Histol Histopathol (1995) 10: 405-416

Histology and Histopathology

The effect of ischemia and reperfusion on mitochondrial contact sites in isolated rat hearts

A. Bakker[1], F. Goossens[2], M. De Bie[1], I. Bernaert[2], H. Van Belle[2] and W. Jacob[1]

[1]Department of Medicine, University of Antwerp, Antwerp and [2]Janssen pharmaceutica, Beerse, Belgium

Summary. Contact sites may be described as energy channels between the mitochondria and the cytosol, created by fusion of the inner and the outer mitochondrial membranes, and their number depends highly on the energy state of the cell.

The aim of the present study was to examine the early changes of ischemia and reperfusion on the number of mitochondrial contact sites. Therefore isolated rat hearts were subjected to short periods of ischemia followed by reperfusion. The left ventricular pressure (LVP), the contractility (dP/dt_{max}) and the heart rate were measured. The number of contact sites was morphometrically evaluated.

As the flow was stopped, LVP, dP/dt_{max} and HR declined rapidly and became undetectable after 2 min of ischemia. The number of contact sites fell to a minimum after 10 min of ischemia after an initial increase (1 min of ischemia). A 15 min ischemic period resulted in a high number of contact sites which decreased again after 20 min of ischemia.

Reperfusion after 2 min of ischemia caused an immediate functional recovery and a high presence of contact sites. After 15 min of reperfusion, all values returned to control values. Reperfusion after 10 min of ischemia resulted in a slow recovery of the number of contact sites and after 15 min of ischemia the number of contact sites remained low upon reperfusion.

We may conclude that mitochondria lose the ability to form contact sites after more than 15 min of ischemia and this might be a first indication of irreversible injury.

Key words: Ischemia, Reperfusion, Isolated rat heart, Mitochondria, Mitochondrial contact sites

Introduction

Contact sites were first described by Hackenbrock (1968) in thin sections of liver mitochondria as places where the inner and outer mitochondrial membranes were in very close apposition. They were further characterised in freeze-fractured mitochondria (Van Venetië and Verkleij, 1982; Knoll and Brdiczka, 1983) and were found to increase during active oxidative phosphorylation. Knoll and Brdiczka (1983) and Brdiczka et al. (1986) postulated that contact sites play an important role in the regulation of the mitochondrial metabolism.

These findings are also affirmed by our studies in which we could show that a stimulation of the energy metabolism, with noradrenaline in vivo (Biermans et al., 1990; Jacob et al., 1992) or Ca^{2+} in isolated hearts (Bakker et al., 1993), led to an increase of the number of contact sites together with a decrease of the number of mitochondrial matrix granules (Jacob et al., 1994).

Although there is not yet consensus about the exact ultrastructure of contact sites (De Kruijff, 1987; Rojo et al., 1991; Wallimann et al., 1992), there are two hypotheses to explain the correlation between the parameters: energy state, Ca^{2+}, contact sites and granules.

In one hypothesis it is suggested that an increase of the energy state of the heart results in a rise of the intracellular calcium content together with the incorporation of the matrix granules in the inner mitochondrial membrane (Jacob et al., 1994). Because of the proposed presence of cardiolipin in the matrix granules (Barnard and Afzelius, 1972) and the increased $[Ca^{2+}]$, membrane fusion may be induced. De Kruijff (1987) showed on artificial membranes that membrane fusion is induced at a certain Ca^{2+} to cardiolipin ratio.

The other hypothesis (Rojo et al., 1991; Wallimann et al., 1992) to explain the above described correlation is via the effect of the energy state and Ca^{2+} on the mitochondrial energy metabolism (McCormack et al., 1990).

Under normoxic conditions, the ATP formed in the mitochondria is converted into creatine phosphate by the activity of the translocase and the mitochondrial isoenzyme of creatine kinase (Mi CK) (Wallimann et al., 1992). So, if the cardiac metabolism is stimulated the mitochondrial ATP formation increases, as does the Mi CK activity. Since Mi CK is active in mitochondrial

Offprint requests to: Dr. A. Bakker, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerp (Wilrijk), Belgium

contact sites (Biermans et al., 1990; Nicolay et al., 1990; Jacob et al., 1992), and can even induce contact site formation (Rojo et al., 1991), the surface density of mitochondrial contact sites in this situation will be high.

In both hypotheses, it is accepted that contact sites are micro-compartments created in order to increase the efficiency of the energy metabolism (Biermans et al., 1990; Wallimann et al., 1992; Bakker et al., 1994).

In the present study we have quantified the surface density of contact sites and the number of mitochondrial granules upon short periods of ischemia followed by reperfusion, in order to obtain a morphological indicator for the early changes induced by ischemia and reperfusion.

Materials and methods

Sixty-five female Wistar rats (250-300 g body weight) were divided into 13 groups of 5 animals. After heparinisation (500 IU), they were anaesthetized with diethylether. The hearts were excised through a left thoracotomy and placed into ice cold St. Thomas' Hospital cardioplegic solution. The aorta was cannulated and the retrograde coronary perfusion, at a constant flow of 9 ml/min, was started with a modified Krebs-Henseleit solution at 37 °C. The perfusate containing in mmol/l: 118 NaCl, 25 Na HCO_3, 2.9 KCl, 1.18 $MgSO_4$, 1.6 $CaCl_2$, 1.28 KH_2PO_4, 11.5 glucose, was gassed with a mixture of 95% O_2 and 5% CO_2.

The left ventricular pressure was measured with a latex balloon (HSE - nr4) inserted into the left ventricle connected to a Statham pressure transducer (p32 Db). Its derivative, dP/dt_{max} served as a measure for contractility.

The cardiac function was evaluated by comparison of the pressure rate (PR) values (PR= left ventricular pressure x heart rate) after ischemia and/or reperfusion with the control PR value.

Experimental protocol

After an equilibration period of retrograde coronary perfusion (20 min), the hearts were subjected to no-flow ischemia (at 37 °C) or no-flow ischemia (at 37 °C) followed by reperfusion. Ischemic periods of 1, 2, 5, 10, 15 and 20 min were applied. After ischemia, the hearts were fixed, either immediately or after different reperfusion periods (1, 5, 15 min). At the end of each experiment, the hearts were processed for electron microscopic investigation. After perfusion fixation for 10 min with 3% glutaraldehyde in 0.1M cacodylate buffer (pH= 7.4), the hearts were rinsed with 7.5% saccharose in 0.1M cacodylate buffer (pH= 7.4) for 10 min, also by perfusion.

To quantify contact sites and matrix granules, we used stereological methods. Stereological methods are tools for obtaining information about three-dimensional microscopic structures, based on observations made on sections. However, most existing stereological procedures require isotropy. Due to the fact that biological structures, like heart muscle for example, are often anisotropic, Baddeley et al. (1986) described a practical solution for anisotorpy, i.e. vertical sections. A vertical section is a plane perpendicular to a given horizontal plane. Therefore the tissue has to be sampled as follows: 1) a horizontal plane has to be chosen a priori, and a number of slabs with the same thickness have to be cut; 2) the horizontal slabs have to be cut into vertical sections in three systematic directions. The first direction may be selected randomly. In our study, the tissue blocks were oriented in such a way that the horizontal plane was chosen perpendicular to the myofibres. The horizontal slabs obtained (approximately 0.5 mm) were fixed by immersion with 1% osmium tetroxide in cacodylate buffer 0.1M (pH= 7.4), rinsed and dehydrated in 70%, 90% and twice in 100% ethanol, each time for 30 min. To improve the penetration of the embedding resin, the samples were twice incubated in propylene oxide for 10 min followed by a 12 h immersion in a 1:1, LX resin: propylene oxide mixture.

From each experiment, six horizontal slabs were flat embedded in LX (Ladd research lab., Burlington, Vermont) and sectioned (50 nm) on an LKB III ultramicrotome perpendicular to this plane in order to obtain vertical sections. On each grid an average of twenty fields were photographed systematically on the same place of the grid meshes at a magnification of 20000. About one hundred pictures per group were taken.

The ratio of surface densities mitochondrial contact sites to surface densities mitochondrial membranes was determined using the method described by Baddeley et al. (1986). To minimize the anisotropic structure of heart muscle, they described a coherent test system consisting of cycloidal curves which were superimposed on the electron micrograph.

The ratio of surface densities is obtained by the equation:

$$S_S = \frac{\sum_{i=1}^{n} I_i}{\sum_{i=1}^{n} S_i}$$

where I_i= the intersection counts of mitochondrial contact sites with the cycloidal test lines ≈ the surface density contact sites.

S_i= the intersection counts of the mitochondrial membrane with the cycloidal test lines ≈ surface density mitochondrial membranes.

The number of granules was estimated as the number per surface density of the mitochondrial profile in the section of heart muscle.

Results

A. Effect of ischemia

As soon as the flow was arrested, the LVP (the left ventricular pressure) and dP/dt_{max} (contractility) decreased. After 1 min of ischemia, the PR value was approximately 70% of the control value. Both left ventricular pressure (LVP) and heart rate (HR) became undetectable after 2 min of ischemia.

If the ischemic period lasted longer than 20 min, we could detect a rise of the resting pressure, leading to rigor contracture of the heart.

The ultrastructure of the heart remained almost intact up to 10 minutes of ischemia: although the matrix granules decreased (control: 6±3 (Fig. 1); 1 min of ischemia (Fig. 2): 2.7±0.5; 2 min 5±2; 5 min: 0; 10 min (Fig. 3): 0), the mitochondria had a normal shape and matrix density; the myofilaments were relaxed and the amount of glycogen was slightly decreased as the ischemia persisted.

After more than 10 min of ischemia, the myofilaments showed some contraction bands; the sarcoplasmic reticulum and the mitochondria were sometimes swollen and the mitochondrial granules were absent.

20 min of ischemia (Fig. 4) or more resulted in serious structural damage: contraction bands; myolysis; swollen T-tubules and sarcoplasmic reticulum (SR); swollen mitochondria without granules; almost no glycogen and an interrupted sarcolemma.

With respect to the effect of the different times of ischemia on the amount of mitochondrial contact sites, we could show that 1 min of ischemia resulted in a significant increase of the ratio of surface densities of mitochondrial contact sites to mitochondrial

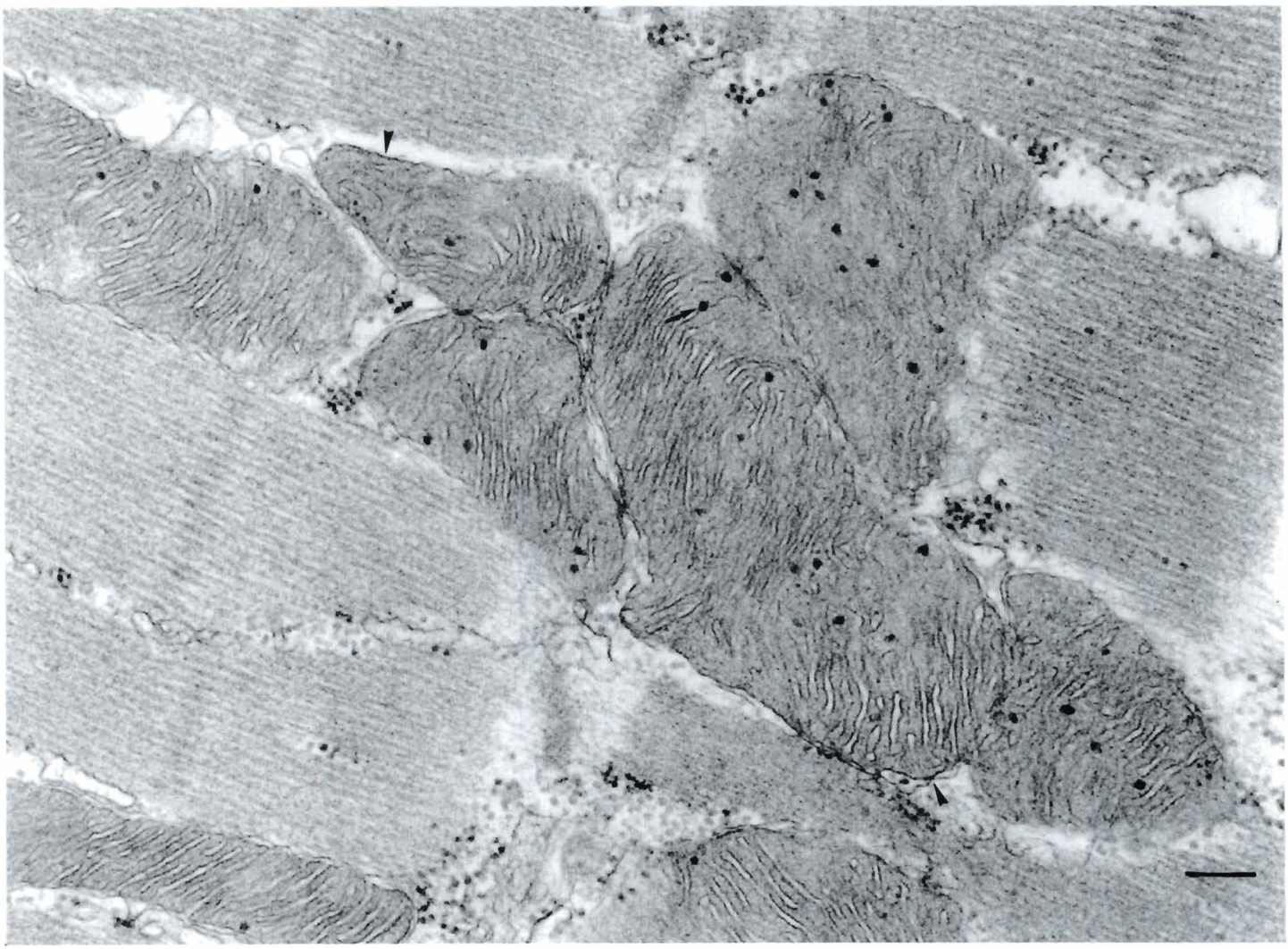

Fig. 1. Ultrastructure of a normal control heart characterised by normal-shaped mitochondria containing granules (arrow) and contact sites (arrowheads), glycogen and relaxed myofilaments. Bar= 200 nm.

Table 1. The effect of ischemia and reperfusion after ischemia on the ratio of surface densities of mitochondrial contact sites to mitochondrial membranes, with S_S the ratio of surface densities of contact sites to mitochondrial membranes (eq.1) and N number of matrix granules per mitochondrial profile.

GROUP	ISCHEMIA (min)	REPERFUSION (min)	PRESSURE-RATE (% of Pre-isc)	N±SEM	S_S±SEM
Control	-	-	100	6±3	0.350±0.020
isc 1	1	-	approx. 70	2.7±0.5	0.430±0.020
isc 2	2	-	approx. 25	5±2	0.370±0.020
isc 3	5	-	undetectable	0	0.330±0.010
isc 4	10	-	undetectable	0	0.310±0.020
isc 5	15	-	undetectable	0	0.380±0.010
isc 6	20	-	undetectable	0	0.300±0.020
isc + rep 1	2	1	approx. 80	3.5±0.8	0.400±0.005
isc + rep 2	2	5	approx. 90	4.1±0.2	0.380±0.010
isc + rep 3	2	15	approx. 90	6±1	0.340±0.020
isc + rep 4	10	1	heart irreg.	0	0.310±0.005
isc + rep 5	10	5	approx. 90	0	0.350±0.010
isc + rep 6	15	5	from 40 to 90	0	0.325±0.005

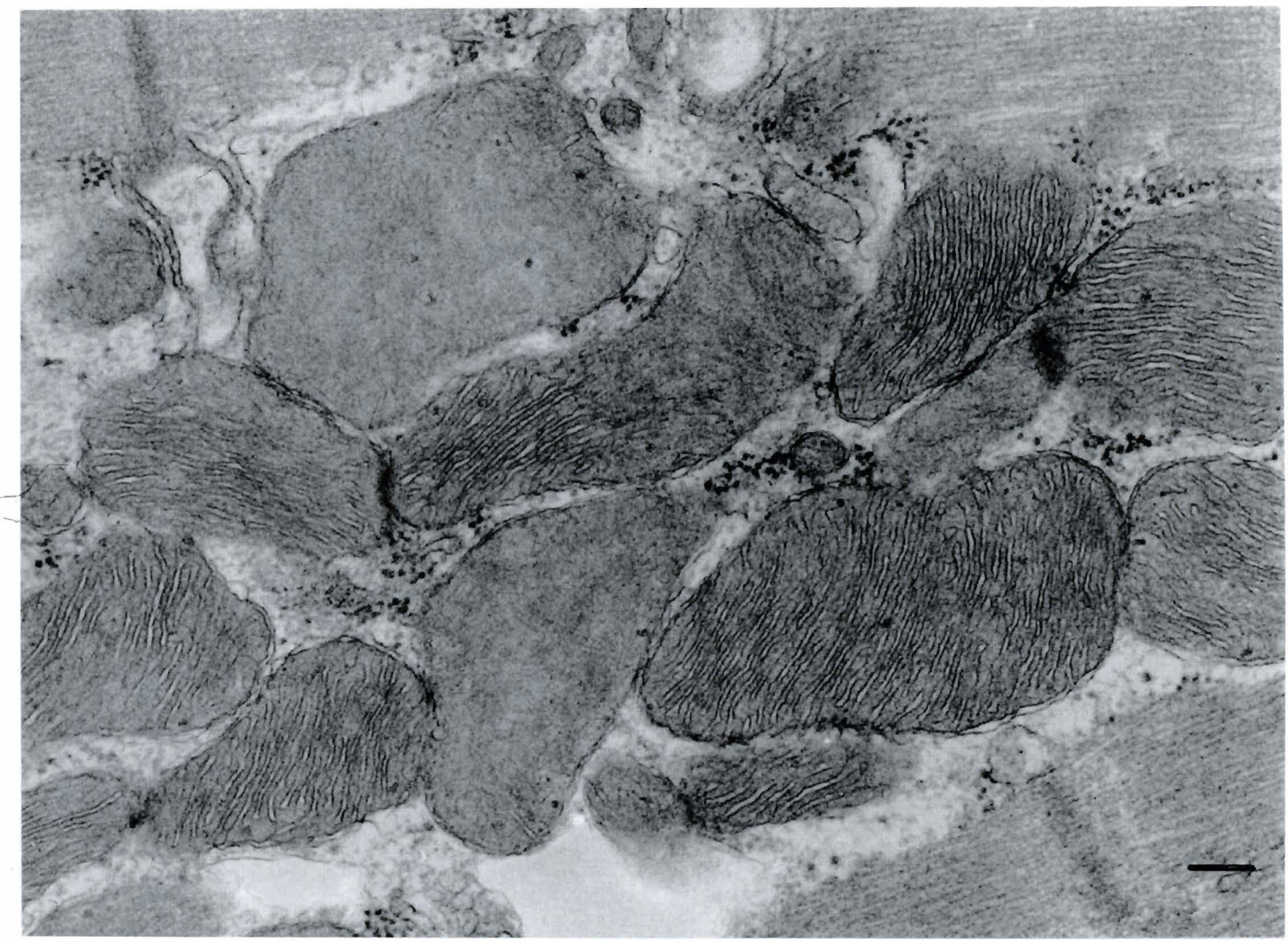

Fig. 2. Ultrastructure of the heart after 1 min of ischemia: the mitochondria have a normal shape, the myofilaments are relaxed, and the number of granules is decreased in comparison to the control heart (Fig. 1) probably because the S_s is significantly higher after 1 min of ischemia. Bar= 200 nm.

membrane (expressed as S_S= 0.43±0.02) in comparison with the control value (0.35±0.02); ischemic periods of 2 min (0.37±0.02), 5 min (0.33±0.01) up to 10 min (0.31±0.02) resulted in a reduction of the ratio; 15 min of ischemia led to a higher ratio (0.38±0.01), and after a 20 min ischemic period the ratio returned to a low value again (0.30±0.02) (Table 1).

B. Effect of reperfusion after ischemia

Reperfusion after 2 min of ischemia (Fig. 5) resulted in an immediate rise of the pressure rate values i.e. 1 min of reperfusion resulted in a pressure rate value of approximately 80% of the pre-ischemic value and of a ratio of surface densities of 0.40±0.01. The ultrastructure remained intact (Fig. 6): the myofilaments were relaxed; the mitochondria were highly coupled (as shown by the high S_S ratio) and the SR and the T-tubules were slightly swollen and the number of mitochondrial granules was diminished (3.5±0.8). If the reperfusion was prolonged, the number of contact sites and granules returned to the pre-ischemic values: S_S= 0.38±0.01 and N= 4.1±0.2 after 5 min of reperfusion and S_S= 0.34±0.02 and N= 6±1 after 15 min of reperfusion. The pressure rate product also returned approximately to the pre-ischemic value (90%).

However, a 1 min reperfusion after an ischemic period of 10 min resulted in an irregular heart with an S_S of 0.310±0.005 and N= 0, but after 5 min of reperfusion the heart recovered completely, except for the absence of the granules and swollen T-tubules (Fig. 7): pressure rate was approximately 90% of the pre-ischemic value and S_S= 0.35±0.01 (Fig. 8). Reperfusing hearts which were subjected to more than 10 min of ischemia (15 min, Fig. 9) sometimes resulted in a good functional recovery (pressure rate= 90% of the pre-ischemic value) and

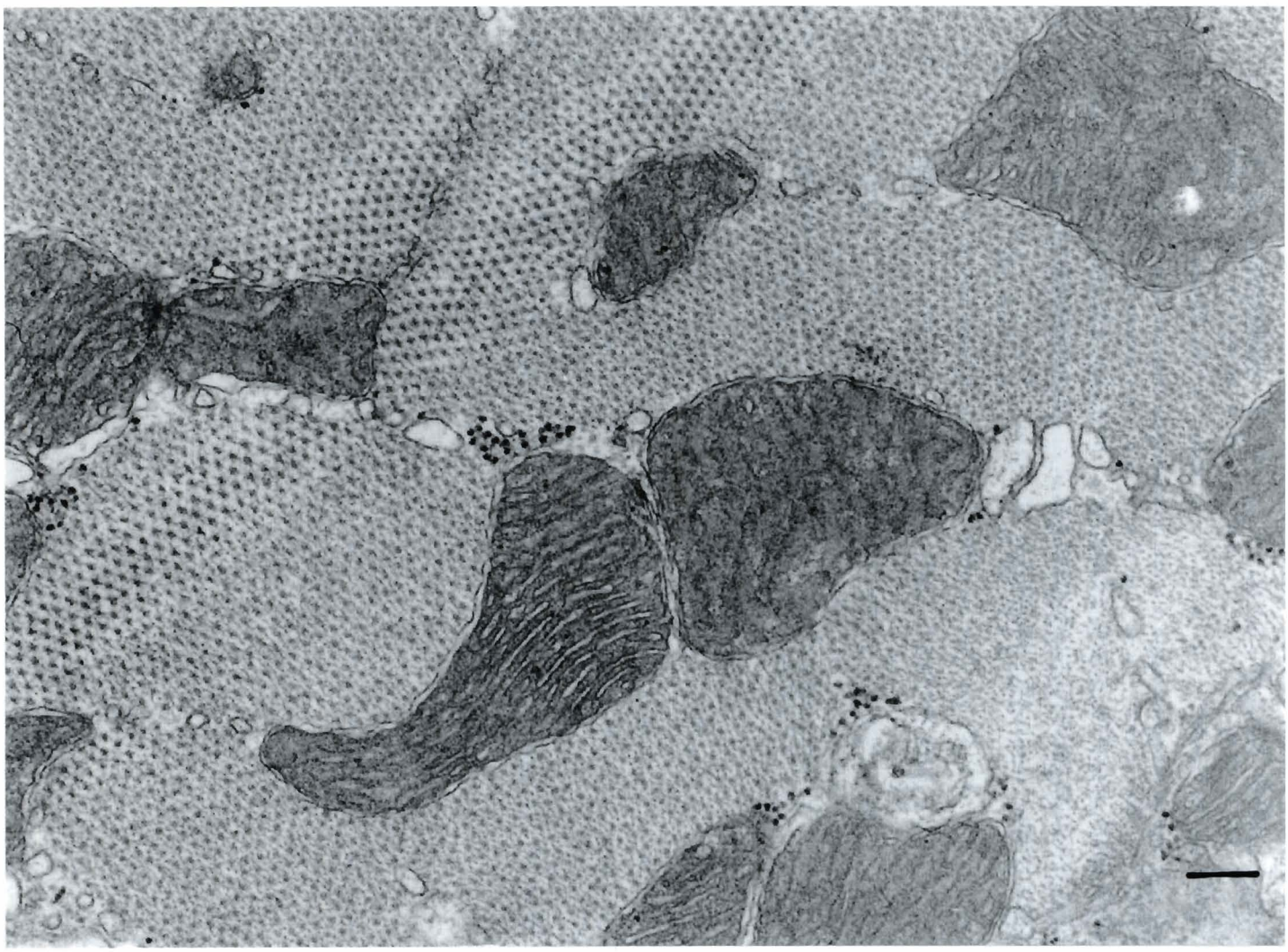

Fig. 3. Ultrastructure of the heart remains almost intact up to 10 minutes of ischemia. Although the matrix granules are absent, the mitochondrial still have a normal shape and matrix density; the amount of glycogen is significantly decreased as the ischemia persists. Bar= 200 nm.

sometimes in a partial functional recovery of the pressure rate value (40% of the pre-ischemic value). However, the number of contact sites was always low (0.325±0.005). With respect to the ultrastructure, some focal damage was visible (Fig. 10): the amount of glycogen was reduced; the SR and the T-tubules were swollen; the mitochondria were swollen, sometimes showing crystolysis and onion like structures (caused supposedly by free radicals); and mitochondrial granules were absent.

From the results of the group of hearts which were reperfused after an ischemic period of more than 10 min, we may conclude that reperfusion could not alter the surface density of contact sites whatever the reperfusion time might be.

More than 20 min of ischemia led to rigor contracture and reperfusion of these hearts resulted in a complete destruction of the ultrastructure, for example: myolysis, an interrupted sarcolemma, swollen mitochondria and crystolysis. In this case we were unable to quantify the surface density of contact sites.

Discussion

Acute ischemia in isolated rat hearts is made up of two components: (a) the abrupt deprivation of oxygen leading to cessation of the oxidative phosphorylation; and (b) the absence of the outflow of metabolites leading to an accumulation of metabolic end products. The cessation of the aerobic metabolism has serious consequences for the ATP production because the breakdown of 1 glucose molecule in the presence of oxygen (via the oxydative phosphorylation) results in the formation of 38 molecules of ATP, whereas under anaerobic conditions (glycolysis) only 2 ATP molecules per glucose molecule are formed.

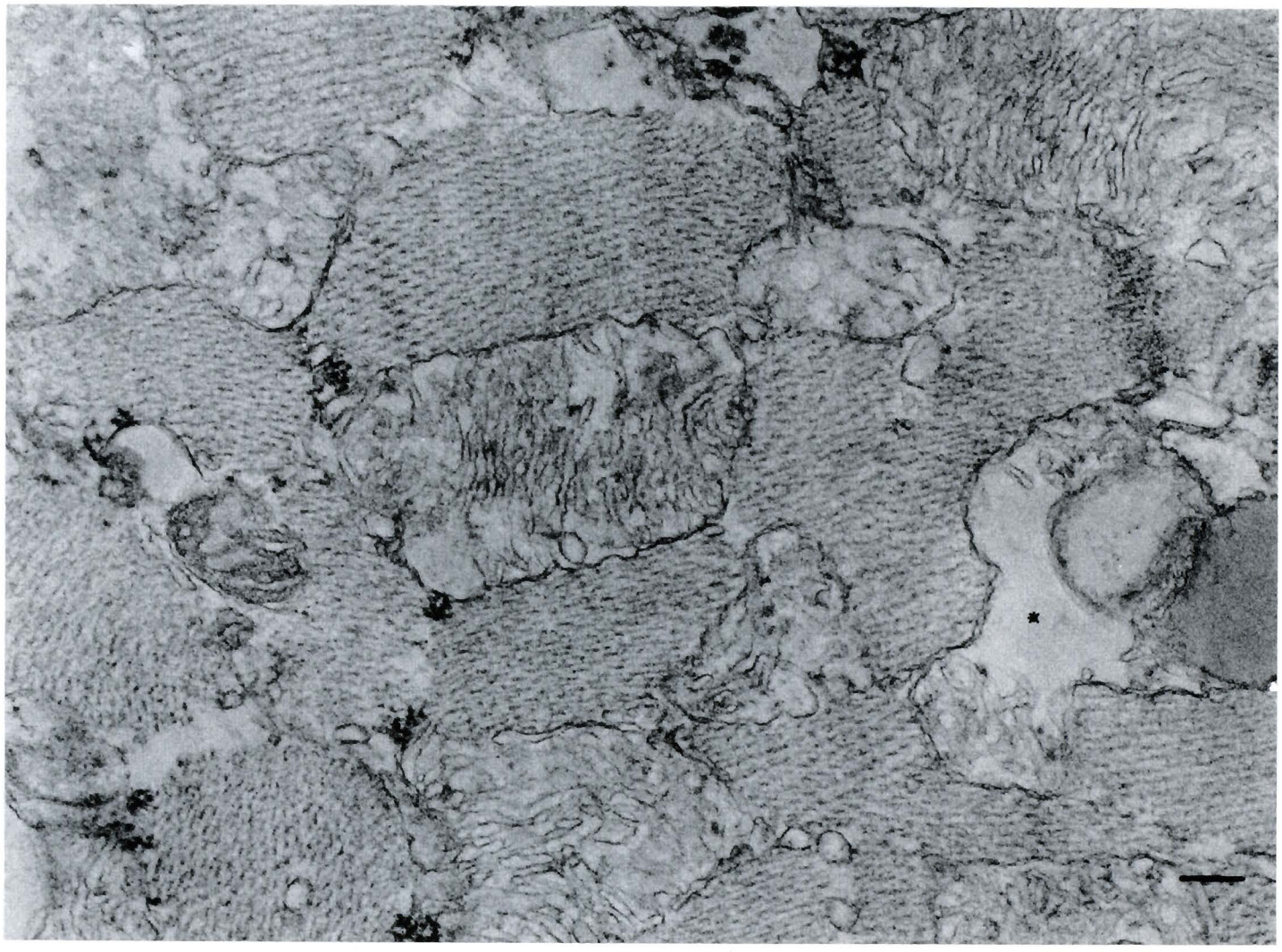

Fig. 4. Ultrastructure of the heart after 20 min of ischemia sometimes shows myolysis, swollen mitochondria, and crystolysis (asterisk) is visible. In this case it is not possible to quantify the surface density of contact sites. Bar= 200 nm.

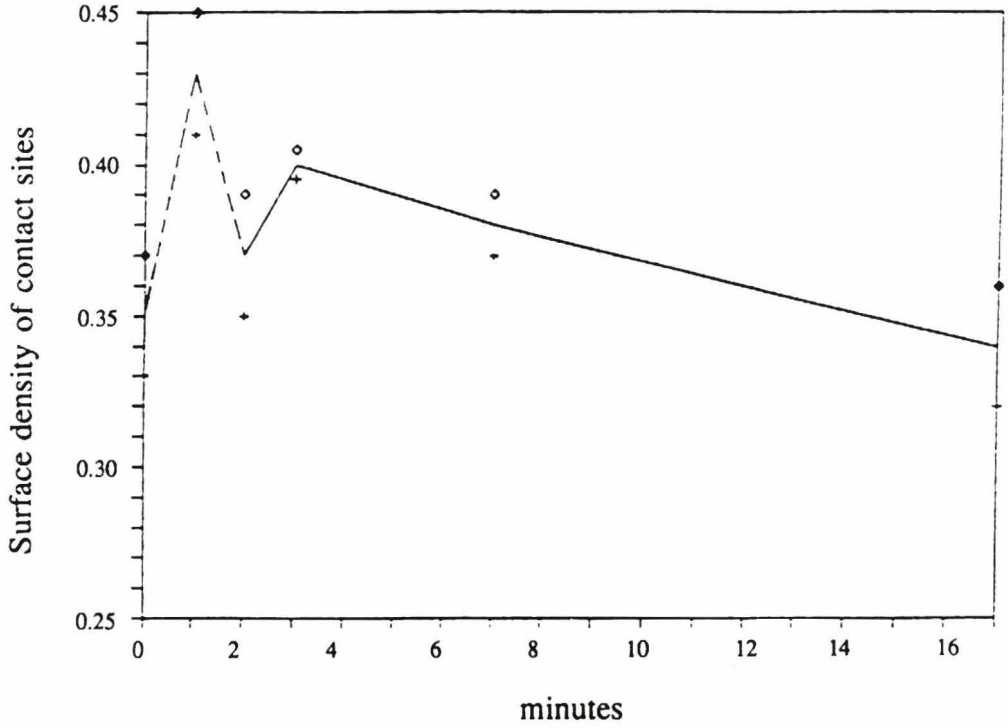

Fig. 5. Effect of reperfusion after 2 min. of ischemia on the ratio of surface densities of contact sites to mitochondrial membranes. Shortly after reperfusion, the surface density of contact sites increases and after a longer reperfusion, the ratio returned to the control value.

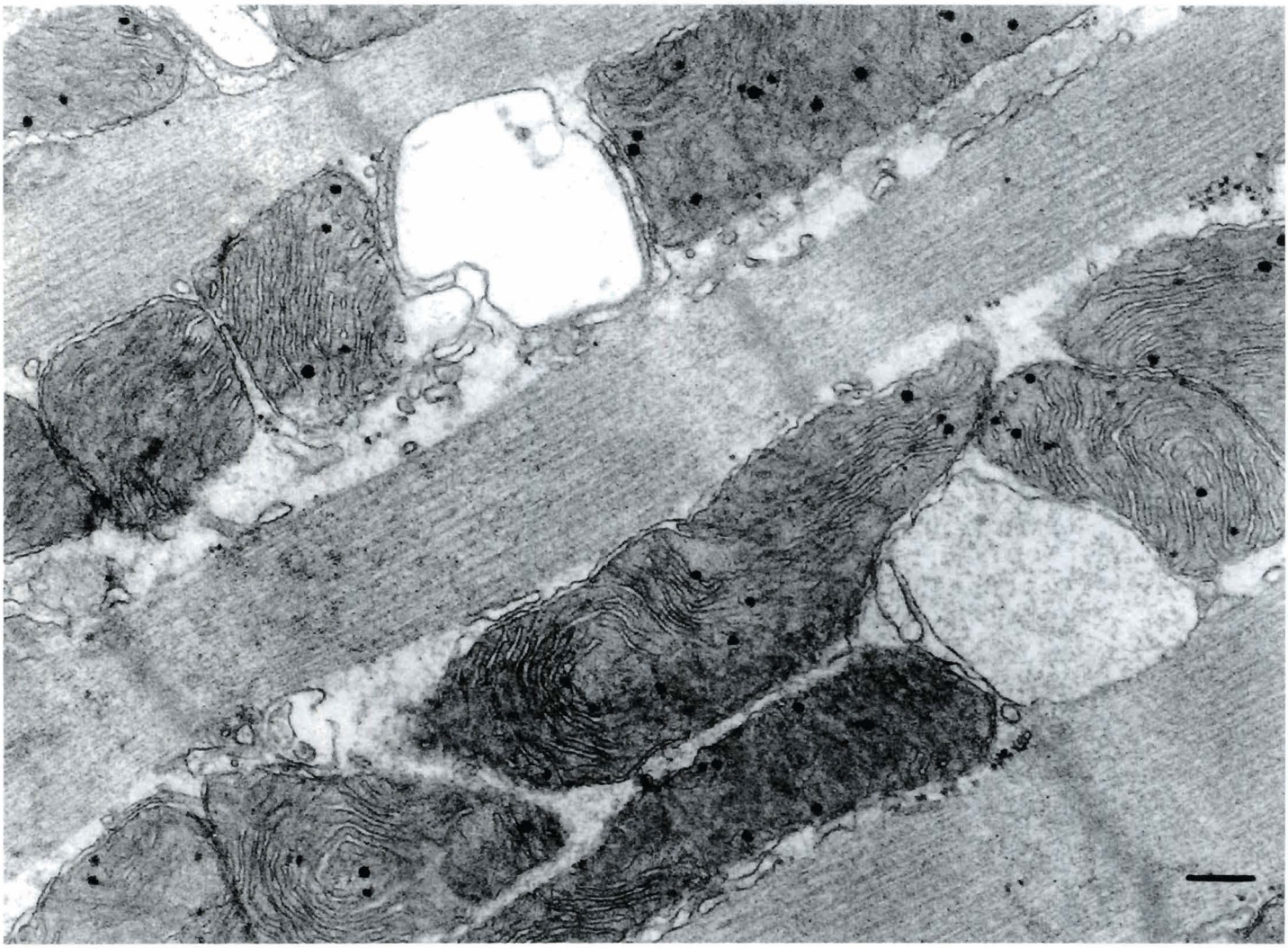

Fig. 6. The ultrastructure of the heart after 2 min of ischemia followed by 1 min of reperfusion remains intact. The surface density of contact sites increases together with a decrease in the number of granules, indicating that the mitochondrial metabolism may be stimulated. Bar= 200 nm.

One of the adaptive mechanisms of the heart to the decreased availability of ATP is the stimulation of ATP production. 30 seconds after onset of ischemia, the extracellular K^+ rises rapidly (Kleber, 1983) and Uchida and Murao (1974) have reported that this increased K^+ enhances the release of catecholamines, independently of the neural stimulation. As shown by Fan and Koenig (1988) and Bean et al. (1984), catecholamine stimulation induces an increase in the intracellular Ca^{2+} concentration, resulting on the one hand in a stimulation of the glycogen degradation (Opie, 1992a,b) and on the other hand in an increase in the number of mitochondrial contact sites (Bakker et al., 1993).

Therefore, although some groups do not observe the short lasting increase of the intracellular Ca^{2+} concentration (Steenbergen et al., 1993), our data, namely the high number of contact sites after 1 minute of ischemia (0.43±0.02) are in agreement with the findings of Mohabir et al. (1991) showing a prompt increase of the peak cytosolic Ca^{2+} concentration soon after ischemia.

Another, at first contradictory, observation is that in spite of an increase of the intracellular Ca^{2+} concentration, Clarke et al. (1987) showed that as soon as the ischemia starts, the cardiac function and the creatine phosphate concentration decline, the ATP level remains constant and the phosphate content increases. Their measurements indicate that the rates at which all these changes occur are different. The loss of creatine phosphate and the increase of inorganic phosphate occurred at similar rates during ischemia, rates approximately 50% slower than the loss of contractile function. As cardiac function declines, the normal ATP utilization is also reduced and so the breakdown of creatine phosphate plus anaerobic glycolysis could maintain an adequate ATP level during the ischemic

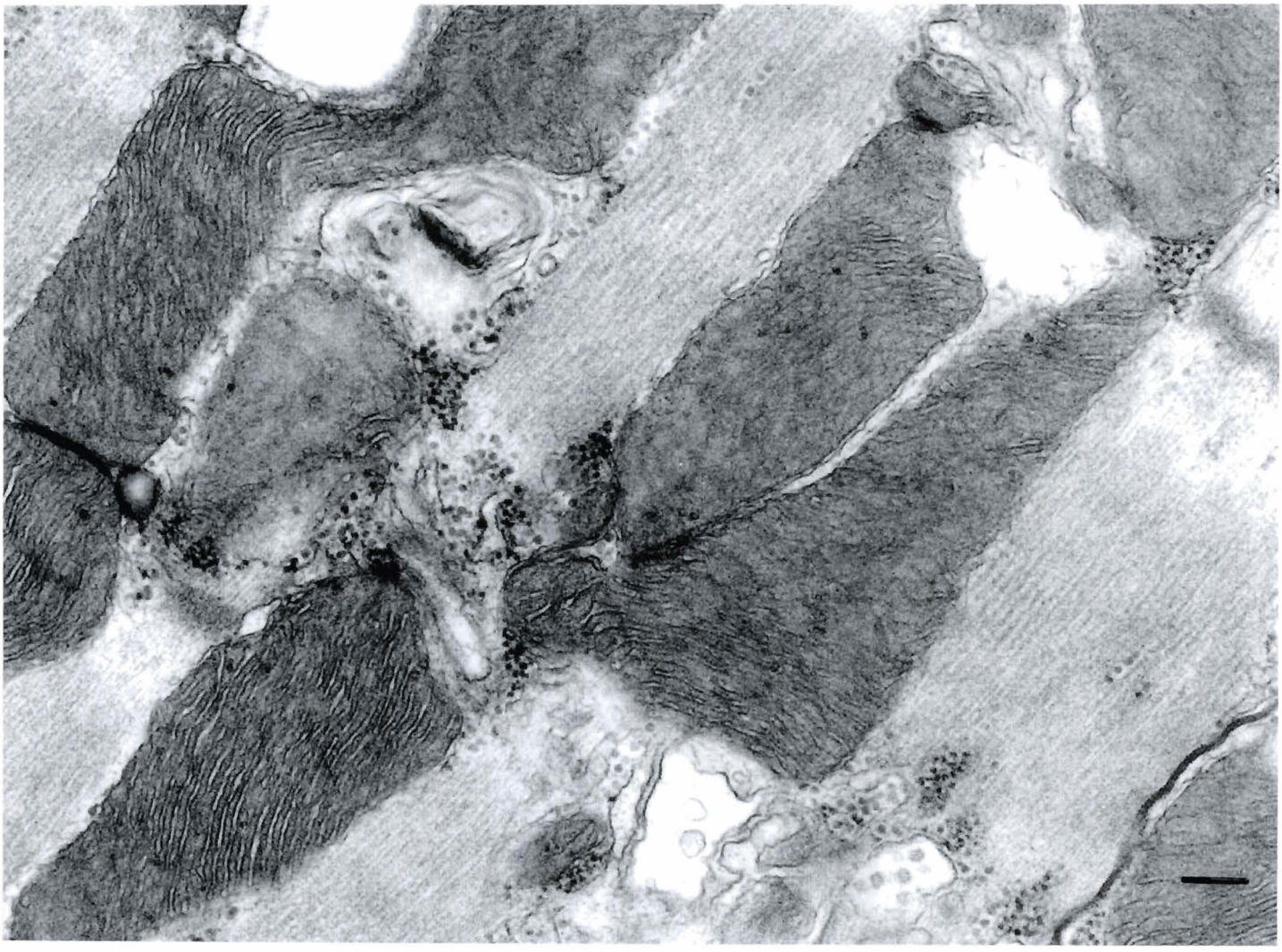

Fig. 7. 5 min of reperfusion after a 10 min ischemic period results in normal mitochondria without matrix granules, a normal amount of glycogen, relaxed myofilaments and swollen T- tubules. Bar= 200 nm.

period.

Although enough energy is present, the contraction decline might be explained by changes of the cytosolic phosphorylation potential. The cytosolic phosphorylation potential is calculated from the equation:

$$\frac{[ATP]}{[ADP] \cdot [P_i]} = \frac{[CrP] \cdot [H^+] \cdot K_{ck}}{[Cr] \cdot [Pi]}$$

with: P_i= inorganic phosphate; Cr= creatine; CrP= creatine phosphate; K_{ck}= equilibrium constant of myofibrillar creatine kinase; and H^+= proton.

The phosphorylation potential is controlled by myofibrillar creatine kinase. A decline of the potential would inhibit the myosin ATPase by preventing the cross-bridge formation and hence decreasing the ATP consumption. A rise of the potential would result in a

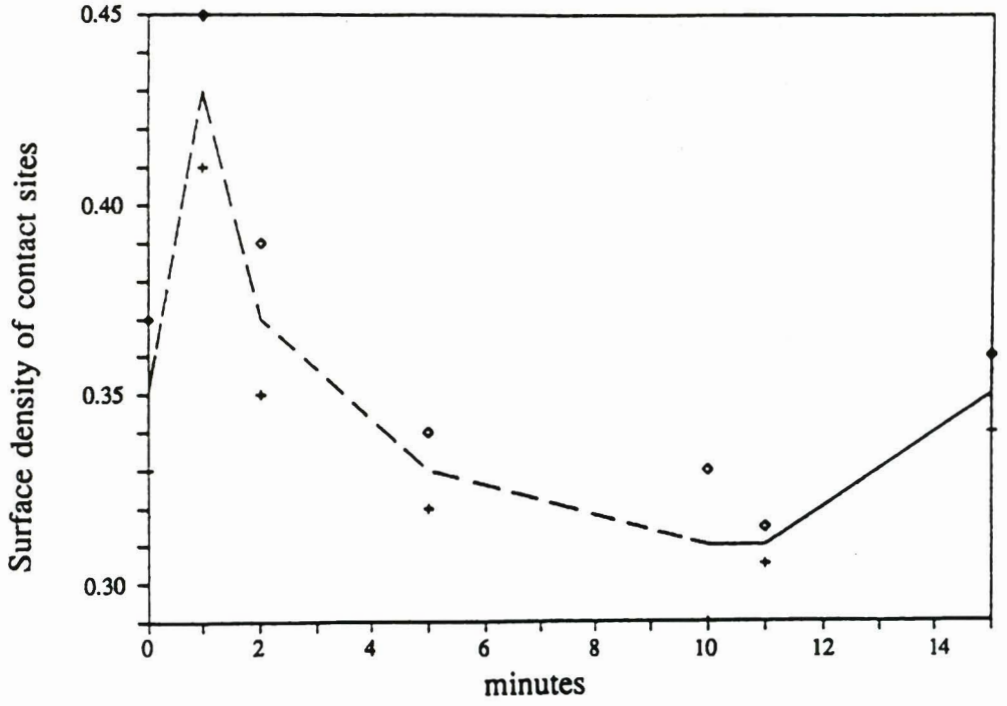

Fig. 8. Effect of reperfusion (——) after 10 min of ischemia (- - -) on the ratio of surface densities of contact sites to mitochondrial membranes. Shortly after reperfusion, the surface density of contact sites remains low, but the value returns to control values upon a longer reperfusion.

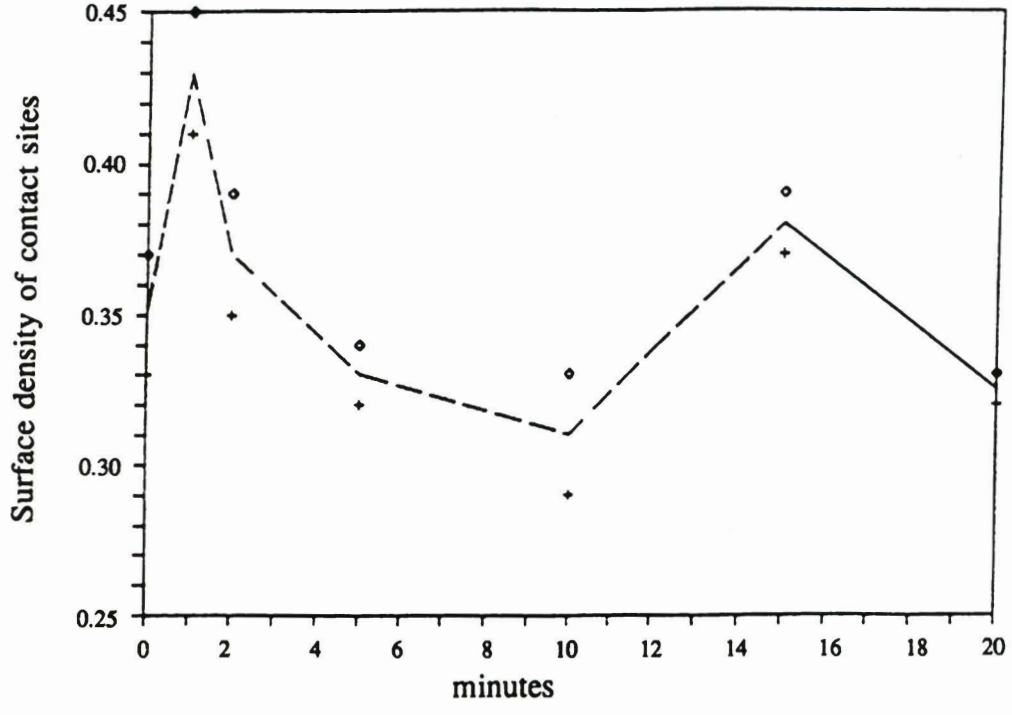

Fig. 9. 15 min of ischemia (- - - -) results in an increase of the ratio of surface densities of contact sites to mitochondrial membranes, probably caused by the increased intracellular Ca^{2+} content. Reperfusion (——) after 15 min of ischemia leads to a decrease of the ratio.

concomitant increase in the ATP consumption.

During ischemia changes of the phosphorylation potential are closely associated to the functional changes in such a way that during the first minute of ischemia, the reduction of phosphorylation potential is mainly responsible for the reduction of the pressure rate product (about 70% of the control value). So, within the first minute of ischemia, we suggest that the increased intracellular Ca^{2+} concentration may increase the ATP production, whereas the reduced phosphorylation potential may at least partially be responsible for the diminished contractile function (which implies a diminished ATP consumption). The maintenance of the ATP level is partially accomplished by the reduced phosphorylation potential and partially by the breakdown of creatine phosphate. An important factor is that the decrease of creatine phosphate is accompanied by an increase of inorganic phosphate. As the ischemic period is prolonged, this increased inorganic phosphate level will result in a decreased intracellular Ca^{2+} availability because phosphate will bind the free Ca^{2+}. The reduced free Ca^{2+} is also reflected by a decrease in the number of contact sites (0.37±0.02 after 2 minutes of ischemia; 0.33±0.01 after 5 minutes of ischemia and 0.31±0.02 after 10 minutes of ischemia). However, the increased number of contact sites after 15 min of ischemia (0.38±0.01) is probably due to the rise of the intracellular Ca^{2+} concentration occurring 10 to 15 min after ischemia (Mohabir et al., 1991; Steenbergen et al., 1993). The increase of Ca^{2+} concentration may be caused by: 1) a decreased ATP level (Jennings and Steenbergen, 1985; Van Belle et al., 1986) because the rate of the glycolytic ATP production slows markedly (accumulation of NADH, lactate and H^+). The reduced

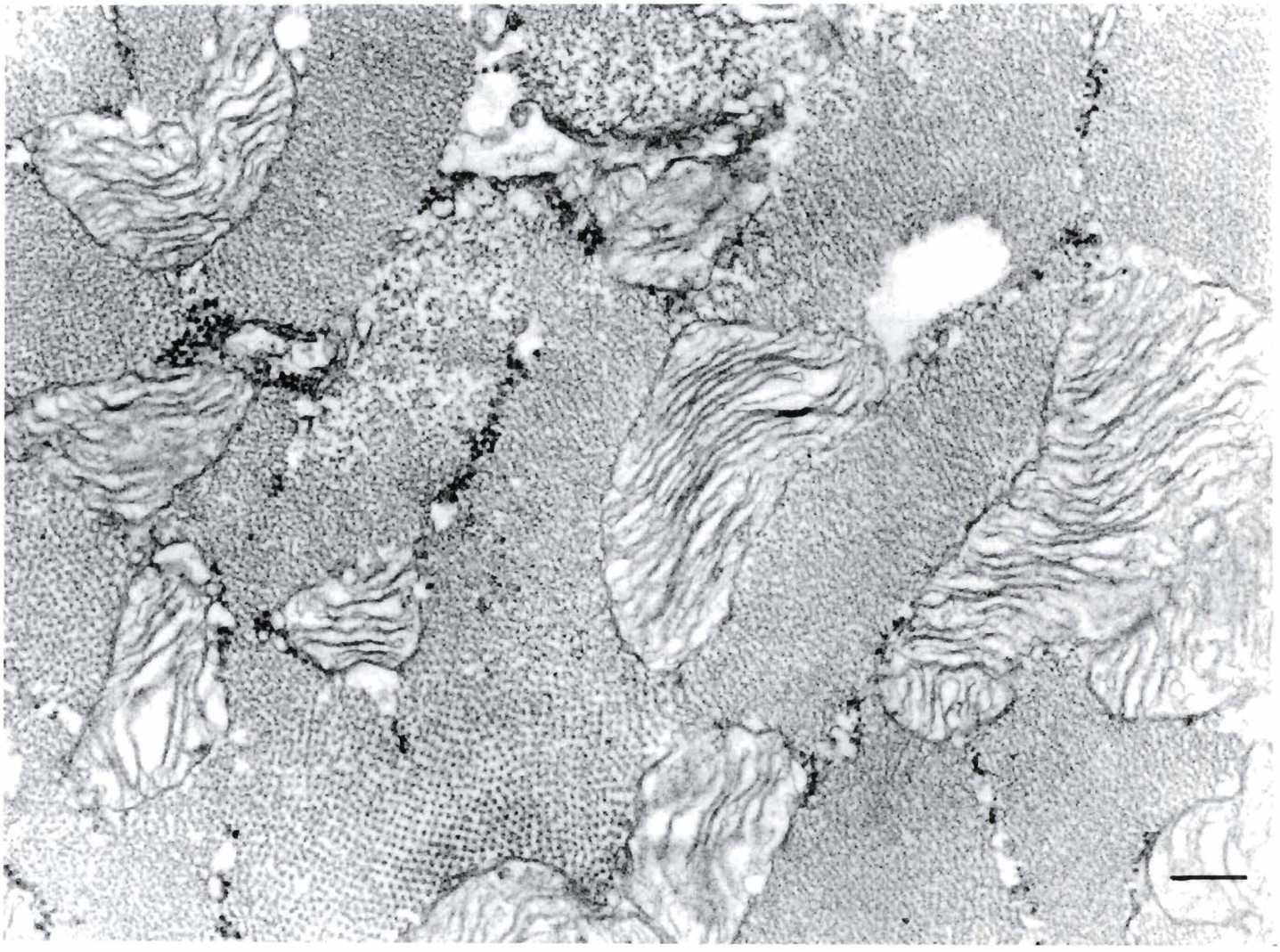

Fig. 10. Ultrastructure after 15 min of ischemia + 5 min of reperfusion: the amount of glycogen is reduced; the SR, the T-tubules and the mitochondria are swollen. The mitochondria sometimes show crystolysis and onion-like structures (caused supposedly by free radicals) and the matrix density is decreased; mitochondrial granules are absent. Bar= 200 nm.

ATP level results in a reduced Ca-ATPase function of the SR and hence in a decreased Ca^{2+} uptake; 2) the reduced buffer capacity of the cytoplasm for Ca^{2+}, since the affinity of troponin for Ca^{2+} decreases during ischemia, due to raised intracellular H^+, Mg^{2+} and Na^+ concentrations (Allen and Orchard, 1987; Allen et al., 1989); and 3) the cytosolic acidification, because acidosis enhances the release of Ca^{2+} from the SR (Orchard et al., 1987; Mohabir et al., 1991). The combination of acidosis and the accumulation of glycolytic catabolites finally results in the complete cessation of the glycolysis (Kobayashi and Neely, 1979). Consequently, no ATP is formed, resulting in an accumulation of intracellular Ca^{2+} and a collapse of the membrane potential. The collapsed membrane potential will result in rigor contracture and rupture of the myocytes (Steenbergen et al., 1990).

To distinguish between reversible and irreversible ischemic injury, we performed ischemia-reperfusion experiments and measured: 1) the functional recovery, by measuring left ventricular pressure, contractility and heart rate, and 2) the ultrastructural effects, by measuring the surface density of mitochondrial contact sites and the presence of matrix granules.

Reperfusion after a short period of ischemia results in a good functional recovery (pressure rate= 80%), an intact ultrastructure and an increase of the surface density of contact sites (0.400±0.005).

As mentioned previously, ischemia causes a reduced creatine phosphate level together with a rise of the mitochondrial ADP content. When the oxygen supply is restored, the oxidative phosphorylation is stimulated and consequently more ATP is available, which is also reflected in a sudden increase of the phosphorylation potential (Clarke et al., 1987), and the Mi CK activity is enhanced as is shown by the high ratio of surface densities. This results in an enhanced shuttle activity and hence an elevated energy supply for contraction. As the reperfusion time is prolonged (15 min), the ratio of surface densities returns approximately to the value under non-ischemic conditions (0.34±0.02) which might imply that the energy demand to supply ratio is again the same as under non-ischemic conditions.

Reperfusion after an ischemic period of 10 min resulted in a slow but complete recovery of both the ratio of surface densities (0.310±0.005 after 1 min and 0.35±0.01 after 5 min) and the pressure rate values (unmeasurable after 1 min and 90% after 5 min) after approximately 5 min.

If the ischemic period is longer than 10 min, the surface density of mitochondrial contact sites remained low after reperfusion, and in these samples the mitochondrial granules were absent.

Therefore, we may conclude that the determination of the ratio of surface densities of contact sites to mitochondrial membranes in isolated rat hearts, does not give additional information about the ischemia/reperfusion mechanism if the ischemic period exceeds 10 min.

The exact mechanism of these observations remains to be elucidated but we assume that in isolated rat hearts, reperfusion after 10 to 15 min of ischemia may result in calcium loading of the mitochondria. The accumulation of calcium by the mitochondria hampers their ATP production which results in the persistence of ischemia (Jennings and Reimer, 1991).

From the results of the present study, we might suggest that in hearts that were reperfused after an ischemic period of 15 min, the relationship between the physiological situation and the presence of contact sites is lost. The loss of the possibility of the mitochondria to form contact sites might possibly be a first indication of irreversible injury.

References

Allen D.G. and Orchard C.H. (1987). Myocardial contractile function during ischemia and hypoxia. Circ. Res. 60, 153-168.

Allen D.G., Lee J.A. and Smith G.L. (1989). The consequences of simulated ischemia on intracellular Ca^{2+} and tension in isolated ferret ventricular muscle. J. Physiol. Lond. 410, 297-323.

Baddeley A.J., Gundersen H.J.G. and Cruz-Oriv L.M. (1986). Estimation of surface area from vertical sections. J. Microsc. 142, 259-276.

Bakker A., de Bie M., Bernaert I., Ravingerova T., Ziegelhöffer A., Van Belle H. and Jacob W. (1993). Increased extracellular calcium concentrations in isolated rat heart: effects on mitochondrial contact sites formation. Eur. J. Morphol. 31, 46-50.

Bakker A., Biermans W., Van Belle H., De Bie M., Bernaert I. and Jacob W. (1994). Ultrastructural localisation of carnitine acetyltransferase activity in mitochondria of rat myocardium. Biochim. Biophys. Acta 1185, 97-102.

Barnard T. and Afzelius B.A. (1972). The matrix granules of mitochondria: A review. Subcell. Biochem. 1, 375-389.

Bean B.P., Nowyiki M.C. and Tsien R.W. (1984). ß-adrenergic modulation of calcium channels in frog ventricular cells. Nature 307, 371-375.

Biermans W., Bakker A. and Jacob W. (1990). Contact sites between inner and outer mitochondrial membrane: a dynamic compartment for creatine kinase activity. Biochim. Biophys. Acta 1018, 225-228.

Brdiczka D., Knoll G., Riesinger I., Weiler U., Klug G., Benz R. and Krause J. (1986). Microcompartmentation at the mitochondrial surface. Its function in metabolic regulation. In: Myocardial and skeletal muscle bioenergetics. Brautbar N. (ed). New York. Plenum Publishing Corp. pp 55-69.

Clarke K., O'Connor A.J. and Willis R.J. (1987). Temporal relation between energy metabolism and myocardial function during ischemia and reperfusion. Am. J. Physiol. 253, H412-H421.

De Kruijff B. (1987). Polymorphic regulation of membrane lipid composition. Nature 329, 587-588.

Fan C-C. and Koenig H. (1988). The role of polyamines in ß-adrenergic stimulation of calcium influx and membrane transport in rat. J. Mol. Cell. Cardiol. 20, 789-799.

Hackenbrock C.R. (1968). Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. Proc. Natl. Acad. Sci. USA. 61, 598-605.

Jacob W., Biermans W. and Bakker A. (1992). Mitochondrial contact sites: a dynamic compartment for creatine kinase activity. In: Guanidino compounds in biology and medicine. De Deyn P.P. and

Marescau B. and Stalon V. (eds). John Libbey and Co Ltd. London. pp 165-174.

Jacob W.A., Bakker A., Hertsens R.C. and Biermans W. (1994). Mitochondrial matrix granules. Their behavior during changing metabolic situations and their relationship to contact sites between inner and outer mitochondrial membranes. Mircosc. Res. Techn. 27, 307-318.

Jennings R.B. and Reimer K.A. (1991). The cell biology of acute myocardial ischemia. Annu. Rev. Med. 42, 225-246.

Jennings R.B. and Steenbergen C. Jr. (1985). Nucleotide metabolism and cellular damage in myocardial ischemia. Annu. Rev. Physiol. 47, 727-749.

Kleber A.G. (1983). Resting membrane potential, extracellular potassium activity, and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig hearts. Circ. Res. 52, 442-450.

Knoll G. and Brdiczka D. (1983). Changes in freeze-fractured mitochondrial membranes correlated to their energetic state. Dynamic interactions of the boundary membranes. Biochim. Biophys. Acta 733, 102-110.

Kobayashi K. and Neely J.R. (1979). Control of maximal rates of glycolysis in rat cardiac muscle. Circ. Res. 44, 166-175.

McCormack J.G., Halestrap A.P. and Denton R.M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. 70, 391-425.

Mohabir R., Lee H-C., Kurz R.W. and Clusin W.T. (1991). Effects of ischemia and hypercarbic acidosis on myocyte calcium transients, contraction, and pH_i in perfused rabbit hearts. Circ. Res. 69, 1525-1537.

Nicolay K., Rojo M., Wallimann T., Demel R. and Hovius R. (1990). The role of contact sites between inner and outer mitochondrial membrane in energy transfer. Biochim. Biophys. Acta. 1018, 229-233.

Orchard C.H., Houser S.R., Kort A.A., Bahinski A., Capogrossi M.C. and Lakkatta E.G. (1987). Acidosis facilitates spontaneous sarcoplasmic reticulum Ca^{2+} release in rat myocardium. J. Gen. Physiol. 90, 145-165.

Opie L.H. (1992a). Cardiac metabolism-emergence, decline and resurgerance. Part II. Cardiovasc. Res. 26, 817-830.

Opie L.H. (1992b). Cardiac metabolism-emergence, decline and resurgerance. Part I. Cardiovasc. Res. 26, 721-733.

Rojo M., Hovius R., Demel R.A., Nicolay K. and Wallimann T. (1991). Mitochondrial creatine kinase mediates contact formation between mitochondrial membranes. J. Biol. Chem. 266, 30, 20290-20295.

Steenbergen C., Murphy E., Watts A. and London R.E. (1990). Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. Circ. Res. 66, 135-146.

Steenbergen C., Fralix T.A. and Murphy E. (1993). Role of increased cytosolic free calcium concentration in myocardial ischemic injury. Basic Res. Cardiol. 88, 456-470.

Uchida Y. and Murao S. (1974). Potassium-induced excitation of afferent cardiac sympatic nerve fibers during coronary occlusion. Am. J. Physiol. 226, 1094-1099.

Van Belle H., Wynants J., Xhonneux R. and Flameng W. (1986). Changes in creatine phosphate, inorganic phosphate and purine pattern in dog hearts with time of coronary artery occlusion and the effect thereon of myoflazine, a nucleoside transport inhibitor. Cardiovasc. Res. 20, 658-664.

Van Venetie R. and Verkleij A.J. (1982). Possible role of non-bilayer lipids in the structure of mitochondria. A freeze-fracture electron microscopic study. Biochim. Biophys. Acta 692, 379-405.

Wallimann T., Wyss M., Brdiczka D., Nicolay K. and Eppenberger H.M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the «phosphocreatine circuit» for cellular energy homeostasis. Biochem. J. 281 (pt1), 21-40.

Accepted January 12, 1995