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19 ABSTRACT (Continue on reverse if necessary and identify by block number) Cardiac muscle subjected to increased hydrostatic pressure from 2 to 150 atmospheres responds with an increase in developed tension, a phenomenon we refer to as high pressure inotropy or HPI. Experiments were performed on papillary muscles isolated from adolescent rabbit hearts to test the possibility that HPI is due to a preferential action of pressure to partially inhibit the activity of the sodium-potassium pump of the cell membrane leading to calcium accumulation through reduced sodium-calcium exchange. Ouabain, a cardiac glycoside known to inhibit pump activity, prevented the development of HPI when applied before pressure was increased. Both ouabain and pressure altered the time course of mechanical restitution and the development of the resting state contraction in a similar manner that could be mimicked by a computer model when the calcium extrusion ordinarily occurring during each cardiac cycle was substantially retarded. Finally, we report on progress made to develop a microfluorimeter for measuring cytosolic calcium in single cardiac myocytes under hyperbaric conditions.				
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ANNUAL REPORT ON OFFICE OF NAVAL RESEARCH CONTRACT
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CONTRACTOR: Research Foundation of State University of New York

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in Cardiac Muscle

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INTRODUCTION

Our previous work has demonstrated that elevated hydrostatic pressure over the range of 2 to 150 atmospheres causes an increase in the force of cardiac muscle contraction (1). In the first year of this project we have explored the possibility that the positive inotropic action of pressure is due to a partial inhibition of the sodium-potassium pump of the cardiac cell membrane and that through coupling to the sodium-calcium exchange mechanism calcium is trapped in the cell leading to augmented contraction force. If this hypothesis is correct the application of ouabain, a cardiac glycoside known to specifically inhibit Na/K-pump activity and to augment mechanical force in heart muscle, should interfere competitively with elevated hydrostatic pressure in altering inotropic state. Our present findings show that, in fact, the rabbit papillary muscle pretreated with ouabain responds with an increase in steady-state twitch tension that precludes the positive inotropic effect of subsequently elevated hydrostatic pressure. Further, ouabain and pressure have similar effects on the cyclical restitution of the inotropic state occurring normally during diastole and on the recovery of mechanical activity following artificially prolonged quiescence. When kinetic data provided by the latter experiments were compared to data from a computer model of the kinetics of calcium flux in cardiac cells, the model predicted the same behavior when the rate of transsarcolemmal calcium extrusion alone was reduced to a degree consistent with that predicted for the tissue following a decrease in Na/K-pump activity. Details of this work are reported below.

Recognizing the possibility that calcium may play a central role in high pressure inotropy, we have set as a major goal for this project the determination of pressure effects on the temporal and spatial distributions of cytosolic calcium for a single contraction cycle in physiologically viable cardiac myocytes. The details of our technical progress on this aspect of the project are reported in Appendix I.

MATERIALS AND METHODS

Papillary muscles were excised from the right ventricle of adolescent rabbits and maintained in Tyrode's solution at 37° C throughout the course of the experiment. The solution was gas equilibrated with a 95% O₂ - 5% CO₂ gas mixture at one atmosphere prior to being pumped continuously into the tissue bath located in the compression chamber. A detailed description of the pressure chamber and its operation may be found in reference 2. The papillary muscle was mounted vertically in the tissue bath such that its base was fixed rigidly and its apex attached to a temperature compensated, force sensing transducer. The muscle was excited using square pulse electrical stimuli applied through field electrodes placed near the base of the muscle. A computer was used to synchronize the application of stimuli with the recording of twitch responses. Muscle force was digitized, stored and subsequently analyzed for several indices of inotropic state of the muscle; specifically, peak developed tension, time to peak, and the rates of contraction and relaxation.

Muscle preparations were passively stretched to a resting tension of 0.5 grams and were allowed to equilibrate at a stimulation frequency of 1 hertz for at least an hour. Each experiment began with the collection of steady-state data at a fixed stimulation frequency. Subsequently, two arrhythmic stimulation protocols were employed to extract information about time-dependent changes in contractile state. In the first, a period of constant stimulation was interrupted for 5 seconds during which an extra stimulus was applied to elicit an extra twitch contraction. This sequence was repeated multiple times with the extra response being evoked at a time further into the rest interval in each cycle. The family of peak twitch tensions so obtained, when plotted against time, indicate the time course of mechanical restitution following a twitch contraction. In the second stimulation protocol, the rest interval following a period of fixed frequency stimulation was extended to 5 minutes. In this procedure, the first contraction following the resumption of stimulation is referred to as the resting state contraction. Under normal circumstances this contraction is of markedly reduced amplitude due to loss of intracellular calcium occurring during the pause. The return to

normal intracellular calcium that occurs over the next several contraction cycles and is reflected in the time course of recovery of peak twitch tension.

Chamber pressure was increased using nitrogen at a compression rate of about 25 atmospheres per minute. Calibrated chamber pressure and tissue bath temperature were recorded automatically whenever muscle tension was recorded. The outflow port of the tissue bath opened into the compression chamber thus allowing chamber pressure to be transmitted hydraulically back into the fluid filled tissue bath. This arrangement prevented the contamination of the perfusion fluid with the gas used for compression.

Ouabain was added to the perfusion fluid at a concentration of $1 \cdot 10^{-7}$ g/ml.

RESULTS

Figure 1 shows the effect of elevated hydrostatic pressure on steady-state twitch tension in rabbit papillary muscle. Increases in hydrostatic pressure in 50 atmosphere increments were accompanied by corresponding incremental increases in peak tension, time to peak and the rates of contraction and relaxation.

Figure 2 shows the effect of pretreatment with ouabain before the application of elevated pressure. The positive inotropic action of ouabain reached a steady-state in about 30 minutes and was of similar amplitude to that produced by pressure alone. Compression to 125 atmospheres in the presence of ouabain had little effect on peak tension but did delay the time to peak.

The effect of pressure on mechanical restitution is shown in figure 3. Under 1 atmosphere control conditions the recovery of inotropic state was at first rapid, reaching a peak in about 1 to 2 seconds, and then began to decline slowly. At 125 atmospheres the entire curve was displaced to a higher level of tension consistent with the increase in steady-state twitch tension. Further, the initial phase of rapid recovery was slowed and the later phase exhibited a gradual increase in inotropic potential rather than the decrease seen under 1 atmosphere control conditions.

The actions of pressure on time-dependent changes in inotropic state were further reflected in the response of the resting state contraction to elevated pressure. Figure 4 shows that at 1 atmosphere the first contraction after a 5 minute interruption in stimulation was markedly attenuated and that the subsequent recovery of twitch tension

was initially rapid and then gradual as the steady-state was approached. At 125 atmospheres, figure 4b, the resting state contraction was attenuated to a lesser extent and subsequent recovery of twitch tension was much slower. This pattern of inotropic change was similar to that caused by ouabain, figure 4c. When pressure and ouabain were applied together, figure 4d, the resting state contraction was augmented rather than attenuated, i.e., the resting state contraction was greater than the pre-rest, steady-state contraction. Further, under the combined influence of pressure and ouabain, contractions subsequent to the resting state contraction were nearly fully recovered and exhibited only a small and slowly developing transition to steady-state.

DISCUSSION

The present findings indicate that elevated hydrostatic pressure enhances cardiac muscle contraction by somehow affecting the disposition of calcium as an agent for electromechanical coupling during each contraction cycle. Dynamic regulation of cytosolic calcium in cardiac cells involves a number of transport mechanisms operating simultaneously across the cell membrane and across the membranes of the sarcoplasmic reticulum. The fact that both elevated hydrostatic pressure and ouabain have similar effects on twitch tension, mechanical restitution and the resting state contraction suggest that the primary pressure-sensitive locus may be the Na/K-pump of the sarcolemma. Given the complexity of the calcium control system and the ubiquitous action of pressure in affecting all physico-chemical reactions of the system, it is intriguing to consider the possibility that a preferential action of pressure on the kinetics of calcium extrusion may suffice to explain both steady-state and transient inotropic phenomena. To assess this possibility we have constructed a mathematical model of calcium flux and contraction in heart cells and have tested the model for perturbations that yield inotropic responses like those seen under conditions of elevated hydrostatic pressure. The model, illustrated in figure 5, is comprised of four differential equations that account for calcium flux into and out of the cytosol during each cardiac cycle. Figure 4e-g shows that a progressive decrease in k_4 , the rate constant for calcium extrusion that reflects the activity of the Na/K-pump-Na/Ca-exchanger complex, yielded a good simulation of pressure and ouabain effects on resting state contraction, post-resting state recovery and steady-state contraction force. No other single or combined change in model parameters was sufficient to mimic the pressure-induced inotropic response of the tissue. It is particularly significant that a reduction in the extrusion rate constant alone resulted in augmentation of the resting state contraction beyond that of the pre-resting-state control, just as observed in the tissue. Again, given

the complexity of this system it is difficult to imagine that this similarity is merely fortuitous.

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FIGURES

Figure 1. Effect of hydrostatic pressure on twitch tension in isolated rabbit papillary muscle at 37^o C. The lower panel shows the first derivative vs. time curves for the twitch tension tracings shown in the upper panel.

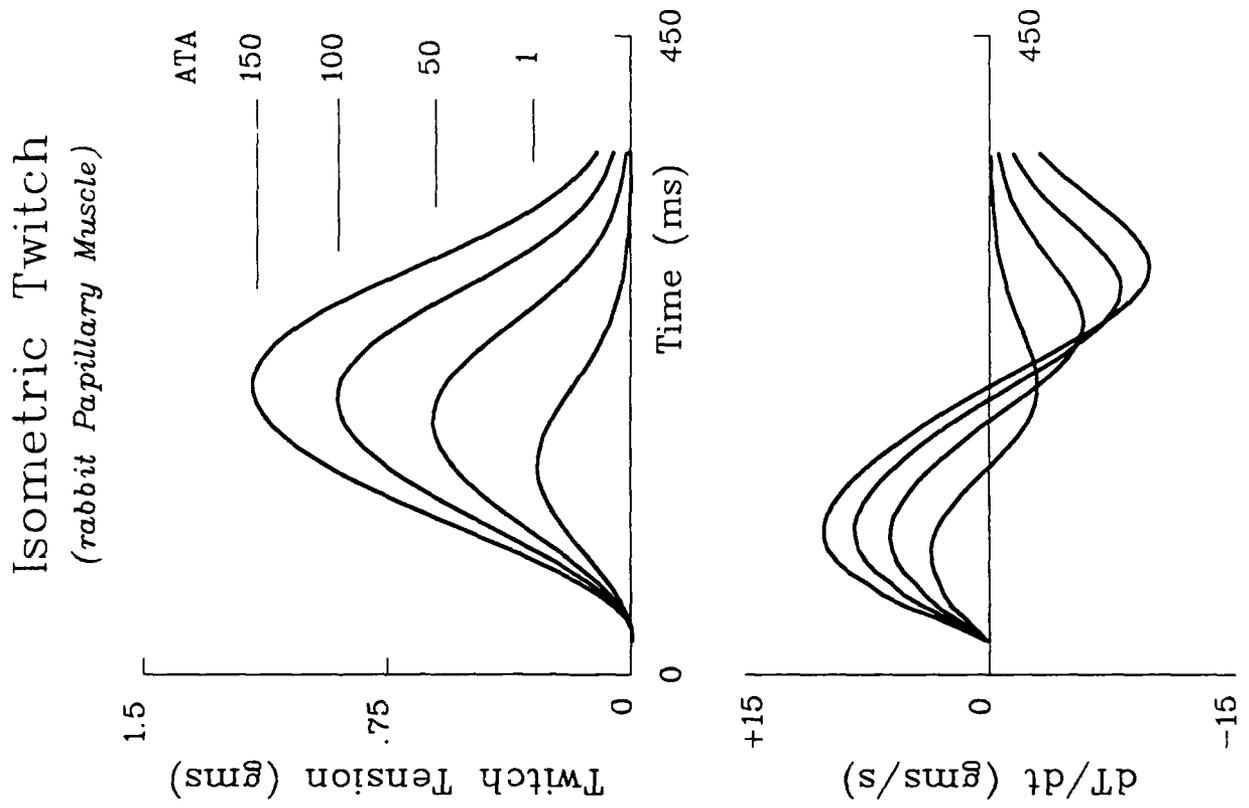
Figure 2. Effect of ouabain pretreatment on the twitch response to elevated hydrostatic pressure. Note that in this experiment pressure was increased after the tissue had equilibrated to the presence of ouabain.

Figure 3. Effect of hydrostatic pressure on mechanical restitution in rabbit papillary muscle.

Figure 4. Effect of hydrostatic pressure and ouabain on the resting state contraction of rabbit papillary muscle. The upper panel shows the response of acutely isolated muscle and the lower panel the response of the computer model conditioned to mimic the tissue response.

Figure 5. Conceptual and mathematical details of the calcium flux model for cardiac cells.

Figure 1



Isometric Twitch (Rabbit Papillary Muscle)

Figure 2

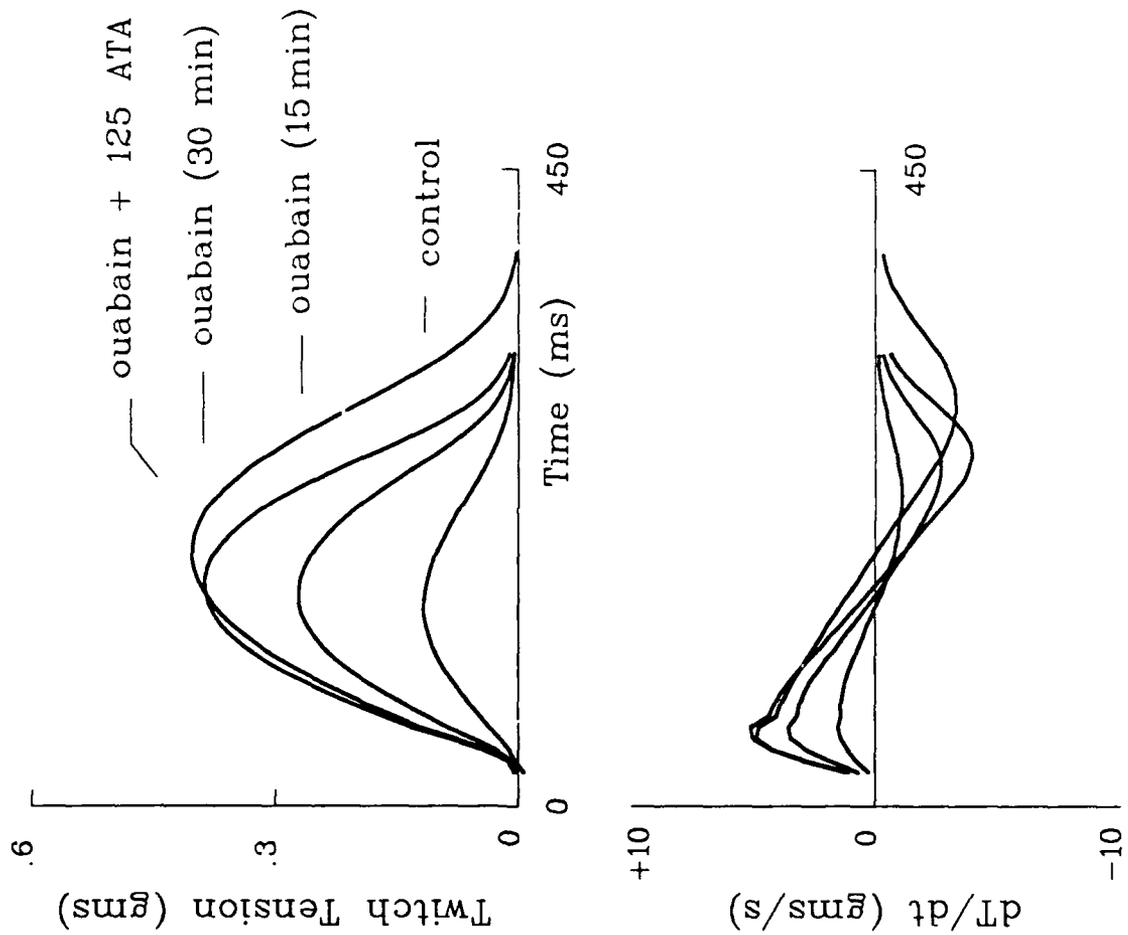


Figure 3

Mechanical Restitution

(rabbit papillary - exp. #mi100887)

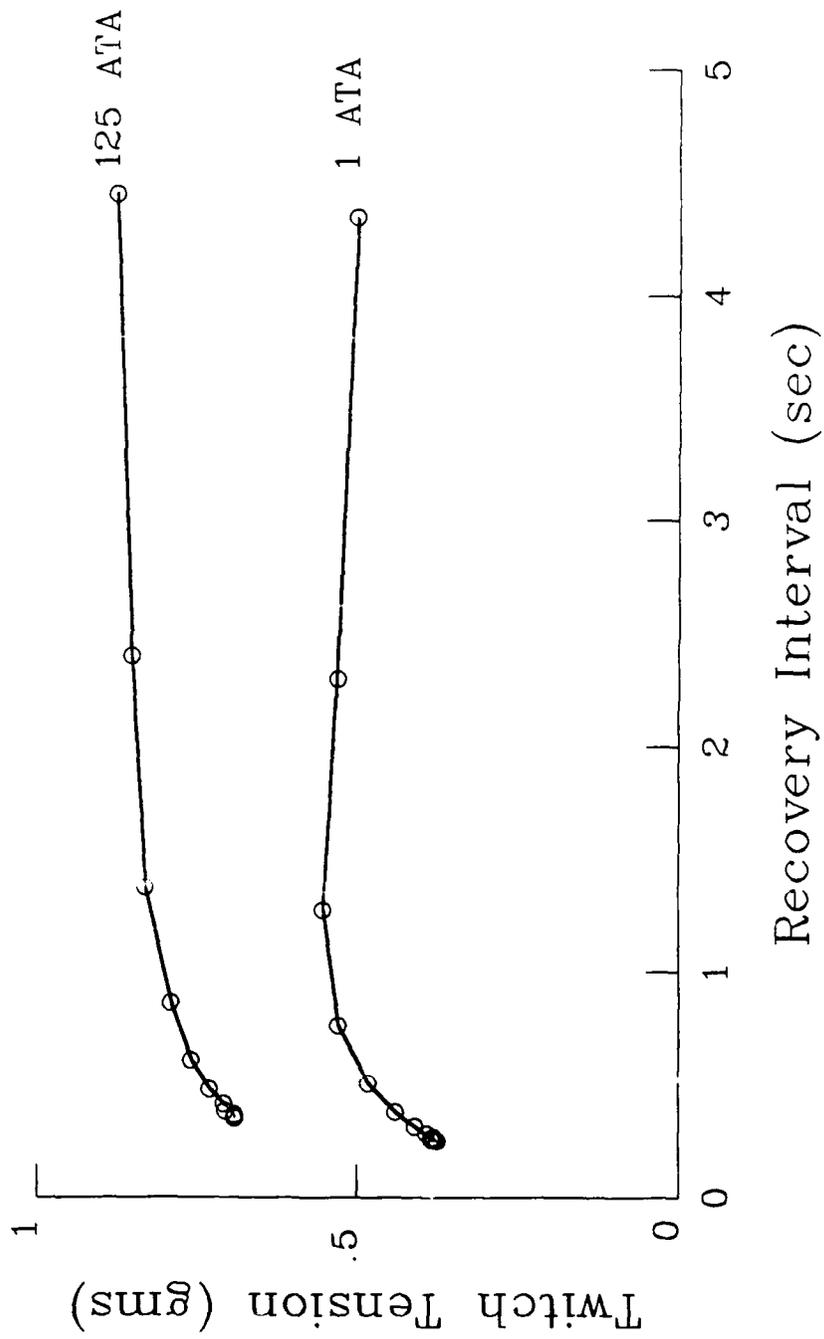
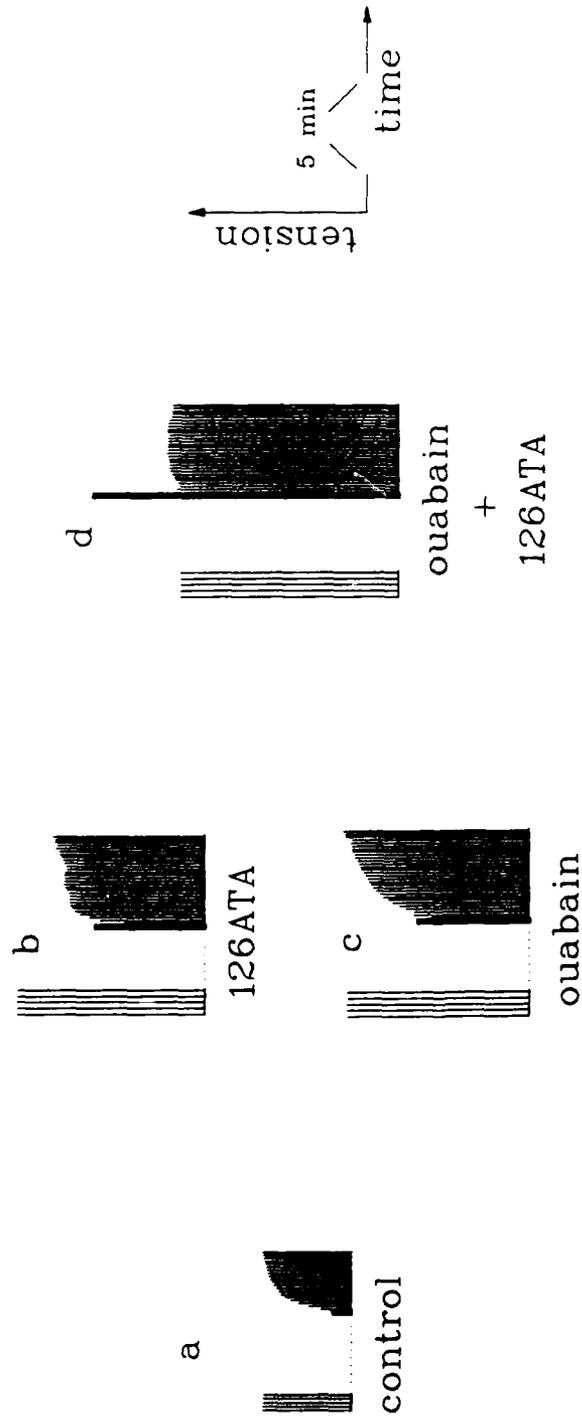


Figure 4

—Tissue



—Model

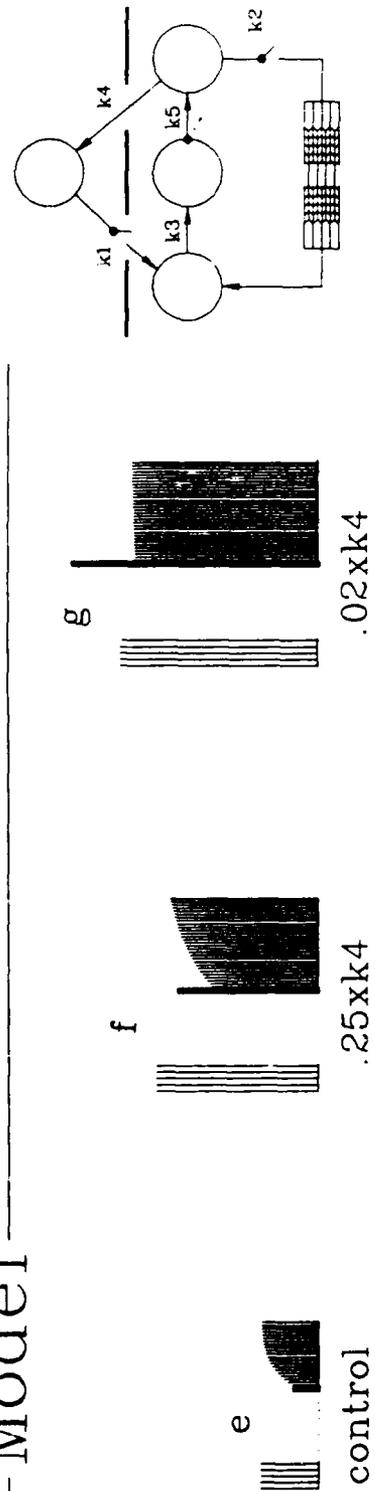
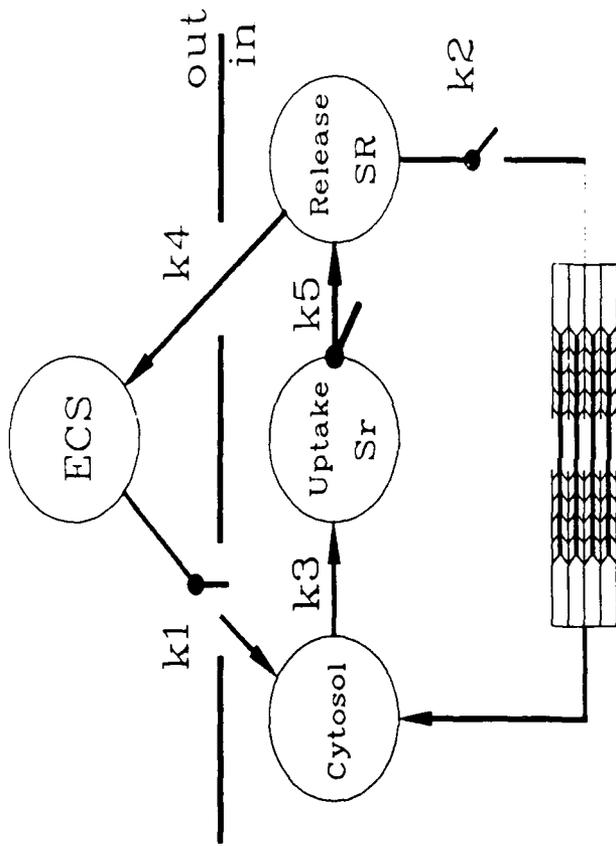
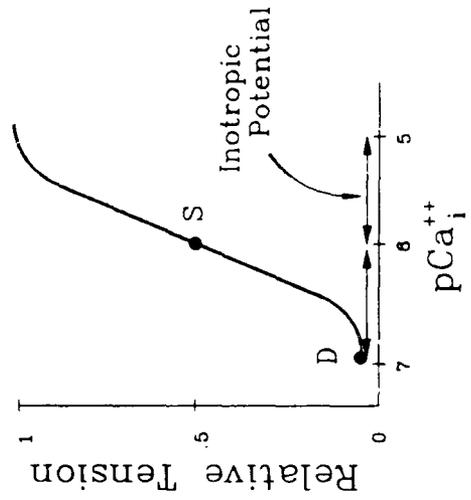


Figure 5



$$\begin{aligned}
 1) \left(\frac{dQ_{Ca}}{dt} \right)_e &= -k_1 \cdot \left(\frac{Q_{Ca}}{V} \right)_e + k_4 \cdot \left(\frac{Q_{Ca}}{V} \right)_r \\
 2) \left(\frac{dQ_{Ca}}{dt} \right)_c &= -k_3 \cdot \left(\frac{Q_{Ca}}{V} \right)_c + k_2 \cdot \left(\frac{Q_{Ca}}{V} \right)_r + k_1 \cdot \left(\frac{Q_{Ca}}{V} \right)_e \\
 3) \left(\frac{dQ_{Ca}}{dt} \right)_u &= -k_5 \cdot \left(\frac{Q_{Ca}}{V} \right)_u + k_3 \cdot \left(\frac{Q_{Ca}}{V} \right)_c \\
 4) \left(\frac{dQ_{Ca}}{dt} \right)_r &= -k_2 \cdot \left(\frac{Q_{Ca}}{V} \right)_r - k_4 \cdot \left(\frac{Q_{Ca}}{V} \right)_r + k_5 \cdot \left(\frac{Q_{Ca}}{V} \right)_u
 \end{aligned}$$



APPENDIX I

Introduction

The primary research objective of our project is to elucidate the mechanism of high pressure inotropy in cardiac muscle cells. To this end, we must measure intracellular calcium transients in relation to the changing force of the muscle twitch with increasing hydrostatic pressure. The proposed method is to record the calcium transient as the fluorescent emission from the intracellular reporter dye FURA-II while simultaneously obtaining contraction information from sarcomere length extracted from differential interference contrast images of the cell. Basically, this involves the development of three pieces of equipment. First, a computer controlled dual wavelength light source; second, suitable optical equipment for data collection; and third, a high pressure chamber suitable for microscopic observation and measurements from living single cardiac cells. Several of these problems are not unique to the present study and their solutions represent generally applicable improvements to the technique of measuring intracellular calcium with fluorescent reporter dyes such as FURA-II.

We have set the following design objectives for this system.

- 1) The dual wavelength epifluorescent light source must be capable of very high switching speeds (15 μ s between wavelengths).
- 2) Fluorescent excitation light must be presented to the tissue as a uniformly illuminated field.
- 3) Exposure of the target cell to excitation light must be kept to an absolute minimum.
- 4) Maximum recovery and utilization of emitted photons must be achieved.
- 5) Transillumination light for the differential interference contrast image must not contaminate the fluorescence measurement.
- 6) The tissue (cell) pressure chamber must be small enough to be mounted on the microscope stage.
- 7) Electrical stimulation of the tissue must be precisely synchronized with data sampling under computer control.

8) The system must be designed to accept spatial imaging hardware (intensified CCD video camera) for later implementation.

Details regarding the implementation of each of these items will be covered in the following paragraphs.

Dual wavelength epifluorescence light source.

The photon source consists of a high pressure helium-xenon arc lamp mounted in an F4.5 elliptical reflector (PTI). When properly adjusted, this provides a small, well focussed, highly intense image of the lamp arc. The non-uniform intensity of the arc, however, is not optimal for fluorescent excitation. Accordingly, the beam is passed through a diverging quartz lens to produce a nearly collimated beam. The defocussed nature of the beam provides a uniform cross sectional field intensity for illumination of the specimen plane of the microscope. Infra-red light is removed by passing the beam through a 46mm column of distilled water.

The "cooled" beam is then split with a dichroic mirror. Each beam is passed through an appropriate interference filter to generate a pair of narrow band beams whose center wavelengths are at 340 and 380 nm. These beams are then masked by slits and chopped to be out of phase by a spinning sector wheel. The wheel has 30 sectors and may be spun as fast as 6000 RPM to produce a wavelength pair every 333 us. The width of the slits are adjustable and typically set to less than 0.4mm. This combination produces a net light utilization ratio of greater than 90%.

Following the chopper wheel, the beams are recombined with a second dichroic mirror and passed into wavelength corrected quartz output optics which focus the images of the slits onto the field stop of the Zeiss epifluorescence attachment, which is conjugate to the specimen plane. Adjustments are provided to overlay the images of the slits in the microscope specimen field. An aperture stop is provided in the output optics to adjust light intensity and the output beam is electro-mechanically shuttered. The shutter and rotational speed of the chopper wheel are both under control of the computer.

At the present time, the design and manufacture of the light chopping device is complete and undergoing final testing and adjustment.

Light Source Electronics Package

The control of the light source requires 2 components: a power supply and control circuit for motor speed which interfaces with the computer, and sensor circuitry to indicate wheel speed to the computer and to provide control signals to the data acquisition hardware. The design and implementation of these components has been completed and are described briefly below.

In order to simplify interfacing the chopper wheel motor to the computer, it was desirable to drive the motor with a circuit which linearizes motor RPM to input control voltage. This was accomplished with an operational amplifier driver circuit, which uses the back EMF of the motor as its feedback voltage. The output from a digital to analog converter in the computer comprises the remainder of the motor control circuitry.

The power supply circuitry resides on the motor speed control circuit board and supplies power to all circuits in the chopper assembly. It supplies +25V and -5V for the on board operational amplifiers in addition to +25, +8 and -8V for the sensor circuit board. Its design is straightforward and requires no further description.

The sensor circuitry consists of two identical channels, each of which is made up of a narrow acceptance angle infra-red photodiode detector, a photodiode amplifier, a slightly leaky peak detector, and a high speed voltage comparator. The excitation light slits are made to extend along the entire length of the cutouts in the rotating sector wheel and a narrow beam infra-red emitter is located on the side of the slit opposite each photodiode. Thus, an emitter-detector pair is placed along the same radial line as each of the two excitation light slits and the emitter-detector coupling will be chopped in exact synchrony with the corresponding excitation light beam.

Three signals are then developed from these two chopped waveforms which are used to control the data acquisition hardware. Each channel generates a gate signal which goes high when the corresponding excitation light slit is fully open. The opposite light slit must always be closed at this point by virtue of the 52%-48% duty cycle of the chopper wheel. These 2 signals are "OR"ed together to provide a third signal which indicates that one or the other of the two windows is fully open. The sensor circuitry is capable of tracking from a low of about 60 RPM up to the maximum of 6000 RPM.

Simultaneous DIC-Epifluorescence system

This consists of several optical components, many of which serve multiple purposes. The differential interference contrast optics were supplied by Zeiss, and consist of a 40X multi immersion lens with a dry numerical aperture of 0.9. The lens will be used immersed in water or oil to further increase the numerical aperture. We custom selected the matching Wollaston prism to optimize the contrast enhancement since Zeiss did not supply this lens with a matching prism and could not make a suitable recommendation. Elimination of cross talk with the fluorescent data was achieved by illuminating through a 650 nm interference filter which had a high degree of blocking added in the 510 nm FURA emission band. The specimen facing side of this filter has an additional dichroic reflective coating which reflects the 490-530 nm band back to the tissue. Overall transmissive blocking of the FURA emission band by a factor of at least 10^{12} was obtained.

From the dual wavelength light source, the epifluorescent excitation light is routed via a modified Zeiss epifluorescence attachment into the standard Zeiss mirror block located just beneath the microscope objective. This block contains a special dichroic mirror with 2 coatings. The first reflects the UV excitation light up into the back of the objective lens. The second reflects the returning fluorescent emission light back into the epifluorescence attachment. This mirror transmits the DIC image light into the microscope without modification.

The modification to the epifluorescence attachment consists of a hole made in the side which looks upon a dichroic mirror placed so as to reflect the returning fluorescent emission light out of the epifluorescence attachment, through a suitable barrier filter and into a photomultiplier tube used in a photon counting mode. The photomultiplier tube and housing (Thorn-EMI) lies in a custom made holder which fits in a port which Zeiss had conveniently place in the side of the microscope body at right angles to the epifluorescence attachment port. This location of the photomultiplier tube minimizes photon loss due to passing through excessive optical components. Power supplies and signal conditioners for the custom selected photomultiplier tube were also supplied by Thorn-EMI. The photon counting registers reside in a laboratory interface board in the computer. At present, the dichroic mirrors have been manufactured (Omega Optical, 3 Grove Street, Brattleboro, Vermont) and the modifications to the epifluorescence attachment are underway. Completion is expected by the second week of August, 1989.

At a later time, when spatial imaging is planned, the photomultiplier tube may easily be replaced with the appropriate imaging array. No plans have been made at present to mount a photomultiplier tube and an imaging array at the same time, although the system could easily be modified to accomplish this.

The final component of the system is the holder and optics for the photodiode array which will record the differential interference contrast image. This consists of an adjustable lens holder which mounts to a video camera port located on the side of the microscope. The focal plane of this camera port is moved away from the microscope with an auxiliary lens which adds a magnification of 2.5 times. The 1024 element, 25 microns/pixel linear photodiode array (EG&G Reticon) is then mounted at the focus of this lens. The net magnification with a 40X objective is 100 times, for an ultimate resolution of 250 nm at the specimen plane, near the resolution limit of the microscope with 650 nm. illumination.

The driving and readout electronics for the photodiode array is supplied by EG&G Reticon as a pair of circuit boards to which photodiode array is mounted directly. All of the components of this system have been purchased. The lens holder has been designed and is awaiting manufacture in our departmental machine shop.

Stage mounted, hyperbaric, single cell tissue bath

The tissue bath is still in the design phase. The preliminary design consists of a narrow bore pressure vessel with a thin (0.17 to 0.20 mm thick) glass window attached over the end. The pressure vessel is sealed in such a manner as to allow rotation for positioning the cell under study into the proper orientation. The technology for much of the experimental control of the chamber environment was developed in our laboratory for past work in a large size (7 liters) pressure bomb. See reference 2 for details. This technology is directly applicable to our present project.

Preliminary experiments have been completed on the feasibility of using such thin windows at pressures in excess of 100 ATA. The use of a small pressure aperture is essential, somewhere in the range of 1 mm diameter. The window is attached using a super high strength thermo-setting epoxy (EpoTek #377, Epoxy Technology). Using a 1 mm diameter aperture, we have achieved withstanding pressures of 215 ATA for over 8 hours using a standard microscope coverslip. Glue line thickness is 13-14 um.

With the pressures available to us on a standard basis, we were unable to burst this window, although microscopic examination of the aperture indicated a gradual degradation of the epoxy joint at the circumference of the aperture which reached a steady state of about 1.6 mm diameter after 9 hours. An attempt to use a larger aperture of 2 mm produced burst pressures of about 100 ATA after 2 minutes of exposure. Microscopic examination indicated an apparent glue joint failure leading to explosive delamination of the window and bursting. The high hydrostatic pressures apparently increase the rate and amount of water uptake into the epoxy glue matrix, causing decompaction and weakening of the joint. We are presently experimenting with modifications to the thermal curing schedule of the epoxy and the application method to minimize the attack of the epoxy by water.

In theory, the use of quartz coverslips, 0.2 mm thick, should provide a higher burst pressure, even with glue joint degradation. In experiments with quartz coverslips we have not been able to obtain burst pressures in excess of 60 ATA, even with the 1 mm diameter pressure aperture. The difficulty appears to be that all commercially available quartz coverslips are ground and polished, leaving microscopic scratches in the surface of the glass which act as focal points for crack growth which is greatly accelerated by water at high hydrostatic pressures. We are presently undertaking experiments to fire polish quartz coverslips and determine if we will be able to capitalize on the improved physical characteristics of quartz in the design of the tissue bath window.

We anticipate the completion of the tissue chamber and attendant apparatus sometime in the fall of 1989.

Computer based functions

The laboratory computer used in this system to control data acquisition, manage data storage and retrieval, and perform data analysis is an 80386/80387 based machine operating in the IBM DOS environment. The computer is equipped with a TECMAR laboratory interface board, with digital I/O, counter functions, and analog to digital conversion capabilities. This board provides the interface to control the epifluorescent light source, photodiode array electronics, photon counting, and tissue stimulation all under the control of custom written software. The software is still under development, requiring for its completion the final assembly of the associated hardware. We expect to have a working version of the software by the fall of 1989.

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