1515 - 1522 .

Katz A M Regulation of myocardial contractility ***J . Am . Coll . Cardiol .*** 1983 ; 1 : 42 - 51 .

Katz A M Cardiac action potential **In :** ***Physiology of the heart*** 2nd edn. (Raven Press , New York) 1992 (d) pp.438 - 472 .

Katz A M Receptors , coupling proteins and second messengers **In:** ***Physiology of the heart*** 2nd edn. (Raven Press , New York) 1992 (b) pp.274 - 302 .

Katz A M The arrhythmias . III . Premature systoles , Tachycardias , flutter and fibrillation **In :** ***Physiology of the heart*** 2nd edn. (Raven Press , New York) 1992 (c) pp.569 - 608.

Katz A M Structure of the heart and cardiac muscle **In :** ***Physiology of the heart*** 2nd edn. (Raven Press , New York) 1992 (a) pp.1 - 37.

Kawai M Mg^{2+} and H^{+} inhibit Sr - function in cardiac function ***J . Mol . Cell . Cardiol .*** 1996 ; 28 : 1401 - 1403 .

Kelman G R Applied cardiovascular physiology **In :** ***The heart*** 2nd edn . (Butterworth and Co . Ltd , New York) 1991 pp. 22 -66 .

Kennedy R H , Seifen E , Wang S N , Fontenot H J , Liu S Effects of aging on Na^{+} - pump current in rat ventricular myocytes ***Mech . Aging . Dev .*** 1996 ; 15 : 59 - 66 .

Kennedy R H , Berlin J R , Ng Y C , Akera T , Brody T M , Amiloride : effects on myocardial force of contraction , sodium pump and Na^{+} / Ca^{2+} exchange ***J . Mol . Cell . Cardiol .*** 1986 ; 18 : 177 - 188 .

Kerns W , Rausons M , Tornaszewski C , Kline J , Raymond R The effects of extracellular ions on β - blocker cardiotoxicity ***Toxicol . Appl . Pharmacol .*** 1996 ; 137 : 1 - 7 .

Khalil S I Magnesium the forgotten cation ***Int . J . Cardiol .*** 1999 ; 68 : 133 - 135 .

Kihara Y , Grossman W , Morgan J P Direct measurement of changes in intracellular calcium transients during hypoxia , ischemia and reperfusion of the intact mammalian heart ***Circ . Res .*** 1989 ; 65 : 1029 - 1044 .

Kim M S , Akera T Oxygen free radicals : cause of ischemia reperfusion injury to Na , K - ATPase *Am . J . Physiol .* 1987 ; 252 : H252 - H257 .

Kjeldsen K , Gron P J Age dependent change in myocardial cardiac glycoside receptor (Na,K - pump) concentration in children *Cardiovas . Pharmacol .* 1990 ; 15 : 332 - 337 .

Koss K L , Grubbs R D Elevated extracellular magnesium increases magnesium buffering through a calcium dependent mechanism in cardiomyocytes ***Am . J . Physiol .*** 1994 ; 267 : C633 - C641 .

Kramer J H , Philips T M , Weglicki W B Magnesium deficiency enhanced post ischemic myocardial injury is reduced by substance P receptor blockade ***J . Mol . Cell . Cardiol .*** 1997 ; 29 : 97 - 110 .

Krebs H A , Henseleit K Untersuchunger uber die Harnstoffbilduing in Tierkorper ***Hoppe - Seylers . Z . Physiol . Chem .*** 1932 ; 210 : 33 - 66 .

Kumar B P , Shivkumar K , Kartha C C , Rathinam K Magnesium deficiency and cerium spromote fibrogenesis in rat heart ***Bull . Environ . Contam . Toxicol .*** 1996 ; 57 : 517 - 524 .

Lakatta E G , Yinc C P Myocardial ageing : functional alterations and related cellular mechanisms ***Am . J . Physiol .*** 1982 ; 24 : H927 - H941

Lakatta E G Starling's law of the heart is explained by an intimate interaction of muscle length and myofilament Ca - activation ***J . Am . Coll . Cardiol .*** 1987 ; 10 : 1157 - 1164 .

Lakatta E G Similar myocardial effects of ageing and hypertension ***Eur . Heart . J .*** 1990 ; G0 : 29 - 38 .

Lakatta E G Changes in excitation - contraction coupling with development and aging ***J . Mol . Cell . Cardiol .*** 1991 ; 23 (suppl V) : S158 .

Lakatta E G Myocardial adaptations in advaned age ***Bas . Res . Cardiol .*** 1993 ; 2 : 125 - 133 .

Landsberg A , Sideman S Mechanical regulation of cardiac muscle by coupling calcium kinetics with cross bridge cycling : A dynamic model ***Am . J . Physiol .*** 1994 ; 267 : H779 - H795.

Langendorff O Untersuchungen am ubelebenden saugethierherzen ***Pflugers . Arch .*** 1895 ; 61 : 291 - 332 .

Langer G A Calcium at the sarcolemma ***J . Mol . Cell . Cardiol .*** 1984 ; 16 : 147 - 153 .

Langer G A , Nudd L M Effects of cations , phospholipases on Ca - binding to gas - dissected membrane from cultured cardiac cells ***Circ . Res .*** 1983 ; 53 : 482 - 490 .

Laniado M E , Abel P A , Lalani E N Ion - channels : New explanations for old diseases ***Br . Med . J .*** 1998 ; 13 : 1221 - 1222 .

Lansman J B , Hess P , Tsien R W Blockade of current through single Ca - channels by Cd^{2+} , Mg^{2+} and Ca^{2+} ***J . Physiol .*** 1986 ; 88 : 321 - 347 .

Lattanzio F A , Bertschart D K The effect of pH on rate constants , ion selectivity and thermodynamic properties of fluorescent calcium and magnesium ***indicators Biochim . Biophys . Res . Commn .*** 1991 ; 177 : 184 - 191 .

Lauler D P Magnesium coming of age ***Am. J . Cardiol .*** 1989 ; 63 : 1G - 4G .

Laver D R , Baynes T M , Dulhunty A F Magnesium inhibition of ryanodine - receptor calcium channels : evidence for two independent mechanisms ***J . Membr . Biol .*** 1997 ; 156 : 213 - 229 .

Leblanc N , Hume J R Sodium current induced release of calcium from cardiac SR ***Science*** 1990 ; 248 : 372 - 376 .

Lee C O , Abete P , Pecker M , Sonn J K , Varsalla M Strophanthidine inotropy : role of intracellular sodium ion activity and Na - Ca exchanger ***J . Mol . Cell . Cardiol .*** 1985 ; 17 : 1043 - 1053 .

Lee H C , Smith N , Mokabir R , Clusin W T Cytosolic calcium transients from the beating mammalian heart ***Proc . Natl . Acad . Sci . USA*** 1987 ; 84 : 7793 - 7797 .

Legato M J The cardiac cell : Myocardial cell structure and relaxation rate . Clinical cardiology **In :** ***Current Cardiology*** I . M . Ferier Ed . (Houghton Mifflin Proffessional Publishers , Berlin) Vol . 1 . 1979 pp . 207 - 233 .

LeGrand B , Deroubaix E , Coulombe A , Coraboeuf E Stimulatory effect of ouabain on T- and L - type Ca - currents in guinea pig cardiac myocytes ***Am . J . Physiol .*** 1990 ; 258 : H1620 - H1623 .

Leibowitz S F , Lucas D J , Leibowitz K L , Jhanwar Y S Developmental patterns of macronutrient intake in female and male rats from weaning to maturity ***Physiol . Behaviour*** 1991 ; 50 : 1167 - 1174 .

Leier C , Dei C L , Metra M Clinical relevance and management of the major electrolyte abnormalities in congestive heart failure : hyponatremia , hypokalemia , hypomagnesemia ***Am . Heart . J .*** 1994 ; 128 : 564 - 574 .

Levesque P C , leblanc N , Hume J R , Role of reverse mode Na - Ca exchange in excitation - contraction coupling in the heart ***Ann . N . Y . Acad . Sci*** . 1991 ; 6390 : 386 - 397 .

Levitsley D L , Fleischer S Isolation and characterization of canine cardiac SR with improved Ca^{2+} - transport properties ***J . Biol . Chem .*** 1983 ; 258 : 6602 - 6609 .

Li F , Wang X , Capasso J M , Gerdes A M Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development ***J . Mol . Cell . Cardiol .*** 1996 ; 28 : 1737 - 1746 .

Li T , Vassalle M The negative inotropic effect of Ca - overload in cardiac purkinje fibres ***J. Mol . Cell . Cardiol .*** 1984 ; 16 : 65 - 77 .

London R E Methods for measurement of intracellular Mg : NMR and fluorescence ***Ann . Rev . Physiol .*** 1991 ; 530 : 241 - 258 .

Lundgren E L , Borg T , Mardh S Isolation , characterisation and adhesion of Ca - tolerant myocytes from adult rat heart ***J . Mol . Cell . Cardiol .*** 1984 ; 16 : 355 - 362 .

Mahouny L Calcium homeostasis and control of contractility in the developing ***heart Semin . Perinatol .*** 1996 ; 20 : 510 - 519 .

Marban E , KitaKaze M , Koretsune Y *et al* Quantification of $[Ca^{2+}]_i$ in perfused hearts. Critical evaluation of the 5F - BAPTA and NMR methods as applied to the study of ischemia and reperfusion ***Circ . Res .*** 1990 ; 16 : 1255 - 1267 .

Marban E , O'Rourke Calcium channels : Structure , function and regulation **In** *: Cardiac electrophysiology - from cell to bedside* 2nd edn . D P Zipes and J Jalife Eds . ( ) 1995 pp . 11 - 21 .

Marban E , Tsien R W Enhancement of Ca - current during digitalis inotropy in mammalian heart : positive feed back regulation by intracellular Ca ? ***J . Physiol .*** 1982 ; 329 : 589 - 614 .

Marier J R Cardioprotective contribution of hard water to Mg - intake ***Rev . Can . Biol . 1978*** ; 37 : 115 - 125 .

Matsumori A Molecular and immune mechanism of pathogenesis of cardiomyopathy . Role of viruses and nitric oxide ***Jpn . Circ . J .*** 1997 ; 61 : 275 - 291 .

McCormark J G , Denton R M Role of calcium ions in the regulation of intramitochondrial metabolism in rat heart ***Biochem . J .*** 1984 ; 218 : 235 - 247 .

McMohan W S , Mukherjee R , Gillette P C , Crawford F A , Spinale F G Right and left ventricular geometry and myocyte contractile process with dilated cardiomyopathy : myocyte growth and β - adrenergic responsiveness ***Cardiovas . Res .*** 1996 ; 31 : 314 - 323 .

McNutt N S Ultrastructure of the myocardial sarcolemma ***Circ . Res .*** 1975 ; 37 : 1 - 13 .

Meissner G , Henderson J S Rapid Ca - release from cardiac SR vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+} , adenine nucleotide and calmodulin ***J . Biol . Chem .*** 1987 ; 262 : 3065 - 3073 .

Millane T A , Camm A J Magnesium and the myocardium ***Br . Heart . J .*** 1992 ; 68 : 441 - 442 .

Mima T , Miyazaki S , Guth B D , Kambuyashi M , Ross J Influence of the force - frequency relation on left ventricular function during exercise in conscious dogs ***Circulation*** 1998 ; 86 : 563 - 577.

Minta T , Miyazaki S , Guth B D , Kambuyashi M , Ross J Influence of the force - frequency relation on left ventricular function during exercise in conscious dogs ***Circulation*** 1998 ; 86 : 563 - 577 .

Mitamura H Ventricular arrhythmias in heart failure ***Keio . J . Med*** . 1996 ; 45 : 1 - 8 .

Mittman C , Eschenhagen T , Scholze H Cellular and molecular aspects of contractile dysfunction in heart failure ***Cardiovas . Res .*** 1998 ; 39 : 267 - 275 .

Moody C J , Dashwood M R , Sykes R M Functional and autoradiographic evidence for endothelin 1 receptors on human and rat cardiac myocytes ***Circ . Res .*** 1990 ; 67 : 764 -769 .

Moravec C S , Bond M Calcium is released from the junctional SR during cardiac muscle contraction ***Am . J . Physiol .*** 1991 ; 260 : H989 - H997 .

Mordes J P , Walker W C Excess magnesium ***Pharmacol . Rev .*** 1978 ; 29 : 273 - 300 .

Morner S E , Wohlfart B Myocardial force inerval relationships : influence of external sodium and calcium , muscle length , muscle diameter and stimulation frequency ***Acta . Physiol . Scand .*** 1992 ; 145 : 323 - 332 .

Mubagwa K , Kaplan P , Flameng W The effects of ryanodine on calcium uptake by the SR of ischemic and reperfused rat myocardium ***Fundam . Clin . Pharmacol .*** 1997 ; 11 : 315 - 321 .

Murphy M Contractile proteins phenotypic variation during development ***Cardiovas . Res .*** 1996 ; 31 : E25 - E33 .

Muscari C , Caldarera I , Rapezze C , Branzi A , Caldarera C M Biochemical correlates with myocardial ageing ***Cardioscience*** 1992 ; 3 : 67 - 78 .

Nair R R , Eapen J T , Radhakumari C , Rajasree S Magnesium deficiency in serum and erythrocyte of children from Kerala ***Nati . Med . J . India*** 1995 ; 8 :

Nassar R , Reedy M C , Anderson P A W Developmental changes in the ultrastructure and sarcomere shortening of the isolated rabbit ventricular myocytes ***Circ . Res .*** 1987 ; 61 : 465 - 483 .

Nilius B , Hess P , Lansman J B , Tsien R W A novel type of cardiac calcium channel in ventricular cells ***Nature*** 1985 ; 316 : 443 - 446 .

Nishiyama S , Saito N , Konishi Y , Abe Y , Kusumi K Cardiotoxicity in Mg - deficient rats fed cadmium ***J . Nutr . Sci . Vitaminol .*** 1990 ; 36 : 33 - 44 .

Nitahara J A , Chang W , Liu Y et al Intracellular calcium , DNAase activity and myocyte apoptosis in aging Fischer 344 rats *J . Mol . Cell . Cardiol .* 1998 ; 30 : 519 - 535 .

Nozawa T , Cheng C P , Noda T , Little W C Effect of exercise on left ventricular effeciency in conscious dogs ***Circulation*** 1994 ; 90 : 3047 - 3054 .

Nuss H B , Houser S R T - type calcium current is expressed in hypertrophic adult feline left ventricular myocytes ***Circ . Res .*** 1993 ; 73 : 777- 782 .

Nyarko - Adomfch C Cardiac β - 2 - adrenoreceptors and the inotropic response to exercise in man ***Clin . Anton . Res .*** 1991 ; 4 : 317 - 321 .

O'Neill S C , Eisner D A The effects of refilling of the SR with calcium on intracellular Ca - concentration in isolated rat ventricular myocytes ***J . Physiol .*** 1990 ; 429 : 20P .

O'Neill S C , Donoso P , Eisner D A The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]$ sensitization in the caffeine contracture of rat myocytes : Measurement of $[Ca^{2+}]_i$ and $[Caffeine]_i$ ***J . Physiol .*** 1990 ; 425 : 55 - 70 .

Oparil S , Bishop S P , Clubb F J Myocardial cell hypertrophy or hyperplasia ***Hypertension*** 1984 ; 6 : 38 - 43 .

Opie L H Reperfusion injury and its pharmacological modification ***Circulation*** 1989 ; 80 : 1049 - 1062 .

Opie L H , Clusin W T Cellular mechanisms for ischemic ventricular arrhythmias ***Ann . Rev . Med .*** 1990 ; 41 : 231 - 238 .

Opie . L . H . Intracellular Ca - fluxes and sarcoplasmic reticulum **In:** ***The heart - Physiology and Metabolism*** second edn . (Raven Press , New York) 1991 (b) pp . 127 - 146 .

Opie . L . H . Myocardial reperfusion : calcium and free radicals **In :** ***The heart - Physiology and Metabolism*** second edn . (Raven Press , New York) 1991 (c) pp . 469 - 483 .

Opie . L . H . Heart cells and organelles **In :** ***The heart - Physiology and Metabolism*** second edn . (Raven Press , New York) 1991(a) pp . 19 - 25

Opie . L . H . ***The heart - Physiology and Metabolism*** second edn . (Raven Press , New York) 1991 (d) pp . 788 - 796 .

Osaka T , Joyner R W Developmental changes in Ca - currents of rabbit ventricular cells ***Circ . Res .*** 1991 ; 68 : 788 - 796 .

Osaka T , Joyner R W , Kumar R Postnatal decrease in muscarinic cholinergic influence on Ca^{2+} currents of rabbit ventricular cells ***Am . J . Physiol .*** 1993 ; 264 : H1916 - H1925 .

Osaka T , Joyner R W Developmental changes in the β - adrenergic modulation of Ca - currents in rabbit ventricular cells ***Circ . Res .*** 1992 ; 70 : 104 - 115 .

Ostadal B , Kolar F , Skorranck J , Ostadalva I Ontogenic development of cardiac inotropic responsiveness In: Heart function in health and disease B Ostadal and NS Dhalla Eds. (Kluwer Academic Publishers, Netherlands) 1993 pp.32-42

Owen P , Dennis S , Opie L H Glucose flux rate regulates onset of ischeming contracture in globally under perfused rat hearts ***Circ . Res .*** 1990 ; 66 : 344 - 354 .

Parikh S S , Zhou S Z , Tung L Contraction and relaxation of isolated cardiomyocytes of the frog under varying mechanical loads ***Circ . Res .*** 1993 ; 72 : 297 - 311 .

Pauletto P , Vescovo G , Scannapleco G , Pessina A C , Dal Palu C Cardioprotection by β - blockers : molecular and structural aspects in experimental hypertension ***Drugs . Exp . Clin . Res*** . 1990 ; 16 : 123 - 128 .

Pearigen P D , Benowitz N L Poisoning due to Ca - antagonists . Experience with verapamil , diltiazem and nifedepine ***Drugs . Saf .*** 1991 ; 6 : 408 - 430 .

Peronnet F , Nadear R A , de Champlien J , Margrassi P , Chatrand C Exercise plasma catecholamines in dogs : role of adrenals and cardiac nerve endings ***Am . J . Physiol .*** 1981 ; 241 : H243 - H247 .

Philipson K D , Nishimoto A Y Stimulation of Na - Ca exchange in cardiac sarcolemmal vesicles by proteinase pretreatment ***Am . J . Physiol .*** 1982 ; 243 : C191 - C195 .

Pignatti C , Tantini B , Zanfanti M L , Sacchi P , Clo C Influence of Mg^{2+} on the invitro responsiveness of adenylate cyclase from hearts of aging rats ***Cardioscience*** 1993 ; 4 : 105 - 109 .

Polimeni P , Page E Magnesium in heart muscle ***Circ . Res .*** 1973 ; 33 : 367 - 374 .

Polimeni P I Ionic distribution in rat ventricle ***Physiologist*** 1973 ; 16 : 424 .

Ponikowski P , Anker S D , Amadi A *et al* Heart rhythms , ventricular arrhythmias and death in chronic heart failure ***J . Cardiol . Fail .*** 1996 ; 3 : 177 - 183 .

Potter J D , Gergely J The calcium and magnesium binding site on troponin and their role in the regulation of myofibrillar adenosine tri phosphate ***J . Biol . Chem .*** 1975 ; 250 : 4626- 4628 .

Qi X L , Roulèau J L Age dependent changes in the effects of endocardial endocardium on the contractile characteristics of its adjacent mycardium in rats ***J . Mol . Cell . Cardiol*** . 1997 ; 29 : 823 - 829 .

Quigley K , Goya R , Nachveiner R , Meites J " Effects of underfeeding and refeeding on growth hormone and thyroid hormone secretions in young , middle aged and old rats ***Exp . Gerentol .*** 1990 ; 25 : 447 - 457 .

Rabenowitz E , Axelsson G , Rylander R Magnesium and calcium in drinking water and death from acute myocardial infarction in women ***Epidemiology*** 1999 ; 10 : 31 - 36 .

Rabenowitz E , Axelsson G , Rylander R Magnesium in drinking water and death from acute myocardial infarction ***Am . J . Epidemiol .*** 1996 ; 143 ; 456 - 462 .

Rakussan K Postnatal growth and restructuring of mammalian hearts **In** *: Heart function in health and disease* B Ostadal and N S Dhalla Eds . (Kluwer Academic Publishers , Netherlands) 1993 pp. 23 - 32 .

Rasmussen C A F , Sutko J L , Barry W H Effects of ryanodine and caffeine on contactility , membrane voltage and Ca - exchange in cultured heart cells ***Circ . Res .*** 1987 ; 60 : 495 - 504 .

Reuter H Calcium channel modulation by neurotransmitters , enzymes and drugs *Nature* 1983 ; 301 : 569 - 574 .

Rich T L , Langer G A , Klassen M G Two components of coupling calcium in single ventricular cells of rabbits and rats ***Am . J . Physiol .*** 1988 ; 254 : H937 - H946 .

Ringer S A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart ***J . Physiol .*** 1883 ; 4 : 29 - 42 .

Romani A , Marfella C , Scarpa A Magnesium homeostasis in cardiac ventricular myocytes **In : *Biochemistry of cell membranes*** S Bapa and J M Tager Eds .(Brkhauser Verlag Besel / Switzerland) 1995 pp . 231 - 244 .

Romani A , Marfella C , Scarpa P Regulation of Mg - uptake in isolated ventricular myocytes ***Circ . Res .*** 1993 ; 72 : 1139 - 1148 .

Roos K P , Leung A E Theoretical Fraunhofer light diffraction patterns calculated from three - dimensional sarcomere arrays imaged from isolated cardiac cells at rest ***Biophys . J .*** 1987 ; 52 : 329 - 341 .

Rousseau E , Smith J S , Meissner G Ryanodine modifies conductance and gating behaviour of single calcium release channel ***Am . J . Physiol .*** 1987 ; 253 : C364 - C368 .

Ruddel H , Langewitz W , Schachinger H *et al* Hemodynamic response patterns to mental stresss : diagnostic and therapeutic implications ***Am . Heart . J .*** 1988 ; 116 : 617 - 618 .

Ruegg J C Towards a molecular understanding of contactility ***Cardioscience*** 1990 ; 1 : 163 - 167 .

Safar M Aging and its effect on the cardiovascular system ***Drugs*** 1990 ; 39 : 1 - 18 .

Sarsero D , Fujiwara T , Molenaar P , Agus J A Human vascular to cardiac tissue selectivity of L - type and T - type Ca - channel antagonists ***Br . J. pharmacol .*** 1998 ; 125 : 109 - 119 .

Sasaki T , Inuii M , Kimura Y , Kuzuya T , Tada M Molecular mechanism of regulation of calcium pump ATPase by phospholamban in cardiac SR ***J . Biol . Chem .*** 1992 ; 267 : 1674 - 1677 .

Schwinger R H G , Bohm M , Uhlmann R *et al* Magnesium restores the altered force - frequency relationship in failing human myocardium ***Am . Heart . J .*** 1993 ; 126 : 1018 - 1021 .

Schwinger R H G , Frank K , Hoischen S , Muller - Ehmsen J , Brixius K Effect of changed extracellular K^+ and Mg^{2+} concentration on intracellular Ca^{2+} - homeostasis , contraction coupling and force - frequency relations in the human myocardium ***Herz*** 1997 ; 22: 18 - 27 .

Schwinger R H G , Erdmann E Heart failure and electrolyte disturbances ***Methods . Find . Exp . Clin . Pharmacol .*** 1992 ; 14 : 315 - 325 .

Schwinger R H G , Bohm M , Uhlmann R , La - Rosee K , Koch A , Erdmann E Increase of the extracellular Mg - concentration reduces cardiac glycoside toxicity in the human myocardium ***J . Pharmacol . Exp . Ther .*** 1992 ; 263 : 1352 - 1359 .

Seelig M Consequences of Mg - deficiency on the enhancement of stress reactions : Preventive and therapeutic implications (A review) ***J . Am . Coll . Nutr .*** 1994 ; 13 : 429 - 446 .

Seelig M The requirement of Mg by the normal adult ***Am . J . Clin . Nutr .*** 1964 ; 14 : 342 - 390 .

Seelig M Magnesium requirements in human nutrition ***Mag . Bull .*** 1981 ; 10 : 225 – 233 .

Seelig M ***Magnesium deficiency in the pathogenesis of disease*** (Plenum Medical Book Co, New York) 1980 pp. 141 - 264 .

Seguchi M , Harding S E , Jarmakani J M Developmental change in the function of SR ***J . Mol . Cell . Cardiol*** . 1986 ; 18 : 189 - 195 .

Seller R H The rolle of magnesium in digitalis toxicity ***Am . Heart . J*** . 1971 ; 82 : 551 - 556 .

Sen L , Smith T W T - type Ca - channels are abnormal in genetically determined cardiomyopathic hamster hearts ***Circ . Res*** . 1994 ; 75 : 149 - 155 .

Shils M E Magnesium in health and disease ***Ann . Rev . Nutr .*** 1988 ; 8 : 429 - 460 .

Shine K I Myocardial effects of magnesium ***Am . J . Physiol .*** 1979 ; 237 : H413 – H423 .

Siebrits F K , Barnes P M The change in the rate of muscle protein metabolism of rats from weaning to 90 days of age ***Comp . Biochem . Physiol . A . Mol . Integr . Physiol .*** 1989 ; 92 : 485 - 488 .

Singh V P , Sharma P L , Wahii P L Effect of hypomagnesemia on ouabain induced cardiac arrhythmias in dogs ***Ind . J . Med . Res .*** 1975 ; 63 : 1029 - 1035 .

Sitasapesan R , Williams A J Mechanisms of caffeine activation of single calcium release channels of sheep cardiac SR ***J . Physiol .*** 1990 ; 423 : 425 - 439 .

Skou J C The energy coupled exchange of Na^+ for K^+ across the cell membrane ***FEBS . Lett*** . 1990 ; 268 : 314 - 329 .

Skou J C , Butler K W , Hansen O The effects of Mg , ATP , Pi , and Na on the inhibition of Na^+, K^+ - activated enzyme system by g - strophanthidine ***Biochim . Biophys . Acta*** 1971 ; 241 : 443 - 461 .

Snowdone K W , Ertel R J , Borle A B Measurement of cytosolic calcium with aequorin in dispersed rat ventricular cells ***J . Mol . Cell . Cardiol*** . 1985 ; 17 : 233 - 241 .

Sommer J R Ultrasructural considerations concerning cardiac muscle ***J . Mol . Cell . Cardiol .*** 1982 ; 14 : 77-83 .

Spah F , Fleckenstein A Evidence of a new preferentially Mg - carrying transport system besides the fast Na and slow Ca - channels in the excited myocardial sarcolemmal membrane ***J . Mol . Cell . Cardiol .*** 1979 ; 11 : 1109 - 1127 .

Srivastava L M , Snehlata Biochemical aspects of aging **In :** ***Aging and heart care*** H . S . Wasir Ed . (Vikas Publishing House , New Delhi) 1993 , pp . 31 - 45 .

Srivastava D K , Mohan M , Dube K P , Katiyar B C Hypomagnesemia and MgSO4 therapy in digoxin intoxication ***J . Assoc . Physicians . India*** 1975 ; 23 : 367 - 372 .

Starling E H ***The Linacre Lecture on the law of the heart*** (Longmans , Green and Co , London) 1918 .

Steenart N A E , Ganim J R , DiSalvo J , Kranias E G The phospholamban phosphatase associated with cardiac SR is a type 1 enzyme ***Arch . Biochem . Biophys .*** 1992 ; 293 : 17 - 24 .

Steenbergen C , Murphy E , Watts J A , London R E Correlation between cytosolic calcium , contracture , ATP and irreversible ischemic injury in perfused rat heart ***Circ . Res .*** 1990 ; 66 : 135 - 146 .

Su J Y , Chang Y I Modulation of SR calcium release channel by caffeine , Ca^{2+} and Mg^{2+} in skinned myocardial fibres of fetal and adult rats ***Pflug . Arch . Eur . J . Physiol .*** 1993 ; 423 : 300 - 306 .

Su N , Dhan J , Moffat M P , Narayanan N Age related changes in electrophysiological responses to muscarinic receptor stimulation in rat myocardium ***Can . J . Physiol .*** 1995 ; 73 : 1430 - 1436 .

Sugushita Y , Iida K , Ohtsuka S , Yamasguchi I Ventricular wall stress revisited . A keystone of cardiology ***Jpn . Heart . J .*** 1994 ; 35 : 577 - 587 .

Sussman M A , Welch S , Cambon N , Klevitsky R *et al* Myofibril degeneration caused by tropomyosin over expression leads to dilated cardiomyopathy in juvenile mice ***J . Clin . Invest .*** 1998 ; 101 : 57 - 61 .

Szlachic J , Hall W D , Tabau J F *et al* Left ventricular hypertrophy reversal with labetalol and propanolol : a perspective , randomized , double - blind study ***Cardiovas . Drugs . Ther .*** 1990 ; 4 : 427 - 434 .

Tackett R L , Holl J E Increased automaticity and decreased inotropism of ouabain in dogs with furosemide induced hypomagnesemia ***J . Cardiovas . Pharmacol .*** 1981 ; 3 : 1269 - 1277 .

Tada M , Yanada M , Kadoma M *et al* Calcium transport by cardiac SR and phosphorylation of phospholamban ***Mol . Cell . Biochem .*** 1982 ; 46 : 73 - 95 .

Talesara C L , Arora R Age related changes in gastrocnemius , diaphragm and heart muscle with special reference to SDH and myofibrillar ATPase ***Ind . J . Exp . Biol .*** 1994 ; 32 : 772 - 780 .

Tanaka H , Sekine T , Nishimaru K , Shigenobu K Role of SR in myocardial contraction of neonatal and adult mice ***Comp . Biochem . Physiol . A . Mol . Integr . physiol .*** 1998 ; 120 : 431 - 438 .

Tani M , Asakura Y , Hasegawa H , Shinmura K , Ebihara Y , Nakamura Y Effect of brief hypoxia on reperfusion arrhythmias and release of Ca^{2+} by rat homogenate blocked by ryanodine ***Cardiovas . Res .*** 1996 ; 31 : 263 - 269 .

Terzic A , Puccat M , Vassort G , Vogel S M Cardiac α - 1 adrenoreceptor : an overview ***Pharmacol . Rev .*** 1993 ; 45 : 147 - 175 .

Thandroyen F T , McCarthy J , Burton K , Opie L H Ryanodine and caffeine prevent ventricular arrhythmias during acute myocardial ischemia and reperfusion in rat heart ***Circ . Res .*** 1988 ; 62 : 306 - 314 .

Tillisch J H , Fung L K , Hom P M , Langer G A Transient and steady - state effects of sodium and calcium on myocardial contractile response ***J . Mol . Cell . Cardiol .*** 1979 ; 11: 137 - 148 .

Tohse N , Meszaros J , Sperelakis N Developmental changes in long - opening behaviour of L - type Ca - channels in embryonic chick heart cells ***Circ . Res .*** 1992 ; 71 : 376 - 384 .

Tominago M , Matsumori A , Horic M , Yoshida H , Okada Y Activation of calcium permeable cation channels by myocarditis associated antibody in guinea pig ventricular myocytes ***J . Clin . Invest .*** 1993 ; 91 : 1231 - 1234 .

Touitou Y , Godard J P , Fermento O et al Prevalance of Mg and K deficiencies in the elderly *J . **Clin . Chem .*** 1987 ; 33 : 518 - 523 .

Triggle D J The physiological and pharmacological significance of cardiovascular T - type , voltage gated Ca - channels ***Am. J . Hypertens .*** 1998 ; 11 : 865 - 875 .

Trosper T L , Philipson K D Effects of divalent and trivalent cations on Na - Ca exchanger in cardiac sarcolemmal vesicles ***Biochim . Biophys . Acta*** 1983 ; 731 : 63 - 73

Trosper T L , Philipson K D Stimulatory effect of Ca - chelators on Na - Ca exchange in cardiac sarcolemmal vesicles ***Cell Calcium*** 1984 ; 5 : 211 - 222 .

Tschudi M R , Luscher T F Age and hypertension differently affect coronary contractions to endothelin - 1 , serotonin and angiotensin ***Circulation*** 1995 ; 91 : 2415 - 2422 .

Van Zweiten P A Cardioprotective activity of mibefradil Cardiovas . Res . 1998 ; 39 : 276 - 277 .

Vandeplassche G , Brenner M , Thone F ***et al*** Singlet oxygen and myocardial injury : ultrastructural , cytochemical and electrocardiographic consequences of photoactivation of rose bengal ***J . Mol . Cell . Cardiol .*** 1990 ; 22 : 287 - 301 .

Vander Hoff M J B , Deprez R H , Monterio M , de Boer P A , Charles R , Moorman A F Developmental changes in rat cardiac DNA , RNA and protein tissue base : Implications for the interpretation of changes in gene expression ***J . Mol . Cell . Cardiol .*** 1997 ; 29 : 629 - 639 .

Vander Velden J , Klein L J , vander Bijl M *et al* Force productiuon in mechanically isolated cardiac myocytes from human ventricular muscle tissue ***Cardiovas . Res .*** 1998 ; 38 : 414 - 423 .

Varro A , Lathrop D A , Hester S B , Nanasi P P , Papp J G Y Ionic currents and action potentials in rabbit , rat and guinea pig ventricular myocytes ***Bas . Res . Cardiol .*** 1993 ; 88 : 93 - 102 .

Villano L G , Dominguez L J , Barbagallo M Alterations of magnesium metabolism in aging ***Riv . Ital . Biol . Med .*** 1997 ; 17 : 63 - 69 .

Vornanen M Excitation - contraction coupling of the developing rat heart ***Mol . Cell . Biochem .*** 1996 ; 163 : 5 -11 .

Vornanen M Contribution of sarcolemmal calcium current to total cellular calcium in postnatally developing rat heart ***Cardiovas . Res .*** 1996 ; 32 : 400 - 410 .

Wacker W E , Parisi A F Magnesium metabolism ***N . Eng . J . Med .*** 1968 ; 278 : 772 - 776 .

Wahler G M Developmental increase in the inwardly rectifying K - current of rat ventricular myocytes ***Am . J . Physiol .*** 1992 ; 262 : C1266 - C1272 .

Wahler G M , Dollinger S J , Smith J M , Flemal K L Time course of postnatal changes in rat heart action potential and in transient outward current is different ***Am . J . Physiol*** . 1994 ; 267 : H1157 - H1166 .

Walker K E , Lakatta E G , Houser S R Age associated changes in membrane currents in rat ventricular myocytes ***Cardiovas . Res .*** 1993 ; 27 : 1968 - 1977 .

Walsh R A , Dorn G W Growth and hypertrophy of the heart and blood vessels **In :** ***Hurst's The Heart*** Vol . 1 R W Alexander , R C Schlant and V Fuster Eds . (McGraw Hill Health Professions Division , New York) 1998 pp . 81 - 124 .

Wang L , Feng Z P , Kondo C S , Sheldon R S , Duff H J Developmental changes in the delayed potassium channels in mouse heart ***Circ . Res .*** 1996 ; 79 : 79 - 85 .

Wang S , George S F , Davis J P , Johnson J D Structural determinants of Ca^{2+} exchange and affinity in the C - terminal of cardiac troponin C *Biochemistry* 1998 ; 37 : 14539 - 14544 .

Waymouth C Osmolality of mammalian blood and of media for culture of mammalian cells *In Vitro* 1970; 6:109-127.

Weber A Caffeine , calcium and activation of contraction ***J . Gen . Physiol .*** 1968 ; 52 : 760.

Wei J X , Sprrgeon .H A , Lakatta E G Excitation - contraction in rat myocardium : alterations with aging ***Am . J . Physiol .*** 1984 ; 246 : H784 - H791 .

Weiss A , Lassare B Should Mg - therapy be considered for the treatment of coronary heart disease ? . A critical appraisal of current facts and hypotheses ***Magnes . Res .*** 1994 ; 7 : 135 - 144 .

Werner J , Piutar K , Simon M A , Motola R , Friedman R Mayman A The effects of prolonged hypomagnesemia on the cardiovascular system in the young bog Am . Heart . J . 1964 ; 67 : 221 - 231 .

Wetzel G T , Chen F , Klitzner T S L - type and T - type Ca - channels in acutely isolated neonatal and adult cardiac myocytes ***Pediatr . Res .*** 1991 ; 30 : 89 - 94 .

Wetzel G T , Klitzner T S Developmental cardiac electrophysiology : Recent advances in cellular physiology ***Cardiovas. Res .*** 1996 ; 31: E52 - E60 .

Whang R , Welt L G Observations in experimental Mg - depletion ***J . Clin . Invest .*** 1963 ; 42 : 305 - 313 .

Whang R , Oei T O , Aikawa J K , Watanabe A , Vannatta J , Fryer A , Markanich M Predictors of clinical hypomagnesemia , hypokalemia , hypophosphatemia , hyponatremia and hypocalcemia ***Arch . Intern . Med .*** 1984 ; 144 : 1794 - 1796 .

White R E , Hartzell H C Magnesium ions in cardiac function : regulator of ion channels and second messengers ***Biochem . Pharmacol .*** 1989 ; 38 : 859 - 867 .

WHO ; Guideline for drinking water quality Vol . 2 . 1984 ; pp . 106 - 110 .

Williams F A , Dry J J A history of the heart and circulation W B Saunders Ed . (Philadelphia) 1948 pp. 1 - 10 .

Winegrad S Regulation of cardiac contractile proteins : correlation between physiology and biochemistry ***Circ . Res .*** 1984 ; 55 : 564 - 574 .

Woods K L , Fletchn S , Roffe C , Haider Y Intravenous $MgSO_4$ in suspected mocardial infarction : results of the second Leicester Intravenous Mg Intervention Trial (LIMIT - 2) ***Lancet*** 1992 ; 339 : 1553 - 1558 .

Wu C C , Su M J , Chi J F , Wu M H , Lee Y T Comparison of aging and hypercholesterolemic effects on the Na - induced currents in cardiac myocytes ***Life . Sci .*** 1997 ; 61 : 1539 - 1557 .

Xiao R P , Lakatta E G Deterioration of β - adrenergic modulation of cardiovascular function with aging ***Ann . N . Y . Sci .*** 1992 ; 673 : 293 - 310 .

Xiao . R . P , Tornhave . E . d , Wang . D . J . et al Age associated reductions in cardiac β1 and β2 adrenergic responses without changes in inhibitory G proteins or receptor kinases ***J . Clin . Invest .*** 1998 ; 101 : 1273 - 1282 .

Xu A , Narayanan N Effects of aging on SR Ca^{2+} - cycling proteins and their phosphorylation in rat myocardium ***Am . J . Physiol .*** 1998 ; 275 : H2087 - H2094 .

Xu L , Mann G , Meissner G Regulation of cardiac Ca - release channel by Ca^{2+} , H^+ , Mg^{2+} and adenine nucleotides under normal and simulated ischemic conditions ***Circ . Res .*** 1996 ; 79 : 1100 - 1109 .

Xu X , Best D M Postnatal changes in T - type Ca - current density in rat atrial myocytes ***J . Physiol*** . 1992 ; 454 : 657 - 672 .

Xu Y Q , Pickoff A S , Clarkson C W Evidence for developmental changes in Na - channel inactivation gating and Na - channel block by phenytoin in rat cardiac myocytes ***Circ . Res.*** 1991 ; 69 : 644 - 656 .

Yamadi S , Fuji J , Katayama H Sarcoplasmic reticular Ca - ATPase : distribution of phosphoenzymes formed from MgATP and CaATP as substrates and interconversion by Mg^{2+} and Ca^{2+} *J . Biochem .* 1986 ; 100 : 1329 - 1342 .

Yanagi N , Maruyama T , Uehata S , Wakimoto Y , Sasaki Y , Arita M Electrical and mechanical abnormalities in the heart of a schizophrenic patient with hyponatremia derived from water intoxication ***J . Cardiol .*** 1998 ; 32 : 197 - 204 .

Yang C Y Calcium and magnesium in drinking water and risk of death from cerebrovascular disease ***Stroke*** 1998 ; 29 : 411 - 414 .

Yang C Y , Chin H F , Chin J F , Wang T N , Cheng M F Magnesium and calcium in drinking water and cerebrovascular mortality ***Magnes . Res .*** 1997 ; 10 : 51 - 57 .

Yang Y , Guo Q , Peng T , Gu Q , Zhao J , Xiong D Effect of verapamil on Ca^{2+} - influx and CVB3 - RNA replication in cultured neonatal rat heart cells infected with CVB3 ***Clin . Med . Sci .*** 1996 ; 11 : 89 - 92 .

Yoshimo H Mechanism of myocardial protection with potassium arrest in isolated ischemic rat heart ***Kokyn . To . Junkan .*** 1991 ; 11 : 1151 - 1157 .

Young D A B Preparation of isolated cells from rat heart ***Experientia*** 1976 ; 32 : 1389 - 1390.

Zakaria O , Shono T , Imajma T , Snita S Fertility and histological studies in a unilateral cryptoched model during early and late adulthood ***Br . J . Urol .*** 1998 ; 82 : 404 - 407 .

Zhang A , Cheng T P D , Altura B M Magnesium regulates free ionized calcium concentration and cell geometry in vascular smooth muscle cells ***Biochim . Biophys . Acta . Mol . Cell . Res .*** 1992 ; 1134 : 25 - 29 .

Zhou Y Y , Lakatta E. G , Xiao R P Age associated alterations in Ca - current and its modulation in cardiac myocytes ***Drugs . Aging*** 1998 ; 13 : 159 - 171 .

Zimmer H - G Some aspects of cardiac heterogeneity ***Bas . Res . Cardiol .*** 1994 ; 89 : 101 - 117 .