Excitation–Contraction Coupling in Myocardium of Nonhibernating and Hibernating Chipmunks: Effects of Isoprenaline, a High Calcium Medium, and Ryanodine

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The electromechanical responses to isoprenaline and a high calcium medium on cardiac muscles from nonhibernating and hibernating chipmunks were studied in the presence and the absence of ryanodine. In nonhibernating animal preparations, isoprenaline (5 × 10⁻⁶ M) caused a marked positive inotropic effect with an increase in the amplitude of the action potential plateau and augmented the slow action potential in muscle depolarized with 26 mM K⁺. In hibernating animal preparations, isoprenaline failed to cause a positive inotropic effect in spite of an increase in the amplitude of action potential plateau and slow action potentials. Similar inotropic effects were caused in the two preparations when extracellular calcium was raised to 6 mM, but action potential plateau and slow action potentials of the two preparations were less affected by this procedure; only in nonhibernating animal preparations was the amplitude of the slow action potential slightly increased. Ryanodine (2 × 10⁻⁴ M) partially inhibited the contraction but augmented the action potential plateau and slow action potentials in nonhibernating animal preparations, while in hibernating animal preparations, it eliminated the contraction and severely inhibited the action potential plateau and slow action potentials. The electrical effects of isoprenaline on the two preparations and of a high calcium medium on nonhibernating animal preparations were more pronounced in the presence of ryanodine than in the absence of it. However, the electrical activity on hibernating animal preparations was unaffected by a high calcium medium in either the presence or absence of ryanodine. In voltage clamp experiments, the slow inward current in hibernating animal preparations was much less than that in nonhibernating animal preparations, while the net outward current was not essentially different between the two preparations. These results indicate that an increase in the transsarcolemmal calcium influx augments cytoplasmic calcium concentration and results in the positive inotropic effect on nonhibernating animal preparations, but an increase in cytoplasmic calcium influx does not have the same effect on hibernating animal preparations. The contraction in hibernating animal preparations is directly controlled by the ryanodine-sensitive internal calcium release. The unique characteristics of excitation–contraction coupling in hibernating animal myocardium are discussed. (Circulation Research 1986;S9:221–228)

It has recently been shown that some characteristics of cardiac excitation–contraction coupling are markedly changed during hibernation. The contraction of the cardiac muscle depends mainly on intracellularly derived calcium in hibernating animals, whereas in myocardium of nonhibernating animals calcium influx across cell membranes makes a greater contribution to the activation of contraction. The cardiac membrane action potential of hibernating animals was also demonstrated to be markedly different from that of nonhibernating animals. In myocardium of hibernating animals, the early plateau phase of the action potential was suppressed and the slow action potential as a measure of the slow inward current showed low amplitude, suggesting less contribution of the slow inward current to the plateau potential. The difference in myocardial characteristics between the two preparations was also clearly observed in the cardiac responsiveness to the drugs. The cardiotonic effects of ouabain and noradrenaline were greatly reduced and the cardio-inhibitory effects of ryanodine and caffeine were markedly enhanced in myocardium of hibernating animals. These data suggest that the cardiac muscle from hibernating animals is an interesting model for studying the excitation–contraction coupling mechanism of the heart.

In the present study, therefore, the electromechanical effects of isoprenaline and a high calcium medium, both of which are known to increase transsarcolemmal calcium influx through Ca²⁺ channels, were compared on isolated papillary muscles from nonhibernating and hibernating animals. Similar experiments were also carried out in the presence of ryanodine in an attempt to examine the involvement of intracellular calcium kinetics in the cardiac responsiveness. To compare the contribution of the slow inward current to the plateau potential in the two preparations, membrane currents were measured.

Materials and Methods

Asian chipmunks (Tamias sibiricus) of both sexes were trapped in September and transferred to individ-
The heart was quickly excised, a papillary muscle, 2-3 mm in length and less than 1 mm in diameter, was used for experiments on hibernating preparations. Others were housed in a darkened cold room (4 ± 1°C) with food, a standard diet of pelleted laboratory rat chow, and water available. Most of them exhibited preliminary times of hibernation within 3 weeks, and subsequently, they all exhibited several consecutive times of hibernation greater than 1 week in duration until the following March. Animals in deep hibernation were used for experiments on hibernating preparations.

Animals were killed following cervical dislocation. The heart was quickly excised, a papillary muscle, 2–3 mm in length and less than 1 mm in diameter, was isolated from the right ventricle. The ends of the preparation were impaled on two hooks with one end attached to a force displacement transducer and mounted in a tissue bath of Krebs-Ringer solution equilibrated with 95% O₂ and 5% CO₂. The composition of the Krebs-Ringer solution, in millimoles per liter, was NaCl, 120; KCl, 4.8; CaCl₂, 1.2; MgSO₄·7H₂O, 1.3; KH₂PO₄, 1.2; NaHCO₃, 24.2; and glucose, 5.5 (pH 7.4). In some experiments, the K⁺ concentration of the Krebs-Ringer solution was raised to 26 mM by substituting KCl for NaCl on an equimolar basis to inactivate the fast Na⁺ channels. The temperature of the superfusate was maintained at 30°C. The preparations were stimulated at 1 Hz with pulses 1 msec in duration and twice the diastolic threshold. In the high [K⁺] solution, a frequency of 0.2 Hz and voltage five times the diastolic threshold. In the high [K⁺] solution, a frequency of 0.2 Hz and voltage five times the diastolic threshold. In the high [K⁺] solution, a frequency of 0.2 Hz and voltage five times the diastolic threshold. In the high [K⁺] solution, a frequency of 0.2 Hz and voltage five times the diastolic threshold.
Excitation-Contraction Coupling of Hibernating Animal Myocardium

Figure 1. Electromechanical effects of isoprenaline on papillary muscles from nonhibernating (a) and hibernating (b) animals. The top trace shows the slow action potential in high K⁺ medium (26 mM K⁺); the middle trace indicates the action potential in normal medium, and the bottom trace shows the developed tension in normal medium. APD₅₀ was measured at 50% repolarization. Isoprenaline (5 × 10⁻⁶ M) was applied for 6-7 minutes. Arrowheads indicate points of electrical response.

(96.6 ± 7.6% of control, n = 4) within 2–3 minutes. Although the amplitude of APp and APs and APD₅₀ were virtually unaffected, the rate of repolarization of APp and APs tended to accelerate (Figure 2b).

Preparations Pretreated with Ryanodine

In order to eliminate the involvement of intracellular calcium kinetics, ryanodine, an inhibitor of calcium release from sarcoplasmic reticulum, was applied to the preparations of nonhibernating and hibernating animals. In these preparations, the effects of isoprenaline and a high calcium medium were further examined. As previously reported by Kondo and Shibata, the application of ryanodine (2 × 10⁻⁶ M) for 15 minutes abolished the developed tension of ventricular muscles of hibernating animals and partially inhibited the contraction in nonhibernating animal preparations. Interestingly, all preparations from hibernating animals in the present study showed a transient positive inotropic effect just after the application of ryanodine.

Figure 2. Electromechanical effects of a high calcium medium on papillary muscles from nonhibernating (a) and hibernating (b) animals. The calcium medium contains 6 mM of Ca²⁺.
FIGURE 3. Electromechanical effects of ryanodine on papillary muscles from nonhibernating (a) and hibernating (b) animals. Ryanodine (2 × 10⁻⁶ M) was applied for 15 minutes.

while those from nonhibernating animals did not. This transient positive inotropic effect of ryanodine has been observed in previous experiments done in this laboratory on hibernating animal myocardium (unpublished observation). Representative results are shown in Figure 3. In the nonhibernating animal preparations, APp and APs were markedly augmented by ryanodine, while in hibernating animal preparations, APp and APs were severely inhibited by this agent. Inhibitory effects of ryanodine on APs in hibernating animal preparations selectively occurred in the plateau phase; the initial phase of APs was unaffected.

In ryanodine-pretreated preparations from nonhibernating animals, isoprenaline (5 × 10⁻⁸ M) caused a marked positive inotropic effect (358 ± 37% of value after exposure to ryanodine; n = 5) with a greater augmentation of APp and APs. This enhancement of APp and APs was characterized by APD of more than two times that before application of isoprenaline (Figure 4a). In hibernating animals, the abolished contraction was not affected by isoprenaline at all (n = 4), but the severely inhibited APp and APs were dramatically increased by isoprenaline (Figure 4b).

Although similar inotropic effects were observed during exposure to a high calcium medium, the change in the electrical responses was less. In ryanodine-pretreated preparations from nonhibernating animals, isoprenaline (5 × 10⁻⁸ M) caused a marked positive inotropic effect (358 ± 37% of value after exposure to ryanodine; n = 5) with a greater augmentation of APp and APs. This enhancement of APp and APs was characterized by APD of more than two times that before application of isoprenaline (Figure 4a). In hibernating animals, the abolished contraction was not affected by isoprenaline at all (n = 4), but the severely inhibited APp and APs were dramatically increased by isoprenaline (Figure 4b).
treated preparations from nonhibernating animals, a marked positive inotropic effect (331 ± 52% of value after exposure to ryanodine; n = 4) was found with an increase in the amplitude of APp and APs (Figure 5a). In hibernating animals, the electrical and the mechanical responses to a high calcium medium faded completely, (n = 4) (Figure 5b).

Membrane Currents

Voltage clamp experiments were conducted to examine the involvement of the slow calcium inward current in the respective plateau potential of three nonhibernating and hibernating animal preparations. The rapid component of inward current (fast sodium inward current) was inactivated by holding the membrane potential at -40 mV. At this holding potential the ryanodine-sensitive inward current that generated the plateau potential in hibernating animal myocardium was also inactivated (unpublished observation). Various depolarization steps, which had a duration of 500 msec each were applied at a frequency of 0.2 Hz. Representative results are illustrated in Figure 6. The depolarization steps from the holding potential elicited the slow inward current followed by a slight transient outward current in nonhibernating and hibernating animal preparations. The net inward and outward currents were estimated by taking the difference between the holding current level and the peak level of the slow inward current or the current level at the end of depolarization step, respectively. The slow inward current in hibernating animal preparations was much less than that in nonhibernating animal preparations, while the slight transient outward current and the net outward current at the end of the depolarization step were similar in the two preparations. The current-voltage relation indicated that the net outward current in hibernating animal preparations was practically superimposable on that in nonhibernating animal preparations, while the peak slow inward current was dramatically attenuated in hibernating animal preparations. Such slight slow inward current in hibernating animal preparations may be inherent, but not due to a possible underestimation by the transient outward current, since the similar transient outward current was observed in the two preparations. Nevertheless, some involvement of the transient outward current in the suppressed APp and APs in hibernating animal preparations may not be eliminated, since the transient outward current is known to contribute to the rapid repolarization of the action potential. This problem will require further studies.

Discussion

A well-known cardiotonic agent, isoprenaline, and a medium containing a high concentration of extracellular calcium increase transsarcolemmal calcium influx through Ca\(^{2+}\) channels but failed to cause the positive inotropic effect in the preparations from hibernating animals. In preparations from nonhibernating animals, a marked positive inotropic effect was observed, as generally found in ventricular muscles of most species.

The positive inotropic effect of isoprenaline in the preparations from nonhibernating animals was accompanied by an increase in the amplitude of the action potential plateau and a prolongation of APD\(_{90}\). The activation of slow inward current is of prime importance in the genesis of the action potential plateau in mammalian ventricular muscle. The slow action potential as a measure of the slow inward current was also markedly augmented by the application of isoprenaline. These results can be explained by an increase in maximum conductance of Ca\(^{2+}\) channels by isoprena-
line via β-adrenoceptors; this results in an increase in Ca\(^{2+}\) influx across cell membranes.\(^{9,10}\) A high calcium medium also caused a marked positive inotropic effect on nonhibernating animal preparations. This effect is due to an enhancement of an electrochemical driving force for inward movement of Ca\(^{2+}\) across cell membranes, which was reflected in an increase in the amplitude of the slow action potential. The increasing effect was pronounced in the presence of ryanodine, a selective inhibitor of the release of Ca\(^{2+}\) from the sarcoplasmic reticulum.\(^{11-12}\) This effect of ryanodine was more clearly observed in the electrical responses to isoprenaline. It can, in part, be explained by the fact that ryanodine decreases transient outward current\(^{1}\) and slows the inactivation of Ca\(^{2+}\) current,\(^{14}\) both of which can be attributed to the decrease in internal Ca\(^{2+}\), which in turn results in reducing the rate of repolarization process of the plateau potential. However, ryanodine did not affect the positive inotropic effects of isoprenaline nor of a high calcium medium. The present results suggest that the contractility of this preparation is mainly controlled through Ca\(^{2+}\) influx across cell membranes, as previously demonstrated.\(^{1}\) This suggestion was also supported by the present voltage clamp data, which revealed the existence of a large slow inward current in the present preparations. Thus, the electromechanical characteristics of nonhibernating animal preparations were quite consistent with those of mammalian cardiac muscle from most species.

In hibernating animal preparations, markedly different effects were observed. The plateau potential as well as the contractile force were abolished by ryanodine, suggesting that these responses are controlled by a mechanism different from that controlling the slow inward current, since ryanodine is known not to block the slow inward current kinetics.\(^{12,14}\) Furthermore, the present voltage clamp data revealed that the slow inward current was much less in hibernating animal preparations than in nonhibernating animal preparations. An increase in the electrochemical driving force for Ca\(^{2+}\) concentration also failed to augment the electrical response in either the presence or absence of ryanodine. These results suggest that only a small number of the voltage-activated Ca\(^{2+}\) channels operate in this preparation. This explains the lack of effect of a high calcium medium on the electrical and the mechanical responses.

Similar results were observed on the mechanical effect of isoprenaline on hibernating animal preparations; no positive inotropic effect was found in the presence or absence of ryanodine. However, this drug markedly increased the amplitude of the slow action potential and the action potential plateau, and this enhancement was more pronounced in the presence of ryanodine. The increasing effect of isoprenaline on these electrical responses was observed in the range above the threshold potential for the slow inward current, suggesting that isoprenaline increased the slow inward current through Ca\(^{2+}\) channels, probably via β-adrenoceptors. This electrical effect of isoprenaline on the present preparations in which Ca\(^{2+}\) channels were less involved may be explained by the suggestion that β-adrenoceptor agonists enhance Ca\(^{2+}\) current by increasing the probability of Ca\(^{2+}\) channels opening during depolarization.\(^{15}\) The finding that cardiac contractility is not increased by isoprenaline in spite of the increase in Ca\(^{2+}\) influx through Ca\(^{2+}\) channels is interesting and can be used in studying the mechanism of excitation-contraction coupling.

Such a phenomenon is not likely to be attributed to the saturation of the capacity of contractile elements, since even after attenuating the contractile force by ryanodine, when contractile elements should be in reserve for further contraction, the present uncoupling of Ca\(^{2+}\) influx and the contractile force was clearly ob-
served. The marked increase in the contractile force that resulted from reducing the driving frequency was previously observed in the same preparations, and also supports the above implications. These indicate that in the present myocardium, Ca\(^{2+}\) influx during the action potential plateau cannot elevate Ca\(^{2+}\) concentration near contractile elements. The contractility of hibernating animal myocardium is not directly activated by Ca\(^{2+}\) influx across cell membranes. The fact that a complete inhibition of contraction resulting from the elimination of intracellular Ca\(^{2+}\) kinetics by ryanodine was unaffected in spite of a greater increase in transsarcolemmal Ca\(^{2+}\) influx by isoprenaline further suggests that the contraction of this preparation fully depends on the ryanodine-sensitive intracellular Ca\(^{2+}\) release.

A lack of positive inotropic effects of isoprenaline and a high calcium medium in the present hibernating animal myocardial preparations is consistent with the previous demonstration that the inotropic responses to noradrenaline and a rising extracellular Ca\(^{2+}\) concentration were depressed in myocardium from hibernating ground squirrel. It is suggested that the present changes in the cardiac excitation–contraction coupling mechanism commonly occur during hibernation.

Based on the above discussion, it seems reasonable to conclude that cardiac excitation–contraction coupling in hibernating animals is different from that in nonhibernating animals. The full dependence of cardiac contractility in hibernating animals on internal Ca\(^{2+}\) release suggests that the two sources of Ca\(^{2+}\), the transsarcolemmal Ca\(^{2+}\) influx and the Ca\(^{2+}\) release from internal stores, are in series rather than parallel. A present uncoupling of Ca\(^{2+}\) entry and cardiac contractility in hibernating animals further suggests that the excitation–contraction process involves at least two distinct intracellular compartments for Ca\(^{2+}\), since a unicompartment system through which the cytoplasmic Ca\(^{2+}\) is increased by isoprenaline would secondarily augment the contractile force and cannot explain this uncoupling. These suggestions in hibernating animal myocardium seem to be interpreted best by the model based on the situation in skeletal muscle. In this model, most of Ca\(^{2+}\) entering the cell through Ca\(^{2+}\) channels is trapped in intracellular compartment I, from which it may be transported to another cellular compartment, II. The transported Ca\(^{2+}\) is released by a subsequent action potential. The released calcium is immediately taken up by compartment I and recycled within the two compartments. Ca\(^{2+}\) may be continuously lost from these compartments through the Ca\(^{2+}\) efflux system. In the present hibernating animal preparations, compartment I may have a sufficient capacity to trap the markedly increased Ca\(^{2+}\) influx across cell membranes, and the rate of Ca\(^{2+}\) transportation from compartment I to compartment II may reach plateau levels under the present conditions, resulting in the lack of positive inotropic effect in spite of further increased calcium influx. This proposed model applies only to hibernating animal myocardium. As this interpretation involves a series of assumptions, further studies on intracellular calcium kinetics will be needed to define the characteristics of the present hibernating animal myocardium.

Finally, the present observation that ryanodine caused a transient positive inotropic effect only in hibernating animal preparations is in good agreement with the recent demonstration that ryanodine produced a transient increase in twitch tension on calcium-overloaded sheep cardiac purkinje fibers, suggesting the possibility that the cardiac muscle from hibernating animals is in a calcium overload-like state. The greatly reduced positive inotropic response to ouabain in myocardium from hibernating ground squirrels also is compatible with this suggestion. However, in the present hibernating animal preparations, the after-contraction and the after-depolarization that commonly occur on calcium-overloaded myocardium were not observed even after the application of the cardiotonic agent, isoprenaline, which would facilitate calcium overload.

As the after-contraction and the after-depolarization are thought to be linked to the oscillatory release of Ca\(^{2+}\) from internal stores, it is assumed that calcium overload in the present preparations are buffered by the internal Ca\(^{2+}\) stores (compartments I and II) characterized by the above hypothesis. The present phenomena may be related to characteristic myocardial changes during hibernation although the precise explanation is not clear. Thus, the present findings can provide a useful model for studying cardiac excitation–contraction coupling mechanism avoiding calcium overload.

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**Key Words**
- nonhibernating and hibernating chipmunks
- myocardium
- action potential
- membrane currents
- contraction
- isoprenaline
- high calcium medium
- ryanodine
Excitation-contraction coupling in myocardium of nonhibernating and hibernating chipmunks: effects of isoprenaline, a high calcium medium, and ryanodine.

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_Circ Res._ 1986;59:221-228
doi: 10.1161/01.RES.59.2.221

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/59/2/221