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Taurine prevents intracellular calcium overload during calcium paradox of cultured cardiomyocytes

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Summary. The effect of taurine on the cellular distribution of $[Ca^{2+}]_i$ during the calcium paradox was examined by digital imaging of a single fura-2-loaded cell. Cardiomyocytes superfused with control medium containing 2 mM Ca^{2+} exhibited typical transients associated with spontaneous beating. When the cells were exposed to Ca^{2+}-free buffer, immediate cessation of both spontaneous contractions and calcium transients was observed as $[Ca^{2+}]_i$ rapidly fell to a level of 3–6 $\times$ 10^{-8} M. Subsequent restoration of medium calcium increased $[Ca^{2+}]_i$ to level 4–7 times normal. Large increases in $[Ca^{2+}]_i$ were observed in most cells and were associated with the development of contracture and bleb formation.

Taurine pretreatment (20 mM) caused no significant effect on $[Ca^{2+}]_i$ during Ca^{2+} depletion. However, it inhibited excessive accumulation of $[Ca^{2+}]_i$ during the Ca^{2+} repletion. Moreover, taurine treated cells recovered their Ca^{2+}-transients and beating pattern earlier than non-treated cells. Finally morphological abnormalities commonly associated with calcium overload were attenuated by taurine treatment.

Keywords: Amino acids – Taurine – Calcium paradox – Cultured myocardial cell – Intracellular free calcium – Beating

Introduction

The "calcium paradox" phenomenon, first described by Zimmerman and Hulsman (1966), occurs when hearts are reperfused with calcium after a short period of calcium-free perfusion. The reintroduction of Ca^{2+} following Ca^{2+} repletion leads to irreversible myocardial damage, characterized by reduced electrical activity, extensive ultrastructural damage, depletion of tissue high-energy phosphate content, massive release of intracellular constituents and an increase in cytosolic Na^+ and Ca^{2+} (Chapman and Tunstall, 1987). However,

the occurrence of the calcium paradox in isolated myocytes is rather controversial since most authors have reported either the absence of the calcium paradox phenomenon or only a limited response (Rudge and Duncan, 1984; Lagerstrand, et al., 1983). Goshima et al. (1980) have reported that the calcium paradox can be evoked in cultured mouse myocytes, even though these cells are more resistant to the phenomenon than the isolated heart. Recent experiments have shown that the presence of amino acids, particularly taurine, in the medium increased the resistance to the calcium paradox of enzymatically isolated cardiac ventricular myocytes (Chapman et al., 1993).

Taurine (2-aminoethanesulfonic acid), a sulfur-containing amino acid, is found in almost all mammalian tissues (Huxtable and Sebring, 1993). Reports have appeared in recent years suggesting that many of the effects of taurine on the cardiovascular system may be related to intracellular Ca^{2+} redistribution (Schaffer et al., 1992; Dolara et al., 1973, 1976). However, the exact mechanism of the cardiac action of taurine has not been fully elucidated.

Use of fura-2, a calcium-sensitive fluorescent dye, has provided a new tool to monitor fluctuations in intracellular Ca^{2+} during the contraction cycle (Bals et al., 1990). This agent has permitted the investigation of normal Ca^{2+} transients and the cellular mechanisms that regulate $[Ca^{2+}]_i$.

We initially carried out the present experiment to visualize spatial and temporal changes in $[Ca^{2+}]_i$ of beating myocytes during the calcium paradox. We also intended to characterize the morphological changes in single cells associated with the alteration in $[Ca^{2+}]_i$ following the calcium paradox procedure. Finally, we set out to explore the protective effect of taurine on beating cardiomyocytes subjected to the calcium paradox.

Materials and methods

Mouse myocardial cell culture

Hearts of 14 to 16 day-old mouse embryos (ICR strain) were minced and digested with 5 ml of 0.125% trypsin – 0.025% collagenase solution at 37°C for 15 to 20 min. The dispersed cardiomyocytes were filtered and then collected by centrifugation at 200 × g for 10 min. Isolated cardiac cells ($2–4 \times 10^5$) were selected and placed into Petri dishes (35 mm i.d.) containing a few glass coverslips. The cells were maintained at 37°C Eagle's minimum essential medium (Eagle MEM) supplemented with 10% newborn calf serum in a humidified environment containing 95% air- 5% CO_2 for 24 hr.

Measurement of intracellular free calcium transients

Fura-2 loading was performed by the addition of fura-2/AM (3 μM dissolved in dimethylsulfoxide with 0.2% cremophor EL) into a petri dish containing 1 ml of culture medium (Eagle's MEM with 10% serum) and cells attached to glass coverslips. After mixing, the dishes were incubated in the dark for 1 hr in humidified 5% CO_2 – 95% air atmosphere at 37°C. The medium with fura-2/AM was subsequently removed, and the cells were rinsed three times with phosphate buffered saline and then reincubated with culture medium. In all experiments a coverslip with fura-2 loaded cells incubated in control medium [modified Eagle MEM without phenol red but supplemented with 5% serum containing 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)

(pH 7.4)] was first placed in a experimental chamber of 37 ± 0.5°C. Calcium paradox phenomenon were introduced by altering the incubation medium without or with Ca^{2+}. During the Ca^{2+} free phase of the calcium paradox, the cells were incubated in Ca^{2+}-free buffer, which contained the following constituents (in mM): NaCl, 137; KCl, 2.7; Na_2HPO_4, 8; KH_2PO_4, 1.5; ethyleneglycol-bis (-aminoethylether)-N,N′-tetracetic acid (EGTA), 2; HEPES, 10 (pH 7.4).

An INTER DEC M-1000 dual-wavelength fluorescent spectromicroscope system (Osaka, Japan) was used to evaluate $[Ca^{2+}]_i$. The results were generally presented as fura-2 ratios. The absolute concentration of intracellular Ca^{2+} was estimated for selected experiments. An *in vivo* calibration for fura-2 fluorescence in cultured myocardial cells was accomplished using the Ca^{2+} ionophore, ionomycin to obtain maximum fluorescence, and EGTA (5 mM) to determine minimum fluorescence. The background was determined using non-loaded cells and was subtracted from the data obtained with fura-2 loaded cells. The $[Ca^{2+}]_i$ concentration was calculating using the equations originally developed by Grynkiwiecz et al. (1985). Dye leakage from the cells was not detected 1 hr after initiating the experiments. During the course of one cycle of contraction and relaxation, the 340/380 fluorescence ratio increased to a maximum value during systole {Max or "a" in Fig. 1(A′)}, and fell to a minimum value during diastole {Min or "b" in Fig. 1(A′)}. The calcium transient (Ca-T) depicted in Fig. 1(A′) accompanies a spontaneous beating and represents the difference between the Max and Min values. The interbeat interval (IV) depicted in "d" of Fig. 1 represents the time interval between one Max point and the next. All data are expressed as Max, Min, Ca-T and IV and represent the mean of 10 beats. The variations in IV are used as a measure of arrhythmic beating.

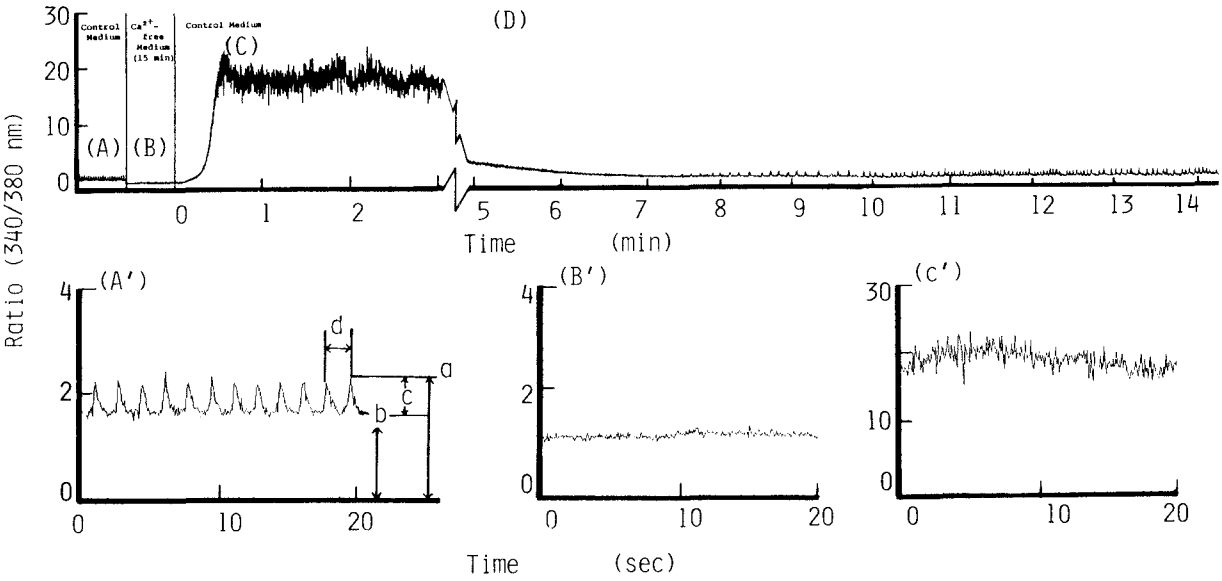

Fig. 1. Time course of intracellular calcium concentration alterations during the calcium paradox. Cardiomyocytes were preincubated in normal medium (**A**), exposed to Ca^{2+}-free medium for 15 min (**B**) and then re-exposed to medium containing normal Ca^{2+} (**C** & **D**). The data are expressed as ratio of 340 nm/380 nm. Inserts (**A′**)–(**C′**) represent the data using an expanded scale of A–C, respectively. In (A′), "**a**" or Max refers to the peak systolic value, "**b**" or Min represents the diastolic value and "**c**" or $[Ca^{2+}]_i$ transients (Ca-T) is the difference between "**a**" and "**b**". The interbeat intervals (IV) depicted in "**d**" represents the time interval between one Max value and the next value. All data are expressed as Max, Min and Ca-T and represent the mean of 10 beats

Morphological evaluation

The morphological status of the cardiomyocytes was monitored with an inverted phase-contrast microscope and videomonitor at magnifications of 150 to 400 in a chamber controlled at 37°C. Morphological changes, such as formation of blebs or ballooning of the cell membrane, were observed upon restoration of Ca^{2+}. The data were expressed as percent of the cells exhibiting morphological changes (the cells showing morphological changes/total observed cells.)

Statistics

Statistical significance was determined by Student's t-test or X^2-test, depending on the design of the experiment. Statistical analysis of IV variance was performed using Bartlett's test. Each value was expressed as mean ± S.E.M. Differences were considered significant when the calculated P values was less than 0.05.

Reagents

Reagents were purchased from the following sources: taurine, cremophor EL, EGTA from Nacalai tesque, Kyoto, Japan; trypsin (1:250) from Difco Laboratories, Detroit, Mich., USA.; collagenase (type I) from Sigma Chemical Co., St. Louis, Mo., USA.; Eagle's MEM medium and Dulbecco phosphate buffered saline (PBS) from Nissui Seiyaku, Tokyo, Japan; newborn calf serum from GIBCO, Madison USA; Fura-2, Fura-2/AM and HEPES from Dojin Chemicals, Kumamoto, Japan. Other agents were of analytical reagent grade.

Results

The spatial and temporal dynamics of $[Ca^{2+}]_i$ of cultured myocardial cells during the calcium paradox

Figure 1(A–D) represents typical data for the change in $[Ca^{2+}]_i$ of cultured cardiomyocytes during the calcium paradox. In these experiments, the calcium paradox refers to the events and reactions which take place within isolated myocytes directly after reintroduction (C, D) of calcium into incubation medium following a period of calcium-free exposure (B). Cardiomyocytes, which are incubated in control medium containing 2 mM Ca^{2+}, beat spontaneously and exhibit typical calcium transients (Fig. 1-A, A′). The average data of calculated Max and Min values were 423 nM and 165 nM, respectively. These values are comparable to the $[Ca^{2+}]_i$ transients reported in isolated cardiac myocytes (Thandroyen et al., 1991). Figure 2 represents a pseudocolor image demonstrating the spatial distribution of $[Ca^{2+}]_i$ in beating, fura-2 preloaded myocytes incubated with control medium. The rainbow color scale on the right of the panel corresponds to the fluorescence ratio of $[Ca^{2+}]_i$. During systole, white and red regions, which represent higher $[Ca^{2+}]_i$, occupy large areas of the cell (Fig. 2A). In contrast, during diastole the fluorescence intensity and the area of white spots, are reduced (Fig. 2A). The fluorescence ratio image reveals discrete, as well as clustered, white regions. When myocardial cells are exposed to Ca^{2+}-free buffer containing EGTA for

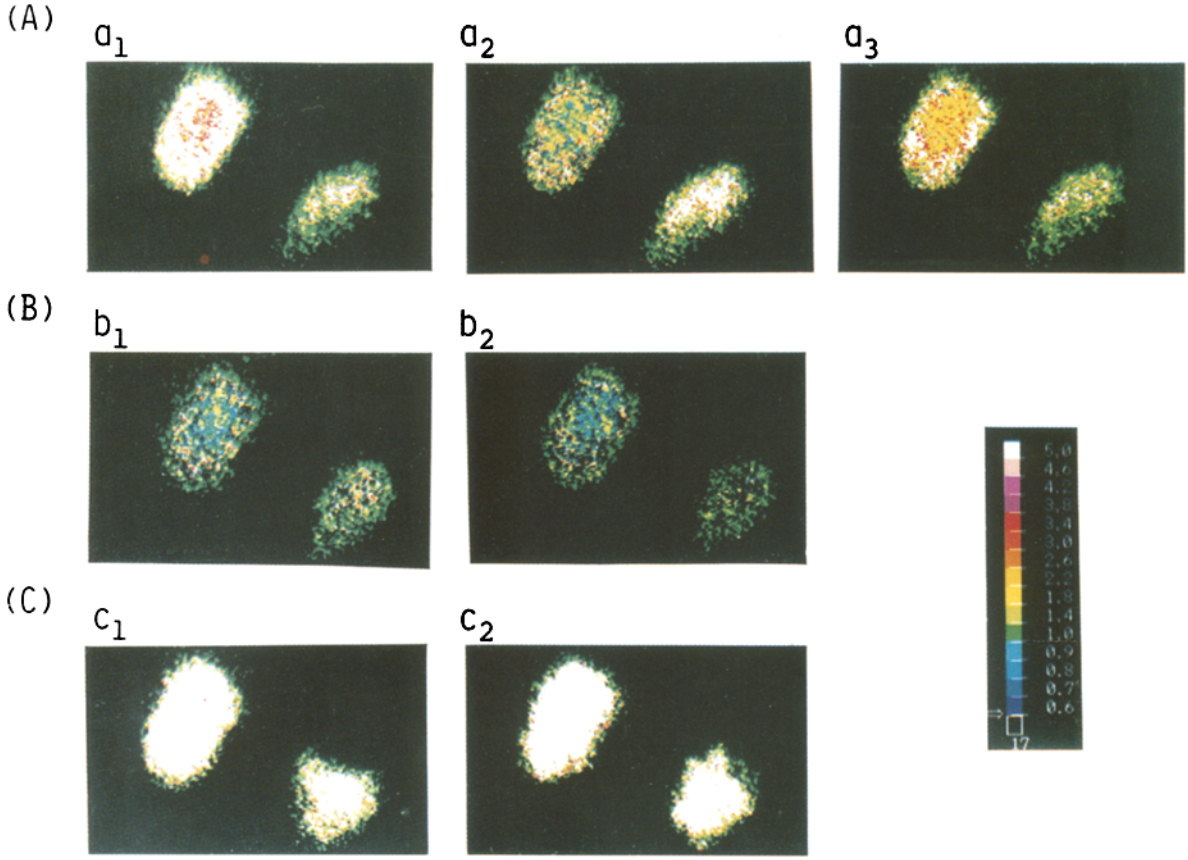

Fig. 2. Pseudocolor fluorescence ratio image (340/380 nm) of fura-2 loaded cardiomyocytes during calcium paradox. Cardiomyocytes were preincubated in normal medium, exposed to Ca^{2+}-free medium for 15 min and then re-exposed to original Ca^{2+} containing medium. (**A**) Control incubation; The myocytes loaded in the left hand frame of $\mathbf{a}_1$ and $\mathbf{a}_2$ is in peak systole and diastole, respectively. The cell loaded in the right hand frame or each block reaches peak systole in $\mathbf{a}_2$ and diastole in $\mathbf{a}_3$. (**B**) Ca^{2+}-depletion phase; 5 sec, ($\mathbf{b}_1$) and 15 min ($\mathbf{b}_2$) after introduction of Ca^{2+} free medium. (**C**) The Ca^{2+}-repletion phase; 7 sec ($\mathbf{c}_1$) and 55 sec ($\mathbf{c}_2$) after re-exposure to Ca^{2+} containing medium. The rainbow color scale on the right corresponds to the fluorescent ratio of $[Ca^{2+}]_i$

15 min, there is immediate cessation of both spontaneous beating and calcium transients (Fig. 1B, B′). Within a short period of time, $[Ca^{2+}]_i$ falls to a level of 1.2–1.4, which corresponds to an intracellular concentration of 3–6 $\times$ 10^{-8} M (Table 1). The degree of Ca^{2+} loss is apparent in the region beneath the cell membrane (Fig. 2B). Restoration of calcium-containing medium increases the $[Ca^{2+}]_i$ values of Max and Min from 3.21 to 18.7 and 1.74 to 13.2 within 1 min, respectively (Table 1). The spatial dynamics of $[Ca^{2+}]_i$ change dramatically. Large increases in $[Ca^{2+}]_i$ are observed in most cells, and are accompanied by the development of contracture and the formation of blebs (Fig. 2C). Moreover, major fluctuations in $[Ca^{2+}]_i$ are observed during the first few minutes of the Ca^{2+} reintroduction step (Fig. 1C). However, all of these changes are reversible. $[Ca^{2+}]_i$ decreases gradually and normal morphology is

Table 1. The effect of taurine on intracellular calcium dynamics of cultured myocardial cells during calcium paradox

Time (min)	Taurine 20 mM	Fluorescence ratio (340/380 nm) Max	(Ratio increase)	Min	(Ratio increase)
Control (0)	−	3.21 ± 0.73		1.74 ± 0.30	
	+	3.09 ± 0.44		1.66 ± 0.33	
Ca^{2+}-depletion	−	1.35 ± 0.26	(0)	1.17 ± 0.24	(0)
(15 min)	+	2.01 ± 0.53	(0)	1.32 ± 0.28	(0)
Ca^{2+}-repletion[a]	−	18.7 ± 1.72	(17.3 ± 1.53)	13.2 ± 1.64	(12.0 ± 1.51)
(less 1 min)	+	9.94 ± 2.12*	(7.92 ± 1.74**)	5.93 ± 1.23**	(4.64 ± 0.98**)
1	−	16.5 ± 1.99	(15.1 ± 1.77)	12.0 ± 1.65	(10.8 ± 1.49)
	+	8.65 ± 1.70*	(6.64 ± 1.27**)	5.38 ± 1.00**	(4.08 ± 0.79**)
2	−	14.8 ± 2.50	(13.5 ± 2.34)	11.1 ± 1.94	(9.80 ± 1.68)
	+	7.69 ± 1.75*	(3.78 ± 0.94*)	5.11 ± 1.18*	(3.78 ± 0.94*)

Myocytes were incubated for 15 min in medium containing 20 mM taurine prior to initiation of the experiment. They were then allowed to undergo the calcium paradox as described in Fig. 1. Max, Min and Ca-T are defined according to the procedure outlined in Fig. 1. *Ratio increase:* Each value represents the mean net increase in the 340/380 nm fluorescence ratio (Max and Min) following the switch to a Ca^{2+}-containing medium as described in Methods.

[a]represents peak $[Ca^{2+}]_i$ which was reached less than 1 min after Ca^{2+} repletion. Each point represents the mean ± S.E.M. of 5 experiments. Asterisks indicate significant difference from the untreated group (*: $P < 0.05$, **: $P < 0.01$).

eventually restored. Recovery of spontaneous beating and of calcium transients is observed after about 7 min (Fig. 1D). These phenomena occur independently of the beating rate over a range of 29–145 beats/min.

The effect of taurine on intracellular free calcium dynamics of cultured myocardial cells during the calcium paradox

The effect of taurine on $[Ca^{2+}]_i$ changes during the calcium paradox is shown in Table 1. We previously reported that taurine uptake by cardiomyocytes reached a maximum by 15 min at a dose of 20 mM (Takahashi et al., 1988). Based on these results, we decided to examine the effect of 15 min of taurine exposure on $[Ca^{2+}]_i$ and myocyte beating rate. The incubation medium was supplemented with 20 mM taurine throughout the course of the experiment. During the Ca^{2+}-free phase, taurine caused no detectable effect on $[Ca^{2+}]_i$. Within 1 min after restoration of Ca^{2+}, $[Ca^{2+}]_i$ reached maximal levels in both taurine-treated and untreated cells. However, taurine promoted a reduction in Max and Min from 18.7 to 9.9 and from 13.2 to 5.9, respectively. After 5 min of Ca^{2+} restoration, $[Ca^{2+}]_i$ in taurine-treated myocytes returned to the initial normal level and all cells recovered their calcium transients and spontaneous beating patterns (Table 2).

In non taurine-treated cells, 10 min of Ca^{2+} restoration, was required for the $[Ca^{2+}]_i$ to reach the normal range while the rhythmical beating pattern only recovered after 15 min of Ca^{2+} restoration. Taurine-treated myocytes recovered their rhythmical beating pattern earlier than non-treated myocytes, as evidenced by differences in IV after 10 min of Ca^{2+} restoration (Table 2).

In addition, we examined the morphological changes of cardiomyocytes at peak $[Ca^{2+}]_i$ during the Ca^{2+} repletion phase of the calcium paradox (Fig. 3). Morphological changes, such as the formation of blebs or ballooning of the cell membrane, were observed in about 50% of cells upon restoration of Ca^{2+} (Fig. 3, Table 3). Taurine significantly decreased the number of morphologi-

Table 2. Improvement in beating pattern of myocytes by exposure to taurine during Ca^{2+}-repletion phase of calcium paradox

Time (min)	Taurine 20 mM	Beating cell ratio (%)	Beating rate (beats/min)	Ca-T Ratio (340/380 nm)	$[Ca^{2+}]_i$ IV (sec)
Control (0)	−	5/5 (100)	70 ± 22	1.47 ± 0.49	1.19 ± 0.30
	+	5/5 (100)	61 ± 19	1.43 ± 0.15	1.73 ± 0.55
5	−	0/5 (0)	0	−	−
	+	5/5** (100)	25 ± 9*	0.85 ± 0.20	2.92 ± 0.67
10	−	4/5 (80)	34 ± 30	0.81 ± 0.20	7.92 ± 4.24
	+	5/5 (100)	62 ± 26	1.18 ± 0.34	2.09 ± 0.76#
15	−	5/5 (100)	47 ± 22	1.06 ± 0.20	2.24 ± 0.69
	+	5/5 (100)	73 ± 26	1.34 ± 0.56	1.75 ± 0.80

Myocardial cells treated with taurine were incubated for 15 min in medium containing 20 mM taurine prior to initiation of the experiment. Data shown represent measurements during exposure to control medium and 5–15 min following Ca^{2+} restoration. The beating cell ratio represents the number of myocytes beating following Ca^{2+} restoration/number of myocytes beating in control medium. The beating rate and the time the interval of beating are expressed as the mean ± S.E.M. of 5 experiments. *Ca-T* with spontaneous beating was defined the difference between the Max and Min values. Interbeat interval (*IV*) is defined in Fig. 1 (A′). Variations in IV were used as a measure of arrhythmic beating. Statistical analysis of IV variance was performed using the Bartlett's test (#: $P < 0.01$). Significant differences from the non-treated cells by X^2-test (*: $p < 0.05$, **: $p < 0.01$).

Table 3. Improvement in myocardial cell morphology by exposure to taurine during the calcium paradox

	Morphological changes (%)
Control	128/259 (49.4)
Taurine 20 mM	128/339* (37.8)

Morphological changes, such as formation of blebs or ballooning of the cell membrane, were observed upon restoration of Ca^{2+}. Myocardial cells were incubated for 15 min in medium containing 20 mM taurine prior to initiation of the experiment. The calcium paradox protocol was carried out as described in Fig. 1 except all buffer contained 20 mM taurine in the taurine treated group. Asterisks indicate a significant difference from the control group (*: $p < 0.01$).

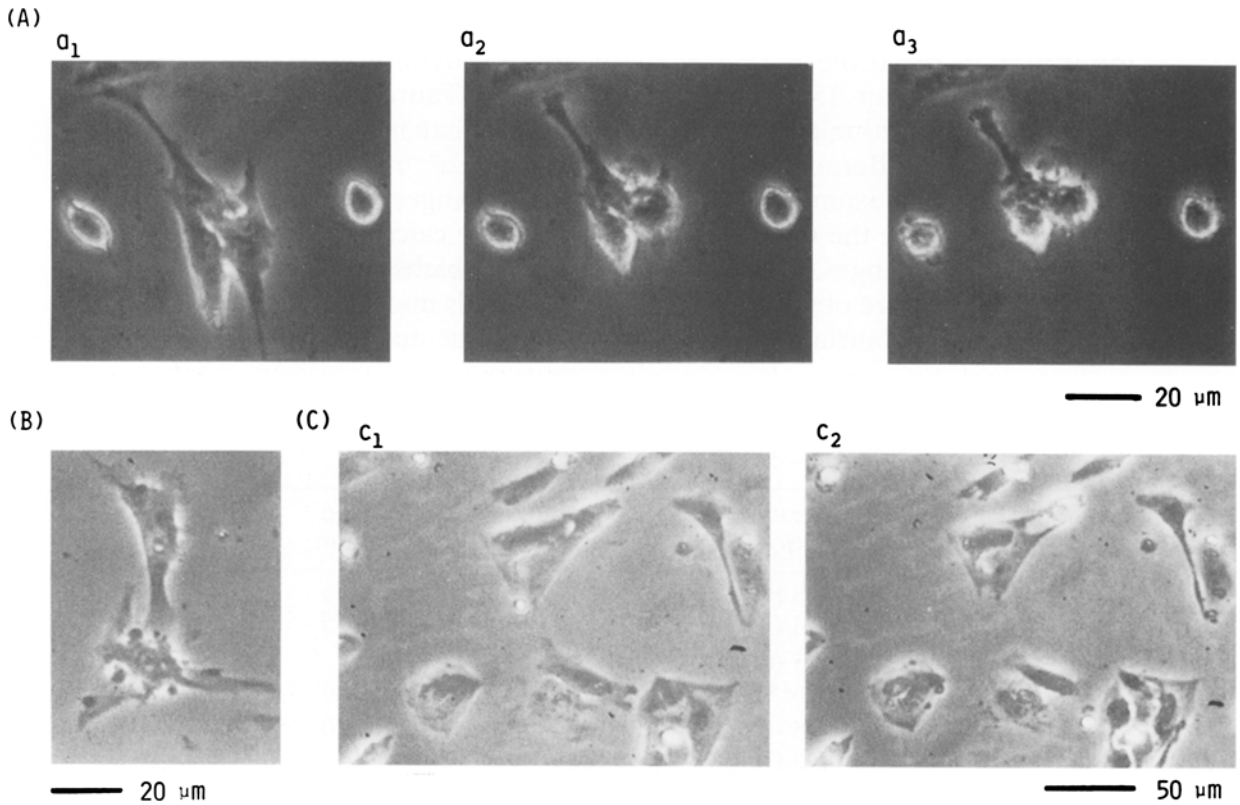

Fig. 3. Characteristic morphological changes induced by high intracellular calcium concentration during the calium paradox. Morphological changes, such as the formation (allows) of blebs or ballooning of the cell membrane, were observed in about 50% of the myocytes upon restoration of Ca^{2+} (**A**,**B** and **C**). Myocytes maintained in control medium are shown in $\mathbf{a}_1$ and $\mathbf{c}_1$ while hearts undergoing the calcium paradox are shown in $\mathbf{a}_2$, $\mathbf{a}_3$, **B** and $\mathbf{c}_2$

cally abnormal cells from 49.4% to 37.8% (Table 3). In both untreated and taurine-treated myocytes, the morphological changes were reversible and the cells gradually regained their normal shape and normal beating pattern.

Discussion

High temporal resolution is required to study physiological and/or pharmacological properties of $[Ca^{2+}]_i$ transients occur so rapidly. In the present study, we have continuously measured dynamic changes in $[Ca^{2+}]_i$ in a fura-2 loaded, single beating myocyte. Although values obtained for $[Ca^{2+}]_i$ in this study were similar to those previously published (Dolara et al., 1976; Burton et al., 1990), variations in calibration and loading between different laboratories are known to cause slight differences in the absolute levels of $[Ca^{2+}]_i$; therefore our data were mainly expressed as the fura-2 340 nm/380 nm fluorescence ratio. Generally, the most useful parameters were Max, Min, Ca-T and IV

[defined in Materials and methods and Fig. 1(A′)], because they revealed the clearest changes in $[Ca^{2+}]_i$.

The calcium paradox in isolated myocytes is characterized by a rapid uncontrolled entry of Ca^{2+} repletion phase. Although the gain in $[Ca^{2+}]_i$ is of critical importance, the source of extra Ca^{2+} has not been fully established. The known routes of Ca^{2+} influx include the voltage-dependent Ca^{2+} channel, the Na^+-Ca^{2+} exchanger, passive diffusion and abnormal sites of Ca^{2+} entry (Chapman and Tunstall, 1987; Ruigrok, 1990; Chatamra and Chapman, 1996).

The present study emphasized the importance of measuring spatial and temporal changes in $[Ca^{2+}]_i$ with a high degree of resolution to clarify the potential role of altered $[Ca^{2+}]_i$ in the development of calcium paradox-induced cell injury. A typical pattern of Ca^{2+} transients was observed using normal cardiomyocytes. Figure 2 clearly demonstrates that the spatial distribution of $[Ca^{2+}]_i$ within beating myocytes was heterogeneous and that the degree of heterogeneity was enhanced during diastole. During Ca^{2+} depletion, there was an immediate decrease in $[Ca^{2+}]_i$ with the largest decline occurring in the region beneath the cell membrane.

It is generally accepted that the initial event triggering the series of reactions leading to the calcium paradox is calcium overload attributed to either an alteration in Ca^{2+} permeability and fluidity of the cell membrane during the Ca^{2+}-free period, damage to key Ca^{2+} transporters or accumulation of $[Na^+]_i$ resulting in Ca^{2+} influx via the Na^+-Ca^{2+} exchanger (Grinwald and Nayler, 1981; Makino et al., 1988; Rodrigo and Chapman, 1991; Chatamra and Chapman, 1996). Although we did not examine the mode of Ca^{2+} accumulation in this study, our results clearly showed that restoration of medium Ca^{2+} dramatically increased $[Ca^{2+}]_i$ and cellular regions containing maximal $[Ca^{2+}]_i$. This enormous increase in $[Ca^{2+}]_i$ was associated with the appearance of contracture and blebbing of the myocytes. However, unlike the isolated heart, in cultured myocytes, these changes were confirmed to be reversible.

The immature hearts are relatively resistant to the calcium paradox (Rudge and Duncan, 1987; Lagerstrand et al., 1983). Although differences in structure, function, metabolism, and pharmacological properties exist between adult and immature myocytes, age-related differences in the architecture of the intercalated disks and Ca^{2+}-handling capacity of the sarcolemma and sarcoplasmic reticulum seem particularly important relative to the calcium paradox (Chapman and Tunstall, 1987; Heylinger et al., 1988; Naylar, 1991) Moreover, we have reported that myocardial taurine content is age-dependent and linked to the severity of calcium paradox-induced injury in post-hatched chicks (Takihara et al., 1988). Intracellular taurine also may be a contributory factor.

In the present study, it was shown that taurine improved the condition of cardiomyocytes subjected to the calcium paradox. Upon reintroduction of Ca^{2+} following a period of Ca^{2+}-free exposure, myocytes which had pretreated for 15 min with 20 mM taurine recovered their spontaneous beating pattern and exhibited calcium transients earlier than untreated cells (Table 2). The taurine-treated myocytes also accumulated less $[Ca^{2+}]_i$, an effect which may account for the early recovery of beating (Table 1, 2). In addition taurine

treatment attenuated the genesis of morphological changes in cardiomyocytes induced by the calcium paradox (Table 3). These results suggest that taurine plays an important role in altering calcium movement during the calcium paradox.

It has been suggested that taurine regulated myocardial Ca^{2+} homeostasis through direct or indirect modulation of several key calcium transports (Schaffer et al., 1992). Our data reveals that taurine causes no detectable effect on $[Ca^{2+}]_i$ during Ca^{2+}-free exposure.

In summary, (a) Taurine has no significant effect on $[Ca^{2+}]_i$ during Ca^{2+} depletion; (b) Taurine inhibits excessive $[Ca^{2+}]_i$ accumulation during the Ca^{2+} repletion phase of the calcium paradox; and (c) Taurine attenuated the development of morphological and beating abnormalities as a result of the calcium paradox.

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