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ORIGINAL PAPER

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# Various divalent cations protect the isolated perfused pigeon heart against a calcium paradox

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**Abstract** The protective effects of various divalent cations against the irreversible damage of myocardium, a phenomenon termed "the $Ca^{2+}$-paradox", were examined in the isolated perfused pigeon heart. All cations examined were added at a concentration of 200 μmol $l^{-1}$ in the "calcium-free" medium. In hearts perfused with low calcium, upon normal calcium repletion, the maximal recovery of the contractile tension (in the 2nd minute) was approximately 115% and the recovery obtained at the end of reperfusion was 81.5% (compared to the equilibration period value). From the other divalent cations examined, the presence of cobalt, nickel, manganese or barium during calcium depletion powerfully protected the pigeon heart. Upon calcium repletion, the maximal recovery of contractile tension was approximately 60%, 76.5%, 100% and 85%, the recovery estimated at the end of reperfusion was 40%, 12%, 70% and 53%, and the resting tension estimated at the end of reperfusion was $2.69 \pm 0.18$ g, $6.40 \pm 0.50$ g, $1.20 \pm 0.10$ g and $1.90 \pm 0.10$ g for cobalt, nickel, manganese and barium, respectively. On the contrary, strontium exerted no protective effects. The protective effects were also indicated by reduced total protein and lactate dehydrogenase activity release into the effluent perfusate and maintenance of electrical activity. The effectiveness of the added divalent cations (with the exception of strontium) showed a strong dependence upon their ionic radius. The most potent inhibitors of this phenomenon in the pigeon heart were the divalent cations having an ionic radius closer to the ionic radius of calcium. These results are discussed in terms of the possible mechanisms involved in the protective effects of these cations.

**Keywords** Calcium paradox · Cardioprotection · Heart · Pigeon, *Columba livia* · Lactate dehydrogenase

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## Introduction

Repletion of $Ca^{2+}$ in the isolated heart perfused for a brief period with $Ca^{2+}$-free medium is known to cause irreversible damage to myocardial cells and loss of contractile function, a phenomenon termed "the $Ca^{2+}$-paradox" (Zimmerman and Hulsmann 1966). The damage consists of a massive release of intracellular components, irreversible loss of electromechanical activity, extensive ultrastructural membrane lesions and depletion of high-energy phosphates.

Several discrepancies exist regarding the mechanism involved in the induction of this phenomenon. One of the major points of controversy has concerned the issue of whether an excessive intracellular $Na^+$ accumulation during perfusion with $Ca^{2+}$-free media is a prerequisite to the cell damage upon $Ca^{2+}$ repletion (Guarnieri 1988; Bhojani and Chapman 1990; Ruigrok 1990; Chapman et al. 1991). According to the $Na^+$ hypothesis, upon extracellular $Ca^{2+}$ removal, $Na^+$ enters the cell via L-type $Ca^{2+}$ channels, which become permeable to monovalent cations under such conditions (Chapman and Tunstall 1987). This causes an increase in intracellular $Na^+$, the extent of which may be limited by an increased activity of the $Na^+/K^+$ pump. Upon $Ca^{2+}$ repletion, $Ca^{2+}$ enters the cell in exchange for $Na^+$, causing a contracture and structural lesions. This hypothesis is supported by the fact that both low $Na^+$ (Alto and Dhalla 1979; Goshima et al. 1980; Guarnieri 1988) and low-temperature perfusion (Alto and Dhalla 1979; Rich and Langer 1982), which prevent the intracellular $Ca^{2+}$ overload, result in cardioprotection against the calcium paradox injury.

According to other investigators, cell injury upon $Ca^{2+}$ repletion, after a period of perfusion with calcium

free-solutions, is not due to intracellular $Na^+$ overload (Ruigrok 1990; Van Echteld et al. 1991; Jansen et al. 1998). Previous studies proposed that $Ca^{2+}$-independent mechanisms of membrane deterioration might also occur during $Ca^{2+}$ overload. Apart from ultrastructural damages (Frank et al. 1977; Dhalla et al. 1983), varying degrees of sarcolemmal (Persad et al. 1993) and sarcoplasmic reticular (Schwartz et al. 1975) derangements have been reported. These disturbances are accompanied by alteration in intracellular electrolytes (Alto and Dhalla 1979). More recent studies suggest the presence in many tissues, including the heart, of a variety of proteins that form cation-permeable non-selective channels, which are potential molecular candidates for the channels underlying a non-selective current, different from the one involving L-type $Ca^{2+}$ channels (Bosteels et al. 1999; Macianskiene et al. 2001). Although the physiological role of these non-selective channels remains unknown, they may constitute a pathway through which $Na^+$ enters to cause $Na^+$ overload during $Ca^{2+}$ depletion and hence lead to $Ca^{2+}$ overload via reverse $Na^+$-$Ca^{2+}$ exchange (Macianskiene et al. 2001).

Calcium influx in the cardiac myocytes during their physiological function occurs mainly through the $Ca^{2+}$-selective sarcolemmal slow channels (Trautein and Cavalie 1985). The activation of these channels depends on the membrane potential and the intracellular calcium levels. The L-type slow calcium channels are blocked by various organic and inorganic antagonists (Baker and Hearse 1983; Nayler 1988). It has been reported therefore, that various divalent cations such as manganese, cobalt and nickel act as potent inhibitors of these channels, possibly by substituting for calcium in its sarcolemmal binding sites. Furthermore, cobalt, manganese and barium exert a protection of rat cardiac myocytes against the calcium paradox (Harrow et al. 1978; Baker and Hearse 1983; Nayler et al. 1983).

In our previous studies we have characterised the calcium paradox and determined the effects of various conditions such as hypothermia, alkalosis and acidosis on its induction in the isolated perfused pigeon heart (Gaitanaki et al. 2002). We have also shown that in the pigeon heart the calcium paradox correlates with the activation of the calpain-calpastatin system and that manganese and barium substituting for calcium prevent the activation of this protease (Gaitanaki et al. 2003). These results provide evidence that despite the fundamental structural and functional differences between the mammalian and avian heart, the mechanisms involved in the induction of the calcium paradox are similar. Furthermore, recent studies by several investigators have shown that the calcium and the oxygen paradoxes are basically similar (Lemasters 1999). Overall, during a prolonged flight the pigeon cardiac output is much higher, giving rise to conditions of anaerobiosis (Smith et al. 2000). Under such conditions in vivo, the resistance of the heart against a calcium paradox injury is very important for this animal. In particular, during ischaemia, calcium depletion to some extent may occur and therefore upon calcium readmission a protection against this phenomenon, possibly via suppression of the calpain-calpastatin system must be very important for the physiological adaptation of the pigeon heart.

Here, we substituted various divalent cations such as barium, nickel, cobalt, manganese and strontium for calcium, in order to examine their protective effects against a calcium paradox in the isolated pigeon heart and the possible relationship between these effects and their ionic radius.

## Materials and methods

### Animals

Isolated hearts of the pigeon *Columba livia* were used. Domestic animals were obtained from a commercial dealer and kept in the laboratory with free access to water and food. All animals received humane care in accordance to the Guidelines for the Care and Use of Laboratory Animals published by the Greek government (160/1991) based on EC regulations (86/609).

### Chemicals

All chemicals were purchased from Sigma Chemical (St Louis, USA).

### Experimental procedure

Pigeons (weight 350–400 g) were anaesthetised with sodium pentobarbital (30 mg per animal) and received heparin (400 IU) i.v. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit's buffer for 2 min. They were then mounted onto the aortic cannula of a conventional two-way non-recirculating Langendorff apparatus. 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 mmHg). The normal perfusion medium was a Krebs-Henseleit's (KH) bicarbonate buffer which consisted of (in mmol $l^{-1}$): 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 pH of the oxygenated KH buffer was adjusted to 7.35–7.40 at 42°C. All KH buffers were equilibrated with 95% $O_2$/5% $CO_2$. In the calcium-free medium calcium was omitted and EGTA was added at a final concentration of 10 μmol $l^{-1}$ to ensure removal of any contaminant calcium. In different sets of experiments 200 μmol $l^{-1}$ of $CaCl_2$ instead of calcium free medium, or 200 μmol $l^{-1}$ of $BaCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, or $SrCl_2$ along with 10 μmol $l^{-1}$ EGTA were added in the calcium-free KH buffer. In all experiments an equilibration period of 10 min was allowed during which the hearts were perfused with the normal KH buffer. This was followed by a 40-min per-

iod of calcium depletion in the presence or absence of added divalent cations and finally by the reperfusion with standard KH buffer for 30 min.

### 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 grams) developed by the heart. Continuous ECG measurements of intracardiac activity were performed as previously described (Gaitanaki et al. 2002).

### 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 (1976). In the same samples lactate dehydrogenase (LDH) activity was determined by the method described by Ward et al. (1969).

### Data analysis

The results are presented as mean ± SE of three or four independent experiments. The cumulative amount of protein and/or LDH activity lost from myocardial cells was determined by specifically multiplying the protein concentration or LDH 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 a low calcium concentration

In a previous paper we had described that in the isolated perfused pigeon heart the calcium paradox is induced upon calcium readmission following a 40-min period of calcium deprivation at 42°C, the normal body temperature of this animal (Gaitanaki et al. 2002).

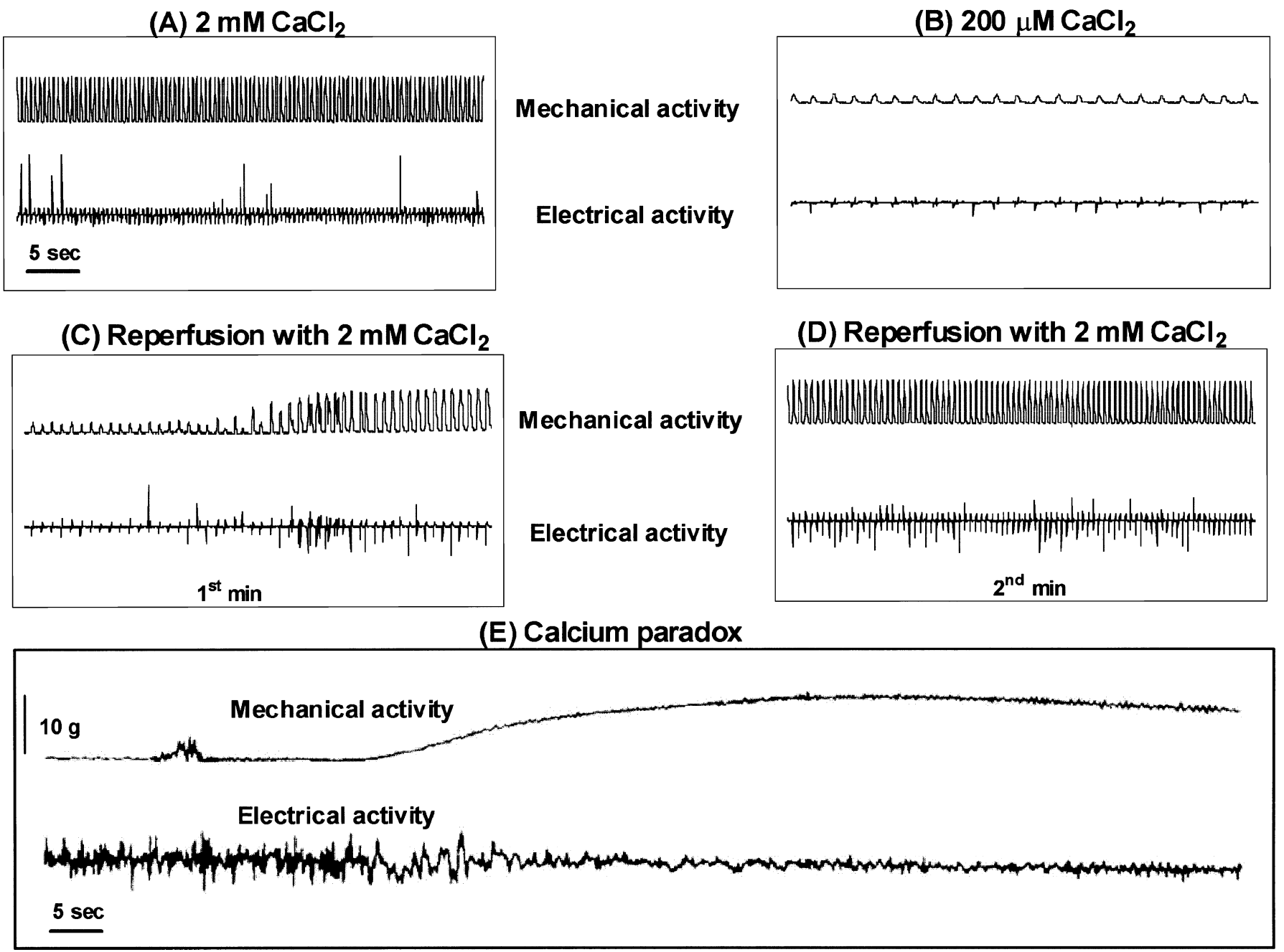
As the first step in the present study, we examined the protective effects of a low calcium concentration (200 μmol $l^{-1}$) against the induction of this phenomenon. In particular, after the equilibration period, the heart was perfused with KH buffer, which contained 200 μmol $l^{-1}$ $CaCl_2$ (instead of calcium-free KH buffer). As controls, pigeon hearts perfused under conditions inducing a calcium paradox were included (Fig. 1E). The results of these experiments clearly showed that even though the calcium concentration tested was low, powerfully protected the heart against the occurrence of a calcium paradox upon readmission of normal calcium containing KH buffer (2 mmol $l^{-1}$ $CaCl_2$). As can be seen in Fig. 1B, during the perfusion with low calcium, the mechanical activity of the heart decreased by approximately 80% compared with the equilibration period value (Figs. 1A, 2A), but did not reach zero. Furthermore, the pulse frequency decreased from 145 to 90 pulses $min^{-1}$ (Figs. 1B, 2B). Upon reperfusion with normal KH buffer, the recovery of contractile tension was approximately 115% in the 2nd minute and 81.5% of the equilibration period value at the end of reperfusion (30th minute) (Figs. 1C, D, 2A). Furthermore, the recovery of electrical activity was approximately 90% of the equilibration period value (Fig. 2B), whereas in hearts perfused under conditions inducing a calcium paradox, no recovery of electrical activity was observed (Fig. 1E). On the other hand, the resting tension gradually increased during both, the low calcium perfusion and normal calcium readmission, reaching a value of approximately 2.70 ± 0.17 g at the end of reperfusion (Fig. 2A). Measurements of total protein and LDH activity release into the effluent perfusate during normal calcium readmission showed that the quantity of total protein lost was 2.01 mg $g^{-1}$ of dry weight and LDH activity 1.98 U $g^{-1}$ of dry weight, values that are much lower compared with the paradox ones (83.47 mg $g^{-1}$ of dry weight and 102.40 U $g^{-1}$ of dry weight, respectively).

### Effects of divalent cations that block the L-type slow $Ca^{2+}$ channels

In this series of experiments, we examined the effect of various divalent cations such as cobalt, nickel and manganese (known L-type slow $Ca^{2+}$ channel inorganic blockers) for their protective effects against the induction of a calcium paradox in the perfused pigeon heart. In all these experiments, a low concentration (200 μmol $l^{-1}$) of each cation was added along with 10 μmol $l^{-1}$ EGTA in the calcium-free buffer.

The results of these experiments revealed that during calcium depletion in the presence of 200 μmol $l^{-1}$ of cobalt (Fig. 3A), nickel (Fig. 3B) or manganese (Fig. 3C), the electrical activity was maintained, whereas contractile activity decreased reaching zero, confirming electromechanical activity uncoupling. Upon calcium repletion, following a 40-min $Ca^{2+}$ deprivation in the presence of $Co^{2+}$, an approximately 60% recovery of contractile activity was observed during the first 3 min, reaching a maximal value of 4.48 ± 0.25 g at 2.5 min (Figs. 4A, 5A). The resting tension also increased during this period reaching a maximal value of 3.38 ± 0.40 g in the 2nd minute. At the end of the reperfusion period, both contractile and resting tension reached approximately values of 2.25 ± 0.15 g and 2.69 ± 0.18 g, respectively (Fig. 5A, B).

Upon calcium repletion, following a 40-min $Ca^{2+}$ deprivation in the presence of $Ni^{2+}$, an initial recovery of contractile tension (76.5% of the equilibration period value) followed by a gradual decrease (to ~12% at the end of reperfusion period) was observed (Figs. 4B and 5A). The resting tension during this period gradually


(A) 2 mM $CaCl_2$
Mechanical activity
Electrical activity
5 sec
(B) 200 μM $CaCl_2$
(C) Reperfusion with 2 mM $CaCl_2$
Mechanical activity
Electrical activity
1st min
(D) Reperfusion with 2 mM $CaCl_2$
2nd min
(E) Calcium paradox
10 g
Mechanical activity
Electrical activity
5 sec

◀
**Fig. 1** Representative recordings of mechanical and electrical activities of the isolated pigeon heart during the three perfusion periods: **A** equilibration period, **B** perfusion with 200 μmol $l^{-1}$ $CaCl_2$, **C** 1st min of reperfusion with normal KH buffer, and **D** 2nd min of reperfusion. As controls, pigeon hearts perfused under conditions that induce a calcium paradox were included (**E**). The experiment was repeated in two further occasions with similar results

increased from 3.04 ± 0.40 g (in the 4th minute) up to 6.40 ± 0.50 g (in the 30th minute) (Fig. 5B). The pulse frequency also increased reaching values of 150 pulses $min^{-1}$ (in the 4th minute) while at the end of reperfusion it reached 360 pulses $min^{-1}$ (data not shown).

Studies on the protective effects of manganese against the calcium paradox in the pigeon heart revealed that this divalent cation protects the pigeon heart more powerfully than cobalt or nickel. In particular, upon calcium repletion, following a 40-min $Ca^{2+}$ deprivation, manganese induced an initial recovery of contractile tension (100% of the equilibration period) followed by a gradual decrease (to ~70% at the end of reperfusion period) and an almost complete recovery of electrical activity (Figs. 4C, 5A), while the resting tension remained low (approximately 1.20 ± 0.10 g) for up to 30 min of reperfusion (Fig. 5B).

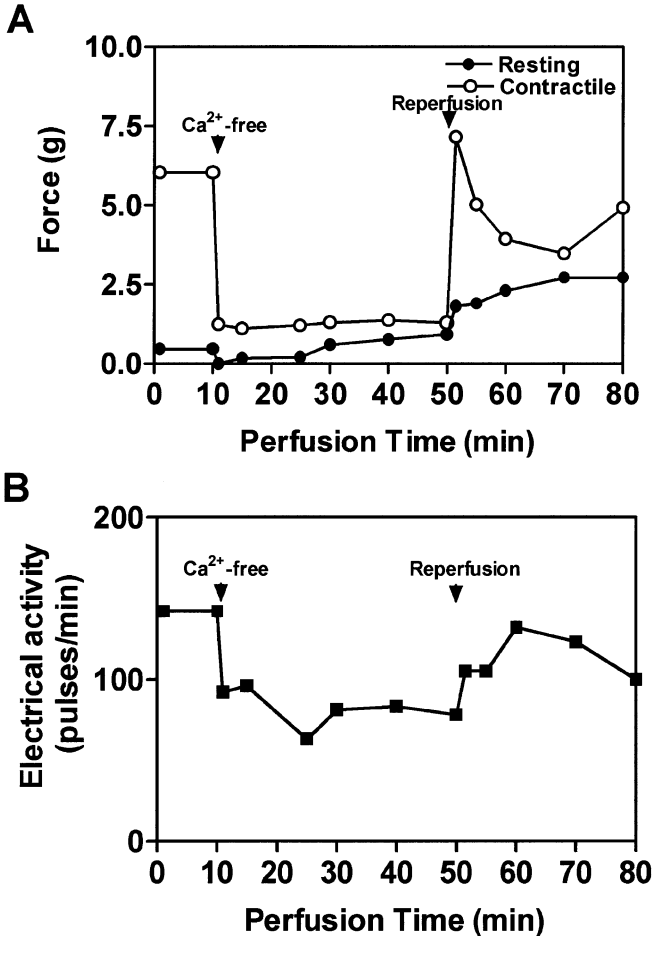



**Fig. 2** Changes in **A** mechanical (contractile force and resting tension in grams) and **B** electrical (pulses $min^{-1}$) activity of the isolated perfused pigeon heart during perfusion with low calcium. After a 10-min equilibration period, the heart was subjected to a 40-min perfusion with a low calcium concentration (200 μmol $l^{-1}$) followed by a 30-min reperfusion with normal KH buffer at 42°C. Values are mean ± SEM of 3–4 independent determinations. In each case SEM is less than 0.02% of the mean value

## Effects of divalent cations that pass through the L-type $Ca^{2+}$ channels

We also examined the protective effects of barium and strontium against the induction of a calcium paradox in the perfused pigeon heart. During $Ca^{2+}$ deprivation, in the presence of barium (200 μmol $l^{-1}$) the mechanical activity decreased reaching zero values in the 3rd minute (Fig. 6A). Upon calcium repletion followed a 40-min deprivation in the presence of this cation, however, a powerful recovery of mechanical activity (approximately 85% of the equilibration period value) in the 2nd minute was observed, while at the end of reperfusion the recovery was approximately 53% (Figs. 5A, 7A). Accordingly, the resting tension was quite low, comparable to the respective of the equilibration period (Fig. 5B), and the pulse frequency after an initial increase finally stabilized at 160 pulses $min^{-1}$ (data not shown).

On the other hand, strontium had a quite different effect on the induction of a calcium paradox. As can be seen in Fig. 6B, during calcium depletion in the presence of 200 μmol $l^{-1}$ of $Sr^{2+}$, the contractile activity gradually decreased reaching zero. On the contrary, the electrical activity changed dramatically during the calcium depletion showing a fibrillatory profile (Fig. 6B). The changes of resting tension observed during this period are also characteristic. In particular, after the 10th minute of $Ca^{2+}$ depletion the resting tension changed between 0 and 8 g. These changes were also observed during the first minutes of calcium repletion, disappearing thereafter (Fig. 7B). In addition, no recovery of contractile activity was observed.

## Comparison of cation effectiveness and their ionic radius

The time-course of contractile tension changes during calcium repletion following a 40-min period of low-calcium perfusion or calcium deprivation in the presence of each cation tested was also studied. The results of this study clearly showed that 200 μmol $l^{-1}$ of $Ca^{2+}$ protected almost completely the heart against the induction of a calcium paradox, whereas from the other divalent cations tested, the order of effectiveness when the contractile activity was taken into account was: $Mn^{2+} > Ba^{2+} > Co^{2+} > Ni^{2+}$ (Fig. 5A). Furthermore, the resting tension during calcium readmission also changed and the increase was in the order of: $Ni^{2+} > Co^{2+} > Ba^{2+} > Mn^{2+}$ (Fig. 5B).

Measurements of the contractile activity recovery during reperfusion clearly showed that with the excep-

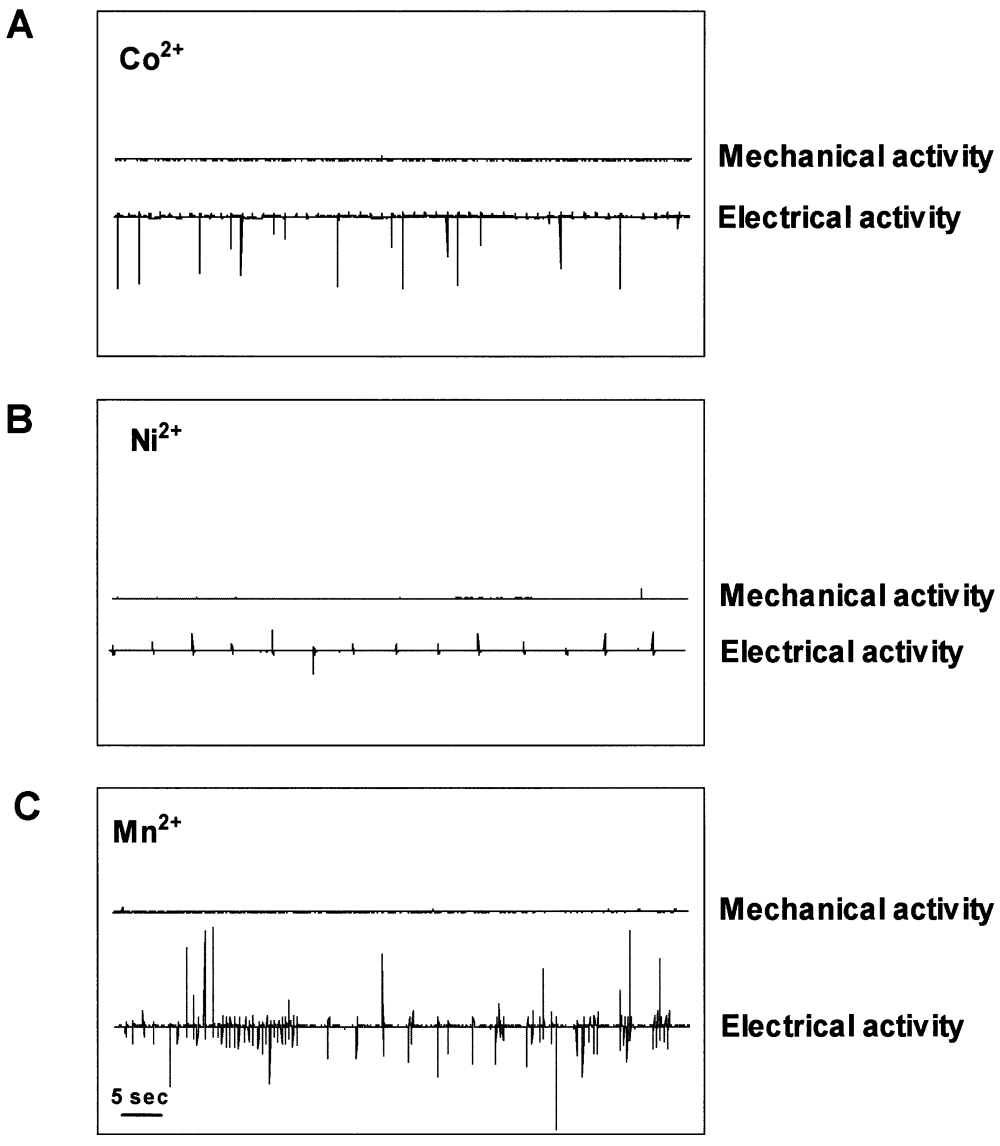



**Fig. 3** Representative recordings of mechanical and electrical activities obtained from the isolated pigeon heart during a 40-min calcium deprivation in the presence of 200 μmol $l^{-1}$ of **A** cobalt, **B** nickel or **C** manganese

tion of $Sr^{2+}$, which exhibited no effectiveness, the recovery was higher in the case of addition of a cation with an ionic radius closer in magnitude to the ionic radius of calcium (Fig. 8A). This order is in accordance with the order of magnitude of the ionic radii of the divalent cations examined. Furthermore, the amounts of total protein and lactate dehydrogenase activity released into the effluent perfusate during calcium repletion following a 40-min deprivation in the presence of the cations mentioned above were quite low compared with the paradox ones and their effectiveness was also in accordance with the order of magnitude of their ionic radii (Fig. 8B).

## Discussion

The calcium paradox as well as the factors affecting its induction has been extensively studied in the mammalian and amphibian heart. Among these factors, lowering the extracellular $Na^{+}$ concentration, extracellular pH, hypothermia, presence of various cations, pharmaceutical and slow calcium channel antagonists can protect the heart against the extensive damages this phenomenon induces (Hunter et al. 1981; Baker and Hearse 1983; Oksental and Jynge 1986; Touraki and Beis 1991; Touraki and Lazou 1992; Harding and Duncan 1997).

The contribution of the present study relates to the protective effects of various inorganic slow calcium channel blockers against the calcium paradox in the isolated perfused pigeon heart. Presence of divalent cations such as manganese, cobalt or nickel in the calcium free perfusion buffer resulted in maintenance of electrical activity and a powerful recovery of contractile activity of the heart during the following reperfusion (Figs. 4, 5, 7). These results clearly showed that each of the divalent cations tested exerted its own distinct inhibitory effect against the induction of a calcium paradox. Although the ion effectiveness was related to the criteria used to

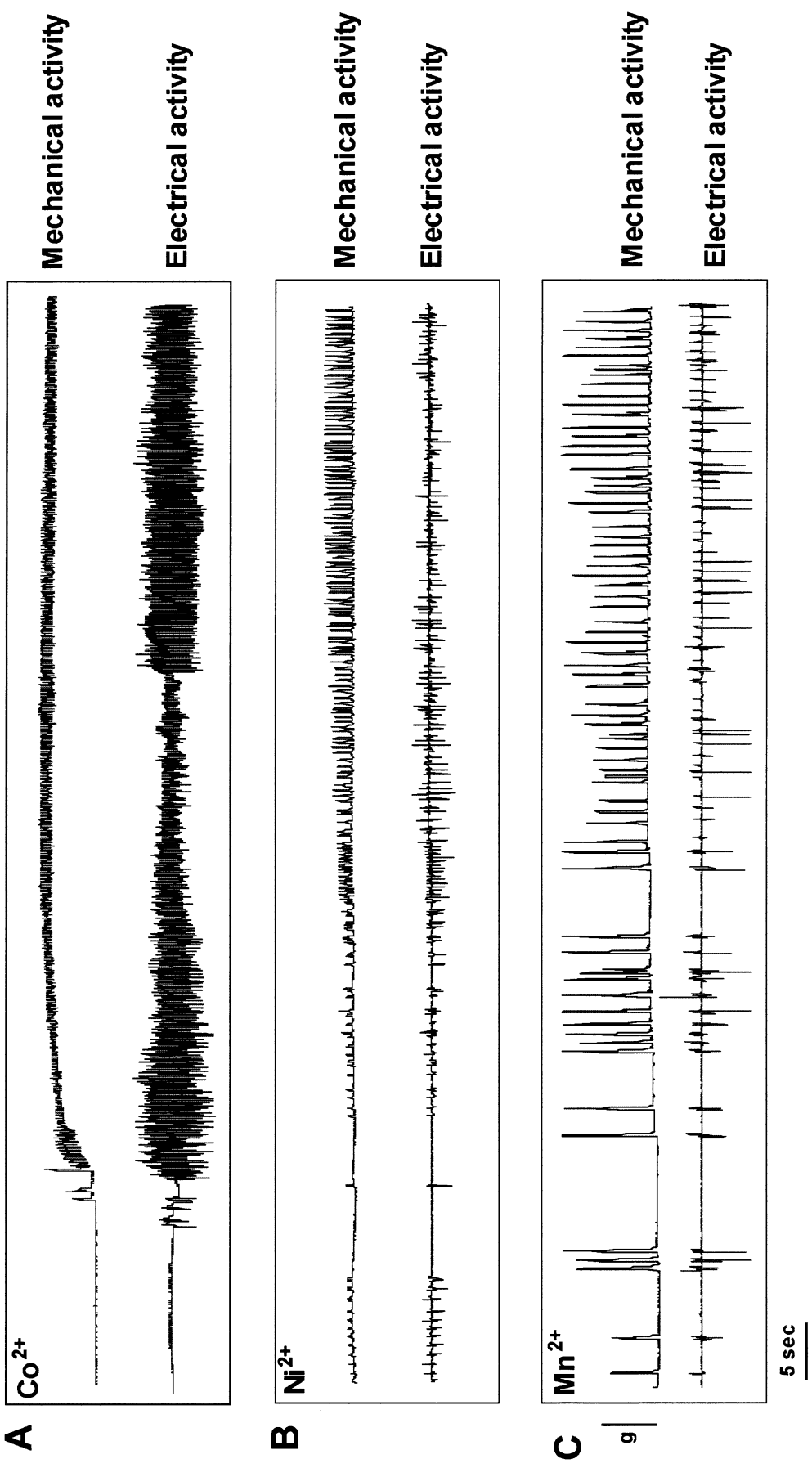
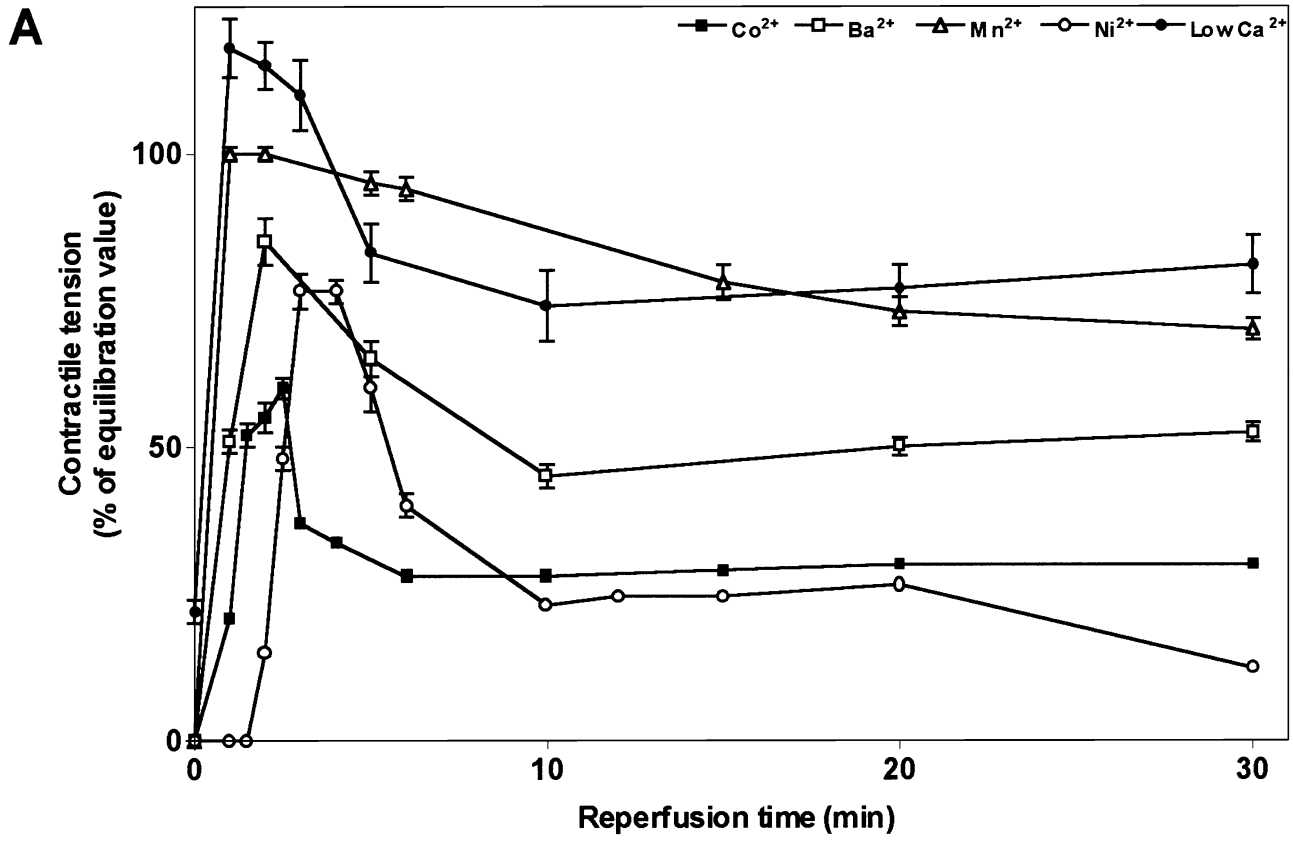
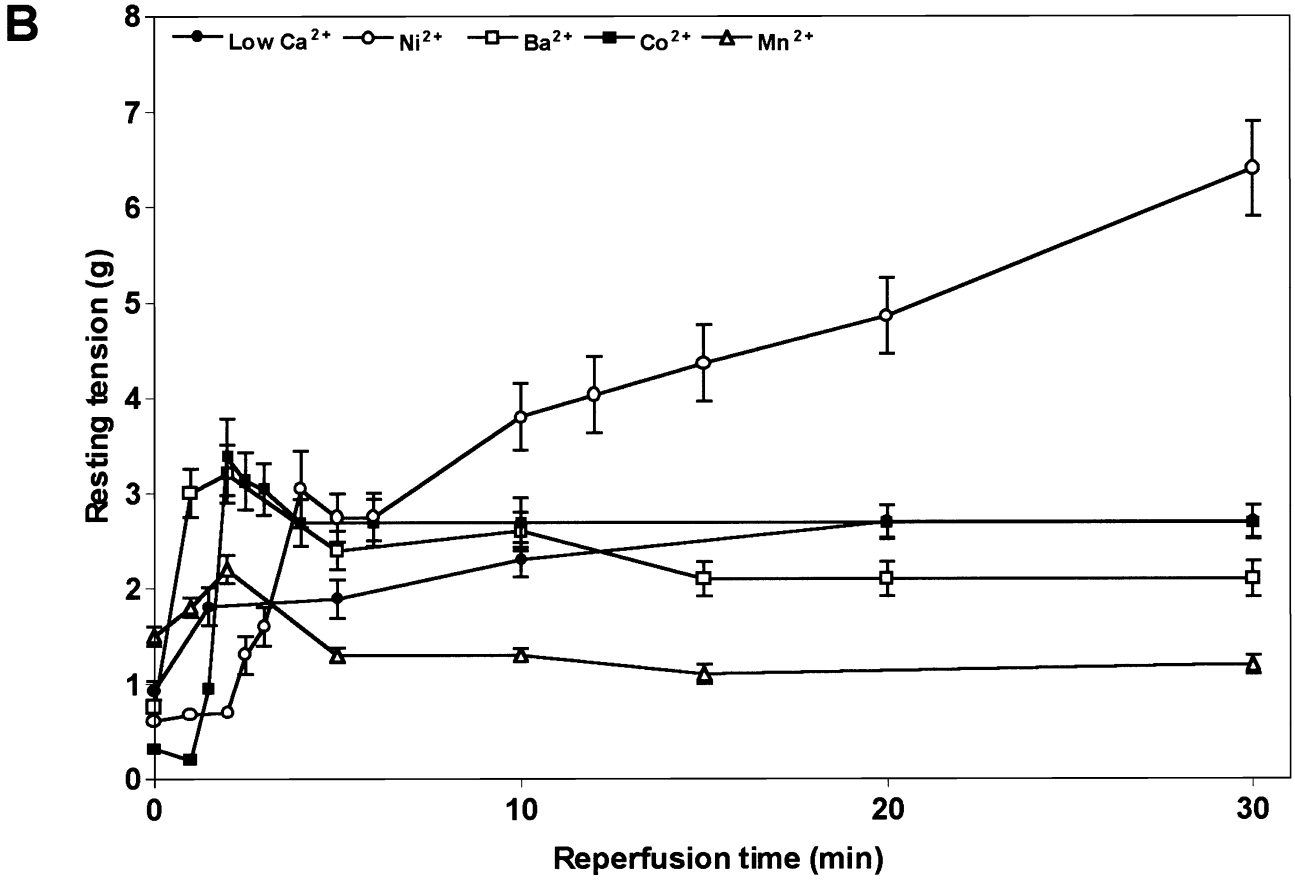



**Fig. 4** Representative recordings of mechanical and electrical activities of the pigeon heart during the first 2 min of calcium repletion followed a 40-min calcium depletion in the presence of 200 μmol $l^{-1}$ of **A** cobalt, **B** nickel or **C** manganese


A
Co2+
Ba2+
Mn2+
Ni2+
Low Ca2+
Contractile tension
(% of equilibration value)
100
50
0
0
10
20
30
Reperfusion time (min)


B
Low Ca2+
Ni2+
Ba2+
Co2+
Mn2+
Resting tension (g)
8
7
6
5
4
3
2
1
0
0
10
20
30
Reperfusion time (min)

◀
**Fig. 5** Time-course of contractile tension (**A**) or resting tension (**B**) changes during reperfusion of the isolated pigeon heart. After a 10-min equilibration period, hearts were subjected to a 40-min perfusion with either 200 μmol $l^{-1}$ calcium or with calcium-free KH buffer in the presence of 200 μmol $l^{-1}$ cobalt, barium, manganese or nickel followed by reperfusion with normal KH buffer. Contractile tension is expressed as a percentage of control, which was taken as the contractile activity of each heart (tension in grams) at the end of equilibration period. Resting tension is expressed in grams. Each point represents the mean ± SEM of 3–4 determinations

define cell damage, such as total protein and LDH activity release and recovery of electromechanical activity, the most potent inhibitor of calcium paradox was manganese. This is in accordance with the fact that the magnitude of the ionic radius of manganese is closest to that of calcium.

The protective effects of various divalent cations against the calcium paradox have also been extensively studied in various mammalian species as well as in the amphibian heart. Addition of divalent cations such as $Mn^{2+}$, $Co^{2+}$ (Rich and Langer 1982; Nayler et al. 1983; Touraki and Beis 1991), $Ni^{2+}$ (Touraki and Beis 1991), $Ba^{2+}$, $Sr^{2+}$ or $Cd^{2+}$ (Nayler and Grinwald 1982; Rich and Langer 1982; Touraki and Beis 1991) in the calcium-free medium prevents the induction of a calcium paradox in the mammalian or amphibian heart. Most investigators use high concentrations of these metals (1.3–4 mmol $l^{-1}$). In the present study we used a low (0.2 mmol $l^{-1}$) concentration of each cation in order to eliminate any of its secondary effects.

Divalent cations can result in an excitation-contractile uncoupling, by blocking the calcium channels according to their ionic radius (Bers and Langer 1979). Eventually, blockade of $Ca^{2+}$ channels by these ions could prevent the complete depletion of intracellular $Ca^{2+}$ stores and consequently the functional coupling of $Ca^{2+}$ release and contractile activity is easier to be achieved upon reperfusion with a normal $Ca^{2+}$ concentration (Nayler 1988; Kohlhard and Haap 1980).

Furthermore, it has been shown that these cations decrease the calcium loading into the cell during reperfusion, exerting an inhibitory effect on the heart sarcolemmal enzymes $Na^{+}$-$K^{+}$ ATPase, $Ca^{2+}$-$Mg^{2+}$ ATPase and adenylate cyclase (Harrow et al. 1978; Bers et al. 1980). This would result in a reduced calcium efflux from cardiac myocytes during calcium depletion, preventing therefore a massive calcium influx upon calcium repletion (Grinwald and Nayler 1981; Alto et al. 2000). On the other hand, inhibition of adenylate cyclase activity results in a decrease of cAMP levels and a decrease of the activated calcium channels during reperfusion (Reuter 1979). The ability of these cations to bind on the sarcolemma at the calcium-binding sites may explain their protective effects.

Another possible mechanism of the inhibitory effects of these divalent cations might be their ability to prevent the calcium influx via the plasma membrane $Na^{+}/Ca^{2+}$ antiporter/pump, which is believed to play a significant role on calcium influx during reperfusion (Bers et al. 1980). The $Na^{+}/Ca^{2+}$ exchange pump is considered to provide an important route for a massive calcium influx under cytotoxic conditions (Volkman et al. 1986).

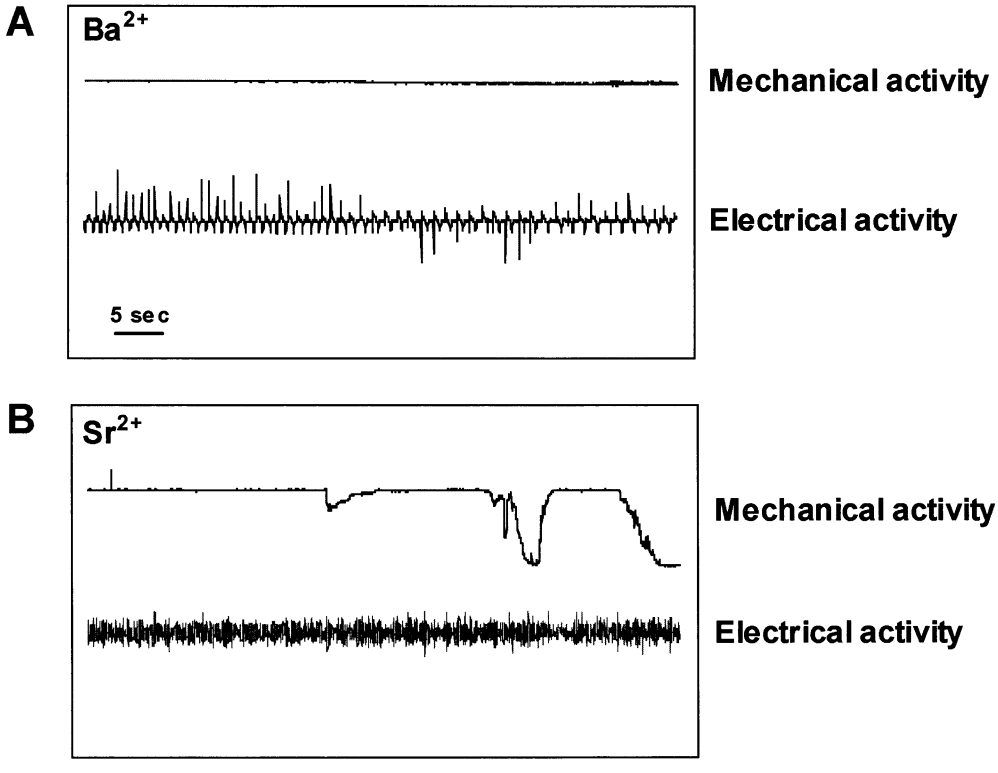



**Fig. 6** Representative recordings of electromechanical activities obtained from the isolated pigeon heart during a 40-min calcium deprivation in the presence of 200 μmol $l^{-1}$ of **A** barium or **B** strontium

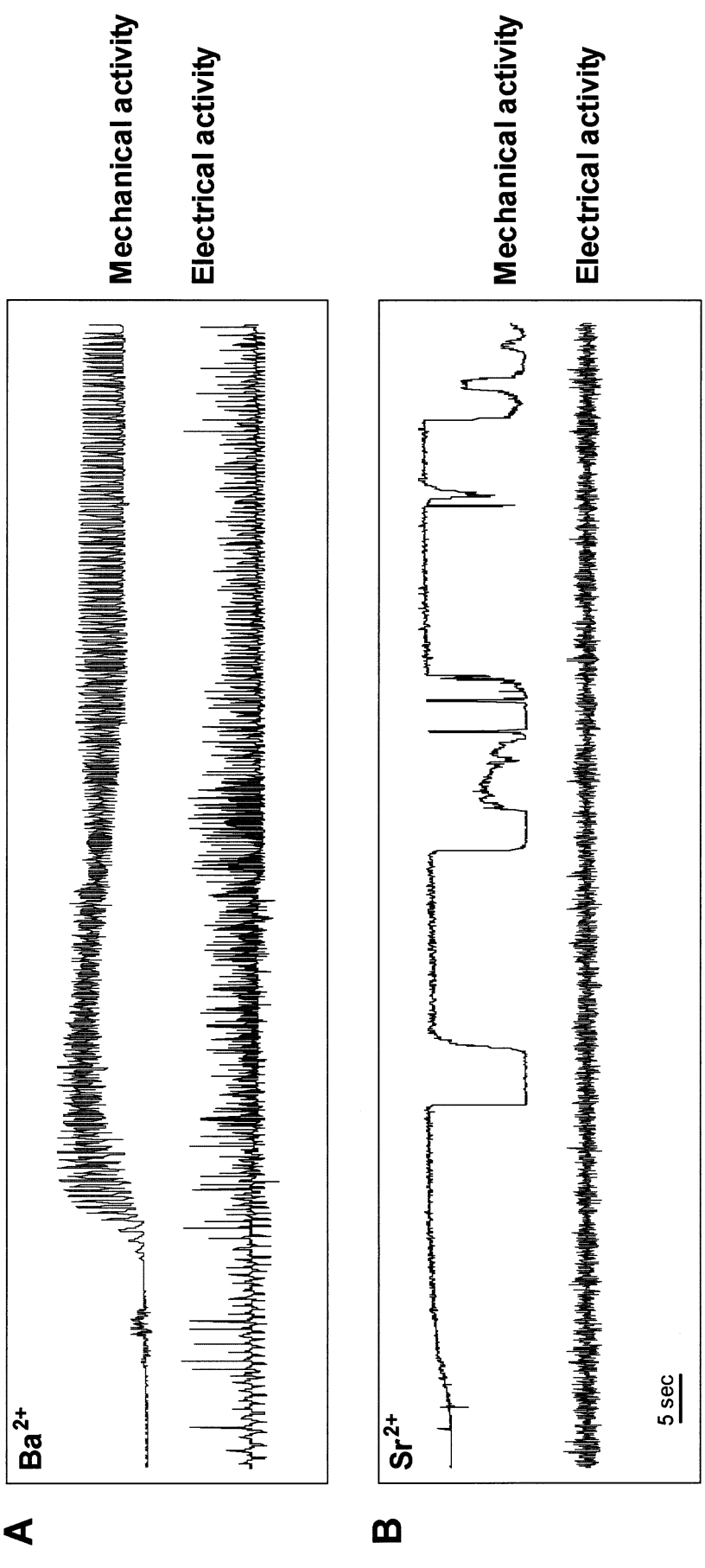



**Fig. 7** Representative recordings of mechanical and electrical activities of the pigeon heart during the first 2 min of calcium repletion followed a 40-min calcium depletion in the presence of 200 μmol $l^{-1}$ of **A** barium or **B** strontium

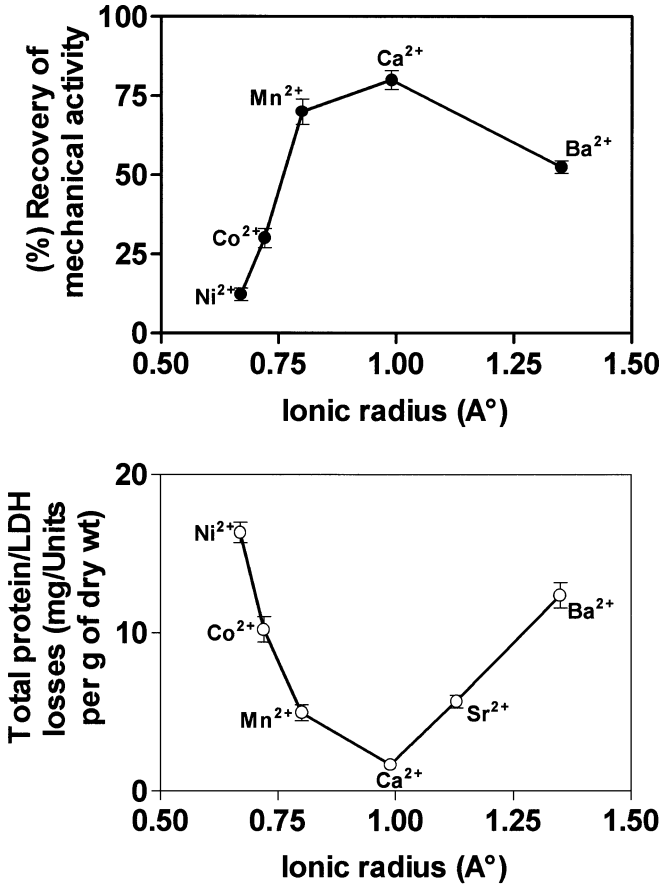



**Fig. 8** Effect of addition of divalent cations during calcium depletion upon recovery of mechanical activity (**A**) or protein and lactate dehydrogenase (*LDH*) activity losses (**B**) during reperfusion. Recovery of contractile function is expressed as a percentage of the equilibration period value. Protein amounts or LDH activity released into the effluent perfusate are expressed as mg or IU per gram of dry weight. Each point represents the mean ± SEM of four determinations

Therefore, inhibition of $Na^+/Ca^{2+}$ exchange by divalent cations might be especially beneficial for the pigeon heart during calcium repletion. The electromechanical recordings (Figs. 3 and 4) as well as the (percentage) recovery of mechanical activity obtained (Fig. 5A) provide evidence that the irreversible contracture, which is a characteristic of the calcium paradox during the first minutes of reperfusion, was absent when these divalent cations were present in the calcium-free buffer. Furthermore, a significant increase in resting tension of the pigeon heart muscle was observed only during reperfusion followed a 40-min period $Ca^{2+}$ depletion in the presence of $Ni^{2+}$ (Fig. 5B), which is less potent slow calcium channel blocker (Bers et al. 1980).

Addition of barium in the calcium-free medium resulted in a powerful protection of the pigeon heart against a calcium paradox (Figs. 5A, 7A) and this is in accordance with previously reported results for mammalian and amphibian hearts (Nayler and Grinwald 1982; Touraki and Beis 1991). The protective effect of barium against a calcium paradox in the pigeon heart could not be explained only by the inhibition of calcium influx (Ochi 1975), since this cation passes through calcium channels. Moreover, barium can displace calcium from its binding sites on the sarcolemmal and mitochondrial membranes and also can prevent the splitting of glycocalyx during calcium repletion, thus protecting the heart during calcium repletion (Nayler and Grinwald 1982; Luckacs and Fonyo 1986; Touraki and Beis 1991).

Presence of strontium in the calcium-free medium resulted in significantly reduced total protein and LDH activity release during reperfusion (Fig. 8B). However, no recovery of electromechanical activity of the pigeon heart was observed (Fig. 7B). The absence of contractile activity and the maintenance of resting tension during reperfusion have been also reported for rabbit heart (Rich and Langer 1982) and could explain the reduced release of intracellular components from the pigeon cardiac myocytes.

Our previous studies had shown that substituting calcium with manganese or barium (at a concentration of 200 μmol $l^{-1}$) during $Ca^{2+}$ depletion resulted in a significant inhibition of calpain activation during reperfusion (Gaitanaki et al. 2003). Furthermore, strontium induced a significant activation of this protease and a synergistic effect with calcium (Gaitanaki et al. 2003). The results of these studies revealed that the calpain-calpastatin system could possibly provide a basis for the elucidation of the calcium paradox mechanism in the pigeon heart, either by degrading myofibrillar or cytoskeletal proteins, indispensable for conservation of contractile capability and sarcolemmal integrity or by activating $Ca^{2+}$-dependent enzymes that initiate an irreversible, self-destructive pathway.

In conclusion, although manganese, cobalt and nickel seem to function in a different manner than barium and strontium, all the cations (with the exception of strontium) examined in the present study exert a marked protective effect against a calcium paradox in the perfused pigeon heart. These divalent cations seem to act at the level of calcium influx in pigeon cardiac myocytes mainly through calcium channels and possibly through $Na^+/Ca^{2+}$ exchange. Overall, the results of the present study clearly showed that the source of the calcium that activates contraction in the pigeon heart is the extracellular space.

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