J. Clin. Chem. Clin. Biochem.
Vol. 24, 1986, pp. 971–979

Intracellular Free Ca^{2+} in the Course of the Ca^{2+} Paradox and during Poisoning
Ca^{2+}-Selective Microelectrode Measurements in the Perfused Rat Heart

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(Received March 13/July 21, 1986)

Summary: The free intracellular Ca^{2+} concentration of perfused rat hearts was measured using Ca^{2+}-selective microelectrodes. In *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+} (controls) the intracellular Ca^{2+} concentration was 0.87 ± 0.07 µmol/l and the membrane potential was −51.5 ± 0.3 mV. Without glucose the membrane potential approached zero after ca. 60 min, whereas the Ca^{2+} concentration during 110 min increased only slowly to 10.0 µmol/l. During Ca^{2+}-free perfusion (5 min) both parameters did not change significantly. With reintroduction of 2.0 mmol/l Ca^{2+} the membrane potential rapidly collapsed and the intracellular Ca^{2+} concentration was elevated above 0.1 mmol/l within two min. Reperfusion with only 0.1 mmol/l Ca^{2+} decelerated both changes. Poisoning by carbonyl cyanide-*p*-trifluro-methoxyphenylhydrazone or antimycin A in Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer increased the intracellular Ca^{2+} concentration to 30.0 and 25.0 µmol/l, and the membrane potential was collapsed after 16 and 10 min, respectively. In antimycin A- and Ca^{2+}-containing sucrose medium the intracellular Ca^{2+} during 16 min increased above 1.0 mmol/l, and the membrane potential began to increase only after 10 min.

The results are consistent with the postulate of a hypothetical mechanism of cell injury, in which noxious membrane-cytoskeleton interactions are induced by an elevated intracellular Ca^{2+} concentration. It is concluded that Ca^{2+} entry via Na/Ca exchange is not fundamentally involved with induction of injury.

Die freie intrazelluläre Ca^{2+}-Konzentration im Verlauf des Ca^{2+}-Paradox und bei Vergiftung
Messungen mit Ca^{2+}-selektiven Mikroelektroden am perfundierten Rattenherzen

Zusammenfassung: Mit Hilfe Ca^{2+}-selektiver Mikroelektroden wurde am perfundierten Rattenherzen die intrazelluläre freie Ca^{2+}-Konzentration gemessen. In *Krebs-Ringer*-Bicarbonatpuffer + 0,1 mmol/l Ca^{2+} (Kontrollen) ergaben sich für die intrazelluläre Ca^{2+}-Konzentration 0,87 ± 0,07 µmol/l und für das Membranpotential −51,5 ± 0,3 mV. Ohne Glucose ging das Membranpotential innerhalb von 60 min auf Null, die Ca^{2+}-Konzentration stieg nur langsam innerhalb von 110 min auf 10,0 µmol/l an. Während der Ca^{2+}-freien Perfusion (5 min) änderten sich beide Kenngrößen nicht wesentlich, bei anschließender Perfusion mit 2,0 mmol/l Ca^{2+} brach das Membranpotential jedoch sehr schnell zusammen, und die intrazelluläre Ca^{2+}-Konzentration war innerhalb von zwei min auf über 0,1 mmol/l erhöht. Bei Perfusion mit nur 0,1 mmol/l Ca^{2+} traten beide Änderungen verlangsamt auf. Auch bei Vergiftung mit Carbonylcyanid-*p*-trifluoromethoxyphenylhydrazon oder Antimycin A in Ca^{2+}-freiem *Krebs-Ringer*-Bicarbonatpuffer war die intrazelluläre Ca^{2+}-Konzentration auf 30,0 bzw. 25,0 µmol/l erhöht, das Membranpotential war nach 16 bzw. 10 min zusammengebrochen. In Antimycin A- und Ca^{2+}-enthaltendem Saccharosemedium stieg Ca^{2+} intrazellulär innerhalb von 16 min auf über 1.0 mmol/l an, während das Membranpotential erst nach 10 min ein Ansteigen erkennen ließ.

Die Resultate stehen im Einklang mit dem im Rahmen einer Hypothese über den Mechanismus der Zellschädigung erhobenen Postulat, daß eine Erhöhung der intrazellulären Ca^{2+}-Konzentration schädigende Interaktionen zwischen Membran und Zytoskelett auslöst. Es wird die Schlußfolgerung gezogen, daß ein Ca^{2+}-Eintritt via Na/Ca-Austausch nicht ursächlich am Zustandekommen einer Zellschädigung beteiligt ist.

Introduction

Recent research on the structure of skeletal and cardiac muscle cells has revealed that the sarcoplasm of adult muscle fibres contains besides the known actomyosin filaments additional filament lattices, which constitute a cytoskeletal matrix ensuring structural continuity in striated muscle (1, 2). Especially the intermediate filament lattice is believed to represent myofibril-sarcolemma transverse connection, and at the level of Z- and M-lines it may be connected to the sarcolemma by special insertion sites (3 – 5).

In previous investigations (6, 7) we have presented evidence that injury of both skeletal and cardiac muscle of the rat may be caused by noxious reactions of a membrane-anchored cytoskeleton which are triggered by an unphysiologically high Ca^{2+} concentration of the sarcosol. Under appropriate conditions a membrane blebbing process is induced by pressure gradients that arise transiently across the sarcolemma, whereby cytosolic constituents are squeezed out through the ruptured membranes of the blebs.

Our aims were to measure the intracellular free Ca^{2+} concentration of myocardial cells with Ca^{2+}-selective microelectrodes to prove the postulate of the above membrane blebbing hypothesis, i. e. that the induction of deleterious cytoskeletal reactions is triggered by a strongly increased Ca^{2+} concentration of the sarcosol. In analogy to the previous enzyme release experiments (7), the perfused rat heart was employed as an experimental model. Cell injury was brought about by the perfusion conditions of the Ca^{2+} paradox and by poisoning free energy production of myocardial cells. The results show that the intracellular Ca^{2+} concentration under all conditions was elevated at an early stage, and so confirm the postulated Ca^{2+} increase of the sarcosol as a necessary prerequisite for induction of membrane damage and enzyme release.

Materials and Methods

Langendorff perfusion

Isolated hearts from male rats (HAN-WISTAR) were perfused (*Langendorff* perfusion) as described elsewhere (7). The perfusion rate was 6 ml/min and the perfusion pressure of beating hearts reached 8 kPa. For microelectrode measurements the hearts were mounted on a stainless steel holding device whithin a perspex perfusion chamber. Generally, isolated myocards were first perfused with *Krebs-Ringer* bicarbonate buffer of normal (1.0 mmol/l) Ca^{2+} concentration for about 5 min to control myocardial contractility. Thereafter, the hearts were perfused with *Krebs-Ringer* bicarbonate buffer containing 0.1 mmol/l Ca^{2+}. Under these conditions contractions disappeared within a few minutes. During the subsequent perfusion interval of 10 min, control measurements were performed to assure cellular integrity. The measuring points of figures 2 – 8 were obtained from 4 hearts, respectively.

Perfusion media

Krebs-Ringer bicarbonate buffer (mmol/l)

NaCl 118.0; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25.0. The Ca^{2+} concentration was adjusted by adding solid $CaCl_2$.

Buffered sucrose solution (mmol/l)

sucrose 250.0; tris (hydroxymethyl)-aminomethane 20.0.

The osmolality of perfusion media was ca 0.3 osmol/kg H_2O, the pH was about 7.4, and the perfusion temperature was kept at 37 ± 0.5 °C. Unless otherwise stated, the glucose concentration was 10 mmol/l. Added toxins are indicated in the respective experiments. Bicarbonate buffers were gassed at 37 °C with 95% O_2 + 5% CO_2, sucrose solutions with 100% O_2.

Construction of microelectrodes

Ca^{2+}-selective liquid membrane microelectrodes were constructed according to the method of *Oehme* (8) and of *Ammann* et al. (9).

Micropipettes were pulled from double-barrelled borosilicate tubing of ca 0.3 cm diameter (Fa. JÜRGENS, Hannover). First the reference barrel was filled from the back with 163.0 mmol/l KCl and then the other in the same manner with 10.0 mmol/l $CaCl_2$. The Ca^{2+} channel was siliconized by repeatedly sucking up a solution of dimethyldichlorosilane (5 vol% in carbon tetrachloride) through the open tip of the pipette. To avoid siliconization of the inner surface of the reference channel, this barrel was continually gassed with N_2 at 400 kPa. After bathing the tip in 163.0 mmol/l KCl and bidistilled water, the sensor (in vol%: 10.0 Ca^{2+} ionophor ETH 1001; 1.0 sodium tetraphenyl-borate; 89.0 *o*-nitro-phenyl-octyl-ether; Fluka) was introduced by suction. The chloride solutions of the two barrels were then electrically connected by chlorided silver wires (silver-silver chloride electrodes). The wires were fixed to the non-inverting pins of high impedance preamplifiers, which were connected to a differential amplifier with a digital voltmeter and a 2-channel recorder (for detailed description of electronic assembly see l. c. (10)). Before calibration the microelectrode was immersed in 163.0 mmol/l KCl + 10.0 mmol/l $CaCl_2$ and "conditioned" for at least two hours.

Calibration of microelectrodes

Since Ca^{2+} impurities are present in salts, bidistilled water and glassware, Ca^{2+} complexing ligands must be introduced to adjust the free Ca^{2+} concentration to low values. Given the stability constant of the Ca^{2+} ligand complex and the acid constants of the ligand, the free Ca^{2+} concentration may be simply calculated from pH determinations with a H^+-selective glass electrode. For calculations we used "mixed constants" from l. c. (11), which were corrected for physiological ionic

strength 0.18 mol/l of the calibration solution, employing the *Davies* equation (12) for activity coefficients. The calibration solution contained (in mmol/l): 163.0 KCl, 5.0 ethylene diaminetetraacetic acid (disodium salt, EDTA) and 1.0 $CaCl_2$. The free Ca^{2+} concentration was calculated by a modification of the α-coefficient method of *Schwarzenbach* (13).

$$K' = \frac{[ML]}{[M'][L']} = \frac{K_{ML}}{\alpha_L \alpha_M}$$

[ML] = concentration of complex
[M] = concentration of metal ion
[L'] = concentration of all ligand species but without ML
[M'] = concentration of all metal ion species but without ML
K_{ML} = thermodynamic stability constant
K' = mixed stability constant
$\alpha_L = 1 + 10^{lg\beta_1 - pH} + 10^{lg\beta_2 - 2pH} + 10^{lg\beta_3 - 3pH} + 10^{lg\beta_4 - 4pH}$
$\alpha_M = 1 + 10^{pH - pK_1}$
β_i = overall formation constants of EDTA
K_1 = first dissociation constant of hydrated metal ion
$[ML] = [M_o] - [M'] = [M_o] - \alpha_M[M]$
$[L'] = [L_o] - [ML] = [L_o] - [M_o] + \alpha_M[M]$

with $Z = [L_o] - [M_o]$ gives

$$K' = \frac{[M_o] - \alpha_M[M]}{\alpha_M[M](\alpha_M[M] + Z)}$$

$$[M] = -\frac{1}{2}\left(\frac{Z}{\alpha_M} + \frac{1}{K'\alpha_M}\right) + \left(\frac{1}{4}\left(\frac{Z}{\alpha_M} + \frac{1}{K'\alpha_M}\right)^2 + \frac{[M_o]}{K'\alpha_M^2}\right)^{\frac{1}{2}}$$

$[M_o]$ = analytical concentration of metal ion
$[L_o]$ = analytical concentration of ligand

Used constants

$lg\,\beta_1 = 10.19$; $lg\,\beta_2 = 16.33$; $lg\,\beta_3 = 19.02$; $lg\,\beta_4 = 21.07$; $lg\,K_{ML} = 10.36$; $pK_1 = 12.6$; $[M_o] = 1.0$ mmol/l; $[L_o] = 5.0$ mmol/l.

Computations were made with a microcomputer. The calculated value for [M] is related to the Ca^{2+} concentration rather then activity, because "mixed contants" were used. In practice, about 80 ml of calibration solution (20 °C) were titrated from pH = 3.9 to pH = 8.5 with ca 0.1 ml 5.0 mol/l KOH and back with HCl. The pH of the solution was monitored by a H^+-selective glass electrode, the Ca^{2+} activity associated potential was measured by the microelectrode (mV). To earth the solution and to control the reference channel of the microelectrode, this channel was electrically connected with the reference of the glass electrode. The same circuit was also used for intracellular recordings. Under these conditions the voltage change between the "outer" and "inner" references represented the membrane potential at the myocardial cell membrane. For pCa = 4 to pCa = 2 ($pCa = -\log_{10}[Ca^{2+}]$) analytical values were taken, which must fit the calculated curve. As mentioned above the Ca^{2+} calibration curve was obtained at a temperature of ca. 20 °C. Intracellular measurements, however, were performed at 37 °C at the inserted tip of the microelectrode. The resulting bias theoretically could amount to 0.05 and 0.2 pCa units in the range of 1.0 mmol/l to 1.0 µmol/l Ca^{2+}, respectively. We did not find, however, any significant change of the electrode signal, when the temperature of a solution of 0.1 mmol/l Ca^{2+} in 163.0 mmol/l KCl was varied between 20 and 37 °C.

Each microelectrode was calibrated before and after an experiment. Usually small deviations between calibration curves could be detected. Data were evaluated by using the second calibration curve.

Results

Control experiments

To avoid myocardial contraction, rat hearts were perfused with *Krebs-Ringer* bicarbonate buffer of only 0.1 mmol/l Ca^{2+}. Figure 1 shows the protocols of the membrane potential and the Ca^{2+} concentration, respectively, during microelectrode impalement of one single cell of the left ventricle. After 33 min at an intracellular Ca^{2+} concentration of 35.0 µmol/l the membrane potential reached about −5 mV, indicating severe membrane deterioration.

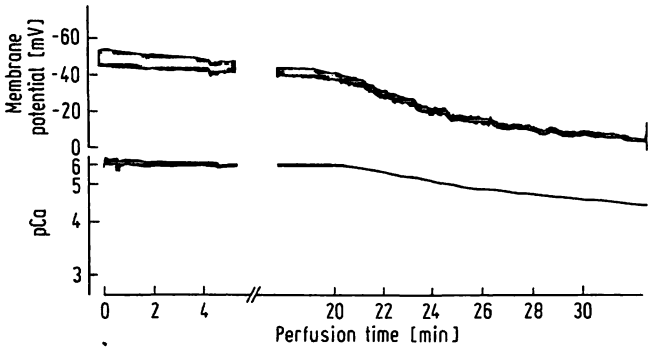

Fig. 1. Intracellular recording of membrane potential and pCa of one single muscle cell of a *Langendorff*-perfused rat heart under control conditions.
Perfusion medium: *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+} (control).
$pCa = -\log_{10}[Ca^{2+}]$

In the following figures, intracellular recordings are represented by points. Two points (membrane potential and Ca^{2+}-concentration) were always obtained from one impaled cell. During the respective perfusion periods, different cells were successively impaled, so that the number, n, of point pairs represents the number of different cells. At given time intervals the respective values of membrane potential and Ca^{2+} concentration were recorded after intracellular values had remained stable for 1−2 min. Under control conditions, during 10 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}, we found −51.5 ± 0.3 mV, ($\bar{x}$ ± SEM; n = 56; 24 hearts), and 0.87 ± 0.07 µmol/l, for the membrane potential and intracellular Ca^{2+}-concentration, respectively.

Glucose withdrawal

The effect of glucose withdrawal from the perfusion medium is demonstrated by comparing figure 2 and 3. In the presence of substrate only the membrane potential slowly increased (became more positive) (fig. 2). Under these conditions the hearts began to

beat again even after two hours of perfusion when Ca^{2+} was increased to 1.0 mmol/l. In the absence of extracellular glucose the membrane potential increased after 20 min and was collapsed after about 60 min. The intracellular Ca^{2+} concentration continuously increased during 110 min from 1.0 to about 10.0 µmol/l (fig. 3). These hearts were unable to recover after two hours when the normal concentration of Ca^{2+} was restored.

Ca^{2+} paradox

When isolated hearts are perfused with a nominally Ca^{2+}-free medium and subsequently are reperfused with a Ca^{2+}-containing buffer, irreversible cell damage can be produced at an early stage of reperfusion. During the Ca^{2+}-free perfusion period, which usually is extended over 20 to 30 min, however, extra- and intracellular alterations may have already occurred. Therefore, to minimize cellular deterioration we reduced this perfusion interval to 5 min. During preperfusion with a nominally Ca^{2+}-free ($(Ca^{2+}) \approx$ 5 µmol/l) *Krebs-Ringer* bicarbonate buffer, the membrane potential only slightly increased and the intracellular Ca^{2+} concentration was not significantly changed (fig. 4 and 5). Reintroduction of 2.0 mmol/l Ca^{2+} to the perfusion medium abruptly collapsed the membrane potential and within 2 min the intracellular Ca^{2+} concentration became elevated above 0.1 mmol/l (fig. 4). With 0.1 mmol/l extracellular Ca^{2+} during reperfusion both parameters increased more slowly (fig. 5), but the intracellular Ca^{2+} concentration of some cells was increased 3-fold over the extra-

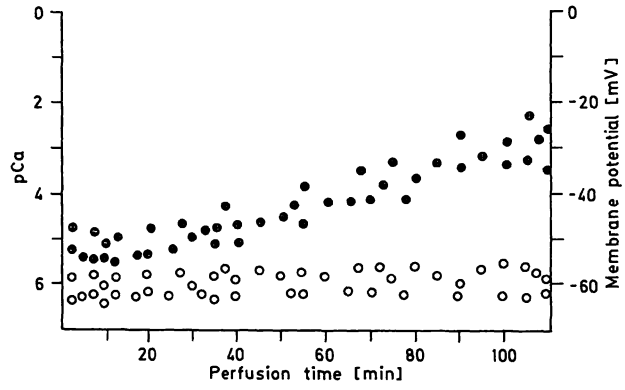

Fig. 2. Membrane potential and intracellular pCa of myocardial cells during control perfusion.
Perfusion medium: *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
$pCa = -\log_{10}[Ca^{2+}]$

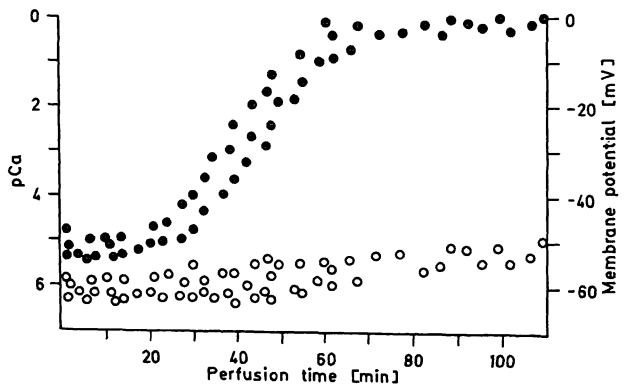

Fig. 3. Membrane potential and intracellular pCa of myocardial cells during glucose-free perfusion.
Perfusion medium: *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+} without glucose.
$pCa = -\log_{10}[Ca^{2+}]$

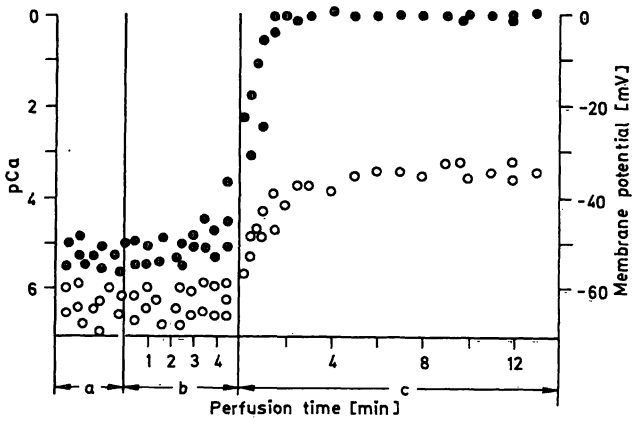

Fig. 4. Membrane potential and intracellular pCa of myocardial cells during the perfusion sequence of the Ca^{2+} paradox.
a: 10 min of control perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
b: 5 min of perfusion with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer.
c: 13 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 2.0 mmol/l Ca^{2+}.
$pCa = -\log_{10}[Ca^{2+}]$

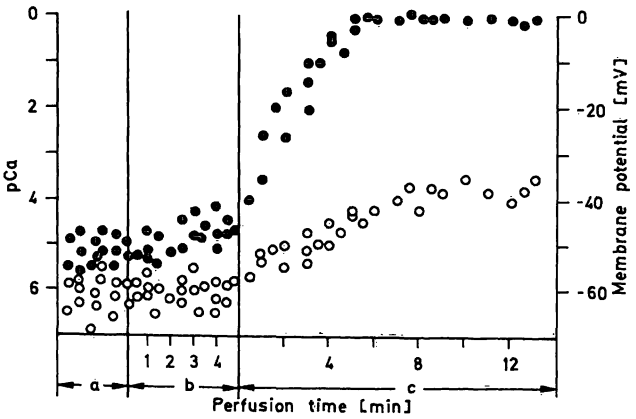

Fig. 5. Membrane potential and intracellular pCa of myocardial cells during the perfusion sequence of the Ca^{2+} paradox.
a: 10 min of control perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
b: 5 min of perfusion with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer.
c: 13 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
$pCa = -\log_{10}[Ca^{2+}]$

cellular concentration. At both extracellular Ca^{2+} concentrations myocards appeared contracted after reintroduction of Ca^{2+} to the perfusion media. Because of continuous cell swelling in Ca^{2+}-free sucrose media, the conditions of the Ca^{2+} paradox in buffered sucrose solution (7) were unsuitable for parallel microelectrode measurements.

Poisoning by uncoupler

Proton carriers, like the classical uncoupler 2,4-dinitrophenol or the more potent carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone, are widely used to dissipate the electrochemical potential difference of protons across the inner membrane of mitochondria, and thus to suppress the energy metabolism of the cell. These chemicals, however, are of limited value for studies with liquid membrane electrodes, because the uncoupler may impair the electrode signal by interaction with the sensor phase. Therefore, to suppress "electrode poisoning", uncoupler-treated myocards were subsequently perfused with unpoisoned medium to wash out the uncoupler from the aqueous phase.

To prove the postulated intracellular Ca^{2+} increase under the conditions of poisoning by uncoupler but in the "absence" of extracellular Ca^{2+}, rat hearts were perfused with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer + 1.0 µmol/l uncoupler. Figure 6 shows the membrane potential and the intracellular Ca^{2+} concentration after a two min wash out with unpoisoned buffer. To adapt the perfusion sequence to that of the enzyme release experiments of l. c. (7), before poisoning, perfusion for 2 min with *Krebs-Ringer* bicarbonate buffer + 1.0 mmol/l Ca^{2+} was included. The Ca^{2+} concentration after 4–5 min (fig. 6, phase c) was increased from 1.0 µmol/l to about 30.0 µmol/l, whereas the membrane potential during 16 min increased more slowly from ca. −30 mV to zero. The intracellular Ca^{2+} concentration was 6-fold higher than the extracellular Ca^{2+} concentration.

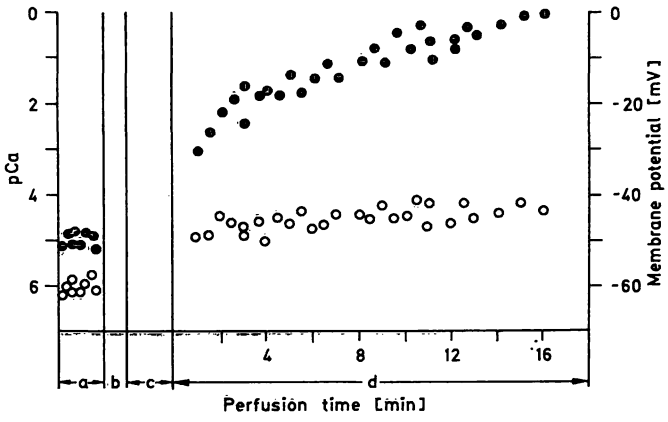

Fig. 6. Membrane potential and intracellular pCa of myocardial cells during poisoning with uncoupler carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone.
a: 10 min of control perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
b: 2 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 1.0 mmol/l Ca^{2+}.
c: 2 min of perfusion with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer without glucose + 1.0 µmol/l uncoupler, thereafter 2 min wash out of uncoupler with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer without glucose.
d: 16 min of perfusion with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer without glucose.
$pCa = -\log_{10} [Ca^{2+}]$

Poisoning by antimycin A

Antimycin A inhibits electron transport between cytochromes b and c_1 of complex III. The substance does not interfere with the microelectrode signal, so that an additional perfusion step to wash out the poison was not necessary. To demonstrate, as in the preceding experiment, an intracellular Ca^{2+} increase in the "absence" of extracellular Ca^{2+}, myocards were perfused with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer + 1.8 µmol/l antimycin A. The intracellular Ca^{2+} concentration was elevated from 1.0 µmol/l to ca 25.0 µmol/l during 8 min of perfusion, and the membrane potential was collapsed after 10 min (fig. 7).

In a previous publication (7) we demonstrated that, despite 2,4-dinitrophenol poisoning, enzyme release from perfused myocards is strongly suppressed, provided cell swelling is hindered by perfusing with Ca^{2+}-containing sucrose media. We suggested an increase

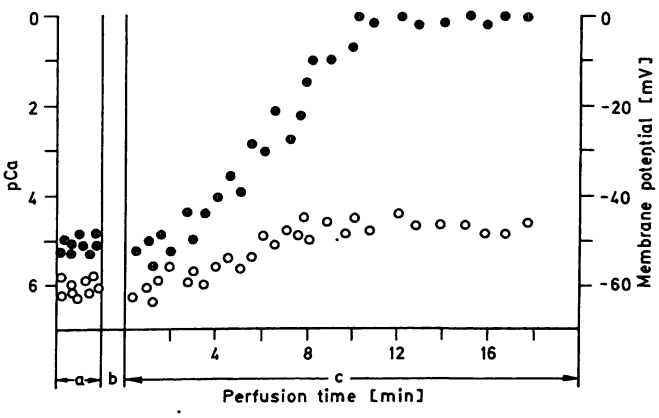

Fig. 7. Membrane potential and intracellular pCa of myocardial cells during poisoning with antimycin A.
a: 10 min of control perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
b: 2 min perfusion with *Krebs-Ringer* bicarbonate buffer + 1.0 mmol/l Ca^{2+}.
c: 18 min of perfusion with Ca^{2+}-free *Krebs-Ringer* bicarbonate buffer without glucose + 1.8 µmol/l antimycin A.
$pCa = -\log_{10} [Ca^{2+}]$

of the intracellular Ca^{2+} concentration and postulated that an elevated Ca^{2+} alone is not sufficient to induce cell injury. An additional cell swelling could rapidly produce a drastic enzyme release under these conditions (7). To prove the above assumption of an increased intracellular free Ca^{2+} concentration, by analogy to enzyme release experiments, rat hearts were perfused with antimycin A-poisoned sucrose solution containing 3.5 mmol/l Ca^{2+}. In the presence of this relatively high extracellular Ca^{2+} concentration the sarcolemma maintained a low permeability, so that swelling by sucrose and water entry could not occur. Because of the absence of extracellular K^+ the membrane potential was strongly hyperpolarised to about −100 mV at the beginning of perfusion (not shown). The hyperpolarisation vanished immediately and during the following 8−10 min the membrane potential remained constant at about −50 mV (fig. 8). After 10 min the membrane potential slowly depolarized and reached zero after 16 min. The intracellular Ca^{2+} concentration increased continuously to about 1.0 mmol/l during this time interval and in some cells approached the Ca^{2+} concentration of the perfusion medium.

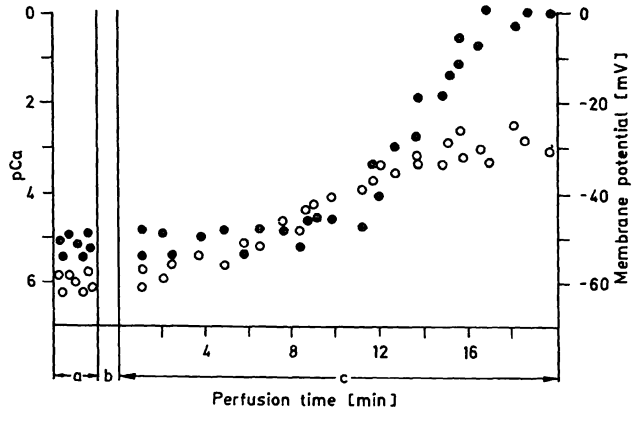

Fig. 8. Membrane potential and intracellular pCa of myocardial cells during poisoning with antimycin A in Ca^{2+}-containing buffered sucrose solution.
a: 10 min of control perfusion with *Krebs-Ringer* bicarbonate buffer + 0.1 mmol/l Ca^{2+}.
b: 2 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 1.0 mmol/l Ca^{2+}.
c: 20 min of perfusion with buffered sucrose solution without glucose + 3.5 mmol/l Ca^{2+} and 1.8 µmol/l antimycin A.
pCa : $-\log_{10}[Ca^{2+}]$

Under the latter three conditions of poisoning drastic cell damage was not observed, although the hearts were strongly contracted.

Discussion

Control experiments

Measurements of the intracellular Ca^{2+} concentration and the membrane potential were carried out using double-barrelled neutral carrier-based liquid membrane microelectrodes. Control values of both parameters were obtained from non-contracting hearts at 0.1 mmol/l extracellular Ca^{2+}. Comparing figures 1 and 2 it is evident that a prolonged impalement of a single myocyte could seriously affect both the membrane potential and intracellular Ca^{2+} concentration. Obviously, insertion of the electrode tip into the cell caused an increase of ionic leaks, which ultimately must be compensated by the energy metabolism of the cell. During short impalements (2 min) this compensation seems to be sufficiently maintained.

During our control conditions at 0.1 mmol/l extracellular Ca^{2+} we measured relative high intracellular Ca^{2+} concentrations of almost 1.0 µmol/l. With the verapamil (Ca^{2+} channel blocker) -arrested rat heart at 1.0 mmol/l extracellular Ca^{2+}, intracellular concentrations of Na^+, H^+ and Ca^{2+} and the membrane potential were significantly reduced compared with those found with 0.1 mmol/l extracellular Ca^{2+} without verapamil (*Diederichs & Wittenberg* in preparation). Especially the intracellular Ca^{2+} concentration was much lower (0.17 µmol/l), and the membrane potential was reduced to −58 mV. *Powell* et al. (14) demonstrated with isolated myocardial cells that a high extracellular Ca^{2+} concentration (5.5−13.5 mmol/l) was necessary to obtain a more negative resting potential and low input resistance. *Philipson* et al. (15) showed that the Na/Ca exchange process of sarcolemmal vesicles could be activated by an increase and partially inactivated by a decrease of phospholipid-bound Ca^{2+}. These latter observations may explain the relatively high intracellular Ca^{2+} concentration of our controls, since a low extracellular Ca^{2+} concentration would also lower the amount of bound Ca^{2+} of the bilayer outer surface and, therefore, could inactivate the Na/Ca exchange process. Ca^{2+} leaks into the sarcosol must then be extruded solely by Ca^{2+} pumps of the sarcoplasmatic reticulum and the sarcolemma.

Glucose withdrawal

When glucose was present, the intracellular Ca^{2+} concentration remained unchanged and the membrane potential increased only slowly (fig. 2). Without added glucose, however, an early increase of the membrane potential was observed (fig. 3). Under similar

conditions during acute ischaemia of guinea pig hearts an increase of membrane potential was shown to be paralleled by a K^+ efflux (16, 17). K^+ release from rabbit septum during anoxia was also demonstrated (18). *Jennings* et al. (19) with the ischaemic dog heart showed that ATP was less than 0.4 µmol/g wet weight after 40 and 90 min of ischaemia in vivo and in vitro, respectively. A coinciding efflux of K^+, lactate and inorganic phosphate was found by *Mathur & Case* (20). At a given resting potential this massive K^+ efflux can only be achieved by a charge compensating influx of cations or efflux of anions as discussed by *Kleber* (17). It is suggested therefore that the initial membrane depolarisation under the conditions of figure 3 was brought about by an electrogenic anion efflux (perhaps phosphate) at decreasing ATP levels of the sarcoplasm. The corresponding K^+ distribution over the cell membrane may be ensured by an equivalent K^+ release. Later, when the ATP levels of the sarcosol are extremely low, Na^+ may be taken up by the cell from the extracellular space.

Compared with poisoned conditions, the intracellular Ca^{2+} concentration increased relative slowly and did not exceed 10.0 µmol/l during 110 min, although the ATP concentration may have been decreased below 0.4 µmol/g wet weight (19) after about 60 min when the membrane potential approached zero (fig. 3). Supposedly, in the presence of oxygen and phosphate, mitochondria, even with a very low supply of substrate, may accumulate Ca^{2+}, especially at a Ca^{2+} concentration > 1.0 µmol/l.

Ca^{2+} efflux via the Na/Ca exchange carrier may also contribute to the lowering of sarcosolic Ca^{2+}, provided the ratio of electrochemical potential differences of Ca^{2+} and Na^+ remains below the coupling ratio, $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na} < n$ (21). This in turn would activate the Na/K pumps by depolarizing the membrane potential (22). However, as we have already discussed above, the Na/Ca exchange reaction may be partially inhibited at 0.1 mmol/l extracellular Ca^{2+}.

Ca^{2+} paradox

It is generally accepted that cell injury during the course of the Ca^{2+} paradox is brought about by a Ca^{2+} overload of the cell. Our results show that not only total cell Ca^{2+} was elevated, as demonstrated by others, but also the free Ca^{2+} concentration of the sarcosol was drastically increased during reperfusion. Therefore, this Ca^{2+} must have entered the myocardial cell from the extracellular space. The route of entry, however, is not yet well understood (23). *Alto & Dhalla* (24) showed that under very similar conditions total myocardial Na^+ was not significantly increased after 5 min of Ca^{2+}-free perfusion. Concerning Ca^{2+} entry via the Na/Ca exchange carrier with an unaltered intracellular Na^+ concentration, an influx of Ca^{2+} would be energetically unfavourable, because $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na} < 3$. Assuming the following values at the beginning of reperfusion: 1.0 µmol/l and 2.0 mmol/l for intra- and extracellular Ca^{2+} concentrations; 15.0 mmol/l (*Diederichs & Wittenberg* in preparation) and 143.0 mmol/l for intra- and extracellular Na^+ concentrations; and a membrane potential of about −45 mV (figs. 4 and 5); then $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ = 293 mV / 105 mV ≈ 2.8 At an extracellular Ca^{2+} concentration of 0.1 mmol/l, $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na} \approx 2.0$, which even more evidently demonstrates that Ca^{2+} could not have entered the sarcosol via exchange carrier under these conditions.

McClellan & Winegrad (25) have demonstrated that ventricular muscle cells have an unspecifically elevated permeability to small ions and molecules at low extracellular Ca^{2+}. Under these conditions we observed pronounced myocardial swelling in isotonic sucrose media (7). It may be concluded, therefore, that Ca^{2+} ions at the onset of reperfusion enter the sarcoplasm through unspecific leaks in the sarcolemma, and that reintroduced Ca^{2+} penetrates these leaks more rapidly than it can restore low permeability. An unspecific increase of permeability of the phospholipid bilayer at a low Ca^{2+} concentration, however, seems unlikely; on the contrary, bilayers of acidic phospholipids become leaky at high Ca^{2+} concentrations in the presence of phosphate by changing the bilayer configuration (26–28). In our opinion, the opening of cell-to-cell channels to the interstitial space at a low extracellular Ca^{2+} concentration (23, 29) could provide unspecific pathways for diffusional fluxes of small ions and molecules. During the Ca^{2+} paradox, reintroduced Ca^{2+} may quickly enter the myocardial cell through these channels by moving down its very steep electrochemical gradient.

During Ca^{2+}-free perfusion the free Ca^{2+} concentration of the sarcosol was not significantly decreased (fig. 4 and 5), although *Alto & Dhalla* have shown that total Ca^{2+} of the myocard is reduced to ca. 68% after 5 min (24). Most of this Ca^{2+} may be released from extracellular binding sites of the bilayer (30), and outward transport by sarcolemmal Ca^{2+} pumps also may contribute to the Ca^{2+} loss. Ca^{2+} efflux by Na/Ca exchange probably is of secondary importance, because the transport reaction is at least partially inhibited by a reduction of Ca^{2+} binding (15). As mentioned above, the increased production of small anions and inorganic phosphate by accelerated

metabolism may induce K^+ release (13% after 5 min (24)) with a corresponding small increase of the membrane potential.

Enzyme release during the Ca^{2+} paradox in tris-buffered sucrose media (pre- and reperfusion) is even more pronounced (7). Under these conditions of a reversed Na^+ electrochemical potential difference (no extracellular Na^+) an additional Ca^{2+} influx via Na/Ca exchange carrier with reperfusion is energetically favourable. Backflux of positive charges across the leaky membrane is then possible.

Poisoning

In a preceding paper we postulated (7) that under the conditions of cell poisoning with no extracellular Ca^{2+}, the intracellular Ca^{2+} concentration is likewise increased by Ca^{2+} efflux from intracellular organelles. An additional cell swelling may cause cell injury with enzyme release. In fact, this postulate was proved under the analogous conditions of poisoning with the uncoupler carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone. The intracellular free Ca^{2+} concentration was 30-fold increased above the control in the nominal absence of extracellular Ca^{2+}. With antimycin A qualitatively similar results were obtained. It is expected that most of this Ca^{2+} was released from the sarcoplasmatic reticulum (31), presumably by a decrease of pump fluxes through Ca^{2+}-ATPases at a reduced sarcosolic ATP concentration. Ca^{2+} accumulation by mitochondria is impossible, because the electric potential difference at the mitochondrial inner membrane was collapsed under these conditions (figs. 6 and 7). At low extracellular Ca^{2+} the intracellular Ca^{2+} increase might be expected to be compensated by Ca^{2+} efflux via the Na/Ca exchange carrier as long as a sufficiently high Na^+ electrochemical potential difference is maintained. *Caroni & Carafoli* (32) have presented evidence, however, that the Na/Ca exchange system of heart sarcolemma is regulated by Ca^{2+}- and calmodulin-dependent phosphorylation and dephosphorylation. At a Ca^{2+} concentration of 3.0 µmol/l the dephosphorylation step becomes privileged and leads to an inactivation of the exchange process. Under our conditions of poisoning at drastically decreased ATP and increased Ca^{2+} concentrations, the Na/Ca exchange carrier may therefore be fully inactivated, so that a decrease of intracellular Ca^{2+} by this transport reaction is rather unlikely. In addition to this intracellular inhibition, the Na/Ca exchange process may be inhibited extracellularly by decreased sarcolemmal Ca^{2+} binding (15).

With regard to the perfusion conditions of figure 8, Ca^{2+} entry via Na/Ca exchange may be inhibited in the same way at low ATP levels. A further inhibition of exchange transport by acidification of the sarcoplasm, resulting from inactivation of Na/H exchange (33), must be considered at a very low extracellular Na^+ concentration (34, 35). Therefore, also during perfusion with Ca^{2+}- and antimycin A-containing sucrose media, the Ca^{2+} increase of the sarcosol, at least during the first 10 min of a maintained membrane potential, was caused by Ca^{2+} efflux from the sarcoplasmatic reticulum. At a later stage, it seems reasonable to suppose that inward transport of Ca^{2+} occurs through voltage-dependent Ca^{2+} channels.

Conclusions

Measurements of the free Ca^{2+} concentration of the sarcosol have revealed that not only during the Ca^{2+} paradox, but also during cell poisoning in the nominal absence of extracellular Ca^{2+}, the intracellular Ca^{2+} concentration is strongly elevated. These results confirm the postulate of our membrane blebbing hypothesis, i. e. that a drastically increased intracellular Ca^{2+} concentration is a prerequisite for induction of cell injury with a concomitant enzyme release. At an elevated Ca^{2+} concentration either additional ATP or swelling is needed to induce membrane blebbing through the reactions of the membrane-anchored cytoskeleton. An increased sarcosolic Ca^{2+} concentration per se is not sufficient to produce severe membrane deterioration.

Concerning the ischaemic or anoxic myocard, at a strongly reduced ATP concentration an uncompensated Ca^{2+} efflux from the sarcoplasmatic reticulum must increase the free Ca^{2+} concentration of the sarcosol. If the Ca^{2+} concentration exceeds 10.0 µmol/l an additional cell swelling may initiate a passive membrane blebbing. If reperfusion or reoxygenation is restored, oxidative phosphorylation by mitochondria rapidly increases the ATP concentration, and in the presence of a high Ca^{2+} concentration an active membrane blebbing can be induced. Additional Ca^{2+} from the extracellular space can then enter the cell through membrane lesions and together with phosphate is massively accumulated by mitochondria. Ca^{2+} entry via the Na/Ca exchange carrier is not thought to be fundamentally involved with induction of injury of the ischaemic or anoxic myocard.

Acknowledgement

The authors thank Mr. *K. M. Pütz* für excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft.

References

1. Wang, K. & Ramirez-Mitchell, R. (1983) J. Cell Biol. *96*, 562–570.
2. Wang, K. (1985) Cell Muscle Motil. *6*, 315–369.
3. Pardo, J. V., Siliciano, J. D. & Craig, S. W. (1983) J. Cell Biol. *97*, 1081–1088.
4. Price, M. G. & Sanger, J. W. (1983) In: Cell and Muscle Motility (Dowben, R. M. & Shay, J. W., eds.) vol. 3, pp. 1–40, Plenum Press, New York.
5. Nelson, W. J. & Lazarides, E. (1984) Proc. Natl. Acad. Sci. USA *81*, 3292–3296.
6. Diederichs, F., Mühlhaus, K., Trautschold, I. & Friedel, R. (1979) Enzyme *24*, 404–415.
7. Diederichs, F., Mühlhaus, K., Wittenberg, H. & Trautschold, I. (1986) J. Clin. Chem. Clin. Biochem. *24*, 3–9.
8. Oehme, M. (1977) Thesis, Swiss Federal Institute of Technology, Zürich.
9. Ammann, D., Morf, W. E., Anket, P., Meier, P. C., Pretsch, E. & Simon, W. (1983) Ion-Selective Electrode Rev. *5*, 3–92.
10. Wittenberg, H. (1985) Thesis, University Hannover.
11. Ringbom, A. (1963) Complexation in Analytical Chemistry, Interscience Publishers, John Wiley & Sons, New York.
12. Butler, J. N. (1964) Ionic Equilibrium, a mathematical Approach. Addison-Wesley Publishing Company, Inc. Reading, Massachusetts, Palo Alto/London.
13. Schwarzenbach, G., Flaschka, H. (1965) Die Komplexometrische Titration Enke Verlag, Stuttgart.
14. Powell, T., Terrar, D. A. & Twist, V. W. (1980) J. Physiol. *302*, 131–153.
15. Philipson, K. D., Langer, G. A. & Rich, T. L. (1985) Am. J. Physiol. *248*, H147–H150.
16. Kleber, A. G. (1983) Circ. Res. *52*, 442–450.
17. Kleber, A. G. (1984) J. Mol. Cell. Cardiol. *16*, 389–394.
18. Shine, K. I. (1981) Am. J. Pathol. *102*, 256–261.
19. Jennings, R. B., Reimer, K. A., Hill, M. L. & Mayer, S. E. (1981) Circ. Res. *49*, 892–900.
20. Mathur, P. P. & Case, R. B. (1973) J. Mol. Cell. Cardiol. *5*, 375–393.
21. Sheu, S. S. & Fozzard, H. A. (1982) J. Gen. Physiol. *80*, 325–351.
22. Coraboeuf, E. (1978) Am. J. Physiol. *234*, H101–H116.
23. Grinwald, P. M. & Nayler, W. G. (1981) J. Mol. Cell. Cardiol. *13*, 867–880.
24. Alto, L. E. & Dhalla, N. S. (1979) Am. J. Physiol. *237*, H713–H719.
25. McClellan, G. & Winegrad, S. (1978) J. Gen. Physiol. *72*, 737–767.
26. Cullis, P. R. & De Kruijff, B. (1979) Biochim. Biophys. Acta *559*, 399–420.
27. Fraley, R., Wilschut, J., Düzgünes, N., Smith, C. & Papahadjopoulos, D. (1980) Biochemistry *19*, 6021–6029.
28. Verkleij, A. J. (1984) Biochim. Biophys. Acta *799*, 43–63.
29. Spray, D. C., White, R. L., Mazet, F. & Bennett, M. V. L. (1985) Am. J. Physiol. *248*, H753–H764.
30. Langer, G. A. (1984) J. Mol. Cell. Cardiol. *16*, 147–153.
31. Somlyo, A. P. (1984) Nature *309*, 516–517.
32. Caroni, P. & Carafoli, E. (1983) Eur. J. Biochem. *132*, 451–460.
33. Philipson, K. D., Bersohn, M. M. & Nishimoto, A. Y. (1982) Circ. Res. *50*, 287–293.
34. Roos, A. & Boron, W. F. (1981) Phys. Rev. *61*, 296–434.
35. Lazdunski, M., Frelin, C. & Vigne, P. (1985) J. Mol. Cell. Cardiol. *17*, 1029–1042.

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