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The respiration and calcium content of heart mitochondria from rats with vitamin D-induced cardionecrosis

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Mitochondria were isolated from the heart and skeletal muscle of rats treated with three consecutive daily doses of 100000 i.u. of calciol (cholecalciferol; 'vitamin D_3'). On the fourth day after the last dose, cardiac necrosis developed. At that time mitochondria isolated from heart displayed a 10-fold higher Ca^{2+} content and a 6-fold lower respiratory rate with pyruvate-plus-malate as substrate as well as with other NAD-dependent substrates. No decrease in respiratory rate with succinate as substrate was observed. EDTA (5mM) added to the medium during the isolation procedure restored both the high respiratory rate with pyruvate + malate and the low Ca^{2+} content of the heart mitochondria. The addition of 1mM-$CaCl_2$ to the medium in which a healthy (control) rat heart had been homogenized caused the same impairment of the mitochondria as did calciol treatment of the animals. No changes of mitochondria isolated from skeletal muscle were observed in rats treated with calciol. It is concluded that the heart mitochondria *in vivo* fail to accumulate Ca^{2+} from the cardiac cell overloaded with Ca^{2+} as the consequence of calciol treatment. Mitochondrial Ca^{2+} accumulation occurs during the isolation procedure unless an appropriate amount of chelating agent is added to the homogenization medium. The implication of these findings for the biochemical sequence of events in the calciol-induced cardiac necrosis is discussed.

A generally accepted hypothesis is that the sequence of biochemical events ending in cell necrosis includes a step in which Ca^{2+} overload of mitochondria occurs, with the subsequent functional and structural damage to mitochondria, energy depletion and death of the cell (Wrogemann & Pena, 1976; Jennings & Hawkins, 1980; Fleckenstein, 1983). This conclusion has been drawn from observations on skeletal muscle and heart of hamsters with hereditary myocardiopathy (Wrogemann *et al.*, 1972; Wrogemann & Nylen, 1978), from experiments with isoproterenol-induced cardionecrosis (Rona *et al.*, 1959), and from experiments on ischaemia-induced myocardial damage (Buja *et al.*, 1983). Although myocardial damage with mitochondrial calcification represents a common pattern of injury that occurs in response to many types of toxic and metabolic insults, this is not the case with vitamin D-induced cardiac necrosis. It has been shown (Wrzołkowa & Żydowo, 1980) that after vitamin D treatment the structure of heart mitochondria remains almost intact in the course of necrosis development, whereas the cytoplasm undergoes severe damage and calcium deposits do not occur in the mitochondria but either in the cytoplasm or between the basement membrane and the plasmalemma (Wrzołkowa & Żydowo, 1980).

An excessive Ca^{2+} content in mitochondria is known to impair their respiratory activity (Slater & Cleland, 1953), and calciol treatment is known to increase the Ca^{2+} concentration in some tissues (DeLuca *et al.*, 1979). However, if mitochondria are isolated from the tissue, their Ca^{2+} content may undergo changes during the course of the isolation procedure (Åkerman & Nicholls, 1983; Reinhart *et al.*, 1984) and usually does not reflect the true intracellular distribution of Ca^{2+} *in vivo*.

In the present paper, Ca^{2+} content in the mitochondria isolated either in the presence or in the absence of chelator from the rat heart in which necrosis was induced by vitamin D treatment is

Abbreviations used: calciol, cholecalciferol ('vitamin D_3'); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

compared with the respiratory ability of these mitochondria. Evidence is presented that the Ca^{2+} overload of mitochondria from the necrotic heart is a consequence *in vitro* of the exposure of mitochondria to excessive Ca^{2+} in the tissue.

Materials and methods

Animals

Wistar albino virgin female rats weighing about 200g, fed standard laboratory diet, were given three intra-oral doses of 100000 i.u. of calciol (dissolved in soya-bean oil) by gastric intubation for 3 consecutive days. This is an approx. 100 times higher dose (as calculated on a body-weight basis) than that administrated to rachitic children. It exceeds three times the minimal dose causing a noticeable Ca^{2+} increase and respiratory-activity impairment of heart mitochondria (Fig. 2 below), and this is the dose producing cardionecrosis from which most rats are able to recover (Wrzołkowa & Żydowo, 1980). Control rats were given adequate amounts of soya-bean oil in the same way. On day 7 after the first calciol dose the rats were killed by decapitation, bled, and the tissues were removed as soon as possible.

Preparation of mitochondria

Three hearts were homogenized by hand in a Potter–Elvehjem homogenizer with 25ml of medium consisting of 250mM-sucrose and 10mM-Tris/HCl, pH7.8. In some experiments the medium also contained 5mM-EDTA as indicated in the Tables. The homogenate was centrifuged successively twice for 3min at 600*g*. The mitochondria were sedimented from the supernatant by centrifuging at 10000*g* for 10min, the pellet was washed once by suspending in 20ml of the medium, and re-centrifuged. A part of the final pellet was fixed in 2.5% glutaraldehyde with 50mM-cacodylate buffer, pH7.4, post-fixed in 2% (w/v) OsO_4 and embedded in Epon 812 resin for electron microscopy. The remaining part of the pellet was suspended in the isolation medium and used for the other measurements.

Mitochondria from skeletal muscle were prepared essentially as described by Świerczyński *et al.* (1975), with slight modifications. About 45g of mixed skeletal muscle from the hind legs were homogenized for 30s in a Waring blender with 10vol. of a medium consisting of 210mM-mannitol, 70mM-sucrose and 10mM-Tris/HCl, pH7.8. The homogenate was centrifuged twice for 5min at 500*g* and the resulting supernatant was centrifuged for 10min at 14000*g*. The sediment was suspended in 20ml of the medium containing 250mM-sucrose and 10mM-Tris/HCl, pH7.8, centrifuged for 5min at 650*g*, and mitochondria were sedimented from the supernatant by centrifugation for 10min at 10000*g*. The pellet was prepared for electron microscopy and other analyses as described above for the heart mitochondrial pellet. No experiments with skeletal muscle are presented in which EDTA was added to the homogenization medium, since this compound had no influence on the respiratory activity of skeletal-muscle mitochondria from the calciol-treated animals.

Oxygen-consumption measurements

The respiratory rate of mitochondria was investigated by measuring oxygen consumption polarographically at 30°C with a Clark oxygen electrode. The incubation medium (final volume 2.5ml) contained 25mM-KCl, 50mM-Tris/HCl, pH7.3, 5mM-potassium phosphate buffer, pH7.3, 1μM-CCCP, approx. 1.5mg of mitochondrial protein and the other additions indicated in the Tables and Figures.

Ca^{2+} determination

Mitochondrial Ca^{2+} content was determined with an AAS IN (Carl Zeiss, Jena, Germany) atomic-absorption spectrophotometer after extraction of approx. 5mg of mitochondrial protein with HNO_3 as described by Jennings *et al.* (1970). Whole-heart Ca^{2+} was analysed as described by Mukherjee *et al.* (1981). Approx. 100mg of the tissue was dried to constant weight at 105°C. The dried samples were digested at room temperature in 5ml of concentrated HNO_3 and 2ml of 70% $HClO_4$ until white fumes were noticed. The samples were then diluted by addition of 5ml of distilled water and analysed by atomic-absorption spectrophotometry.

The protein in the mitochondria and postmitochondrial supernatant was determined by the biuret method (Layne, 1957).

Chemicals

Calciol, pyruvate, malate, glutamate, 2-oxoglutarate, NAD and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CCCP was from Calbiochem (Los Angeles, CA, U.S.A.); suprapure HNO_3 was from Merck (Darmstadt, Germany); sucrose, lanthanum chloride and $HClO_4$ were from BDH Chemicals (Poole, Dorset, U.K.); $CaCl_2$ (as standard) was from Hicol b.v. chemicals (Rotterdam, Holland). All other chemicals were of the highest purity available from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Results

EDTA (5mM) was required in the isolation medium to maintain the respiratory rate of the

mitochondria from necrotic heart at the same level as that of healthy heart mitochondria (Table 1). If 2mM-EDTA was added (not shown), the respiration of the necrotic heart mitochondria with pyruvate+malate as substrate was decreased almost to the same extent as that of the necrotic-heart mitochondria isolated without added EDTA (see Table 2). From the data presented in Tables 1 and 2 it may be seen that 5mM-EDTA was able to prevent both the decrease in the oxidation of pyruvate+malate by the mitochondria from necrotic heart and the excess Ca^{2+} entry into the mitochondria. Only the addition of EDTA at the time of homogenization prevented mitochondrial damage. EDTA added to the isolated mitochondria did not alter their decreased respiratory ability (not shown). The deterioration in the ultrastructure of isolated heart muscle mitochondria could also be prevented by the presence of 5mM-EDTA in the homogenization medium (Fig. 1*d*).

Table 2 presents the respiratory rate and Ca^{2+} content of mitochondria isolated without added EDTA, from the heart and skeletal muscle of control and calciol-treated rats. At day 4 after calciol treatment, the Ca^{2+} content increased about 10-fold, whereas the pyruvate-plus-malate oxidation rate exhibited about a 6-fold decrease. No decrease in succinate oxidation could be observed in the mitochondria from necrotic heart. The mitochondria (isolated without added EDTA) from skeletal muscle of rats treated with calciol showed changes neither in the Ca^{2+} content nor in the respiratory rate with any of the substrates used. Neither did they show substantial structural changes when examined in the electron microscope.

An elevated Ca^{2+} content is known to cause leakage of NAD^+ from the mitochondria (Wrogemann *et al.*, 1973), which could explain the decreased pyruvate-plus-malate oxidation by the necrotic-heart mitochondria presented in Table 2. This would also produce a decrease in the oxidation by these mitochondria of other NAD-dependent substrates. In fact we have found in two experiments that the respiratory activity of mitochondria from necrotic heart with 2-oxoglutarate was only 33% of that of control mitochondria (56 and 167ng-atoms of O_2/min per mg of mitochondrial protein respectively); the corresponding values for the oxidation of 5mM-glutamate+5mM-malate were 74 and 185ng-atoms of O_2/min per mg with the necrotic- and control-heart mitochondria respectively. If the incubation medium in which the oxidation of pyruvate+malate by the deficient

Table 1. *Mitochondrial respiratory rate and Ca^{2+} distribution within the rat heart subcellular fractions isolated in the presence of 5mM-EDTA from control and necrotic rat heart*

Values are (±S.D.) for the number of experiments indicated in parentheses. For details of the experimental conditions, see the text.

	O_2 uptake (ng-atom/min per mg of protein)		Ca^{2+} content (nmol/mg of protein)	
	1mM-Pyruvate +1mM-malate	10mM-Succinate	Mitochondria	Postmitochondrial supernatant
Control heart	443±32.8 (4)	213±19.2 (3)	5.0±1.25 (3)	17.5±2.25 (5)*
Necrotic heart	400±50.4 (4)	218±28.2 (5)	2.7±1.25 (3)	185.0±17.5 (3)

* No detectable Ca^{2+} was present in the control postmitochondrial supernatant when isolated without added EDTA.

Table 2. *Effect of intra-oral calciol treatment on the respiratory rate and Ca^{2+} content of mitochondria isolated from rat heart and skeletal muscle isolated without added EDTA*

Values are means±S.D. for the numbers of experiments given in parentheses. For further details, see the text.

	O_2 uptake (ng-atoms/min per mg of protein)		Ca^{2+} content (nmol/mg of mitochondrial protein)
	1mM-Pyruvate +1mM-malate	10mM-Succinate	
Heart			
Control	360±16.5 (11)	181±7.4 (11)	10.0±1.00 (11)
Necrotic*	60±16.1 (8)	182±8.6 (8)	105.5±12.5 (8)
Necrotic recovered†	177±30.6 (3)	180±12 (3)	26.5±10.75 (3)
Skeletal muscle			
Control	243±46.2 (3)	146±53.9 (3)	10.0±2.50 (3)
Calciol-treated*	247±26.4 (3)	138±32.6 (3)	9.2±2.50 (3)

* Mitochondria isolated 4 days after calciol treatment.
† Mitochondria isolated 6 weeks after calciol treatment.

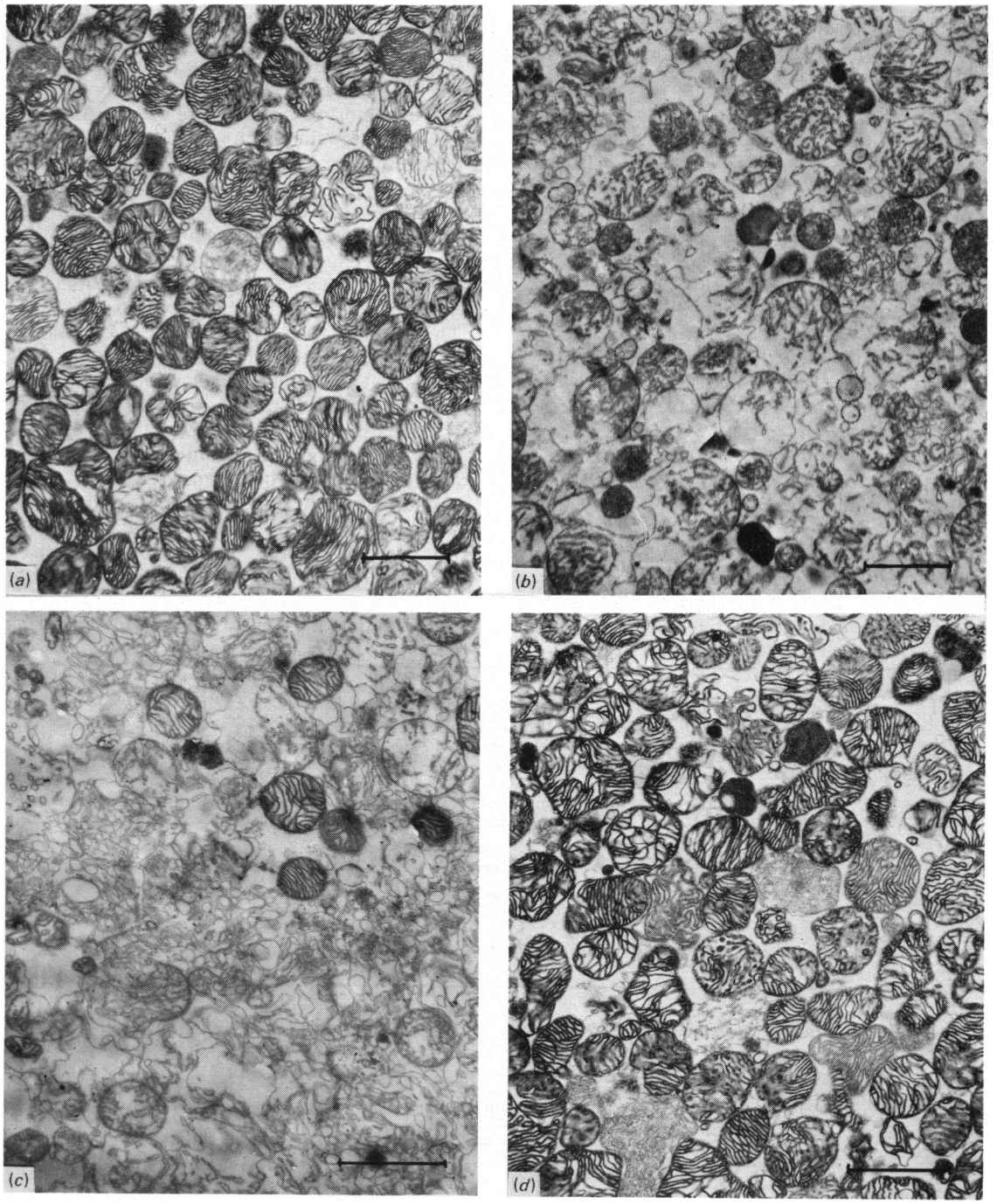

Fig. 1. *Electron micrographs of mitochondria isolated from rat heart*
(*a*) Mitochondria from the heart of control rats. The structure of most of the isolated mitochondria is well preserved. (*b*) Heart mitochondria isolated from the rat treated with three doses of 100000 i.u. of calciol. All mitochondria are swollen, some are disrupted. (*c*) Control rat heart mitochondria subjected to the action of 1 mM-$CaCl_2$ during the tissue-homogenization procedure. Almost all the mitochondria are either swollen or disrupted. (*d*) Heart mitochondria from the calciol-treated rats isolated in the medium containing 5 mM-EDTA. The structure of almost all the mitochondria is well preserved. In all cases the scale bar represents 1 μm.

mitochondria from necrotic heart was measured had been supplemented with 1mM-NAD, 1mM-thiamin pyrophosphate, 0.25mM-CoA and 5mM-$MgCl_2$, the oxidation rate recovered to a value of 217ng-atoms of O_2/min per mg of mitochondrial protein as compared with 51 without the addition of supplementing cofactors. Among these cofactors, only NAD was fully effective, as found in the experiments in which one by one of the cofactors mentioned above had been omitted from the incubation medium.

At 6 weeks after the calciol treatment, the rats recovered, and scars could have been observed on their hearts in sites of the former necrotic foci as described previously (Wrzołkowa *et al.*, 1979; Wrzołkowa & Żydowo, 1980). At that time the isolated heart mitochondria displayed the respiratory ability and Ca^{2+} content shown in Table 2. The pyruvate-plus-malate oxidation rate was restored to about half the control value, and the Ca^{2+} content of the isolated mitochondria was only twice that of the control. In the heart of calciol-treated rats the ultrastructure of mitochondria was shown previously to be almost intact (Wrzołkowa & Żydowo, 1980). However, if mitochondria were isolated from the necrotic heart, the damage to their ultrastructure presented in Fig. 1 occurred.

The Ca^{2+} content of whole-heart tissue increased about 100-fold-after the rats had been treated with 300000 i.u. of calciol, the average content in the heart of control animals being $4.7 \pm 0.04 \mu$mol of Ca^{2+} per g of dry tissue, and in the hearts of 14 animals treated with calciol it ranged from 50 to 1200μmol per g of dry tissue. Thus one suspects that both structural and functional impairment of the mitochondria from necrotic heart occurred during the isolation procedure as a consequence of Ca^{2+} accumulation in the mitochondria.

If the deterioration of the necrotic-heart mitochondrial function and structure presented in Table 2 and Fig. 1 is caused by the entry of Ca^{2+} into the mitochondria from their environment during the isolation procedure, it should be possible to mimic these conditions by adding Ca^{2+} to the medium in which control heart is homogenized. The results of this kind of experiment are presented in Table 3. The addition of 1mM-$CaCl_2$ to the homogenization medium caused almost the same decrease in the pyruvate-plus-malate oxidation rate and the increase in Ca^{2+} content in the heart mitochondria as did the treatment of animals with a high dose of calciol. The electron micrograph of the mitochondrial pellet from the control heart homogenized in the presence of 1mM-$CaCl_2$ disclosed the damage to the mitochondrial structure shown in Fig. 1(*c*).

The question arises as to how large is the dose of calciol that causes Ca^{2+} accumulation in the heart sufficient to produce the damage to isolated mitochondria presented in Table 2 and Fig. 1. The experiments presented in Fig. 2 indicate that even a dose of 100000i.u. of calciol per rat was

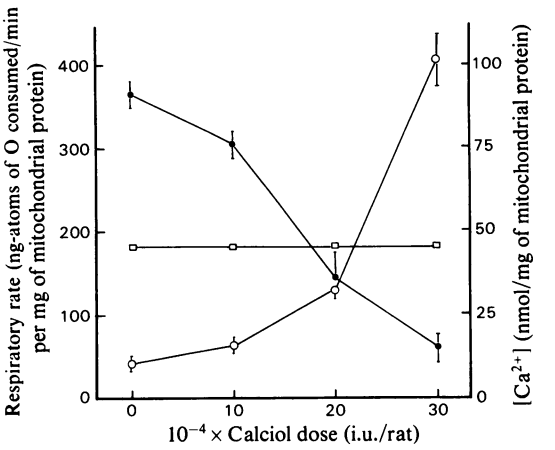

Fig. 2. *Dependence of heart mitochondrial respiratory rate and Ca^{2+} content on the calciol dose*

●, Oxygen uptake with pyruvate+malate; □, with succinate. ○, Ca^{2+} content. The values are means from three to four experiments carried out in duplicate. The vertical bars represent ±S.D. For experimental details, see the text.

Table 3. *Influence of $CaCl_2$ added to the isolation medium on the mitochondrial respiratory rate and on the Ca^{2+} content in rat heart mitochondria*

The control mitochondria were isolated in 250mM-sucrose/10mM-Tris/HCl, pH7.8. $CaCl_2$ (1mM) was added to this medium only during the rinsing and homogenizing of the hearts from the second group of rats. The final rinsing of mitochondria was carried out with the isolation medium without $CaCl_2$ added. For other experimental details, see the text. Values are means±S.D. for the numbers of experiments shown in parentheses.

	O_2 uptake (ng-atom/min per mg of protein)		Ca^{2+} content (nmol/mg of mitochondrial protein)
	1mM-Pyruvate +1mM-malate	10mM-Succinate	
Control	376±21.1 (4)	194±10.0 (4)	9.0±0.75 (4)
1mM-$CaCl_2$ added	42±4.6 (4)	172±11.8 (4)	124.5±27.5 (4)

producing an appreciable decrease in pyruvate-plus-malate oxidation by the isolated heart mitochondria. It is noteworthy that the decrease in the pyruvate-plus-malate oxidation rate was accompanied by an almost proportional increase in the Ca^{2+} content of the isolated mitochondria.

Discussion

Several investigators add EDTA as chelating agent to the homogenization medium when isolating mitochondria from skeletal or heart muscle; however, the concentration of this agent is usually 0.1–1.0mM (e.g. Wrogemann *et al.*, 1973; Jacobus *et al.*, 1975). This is too low a concentration to prevent redistribution of Ca^{2+} within the cell compartments at the time of plasma-membrane disruption in the tissue overloaded with Ca^{2+} as is the case in the heart of calciol-treated animals. Slater & Cleland (1953) reported that 0.07 μmol of EDTA/mg of mitochondrial protein was sufficient to stabilize normal heart mitochondria in the course of the isolation. In our experiments 5mM-EDTA was required to prevent mitochondrial Ca^{2+} accumulation in the disrupted heart cell of the calciol-treated rat. This means that we had to apply 8.3 μmol of EDTA per mg of mitochondrial protein to prepare intact mitochondria from the necrotic heart, i.e. over 100 times larger amount of EDTA than that recommended by Slater & Cleland (1953) for normal heart mitochondria. As Table 1 shows, heart mitochondria prepared in this way from control animals contain 5nmol of Ca^{2+}/mg of protein, which is exactly the same as that found by Williamson *et al.* (1983) in the mitochondria of isolated heart cells.

Ca^{2+} overload in the heart as a consequence of treatment with high doses of calciol has been reported by several authors (Urbanek *et al.*, 1975; Fleckenstein *et al.*, 1975; Wrzołkowa & Żydowo, 1980). However, in contrast with the suggestions of some authors (Fleckenstein *et al.*, 1975; Fleckenstein, 1983), the evidence presented here indicates that the Ca^{2+} accumulated in the cardiac cell does not accumulate in the mitochondria as long as the tissue is not being homogenized. Although in the case of cardionecrosis induced by other methods calcium-salt precipitates were observed within the heart mitochondria *in situ* (Buja *et al.*, 1976; Heggtveit *et al.*, 1964; Jennings & Hawkins, 1980), no calcium deposits could be found in the mitochondria after calciol treatment (Wrzołkowa & Żydowo, 1980). The evidence presented in Table 1 and 3 strongly suggests that the Ca^{2+} accumulation in the heart mitochondria (Table 2) is taking place only at the stage of isolation from Ca^{2+}-overloaded heart. Skeletal-muscle mitochondria were also severely damaged if the 500*g* supernatant was subjected to the presence of 1mM-$CaCl_2$ during further isolation procedures (not shown). However, the treatment of rats with calciol did not produce Ca^{2+} accumulation in the skeletal-muscle cell and thus the mitochondria isolated from the skeletal muscle of calciol-treated rat were intact even without EDTA added to the isolation medium. Calciol seems to disturb Ca^{2+} homoeostasis more in the heart than in skeletal muscle.

The intracellular distribution of Ca^{2+} is different in various tissues (Borle, 1981), and no unequivocal opinions exist about the proportion of Ca^{2+} present in the heart mitochondria. Fiskum & Lehninger (1982) suggest that as much as 60% of the cardiac cell Ca^{2+} is located in mitochondria, which have to accumulate even more Ca^{2+} during ischaemia-induced myocardial damage (Steen *et al.*, 1983). However, a careful recent investigation of the sequestration of Ca^{2+} by mitochondria in rat heart cells (Kessar & Crompton, 1983) revealed that only about 13% of the total cell Ca^{2+} may be mitochondrial *in vivo*. Our experiments suggest that calciol treatment, which produces a very high Ca^{2+} overload of the heart and subsequent cardiac necrosis, does not cause Ca^{2+} accumulation in heart mitochondria *in vivo*. This phenomenon may be due to the unique properties of heart mitochondrial Ca^{2+} transport, which is different from that of liver mitochondria (Jacobus *et al.*, 1975). Although Reinhart *et al.* (1984) have shown that the liver mitochondrial Ca^{2+} content may vary considerably, depending on the Ca^{2+} content of the liver perfusion medium, the heart mitochondria appear to be less sensitive to the variations of Ca^{2+} content in their environment. It is possible that the appreciable capacity of sarcoplasmic reticulum and other extramitochondrial cardiac-cell components to bind Ca^{2+} contributes to the maintenance of a low mitochondrial Ca^{2+} content in the Ca^{2+}-overloaded heart of the rat treated with high doses of calciol.

Thus the sequence of biochemical events ending in cardiac-cell death after calciol treatment evidently does not involve primary mitochondrial damage and energy depletion. One of the possibilities is that a sudden activation by Ca^{2+} of some intracellular proteinases occurs in the heart (Bird & Carter, 1980), which may destroy contractile proteins of the cardiac cell. Some changes of the properties of an extramitochondrial enzyme, AMP deaminase, from the heart of calciol-treated rats have been described (Żydowo *et al.*, 1980) as well as a vivid proliferation of sarcoplasmic reticulum in the cardiomyocytes (Wrzołkowa & Żydowo, 1980).

The results presented here are one more example that the effect of an agent applied to the whole animal on the properties of a cell component

investigated after its isolation may be the result of the changed environment influencing the isolated component during the isolation procedure.

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