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RAPID COMMUNICATION

^{31}P NMR Study of Intracellular pH During the Calcium Paradox

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Reperfusion of an isolated mammalian heart with a calcium-containing solution after a brief calcium-free perfusion results in irreversible cell damage: the calcium paradox. It has been suggested that acidification of the cytosol, as a result of hydrolysis of ATP and accumulation of calcium by mitochondria, is an important factor in the development of the calcium paradox. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectroscopy was used to investigate the course of intracellular pH during the calcium paradox in isolated rabbit heart at 37°C. Intracellular pH was measured from the chemical shift of the intracellular inorganic phosphate (P_i) peak. During control perfusion and the subsequent calcium-free period intracellular pH amounted to 7.1. After induction of the calcium paradox by readmitting calcium to the perfusion fluid, intracellular pH amounted to 7.0. It is concluded that acidification of the cytosol does not play a causal role in the development of the calcium paradox.

Contraction of the heart rapidly ceases when calcium is removed from the extracellular fluid [*12*]. Twenty years ago Zimmerman and Hülsmann reported that reintroduction of calcium into the extracellular fluid does not result in recovery of contraction of the heart, but in irreversible cell damage: the calcium paradox [*15*]. The calcium paradox is characterized by an excessive influx of calcium into the cells [*1*], rapid loss of cytosolic components [*15*], depletion of tissue high-energy phosphates [*2*], and severe contracture of the myofibrils [*4*]. The influx of calcium may be divided into an early, relatively small gain in cytosolic calcium, and a subsequent, massive influx [*9*]. Factors thought to be directly or indirectly responsible for the primary gain in calcium are the sarcolemmal sodium–potassium and calcium pumps, and the calcium pump of the sarcoplasmic reticulum, whose activities are decreased by calcium-free perfusion [*7, 8, 11*]. The raised cytosolic calcium may then trigger a number of events, including energy-dependent mitochondrial calcium accumulation [*13*] and activation of various ATPases [*6*], contracture-mediated disruption of intercalated disc junctions [*4*], disruption of the sarcolemma [*10*], loss of cytosolic components [*15*], and a secondary uncontrolled entry of calcium [*9*]. Neither the precise sequence of these events nor their relative importance is known at present. Hydrolysis of ATP and accumulation of calcium by mitochondria with deposition of insoluble calcium phosphate, are accompanied by a release of protons [*3, 5*]. Hence, it has been suggested that acidification of the cytosol with a consequent stimulation of cytosolic and lysosomal (phospho)lipases and proteases, plays an important role in the development of the calcium paradox damage [*6, 13*].

^{31}P NMR spectroscopy was used to investigate the course of intracellular pH during the calcium paradox in rabbit heart. Six isolated rabbit hearts were perfused at 37°C by the method of Langendorff at a constant pressure of 80 cmH_2O. The standard perfusate (+Ca) had the following composition (mmol/l): NaCl, 124.0; KCl, 4.7; $MgCl_2$, 1.0; $NaHCO_3$, 24.0; Na_2HPO_4, 0.5; $CaCl_2$, 1.3;

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glucose, 11.0. During calcium-free perfusion (−Ca) calcium was omitted from the standard perfusate and no correction was made for the small change in osmolarity. The perfusion fluids were equilibrated with 95% O_2 and 5% CO_2, and the resulting pH was 7.4 at 37°C. The perfusion sequence was: 10 min +Ca, 10 min −Ca, 5 min +Ca. ^{31}P NMR spectra were recorded on a Bruker MSL 200 spectrometer equipped with a wide bore (150 mm) 4.7 Tesla superconducting magnet, using a pulse repetition rate of 2.32 s and a pulse angle of 90°. Accumulated free induction decays were obtained from 6 or 128 transients on submerged rabbit hearts in a total volume of 55 ml in a 30 mm tube and exponentially multiplied, resulting in a line broadening of 20 Hz and 10 Hz, respectively. Zero ppm was assigned to the resonance position of creatine phosphate at pH 7.0. Intracellular pH was measured from the chemical shift of the intracellular P_i peak, using a titration curve obtained from a solution containing ATP (10 mmol/l), creatine phosphate (10 mmol/l), P_i (10 mmol/l), NADPH (10 mmol/l), glucose-6P (10 mmol/l), and $MgCl_2$ (10 mmol/l). Results are expressed as mean ± s.d. A separate series of five rabbit hearts was subjected to the above perfusion sequence to determine creatine kinase (CK) release during reperfusion with calcium-containing solution. The effluent fluid, collected in sequential 20 s samples, was analyzed for CK activity at 30°C, using a Vitatron Automatic Kinetic Enzyme System (AKES) and a Baker CK NAC-activated kit. Enzyme activity was expressed in IU released during 20 s per g dry heart tissue and represented the mean ± s.d.

Until the end of the calcium-free period intracellular pH amounted to 7.1 in all hearts. Figure 1 shows ^{31}P NMR spectra of one of the hearts, taken during control perfusion (+Ca), and during the subsequent calcium-free perfusion (−Ca). From the position of the intracellular P_i peak, intracellular pH during control perfusion was calculated to be 7.1. In this experiment the intracellular P_i peak was absent in the spectrum that was taken during calcium-free perfusion, most likely as a result of incorporation of P_i into high-energy phosphates since myocardial creatine phosphate and ATP levels increase during calcium-free perfusion [*2*]. Reperfusion with the calcium-containing solution resulted in a rapid decline of intracellular creatine phosphate and ATP levels. During the first 100 s of reperfusion intracellular pH in the heart used for Figure 2 varied from 7.1 to 6.9. In the subsequent spectra the intracellular P_i peak, and also the creatine phosphate and ATP peaks, were no longer perceptible. Mean intracellular pH values of all hearts amounted to 7.1 ± 0.1 (0 to 20 s); 7.1 ± 0.1 (40 to 60 s); 7.0 ± 0.1 (80 to 100 s). Creatine kinase release was maximal between 40 and 80 s of reperfusion: 305 ± 80 (40 to 60 s) and 310 ± 60 (60 to 80 s) IU/g dry heart tissue. Total creatine kinase release during the 5 min of reperfusion with calcium-containing solution amounted to 2870 ± 330 IU/g dry heart tissue.

These results demonstrate that there was no appreciable fall of intracellular pH once calcium was readmitted to the perfusion fluid. It may be argued that pH data were obtained only during the first 100 s of reperfusion, i.e. the period that the intracellular P_i peak was perceptible. By comparison, using ^{31}P NMR spectroscopy a gradual fall of intracellular pH from 7.1 to 6.0 was observed over a 15 min period of ischemia [*14*]. It should be noted, however, that the calcium paradox damage develops so rapidly that the release of enzymes, which is one of the characteristics of the calcium paradox, is maximal between 40 and 80 s of reperfusion. The almost simultaneous disappearance of the high-energy phosphate peaks and the P_i peak is in contrast with the situation during ischemia, where depletion of high-energy phosphates is accompanied by a marked increase of the P_i peak. Leakage into the extracellular space and uptake by mitochondria together with calcium may be responsible for the rapid disappearance of P_i from the cytosol during the calcium paradox. As mentioned before, both ATP breakdown and mitochondrial calcium accumulation are accompanied by a release of protons. A third event, however, that has to be taken into account is the breakdown of creatine phosphate, which is a proton-consuming reaction [*5*, *6*]. Apparently these opposite effects cancel during the calcium paradox and the result is little or no net change in intracellular pH. The buffering capacity of the cell and leakage of protons into the extracellular

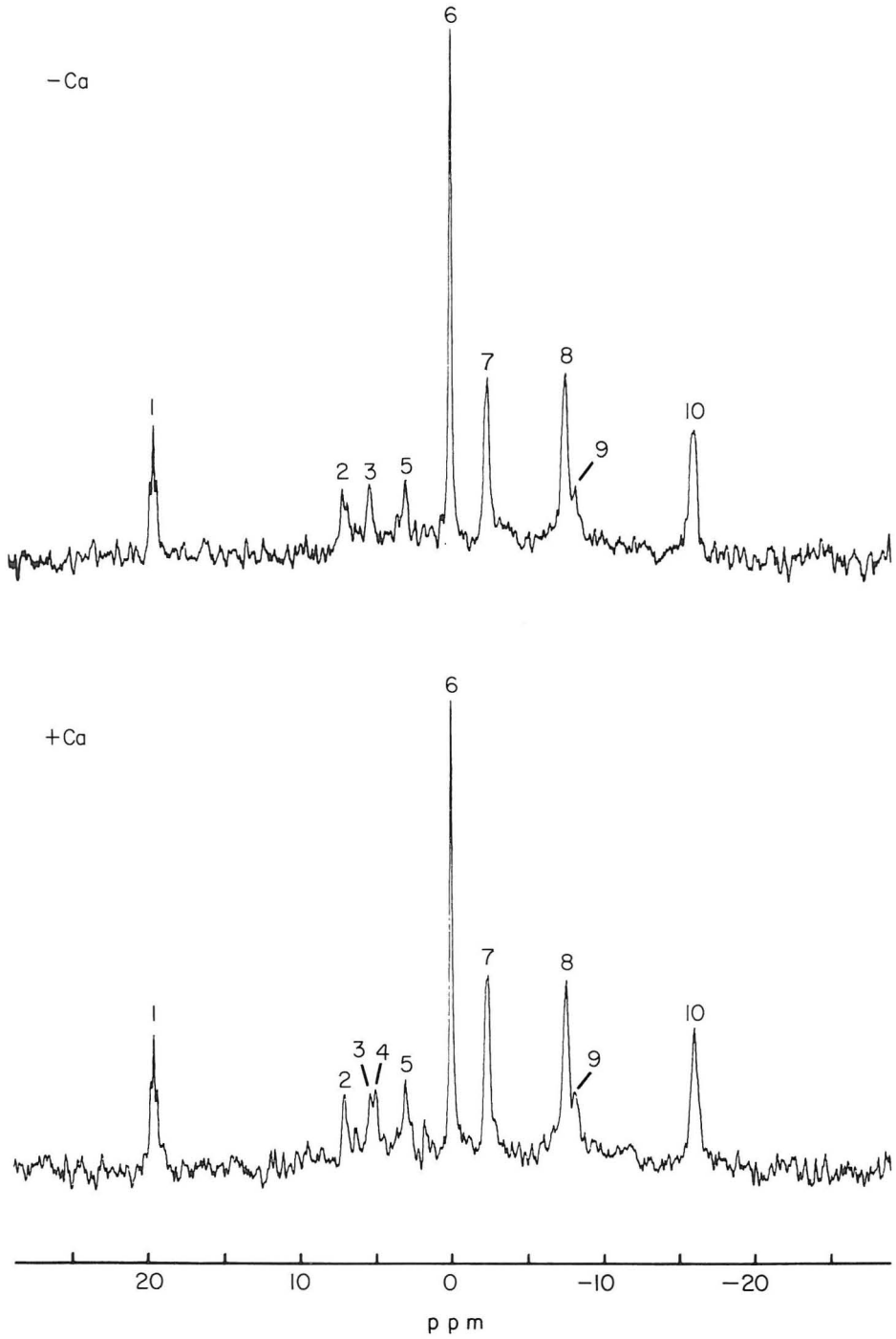

FIGURE 1. ^{31}P NMR spectra of a rabbit heart obtained from 128 scans and taken between 5 and 10 min of control perfusion (+Ca), and between 5 and 10 min of the subsequent calcium-free perfusion (−Ca). Resonances are assigned as follows: 1, methylene diphosphonate (external reference compound); 2, sugar phosphates; 3, extracellular P_i; 4, intracellular P_i; 5, glycerophosphorylcholine; 6, creatine phosphate; 7, γ phosphate of ATP; 8, α phosphate of ATP; 9, NAD(H); 10, β phosphate of ATP.

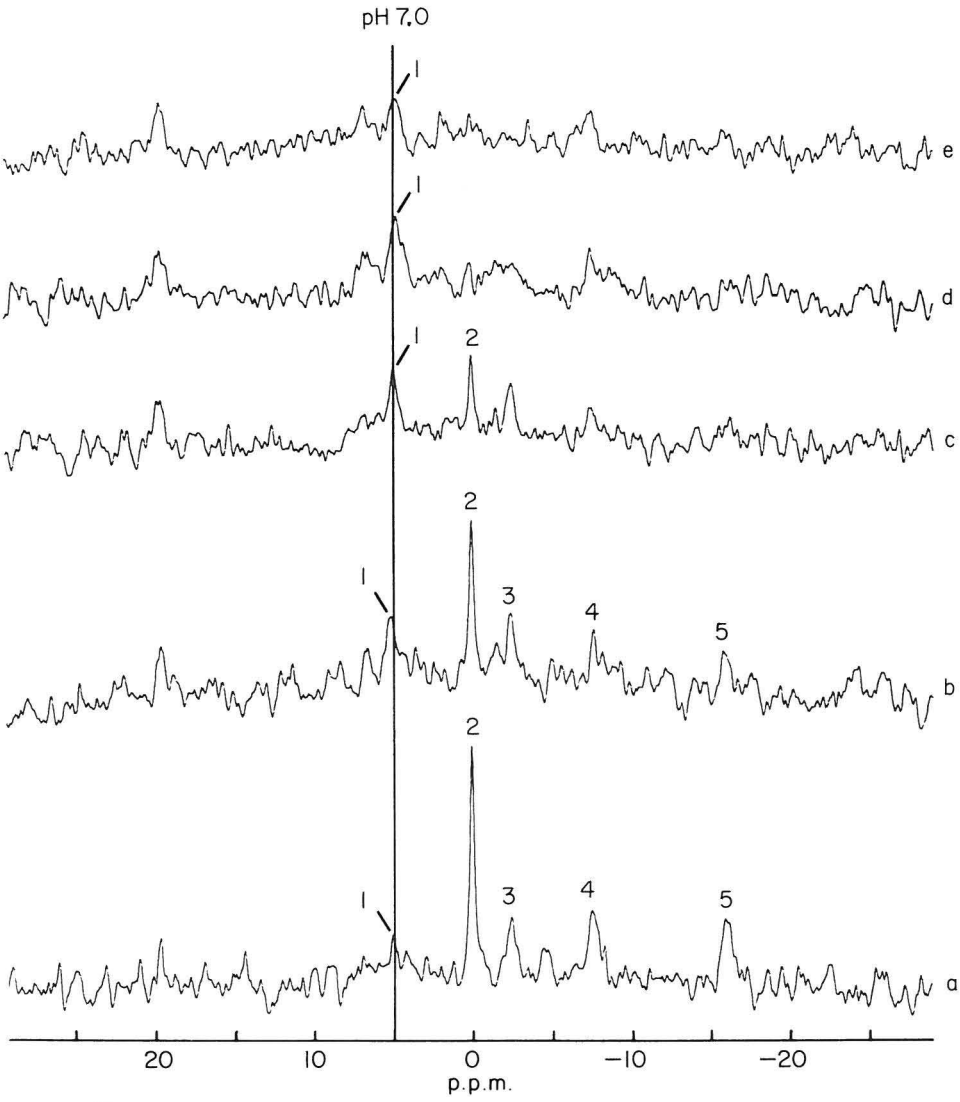

FIGURE 2. ^{31}P NMR spectra of a rabbit heart obtained from 6 scans and taken between 0 to 20 s (a); 20 to 40 s (b); 40 to 60 s (c); 60 to 80 s (d) and 80 to 100 s (e) of reperfusion with calcium-containing solution. Resonances are assigned as follows: 1, intracellular P_i; 2, creatine phosphate; 3, 4 and 5, the γ, α and β phosphate groups of ATP. Intracellular pH was measured from the chemical shift of the intracellular P_i peak, and amounted to 7.1 (a); 7.2 (b); 7.0 (c); 6.9 (d) and 6.9 (e). The vertical line indicates the position of the P_i peak at pH 7.0.

space may have contributed as well. In conclusion, acidification of the cytosol does not play a causal role in the development of the calcium paradox.

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