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Characterisation of the Calcium Paradox in the Isolated Perfused Pigeon Heart: Protection by Hypothermia, Acidosis and Alkalosis

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Key Words

Calcium paradox • Pigeon heart • Acidosis • Alkalosis • Hypothermia • Protection

Abstract

The aim of the present investigation was to examine the conditions inducing a calcium paradox in the isolated perfused pigeon heart. Loss of mechanical and electrical activity, creatine phosphokinase and total protein release were used to define cell damage. Perfusion was performed at 36, 38, 40 and 42°C and calcium deprivation lasted 5, 10, 20 or 40 min. At low temperatures even prolonged calcium depletion failed to induce a calcium paradox. After a 40 min calcium depletion at normal body temperature (42°C) ventricular activity ceased and a major contraction occurred followed by an increase in resting tension. During the 20-min reperfusion period the release of creatine phosphokinase was 267.18 ± 0.8 IU/g of dry wt and the total amount of protein loss was 109.3 ± 1.0 mg/g of dry wt, while lower temperatures resulted in a decreased loss of protein and creatine phosphokinase. Using two different Tyrode's perfusion buffers instead of normal bicarbonate ones, a protection of the pigeon heart against the induction of this phenomenon was observed. Furthermore, acidosis as well as alkalosis protected the heart as estimated by the significant recovery of electromechanical activity, and the quite low total protein and creatine phosphokinase losses. The results of this study suggest that the basic mechanisms and damaging effects of calcium overloading are common in mammalian and pigeon hearts.

Introduction

The calcium paradox, as originally defined by Zimmerman and Hulsmann (1966) [1] is induced by the successive perfusion of the isolated mammalian heart with calcium-free and calcium-containing medium and initiates the development of a severe tissue necrosis upon re-admission of calcium [2-7]. This phenomenon, is characterised by a massive leakage of various intracellular compounds, an immediate contracture and contractile failure, a loss of excitability, a rapid depletion of cellular high-energy phosphate compounds, mitochondrial and sarcolemmal disruption, and a marked rise in tissue Ca^{2+} [1-2, 6-11].

It is generally accepted that the induction of the calcium paradox in rat depends on the perfusion temperature and the duration of calcium depletion [12-13]. This

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phenomenon has been studied in the heart of various other mammalian species than rat such as dog, cat, rabbit, guinea pig, and mouse [10, 14-15] as well as in isolated cardiac myocytes of neonatal mouse, adult rat and in human myocardial strips [16-17]. It has been also studied in the heart of various ectotherms such as snake, fish and frog [18-20]. The results of these studies, although controversial, suggest that the mechanism of the calcium paradox induction in the heart appears to be common to ectotherms and mammalian hearts.

In spite of the systematic electrophysiological, metabolic, biochemical, and electron microscopy studies on the calcium paradox induction, very little is known to date about the precise molecular mechanisms involved in the occurrence of this phenomenon. However, the excessive accumulation of intracellular calcium by influx through the Na^+/Ca^{2+} electrogenic exchange [21] and the Ca^{2+}-selective sarcolemmal channels [22] as well as the contracture development are believed to be the two major factors which lead to a series of biochemical changes that culminate in cell death [6, 10].

Recent studies by several investigators have shown that hypothermia, various inorganic and organic calcium antagonists, low pH and adenosine, protect the heart of both, mammals and amphibians, against the calcium paradox occurrence [23-27].

In spite of the enormous studies on the characterisation of the calcium paradox as well as the protection mechanisms in the mammalian heart, none of the aspects concerning this phenomenon has been investigated in the avian heart. Pigeon is an endotherm animal, with body temperature of 42°C; is characterised by high systolic *(135 mm Hg) and diastolic (105 mm Hg) pressures and* by high resting cardiac rate (166 pulses/min) [28]. In contrast to mammals, cardiac output for a given body mass is higher in birds, whereas stroke volume is lower per gram of heart [29]. Overall, during a prolonged flight the pigeon cardiac output is much higher, giving rise to conditions of anaerobiosis. Under such conditions in vivo, the resistance of the heart against a calcium paradox is very important for this animal. In particular, during ischaemia, calcium depletion to some extent can occur and therefore upon calcium readmission a protection against this phenomenon must be very important as a physiological adaptation of the pigeon heart [30]. The aim of the present study was the characterisation of the calcium paradox in the isolated perfused pigeon heart as well as the protective role of hypothermia, acidosis and alkalosis, as the first step in the study of this phenomenon in birds.

Materials and Methods

Animals

Isolated hearts of the pigeon *Columba livia* were used. Domestic animals were kept in the laboratory with free access to water and food.

Experimental model

Pigeons (350-400 g of weight) were anaesthetised with sodium pentobarbital (30 mg per animal) and received heparin (400 IU) i.v. The hearts were excised and mounted onto the aortic cannula of a conventional Langendorff perfusion system. The temperature of the perfusates and the heart was maintained constant by the use of water-jacketed chambers. All hearts were perfused at a pressure of 10 kPa (70 mm Hg). Following the equilibration period for 15 min with Krebs-Henseleit's (KH) bicarbonate buffer solution which consisted of (in mM): 118 NaCl, 2.96 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose and 1 sodium pyruvate, the perfusion was switched for increasing times to another reservoir for calcium depletion. In the calcium-free medium calcium was omitted and EGTA was added at a final concentration of 10 μM to ensure removal of any contaminant calcium. After the end of calcium depletion, the hearts were re-perfused for 20 min with normal KH. All KH buffers were equilibrated with 95% O_2-5% CO_2.

In another series of experiments, the hearts were perfused with Hepes or Tris Tyrode's modified medium instead of normal KH buffer. When Hepes was buffer, a solution containing (in mM):10 Hepes, 120 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 1 sodium pyruvate was adjusted at 42°C to the desired pH by using 10 M-NaOH and a pH-meter calibrated between and 7.00 and 10.00. Solid NaCl was then added to give a total Na^+ concentration of 140 mM. When Tris was buffer, a solution containing (in mM): 10 Tris-base, 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 *$CaCl_2$, 10 glucose, and 1 sodium pyruvate was adjusted at 22-*25°C to the desired pH by using 12 M-HCl. In the calcium-free medium calcium was omitted and EGTA was added at a final concentration of 10 μM. All Tyrode's buffers were equilibrated with 100% O_2.

In these experiments hearts were equilibrated by perfusion with normal either Hepes or Tris/HCl Tyrode's buffer, pH 7.3 for 15 min. Subsequently, the hearts were subjected to calcium depletion by perfusion with the same Tyrode's buffer of the desired pH (7.0, 7.3 or 8.0) for 40 min. Finally, the hearts were re-perfused for 20 min with the respective normal Tyrode's buffer.

Electromechanical recordings

Contractile activity was measured by means of a force displacement transducer (Grass FT03C), which was connected to the apex of the heart. Mechanical activity was quantitated in terms of tension (in g) developed by the heart. Continuous ECG measurements of intracardiac activity were performed as previously described [20].

Biochemical methods

Samples of the effluent perfusate were collected at timed intervals and the protein content of the samples was estimated by using the method of Bradford [31]. In the same samples creatine

phosphokinase activity was determined by the method previously described [32].

Data analysis

The results are presented as mean±SE of 4-6 independent experiments. The cumulative amount of protein and/or creatine phosphokinase activity lost from myocardial cells was determined by specifically multiplying the protein concentration or creatine phosphokinase activity with the effluent volume during each time interval and dividing by tissue dry weight. The statistical significance was determined by using the Student's t-test at $P<0.05$ level of confidence.

Results

Effect of duration of Ca^{2+}-depletion

In control experiments, pigeon hearts were equilibrated with normal KH buffer, at 42°C, for 15 min, followed by Ca-free perfusion which lasted 5, 10, 20, or 40 min and the protein content and creatine phosphokinase activity were measured in the effluent perfusate. During the equilibration period, and the following Ca^{2+}-free period, only small amounts of protein were detected in the effluent perfusate, while creatine phosphokinase activity was not detectable (Data not shown). Upon reperfusion with calcium-containing medium, the amounts of total protein loss increased from 1.5±0.2 (5 min calcium depletion) to 4.5±0.3 (10 min), 20.3±0.35 (20 min depletion) up to 109.5±0.7 mg/g of dry wt (40 min depletion) (Fig. 1). Respective amounts of creatine phosphokinase were also released under these conditions (Fig. 1).

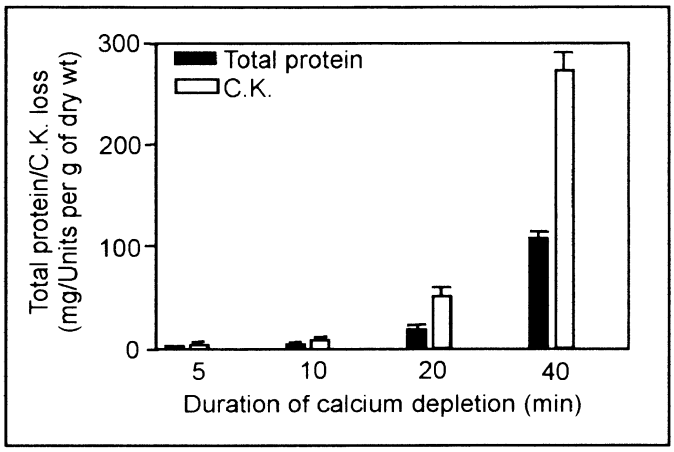

Fig. 1. Total protein and creatine phosphokinase losses in the effluent perfusate during a 20 min reperfusion period that followed a calcium depletion of increasing time intervals, at 42°C. Values are mean±SE of 4 different experiments.

The contractile activity of the hearts was normal during the equilibration period (Fig. 2A, i) whereas no contractile activity was observed during the Ca^{2+}-free perfusion (Figs. 2A ii, iii). Upon reperfusion with calcium-containing medium, after a 40 min Ca^{2+} depletion, an increase in resting tension was observed, which was equal to the contractile tension measured during the equilibration period (Fig. 2A, iv). Furthermore, the contractile activity of the heart during reperfusion was recovered by approximately 95% (5 min calcium depletion), 55% (10 min calcium depletion), 25% (20 min calcium depletion) compared to the control values, whereas no recovery was observed after a 40 min calcium depletion (Fig. 2B).

The electrical activity of ventricles and atria of the perfused pigeon heart was recorded through out the experiments. As can be seen in Fig. 3, during the equilibration period stable extracellular electrograms were obtained and the pulse frequency of both, ventricles and atria, was estimated to be ~250 pulses/min (Fig. 3A). During the Ca-depletion, a decrease to ~66 pulses/min was initially observed followed by an increase reaching a value of 168 pulses/min, a result indicative of the electromechanical uncoupling (Fig.3B). During the calcium readmission however, under conditions inducing a calcium paradox (42°C, 40 min Ca-free), an initial increase to ~180-190 pulses/min and a loss of electrical activity thereafter in both, ventricles and atria, were observed (Fig. 3C).

Effect of temperature

In a series of experiments, we examined the effect of temperature on the Ca-paradox induction in the perfused pigeon heart. The temperature was maintained at the desired value through out each experiment. The time course of protein release into the perfusate showed that during the 3rd up to 4th min of reperfusion a maximal quantity of total protein loss was obtained (Fig. 4A). At 36°C, no measurable amounts of creatine phosphokinase were detected in the effluent samples, while the total protein release was 11.67 ± 0.5 mg/g of dry wt (Figure 4B). Increasing the temperature to 38°C resulted in higher amounts of creatine phosphokinase and total protein loss, reaching values of 61.11 ± 0.8 IU/g of dry wt and 23.21 ± 0.6 mg/g of dry wt, respectively. At 40°C, much higher amounts of creatine phosphokinase and total protein were detected in the effluent samples, reaching values of 88.19 ± 0.6 IU/g of dry wt and 56.79 ±0.8 mg/g of dry wt, respectively. When the perfusion temperature was raised to 42°C, the normal body tempe-

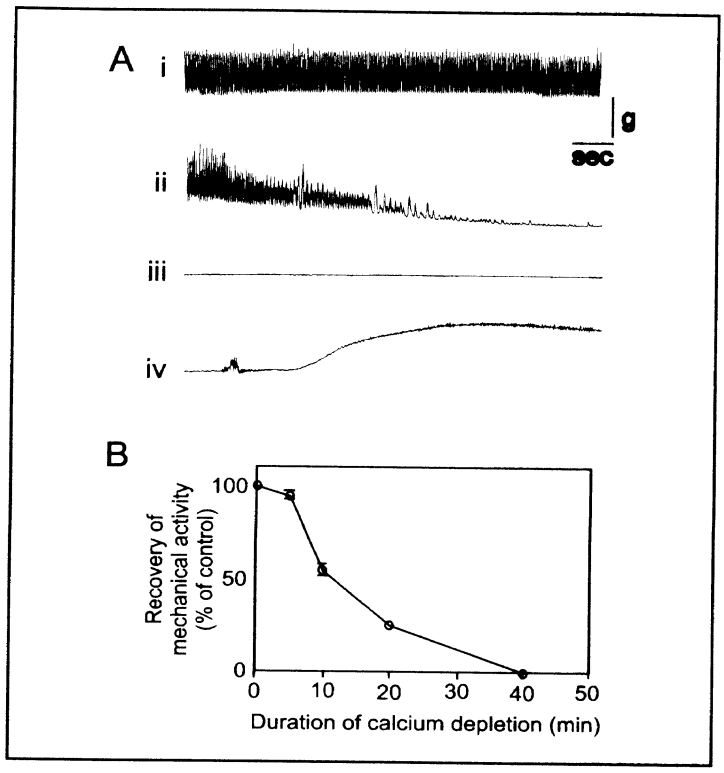

Fig. 2. (**A**) Representative recordings of the mechanical activity of the isolated pigeon heart during the equilibration period (**i**), the 1st and the 2nd min of calcium depletion (**ii** and **iii**, respectively) and the 1st min of reperfusion (**iv**). The heart was subjected to a 40 min calcium free perfusion at 42°C. (**B**) Effect of calcium depletion duration on the recovery of contractile activity of the perfused pigeon heart. Recovery of contractile activity is expressed as contractile force (%) of control. Control was taken as the contractile activity of each heart (tension in g) at the end of the equilibration period. Each point represents the mean ± SE of four determinations.

rature of the animal, a 40 min Ca-free period resulted in a maximal loss of creatine phosphokinase activity and total protein during reperfusion reaching values of 267.18 ± 0.8 IU/g of dry wt and 109.3 ± 1.0 mg/g of dry wt, respectively (Fig. 4B).

Studies on the electromechanical activity showed that hypothermia protected the heart against the induction of a calcium paradox. In particular, mechanical activity was recovered by approximately 100% (at 36°C), 35% (at 38°C), or 10% (at 40°C), whereas no recovery was observed at 42°C (Fig. 4C). Furthermore, the electrical recordings obtained clearly show that during the calcium readmission a complete recovery of electrical activity in both ventricles and atria occurred (Data not shown).

Effect of high and low pH

In order to study the effect of various biological buffers used in heart perfusions in vitro, we examined in detail the electromechanical activity of the perfused pigeon heart under standard conditions inducing a Ca-paradox (42°C, 40 min Ca-depletion).

In control experiments, these conditions induced a Ca-paradox in the pigeon heart with the characteristics described above. When we used Tyrode's Hepes pH 7.3

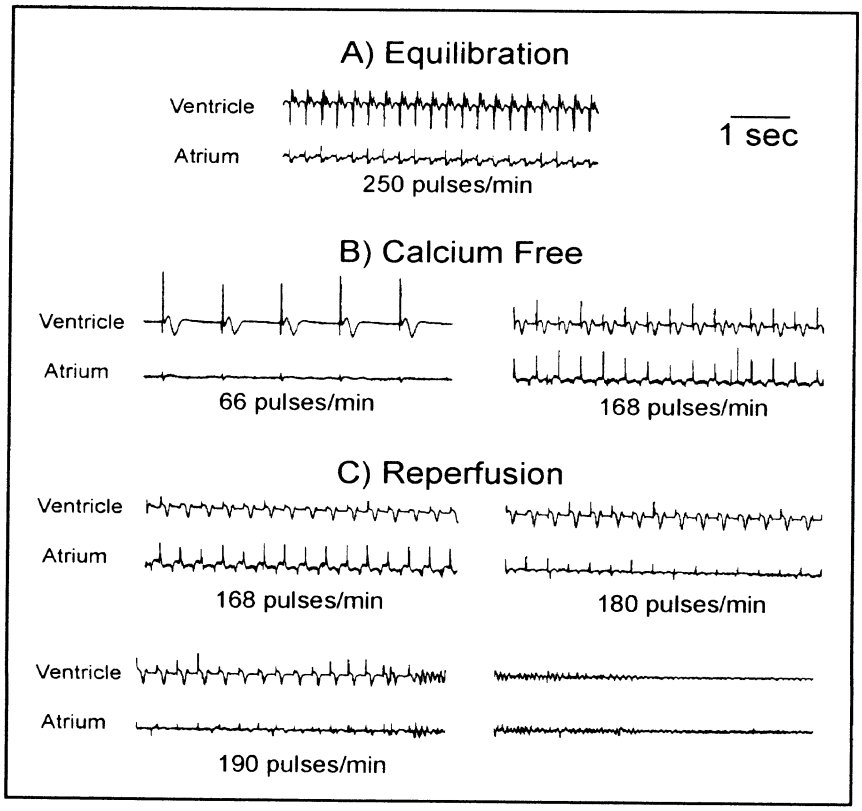

Fig. 3. Representative extracellular electrograms obtained from an isolated pigeon heart during the equilibration period (A), the 1st and the 5th min of calcium depletion (B) and the 1st, 2nd, 3rd and 4th min of reperfusion. The heart was subjected to 40 min calcium-free perfusion at 42°C.

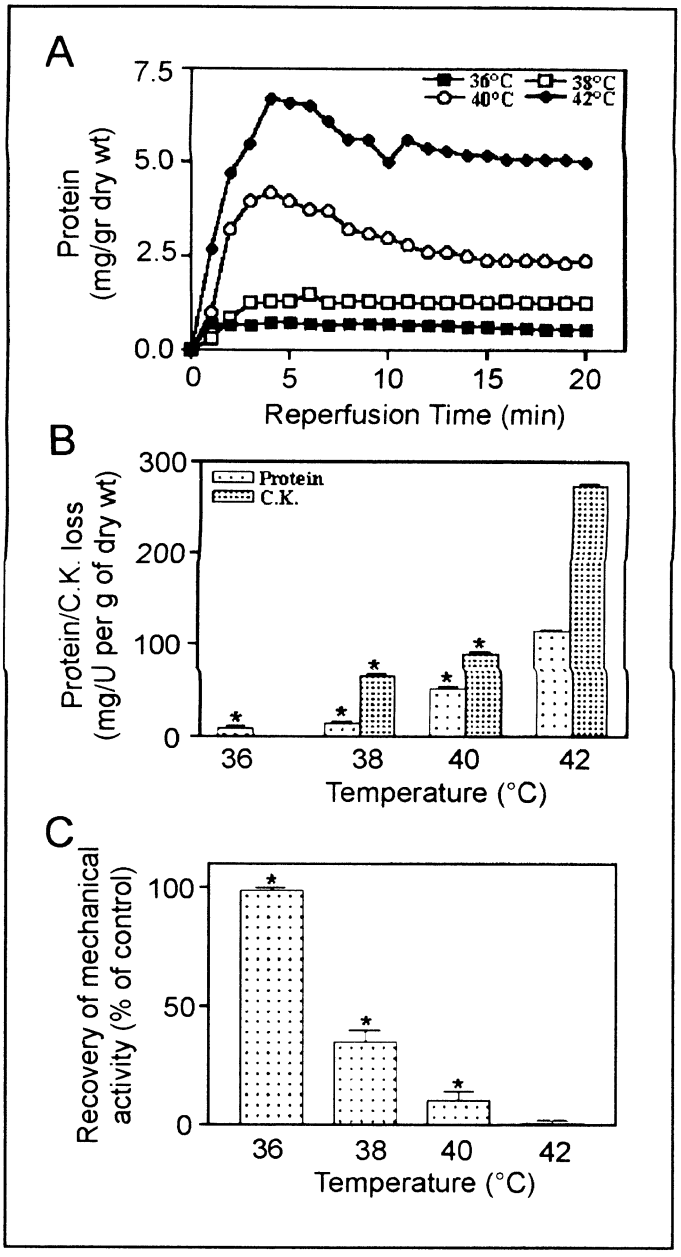

Fig. 4. Effects of perfusion temperature on total protein and creatine phosphokinase activity losses, and the recovery of mechanical activity during reperfusion. (A) Time course of protein loss during reperfusion at the temperatures indicated. (B) Total protein and creatine phosphokinase activity release into the perfusate during reperfusion following a 40 min calcium depletion at the temperatures indicated. (C) Recovery of contractile activity during reperfusion after a 40 min calcium depletion at the temperatures indicated. Mechanical activity is expressed as contractile force (%) of control. Control was taken as the contractile activity of each heart (tension in g) at the end of the equilibration period. Each point represents the mean ± SE of five determinations. Asterisks indicate values significantly different from the calcium paradox ones ($P<0.01$).

as a perfusion buffer instead of normal KH, we found that this buffer significantly protects the heart against a Ca-paradox induction (Fig. 5). As can be seen in Figure 5, the mechanical activity of the heart was normal during the equilibration period (Fig. 5i), whereas during the calcium depletion the contractile activity reached zero (Fig. 5ii). During the calcium readmission, a significant recovery (approximately 77.5% of the equilibration period value) of the contractile activity was observed (Fig. 5iii, 5iv). Furthermore, measurements of the contractile tension, resting tension and pulse frequency showed that in comparison to the equilibration period values (12 g, 1 g, and 165 pulses/min, respectively), during the Ca-depletion all these values decreased reaching zero. Upon reperfusion however, the contractile tension recovered rapidly reaching a maximal value of approximately 9 g, the resting tension was still low (less than 1 g) and the pulse frequency increased gradually up to ~150 pulses/min (Fig. 6A, 6B).

In Fig. 7A, the effect of high and low extracellular pH on the recovery of mechanical activity is shown. For these series of experiments two different Tyrode's buffers instead of KH were used. In particular, Tyrode's Hepes buffer of pH 7.30 or pH 7.00 was used for the examination of the effect of acidosis, whereas Tyrode's Tris/HCl of pH 7.30 or 8.00 was used for the examination of the effect of alkalosis. Acidosis resulted in the complete recovery of mechanical activity at the 4th min of reperfusion (110.5 % vs 77.5% for the respective control, $p<0.001$) whereas at the end of reperfusion the recovery of mechanical activity observed was approximately 87.7%. On the contrary, alkalosis resulted in a lower recovery of mechanical activity at the 4th min of reperfusion (70.4% vs 95.4% of the respective control, $p<0.001$) whereas at the end of reperfusion the recovery was approximately 47%. Measurements of creatine phosphokinase activity and total protein loss in the effluent samples, showed that creatine phosphokinase activity was less that 15 IU/g of dry wt, while total protein loss was less than 5 mg/g of dry wt (Fig. 7B). The above results clearly show that acidosis protects more powerfully the heart than alkalosis against a Ca-paradox.

Discussion

In the present study we demonstrate that the calcium paradox, a phenomenon well established in the mammalian heart, also exists in the avian heart. The criteria used in the present study, such as total protein as

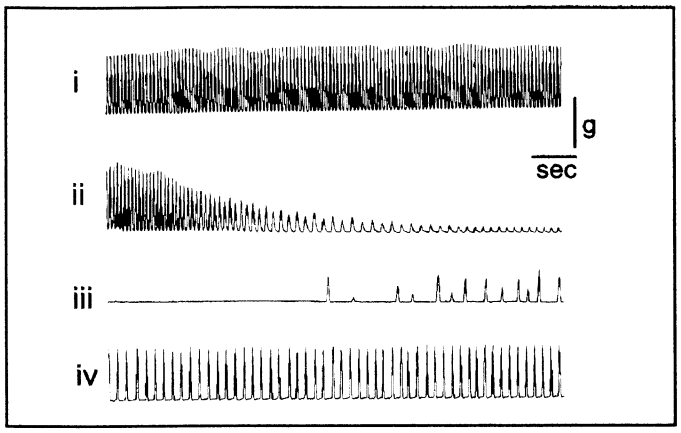

Fig. 5. Representative recordings of the mechanical activity of the isolated pigeon heart during the equilibration period (**i**), the 1st min of calcium depletion (**ii**) and the 1st and the 2nd min of reperfusion (**iii** and **iv**, respectively). The heart was subjected to a 40 min calcium free perfusion at 42°C, using a Tyrode's Hepes pH 7.3 as the perfusion buffer.

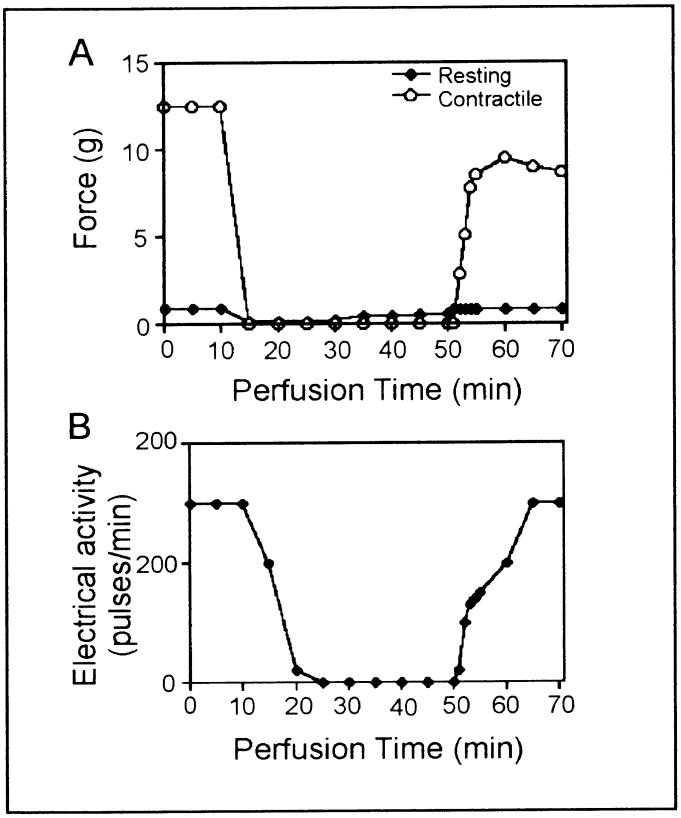

Fig. 6. Changes in mechanical (contractile force and resting tension in g) (A) and electrical (pulses/min) (B) activity of the isolated pigeon heart during perfusion with a Tyrode's Hepes pH 7.3 buffer. The heart was subjected to a 40 min calcium depletion followed by a 20 min reperfusion with the same buffer, at 42°C.

well as creatine phosphokinase release, irreversible loss of electromechanical activity, and an increase in resting tension during reperfusion with normal Ca^{2+}-containing buffer, are common with those used in studies on mammalian hearts. Enzyme release in the perfusate is the strongest criterion, since it is indicative of several ischaemic or anoxic conditions of rat heart [33], as well as of various pathological conditions of human heart diseases.

The dependence of a calcium paradox induction on the perfusion temperature and the time of calcium depletion had been previously described by several investigators in the heart of both mammals and ectotherms [12, 20]. It has been reported that depriving rat hearts of calcium for periods longer than 10 min predisposes them to the calcium paradox, even when the perfusion temperature is 20°C [12], whereas in the frog heart the induction of this phenomenon depends on the temperature (37°C) and the duration of calcium deprivation (30 min) [20]. In the present study, we found that in the avian heart, the induction of a calcium paradox depends on the perfusion temperature (42°C) and the duration of calcium depletion (40 min). Only under these conditions a complete loss of the electromechanical activity, a significant increase in resting tension and a significant loss of total protein and creatine phosphokinase activity are detected (Figs. 1, 2, 3). The diverse conditions inducing a calcium paradox reflect the different sensitivity of cardiac cells between species. In particular, the higher resistance of the pigeon heart against this phenomenon possibly reflects its physiological adaptive mechanisms in vivo.

Hypothermia (36°C) or decrease in calcium depletion duration (5 min), both protect the heart against the induction of a calcium paradox as indicated by the non detectable levels of total protein or creatine phosphokinase release and the 100% recovery of the heart mechanical activity (Fig. 4). It should be noted that a decrease by only 2°C (from 42 to 40°C) or by calcium depletion duration (from 40 to 20 min) results in 80% or 50% decrease in total protein release respectively, compared to the calcium paradox values.

It has been reported that various factors such as a decrease of extracellular Na^+ concentration, extracellular pH, temperature decrease during the calcium depletion, presence of various divalent cations or various an-

tagonists of the slow calcium channels, protect the heart against a calcium paradox [23, 27, 34-35]. The regulation of many intracellular functions by intracellular pH has been previously studied. It is widely known that intracellular pH affects the metabolic activity, the c-AMP levels, the conductivity of some ionic channels but also the intracellular calcium levels in cardiac muscle and other tissues [36-37]. In the present study, the effect of extracellular pH on the calcium paradox induction was studied by perfusing the heart with Tris/HCl or HEPES buffers, in the absence of bicarbonate ions. Our results show that both, lower pH (7.0) and higher pH (8.0) protect the pigeon heart against a calcium paradox. Surprisingly, by using the above buffers instead of bicarbonate ones of identical pH (7.30), the pigeon heart is significantly protected from the induction of this phenomenon (Figs 5, 6 and 7). In all these experiments, the mechanical and electrical activity of the heart recovered by at least 70% in comparison to the control hearts (perfused with normal bicarbonate buffer) and the protein and creatine phosphokinase losses were quite low. Although the mechanisms involved are still unknown, it seems that the dramatic fall/attenuation of intracellular pH observed during reperfusion may have a significant role. In particular, the massive Ca^{2+}-influx during reperfusion induces ATP hydrolysis by the mitochondrial, sarcoplasmic reticulum and myofibrillar ATPases, which in turn induce the H^+ release into the cytoplasm [38]. This acute H^+ release is believed to play an important role in the cell membrane damage and therefore in the loss of intracellular components, since the activation of cytoplasmic and lysosomal phospholipases and proteases depends on the Ca^{2+} and H^+ levels. In addition, acidosis results in a direct damage of cell membranes and in a delay of the inactivation rate of the Na^+-channels [39].

According to various reports there are three main mechanisms regulating the intracellular pH: Na/H, the Na-dependent Cl/HCO_3 and the Hamburger exchanges. The only mechanism functioning in the absence of bicarbonate ions is the Na/H pump, which is specifically inhibited by amyloride. By this mechanism, the intracellular pH during calcium depletion increases and seems possible that during reperfusion its attenuation is less dramatical [40]. Furthermore, it has been previously reported that alkalosis leads to an increase of protein synthesis rate in cardiac cells and possibly therefore to a better state of these cells in terms of their energy metabolism [41].

According to Jacobson and Papahadjopoulos (1975) [42] and Grinwald and Nayler (1981) [6] a slight drop in extracellular pH during calcium depletion protects the heart possibly by changing the fluidity of cell membrane. Further investigation of these mechanisms could give answer in a basic question, concerning the calcium homeostasis in cardiac cells under non-extreme physiological conditions.

In conclusion, both temperature and calcium depletion duration appear to be critical for the occurrence of the calcium paradox in the isolated pigeon heart. Furthermore, acidosis as well as alkalosis seem to protect powerfully the pigeon heart against the induction of this phenomenon. Although the mechanisms involved in the induction of this phenomenon remain obscure, it seems that they must be similar in the mammalian and avian heart in spite of their structural and functional differences.

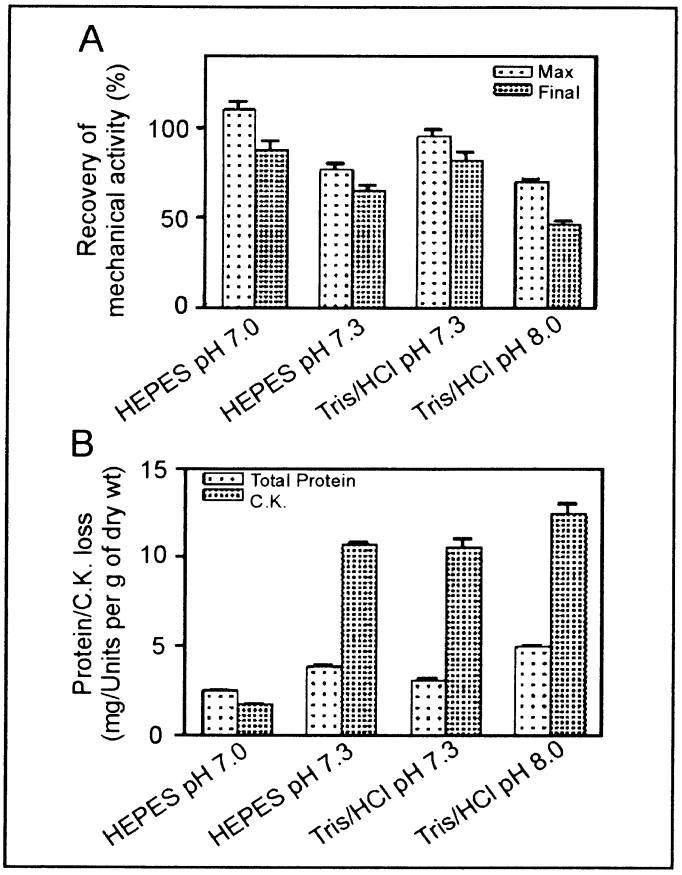

Fig. 7. Effect of extracellular pH on the recovery of contractile activity upon reperfusion following a 40-min calcium depletion (A) and the total protein and creatine phosphokinase activity losses (B). Recovery of contractile activity is expressed as contractile force (%) of control at the 4th (max) and the 20th (final) min of reperfusion.

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