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TAURINE PREVENTION OF CALCIUM PARADOX-RELATED DAMAGE IN CARDIAC MUSCLE

ITS REGULATORY ACTION ON INTRACELLULAR CATION CONTENTS

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Abstract—The present study was designed to investigate in chick heart whether oral pretreatment with taurine or taurine added directly to the perfusate has any effect upon calcium paradox-induced heart failure. In both protocols, taurine significantly reduced the mechanical dysfunction resulting from the calcium paradox. Taurine pretreatment partially inhibited the excess accumulation of calcium in the myocardium that occurs upon calcium repletion, and microscopy revealed almost normal structure. This protective effect of taurine was accompanied by (a) reduction of the gain of sodium content that occurs during calcium depletion, and (b) reduction of the late gain in calcium that occurs during calcium repletion. It is proposed that taurine plays a role in the regulation of calcium homeostasis and membrane stabilization.

Intracellular calcium overload is a common feature of irreversibly damaged tissue [1]. The "calcium paradox" phenomenon, first described by Zimmerman and Hulsmann [2], occurs when hearts are reperfused with calcium after a short period of calcium-free perfusion. The Ca^{2+} repletion causes irreversible myocardial damage, characterized by loss of electrical activity, extensive ultrastructural damage [3, 4], depletion of high-energy phosphates [5], massive release of intracellular constituents [2], and gain in Na^+ and Ca^{2+} [1, 6]. Several interventions, e.g. hypothermia [7], lowered extracellular pH [8], reduced extracellular Na^+ [4, 6, 9], addition of other divalent cations (Ba^{2+}, Cd^{2+}, Mg^{2+}, Mn^{2+}) [10, 11], or addition of some pharmacological agents (verapamil, diltiazem, taurine, phenothiazines) [12–15], have been shown to provide some protection against Ca^{2+} overload.

Taurine (2-aminoethanesulfonic acid) exists in relatively high concentration in the hearts of animals [16]. Taurine has been proposed to maintain cardiac osmolarity [17], to be antiarrhythmic in isoproterenol- and digoxin-induced arrhythmia [18], and to antagonize the positive inotropic action of high Ca^{2+} medium [19] and the negative inotropic action of low Ca^{2+} medium [20]. The effect of taurine on ion movements, such as Ca^{2+} [19] and K^+ [21], has been considered as a possible means through which taurine exerts its effect.

A decreased taurine level is found in hearts subjected to the calcium paradox. The degree of taurine depletion correlates with the severity of mechanical dysfunction after Ca^{2+} repletion [13]. It was also reported that the addition of taurine to the perfusate protected against the loss of mechanical function and prevented both the large decline in sarcolemmal ATPase activities and the increase in sarcolemmal Ca^{2+} binding [13]. In hatched chick heart, myocardial taurine content has been found to have a close relationship with an age-dependent response to the calcium paradox [22].

In this study, we examined how myocardial taurine is linked to the calcium paradox-induced failure in perfused chick hearts. Our findings show that the protective effect of taurine is accompanied by attenuation of the intracellular Na^+ elevation which occurs during the Ca^{2+}-free perfusion period and by reduction of the intracellular Na^+ and Ca^{2+} gain which occurs during Ca^{2+} repletion.

METHODS

Perfusion techniques. Hearts from 2- and 7-day-old post-hatched chicks, maintained on a standard diet, were used. The hearts were perfused by the conventional Langendorff method, using reservoirs (located 60 cm above the heart) containing the perfusing solution at 37°. The hearts were also bathed in the effluent from the coronary sinus (also heated to 37°). The normal perfusing solution had the following constituents (in mM): NaCl, 137; KCl, 3.8; $MgCl_2$, 1.2; NaH_2PO_4, 1.06; $NaHCO_3$, 20; $CaCl_2$, 1.8; and glucose, 5.55; and was equilibrated with a

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95% O_2–5% CO_2 gas mixture (pH 7.35). For Ca^{2+}-free perfusion, 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) was added to the Ca^{2+}-free solution to ensure that it was free of contaminant Ca^{2+}. No correction was made for the small difference in osmolarity when Ca^{2+} was omitted from the normal perfusing solution. A 15-min period of control perfusion was followed by 10–18 min of Ca^{2+}-free perfusion; subsequently, 0–10 min of Ca^{2+} repletion was done according to the experimental protocol (with normal perfusing solution containing 1.8 mM Ca^{2+}). All hearts were electrically paced throughout this procedure at 250 beats/min.

A force displacement transducer (Nihon Koden TB-611) was used to measure contractions by means of a thread sutured to the apex of the ventricle. The resting tension was adjusted 1 g at the start of each experiment. The mechanical recovery during Ca^{2+} repletion was determined by the percentage of the value for the same heart immediately prior to the Ca^{2+} depletion.

When taurine was being tested, 10 mM taurine was added to the perfusing solution during both the Ca^{2+} depletion and repletion periods. For taurine-pretreated chicks, a 5% (w/v) taurine solution (2 mg/kg) was administered daily from the fourth to the seventh day after hatching by means of a cannula inserted into the esophagus; the control chicks received a 5% (w/v) sucrose solution.

Ca^{2+}, Na^+ and H_2O determinations. At the end of the appropriate perfusion sequence, the coronaries were flushed through with 10 ml of ice-cold sucrose (0.35 M)–histidine (5 mM) solution, pH 7.4, as described by Alto and Dhalla [6] to minimize contamination from the extracellular compartment. After flushing, the atria and visible connective tissue were removed. The ventrical was blotted, weighed, and dried at 100° for 24 hr, and tissue H_2O content was calculated.

Tissue cation content was determined by atomic absorption spectrophotometry (Hitachi model 170-50) using the method of Sanui [23]. Briefly, dried ventricles were subjected to concentrated HNO_3 and $HClO_4$, and then evaporated to dryness. For the Ca^{2+} determination, $LaCl_3$ was added; for the Na^+ determination, CsCl was added to the residue.

Analytical reagent grade chemicals were used throughout the study, and care was taken to avoid Ca^{2+} contamination.

Taurine determination. Chicks were killed by decapitation, and the hearts were removed quickly. The ventricles were excised, washed in 0.9% (w/v) NaCl (ice-cold), and rapidly frozen. Homogenates were centrifuged at 3000 rpm for 10 min at 4° with 2 vol. of 10% (v/v) sulfosalicyclic acid. Taurine content in the supernatant solution was determined using a Hitachi 835-amino acid analyzer.

Electron microscopy. Seven-day-old chick hearts were used for ultrastructural analysis. Hearts were fixed by perfusion with 3% (v/v) glutaraldehyde prepared in 0.1 M sodium cacodylate buffer (pH 7.4). After 10 min of glutaraldehyde perfusion, the hearts were removed from the Langendorff apparatus, and biopsy specimens of left ventricle free wall were excised and cut into approximately 1-mm cubes. These were fixed further by immersion in the glutaraldehyde solution for 2 hr at 4°, and then post fixed in 1% (v/v) OsO_4 for 2 hr. Samples were stained *en bloc* with 3% (v/v) uranyl acetate, dehydrated in graded ethanol series, and embedded in epoxy resin. Ultrathin sections (50–70 nm thick) were cut on an LKB-7800 Ultratome, mounted on copper grids, and stained with uranyl acetate and lead citrate. Sections were examined with a JEM-100 CX microscope. Three hearts were used for each group and at least three blocks were taken from each heart. Five grids were cut per block, and ten fields per grid were photographed.

Statistical analysis. The results are presented as the mean ± SEM of N experiments. Test of significance was calculated by Student's *t*-test, or analysis of variance (Bonferroni's method was used to compare individual data when a significant F value was shown), depending on the design of the experiments. Differences were considered significant when the calculated P value was less than 0.05.

RESULTS

Mechanical function. In agreement with Yates and Dhalla [4], the degree of mechanical dysfunction resulting from the calcium paradox was dependent upon the duration of the Ca^{2+}-free perfusion (Table 1). In 2- and 7-day-old chick hearts, the recovery of contractile force after Ca^{2+} repletion was less, the greater the period of Ca^{2+}-free perfusion. No recovery of contractility was found when the Ca^{2+}-free perfusion period was longer than 15 min in 7-day-old hearts. In contrast, substantial recovery of contractility was observed in 2-day-old chick hearts subjected to a 15-min period of Ca^{2+}-free perfusion. These results support a previous study which reported that the mechanical recovery from calcium paradox is age dependent [24].

Effect of taurine in the perfusate on calcium paradox. When 10 mM taurine was present in the perfusate during both the Ca^{2+} depletion and repletion periods, as shown in Tables 1, the percent recovery of contractile force upon Ca^{2+} repletion was increased significantly after 10, 12 and 15 min of Ca^{2+}-free perfusion ($P < 0.05$ vs control 7-day-old chick hearts respectively). The length of Ca^{2+}-free perfusion to provide 30% recovery was increased if taurine were added to the perfusate. Taurine had little effect when added to the perfusate of 2-day-old chick hearts, i.e. there was no significant difference in the percent recovery on contractile force between control and taurine-exposed chick hearts, measured after 10 min of Ca^{2+} repletion.

Effects of pretreatment with taurine on calcium paradox. To test whether an effect on taurine pretreatment on the calcium paradox could be demonstrated, hearts were used from 7-day-old post-hatched chicks pretreated with taurine. Taurine pretreatment significantly improved the mechanical recovery compared to the control (non-treated) 7-day-old chicks upon Ca^{2+} repletion after 12 min of Ca^{2+}-free perfusion (43.3 ± 10.3 vs 5.1 ± 2.5%; $P < 0.05$). Substantial recovery of contractions occurred upon Ca^{2+} repletion even after 18 min of Ca^{2+}-free perfusion.

Table 1. Recovery of contractile force in chick heart upon Ca^{2+}-repletion for 10 min after a variable period of Ca^{2+}-free perfusion

	Percent recovery of contractile force (%)			
	Time of Ca^{2+}-free perfusion			
	10 min	12 min	15 min	18 min
2-Day-old				
Control	50.0 ± 8.2 (8)	43.2 ± 10.0 (5)	30.8 ± 6.8 (8)	8.0 ± 4.7 (4)
Taurine in perfusate	ND	66.5 ± 15.4 (4)	47.7 ± 5.6 (7)	13.0 ± 6.7 (3)
7-Day-old				
Control	29.2 ± 9.4* (10)	5.1 ± 2.5* (18)	0* (4)	0* (3)
Taurine in perfusate	63.2 ± 13.1† (6)	30.4 ± 9.5† (7)	17.7 ± 8.9† (3)	ND
Taurine pretreated	ND	43.3 ± 10.3† (7)	35.5 ± 13.6† (6)	27.7 ± 14.2† (3)

Each value is the mean ± SE for the number of chicks given in parentheses. ND = not done.
* $P < 0.05$ vs 2-day-old control.
† $P < 0.05$ vs 7-day-old control.

Effect of taurine pretreatment on tissue cation and H_2O *contents during calcium paradox.* The time course of the increase in myocardial Ca^{2+} content during Ca^{2+} repletion, following a 15-min Ca^{2+} depletion period, is presented in Fig. 1. After 15 min of Ca^{2+} deprivation, both taurine-pretreated and non-treated hearts were decreased significantly in Ca^{2+} content from their control value (3.61 ± 0.32 vs 1.61 ± 0.19 μmol/g dry weight and 3.38 ± 0.14 vs 1.75 ± 0.91 μmol/g dry weight respectively; $P < 0.001$). Reintroducing normal perfusate containing 1.8 mM Ca^{2+} to these hearts resulted in a significant gain in Ca^{2+}. During the initial 0–7 min repletion period, a rate of Ca^{2+} gain into taurine-pretreated chick hearts was not significantly different from the control hearts, though the Ca^{2+} content was always lower in the taurine-pretreated hearts than in the control hearts. However, at the end of the 10 min repletion period with normal perfusate, the Ca^{2+} content of the taurine-pretreated chick hearts was substantially less than that of the control (6.42 ± 1.02 vs 12.19 ± 0.91 μmol/g dry weight; $P < 0.01$).

The tissue Ca^{2+}, Na^+ and H_2O contents of 2- and 7-day-old and taurine-pretreated 7-day-old chick hearts perfused with Ca^{2+}-free solution for 15 min and then reperfused with normal Ca^{2+} (1.8 mM) containing solution for 10 min are summarized in Table 2. The slightly different values observed by us in comparison to those previously reported for adult rabbit heart septum [9] or rat heart [6] are probably due to the different species and ages used. Taurine pretreatment of 7-day-old chicks did not alter significantly the tissue Ca^{2+}, Na^+ and H_2O contents in the control unperfused and control perfused hearts.

After a Ca^{2+}-free period of perfusion, a small, but

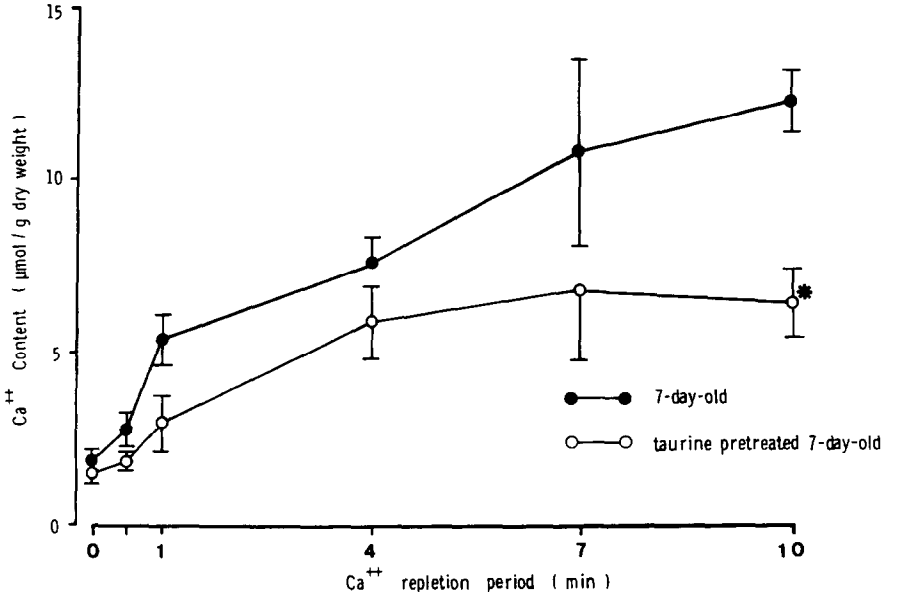


Fig. 1. Effect of taurine pretreatment on myocardial calcium content during calcium repletion after 15 min of calcium depletion. Each value is the mean ± SE of four to seven experiments. Key: *Significantly ($P < 0.01$) different from the 7-day-old control.

Table 2. Myocardial content of H_2O, N^+ and Ca^{2+}

	H_2O (g/100 g wet wt)	Na^+ (μmol/g dry wt)	Ca^{2+} (μmol/g dry wt)
2-Day-old			
Unperfused	81.5 ± 1.1 (10)	97.5 ± 7.7	3.62 ± 0.35
Control perfusion	82.8 ± 0.7 (4)	120.4 ± 17.0	4.67 ± 0.39
Ca^{2+}-free perfusion (15 min)	85.5 ± 0.4* (4)	236.1 ± 21.5†	1.54 ± 0.28‡
Ca^{2+} re-introduction (10 min)	83.0 ± 0.9 (4)	150.8 ± 8.5	9.31 ± 2.04
7-Day-old			
Unperfused	80.9 ± 0.6 (5)	91.3 ± 6.6	3.01 ± 0.16
Control perfusion	80.8 ± 0.5 (6)	128.6 ± 15.3	3.38 ± 0.14
Ca^{2+}-free perfusion (15 min)	84.8 ± 1.0† (7)	250.2 ± 18.1‡	1.75 ± 0.91‡
Ca^{2+} re-introduction (10 min)	84.4 ± 0.5† (7)	220.0 ± 10.3‡	12.19 ± 0.19‡
Taurine pretreated (7-day-old)			
Unperfused	80.6 ± 0.5 (5)	106.3 ± 12.2	3.12 ± 0.30
Control perfusion	83.7 ± 0.5 (7)	128.2 ± 8.0	3.61 ± 0.32
Ca^{2+}-free perfusion (15 min)	83.7 ± 0.8 (7)	183.4 ± 13.6†§	1.61 ± 0.19‡
Ca^{2+} re-introduction (10 min)	83.0 ± 1.0 (5)	181.1 ± 12.8†§	6.42 ± 1.02†‖

Each value is the mean ± SE for the number of chicks given in parentheses.
*–‡ Significantly different from the perfused control: *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.
§‖ Significantly different from the 7-day-old: §$P < 0.05$, and ‖$P < 0.01$.

significant, increase in H_2O occurred in 2- and 7-day-old control chick hearts, both of which had also gained Na^+ significantly during the Ca^{2+}-free perfusion [97% ($P < 0.01$) and 95% ($P < 0.001$) increases above control respectively]. In contrast, tissue H_2O did not change after Ca^{2+} depletion in taurine-pretreated chick hearts, and Na^+ content increased only 43% of control (95% increase in non-treated hearts). Following 10 min of Ca^{2+} repletion, H_2O dropped to control level only in the 2-day-old chick hearts, whereas it remained elevated in the 7-day-old chick hearts.

Upon Ca^{2+} repletion, tissue Ca^{2+} content further increased in 7-day-old chick hearts both in taurine-pretreated and non-pretreated hearts. Taurine pretreatment significantly lowered the tissue Ca^{2+} level after the Ca^{2+} repletion as previously described in Fig. 1. The 2-day-old chick hearts did not have a significant increase in Ca^{2+} and Na^+ contents upon Ca^{2+} repletion (compared to the perfused control) (Table 2).

Taurine content. There was an age-related decrease in myocardial taurine content in the post-hatched chicks (Table 3). Taurine-pretreated chicks had about a 20% increase in taurine level of the ventricular tissue compared to the non-treated 7-day-old chick hearts. Thus, orally-administered taurine antagonized the age-related decrease in myocardial taurine content.

Ultrastructural results. At the end of the 15-min stabilization period of perfusion with normal perfusing solution, the ultrastructure of the hearts was normal. The myofibrils were relaxed and well-aligned, the mitochondria were well preserved, and, the intercalated disks were closely apposed with tight junctions. Hearts perfused for 15 min with Ca^{2+}-free solution and reperfused for 10 min with 1.8 mM Ca^{2+} solution exhibited a very pale appearance upon gross examination. The hearts showed ultrastructural changes similar to those reported previously [4], including severe contraction bands with disruption of myofibrils, edema, and separation of the cell-to-cell junctions. Most mitochondria exhibited marked swelling and contained ruptured cristae (Fig. 2A). At higher magnification (×59,000), separation of the external membrane from the surface coat of the glycocalyx was shown, resulting in the formation of cell surface blebs (Fig. 2B).

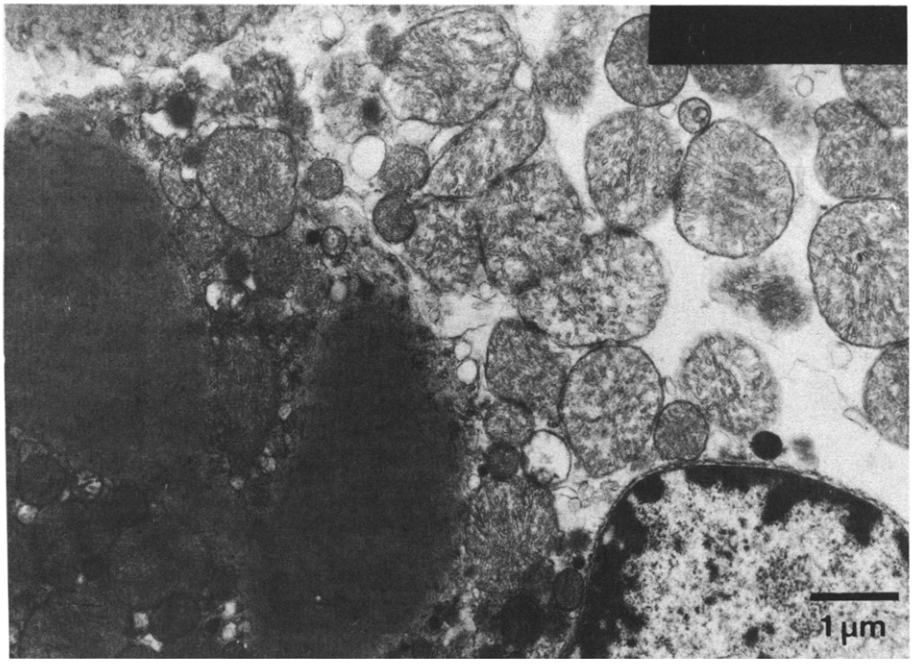
The gross appearance of the tissue from taurine-pretreated chick hearts following Ca^{2+} repletion was normal. Electron microscopy revealed that many myocardial cells had almost completely normal ultrastructure except for the slight interstitial edema (Fig. 3A). Myofibrils were in register, the mitochondria appeared normal, and the glycogen granules were

Table 3. Taurine content of post-hatched chick hearts

	Taurine content (μmol/g dry weight)
2-Day-old	268.8 ± 8.9 (13)
7-Day-old	222.5 ± 3.7* (11)
Taurine pretreated (7-day-old)	265.1 ± 5.3† (10)

Each value is the mean ± SE for the number of chicks given in parentheses.
* $P < 0.05$ vs 2-day-old.
†$P < 0.05$ vs 7-day-old.

(A)



(B)

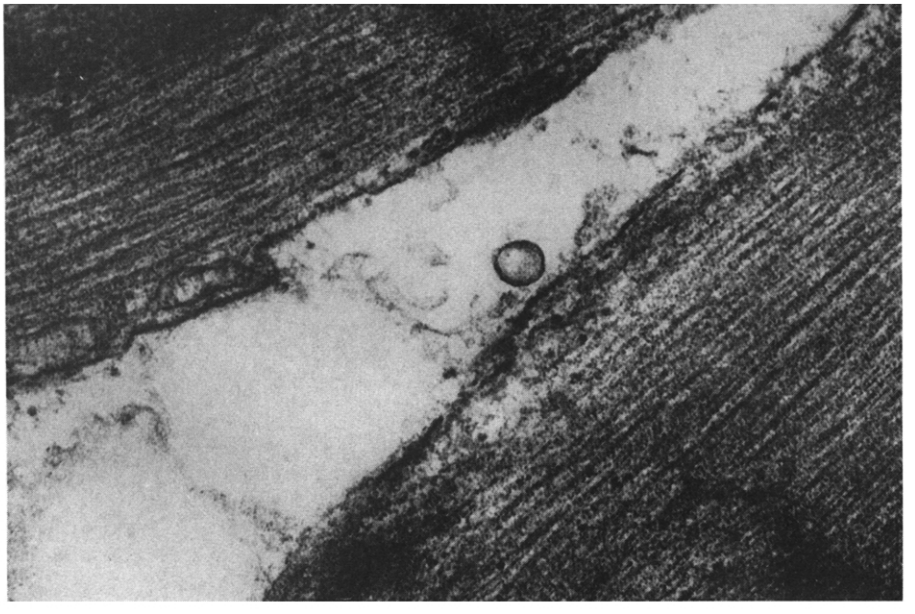
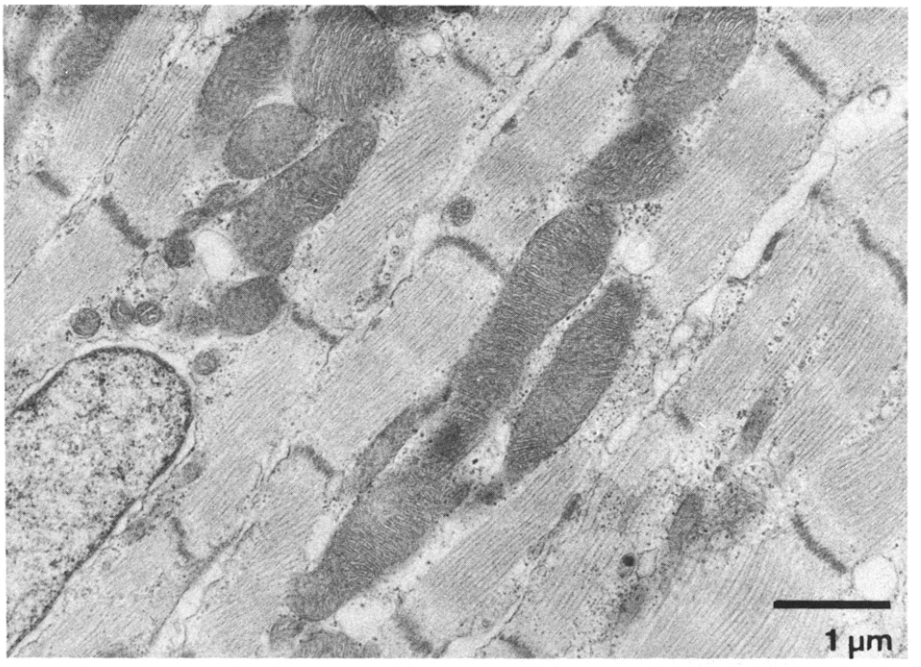


Fig. 2. Electron micrograph of a typical section from an isolated 7-day-old chick heart perfused with Ca^{2+}-free medium for 15 min and reperfused for 10 min with medium containing 1.8 mM Ca^{2+}. The figure shows severe structural damage. (A) ×8,300, and (B) ×59,000.

(A)



(B)

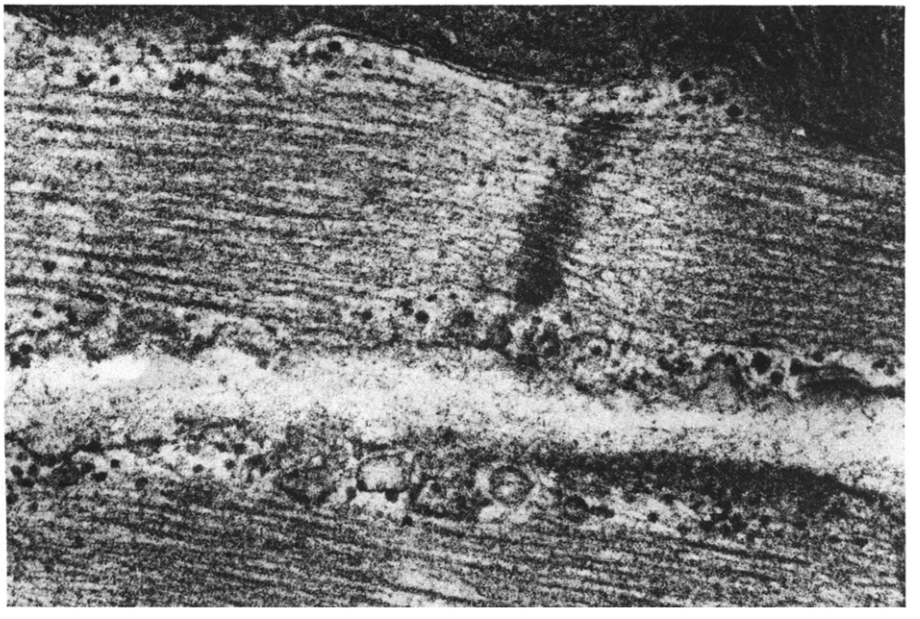


Fig. 3. Electron micrograph of a typical section from an isolated 7-day-old chick heart treated with 10 mM taurine throughout the 15 min Ca^{2+}-free and 10-min reperfusion period. (A) ×9,000, and (B) ×59,000.

present. There was no disruption in the integrity of the sarcolemma, which consisted of continuous unit membrane and glycocalyx (Fig. 3B).

DISCUSSION

Hearts perfused for 10 min with 1.8 mM Ca^{2+} after 10–18 min of Ca^{2+}-free perfusion had a slower recovery of contractile force. Massive Ca^{2+} overload after Ca^{2+} repletion was accompanied by extensive cellular and functional damage. These findings on avian hearts agree with the initial observation of Zimmerman and Hulsmann [2] on mammalian hearts; these authors termed this response the calcium pardox and attributed the phenomenon to excessive Ca^{2+} influx. In the present study, it was clearly shown that taurine treatment *in vitro* and pretreatment *in vivo* improved the state of the myocardium of hearts subjected to the calcium paradox. In the control non-treated hearts, both Ca^{2+} and Na^{+} contents were significantly higher upon Ca^{2+} repletion than the initial control levels. In taurine-pretreated hearts, the mechanical dysfunction produced was reduced, and accumulation of Ca^{2+} and Na^{+} was curtailed significantly compared with the control non-treated hearts. In addition, taurine pretreatment attenuated the extent of the gross morphological changes that resulted from the calcium paradox condition.

The calcium paradox has been studied and compared in isolated rat, rabbit, mouse, and guinea pig hearts [25]. Calcium paradox could be induced also in frog heart after 30 min of Ca^{2+}-free perfusion [26]. In the present study on 7-day-old post-hatched chick hearts, at least 15 min of Ca^{2+}-free perfusion was required to prevent completely the myocardium from generating contractile force upon Ca^{2+} repletion; however, impairment of recovery of contractile force was apparent after 10 min. The species and age used in this study may be the reason for the longer period of Ca^{2+}-free perfusion required to depress the contraction following Ca^{2+} repletion.

Although the gain in Ca^{2+} that occurs during Ca^{2+} repletion is of critical importance, its route of entry has not been fully established. The known routes of Ca^{2+} entry include the voltage-dependent Ca^{2+} slow channels, the Na^{+}–Ca^{2+} exchange mechanism, passive diffusion, and abnormal sites of Ca^{2+} entry [1]. Nayler *et al.* [27] proposed that the gain in Ca^{2+} that occurs during Ca^{2+} repletion has two phases (early and late); the early phase contains slow channel blocker-sensitive and -insensitive components, the latter probably involving Na^{+}–Ca^{2+} exchange. The late phase of Ca^{2+} gain was reported to be neither slow channel blocker nor Na^{+}–Ca^{2+} exchange sensitive, and is presumed to be passive diffusion through pathologically-altered membrane. In the present study, taurine significantly inhibited the late gain in Ca^{2+} on reperfusion, suggesting that taurine reduced the late entry of Ca^{2+} through non-physiological pathways that developed in association with the ultrastructural damage triggered by the early rapid increase of intracellular Ca^{2+}.

In fish ventricles, the rise in $[Na]_i$ during Ca^{2+} depletion is inhibited by Ca^{2+} channel blockers at concentrations that also block the development of Ca^{2+} repletion contracture [28]. Ca^{2+} channel blockers, however, are without effect on the Ca^{2+} repletion contracture if added after $[Na]_i$ has been raised [28].

What happens during Ca^{2+}-free perfusion is also of critical importance. The cellular Na^{+} ($[Na]_i$) increases during Ca^{2+}-free perfusion in rabbit interventricular septum, and the magnitude of the Na^{+} gradient at the end of the Ca^{2+}-free period, are important determinants of the extent of cell Ca^{2+} gain and reduction of contractile function upon Ca^{2+} repletion [9]. Low Na^{+} perfusion during Ca^{2+} depletion has a protective effect against the depression in contractile force and changes in cellular electrolytes upon Ca^{2+} repletion [4, 9]. The protective effect of taurine on the gain in Ca^{2+} during Ca^{2+} repletion was accompanied by a significant lowered level of Na^{+} (Table 2).

Kramer *et al.* [13] reported that taurine had no effect when present only in the Ca^{2+}-free buffer, but had a beneficial effect on calcium paradox if taurine was present either throughout the perfusion or only in the reperfusion buffer. They concluded that taurine does not protect against the biochemical changes that occur during the Ca^{2+}-free period. Since taurine uptake is inhibited markedly in Ca^{2+}-free medium [29], we employed hearts whose taurine content had been prior elevated by pretreatment. Pretreatment with taurine was found to have a superior protective effect compared to that when taurine was added to the perfusate during Ca^{2+} repletion. Thus, taurine may exert some additional effect when pre-elevated during the Ca^{2+}-free period.

Taurine was reported to play an important role in the maintenance of intracellular osmolar concentration in marine invertebrate [30], amphibian [31], and mammalian hearts [17]. However, in the present experiments, taurine pretreatment did not show any significant increase in H_2O content during Ca^{2+}-free perfusion compared to untreated chick hearts.

It is widely thought that the initial event that triggers the series of reactions that lead to the calcium paradox is an alteration in Ca^{2+} permeability of the cell membrane during the Ca^{2+}-free perfusion period [1]. The same time is required to flush Ca^{2+} out of the extracellular space and to remove Ca^{2+} from the membrane itself [2, 32]. Removal of Ca^{2+} from the lipid bilayer results in increased membrane fluidity [33]. In the present study, total Ca^{2+} content of the taurine-pretreated chick heart after 15 min of Ca^{2+} depletion was not significantly different from the untreated hearts (Table 2). Taurine was reported to delay the loss of Ca^{2+} from guinea pig heart during Ca^{2+}-free perfusion [19], and taurine was reported to modulate Ca^{2+} homeostasis in hearts through its interaction with the sarcolemma [34]. Therefore, another possible mechanism of action of taurine against the calcium paradox could be prevention of the non-specific permeability changes (resulting from Ca^{2+} loss from the sarcolemma during Ca^{2+}-free perfusion) and hence of the non-specific massive influx of Ca^{2+} upon Ca^{2+} repletion.

In conclusion, we have demonstrated by contractile, biochemical, and morphological measurements that taurine protects against myocardial damage caused by calcium paradox. Our studies are con-

sistent with the hypothesis that taurine plays a role in the regulation of calcium homeostasis and in membrane stabilization.

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