Excitation-Contraction Coupling in Developing Mammalian Myocardium: Evidence from Voltage Clamp Studies

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ABSTRACT. The single sucrose gap voltage clamp technique was used to study excitation-contraction coupling processes in right ventricular papillary muscles from New Zealand White rabbits at various stages of development. In response to voltage clamp controlled depolarizations, muscles from newborn rabbits were found to exhibit a monotonically increasing tension response reaching a steady state level that was maintained for the duration of depolarization. In contrast, more mature myocardium responded to similar depolarizations by developing an early peak of tension before relaxing to a steady state level. Measurement of the ratio of early peak or phasic tension to steady state or tonic tension revealed a statistically significant increase in the phasic tension component with maturation. In addition, Ca^{2+} loading of immature myocytes via a conditioning voltage clamp step resulted in enhancement of phasic tension in subsequent test depolarizations. Finally, the voltage dependence of tonic tension was found to be the same in all age groups. In contrast, the voltage dependence of phasic tension, seen only in the more mature myocardium, differed from that of tonic tension. The results of this investigation suggest that tension development in the immature myocardium is supported largely by the influx of Ca^{2+} across the sarcolemma. As the myocardium matures, intracellular Ca^{2+} uptake and rerelease by the sarcoplasmic reticulum plays an increasingly important role in tension development. A developmental schema is presented to account for the observed maturational changes in excitation-contraction coupling. (*Pediatr Res* 23: 428-432, 1988)

Abbreviations

P/T, ratio of phasic to tonic

Myocardial contraction results from an increase in free myoplasmic calcium brought about by the combination of an influx of extracellular Ca^{2+} and the release of Ca^{2+} from internal stores (1-3). In the adult myocardium it has been estimated that the percentage of activator Ca^{2+} which is made available to the myofilaments via transsarcolemmal Ca^{2+} influx varies from 30 to 70% with the remainder supplied by release of Ca^{2+} from the sarcoplasmic reticulum (2). In addition, it has been shown that influx of Ca^{2+} from the extracellular space in adult myocardium

may be responsible, in part, for the replenishment of internal Ca^{2+} stores (2).

Recently, evidence has become available to suggest that immature myocardium relies largely on transsarcolemmal Ca^{2+} influx to provide the activator Ca^{2+} required to support contraction (4-7). In this regard the immature myocyte does not possess the well-developed t-tubular system and sarcoplasmic reticulum of the adult (7-11); myofibrils are less well organized and tend to lie closer to the sarcolemma than in the adult (9, 10); and mitochondria differ in configuration and occupy less total volume than in mature myocardium (8).

The single sucrose gap voltage clamp technique provides the opportunity to study intracellular Ca^{2+} regulation in a quantitative fashion. Using this technique it is possible to control the membrane potential while simultaneously measuring developed tension in cardiac tissue preparations (12). The membrane potential, rather than following the course of a normal action potential, may be rapidly increased to a predetermined level for a variable period of time. Thus, the level and duration of depolarization may be independently controlled, and the voltage and time dependence of tension generation studied. Accordingly, the purpose of the present investigation was to use the single-sucrose gap voltage clamp technique to evaluate the relative contribution of transsarcolemmal Ca^{2+} influx and release of Ca^{2+} from intracellular stores in the generation of tension in mammalian myocardium as it matures from newborn to adult.

METHODS

Preparation. Right ventricular papillary muscles 0.3-0.7 mm in diameter were dissected from New Zealand White rabbits from three age groups: newborns (0-10 days), juveniles (18-29 days), and adults. Muscles were placed in a three compartment, single sucrose gap chamber (Fig. 1). The tendinous end of the papillary muscle was fastened with fine suture to an isometric tension transducer in the physiologic chamber and the cut end was pulled through adjustable holes in the rubber membranes separating the larger outer compartments from the central chamber. The cut end was left loose in the outer chamber containing KCl-Tyrodes solution (KCl 128 M; KHCO_3 24 M). Typically 0.4-1.0 mm of the tendinous end of the muscle protruded into the physiologic compartment through which oxygenated tyrodes solution (NaCl 118 M; CaCl_2 1.0 M; glucose 6 M; MgCl_2 1.0 M; KCl 4.0 M; NaHCO_3 24 M; NaH_2PO_4 0.435 M) flowed at a constant rate (2-3 ml/min). Oxygenated, isosmotic sucrose (sucrose 280 M—high purity, Grade I, Sigma Chemical Company, St. Louis, MO) flowed at a rapid rate (2-6 ml/min) through the middle compartment. Preparations were stimulated at six shocks/min by passing 5 ms current pulses to raise the membrane potential to twice threshold and were allowed to stabilize in the experimental chamber for 45 min before the start of an experi-

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Fig. 1. Experimental chamber—a schematic representation of the single sucrose gap voltage clamp chamber is shown. A papillary muscle is attached by fine suture to the tension transducer and pulled through adjustable holes in the latex rubber membranes (dotted lines) separating the two outer chambers from the middle chamber. The length of the muscle in the physiologic chamber is adjusted using the micromanipulator. A glass microelectrode impales a cell in the center of the physiologic portion of the preparation and measures the intracellular membrane potential. This potential is fed through the voltage clamp circuitry that supplies the "clamping" current into the KCl chamber to hold the physiologic portion of the preparation at a predetermined clamp potential. The clamping current is forced by the insulating properties of the extracellular sucrose solution to flow intracellularly through the preparation in the sucrose "gap" and uniformly depolarize the physiologic portion of the preparation.

ment. The control tyrodes solution was equilibrated with 95% O_2 and 5% CO_2 , and all experiments were conducted at 20–23° C.

Stimulating or clamping current was applied to the preparation across the sucrose gap employing Ag/AgCl electrodes close to the muscle. The potential of the surface of the muscle in the physiologic compartment was held to ground potential by a separate circuit (virtual ground). Transmembrane potential was measured relative to virtual ground with conventional 3 M KCl filled glass microelectrodes connected to the input of a solid state negative capacitance preamplifier (World Precision Instruments). In voltage clamp experiments the output of the preamplifier was fed into the voltage clamp circuitry which supplied the necessary "clamping" current to the KCl pool. The insulating properties of the extracellular sucrose caused this current to flow intracellularly through the muscle in the sucrose compartment to uniformly depolarize the muscle in the physiologic bath.

Measurement of tension. Tension was measured using a highly stable, temperature insensitive, capacitive-coupled transducer (Dynagauge). A micromanipulator was used to adjust the length of the segment of muscle in the physiologic compartment to bring it close to the apex of its length tension relation. The segment of muscle bathed in sucrose was kept slack to prevent it from contributing to the measured tension. This arrangement allowed for some internal shortening of the myofilaments in the portion of muscle in the physiologic bath and, hence, the measured tension was very nearly, but not perfectly, isometric.

Measurement of current. The "clamping" current, representing the transmembrane current (plus small leakage currents), was measured by the virtual ground circuit. Current was recorded during all voltage clamp experiments as the current record provided a check on the homogeneity of voltage clamp control.

Approximately 40% of all papillary muscles met the criteria for good voltage clamp control. The validity and limitations of the single sucrose gap voltage clamp technique have been discussed previously (12, 13).

Statistical methods. Results within age groups were analyzed using paired *t* tests allowing each preparation to serve as its own control. Comparisons between age groups were made using the Student's *t* test for pairwise comparisons or analysis of variance to analyze changes across all three age groups.

Because of the variable size and taper of the physiologic portion of different papillary muscles, accurate normalization of tension measurements by weight or cross-sectional area was not possible. Therefore, statistical analysis was performed only on nondimensional ratios.

RESULTS

Comparison of the immature and mature tension response. Mature mammalian ventricular muscle responds to long, voltage clamp controlled depolarizations with a biphasic tension response (2, 14, 15). As seen in the bottom right panel of Figure 2, when a 2 s voltage clamp step to the plateau level was imposed on an adult right ventricular papillary muscle, the muscle first developed an early peak tension (phasic tension), which then relaxed to a lower steady state level (tonic tension). By comparison, the response of newborn myocardium to a long voltage

Fig. 2. The upper and lower left-hand panels show an action potential and the accompanying tension response in a newborn (top) and adult (bottom) papillary muscle. Note that despite the apparent similarity of the tension response in the two age groups, the tension response in the newborn begins to relax immediately on repolarization of the action potential, whereas in the adult, the peak of tension occurs after repolarization is complete. The response to a 2 s voltage clamp step to +10 mV is shown for the two age groups in the left-hand panels. Three traces are displayed in each panel. Membrane potential is taken from rest (−80 mV) to +10 mV by the voltage clamp circuitry. After a 2 s depolarization, the membrane potential is allowed to return to rest. The current trace (top of each panel) represents the net membrane current that flows during the clamp step. The line at 0 mV represents zero current flow (inward current is displayed as negative). The tension response is shown at the bottom of each panel. Note that the adult responds to the clamp step with both early peak (phasic) and steady state (tonic) tension whereas the newborn develops only tonic tension. The magnitude of tonic tension in the newborn is equal to that of the twitch tension while in the adult, tonic tension represents slightly more than half the twitch height. Also of interest is the fact, that in both age groups, the tonic tension component begins to relax immediately on repolarization.

clamp step was not biphasic; no early peak of tension was seen (Fig. 2, *top right panel*). Rather, tension increased monotonically to a final steady state level where it was maintained for the duration of the clamp step. This distinction between the mature and immature tension response was not evident in the twitch response accompanying an action potential which appeared very similar in the neonate and in the adult (*upper and lower left-hand panels* of Fig. 2). The only difference was that the peak of tension in the adult followed the rapid repolarization phase of the action potential, while in the newborn, tension began to relax immediately on repolarization. Comparison of the amplitude of the twitch and clamp-induced tension revealed that twitch tension and steady state tension were of the same approximate magnitude in the newborn. In contrast, in the adult, both twitch tension and phasic tension were larger than steady state tension.

Changes in the identified tension components with development. To delineate the changes that take place in the relative magnitude of phasic and tonic tension with development, long voltage clamp steps to the plateau level were imposed on papillary muscles from newborn, juvenile, and adult rabbits. In Figure 3 the original records of a typical experiment on a juvenile rabbit papillary muscle were combined with the records from the newborn and adult preparations shown in Figure 2. Note that phasic tension was absent in the newborn, present but small in the juvenile, and largest in the adult. In order to quantify the change in the relative contributions of these two tension components with age, a ratio of phasic to tonic tension was measured during a clamp step to the plateau level. This P/T ratio was measured in six newborn, five juvenile, and five adult rabbits. The newborn group showed little or no early peak tension ($P/T = 0.9 \pm 0.3$; mean $\pm$ SD); whereas phasic tension began to develop in the juvenile group ($P/T = 1.4 \pm 0.3$) and was maximal in the adults ($P/T = 2.6 \pm 0.6$). This increase in the P/T ratio with age is statistically significant ($p < 0.05$).

Effects of calcium loading. Experiments on adult mammalian myocardium suggest that the phasic component of tension results from release of a bolus of Ca^{2+} from the sarcoplasmic reticulum (2). Thus, a conditioning clamp step might load the sarcoplasmic reticulum with Ca^{2+} and result in an increase in phasic tension. A typical experiment in a newborn rabbit is shown in Figure 4. A train of action potentials was interrupted by two consecutive clamp steps near the plateau potential. The conditioning clamp step (*left panel*) was accompanied by a tension record that showed little or no phasic tension. However, the prolonged tension response that accompanied this conditioning clamp step indicates an increased intracellular Ca^{2+} load. The test clamp step, which immediately followed the Ca^{2+} -loading conditioning clamp step, was accompanied by an increase in the phasic

Fig. 4. The effect of a conditioning clamp step in the newborn—a train of stimulated action potentials is interrupted by two consecutive clamp steps to the plateau potential in a papillary muscle from a newborn rabbit. In response to the first depolarization (*left panel*) virtually no phasic tension develops. The prolonged tension response to this first voltage clamp step, however, indicates an increase intracellular Ca^{2+} load. This increase in intracellular Ca^{2+} is reflected in the subsequent clamp step by the appearance of a phasic component of tension (*right panel*). The phasic tension seen in response to the second clamp step is thought to result from the release of Ca^{2+} from functionally immature sarcoplasmic reticulum loaded with Ca^{2+} during the conditioning clamp step.

component of tension (compare *right and left panels*). Table 1 shows the results of this type of experimental protocol for five newborn and five juvenile rabbit papillary muscles. In both age groups the Ca^{2+} -loading conditioning clamp pulse significantly increased the phasic component of tension.

The voltage dependence of the identified components of tension. The voltage dependence of tension generation may be studied using the voltage clamp technique by repeating clamp steps, such as those shown in Figure 2, and varying the degree of depolarization by altering the clamp potential. Measurement of phasic and tonic tension at each clamp potential allows construction of a graphical tension-voltage relation for each component of tension (Fig. 5). The *top panel* of Figure 5 shows the tension-voltage relation for the tonic component of tension in a typical newborn papillary muscle. The voltage threshold for the development of tonic tension was between -50 and -60 mV. Above this threshold, tonic tension was a monotonically increasing function of membrane potential up to the highest potential tested. The *lower graph* of Figure 5 shows a typical tension-voltage relation for both the phasic and tonic tension of the adult rabbit papillary muscle. Of note is the fact that tonic tension showed a similar voltage dependence in the adult as in the newborn. Thus, tonic tension began to develop at -60 to -50 mV and had a monotonically increasing dependence on voltage up to the highest potential tested. However, in contrast to tonic tension, phasic tension increased with increasing membrane potential from -60 to 0 mV and then declined with increasing clamp amplitude.

DISCUSSION

Our experiments disclosed a fundamental difference between newborn and adult myocardium in response to voltage clamp steps. Neonatal myocardium developed no significant phasic tension in contrast to heart muscle from both juvenile and adult rabbits. In addition, the ratio of phasic to tonic tension increased with age. Ca^{2+} -loading of the newborn or juvenile myocardial cell via a conditioning clamp step increased the magnitude of phasic tension. Muscles from all age groups displayed tonic tension that was maintained for the duration of long (2 s) voltage clamp steps and relaxed immediately on repolarization. At all ages the relationship was similar between membrane voltage and final tonic tension. However, the voltage dependence of phasic

Fig. 3. Voltage clamp steps to the plateau level are shown for papillary muscles from newborn, juvenile, and adult rabbits along with the accompanying current and tension records. Note that the phasic tension is virtually absent in the newborn, present but small in the juvenile, and quite prominent in the adult. Tension bars to the right of the figure represent 60 mg for the newborn and 30 mg for the juvenile and adult. Zero current is represented by the bottom of the current bar to the right of the figure representing $10 \mu A$ for all current traces.

Table 1. Effect of Ca^{2+} loading on ratio of phasic to tonic tension in newborn and juvenile myocardium (mean $\pm$ SD)

P/T ratio	Control	Test
Newborn ($n = 5$)	0.9 ± 0.3	$1.6 \pm 0.3^*$
Juvenile ($n = 5$)	1.4 ± 0.3	$2.5 \pm 0.8^*$

* Differences significant at the $p < 0.01$ level.

Fig. 5. The relationship between tension and voltage is plotted for 2-s voltage clamp steps in typical newborn (top) and adult (bottom) papillary muscles. Tension-voltage relations are generated by varying the clamp potential (plotted on the X axis) and measuring the developed tension (plotted on the Y axis). Tonic tension displays a similar voltage dependence in the newborn and adult. Only the adult displays measurable phasic tension. Note that the voltage dependence of phasic tension seen in the adult is different from that of tonic tension.

tension, seen only in the mature myocardium, was different from that of tonic tension.

Phasic tension and the sarcoplasmic reticulum. The results of this investigation are consistent with the hypothesis that the contribution of the sarcoplasmic reticulum to excitation-contraction coupling in mammalian myocardium increases with age. Previous investigations have indicated that the phasic component of voltage clamp controlled tension is generated by release of Ca^{2+} from the sarcoplasmic reticulum triggered by depolarization (2). Other mechanisms of generation of the phasic component of tension, however, must be considered. Most importantly, tension records such as those reported herein might be generated

because voltage clamp control was not optimal at the beginning of long clamp steps. Escape action potentials within the physiologic segment of the papillary muscle might then cause the transient development of the twitch-like tension measured in this series of experiments. The measured increase in phasic tension with development, then, might reflect a decreased ability to achieve voltage clamp control in the more mature preparations. The Ca^{2+} -loading experiments provide evidence against this possibility. In these experiments, two identical voltage clamp steps are imposed sequentially on the papillary muscle (Fig. 4). Whereas the first produces little or no phasic tension in the neonate, the second produces a more adult-like response. There is no *a priori* reason to suppose that the voltage control of the second clamp step is less good than that of the first. It is therefore unlikely that clamp control is adequate in the conditioning clamp step but inadequate in the test clamp step, allowing escape action potentials and twitch-like tension. A more likely explanation is that the sarcoplasmic reticulum which is present, but not functional to any significant extent, in the newborn myocardium, is loaded with Ca^{2+} by the conditioning clamp step and that the release of this Ca^{2+} load during the test clamp produces phasic tension not seen under control conditions. Consistent with this explanation, the phasic tension of the juvenile myocardium is also increased by Ca^{2+} loading (Table 1). These results are consistent with the notion that the sarcoplasmic reticulum is present but functionally immature in the newborn rabbit. Under conditions of Ca^{2+} loading, however, the immature sarcoplasmic reticulum may play a role in excitation-contraction coupling.

Mechanisms tension generation. Our experiments indicate that, in all age groups, tonic tension is under direct control of the membrane potential, similar to the tonic tension component of amphibian ventricle that is supported by the transport of Ca^{2+} into the cell from the extracellular space (16). If tonic tension in mammalian myocardium represents transsarcolemmal Ca^{2+} influx, then the similarity between the tension voltage relation for tonic tension in the neonate and adult (see Fig. 5) suggests that the voltage dependence of the mechanism by which Ca^{2+} is transported into the cell does not change with age.

The relative contributions of transsarcolemmal Ca^{2+} influx and the release of Ca^{2+} from the sarcoplasmic reticulum has been evaluated by measuring the ratio of phasic to tonic tension. The increase in this ratio in response to Ca^{2+} loading has already been discussed. The ratio, however, also has been demonstrated to increase with development (Fig. 3), consistent with a maturational increase in Ca^{2+} sequestration and rerelease by the sarcoplasmic reticulum. In the immature myocardium, it would appear that Ca^{2+} transport across the sarcolemma from the extracellular space is the primary mechanism responsible for tension generation. Several observations support this notion. Importantly, the immature myocardium displays only tonic or membrane potential controlled tension in response to voltage clamp steps (Fig. 2). Further, only in the neonate was the steady state component of tension generated by a voltage clamp step to the plateau potential of sufficient magnitude to account for the twitch tension accompanying an action potential. In addition, influx of Ca^{2+} from the extracellular space as a mechanism of tension generation is well suited to the neonatal myocyte. Its smaller size (10) allows Ca^{2+} transported across the membrane to easily reach the interior of the cell. The subsarcolemmal arrangement of myofilaments further reduces diffusion distances for Ca^{2+} crossing the membrane to reach the contractile and regulatory proteins. Finally, if mitochondrial energy production advances with progressive maturation, it should be recognized that either diffusion or coupled transport of Ca^{2+} from the extracellular space into the cell does not directly require ATP.

Our results are compatible with a developmental scheme (Fig. 6) for excitation-contraction coupling in which immature myocardium relies on membrane depolarization to directly control Ca^{2+} influx and deliver Ca^{2+} to the myofilaments to activate contraction. With development, as the ability to store and rere-

Fig. 6. A model of excitation-contraction coupling is schematically depicted. The action potential as it propagates through the myocardium causes membrane depolarization and modulates Ca^{2+} influx and delivery to the myofilaments. This mechanism is thought to be the primary mechanism of excitation-contraction coupling in the immature myocardium. With development, as the t-tubular system appears and the sarcoplasmic reticulum becomes functionally mature, action potential triggered release of Ca^{2+} from the sarcoplasmic reticulum plays an increasingly important role in excitation-contraction coupling.

lease intracellular Ca^{2+} increases, this second mechanism augments the rise in intracellular free Ca^{2+} which accompanies the action potential and results in tension generation. This scheme is consistent with the observations that immature myocardium does not possess the well developed t-tubular system and sarcoplasmic reticulum of the adult (7-11); that the sarcoplasmic reticulum of the immature myocardium is functionally less able to sequester and rerelease Ca^{2+} than that of the adult (17-19); that the newborn and fetal myocardium are less sensitive to changes in stimulation rate (7, 20); and that the dependence on the extracellular concentration of Ca^{2+} is greater for newborn myocardium than adult (4). This model, which is similar to previous proposals (2, 5-7, 21), would explain the direct control of membrane potential over steady state tension and the absence of phasic tension seen in the newborn. Additionally, the increase in phasic tension seen with maturity may serve as a marker for

the development of the functional role of the sarcoplasmic reticulum in excitation-contraction coupling.

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