Recording of calcium transient and analysis of calcium removal mechanisms in cardiac myocytes from rats and ground squirrels

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Abstract With confocal microscopy, we recorded calcium transients and analyzed calcium removal rate at different temperatures in cardiac myocytes from the rat, a non-hibernator, and the ground squirrel, a hibernator. The results showed a remarkable increase of the diastolic level of calcium transients in the rat but no detectable change in the ground squirrel. Calcium transient of the ground squirrel, compared with that of the rat at the same temperature, had a shorter duration and showed a faster calcium removal. As indicated by the pharmacological effect of cyclopiazonic acid, calcium uptake by sarcoplasmic reticulum (SR) was the major mechanism of calcium removal, and was faster in the ground squirrel than in the rat. Our results confirmed the essential role of SR in hypothermia-tolerant adaptation, and negated the importance of Na-Ca exchange. We postulated the possibility to improve hypothermia-tolerance of the cardiac tissue of non-hibernating mammals.

Keywords: **cardiac cells, calcium transient, calcium removal, sarcoplasmic reticulum, hibernation, hypothermia, confocal laser-scanning microscopy.**

It is well known that the myocardium of non-hibernating mammals, including the human, cannot work at low body-temperatures, which is in contrast to the well-maintained contractility of hibernator myocardium under the same condition[1,2]. Our recent research revealed that low temperature caused a marked increase of intracellular free calcium ($[Ca^{2+}]_i$) in cardiac myocytes from the rat but not in those from the ground squirrel, a hibernator[3]. However, since these data were collected from resting myocytes, they were still insufficient to correlate intracellular calcium with the hypothermia-induced change of contractility.

Further, if hibernator cells do successfully keep from calcium overload during hypothermia, they must have special adaptation in calcium removal mechanisms. Some investigations showed that sarcoplasmic reticulum (SR) vesicles isolated from hibernator hearts had a higher calcium uptake rate than those from non-hibernators[4]. Notable increases of both cellular SR density and *in vitro* calcium uptake capacity of SR were found in hibernators during their entrance into hibernation[5,6]. While the evidence suggested an important role of SR in cold-adaptation of heart myocardium, some investigators, however, argued a special role of Na-Ca exchange in intracellular calcium homeostasis during hypothermia. Since Na-Ca exchange does not consume

ATP directly, it was postulated that Na-Ca exchange could help buffering the ineffective energy supply at low temperatures. This viewpoint was supported by a low-amplitude plateau of cardiac action potential in hibernating chipmunks[7], different intracellular ionic response to cryo-treatment in a hibernator and a non-hibernator[8], and high sensitivity of repolarization to extracellular Na/Ca in hibernators (unpublished data). The key point of this controversy, we believe, is that the measurement of SR uptake was done *in vitro*, and thus could not account for their *in situ* contribution, let alone compare with Na-Ca exchange.

In the present study, we recorded the temperature-dependent change of calcium transient in driven cardiac myocytes of rats and ground squirrels with confocal microscopy, analyzed their calcium removal rate by exponential fitting of the recovery phase of calcium transients, and determined the *in situ* contribution of SR in uptake calcium by the aid of a selective SR Ca-pump blocker cyclopiazonic acid (CPA). The results revealed the adaptation of hibernator myocardium in avoiding intracellular calcium overload during hypothermia.

1 Methods

1.1 Cell preparation

Adult rats and ground squirrels (*Cittelus dauricus*) of either sex were used in our experiments. Ventricular myocytes were prepared and loaded with indo-1 as previously reported[3]. Briefly, the hearts were rapidly excised from the animals under ether anesthesia and perfused for 5 min at 37°C with a calcium free buffer containing (in mmol/L): 110 NaCl, 4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 30 taurine, 10 glucose, aerated with 95% O_2 + 5% CO_2 , pH 7.4. The tissue was then digested in a buffer containing 0.5 mg/mL collagenase (Sigma, Type 1A), 1% bovine serum albumin (Sigma, fraction V, essentially fatty acid free) and 75 μmol/L Ca^{2+}. About 15 min later, the ventricle was cut into small pieces and incubated in digesting solution. Finally the myocytes were harvested and stored in Tyrode solution which contained (in mmol/L): 140 NaCl, 4 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 5 HEPES, pH 7.4 adjusted by NaOH. Indo-1 was loaded by exposing the cells to 2.5 μmol/L indo-1/AM (Molecular Probes) in Tyrode solution for about 10 min in the dark at 37°C.

1.2 Temperature-controlled chamber

The experimental chamber was a glass-bottomed petri culture dish fixed inside with a circular glass canula of 1 cm diameter to form a 0.5 mL solution container (fig. 1). Temperature was controlled by perfusing the circular glass canula with a Cole-Parmer refrigerated circulator. A tiny bithermal thermistor probe was placed on the glass bottom of the dish to monitor the temperature. When there was 0.3 mL or more solution in the chamber, the temperature difference between the center and the periphery of the glass bottom was less than 0.5°C. Cells were driven by 0.5-Hz and 2-ms pulse with a pair of silver electrodes, which were placed parallelly in the solution container and connected to an electronic stimulator. In order to stabilize the cell position,

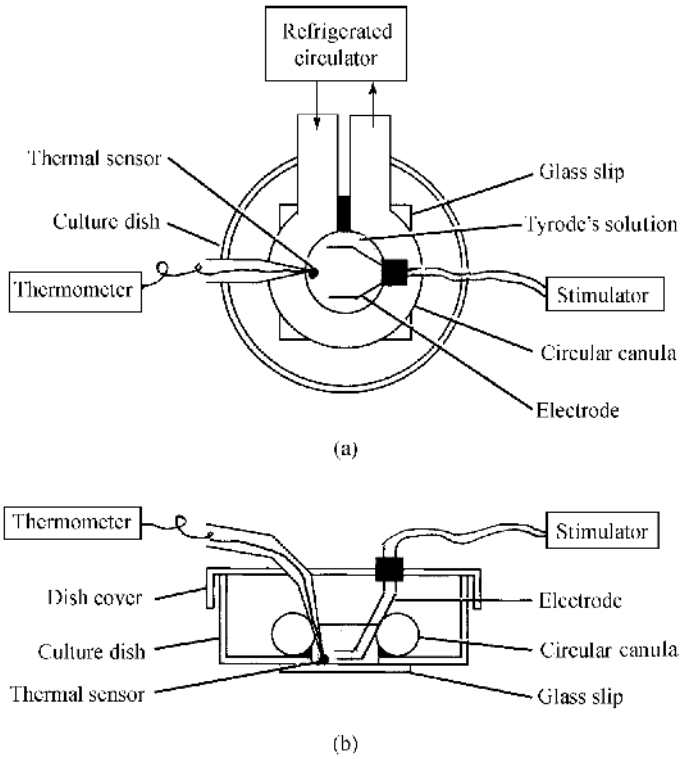

Fig. 1. Experimental chamber and setups for temperature control and stimulation. (a) Overlooking profile; (b) central section.

dish bottom was pretreated with 0.5% gelatin before experiment.

1.3 Fluorescence recording and calculation

Indo-1 fluorescence was visualized and measured by a confocal laser scanning microscope (ASCS meridian 575 UV) with hardware setting the same as our previous report[3]. With line-scan of this system being too slow, calcium transients were recorded in point mode at 100 Hz acquisition rate. To improve the signal/noise ratio, eight traces were averaged for each calcium transient. $[Ca^{2+}]_i$s were calculated from fluorescent data with formula (1)[9]

$$[Ca^{2+}] = K_d \frac{FR - FR_{min}}{FR_{max} - FR} \cdot \beta , \quad (1)$$

in which FR is the ratio of the fluorescence recorded from detector PMT2 (405 nm) vs. detector PMT1 (480 nm), FR_{max} and FR_{min} are the FR in calcium free and calcium-saturated buffers, respectively, and β is the ratio of fluorescence at 480 nm in calcium-free vs. calcium-saturated buffers. The dissociation constant K_d at different temperatures was calibrated *in situ* and at alpha-stat as our recent report[3,10]. When the calcium removal process was analyzed, the rate constant v of calcium removal was determined by fitting the recovery phase of calcium transient to equation

$$[Ca^{2+}]_i = A \cdot \exp(-t+B), \quad (2)$$

in which t is time, A and B are fitting constants.

1.4 Statistics

The data are presented as means ± S.E. One-way or two-way analysis of variation with repeated measures (ANOVA-RM) was used to analyse the effects of temperature, species and other factors. $P < 0.05$ was considered significant. Some S.E., when appropriate, was calculated following formula (3). If $Q \pm \delta_Q$ is calculated from $X \pm \delta_X$ and $Y \pm \delta_Y$ according to the function $f(X,Y)$, then

$$\delta_Q = \sqrt{\left[\frac{\partial f(X,Y)}{\partial X}\right]^2 \delta_X{}^2 + \left[\frac{\partial f(X,Y)}{\partial Y}\right]^2 \delta_Y{}^2}\,. \tag{3}$$

2 Results

2.1 Recording and comparison of calcium transients

Calcium transients were recorded in point mode. A typical recording is illustrated in fig. 2. The fluorescence from PMT1 and PMT2 changed symmetrically. The calcium transient trace shown as FR is their ratio PMT2/PMT1. The last trace shows an average of eight, whose signal/noise ratio was greatly improved. We observed that calcium transients recorded at different point of a cell, especially after signal average, were usually similar in amplitude, diastolic level and duration. Our previous investigation also supported a general homogeneity of $[Ca^{2+}]_i$ in

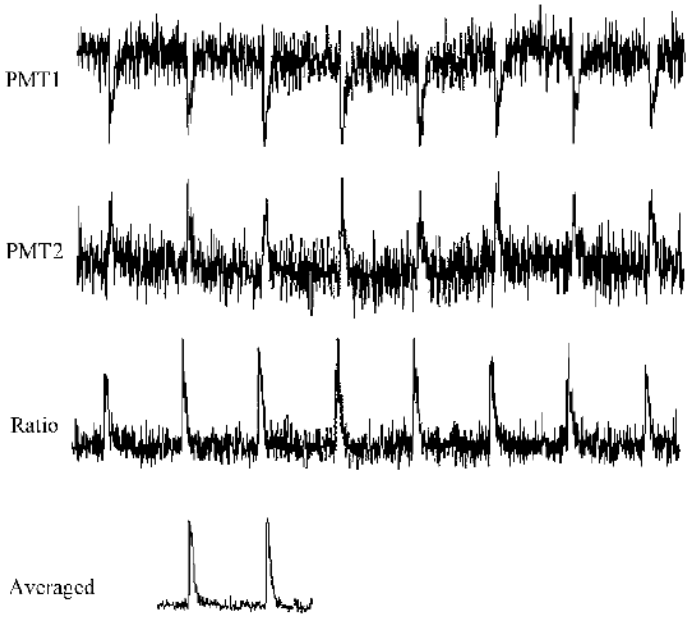

Fig. 2. Typical calcium trancients recorded in point mode with confocal microscopy. Fluorescences at 480 nm and 405 nm were recorded by detectors PMT1 and PMT2, respectively. The third trace was their ratio (PMT2/PMT1). The fourth trace was an average of eight.

resting cells[3]. We believe that, the averaged point-mode recording, although from local area of a cell, was a reflection of the unitary property of the whole cell.

Temperature dependence of calcium transient was compared between cardiac myocytes from rats and ground squirrels. With the decrease of temperature, calcium transients of both animals showed similar increase in peak $[Ca^{2+}]_i$ and duration, but not in diastolic $[Ca^{2+}]_i$ (fig. 3). The diastolic $[Ca^{2+}]_i$ increased only in the rat, giving rise to the decrease of the dynamic amplitude of calcium transients, but was almost unchanged in the ground squirrel so that the amplitude of calcium transients exhibited some increase. Although the half-peak duration increased in both

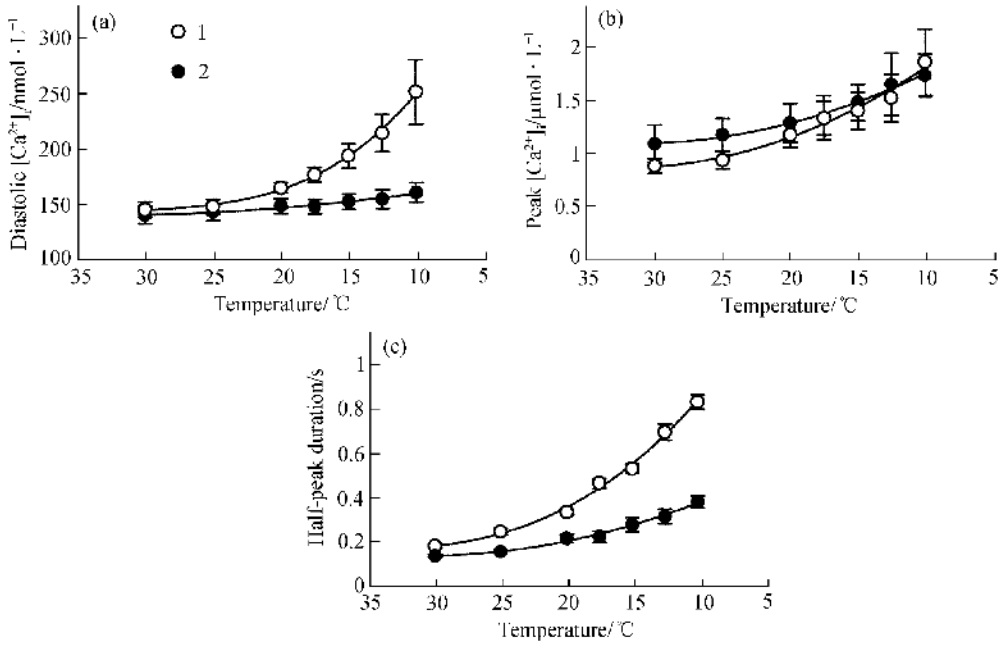

Fig. 3. Temperature dependence of diastolic $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and half-peak duration of the calcium transients in cardiac myocytes of the rat (n = 9) and the ground squirrel (n = 5). There are significant interactions in diastolic $[Ca^{2+}]_i$ (P<0.05) and half-peak duration (P<0.01), but not in peak $[Ca^{2+}]_i$ (P>0.05), tested by two-way ANOVA-RM. 1, Rat; 2, ground squirrel.

animals, that of the ground squirrel was shorter than that of the rat at each temperature, indicating a faster calcium removal.

2.2 Analysis of calcium removal rate

The rate constant v of calcium removal process was determined by fitting the recovery phase of calcium transient with eq. (2) (for fitting see fig. 4). Previous studies showed that the diastolic tension and $[Ca^{2+}]_i$ in

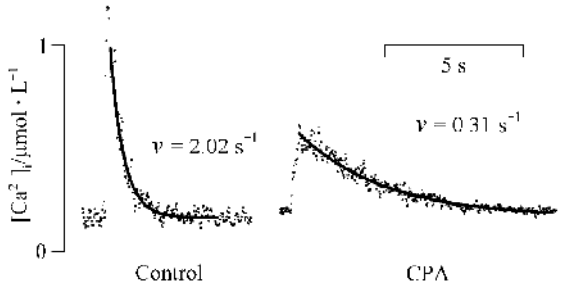

Fig. 4. Typical records of calcium transients of a rat cardiac myocyte before and after blockage of SR by CPA at 15°C. The superimposed thick lines show the fit of eq. (2) to the recovery phase.

cardiac myocytes of the rat did not change much above 25°C[1,3]. Change of calcium transient was similar as shown in fig. 3. Since the calcium transient became unstable below 15°C in the rat, especially after CPA treatment, calcium removal rate was analyzed and compared at 25°C and 15°C in the present study. Between these two temperatures, v decreased with lowering temperature at Q_{10} of 2.9 in the rat or 2.4 in the ground squirrel. At each temperature, v was higher in the ground squirrel than in the rat (fig. 5(a)).

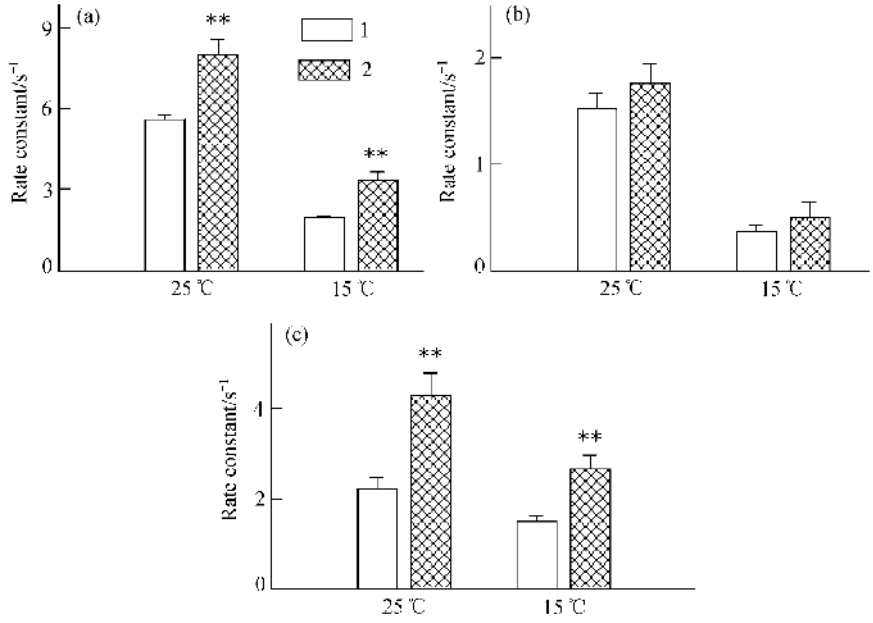

Fig. 5. Calcium removal rate constant (a) and its CPA-sensitive and CPA-insensitive fractions in cardiac myocytes of the rat (white, $n = 12$) or the ground squirrel (gray, $n = 6$). ** $P < 0.01$ by t-test.

In order to analyze the contribution of SR in the total calcium removal rate, 50 μmol/L CPA was used to selectively block the SR Ca-pump. The dosage we used, which was 5-fold as high as that used at 23°C by Balke et al.[11], ensured the effectiveness at lower temperature. In the presence of CPA, the recovery phase of calcium transient, though much slower, still fitted well mono-exponential kinetics (fig. 4). Fitting results showed that CPA decreased v by 72.7% at 25°C and 81.3% at 15°C in the rat, and by 78.0% and 84.9%, respectively, in the ground squirrel, suggesting the dominant role of SR in calcium removal in both animals. The percentage in rats at 25°C agreed well with literatures[11,12], and confirmed the liability of our data. After CPA treatment, species difference was not significant (fig. 5(b)). By subtraction of the CPA-insensitive v from the total (control) value, we got the calcium uptake rate constant of *in situ* SR, which is about 2-fold as high in the ground squirrel as in the rat (fig. 5(c)). Between 25°C and 15°C, Q_{10} of CPA insensitive fraction were 4.1 in the rat and 3.6 in the ground squirrel, but those of CPA sensitive fraction are 2.0 and 1.6, respectively, indicating less thermal sensitivity of SR calcium uptake.

3 Discussion

3.1 Methodological considerations to measure calcium removal process

Methods to measure calcium removal rate can be either *in vitro* or *in situ*. *In vitro* measurement has been widely used to study the calcium uptake of isolated SR[5,6]. However, this method is rather limited to account for the calcium removal function in a living cell, since the intracellular density and distribution of SR or other removal mechanisms vary with preparations and experimental treatments[6,11]. In order to compare the calcium removal function of cells from different species, we determined the rate constant v of calcium removal by exponential fitting of the recovery phase of calcium transient. Since our experimental condition (e.g. temperature) was quite different from the commonly designed one, careful consideration was taken below to ensure the reliability of the results.

Given that n kinds of processes participate in calcium removal, that the rate of the jth process is V_j, and that the rate of calcium influx into cytosol is L, then $[Ca^{2+}]_i$ is a function of time t:

$$-\frac{d}{dt}[Ca^{2+}]_i = \sum_{j=1}^{n} V_j - L . \tag{4}$$

Set $C_0 \equiv [Ca^{2+}]_{i\,(t=0)}$ and $C_\infty \equiv [Ca^{2+}]_{i\,(t\to\infty)}$. If $L << \Sigma V$ and that V has a linear relationship with $[Ca^{2+}]_i$, i.e. $V = v \cdot ([Ca^{2+}]_i - C_\infty)$, the solution of (4) can be

$$[Ca^{2+}]_i = (C_0 - C_\infty) \cdot \exp\left[-\left(\sum_{j=1}^{n} v_j\right) t\right] + C_\infty . \tag{5}$$

Formula (5) is an analytical form of formula (2). The above analysis suggests that two preconditions should be considered when using fitting method to determine calcium removal rate. (ⅰ) $L << \Sigma V$. On account of the inactivation of calcium channels, fitting the late stage of calcium transient should be more reliable. (ⅱ) V has a linear relationship with $[Ca^{2+}]_i$. This demands that all removal processes meet first-order kinetics. At 5—37°C, calcium uptake of SR turns to saturated kinetics when $[Ca^{2+}]_i$ > 1 μmol/L[4]; half-maximum rate of Na-Ca exchange was found when $[Ca^{2+}]_i$ is 1—5 μmol/L [13]. In the present study, only the part of calcium transients that was lower than 1 μmol/L was fitted to meet this consideration.

Formula (5) also means that total v is a sum of fractional v. This is the theoretical basis to calculate CPA sensitive fraction of v in the present study.

3.2 Homeostatic mechanisms of intracellular calcium during hypothermia

We have recently reported that lowering temperature caused a remarkable increase of $[Ca^{2+}]_i$ in resting cardiac myocytes from rats, but not in those from ground squirrels[3]. Here we extended the investigation to beating cells to account for the hypothermia-induced functional change of working cardiac tissue. The major difference of calcium transient between these two species, as we observed, was that rat cardiac myocytes could not maintain the diastolic level of $[Ca^{2+}]_i$ at low

temperatures. This finding explained the cellular mechanism underlying the incomplete diastole and loss of contractility in non-hibernator myocardium during hypothermia[1,2], and demonstrated that intracellular calcium overload is a major process in hypothermia-induced cardiac dysfunction in non-hibernating mammals. The key point that hibernator myocardium can keep active function at low temperature is their ability to maintain a homeostatic basic (resting or diastolic) level of $[Ca^{2+}]_i$.

During an excitation-contraction cycle, $[Ca^{2+}]_i$ is restored to its basic level by calcium removal mechanisms, in which SR calcium uptake and Na-Ca exchange are predominant, and mitochondria and sarcolemmal Ca-pump contribute little[11,12]. In our results, the quicker relaxation phase of calcium transient in ground squirrel cells suggested that hibernator myocardium has an adaptation in calcium removal process. The effect of CPA demonstrated that this adaptive mechanism principally is SR calcium uptake rather than other calcium removal mechanisms.

Since Na-Ca exchanger works without direct consumption of ATP, some investigators believed that it is a preferred mechanism by which hibernator myocardium handles intracellular calcium at low temperature. However, the present work showed that the total contribution of CPA-insensitive mechanisms, including Na-Ca exchange, was less than 1/4. Moreover, Na-Ca exchange is even more temperature-sensitive than SR calcium uptake as seen in our data and in literatures[14]. With the decrease of temperature, its role in calcium removal does decrease rather than increase. Na-Ca exchange does not play an adaptive role in intracellular calcium homeostasis of hibernator myocardium.

We have previously investigated the seasonal regulation of hibernation. Associated with an increased hypothermia-tolerance during hibernating season[15], cardiac SR of hibernators increases, both in volume density and in capacity of calcium uptake[5,6]. Comparing with non-hibernators, we showed in the present study that the cardiac myocytes of ground squirrel, even during non-hibernating season, have quicker SR uptake with a smaller temperature coefficient. We also tested that inhibition of SR function by caffeine resulted in losing hypothermia-tolerance in myocardium of ground squirrels[1]. All the evidence tightly related hypothermia-tolerance of mammalian myocardium to their SR calcium uptake. Now that this principle is efficacious in hibernators, it is possibly applicable in medical purpose. Up-regulation of SR function and other mechanisms that helps handle intracellular calcium may be a useful strategy to treat cardiac disease or disorders concerning hypothermia and calcium overload.

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References

1. Wang, S. Q., Huang, Y. H., Zhou, Z. Q., Dependence of myocardial hypothermia resistance on sources of activator calcium, Cryobiology, 1997, 35: 193.
2. Liu, B., Wohlfart, B., Johansson, B. W., Effects of low temperature on contraction in papillary muscles from rabbit, rat and

hedgehog, Cryobiology, 1990, 27: 539.
3. Wang, S. Q., Zhou, Z. Q., Qian, H., Temperature dependence of intracellular free calcium in cardiac myocytes from rat and ground squirrel measured by confocal microscopy, Science in China, Ser. C, 1999, 42(3): 293.
4. Liu, B., Belke, D. D., Wang, L. C. H., Ca^{2+} uptake by cardiac sarcoplasmic reticulun at low temperature in rat and ground squirrel, Am. J. Physiol., 1997, 272: R1121.
5. Belke, D. D., Milner, R. E., Wang, L. C. H., Seasonal variations in the rate and capacity of cardiac SR calcium accumulation in a hibernating species, Cryobiology, 1991, 28: 354.
6. Tang, Y. J., Wang, S. Q., Zhou, Z. Q., Seasonal variation in the ultrastructure and calcium uptake rate of cardiac sarcoplasmic reticulum in ground squirrel, Acta Physiologica Sinica (in Chinese), 1995, 47: 478.
7. Kondo, N., Electrophysiological effects of Ca antagonists, tetrodotoxin, $[Ca]_o$ and $[Na]_o$ on myocardium of hibernating chipmunks: possible involvement of Na-Ca exchange mechanism, Br. J. Pharmacol., 1987, 91: 315.
8. Smith, T. W., Yamaoka, K., Twist, V. W. et al., Membrane transport in isolated cardiac myocytes of ground squirrels: cold sensitivity of pumps and channels, in Living in the Cold Ⅱ (eds. Malan, A., Canguilhem, B.), London: John Libbey Eurotext, 1989, 177.
9. Grynkiewicz, G., Poenie, M., Tien, R. Y., A new generation of Ca^{2+} indicators with greatly improved fluorescent properties, J. Biol. Chem., 1985, 260: 3440.
10. Wang, S. Q., Zhou, Z. Q., Alpha-stat calibration of indo-1 fluorescence and measurement of intracellular free calcium in rat heart cells at different temperatures, Life Sciences, 1999, 65(9): 871.
11. Balke, C. W., Egan, T. M., Wier, W. G., Processes that remove calcium from the cytoplasm during excitation-contraction coupling in intact rat heart cells, J. Physiol. (London), 1994, 474: 447.
12. Puglisi, J. L., Bassani, R. A., Bassani, J. W. et al., Temperature and relative contributions of Ca transport systems in cardiac myocyte, Am. J. Physiol., 1996, 270: H1772.
13. Zhang, Y., Xue, S., Calcium and cell function, in Progress in Cellular Biology (eds. Zheng, G., Zhai, Z.) (in Chinese), Vol. 2 , Beijing: Higher Education Press, 1991, 160.
14. Marengo, F. D., Wang, S. Y., Langer, G. A., The effects of temperature upon calcium exchange in intact cultured cardiac myocytes, Cell Calcium, 1997, 21: 263.
15. Wang, S. Q., Zhou, Z. Q., Gao, T. L., Features of the cardiovascular function in mammalian hibernators, Acta Zoologica Sinica (in Chinese), 1997, 42: 203.