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THE EFFECTS OF RYANODINE ON THE FORCE-INTERVAL RELATION OF RAT CARDIAC MUSCLE

by

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ABSTRACT

In this thesis, the role of the sarcoplasmic reticulum in the force-interval relation of rat cardiac trabeculae was studied both in the absence and presence of ryanodine, a selective inhibitor of Ca^{2+} release from the sarcoplasmic reticulum. Sarcomere length and force were measured by a laser diffraction technique and silicon strain gauge, respectively.

The results show that the negative inotropic effect of ryanodine is dose-dependent and can be enhanced in a use-dependent way. Two parameters which characterize each twitch, time to peak force (T_{peak}) and relaxation time (RT_{10}), were changed by ryanodine. T_{peak} was shown to decrease in the presence of ryanodine, whereas RT_{10} increased in the presence of ryanodine. Ryanodine completely inhibited the potentiated force of the first beat normally observed (i.e. control solution) after a decrease in stimulation frequency, and it abolished the depressed force of the first beat normally observed (i.e. control solution) after an increase in stimulation frequency. The rest-depression phase of the force-interval relation began after shorter rest-intervals in the presence of ryanodine. Elevating $[Ca^{2+}]_i$, by increasing $[Ca^{2+}]_o$ or by extrasystole potentiation, counteracted the inhibitory effect of ryanodine on twitch force; however, the rest-interval required for optimal rest-potentiation was not changed by increased $[Ca^{2+}]_i$.

The results of this study are consistent with the proposal that leak of calcium from the sarcoplasmic reticulum may underly the rest-depression phase of the force-interval relation, and may modulate steady-state twitch force parameters and characteristics of the Bowditch staircase.

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Difficulties do not crush men, they make men.
authorship unknown

During the course of attaining this degree, I encountered many challenges. I would like to dedicate this degree to my mother Sarla and my brother Rajiv for their continued love and support, which helped me to meet these challenges.

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CHAPTER 1

INTRODUCTION

The contraction of heart muscle has been and continues to be the focus of much experimental and theoretical work. Although our knowledge of this subject is continually expanding, many fundamental questions have yet to be answered.

In this thesis, one aspect of heart muscle contraction is investigated in a study of the role of the sarcoplasmic reticulum in excitation-contraction coupling. To investigate the role of the sarcoplasmic reticulum, the force-interval relation of rat cardiac trabeculae has been measured in both the absence and presence of ryanodine, a selective blocker of sarcoplasmic reticulum function.

1.1 Present Understanding of Excitation-Contraction Coupling

When electrophysiological events at the sarcolemma of heart cells directly or indirectly increase intracellular calcium, the contractile proteins (i.e. actin and myosin) interact to cause muscle contraction. This process has been labelled excitation-contraction coupling (E-C coupling).

1.1.1. *Excitation and Calcium Influx*

The sarcolemma is a biomolecular leaflet (lipid and protein) that is semi-permeable. Suspended within the sarcolemma are ion channels, consisting of glycoproteins surrounding an aqueous pore. Ion channels facilitate the movement of

ions through the high resistance membrane, resulting in an ionic current. These channels can be highly selective for a specific ion (eg. Ca^{2+}, Na^+, K^+). Currents flow because there are electrochemical gradients across the sarcolemma. Electrochemical gradients are maintained by the semi-permeable nature of the membrane and energy requiring membrane ion pumps. The action potential is generated by different ionic currents. Although each region of the heart exhibits a distinct action potential, the major ionic currents underlying the action potential are similar (Coraboeuf, 1978).

The rat action potential has five phases (see Figure 1.1). The first phase is the rapid-upstroke phase, and is generated by a transient inward Na^+ current (I_{Na}). This current is the largest and fastest current in the heart. By the time of the 'notch' of the action potential, I_{Na} has inactivated almost completely, resulting in a rapid, initial repolarization of the action potential. This repolarization is due to two transient outward currents: I_t and $I_{K(Ca)}$. Both I_t and $I_{K(Ca)}$ are K^+ currents, however $I_{K(Ca)}$ requires calcium for its activation. The significance of $I_{K(Ca)}$ in healthy rat ventricle is uncertain. The plateau of the action potential is maintained by a transient inward current I_{Ca}, which corresponds to movement of calcium ions through time and voltage-dependent L-type calcium channels. Final repolarization is initiated by a third K^+ current known as the delayed rectifier (I_K). I_K is a K^+ current that slowly activates, and brings about repolarization. It increases with depolarization in a time and voltage-dependent fashion. The contribution of this current to the rat action potential is small. Final repolarization in rat heart is controlled by three currents: I_{K1}, I_{NaCa}, and $I_{pump(Na/K)}$. The two outward currents, I_{K1} and $I_{pump(Na/K)}$ counteract the inward current due to Na-Ca exchange (I_{NaCa}). Final repolarization will be prolonged if I_{NaCa} predominates, whereas

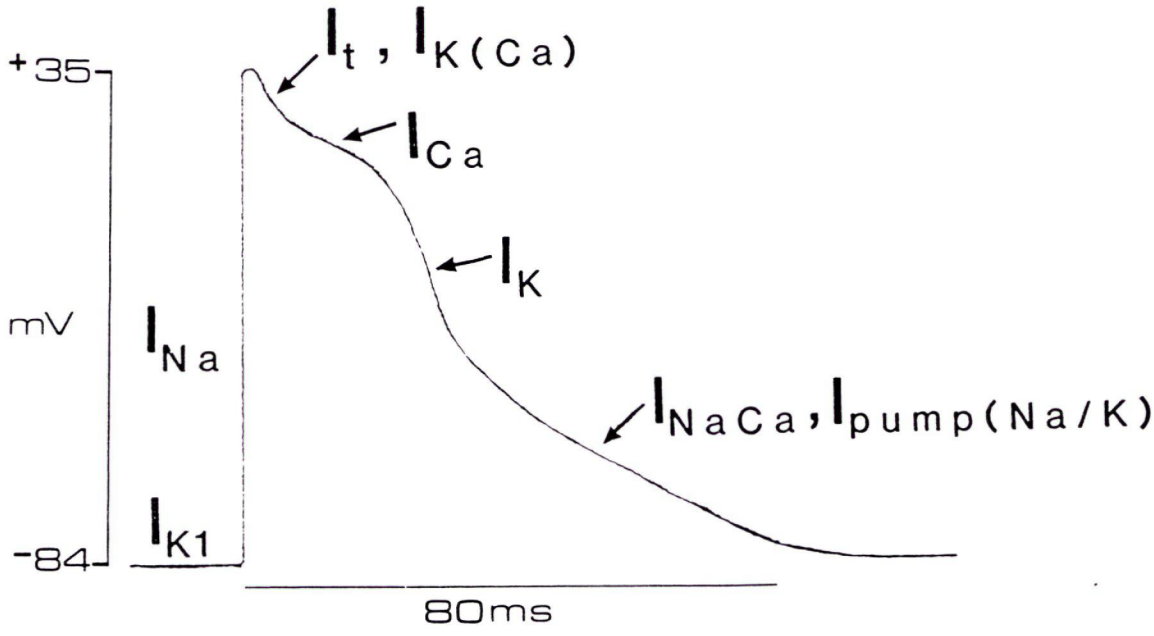


Figure 1.1. Illustration of Rat Action Potential.

The rat action potential has five phases. The first phase is the rapid upstroke phase, and is generated by a transient inward Na^+ current (I_{Na}). By the time of the 'notch' of the action potential, I_{Na} has inactivated almost completely, resulting in a rapid, initial repolarization. This repolarization is due to two transient outward currents: I_t and $I_{K(Ca)}$. Both I_t and $I_{K(Ca)}$ are K^+ currents, however $I_{K(Ca)}$ requires calcium for its activation. The plateau of the action potential is maintained by a transient inward current I_{Ca}, which corresponds to the movement of calcium ions through time- and voltage-dependent L-type calcium channels. Final repolarization is initiated by a third K^+ current known as the delayed rectifier (I_K). I_K is a K^+ current that slowly activates and brings about repolarization. Final repolarization is controlled by three currents: I_{K1}, I_{NaCa}, and $I_{pump(Na,K)}$. The two outward currents, I_{K1} and $I_{pump(Na,K)}$ counteract the inward current due to Na-Ca exchange (I_{NaCa}). The resting membrane potential is maintained by I_{K1}, a K^+ current that decreases with depolarization.

the action potential duration will be shortened if $I_{pump(Na/K)}$ (the Na-K pump current) predominates. The conditions inside and outside the muscle will determine the dominant current in this last phase. Normally, rat ventricle action potential duration is approximately 80 ms (Schouten, 1985). The resting membrane potential (-85 mV in rat ventricle) is maintained by I_{K1}, a K^+ current known as the inward rectifier. Unlike I_K, I_{K1} decreases with depolarization.

E-C coupling is initiated when an action potential spreads over the sarcolemma and results in calcium influx via I_{Ca} (see McDonald, 1982 for Review of properties of I_{Ca}). Calcium influx is controlled by extrinsic and intrinsic factors (see Table 1 of Sperelakis, 1984). From their photoinactivation of dihydropyridine calcium antagonists (nifedipine and nisoldipine) experiments and extracellular calcium depletion experiments on cat ventricular muscle, Morad and Cleeman (1987) concluded that 5-15% of rise in intracellular calcium can be attributed to sarcolemmal calcium influx during an action potential, and that calcium efflux during relaxation balances this influx in the steady state. This transmembrane influx is essential for E-C coupling (Morad and Cleeman, 1987; Fabiato, 1983); however, the relative importance of this influx is species-dependent. Contraction in rat cardiac muscle appears to be the least sensitive to interventions that interfere with Ca^{2+} entry, whereas force development in rabbit ventricle is very sensitive to interventions that interfere with calcium entry (Horackova, 1986). In addition, there also appears to be regional (eg. atrium vs. ventricle) and developmental (eg. neonate vs. adult rat) differences in the relative contributions of Ca^{2+} influx to the activation of the myofilaments (Bers, 1987).

1.1.2. *Mechanism of Sarcoplasmic Reticulum Calcium Release*

It is now accepted that an action potential across the heart sarcolemma can increase $[Ca^{2+}]_i$. However, the precise mechanism by which depolarization of the sarcolemma increases intracellular calcium is not known. Since I_{Ca} can account for approximately only 5-15% of the increase in $[Ca^{2+}]_i$ (Morad and Cleeman, 1987), there must be another source of calcium for contraction. This source is the sarcoplasmic reticulum (SR). The importance of the SR in providing a major portion of the calcium for activation of the contractile system has been demonstrated using agents (eg. ryanodine) that selectively affect tension development by altering SR function (Marban and Wier, 1985). The contribution of SR Ca^{2+} for contraction is species dependent (Hoste et al, 1988; Horackova, 1986).

Currently, there are three theories regarding the mechanism of SR calcium release. These theories are: calcium-induced calcium release (CICR) (Fabiato, 1983), charge-coupled release mechanism (CCRM) (Lederer et al, 1989), and a cAMP-dependent release mechanism (Boller and Pott, 1989).

The most widely accepted theory for SR calcium release is CICR. This theory postulates that the influx of calcium through sarcolemmal L-type calcium channels raises intracellular calcium levels rapidly enough to trigger SR calcium release. Fabiato hypothesizes that the two components of I_{Ca} (see Sipido and Wier, 1989 for a recording showing the two components of I_{Ca}) play a major role in coupling the excitation event to contraction. He suggests that the fast component of I_{Ca} induces SR calcium release, whereas the slow component loads the SR with calcium for release on subsequent beats. Inactivation of CICR is proposed to be caused by the large increase of free Ca^{2+} resulting from SR calcium release inhibiting further release (i.e. negative feedback).

An alternative theory is CCRM. According to this theory, charge movements resulting from a change in membrane potential (i.e. an action potential) can cause SR calcium release. The amount of calcium released should be proportional to the amount of charge movement during depolarization. Charge movements have been studied extensively in skeletal muscle (Melzer et al, 1986), and have recently been also studied in cardiac muscle (Field et al, 1988). Intramembrane charge movements may couple excitation to calcium release via a "linking protein" (Lederer et al, 1989).

A third hypothesis is that SR calcium release occurs by a cAMP-dependent mechanism. Boller and Pott (1989) have suggested that since one substrate (i.e phospholamban) for a cAMP-dependent protein kinase is localized in the membrane of the SR, then changing cAMP levels should alter SR function. Support for this hypothesis is based on the observation that calcium currents too small to trigger SR calcium release can evoke calcium release after augmentation of intracellular cAMP by a B-adrenergic agent (Boller and Pott, 1989).

The mechanism of CICR has received extensive experimental support (Fabiato, 1983; London and Krueger, 1986; Beuckelmann and Wier, 1988; Kentish et al, 1990). Recently, Kentish et al (1990) have shown with "caged Ca^{2+}" experiments that CICR plays a major role in cardiac activation. They also show that Ins(1,4,5)P_3 can induce calcium release, although this response is very small. Therefore, they suggest a role for Ins(1,4,5)P_3 in increasing calcium sensitivity of CICR, when [Ca^{2+}] are too low to produce SR calcium release. Nevertheless, there are some data that cannot be readily explained by the CICR mechanism (Cannell et al, 1987; Lederer et al, 1989).

1.1.3. *Sarcoplasmic Reticulum Calcium Release Channel*

The calcium release channel of the SR, which mediates calcium release in E-C coupling, has been isolated, purified, characterized with biological techniques and reconstituted into planar lipid bilayers to investigate its properties (Anderson et al, 1989). The SR Ca^{2+} release channel is a high conductance Ca^{2+} channel that is activated by micromolar Ca^{2+} and millimolar ATP (Anderson et al, 1989). The channel is an oligomer of four subunits (400-450 kD) of a high MW protein (Lai et al, 1988) that specifically binds ryanodine, a plant alkaloid which affects the SR calcium release channel in a dose-dependent manner (Rardon et al, 1989; Nagasaki and Fleischer, 1988). The SR calcium release channel and the ryanodine receptor are the same entity (Smith et al, 1988), and the cardiac ryanodine receptor is similar to the "foot" structures connecting the T-tubules and terminal cisternae of the SR in skeletal muscle (Anderson et al, 1989; McGrew et al, 1989). Recently, the ryanodine receptor has been shown to consist of two main parts: a C-terminal channel region and a large cytoplasmic region that corresponds to the foot structure (Takeshima et al, 1989). Homology between the ryanodine receptor and inositol 1,4,5-triphosphate receptor has also been shown (Furuichi et al, 1989). A tabulated summary of the presently known properties of the SR ryanodine receptor/Ca^{2+} release channel is provided in Lai and Meissner (1989). The SR calcium channel can be modulated by a number of drugs and compounds (see Table 2 in Fleischer and Inui, 1989).

1.1.4. *Calcium Uptake by the Sarcoplasmic Reticulum and Calcium Extrusion*

Once contraction has occurred, the cell must decrease $[Ca^{2+}]_i$. Two mechanisms are present for decreasing intracellular calcium: calcium uptake by the SR, and calcium

extrusion via the Na-Ca exchanger and sarcolemmal Ca-ATPase.

SR Calcium Uptake

Approximately 75% of calcium sequestration can be attributed to active calcium transport by the SR Ca-ATPase (Bers and Bridge, 1989). The ATPase is abundant in the SR membrane. It interacts with Ca^{2+} with high affinity (Km = 0.5 uM) (Carafoli, 1988), and transports Ca^{2+} with a stoichiometry of $2Ca^{2+}$:1ATP (Carafoli, 1988). This Ca-ATPase is regulated by an SR membrane-bound protein called phospholamban (Tada et al, 1975). Phospholamban can be phosphorylated at distinct sites by three different protein kinases (PK): a cAMP-dependent PK, a Ca^{2+}/Calmodulin-dependent PK, and a Ca^{2+}/Phospholipid-dependent PK (Simmerman et al, 1980). Phosphorylation of phospholamban by any of these PK increases the rate of active calcium transport by the SR (see Edes and Kranias, 1989 for review of phospholamban regulation). Very little is known about the molecular details of how phosphorylated phospholamban interacts with the Ca-ATPase to bring about calcium uptake. Several models have been suggested (see Fowler et al, 1989 for review of models).

Calcium Extrusion

Muscle relaxation is also mediated by two calcium extrusion systems located at the sarcolemma. These extrusion systems are the Ca-ATPase and Na-Ca exchanger. Each extrusion mechanism is conferred with different calcium transport capacities and calcium affinities (Caroni and Carafoli, 1981).

A) Sarcolemmal Ca-ATPase

The Ca-ATPase utilizes the free energy from ATP hydrolysis to extrude calcium. It interacts with calcium with a high affinity (Km < 0.5 uM), but transports calcium at a relatively low rate (Carafoli et al, 1983). Since the maximal transport rate of this

pump is too slow to contribute significantly to calcium efflux during relaxation (Sheu and Fozzard, 1982), it has been suggested that the Ca-ATPase plays its most important role in maintaining the 10^4-fold calcium gradient between the cytosol and extracellular medium during diastole. The Ca-ATPase transports calcium with a 1Ca:1ATP stoichiometry (Carafoli, 1988). A summary of some essential properties of the calcium pump is provided in Table 1 of Carafoli et al (1983).

B) Na-Ca Exchange

As mentioned earlier, approximately 75% of the decrease in $[Ca^{2+}]_i$ responsible for muscle relaxation is achieved by calcium uptake by the SR Ca-ATPase. Since the sarcolemmal Ca-ATPase is considered to play a minor role during relaxation, the remaining 25% of activator calcium is decreased by Na-Ca exchange (Bers and Bridge, 1989). The Na-Ca exchanger derives energy for the uphill transport of calcium from the inwardly-directed electrochemical Na gradient produced by the Na-K pump (Mullins, 1981). Hence, calcium regulation by Na-Ca exchange is sensitive to changes in both sodium concentration and membrane potential. The Na-Ca exchange system interacts with calcium at a lower affinity (Km: 1-5 uM) than the sarcolemmal Ca-ATPase, but transports Ca^{2+} with a much larger total capacity than the Ca^{2+} pump (Carafoli, 1988). The affinity of the exchanger for Ca^{2+} is increased by a calmodulin-dependent phosphorylation system (Caroni and Carafoli, 1983).

i. Stoichiometry

Direct measurements of Na-Ca exchange stoichiometry have been attempted by several investigators, implementing various techniques. A substantial proportion of these studies have utilized sarcolemmal vesicle preparations. Vesicle preparations allow manipulation of ionic compositions on either side of the membrane, while

minimizing compartmentation effects (Reeves and Sutko, 1979). However, such preparations may be contaminated with intracellular membrane components (i.e. sarcoplasmic reticulum membrane and/or mitochondrial membrane) (Sulakhe and St.Louis, 1980), and it is sometimes difficult to separate inside-out vesicles from outside-in vesicles (Philipson, 1985). The relationship of and comparisons between measurements obtained from vesicles with those from intact cells have been discussed by Philipson and Ward (1986).

Pitts (1979) provided the first evidence for a $3Na^+:1Ca^{2+}$ stoichiometry, using cardiac sarcolemmal vesicles for the coupling between ^{22}Na-influx and ^{45}Ca-efflux. Since then, several other investigators have also estimated a $3Na^+:1Ca^{2+}$ stoichiometry (Bridge and Bassingthwaighte, 1983; Reeves and Hale, 1984; Yau and Nakatani, 1984; Lagnado and McNaughton, 1988). A recent study (Schnetkamp et al, 1988) using bovine rod outer segments presents evidence for a variable Na^+/Ca^{2+} exchange ratio. In this preparation, K^+ is required to increase the driving force for calcium extrusion, and the exchange stoichiometry is $3Na^+:1Ca^{2+} + 1K^+$.

Stoichiometries of $4Na^+:1Ca^{2+}$ have been suggested by Mullins (1981) and Chapman et al (1981). From measurements of the decrease in $[Na^+]_i$ in guinea-pig or ferret trabeculae in Na-free solutions, Chapman et al concluded that intracellular Na levels decrease faster than expected from a $3Na^+:1Ca^{2+}$ stoichiometry. Recently, a stoichiometry of $5Na^+:1Ca^{2+}$ has been proposed based on data from synaptosomal membrane vesicles (Barzilai and Rahamimoff, 1987).

From the above discussion, it is evident that the stoichiometry of the Na-Ca exchanger has not been unequivocally defined. However, there is a consensus that the Na-Ca exchanger operates with a ratio greater than $2Na^+:1Ca^{2+}$, because a coupling

ratio of $2Na^+:1Ca^{2+}$ is inadequate to reduce intracellular calcium to diastolic levels known to exist in intact cells (Sheu and Fozzard, 1982). The consequence of a stoichiometry that is greater than 2:1 is that each countertransport event via Na-Ca exchange is associated with the net movement of at least one positive charge. This implies that the Na-Ca exchanger is electrogenic.

Evidence for the electrogenic nature of the Na-Ca exchanger has been obtained in two ways:

(a) Indirectly
-from changes in membrane potential i.e. hyperpolarization or depolarization (Horackova and Vassort, 1979; Reeves and Sutko, 1980; Jacob et al, 1987)

(b) Directly
-by identification of the Na-Ca exchange current (Kimura et al, 1986; Mechmann and Pott, 1986; Kimura et al, 1987).

The reversal potential (i.e. the membrane potential at which the current changes direction, and there is no net flux of Na or Ca) for I_{NaCa} is given by (Mullins, 1981):

$$V_r = \frac{nV_{Na} - 2V_{Ca}}{n - 2} \qquad (1)$$

where,

V_{Na} and V_{Ca} are the equilibrium potentials for Na^+ and Ca^{2+}, respectively
n: stoichiometry

When the membrane potential (V_m) is more positive than V_r, calcium influx is favored (i.e. Na efflux); and when V_m is more negative than V_r, calcium efflux is favored (i.e. Na entry).

ii. Voltage Dependency

Because the stoichiometry is greater than 2:1, the Na-Ca exchanger is sensitive to changes in membrane potential. Therefore, the Na-Ca exchanger is capable of altering membrane potential (via its current); and is capable of altering its operation

in response to changes in membrane potential. The net response of the Na-Ca exchanger to membrane potential changes depends on $[Ca^{2+}]$ and $[Na^{+}]$, stoichiometry, and the activity of other calcium transport pathways (Philipson, 1985).

1.1.5. *Rat Muscle*

The experiments for this thesis have been performed on rat ventricular trabeculae. Although the above described mechanisms for E-C coupling are applicable to rat heart, rat heart uniquely manifests certain aspects of the E-C coupling process. For example:

1) the action potential duration in rat heart is short (about 80 ms) (Horackova, 1986; Schouten, 1985),

2) the contraction of rat heart relies primarily on SR calcium release (Bers, 1985),

3) rat ventricle displays rest-potentiation (Shattock and Bers, 1989; Bers, 1985; Schouten, 1985; Wohlfart, 1982),

4) peak force is nearly maximal at almost all stimulation frequencies when $[Ca^{2+}]_o$ = 2.5 mM (Schouten, 1985); whereas in other species, saturation generally occurs at higher $[Ca^{2+}]_o$ (Allen and Kurihara, 1980).

Using this animal model to study the E-C coupling process may reveal other important elements in the process that have not yet been identified from studying other species.

1.2 Use of the Force-Interval Relation to Probe Excitation-Contraction Coupling

Bowditch (1871) first described the influence of stimulus frequency on contractile strength in heart; and drew attention to the fact that alterations in rate and pattern of stimulation can provide an intrinsic mechanism by which the heart can regulate its force development (see Figure 1.2). Modern concepts of this mechanism are founded

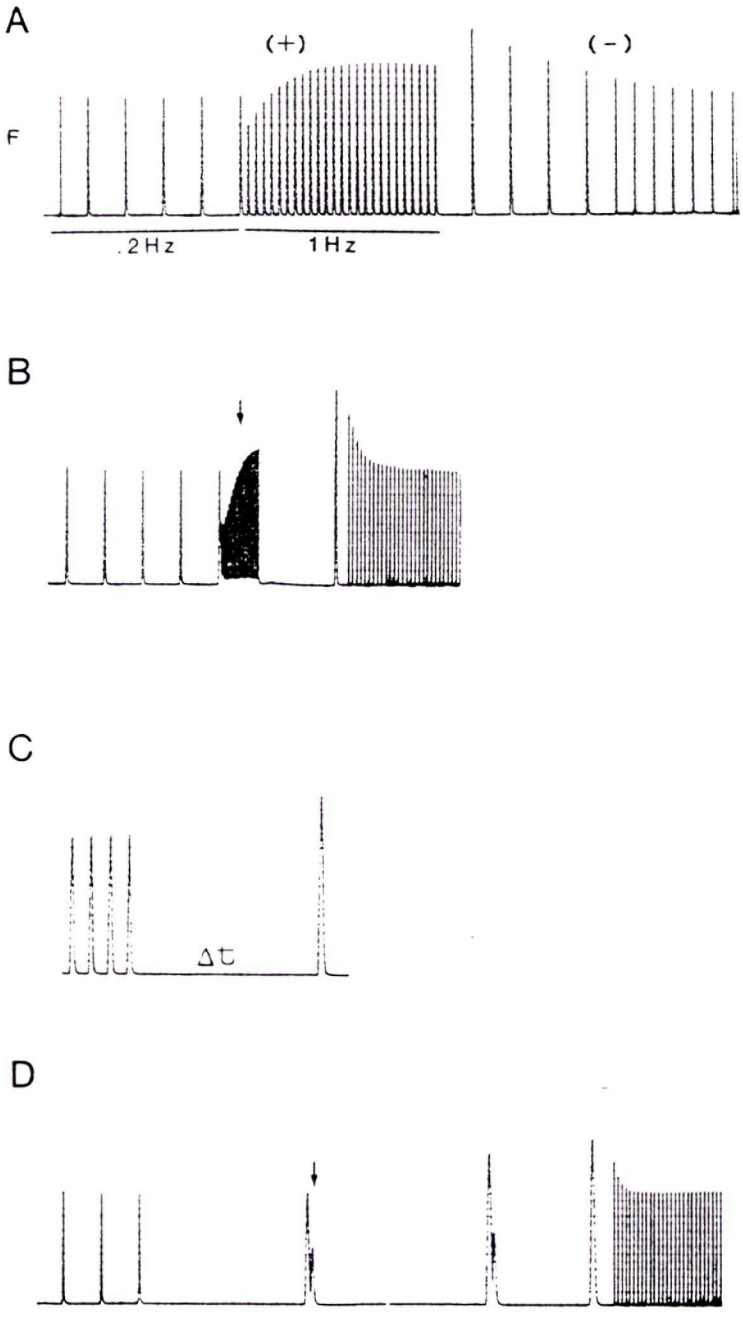
Figure 1.2. Recordings of four types of interval dependencies of force development.

A) Staircase. Increasing stimulation frequency, increases force (the '+' staircase), whereas decreasing stimulation frequency, decreases force (the '-' staircase).

B) Post-extrasystolic potentiation. Individual beats or a series of extrasystoles (arrow) are interposed within a regular stimulation pattern to enhance subsequent force. Potentiated force decreases exponentially to the same steady-state force after the extrasystole(s).

C) Mechanical Restitution (Force-Interval Relation). A test beat is measured after a variable period of rest that follows a regular pattern of steady-state stimulation.

D) Paired-pulse potentiation. One extrasystole (arrow) is paired with each twitch to potentiate subsequent twitches.


A
(+)
(-)
F
.2Hz
1Hz
B
C
Δt
D

upon theories of E-C coupling. Hence, studying the effects of altered rates and patterns of stimulation on force development provides a powerful method for investigating E-C coupling. One expression of this intrinsic mechanism is depicted by the force-interval relation. The force-interval relation (also known as mechanical restitution) is a plot of the amplitude of a test beat that follows a variable period of rest interposed within a steady-state stimulation pattern (see Figure 1.2, panel C).

1.2.1. *History*

The history of interval dependency on force development can be dated back to Bowditch's observation of a "staircase" phenomenon (see Figure 1.2, panel A), which is produced when heart preparations are exposed to changes in stimulation frequencies. The "typical" staircase pattern displays a rising staircase when stimulus frequency is increased, and a declining staircase when stimulus frequency is decreased. Rat myocardium is atypical because a declining staircase is observed with increasing frequencies (Hoffman and Kelly, 1959). One possible explanation for the unusual staircase pattern in rat is that there is inadequate oxygen supply to the tissue (Koch-Weser, 1963). Henry (1975) suggested that glucose deficiency is responsible for the negative staircase. In 1959 however, Hoffman and Kelly had already shown that inadequate diffusion of O_2 and/or deficiency of a substance necessary for contraction were not the reasons for the negative staircase. Schouten and ter Keurs (1986) suggest that the diameter of the preparation determines whether the force will be reduced at higher frequencies (1.0 Hz and greater). Another study states that the negative staircase pattern in rat can only be evoked by "special measures" (eg. adding ACh to the perfusion medium) (Meijler, 1962). It should be noted that Meijler used whole rat

heart preparations, which contrasts with the isolated rat papillary preparations used in the previously mentioned studies (i.e. Henry, Hoffman). Differences do exist between whole and isolated preparations (Wohlfart and Elzinga, 1982). A recent study (Orchard and Lakatta, 1985) using rat papillary muscle suggests that the SR is incompletely primed with calcium at high frequencies. Priming refers to the extent of calcium loading and recycling to the release site of the SR. By changing the priming rate of the SR with raised levels of $[Ca^{2+}]_o$ (8.0 mM), Orchard and Lakatta were able to nullify the negative staircase.

The notion that the strength of a contraction, irrespective of rate and pattern of stimulation, is the result of a cumulative interaction between two factors was introduced by Kruta and Braveny (1960). They proposed that gradual recovery of contractility increases with progressively larger rest periods (restitution), and is augmented by very short rest periods (potentiation). Blinks and Koch-Weser (1961) suggested that the strength of contraction is determined by the complex interaction between the production and decay of a negative (NIEA) and positive (PIEA) inotropic effect of activation; and that a rested-state contraction (RSC) represents a condition where the influence of previous activity has disappeared. Thus, the strength of contraction after any quiescent interval is given by:

$$\text{strength of contraction} = \text{RSC} + \text{PIEA} - \text{NIEA}$$

In contrast, Edman and Johannsson (1976) hypothesized that activation of a contractile response involves only a positive inotropic effect (PIEA), and the force-interval relation illustrates the time course for the development and disappearance of PIEA. The disappearance of PIEA results in a decrease in force in parts of the force-interval relation. Using rabbit papillary muscle, Edman and Johannsson (1976) showed

that the decay was biphasic, and suggested the involvement of two transport mechanisms for calcium extrusion. This inference is in agreement with modern excitation-contraction coupling models, which postulate the involvement of a Ca-ATPase and Na-Ca exchanger for calcium extrusion to the extracellular space (Wohlfart and Noble, 1982). Allen et al (1976) also noted a decay in their cat papillary "rest-decay curves", and attributed the decline to a progressive loss of calcium from the SR to the extracellular space, presumably by Na-Ca exchange. By increasing extracellular calcium or lowering extracellular sodium, they found the decay could be slowed. Schouten (1985) demonstrated a similar finding in rat ventricular trabeculae. Alterations in priming frequency can also affect the decay of the force-interval relation (Allen, 1976). When priming frequencies are increased, the "optimal contractile response" (OCR) is enhanced and the force-interval relation shifts upwards (Edman and Johannsson, 1976; Pidgeon et al, 1980; Schouten, 1985). The OCR defined by Edman and Johannsson in rabbit papillary muscles is similar to the plateau attained by the early recovery phase of the force-interval relation of rat myocardium. Paired-pacing has also been shown to increase the OCR (Elzinga et al, 1981). However, steady-state beats are minimally affected or slightly depressed when frequency is elevated (Pidgeon et al, 1982; Franz et al, 1983; Burkhoff et.al., 1984; Orchard and Lakatta, 1985). Augmentation of OCR by higher frequencies is attributed to increased $[Ca^{2+}]_i$, when calcium inflow is enhanced during the extended time the membrane spends depolarized (Edman and Johannsson, 1976; Elzinga et.al., 1981). Transiently, increased frequency causes,

$$\text{calcium influx} > \text{calcium efflux}$$

However, beat dependent efflux (via Na-Ca exchange and the calcium pump) increases

until influx and efflux are equalized (ter Keurs et al, 1987). A steady-state is then reached with the recirculating calcium fraction unaltered by the frequency perturbation (Ragnarsdottir et al, 1982).

The influence of muscle length on the force-interval relationship has also been investigated. Changing muscle length is an inotropic intervention (like altered bathing calcium or stimulus frequency) since the level of activation of the contractile system is dependent on muscle length (Lakatta and Jewell, 1977). Aequorin studies have shown that following increases in muscle length, $[Ca^{2+}]_i$ levels rise (Allen and Kurihara, 1982). The general finding is that changes in muscle length scale the force-interval relation, but do not alter its shape (Anderson et al, 1979; Pidgeon et al, 1980; Elzinga et al, 1981).

Changes in temperature are believed to profoundly influence the force-interval relation (Kruta, 1964). The inotropic effects of temperature are assumed (Mattiazzi and Nilsson, 1976) to arise from an alteration in:

1. the rate of calcium release into the myofibrillar space,
2. the duration of calcium release, and
3. SR sequestration of activator calcium.

Although a study by De Clerck et.al. (1981) examining the effects of decreased temperature (35-16°C) on skinned cardiac cells shows that force decreases when temperature is lowered, the general finding is decreased temperature (37-25°C) enhances force (Koch-Weser, 1963; Penefsky et al, 1972; Mattiazzi and Nilsson, 1976). This positive inotropic effect of cooling is attributed to slow SR sequestration of activator calcium (Mattiazzi and Nilsson, 1976) and decreased rate of calcium efflux via the Na-Ca exchanger (Reuter and Seitz, 1968).

The effects of certain drugs (eg. ouabain, pentobarbital, acetylstrophanthidin) on

the force-interval relation has also been studied (Hoffman and Kelly, 1959; Tuttle and Farah, 1962; Koch-Weser and Blinks, 1963; Koch-Weser et al, 1964; Anderson et al, 1977). For example, the effect of acetylstrophanthidin on the force-interval relation of kitten left atrial strips has been shown to increase the strength of contraction at all rest-intervals (Koch-Weser and Blinks, 1962).

1.2.2. *Models describing the Force-Interval Relation*

In the past twenty years, several mathematical models have been derived to describe the underlying mechanisms of the force-interval relation (Bautovich et al, 1962; Posner and Berman, 1967; Manring and Hollander, 1971; Edman and Johannsson, 1976). Essentially, these models postulate the presence of two compartments for the metabolism of activator calcium. However, certain findings have made it necessary to broaden the two-component model (Kaufmann et al, 1974; Adler et al, 1985). It has been shown that produced tension can exceed the steady-state force, suggesting extra loading of the "release-compartment" (RC) of the SR (Allen et al, 1976). Also, if the action potential is delayed for time intervals longer than the optimal time interval, (i.e the rest-interval that produces the maximal force, under a given set of conditions) the resulting decrease in force has been attributed to a loss of calcium from the RC (Pidgeon et al, 1982). The current model (Schouten et al, 1987) proposes the involvement of the Na-Ca exchanger to account for these phenomena.

1.2.3. *Force-Interval Relation of Rat Myocardium*

Recently, the force-interval relation of rat ventricular myocardium has been studied in detail by Schouten (1985). It is triphasic (see Figure 1.3), and Schouten has

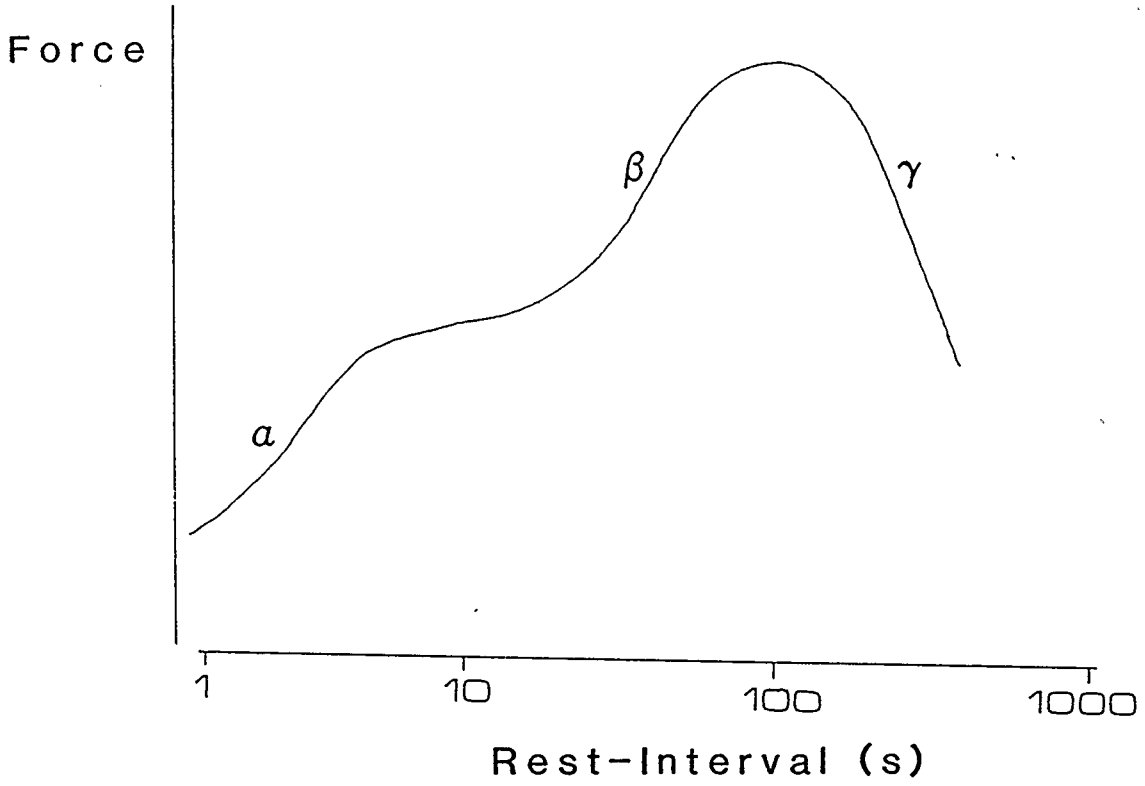


Figure 1.3. Illustration of the Force-Interval Relation from Rat Ventricular Myocardium. The three exponential phases of the relation have been labelled as alpha, beta, and gamma in the current mathematical model describing this relation. Phase alpha and beta correspond to rest-potentiation, and phase gamma corresponds to rest-depression.

suggested that three separate exponential processes are responsible for the overall recovery of force. These can be described as the sum of three exponential processes. The most rapid phase is completed within 10 seconds, a secondary, slower recovery of force occurs within 100 seconds ("rest-potentiation"), and the third phase is the decline of force that occurs when quiescent intervals exceed 100 seconds ("rest-depression"). The three exponential phases of the force-interval relation of rat myocardium have been described in a mathematical model of the force-interval relation (Schouten et al, 1987). This 6-parameter model postulates that three rate constants (α, β, γ), 2 influx quantities ($\Delta^u, \Delta^\varepsilon$), and a recirculating fraction (r) can characterize the rat force-interval relation. The parameters of the model have been related to a current view of E-C coupling in cardiac muscle published by ter Keurs et al (1987).

1.3 Ryanodine

Ryanodine is a neutral alkaloid obtained from the plant Ryania speciosa. Ryanodine has been used as a tool to assess the role of the SR in various responses:

- biphasic contractions (Bers, 1987),
- inotropic response to hypothermia (Shattock and Bers, 1987),
- spontaneous light intensity fluctuations (SLIF) (Lakatta et al, 1985),
- activating contraction (Marban and Wier, 1985).

Ryanodine is an excellent tool to study the role of the SR in heart preparations, since it selectively alters SR function without directly affecting: Na-Ca exchange, sarcolemmal Ca-ATPase, SR Ca-ATPase, myofilament sensitivity, or I_{Ca} (Sutko et al, 1985; Saxon and Kobrinsky, 1988).

Ryanodine has been shown to have two effects (Rousseau et al, 1987; Lattanzio et al, 1987). At low concentrations (nM-10 uM), ryanodine opens the SR calcium release channel and enhances passive SR calcium leak. At high concentrations (> 10 uM),

ryanodine locks the calcium release channel in a closed state and prevents SR calcium release. In addition to acting in a concentration-dependent manner, the effects of ryanodine are also time-dependent (Hansford and Lakatta, 1987) and species-dependent (Sutko and Willerson, 1980).

When stimulation is terminated, low concentrations of ryanodine deplete the SR rapidly without a transient rise of tension (Bers et al., 1987). Calcium efflux has been shown to increase in the presence of ryanodine (Hilgemann, 1986). Ryanodine does not, however, prevent the SR from accumulating calcium to allow for relaxation, and therefore the muscle relaxes (Bers and Bridge, 1989).

Ryanodine does not block calcium entry via sarcolemmal calcium channels, but may affect the inactivation of these channels (Wier et al, 1985; Mitchell et al, 1984). Ryanodine has also been shown not to affect the calcium sensitivity of the myofilaments (Fabiato, 1985).

Although the effects of ryanodine are irreversible (Wier et al, 1985), the attractiveness of this drug as a tool to investigate E-C coupling is based on the fact that ryanodine has a specific site of action (i.e. ryanodine receptor/SR calcium release channel) (Nagasaki and Fleischer, 1988). Hence, ryanodine allows for the role of the SR in a particular response or mechanism to be specifically investigated. For this reason, ryanodine was used in the experiments for this study.

1.4 Goal of the Study

The mechanisms underlying the force-interval relation are not completely understood. Studying the cellular basis for this relation may provide information about the E-C coupling process generally.

The aim of this thesis was to investigate one aspect of the force-interval relation: the role of the sarcoplasmic reticulum in rest-potentiation and rest-depression. Not all species display both phenomena (Bose et al, 1988). The experiments for this thesis were performed on rat ventricular trabeculae, which display both rest-potentiation and rest-depression in its force-interval relationship (Schouten, 1985). Rat heart was also used because it appears that the SR plays a major role in its E-C coupling process (Horackova, 1986; Sutko and Willerson, 1980).

1.4.1. *Hypotheses*

It has been shown that $[Na^+]_i$ exponentially decrease with a time-constant of a few minutes following an action potential (Cohen et al, 1982). Based on this finding, two hypotheses were proposed to explain the underlying mechanism for rest-potentiation and rest-depression in the rat force-interval relation. These hypotheses were:

1) when $[Na^+]_i$ declines, cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) will also decrease due to calcium efflux via the Na-Ca exchanger,

2) the amount of calcium in the SR is the net result of two $[Ca^{2+}]_i$-dependent fluxes across the SR membrane i.e. calcium sequestration by the SR calcium ATPase and passive calcium leak from the SR.

1.4.2. *Model*

These hypotheses were incorporated into a model (ter Keurs et al, 1987) describing rest-potentiation (RP) and rest-depression (RD) of the force-interval relation. The premises of this model are as follows:

a) as $[Na^+]_i$ decreases exponentially following stimulation, $[Ca^{2+}]_i$ also decreases because the steepening Na-gradient is favoring calcium extrusion via the Na-Ca exchanger,

b) the $[Ca^{2+}]_i$ governs the net calcium flux across the SR membrane i.e. calcium

sequestration or passive calcium leak,

c) RP is a manifestation of **net calcium entry** into the SR, when at relatively high $[Ca^{2+}]_i$ the rate of SR calcium sequestration exceeds the rate of SR calcium leak,

d) RD is a manifestation of **net calcium loss** from the SR, when $[Ca^{2+}]_i$ has declined to a level where the calcium gradient between the SR and cytosol steepens and passive SR calcium leak exceeds SR calcium uptake.

1.4.3. *Test*

To test the ideas presented in this model, ryanodine was used to shift the SR calcium flux towards net calcium loss, by enhancing calcium leak (Hunter et.al., 1983). It has been shown that the large, rapid loss of SR calcium following ryanodine treatment does not activate tension appreciably (Bers et.al., 1987). Thus, Bers and his colleagues suggest the primary calcium extrusion mechanism (i.e. Na-Ca exchange) is keeping up with the enhanced calcium leak, and decreasing $[Ca^{2+}]_i$. Therefore, the calcium gradient between the SR and cytosol will be steep, and passive SR calcium leak will exceed SR calcium uptake. The increased calcium leak from the SR should be apparent as an earlier onset of rest-depression, in comparison to control force-interval curves.

1.5 Relevance of the Study

It is important to understand the cellular basis of the force-interval relationship since it may provide information about E-C coupling. Furthermore, comparison of this relationship obtained from normal and diseased hearts could provide a method for identifying the dysfunctional cellular component(s) causing the disease. This knowledge can then be useful in selecting appropriate treatment paradigms.

CHAPTER 2

METHODS

2.1 *Muscle Preparation*

The experimental work for this thesis was performed on right ventricular trabeculae dissected from the hearts of Sprague-Dawley rats (200-300 g) anesthetized with ether.

There were three main reasons for using the rat as the animal model. The primary reason was that since the goal of this thesis is to study the role of the SR in the force-interval relation, and therefore gain further understanding of its role in E-C coupling, experiments were performed on a species (i.e. rat) that relies substantially on the SR for myocardial contraction. Also, since rat heart muscle displays several unique properties of tension development/recovery (see Chapter 1), it seemed worthwhile to determine whether these factors could supplement our current knowledge of E-C coupling. A third reason for using rat trabeculae was that the laboratory in which these experiments were performed (Dr. H. ter Keur's laboratory) has developed a detailed protocol for studying rat preparations.

Thin, unbranched trabeculae (2-4 mm long, 100-250 um wide, 40-70 um thick) attached between the tricuspid valve and free wall of the right ventricle were selected. Following excision, the rat heart was perfused with a modified Krebs-Henseleit solution and placed in a dissection bath. This perfusion medium was at room temperature, equilibrated with 95% O_2 + 5% CO_2 (pH: 7.35-7.45), and contained elevated $[K^+]_o$ (15-20 mM) to depolarize the heart, render it quiescent, and therefore allow for easier dissection.

Once dissected, the preparations were mounted in an experimental setup and

stretched to a sarcomere length of 2.1 um (to eliminate prominent effects of the series elastic component). The preparations were equilibrated at 1 Hz for 1 hour at 26°C, in control Krebs-Henseleit solution ($[Ca^{2+}]_o$ = 0.7 mM). Normally, an $[Ca^{2+}]_o$ of 1.5 mM would be used, however 0.7 mM $(Ca^{2+})_o$ was selected because it establishes optimal conditions for separation of the different phases of the rat force-interval relation (Schouten, 1985). During the equilibration period, the passive elements of the preparation relax, and therefore decrease peak twitch force. Thus, following the equilibration period, the muscle was restretched to 2.1 um and stimulated for 1 hour at 0.2 Hz. If the force was stable after this period, the preparation was used for an experiment.

2.2 *Solutions*

The solution used in these experiments was a modified Krebs-Henseleit (KH) medium of the following composition (in mM): Na^+, 140.5; K^+, 5.0; Cl^-, 127.5; Mg^{2+}, 1.2; $H_2PO_4^-$, 2.0; SO_4^{2-}, 1.2; HCO_3^-, 19; D-glucose, 10.0; Ca^{2+}, 0.7 (unless otherwise indicated). All chemicals were of the highest purity available (Analar grade, BDH Chemicals). The solution was equilibrated with a 95% O_2 + 5% CO_2 gas mixture (pH: 7.35-7.45). The KH solution for trabeculae dissection contained elevated external K^+ (15-20 mM) and was at room temperature; whereas the KH solution for superfusing the trabeculae in the experimental setup contained normal K^+ (5.0 mM), and was maintained at a temperature of 26°C. This temperature was selected since rat preparations are more stable at this temperature than at 37°C (Schouten, 1985); and there is minimal if any spontaneous activity observed. Furthermore, if the temperature is lowered below 26°C, then the efficacy of ryanodine has been shown to

decline (Shattock and Bers, 1987).

Ryanodine was a gift from J.L. Sutko. A 0.01 mM ryanodine stock was made and stored in 400 uL aliquots in the freezer. The amount of ryanodine added to the control KH perfusion solution depended on the ryanodine concentration desired.

2.3 *Experimental Setup* (see Figure 2.1)

The preparations were mounted horizontally in an experimental chamber (vol = 3ml) between a force transducer (AE801; Sensonor, Norway) and a stainless steel hook. The temperature in the chamber was measured with a teflon-coated thermocouple (Digi-Sense, 8528-20, Cole and Parmer) and maintained by a water bath (Mgw Lauda RM6). The perfusion rate of the chamber was approximately 5mL/min.

Viewing of the preparation was possible with an inverted microscope (Nikon Diaphot TMD) and a TV camera (Panasonic WV-1500) with video monitor (Panasonic TR-930UC). A permanent image of the preparation was obtained at the end of the experiment with a video printer (Mitsubushi, P50C).

2.4 *Sarcomere Length Measurement*

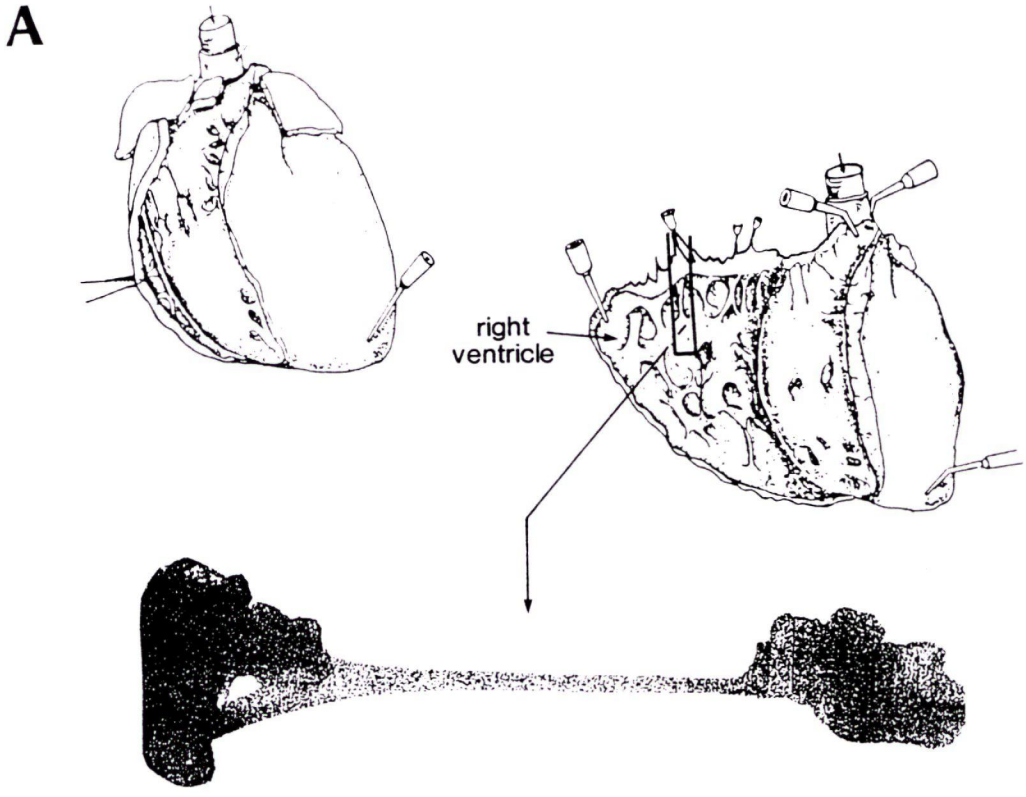
Sarcomere length (SL) of the preparations was measured using laser diffraction techniques. An extensive description of the technique is provided in Daniels et al (1984). Briefly, the striations of heart muscle act as an optical grating to incident light and diffract the light into zero-order and multiple higher-order band pairs. The angle between the zero and first-order bands can be related to SL by:

$$\sin \theta = \frac{k\lambda}{SL}$$

Figure 2.1. Illustration of preparation and experimental setup for measuring force-interval relation.

A) Heart from a Sprague-Dawley rat is removed and the right ventricle is opened. The preparation selected is a thin, unbranched trabecula connected between the tricuspid valve and free wall of the right ventricle.

B) The muscle is mounted horizontally between a force transducer and stainless steel hook in an experimental chamber positioned on the stage of an inverted microscope. A He-Ne laser is passed through the muscle and diffracted. The first-order band is scanned by a photodiode array and SL is computed electronically. A TV monitor provides for continuous viewing of the preparation.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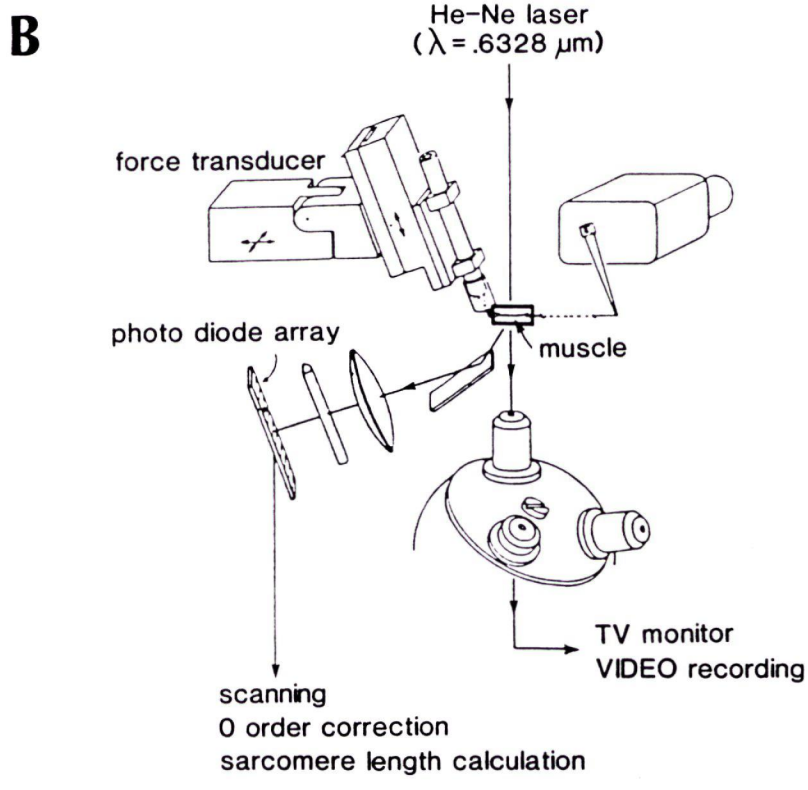

B
He-Ne laser
(λ = .6328 μm)
force transducer
photo diode array
muscle
TV monitor
VIDEO recording
scanning
0 order correction
sarcomere length calculation

A 5 mW He-Ne laser (Spectra-Physics # 105) beam (λ = 632.8 nm) is passed through the preparation to be diffracted into zero and first-order bands. One of the first-order diffraction bands is scanned by a photodiode array (Reticon RL256EC), and the median position of the first-order intensity distribution is used to compute the SL electronically. Correction is made for the zero-order band and scatter (Iwazumi and Pollack, 1979). The circuitry is calibrated with gratings at the beginning of each experiment.

It is advantageous to measure SL because it provides information on the shortening pattern, and also indicates damage of the preparation as manifested by spontaneous activity within the preparation and compliance at the ends of the preparation. Compliance occurs because the central region of the trabeculum is shortening at the expense of the damaged ends (damage due to dissecting and mounting the preparation), during an isometric contraction (ter Keurs et al, 1980). Compliance causes variations in sarcomere length. Variations in sarcomere length that exceed 25 % introduce an error in the measurement of peak force (Schouten, 1985). In summary, sarcomere length was measured in this study in order to monitor all of these variables.

2.5 *Force Measurement*

Twitch force was measured with a silicon strain gauge (AE801, Sensonor, Norway). A light weight basket (0.5 mm wide) was made from platinum wire (100 um diameter) and glued onto the tip of the silicon beam with epoxy. The basket was a cradle for mounting the right ventricular end of the trabeculum. The entire silicon beam was covered with silicone glue mixed with carbon powder and toluene, the latter for smooth application of the glue. This was done to ensure light and water tightness of the

transducer.

2.6 *Electrical Stimulation*

The preparation was stimulated (Grass SD9 stimulator) with a punctate electrode arrangement consisting of platinum electrodes (0.4 mm diameter) (A-M Systems, 7110) mounted along each side of the experimental chamber. The stimulus strength was 25% greater than threshold (usually about 9V), and the duration of the stimulus was 5 ms.

2.7 *Data Acquisition*

Twitch force and sarcomere length were displayed on a storage oscilloscope, and converted by an analog to digital converter (Datatranslation, 2801 A: maximum throughput 27 kHz) for storage into a personal computer (see Figure 2.2). Force and SL were also recorded onto a chart recorder (Gould, RS3400 - 4 channels).

2.8 *Force-Interval Relation*

The stimulus protocol for the force-interval curves consisted of a priming series (30 pulses, 0.2 Hz) that preceded randomized rest-intervals from 100 ms - 600 s (see Figure 2.3A); followed by a stimulus to trigger a test contraction. A priming frequency of 0.2 Hz was selected in order to observe whether spontaneous activity would develop in each preparation. If spontaneous activity was present, then the preparation was discarded. Also, ryanodine effects on force development are more noticeable at lower frequencies, since ryanodine enhances Ca^{2+} leak from the SR during diastole (Bose et al, 1988). The protocol was run by a computer program (see Figure 2.4) that triggered the stimulator and chart recorder and simultaneously stored peak twitch force and SL

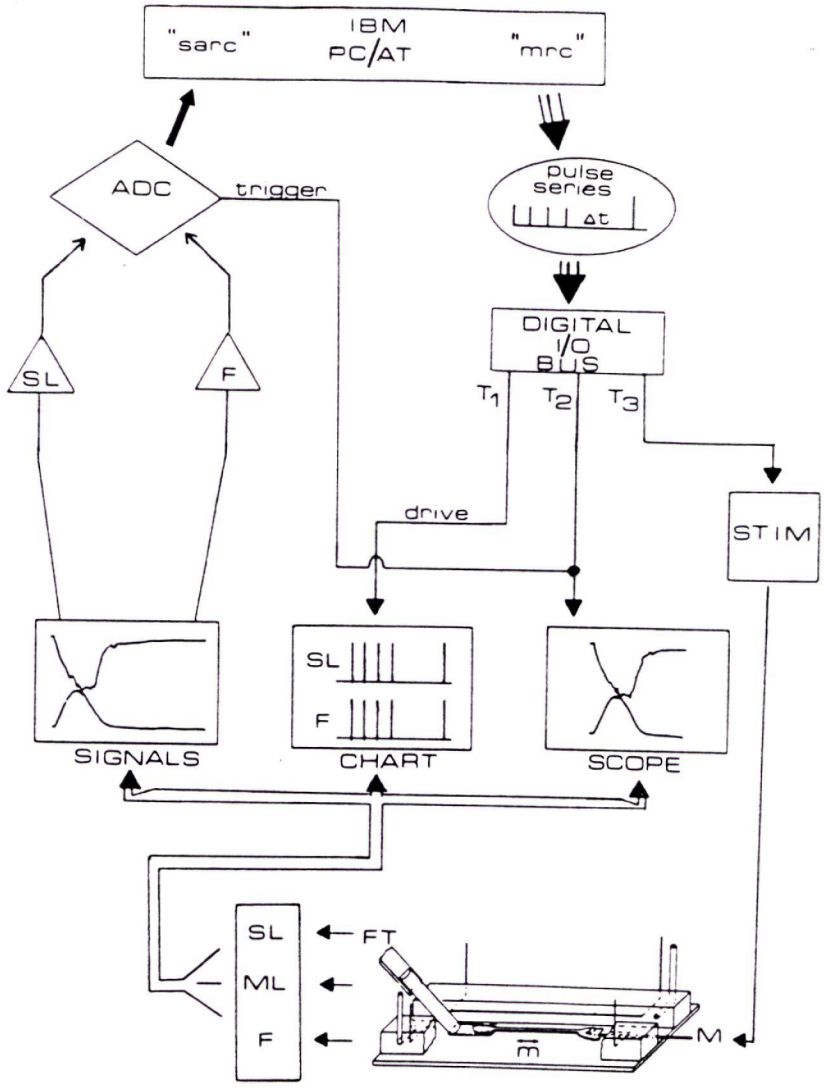


Figure 2.2. Illustration of the components of the computerized system for determination of the force-interval relation (mrc). From a digital input/output bus three separate triggers are sent to a stimulator, oscilloscope and chart recorder. Upon stimulation, force and SL signals are recorded and analog to digital converted for storage into a computer (sarc).

of the test contraction into the computer for subsequent data analysis.

Force-interval curves were obtained in the absence (control) and presence of ryanodine (0.1 - 10 nM). After the control force-interval relation was measured, the preparation was equilibrated at 0.2 Hz for 1 hour at a selected ryanodine concentration. During the equilibration period, both relaxation time (RT_{10}) and time to peak force (Tpeak) were continually measured and stored in the computer. RT_{10} and Tpeak were measured to determine whether ryanodine affected these twitch parameters. After 1 hour of ryanodine equilibration, the twitch force was stable and the ryanodine force-interval relation was obtained.

The peak force of the test contractions (in both control and ryanodine solutions) were normalized with respect to a potentiated force (F_{pot}) that was measured just before the force-interval relation stimulation protocol was initiated. Fpot for each preparation was determined in control KH solution by measuring the last beat in a train of 20 pulses at 4 Hz (see Figure 2.3B). Normalization was done to compare force-interval relations from different preparations.

2.9 *Staircases*

Staircase patterns were measured in both control and ryanodine KH solutions. Alterations in frequency between 0.2 Hz and 1 Hz elicited the staircase patterns.

2.10 *Dose-Response of Ryanodine*

Steady-state twitch force at 0.2 Hz, after 1 hour of superfusion at a given ryanodine concentration, was measured as a percentage of control twitch force (Ft). ED_{50} from this sigmoidal relation is 0.7 nM.

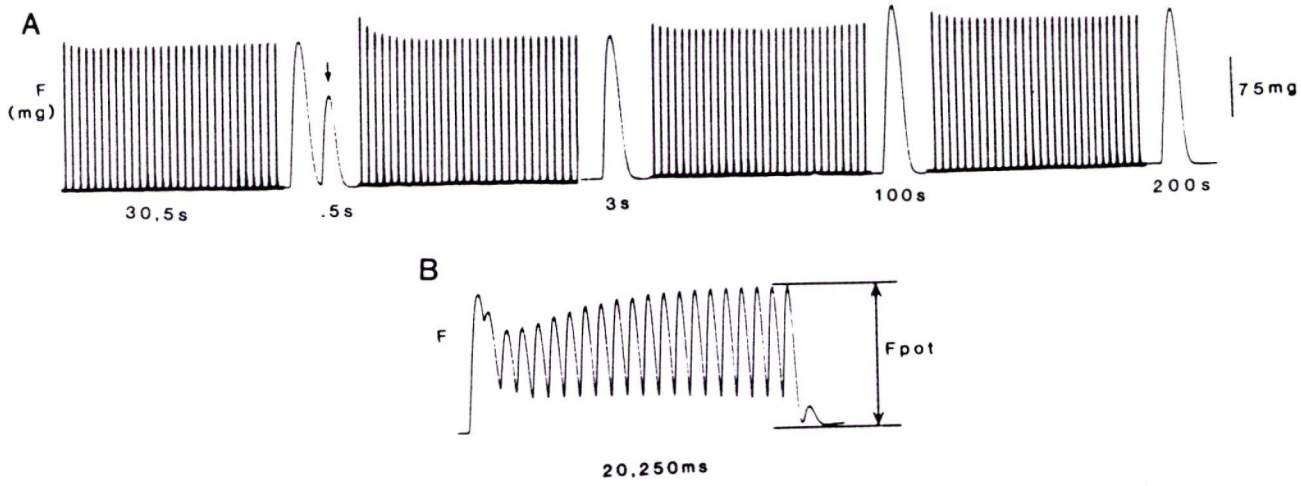


Figure 2.3. Twitches recorded for force-interval relationship and potentiated force (F_{pot}).

A) Recording of twitches measured for force-interval relation. A priming series of stimuli (30 pulses, 0.2 Hz) is followed by a variable period of rest (eg. .5, 3, 100, 200 seconds), and then a test contraction (arrow) is measured.

B) Twitches recorded during the F_{pot} protocol. The last beat in a train of 20 pulses at 4 Hz is measured. The small twitch at the very end of the train is a result of turning off the stimulator.

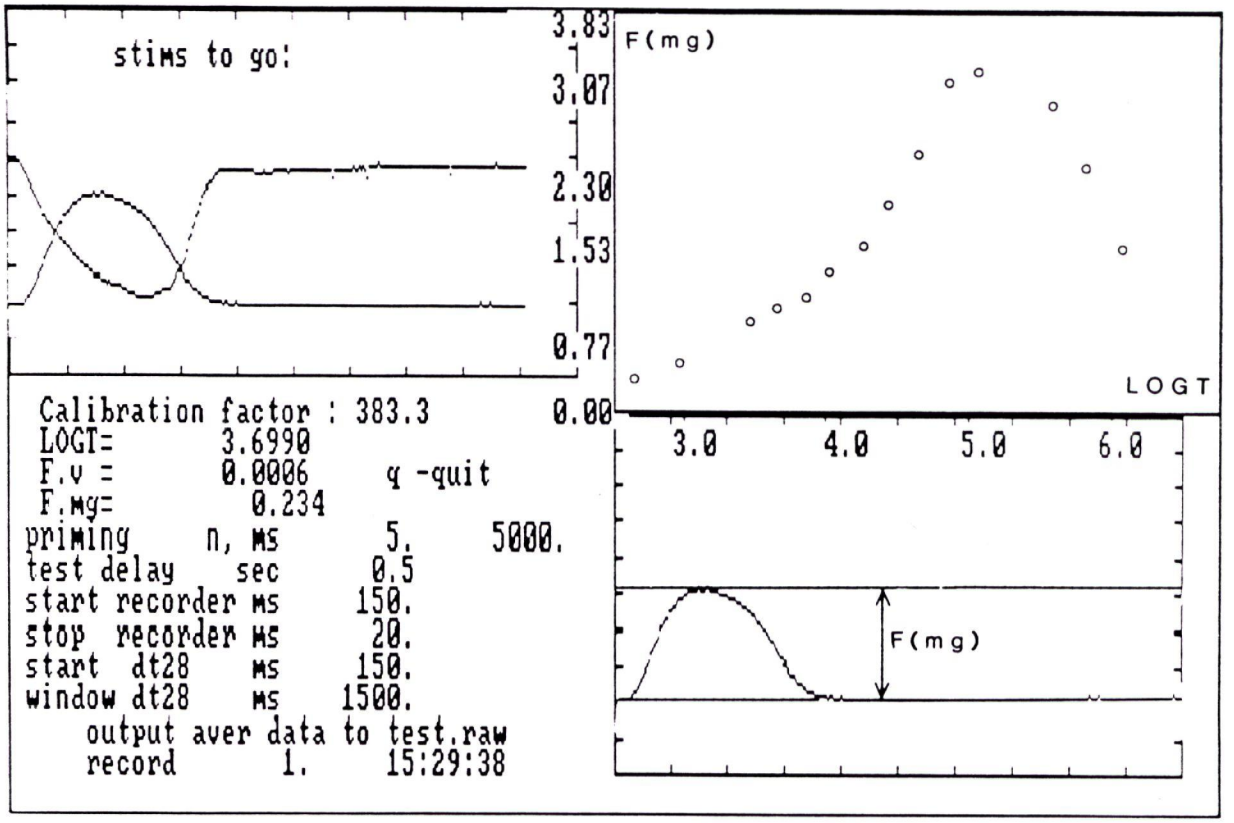


Figure 2.4. Graphics screen display of the program (mrc) for measuring force-interval relations.

Left, upper grid shows force (upward deflection) and SL (downward deflection) that are continuously monitored. Peak force (Fpeak) of test contraction is measured (right, bottom grid). Fpeak is plotted against the log of the rest-interval (Log T) for force-interval relation.

2.11 *Use-Dependence of Ryanodine*

In a recent paper, Sutko et al, (1986) suggested that the actions of ryanodine may be "use-dependent" (i.e. dependent on membrane polarization), since they found in rat papillary muscle that ryanodine's negative inotropic effect could be accelerated by elevated potassium levels. This observation is significant because it highlights the notion that experiments which study alterations in rate and pattern of stimulation using ryanodine must determine whether the preparation being studied responds to ryanodine in a use-dependent manner. Otherwise, analysis and interpretation of data could lead to inappropriate conclusions, because the fact that alterations of rate and pattern of stimulation do affect the electrical activity of the heart (i.e. resting potential, upstroke and repolarization phases of the action potential) (Boyett and Jewell, 1980) is not being considered. For this reason, it was determined whether rat ventricular trabeculae respond to ryanodine in a use-dependent manner.

In 10 nM ryanodine, the preparation was alternated between a stimulus frequency of 1 Hz and 0.2 Hz every other minute for 1 hour. The force was measured continuously throughout the 1 hour interval and compared with the forces from a preparation superfused with 10 nM ryanodine stimulated at a frequency of 0.2 Hz for the entire hour.

2.12 *Increasing* $[Ca^{2+}]_i$

To test whether elevated $[Ca^{2+}]_i$ could counteract the effects of ryanodine, two methods were implemented. The first technique was potentiation. In the presence of 10 nM ryanodine, the priming series (30 pulses, 0.2 Hz) was immediately followed by a train of stimuli (varied for each preparation) that potentiated force to the same

magnitude as control force. The rest-interval and the test beat then followed this potentiation period, and the peak force and SL of the test contraction was measured and stored. The second method applied to increase $[Ca^{2+}]_i$ was increasing $[Ca^{2+}]_o$ to 1.5 mM from 0.7 mM (Wier et al, 1985).

2.13 *Data Analysis*

Peak force (Fpeak) of the test contraction was measured and normalized with respect to Fpot of the preparation. For individual experiments, normalized force was plotted against the log of milliseconds of rest. The mean + /- SE of normalized force from several experiments was also plotted against the log of milliseconds of rest. Fitting of the data points was by Sigma-Plot regression.

CHAPTER 3

RESULTS

3.1 *Dose-Response Curve of Ryanodine*

Figure 3.1 shows the negative inotropic effect of ryanodine on steady-state twitch force. Panel A shows steady-state twitches (F) during 0.2 Hz stimulation obtained from a rat trabecula in control Krebs-Henseleit solution (the leftmost twitch), and one hour after ryanodine perfusion at .1, .5, 1, and 10 nM ryanodine (moving to the right). Lower traces of Panel A show the accompanying sarcomere shortening pattern (SL) for each twitch. In addition to indicating the extent of sarcomere shortening, the sarcomere shortening pattern also shows whether the resting sarcomere length has changed. This information is important because in rat ventricular trabeculae it has been shown that a change in resting sarcomere length affects twitch force (ter Keurs et al., 1987). Hence, if resting sarcomere length changes during ryanodine superfusion, it is not certain whether alterations in force are due to ryanodine or to a change in resting sarcomere length. For these reasons, sarcomere shortening was recorded. The lower traces of panel A show that the resting SL is constant (2.10 um), therefore force changes cannot be attributed to this factor. From these recordings, it is difficult to reach a conclusion about whether ryanodine affects the extent and/or duration of shortening.

Panel B shows a dose-response curve measured from steady-state twitches during 0.2 Hz stimulation, obtained after one hour of superfusion at a selected ryanodine concentration. The number of data points shown at each ryanodine concentration is the number of experiments which tested that particular ryanodine concentration.

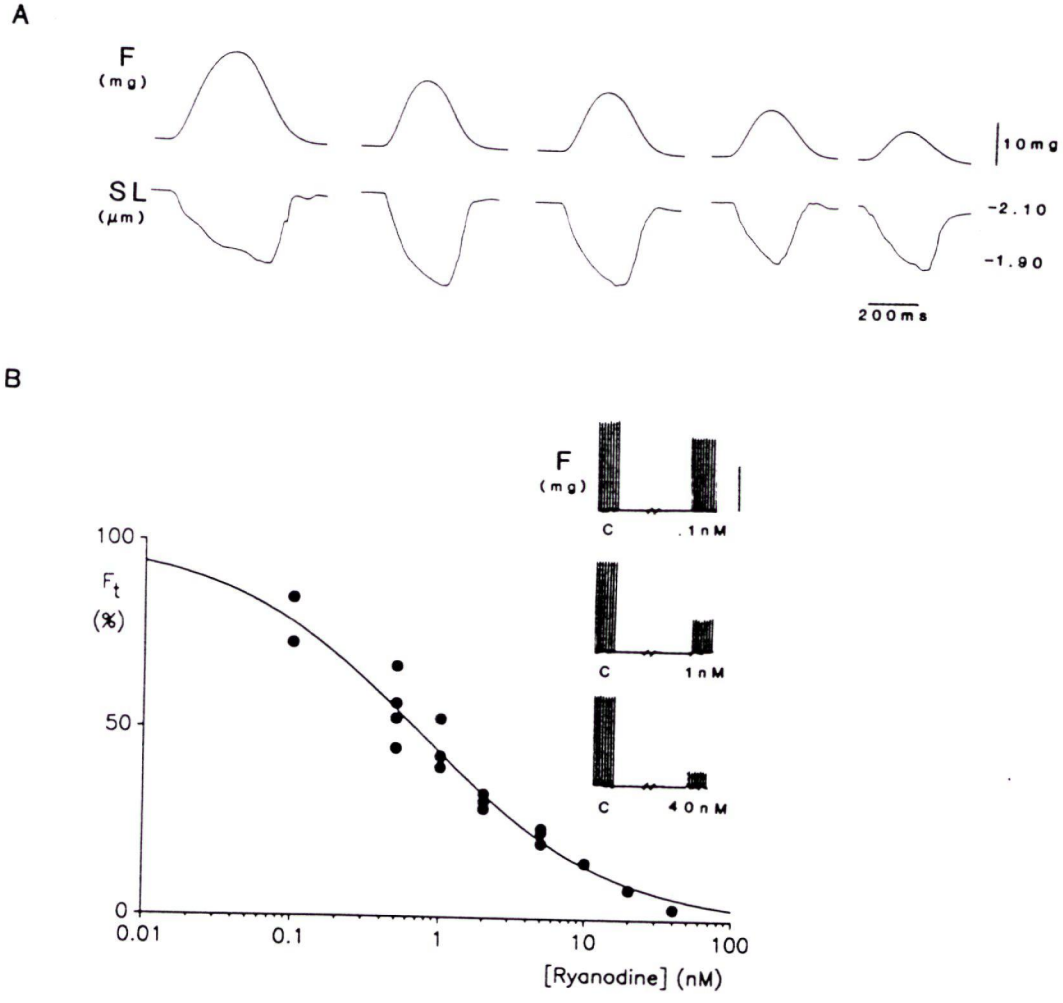


Figure 3.1. Negative Inotropic Effect of Ryanodine on Steady-State Twitch Force.

A) Individual steady-state twitches (F) and their respective sarcomere shortening patterns (SL) from a rat ventricular trabecula. Twitches were recorded at 0.2 Hz, with $[Ca^{2+}]_o$ = 0.7 mM. The twitch at the extreme left was recorded in control Krebs-Henseleit solution. Moving to the right, twitches in the presence of increasing ryanodine concentration (.1, .5, 1, 10 nM) are shown. All F and SL recorded at 50 mm/sec.

B) Dose-Response Curve of Ryanodine in Rat Ventricle Trabeculae. Steady-state twitch force at 0.2 Hz stimulation frequency and $[Ca^{2+}]_o$ = 0.7 mM, after 1 hour of superfusion at a given ryanodine concentration, was measured as a percentage of control twitch force (F_t). ED_{50} is 0.7 nM. The inset shows the protocol for dose-response measurement. In this protocol, a series of control twitches (C) at 0.2 Hz was followed by steady-state twitches after 1 hour at a given ryanodine concentration (eg. .1, 1, 40 nM). The paper speed for the inset recording was 10 mm/sec.

Twitch force in ryanodine was expressed as a percentage of control twitch force (F_t). The dose-response curve shows that steady-state twitch force is a sigmoidal function of ryanodine concentration, with an ED_{50} of 0.7 nM. At 10 nM ryanodine, steady-state twitch force decreased by 85 %. At 40 nM ryanodine, only 5 % of control steady-state twitch force remained.

3.2 *Staircase*

Figure 3.2 presents a series of twitches recorded from a rat ventricular trabecula when stimulation frequency alternated between 0.2 Hz or 1 Hz. Bowditch (1871) first observed the patterns of force changes arising from a change in frequency and named them staircases.

The left, upper recording shows the "typical" Bowditch staircase obtained in control Krebs-Henseleit solution. Increasing frequency from 0.2 Hz to 1 Hz depressed the first beat (left arrow) in the 1 Hz train. Force increased exponentially to a steady-state level within 10 beats. When frequency decreased from 1 Hz to 0.2 Hz, the first beat (right arrow) in the 0.2 Hz train was potentiated, thereafter force exponentially decreased to a steady-state level within 10 beats. When the trabeculum was superfused with 0.1 nM ryanodine (right, upper recording), the staircase pattern was not affected. The steady-state twitch force in this preparation did not show any decline in force during 0.1 nM ryanodine superfusion, unlike the preparations tested (n = 2) in Figure 3.1.

Increasing ryanodine concentration to 2 nM (lower, left recording) decreased steady-state twitch force, but did not change the staircase pattern. However, the first beat after an increase in frequency was depressed by only 20 %, in comparison to the

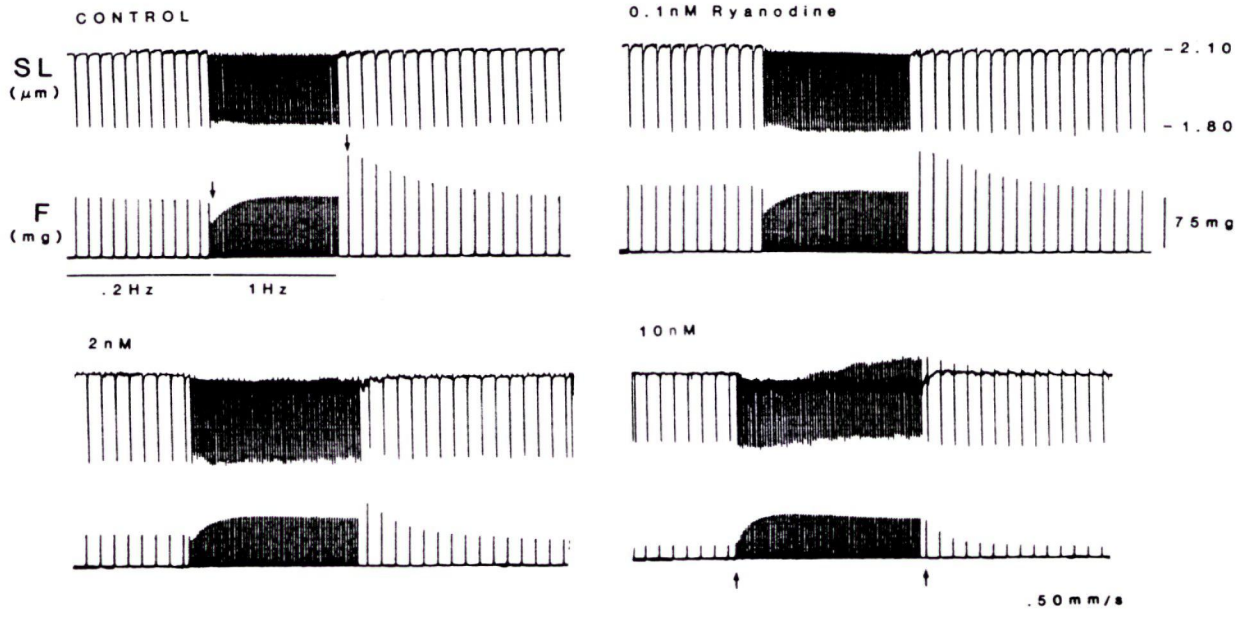


Figure 3.2. Staircases.

Force (F) of steady-state twitches at indicated frequency and respective sarcomere shortening (SL) were recorded in four different conditions (control and .1, .2, and 10 nM ryanodine). The frequency of stimulation was alternated between 0.2 Hz and 1 Hz. Arrows indicate first beat after a frequency change. All data were recorded at a paper speed of .50 mm/sec. Recording made from one preparation.

decrease of force by 38 % in control solution and 33 % in 0.1 nM ryanodine. The first beat after the decrease in frequency is potentiated by 29 % in 2 nM ryanodine, however the extent of potentiation is less than that measured in control solution (75 %) and 0.1 nM ryanodine (65 %). In addition, at 2nM ryanodine the recovery of force at 0.2 Hz stimulation, following a short duration of 1 Hz stimulation, was incomplete. This effect is more evident in Figure 3.3.

When the preparation is superfused with 10 nM ryanodine (Figure 3.2 lower right panel), a depression of force following an increase in stimulus frequency was not observed (left arrow); and potentiation of force when stimulus frequency was decreased was also absent (right arrow). Steady-state force was further depressed at this higher ryanodine concentration.

The upper trace in each recording shows the sarcomere length change accompanying the force. The extent of shortening appears to be slightly decreased only in 10 nM ryanodine, although the force had already substantially decreased during 2 nM ryanodine superfusion.

3.3 *Use-Dependence of Ryanodine*

Use-dependent effects of ryanodine are shown in Figure 3.3. In Panel A, the protocol for demonstrating use-dependence is illustrated. In 10 nM ryanodine, the stimulation frequency alternated between 0.2 Hz and 1 Hz, with one minute periods at each frequency. Steady-state twitch force at 0.2 Hz did not completely recover after a period of 1 Hz stimulation (recall lower, right recording in Figure 3.2). A more striking example of this observation is presented in panel B.

A plot of the decrease of steady-state twitch force at 0.2 Hz during ryanodine

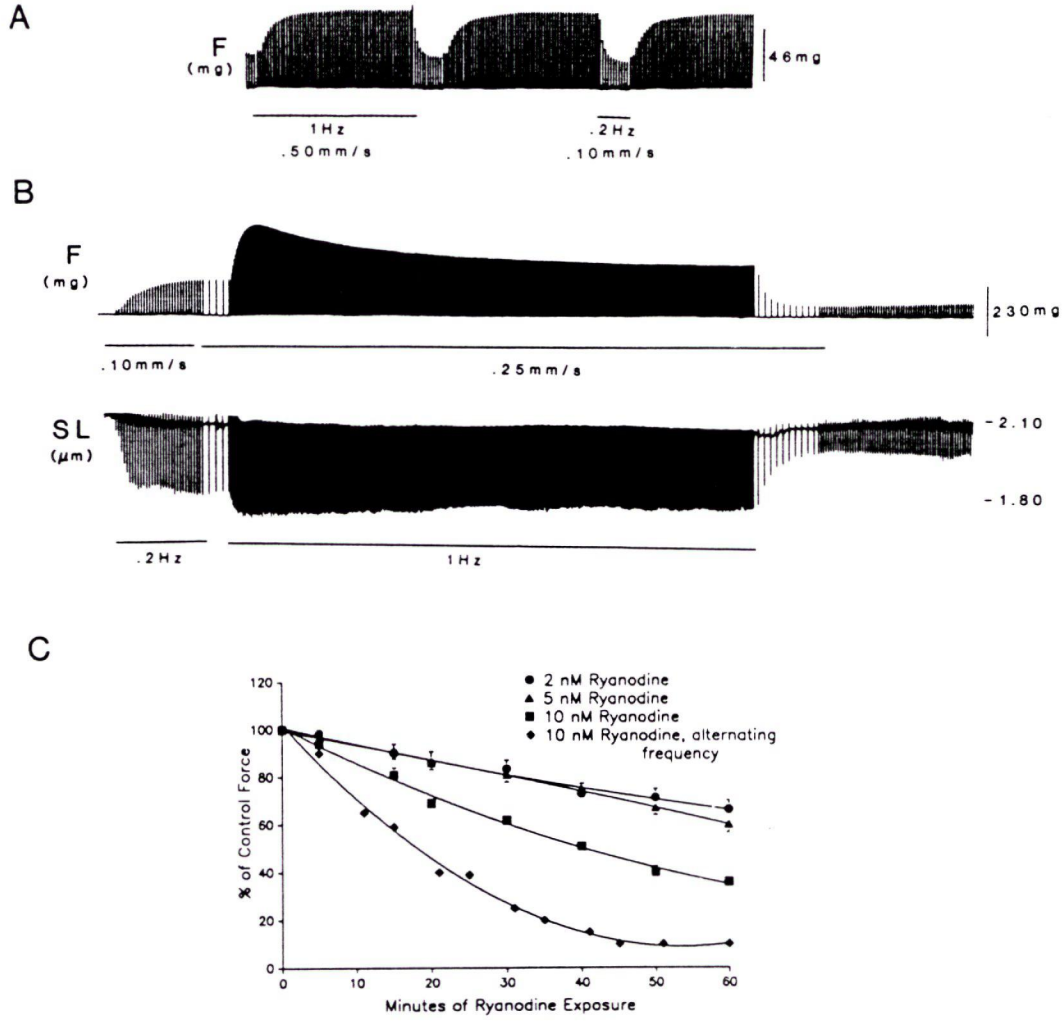


Figure 3.3. Use-Dependence of Ryanodine Effects.

A) Protocol for assessing use-dependence of ryanodine effects. The trabecula was exposed to alternating stimulus frequencies of 1 Hz and 0.2 Hz every other minute. A series of steady-state twitch forces (F) at a given frequency, during the 1 hour equilibration period of 10 nM ryanodine superfusion, is shown.

B) An example of incomplete twitch force recovery at a stimulus frequency of 0.2 Hz, following 1 Hz stimulation. Twitch force (F) and accompanying sarcomere shortening (SL) was recorded when stimulation frequency was alternated between 0.2 Hz and 1 Hz, in 10 nM ryanodine.

C) Plot of % of control twitch force as a function of time of exposure to ryanodine. Control force was 100 %, and force at a given ryanodine concentration was expressed as a percentage (mean + /- SE) of control force. 2 nM ryanodine (n = 5), 5 nM (n = 6), 10 nM (n = 9), 10 nM with alternating frequency (n = 1).

superfusion is shown in panel C. Steady-state twitch force in ryanodine is expressed as a percentage of control force. The rate of decrease in force (.0117 s^{-1}) is virtually identical in 2 and 5 nM ryanodine. At 10 nM ryanodine, the rate of decline of force is .025 s^{-1}. When stimulation frequency was changed between 0.2 Hz and 1 Hz every other minute, in the presence of 10 nM ryanodine, the rate of decrease of force is estimated (by taking the slope of the tangent at 50 % control force) to be .0472 s^{-1}. In addition, the declining force during 10 nM ryanodine with alternating frequency decreases in an exponential manner.

3.4 *Effects of Ryanodine on Relaxation time and Time to Peak Force*

The effects of ryanodine on relaxation time (RT_{10}) and time to peak force (T_{peak}) are shown in Figure 3.4. Individual steady-state twitches at 0.2 Hz, with comparable forces (force values indicated in the figure), in the absence and presence of 10 nM ryanodine are shown in panel A. T_{peak} was measured from 10 % active force to peak force (F_{peak}). RT_{10} was measured from peak force to 10 % contraction. T_{peak} is slightly shorter in 10 nM ryanodine solution. With respect to the small twitches (i.e. F_{peak}: 169 mg or 149 mg), T_{peak} was 114 ms in control Krebs-Henseleit solution, and 100 ms in 10 nM ryanodine. In the larger twitches (i.e. F_{peak}: 399 mg), T_{peak} decreased from 135 ms in control solution to 116 ms in 10 nM ryanodine.

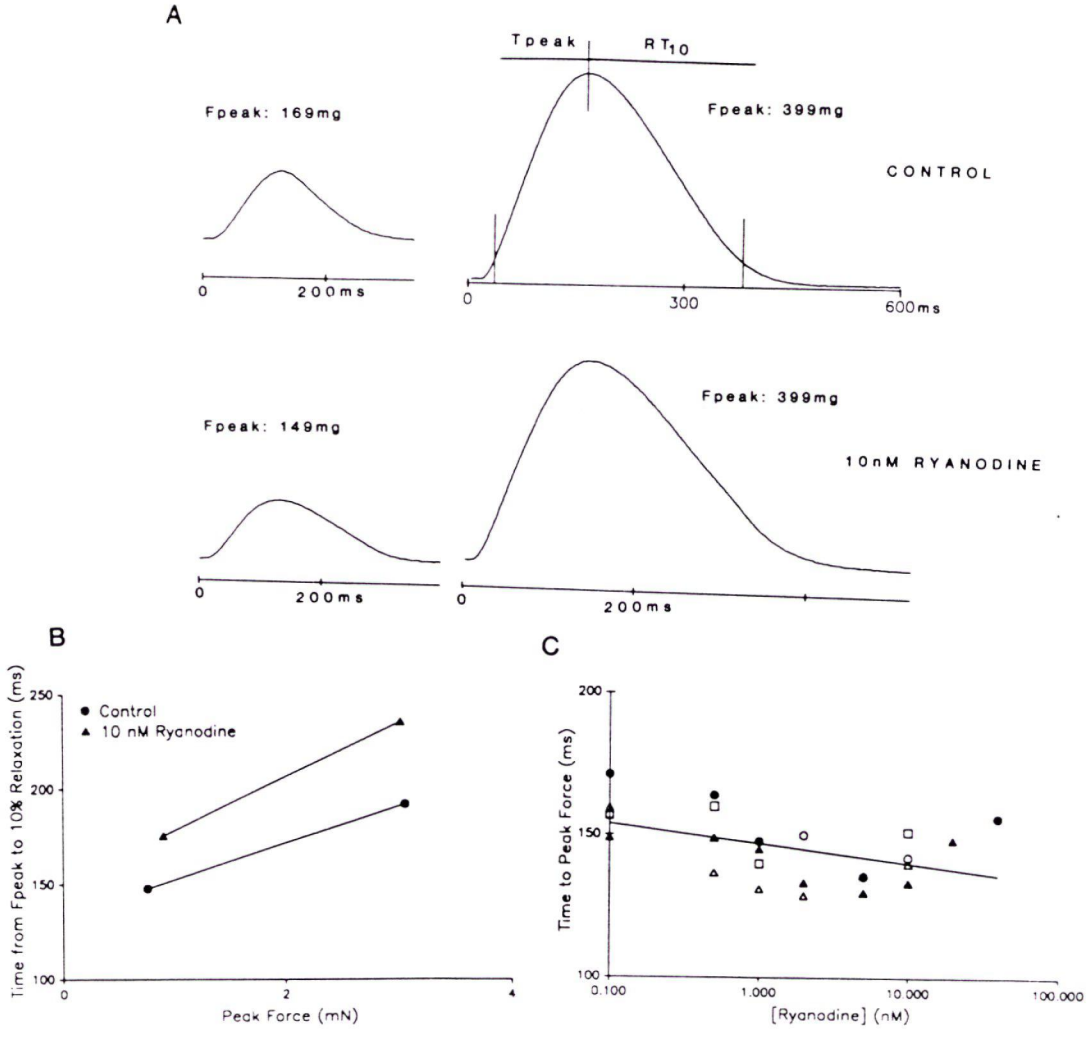
RT_{10}, however, increased in 10 nM ryanodine. RT_{10} of the small control twitch (F_{peak}: 169 mg) was 143 ms in control Krebs-Henseleit solution, whereas in 10 nM ryanodine RT_{10} was 171 ms. In the larger twitches, RT_{10} increased from 225 ms in control solution to 232 ms in 10 nM ryanodine. In panel B, RT_{10} is plotted as a function of peak force. Two points of the regression lines fitted through the data

Figure 3.4. Effect of Ryanodine on Relaxation Time (RT_{10}) and Time to Peak Force (T_{peak}).

A) Individual steady-state twitches at 0.2 Hz and $[Ca^{2+}]_o$ = 0.7 mM, with comparable forces (force values indicated) in the absence and presence of 10 nM ryanodine. The upper row twitches are from a rat ventricular trabecula superfused with control Krebs-Henseleit medium. The lower row of twitches have been recorded from a trabecula superfused with 10 nM ryanodine. T_{peak} is measured from 10 % active force to peak force. RT_{10} is measured from peak force to 10 % contraction. Please note the different time scales between the upper and lower row of twitches.

B) RT_{10} as a function of peak force in the absence and presence of 10 nM ryanodine. Two points of the regression lines fitted through the data points (from 5 experiments), obtained in both conditions, are shown. RT_{10} of steady-state twitches at 0.2 Hz, at three $[Ca^{2+}]_o$ (0.3, 0.7, 1.5 mM), was measured in control Krebs-Henseleit solution. At each $[Ca^{2+}]_o$, sarcomere length was progressively decreased from 2.15 um to 1.85 um (slack length), and the RT_{10} of each force at a given sarcomere length was recorded. RT_{10} with its respective force was then plotted and a regression line was fit through the points. In 10 nM ryanodine, RT_{10} of steady-state twitch force at 0.2 Hz and $[Ca^{2+}]_o$ = 1.5 mM, after 1 hour of ryanodine superfusion, was measured. Thereafter, $[Ca^{2+}]_o$ was titrated up and RT_{10} of the force at each $[Ca^{2+}]_o$ was measured. RT_{10} and its respective force was then plotted and a regression line was fit through the points.

C) T_{peak} as a function of ryanodine concentration. T_{peak} was measured from steady-state twitches at 0.2 Hz stimulation frequency and $[Ca^{2+}]_o$ = 0.7 mM, after 1 hour of superfusion at a given ryanodine concentration. The different symbols represent 5 individual experiments. A regression line has been fitted through the points.


A
Tpeak
RT10
Fpeak: 169mg
Fpeak: 399mg
CONTROL
0
200ms
0
300
600ms
Fpeak: 149mg
Fpeak: 399mg
10nM RYANODINE
0
200ms
0
200ms
B
Control
10 nM Ryanodine
Time from Fpeak to 10% Relaxation (ms)
250
200
150
100
0
2
4
Peak Force (mN)
C
Time to Peak Force (ms)
200
150
100
0.100
1.000
10.000
100.000
[Ryanodine] (nM)

points (from 5 experiments) obtained in the absence and presence of ryanodine are shown. From this plot, it can be seen that 10 nM ryanodine increased relaxation by approximately 15 %. This finding coincides with the small twitch data presented in panel A.

T_{peak} as a function of ryanodine concentration is shown in panel C. A regression line has been fitted through the data points (from 5 experiments). It appears that T_{peak} slightly decreased in the presence of ryanodine. This finding is also in agreement with the single twitch data in panel A.

3.5 *The Force-Interval Relation of Rat Ventricular Trabeculae in the presence of Ryanodine*

Force-interval relations measured in the absence and presence of ryanodine are shown in Figures 3.5 and 3.6. The normalized force was plotted as a function of the log of milliseconds of rest.

In Figure 3.5, the left, upper panel shows a scatter plot of twenty experiments. The upper and lower curves fit points from single experiments, whereas the middle curve fits the data points from all the experiments. These data show that in control Krebs-Henseleit solution, rest-potentiation (RP) of force occurred in two phases (see Figure 3.6 also). An initial plateau of normalized force was reached following one second of rest, and normalized maximal force was reached following 100 seconds of rest. Rest-depression (RD) was evident after 100 seconds of rest. When the trabeculae were superfused with ryanodine (2, 5, 10 nM), RD was detected after shorter rest-intervals (20, 3 and 1 second, respectively). In addition, normalized force decreased three-fold in the presence of ryanodine. The shape of the force-interval relation was also affected by ryanodine.

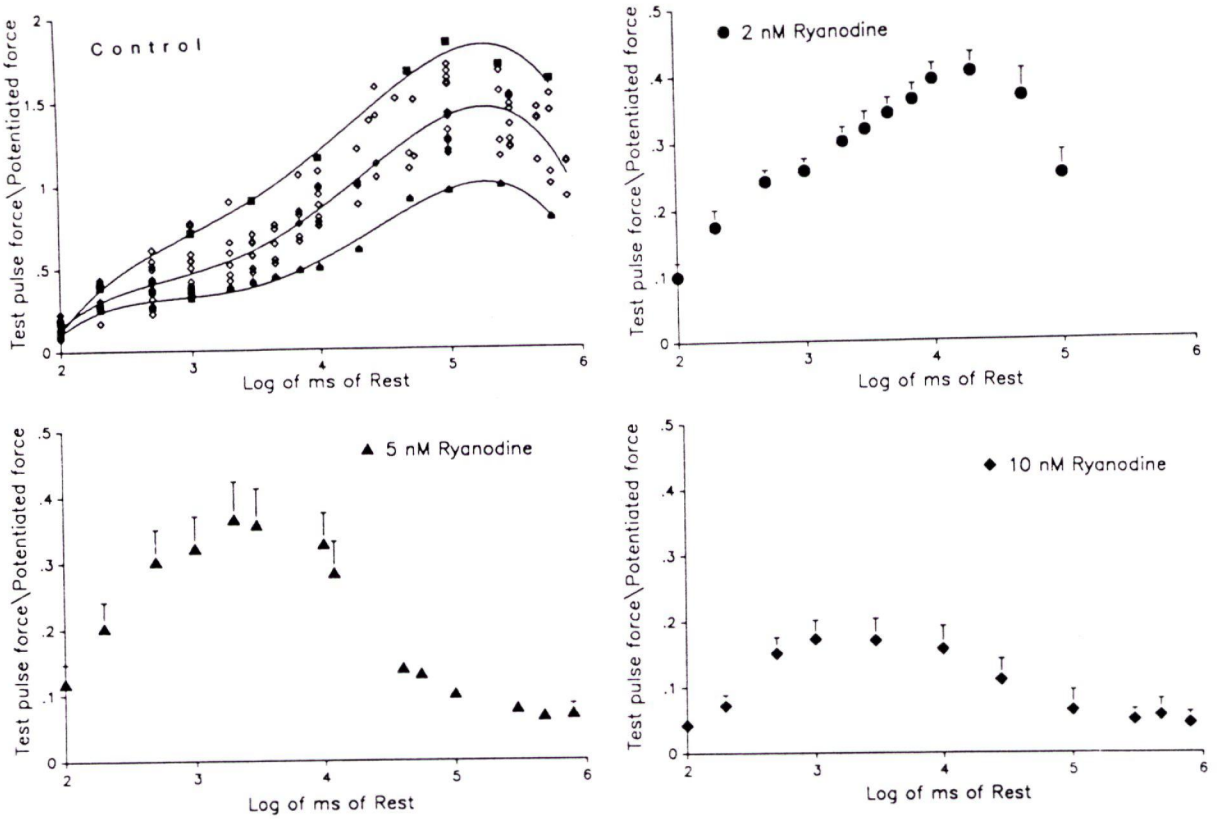


Figure 3.5. Force-Interval Relations in the absence and presence of ryanodine.

Normalized force was plotted as a function of the log of milliseconds of rest. The left, upper panel shows a scatter plot of 20 experiments. Three alternate fits of the points are shown. The upper and lower curves fit the data points from two experiments; whereas the middle curve is a fit of all the data points. The upper panel on the right shows the force-interval relation measured in the presence of 2 nM ryanodine (n = 5). Lower left and right panels show force-interval relations measured during 5 nM (n = 6) and 10 nM (n = 9) ryanodine superperfusion respectively. All data points in ryanodine are designated as mean + /- SE. $[Ca^{2+}]_o$ = 0.7 mM.

Figure 3.6 is a cumulative plot of normalized force as a function of the rest-duration in one preparation. These data show that increasing ryanodine concentration shifts the normalized maximal force leftwards, and decreases the magnitude of maximal force.

3.6 *Counteracting Ryanodine Effects on the Force-Interval Relation with Increased* $[Ca^{2+}]_i$

Two different protocols were used to test whether increasing $[Ca^{2+}]_i$ could counteract the effects of ryanodine on the force-interval relation. The first protocol is shown in panel A of Figure 3.7. This protocol is referred to as a potentiation experiment. After the priming period (30 pulses, 0.2 Hz), a variable period of extrasystole potentiation was used to potentiate the steady-state force in ryanodine to the same magnitude as control steady-state twitch force. Following the potentiation period, there was a variable period of rest (Δt), and then a test contraction (TC) was elicited. Panel B shows the effects of the potentiation protocol on the force-interval relation. Potentiation scaled up the force-interval relation by two, i.e. normalized force was doubled at virtually all rest-intervals; however, the shape of the force-interval relation did not appear to be affected.

The second protocol for increasing intracellular calcium involved raising extracellular calcium concentration. Panel C shows the effect of increasing $[Ca^{2+}]_o$ from 0.7 mM to 1.5 mM in the presence of 10 nM ryanodine. Once again, force was enhanced at all rest-intervals tested; however, the increase in magnitude of normalized force was less than that observed with the potentiation protocol. The shape of the force-interval relation did not appear to change.

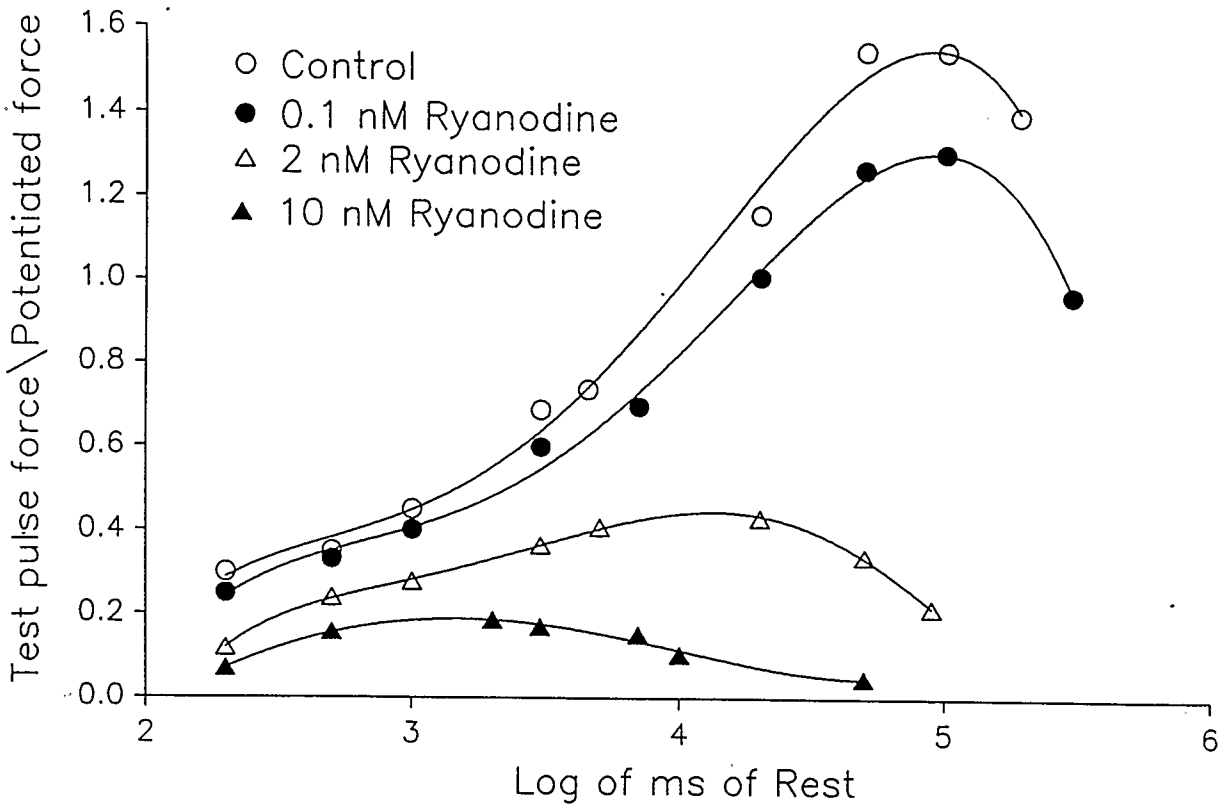


Figure 3.6. Cumulative force-interval relation of control and various ryanodine concentrations, measured in one trabecula. Normalized force was plotted as a function of the log of milliseconds of rest.

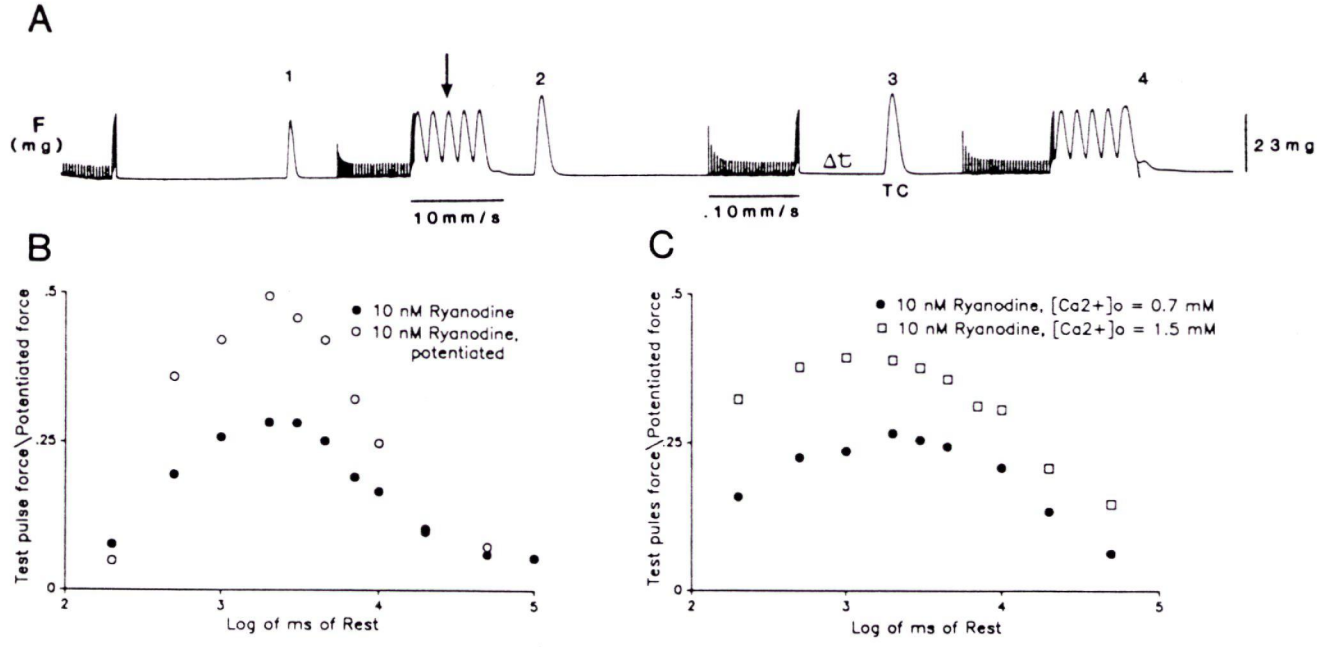


Figure 3.7. Counteracting Ryanodine Effects on the Force-Interval Relation with Increased $[Ca^{2+}]_i$.

A) Protocol used for potentiating force in rat ventricular trabeculae. The force of the preparation in 10 nM ryanodine was potentiated to attain the same magnitude of steady-state twitch force at 0.2 Hz in control solution. After the priming period (30 pulses, 0.2 Hz), a variable period of potentiation was inserted to potentiate ryanodine steady-state twitch force to control steady-state twitch force. The arrow indicates the last 4 beats of a potentiation train. Following the potentiation period, a variable period of rest (Δt) was inserted, and then a test contraction (TC) was elicited. Twitches 1, 2, 3, and 4 are test contractions following rest periods of 4.5, 1, 2, and .1 second, respectively.

B) Force-interval relation before and after the potentiation protocol. Normalized force was plotted as a function of the log of milliseconds of rest, when the preparation (n = 1) was or was not potentiated during 10 nM ryanodine superfusion.

C) Force-interval relation before and after an increase in $[Ca^{2+}]_o$. Normalized force was plotted as a function of log of milliseconds of rest when the preparation (n = 1) was superperfused with 10 nM ryanodine, at either $[Ca^{2+}]_o$ of 0.7 mM or 1.5 mM.

CHAPTER 4

DISCUSSION

4.1 Summary

In 1963, Koch-Weser and Blinks pointed out the usefulness of the pharmacologic approach for studying the mechanism of the force-interval relation. Since that time, a large number of investigators have used this approach (Koch-Weser et al, 1964; Anderson et al, 1977; Sutko et al, 1986; Pogessi et al, 1987; Bose et al, 1988). In this study, the drug ryanodine was used in an attempt to determine the role of the sarcoplasmic reticulum in the force-interval relation of rat ventricular myocardium. It was shown that the negative inotropic action of ryanodine is dose-dependent (Figures 3.1, 3.2, 3.5 & 3.6), and can be enhanced in a use-dependent way (Figure 3.3). The twitch parameters, time to peak force (T_{peak}) and relaxation time (RT_{10}), were affected by ryanodine (Figure 3.4). T_{peak} decreased in the presence of ryanodine, whereas RT_{10} increased. Furthermore, ryanodine completely inhibited the potentiated force of the first beat after a decrease in stimulation frequency, and it completely abolished the depressed force of the first beat recorded immediately after an increase in stimulation frequency (Figure 3.2). In addition, the rest-depression phase of the force-interval relation began after shorter rest-intervals in the presence of ryanodine (Figures 3.5 & 3.6). Elevating $[Ca^{2+}]_i$ counteracted the inhibitory effect of ryanodine on twitch force; however, the rest-interval required for maximal force development was not changed by increasing $[Ca^{2+}]_i$ (Figure 3.7). The results of this study are consistent with the proposal that leak of calcium from the sarcoplasmic reticulum may underly the rest-depression phase of the force-interval relation, and may modulate steady-state

twitch force parameters and characteristics of the Bowditch staircase.

4.2 Relation of the Findings in this Study to Previous Investigations of Rat Ventricle Force-Interval Relation

When changes in rate and/or pattern of stimulation are made, both electrical and mechanical activity of the heart are affected (Boyett and Jewell, 1980). These changes can be observed as alterations of action potential duration, developed force or both. Alteration of action potential duration is due to time- and voltage-dependent transmembrane ionic currents as well as changes in intra and/or extracellular ion concencentrations (Boyett and Jewell, 1980). In rat ventricle, action potential duration can strongly influence developed force (Schouten, 1985; Stern et al, 1988). The details of the relationship between action potential duration and force in rat heart depends on the phase of the action potential being considered (Schouten, 1985). Schouten (1985) showed that there is a negative correlation between action potential duration measured at the plateau and force; whereas there is a positive correlation between action potential duration measured at the late phase of the action potential and force. In rat heart, it has been shown that increasing stimulation frequency increases action potential duration, whereas decreasing stimulation frequency decreases action potential duration (Ragnarsdottir et al, 1982; Schouten, 1985; Pogessi et al, 1987; Johannsson et al, 1989). Although the effects of altered stimulation frequency on action potential duration have been well documented, the effect of altered stimulation frequency on on developed force is less clear. Some investigators have shown that increasing stimulation frequency decreases developed force (Hoffman and Kelly, 1959; Henry, 1975), whereas others have shown that increasing stimulation frequency increases force (i.e. "positive" staircase) (Henry, 1975; Schouten, 1985). The contradiction in these

findings has been attributed to the size of the preparation and the metabolic consequence, e.g. O_2 supply to the preparation (Schouten and ter Keurs, 1986). In the experiments for this thesis, I consistently observed a positive staircase pattern.

In this study, the role of the sarcoplasmic reticulum (SR) in force alteration as a result of rate and/or rhythm perturbation was investigated. The main hypothesis (called the "Ca^{2+} - leak hypothesis" in this study) of this study is based on the work of Cohen et al (1982), who suggested that the frequency-dependent effects on action potential duration and force can be attributed to $[Na^+]_i$ and its effect on $[Ca^{2+}]_i$, via Na-Ca exchange. Their underlying premise was that myoplasmic calcium $(Ca^{2+})_i$, which is dependent on $[Na^+]_i$, affects the amount of calcium in the SR by governing the net transmembrane calcium flux across the SR. The two unidirectional calcium fluxes across the SR membrane are calcium pump-mediated Ca^{2+} uptake and passive Ca^{2+} leak. The unidirectional flux that predominates in diastole will be dependent on $[Ca^{2+}]_i$, which is dependent on $[Na^+]_i$. Interventions which increase $[Na^+]_i$ (eg. increase in stimulation frequency or blocking the Na/K pump) should increase $[Ca^{2+}]_i$ and result in SR calcium uptake exceeding passive calcium leak. Hence, force should be enhanced. The converse should occur with interventions that decrease $[Na^+]_i$.

To evaluate this hypothesis, ryanodine was used to enhance SR calcium leak, and therefore partially deplete the SR of calcium (Sutko et al, 1986; Hansford and Lakatta, 1987). However, although the SR is losing its calcium, $[Ca^{2+}]_i$ actually decreases in the presence of ryanodine (Sutko et al, 1986). Hansford and Lakatta (1987) suggest that ryanodine causes calcium to leak from the SR slowly enough, so that sarcolemmal calcium extrusion mechanisms (i.e. primarily Na-Ca exchange) are able to extrude $(Ca^{2+})_i$, preventing it from rising significantly. This suggestion agrees with the findings

that resting tension does not increase (Sutko et al, 1986; Bers et al, 1987) and that spontaneous light intensity fluctuations (SLIF) decrease in the presence of ryanodine (Sutko et al, 1986; Kort and Lakatta, 1988).

Small doses of ryanodine have been shown to be very effective on rat heart muscle (Sutko and Willerson, 1980; Horackova, 1986). The dose-response data in this study confirm these findings.

Ryanodine shortens action potential duration in rat heart muscle (Shattock and Bers, 1987; Mitchell et al, 1984b), by decreasing the late phase of the action potential (Mitchell et al, 1984b) without shortening the plateau phase of the action potential (Saxon and Kobrinsky, 1988). In fact, the plateau phase can lengthen in the presence of ryanodine (Horackova, 1989). This observation can be explained with the finding that I_{Ca} is prolonged in the presence of ryanodine (Mitchell et al, 1984a; Mitchell et al, 1987), because inactivation of I_{Ca} is slowed (Mitchell et al, 1984a; Wier et al, 1985). The mechanism for the inactivation of I_{Ca} may be dependent on $[Ca^{2+}]_i$. Alternatively, perhaps, ryanodine binding to its receptor (i.e. the SR calcium release channel) mediates the slow inactivation of the sarcolemmal (SL) calcium channel via a hypothetical link protein, which connects the SL calcium channel to the ryanodine receptor (Lederer et al, 1989; Bers et al, 1990).

The effects of ryanodine on a single twitch, a series of twitches during frequency alterations (i.e. staircase), and a twitch following a period of rest (i.e. the force-interval relation) have been recorded in this study. The majority of these results can be explained by the Ca^{2+} -leak hypothesis, as will be described in subsequent sections of this Discussion.

4.2.1. *Single Twitch Parameters*

The effects of ryanodine on the twitch parameters time to peak force (T_{peak}) and relaxation time (RT_{10}) (see Figure 3.4) have been demonstrated previously in rat cardiac muscle (Sutko and Willerson, 1980; Hoste et al, 1988). Ryanodine has a minimal effect (i.e. slightly decreases it) on T_{peak} and prolongs RT_{10}. The minimal effect of ryanodine on T_{peak} can be explained by data showing that contractile filament sensitivity is not changed by ryanodine (Fabiato, 1985). If binding of calcium to tropinin-C were reduced (i.e. decreased sensitivity), then it would be expected that T_{peak} would increase rather than slightly decrease. Prolongation of relaxation by ryanodine has also been demonstrated in rat cardiac muscle (Sutko and Willerson, 1980; Tanaka and Shigenobu, 1989). This result is consistent with the Ca^{2+} leak hypothesis. Ryanodine-enhanced SR calcium leak reduces the net calcium uptake by the SR which is necessary for twitch relaxation. Therefore it slows the rate of $[Ca^{2+}]_i$ decline, resulting in prolonged relaxation. If ryanodine doses are low, such that the rate of SR calcium uptake can exceed the rate of ryanodine-enhanced SR calcium leak, then it is reasonable to expect that twitch relaxation will not be prolonged. Prolonged relaxation cannot be explained by ryanodine altering the functioning of the SR Ca-ATPase, the Na-Ca exchanger, or the sarcolemmal Ca-ATPase, since ryanodine does not significantly affect these systems (Sutko et al, 1985). Thus, it appears that SR calcium leak is the main ryanodine-sensitive variable that modulates twitch relaxation in rat ventricle.

4.2.2. *Staircase*

It has been shown in this study and previously (Hadju and Leonard, 1961) that ryanodine ([ryanodine] > 2 nM in this study) causes an immediate increase in force

when stimulation frequency is increased, and an immediate decrease in force when stimulation frequency is decreased. These observations differ from the "typical" staircase pattern (see Figure 3.2, upper-left panel).

The effects of ryanodine on the staircase pattern (see Figure 3.2) can also be explained by the Ca^{2+} - leak hypothesis (see Figure 4.1). Cohen et al (1982) have shown that stimulation increases $[Na^+]_i$. In addition, ion-sensitive microelectrode data show that diastolic intracellular sodium activity (a^i_{Na+}) is significantly higher in rat heart than in other species (eg. rabbit) (Shattock and Bers, 1989). When stimulation frequency is increased, $[Na^+]_i$ increases. The increased $[Na^+]_i$ slows the decrease of $[Ca^{2+}]_i$ via Na-Ca exchange (Cohen et al, 1982). Furthermore, action potential duration is increased (Ragnarsdottir et al, 1982; Schouten, 1985; Johannsson et al, 1989) and calcium influx via the sarcolemmal calcium channels is enhanced (Bers, 1985). This increase in $[Ca^{2+}]_i$ is eventually sequestered by the SR (Capagrossi and Lakatta, 1985), hence SR calcium uptake can exceed ryanodine-enhanced SR calcium leak, and therefore force increases. The decrease in force observed when stimulation frequency is decreased, may be due to the rate of ryanodine-enhanced SR calcium leak exceeding the rate of SR calcium uptake. In addition, there is less calcium influx at lower frequencies and more time between stimuli for leak to occur. The results of this study are consistent with the Ca^{2+} - leak hypothesis, and demonstrate that SR calcium leak can influence the staircase phenomenon.

4.2.3. *The Force-Interval Relation*

Wang et al (1988) have shown that $[Na^+]_i$ decreases exponentially when stimulation is stopped (see Panel A, Figure 4.1). The exponential decline in a^i_{Na+} has been

Figure 4.1. Model Describing Ca^{2+} Leak Hypothesis.

A) Left panel shows the time course of $[Na^+]_i$ decline (by the Na-K pump) once stimulation is stopped. Values plotted have been calculated from measurements made by Wang et.al. (1988).

Right panel shows the corresponding decrease in $[Ca^{2+}]_i$ (primarily by Na-Ca exchange) as $[Na^+]_i$ declines. All $[Ca^{2+}]_i$ values have been calculated with the following equation:

$$[Ca^{2+}]_i = [Ca^{2+}]_o * ([Na^+]_i/[Na^+]_o)^n * \exp [(n-2)VF/RT]$$

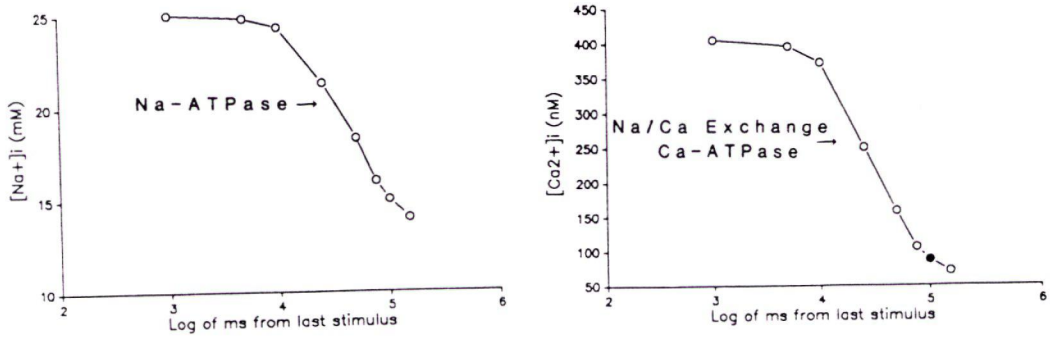
using the $[Na^+]_i$ values shown in the left panel and external ion concentrations used by Wang et.al. (1988).

B) Ca^{2+} Leak Hypothesis and Force-Interval Relation of Rat Ventricular Trabeculae.
The exponential decrease of $[Na^+]_i$ following stimulation affects $[Ca^{2+}]_i$ via Na-Ca exchange. $[Ca^{2+}]_i$ governs the net calcium flux across the sarcoplasmic reticulum (SR) membrane i.e. Ca-uptake or passive Ca leak. When quiescent intervals are less than 100 seconds, force increases (rest-potentiation) because $[Ca^{2+}]_i$ is stimulating the SR calcium pump such that the rate of SR calcium uptake surpasses the rate of passive SR calcium leak (U > L). Hence, there is **net calcium entry** into the SR. At approximately 100 seconds, a "critical" $[Ca^{2+}]_i$ is reached (via Na-Ca exchange calcium efflux) and shifts the net calcium flux across the SR membrane towards calcium leak. When quiescent intervals are longer than 100 seconds, the low $[Ca^{2+}]_i$ does not stimulate SR calcium uptake and the calcium gradient between the SR and cytosol is steep; therefore, the rate of SR calcium leak exceeds the rate of SR calcium uptake (L > U). Thus, there is **net calcium loss** from the SR and the force decreases (rest-depression).

C) Schematic of Rat Ventricular Myocyte.
At the sarcolemma, the Na-K ATPase, the Ca-ATPase, the Na-Ca exchanger are located. The Na-K ATPase maintains the resting membrane potential of the cell, whereas the Ca-ATPase and the Na-Ca exchanger extrude calcium from the cell. The sarcoplasmic reticulum (SR) and myofilaments (SARC) are located inside the cell. Across the SR membrane, there are two unidirectional calcium fluxes: Ca-uptake (Ca-ATPase) and Ca-leak.

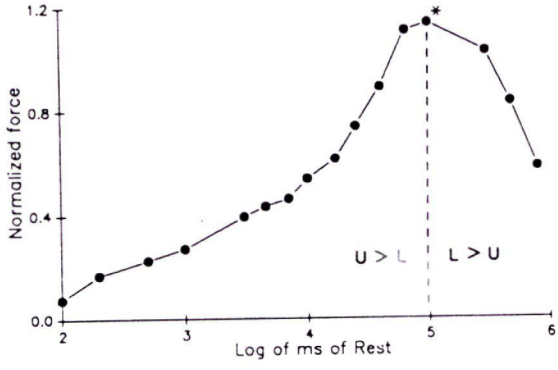
MODEL

A

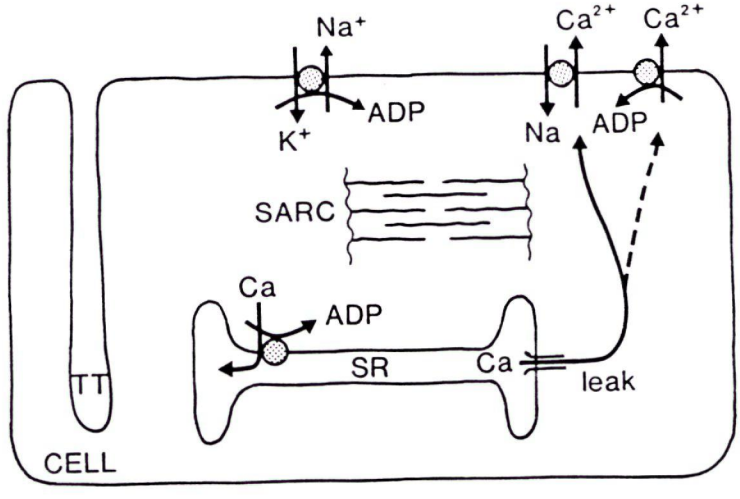


* critical $[Ca]_i$

B



C



attributed to Na^+ extrusion via the Na/K pump (Wang et al, 1988). In rat heart, periods of rest result in SR calcium loading, whereas stimulation leads to SR calcium depletion (Shattock and Bers, 1989). SR calcium loading during rest has been demonstrated with rapid cooling contractures (Bers, 1989) and is manifest in the rest-potentiation phase of the force-interval relation. The Ca^{2+} -leak hypothesis may therefore provide an explanation for SR calcium loading during rest, and hence rest-potentiation.

The rest-depression phase of the force-interval relation can also be explained by the Ca^{2+} - leak hypothesis. In control conditions, maximal force is attained after approximately 100 seconds of rest (see Figures 3.5 & 3.6). When rest-intervals last longer than 100 seconds, rest-depression occurs (see Figures 3.5 & 3.6). These findings can be attributed to the effects of exponentially declining $[Na^+]_i$ on $[Ca^{2+}]_i$. As $[Na^+]_i$ decreases, $[Ca^{2+}]_i$ also decreases (see Panel A, Figure 4.1) because the increasing electrochemical Na gradient promotes calcium extrusion via Na-Ca exchange. However, when rest-intervals are less than 100 seconds, $[Ca^{2+}]_i$ is high enough to strongly stimulate SR calcium uptake, and thus the rate of SR calcium uptake exceeds the rate of SR calcium leak (U > L) (see Panel B, Figure 4.1). Hence, force is increased (rest-potentiation). At approximately 100 seconds, a "critical" $[Ca^{2+}]_i$ is reached and the net SR calcium flux shifts from SR calcium uptake to SR calcium leak. As a result, when quiescent intervals last longer than 100 seconds, the rate of SR calcium leak can exceed the rate of SR calcium uptake (L > U) (see Panel B, Figure 4.1) because the $[Ca^{2+}]_i$ is too low to stimulate SR calcium uptake; since Na-Ca exchange has extruded $(Ca^{2+})_i$. Hence, twitch force is decreased (rest-depression) due to reduced SR calcium release.

The effect of ryanodine on the force-interval relation of rat ventricle can be explained by its enhancement of SR calcium leak (Hansford and Lakatta, 1987), decreases $[Ca^{2+}]_i$ (Sutko et al, 1986), which enables SR Ca^{2+} leak to override SR Ca^{2+} uptake after relatively shorter rest-intervals. As a result, rest-depression is observed following shorter rest-intervals. This effect of ryanodine on rest-depression is dose-dependent (see Figures 3.5 & 3.6) . The effects of ryanodine on the force-interval relation can be counteracted with interventions that increase $[Ca^{2+}]_i$ (Figure 3.7). However, the rest-interval after which maximal force is attained and the shape of the force-interval relation in ryanodine are not changed by the increased $[Ca^{2+}]_i$. The major effect of increased $[Ca^{2+}]_i$ on the force-interval relation is a simple scaling up of the curve. The results of this study are consistent with the notion that SR calcium leak underlies the rest-depression phase of the force-interval relation.

4.3 Limitations of the Study

One intrinsic limitation of all studies of this kind is whether the findings obtained from a particular animal model with selected experimental conditions can be related to other animal preparations and conditions. There were three main reasons for using the rat as the animal model for these experiments. The primary reason was that since the goal of this thesis is to study the role of the SR in the force-interval relation, and therefore gain further understanding of its role in E-C coupling, experiments were performed on a species (i.e. rat) that relies substantially on the SR for myocardial contraction. Also, since rat heart displays several unique properties of tension development/recovery (see Chapter 1), it seemed worthwhile to determine whether these factors could supplement our current knowledge of E-C coupling. A third reason

for using rat trabeculae was that the laboratory in which these experiments were performed (Dr. H. ter Keur's laboratory) has developed a detailed protocol for studying rat preparations. With respect to the experimental conditions, a $[Ca^{2+}]_o$ of 0.7 mM was used because it established optimal conditions for the separation of the different phases of the rat force-interval relation (Schouten, 1985). A temperature of 26°C was selected because rat preparations are more stable at this temperature than at 37°C. (Schouten, 1985); and very few, if any, spontaneous activity is observed at this temperature. Furthermore, the efficacy of ryanodine declines when temperature is lower than 26°C (Shattock and Bers, 1987).

In this study, mechanical measurements (i.e force) were made from rat cardiac trabeculae, without simultaneous electrophysiological measurements. This is a significant limitation, since electrophysiological and mechanical phenomena in the heart are interdependent (Boyett and Jewell, 1980). In addition, perturbations in rate and/or pattern of stimulation affect transmembrane currents which generate the action potential (eg. I_t). Therefore, action potential duration can be altered and this can affect developed force in rat ventricle (Schouten, 1985; Stern et al, 1988). Furthermore, the data in this thesis have been interpreted on the basis of electrophysiological changes which are thought to be occurring. It would have been preferable to record these changes during the experimental interventions.

A further limitation in this study arises from the lack of a specific Na-Ca exchange blocker. Since Na-Ca exchange is proposed to play an essential role in the Ca^{2+} - leak hypothesis, it would have been helpful if a drug or antibody were available that specifically affects Na-Ca exchange (just as ryanodine specifically affects the SR).

Despite these limitations, significant effects of ryanodine on a steady-state twitch,

a series of twitches after a change in stimulation frequency, and the force-interval relation were demonstrated. These results are consistent with a Ca^{2+} - leak hypothesis, which emphasizes the importance of sarcoplasmic reticulum calcium leak as a factor in modulating frequency-dependent changes of mechanical output in rat heart muscle.

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