Molecular and Cellular Biochemistry **226:** 119–128, 2001.

Concomitant accumulation of intracellular free calcium and arachidonic acid in the ischemic-reperfused rat heart

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Received 6 March 2001; accepted 31 July 2001

Abstract

This study was designed to elucidate the relationship between enhanced cytoplasmic calcium levels (Ca^{2+}_i) and membrane phospholipid degradation, a key step in the loss of cellular integrity during cardiac ischemia/reperfusion-induced damage. Isolated rat hearts were subjected to 15 min ischemia followed by 30 min reperfusion. Ca^{2+}_i was estimated by the Indo-1 fluorescence ratio technique. Degradation of membrane phospholipids as indicated by the increase of tissue arachidonic acid content was assessed in tissue samples taken from the myocardium at various points of the ischemia/reperfusion period. The hemodynamic parameters showed almost complete recovery during reperfusion. Fluorescence ratio increased significantly during ischemia, but showed a considerable heart-to-heart variation during reperfusion. Based upon the type of change of fluorescence ratio during reperfusion, the hearts were allotted to two separate subgroups. Normalization of fluorescence ratio was associated with low post-ischemic arachidonic acid levels. In contrast, elevated fluorescence ratio coincided with enhanced arachidonic acid levels. This observation is suggestive for a relationship between the Ca^{2+}-related fluorescence ratio and arachidonic acid accumulation probably due to a calcium-mediated stimulation of phospholipase A_2. (Mol Cell Biochem **226**: 119–128, 2001)

Key words: intracellular calcium, ischemia, lipid metabolism, myocytes, reperfusion

Introduction

Intracellular free calcium (Ca^{2+}_i) plays a prominent role in the regulation of multiple cellular events. Under normal conditions, the level of Ca^{2+}_i in cardiomyocytes is tightly regulated, and any disturbance of the underlying control mechanism will inevitably lead to elevation of Ca^{2+}_i [1, 2]. Substantial increase of Ca^{2+}_i in the cytoplasm results in complex functional disorders [3–6]. Activation of calcium dependent phospholipases can damage the cell membrane resulting in elevated levels of cytotoxic fatty acids and lysophospholipids [7]. High Ca^{2+}_i promotes scavenging of Ca^{2+} by mitochondria, thereby uncoupling oxidative phosphorylation [8–11]. Moreover, high levels of Ca^{2+}_i may induce hypercontracture of sarcomeres. These events are thought to be major mediators of the ischemic/post-ischemic tissue injury, but the exact relationship between elevated Ca^{2+}_i and irreversible structural disintegration of the myocytes under ischemic/reperfusion condition remains to be elucidated.

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The accumulation of arachidonic acid, a fatty acid abundantly present in cardiac phospholipids, was found to be a sensitive marker of membrane degradation [7, 12–14]. Although circumstantial evidence indicates that a Ca^{2+}-dependent non-specific phospholipase A_2 (PLA_2) may play an important role in phospholipid hydrolysis during ischemia/reperfusion [15], the correlation between Ca^{2+}_i and PLA_2 activity in the intact heart and, hence, membrane phospholipid degradation is basically unknown.

It was the aim of the present study to investigate the effect of short-term ischemia and reperfusion on myocardial Ca^{2+}_i homeostasis, and to delineate the relationship between changes of Ca^{2+}_i levels and myocardial arachidonic acid content as a measure of PLA_2 activity under these circumstances. The effect of alterations in the Ca^{2+}_i homeostasis on hemodynamic recovery during reperfusion was also studied. To this end, changes in Ca^{2+}_i levels were measured with the use of Indo-1 fluorescence in isolated, perfused hearts, subjected to 15 min ischemia, followed by 30 min reperfusion. Degradation of membrane phospholipids was assessed by the measurement of the myocardial content of arachidonic acid in tissue samples taken at the end of ischemia or the reperfusion period. Since there is a considerable controversy in the literature regarding the origin of the fluorescence signals in these types of experiments [16, 17], an additional – methodological – goal of this study was to classify the types of cells contributing to the Ca^{2+}_i-related Indo-1 fluorescence signals in the myocardium, and furthermore, to delineate the intracellular distribution of these signals. In a subgroup of experiments, either sections of the myocardial tissue were examined to show the distribution of Indo-1 in the heart, or mitochondria were isolated from Indo-1 loaded hearts to determine the level of sequestration of Indo-1 into these cell organelles, if any.

Materials and methods

Animals and isolated heart preparation

Hearts were obtained from 300–350 g male Sprague–Dawley rats anaesthetized by 40 mg/kg pentobarbital i.p. and perfusion of the isolated hearts was started according to Langendorff with a modified Krebs–Henseleit solution (118 mM NaCl, 4.3 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM NaEDTA, 2.0 mM Ca_2Cl, 11 mM glucose, 5 mM pyruvate) at 37°C and pH = 7.4. The perfusion apparatus was manufactured by Experimetrica, Budapest, Hungary. The hearts were immersed in the Krebs–Henseleit solution kept at 37°C. The perfusate was equilibrated with a mixture of 95% O_2–5% CO_2 and the perfusion pressure was set to 70 mmHg. Coronary flow was measured with an ultrasonic transducer (T-106, Transonic Systems, Ithaca, NY, USA) inserted into the perfusion line. A balloon catheter positioned in the left ventricle and connected to a pressure transducer (Electromedics, Englewood, CO, USA) was used to assess left ventricular pressure (LVP). The diastolic LVP was set routinely at 7.5 mmHg at the beginning of the experiment. From LVP heart rate (HR) and LVdP/dt were calculated with the aid of subsequent analogue units (Grass Model 7D, Grass, Quincy, MA, USA).

Following a 30 min equilibration period, the hearts were loaded in recirculating mode with the calcium-sensitive fluorescent dye, Indo-1 AM containing solution with a peristaltic pump (Cole-Palmer, Chicago, IL, USA) connected to the aortic cannula. To this end, 250 μg Indo-1 AM was first dissolved in 250 μL dimethyl-sulfoxide (DMSO), then it was diluted in normal Krebs–Henseleit solution to a final concentration of 6.25 μM containing 15% Pluronic F-127 and DMSO (0.006 vol. %). Pluronic F-127 was present to increase the solubility of Indo-1 AM. The temperature of this solution was maintained at 30°C and the flow rate was set to 8 ml/min. The loading period lasted to about 20 min; during this time period fluorescence reached maximal levels. Then a washout period was started in normal Langendorff mode for 20 min with the modified Krebs–Henseleit buffer to eliminate fluorescence originating from Indo-1 AM present in the extracellular space.

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Chemicals

Pentobarbital (Nembutal) was purchased from Sanofi, Budapest, Hungary and Indo-1 AM and Pluronic F-127 from Molecular Probes, Eugene, OR, USA. All other chemicals were obtained from Sigma (Budapest, Hungary).

Optical measurement

A custom-made 4-channel fluorometer system equipped with fused silica optics was used to carry out the optical measurements. A randomized multifurcated fiber optic light guide provided the connection between the light source, the perfused heart and the photomultiplier tubes of the fluorometer. The epicardial surface of the left ventricle and the fluorescence dye were excited at 340 nm with a 100 W DC mercury arc lamp. Tissue fluorescence was measured at two wavelengths (400 nm, 506 nm) and reflectance was detected at 340 nm. The Ca^{2+}_i level was estimated by the ratios of the 400 (Ca^{2+}-dye) nm and 506 (Ca^{2+}-free dye) nm signals. The reflected signal was used to estimate non-specific changes in tissue density (optical artifacts). Illumination of the heart was

kept to minimum to avoid photobleaching of the intracellular dye. Therefore, hearts were illuminated for about 1 min in every 5 min of the experiment. The fluorescence and reflected signals were continuously recorded and stored with a multichannel AD converter (Adventec, PCL 718) coupled to an IBM-based computer.

Experimental protocols

The fluorescence measurements were carried out in five experimental groups.

Group 1 (Autofluorescence)
Changes in the fluorescence ratio values during ischemia may be affected by an increase in tissue autofluorescence at both 400 and 506 nm due to an NAD(P)H shift in the mitochondria [18]. Therefore, the influence of NAD(P)H level changes on the Indo related fluorescence intensities were determined. Hearts (n = 3) were perfused with a 'loading' solution lacking Indo-1 AM, and then were subjected to 15 min ischemia and 30 min reperfusion. The changes in autofluorescence at 400 and 506 nm during ischemia were used to correct the Indo-1 related fluorescence intensities before ratio calculation.

Group 2 (Control dye-leakage):
After the equilibration period these hearts (n = 3) were loaded with Indo-1 AM, then the washout period was followed by 45 min perfusion under normoxic conditions. Fluorescence (400 and 506 nm) and reflectance (340 nm) signals were recorded and used to determine the stability of the Ca^{2+}_i related ratio values during this period of time. Thereafter, hearts were rapidly frozen with a Wollenberger clamp prechilled in liquid nitrogen and stored at –80°C prior to lipid analysis.

Group 3 (Normoxic arachidonic acid levels)
Seven hearts were normoxically perfused in the absence of Indo-1 AM for 120 min. No fluorescence measurement was carried out in this group. At the end of the perfusion period these hearts were also snap frozen for lipid analysis.

Group 4 (Ischemia)
Hearts (n = 8) were loaded with Indo-1 AM and subjected to no-flow ischemia for 15 min after the washout period of 20 min. The temperature of the hearts was maintained at 37°C during the ischemic period by immersing the hearts into Krebs–Henseleit solution. At the end of ischemic period, hearts were rapidly frozen and stored at –80°C prior to lipid analysis.

Group 5 (Ischemia and reperfusion):
Hearts (n = 13) were loaded with Indo-1 AM and rendered ischemic for 15 min by complete cessation of the perfusion. The temperature of the hearts was maintained at 37°C during the ischemic period (see above). Ischemia was followed by a 30 min reperfusion period and the hearts were rapidly frozen at the end of this period. Hearts were stored at –80°C prior to lipid analysis.

Analysis of tissue arachidonic acid content

Aliquots of rat cardiac tissue ranging in weight from 200–300 mg wet weight were pulverized in an aluminum mortar with a stainless steel pestle, both previously cooled in liquid nitrogen. Extraction of lipids and determination of the content of unesterified arachidonic acid by capillary gas chromatography have been extensively described by Van der Vusse *et al*. [19].

Isolated mitochondria study

In a separate set of experiments an attempt was made to elucidate the intracellular origin of the fluorescence signal. To this end, tissue homogenates were prepared from Langendorff hearts (n = 5) loaded with Indo-1 AM to measure the fluorescence signal characteristic for both the acetoxymethylester and free acid in the mitochondrial and cytosolic fraction. The loading procedure and fluorescence measurements were performed as described above. Mitochondria were isolated according to Lukács and Fonyó [20]. In brief, ventricular tissue was chopped with scissors, and homogenized gently in an extraction medium, containing 225 mM mannitol, 75 mM sucrose, 0.4 mM EDTA, 0.4 mM EGTA, 5 mM Tris-HCI (pH 7.4) and 0.2 mg/ml bovine serum albumin with a loose teflon-glass homogenizer in 3 strokes. The homogenate was centrifuged at 500 × g for 10 min. The precipitate was resuspended with the extraction medium and centrifuged again at 500 × g for 10 min. The supernatants of the first two centrifugation steps were combined (20 ml). The precipitate containing the non-disintegrated cells, red blood cells and cell debris was discarded. The supernatant was centrifuged at 10,000 × g for 10 min. The fluorescence intensity of the supernatant was measured. The mitochondrial pellet was then resuspended in 5 ml of washing solution containing mannitol, sucrose and Tris-HCI (pH = 7.4) in the same concentration as the extraction medium and was centrifuged at 10,000 × g for 10 min. The fluorescence intensities of supernatant fluid and the resuspended mitochondrial pellet were measured at 400 and 506 nm.

Fluorescence microscopy

To investigate the cellular distribution of Indo-1 loading in the hearts, one loaded and one unloaded heart were quick-frozen in a bath of liquefied nitrogen and isopentane (2-methyl-

butane). From the left ventricles of both frozen hearts 25 μm thick sections were cut with a cryomicrotome (Reichert-Jung, cryocut E, Austria). Unstained dry sections were examined with a fluorescent microscope equipped with a cooled slow-scan camera (TE/CCD-512TKB/1, Princeton Instruments, Princeton, NJ, USA equipped with a TK-512CB type CCD chip) at an illumination wavelength of 365 nm according to Tóth *et al*. [8]. In brief, fluorescent images of the slides were captured at 400 and 506 nm for 10 sec and stored for off-line determination of fluorescence ratios and analysis of the distribution of Indo-1 fluorescence.

Data analysis

The stored fluorescence (400 and 506 nm) and reflectance (340 nm) signals were analyzed off-line using a Labtech Notebook software package. Single wavelength signals were corrected for closed shutter background and tissue autofluorescence (i.e. unloaded tissue). These corrected single wavelength signals were used to calculate the 400/506 nm ratio. During ischemia both 400 and 506 nm signals are influenced by a Ca-independent increase in autofluorescence due to NAD(P)H shift. This phenomenon was investigated in Group 1 and corrections for Indo-1 fluorescence were made accordingly in the other experimental groups. The 400/506 nm ratio corresponding to the onset of ischemia was considered to be unity in each of the experiments, the consecutive ratio values are representing changes compared to 1.

Statistics

Data are given as means ± S.D. Statistical analysis was carried out using one-way analysis of variance followed by Duncan's *post hoc* test. The corresponding means of ratios of the subgroups in Group 5 during ischemia/reperfusion were compared using Student's *t*-test. A statistical significance level of $p < 0.05$ was accepted throughout.

Results

Validation of the technique

The results obtained in *Group 1 (Autofluorescence)* show that there was a rapid, 35% (range: 32–37%) increase in autofluorescence at 506 nm after the onset of ischemia. Therefore, the 506 nm signals collected during ischemia were corrected for this increase in autofluorescence prior to ratio calculation. Since the effect of increase in autofluorescence on the 400 nm signal was negligible no correction was made on this signal.

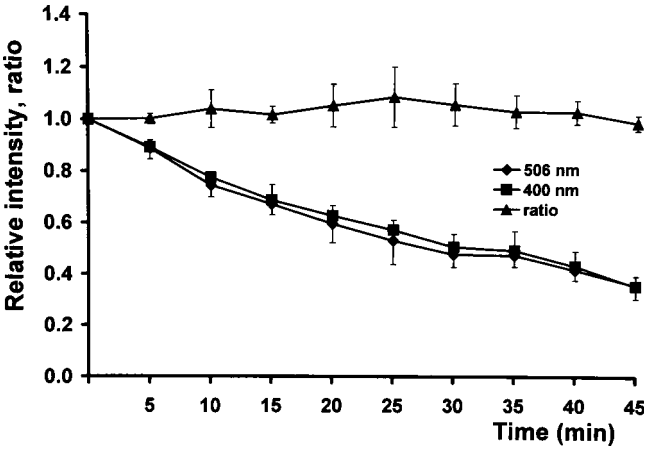

Fig. 1. Stability of the fluorescence ratio values in Group 2 (Control dye-leakage). In this group fluorescence of Indo-1 AM loaded hearts was followed for 45 min with no interventions. Ratio values (n = 3) were calculated from tissue fluorescence values at 400 and 506 nm and were normalized to those obtained in the loaded state.

Figure 1 shows the stability of the fluorescence ratio values for up to 45 min of normal perfusion without interventions. Despite a significant decrease in the individual fluorescence intensities at 400 and 506 nm indicating a steady dye-leakage from the individual myocytes, the calculated ratio values remained basically unaffected by the decrease of the intracellular dye concentration. These results indicate that during this period of observation the preparation retained enough dye intracellularly to allow reliable calculation of the Ca^{2+}_i related fluorescence ratio values.

The analysis of the Indo-1 related fluorescence ratio images of ten thin sections showed that the dye was evenly distributed in the myocardium (see Fig. 2 for a representative example). Qualitative assessment of the slides showed that the myofibrils occupied more than 80% of the tissue, while vascular elements occupied 5 to 10% with 10% interstitial space, hence the majority of fluorescence originated from myocytes.

The results of the isolated mitochondria studies show that Indo-1 could only be detected in the first supernatant of the homogenate of the heart cells gained at 10,000 × g, i.e., Indo-1 is present in the combined interstitial and intracellular fluids The supernatant after the second centrifugation at 10,000 × g and the final resuspended mitochondrial pellet contained no measurable fluorescence intensity compared to the blank (washing medium) solution. The mean protein content of the mitochondrial suspension was 1.8 mg/ml. To exclude the inner filter effect of the high mitochondrial content of the suspension, further dilution was applied (protein concentration: 0.3 mg/ml) and the fluorescence intensity was measured again. This procedure caused no increase in the intensity of the measured signals. In studies carried out on hearts not loaded with Indo-1, fluorescence intensity in any solution

Fig. 2. Ratio image of myocardial section of Indo-1 loaded heart. A 25 µm thick unstained dry section of the left ventricle is shown. For collection of fluorescent data (400 and 506 nm) a cooled, slow scan camera was used. The Indo-1 loaded individual muscle fibres can easily be distinguished, the intensity of which are essentially the same as that of other cellular elements. Note the fascia surrounding the elementary muscle fibres (dark lines).

during the isolation procedure was not different from that of the blank solution.

Effect of ischemia

In Group 4 (Ischemia) the Indo-1 loaded hearts were subjected to 15 min ischemia without reperfusion. Contractile activity of the hearts stopped within 1–2 min after the start of ischemia. No contracture developed during the ischemic period. The hearts displayed a significant elevation of fluorescence ratio (190 ± 31% of control, $p < 0.05$) during ischemia. The pre-ischemic hemodynamics of the hearts in this group were similar as seen in the Ischemia and reperfusion Group 5 (see below).

Effect of ischemia and reperfusion

Figure 3 summarizes the results obtained in Group 5 (Ischemia and reperfusion). A substantial inter-individual difference in response could be observed in the Ca^{2+}_i-related fluorescence ratio values in the reperfusion period. The individual

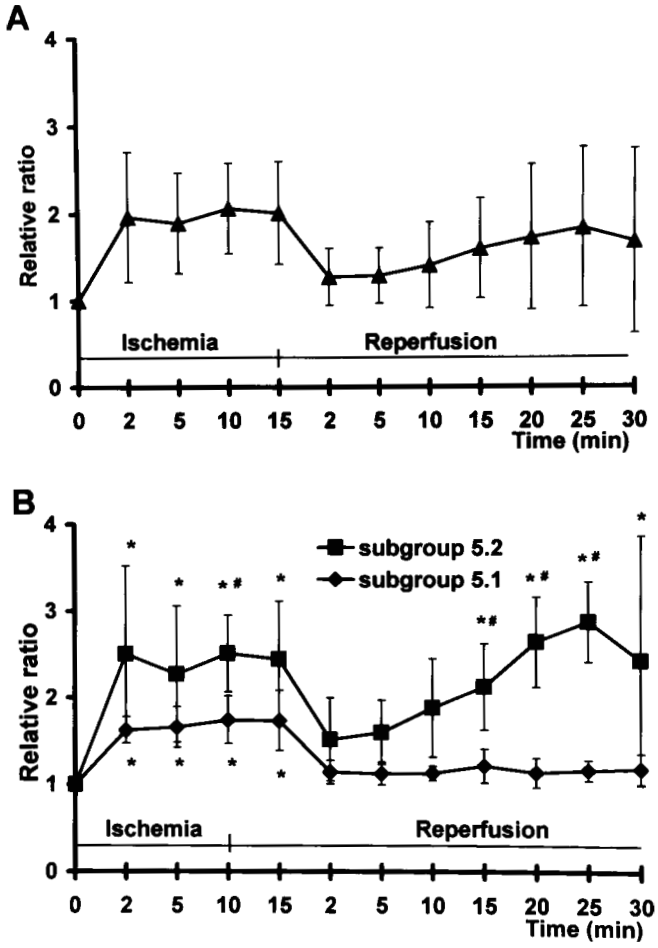

Fig. 3. Ratio values (400/506) obtained in Group 5 (Ischemia and reperfusion). 400/506 nm ratio values were corrected for autofluorescence due to NAD(P)H changes and normalized to those obtained in the normoxic state. (A) The mean fluorescence ratio of all 13 hearts increased during 15 min ischemia, but it showed a considerable heart-to-heart variation during reperfusion as indicated by the S.D. values. (B) In subgroup 5.1 (n = 8) the ratio increased significantly during ischemia, then it returned to near control level in the reperfusion period. In subgroup 5.2 (n = 5) however, the ischemic increase of the Ca^{2+}-related fluorescence ratio was followed by a secondary, gradual fluorescence ratio elevation in the latter half of the reperfusion period. *($p < 0.05$) indicates values significantly different from control. #($p < 0.05$) indicate significant differences between the corresponding means of ratios in the two subgroups.

experiments could be classified into two separate subgroups based on the type of change of the fluorescence ratio during reperfusion. In subgroup 5.1 (n = 8) the ratio returned to near control levels in the reperfusion period. In subgroup 5.2 (n = 5), however, a secondary, gradual elevation of the fluorescence ratio in the latter half of the reperfusion period (287 ± 46% of pre-ischemic values, $p < 0.05$) could be observed (Fig. 3). Also, the ischemic increase of fluorescence ratio in the second subgroup appeared to be considerably higher as compared to the first subgroup, although the difference between

the fluorescence ratio values were only statistically significantly different at the 10th min of ischemia ($p < 0.05$).

Hemodynamics

Figure 4 shows the pre- and post-ischemic systolic LVdP/dtmax values and the coronary flow in the two subgroups 5.1 and 5.2 during the course of the experiment. Pre-ischemic hemodynamic values did not differ between the two subgroups, hence, the data were pooled. No obvious differences could be found between pre- and post-ischemia, indicating that this short term ischemia was well tolerated by the hearts. Also, no difference in these hemodynamic parameters between the two subgroups (i.e. hearts with low and high post-ischemic fluorescence ratio) was found. The analysis of the other hemodynamic parameters also revealed no major differences between the two subgroups. The pre-ischemic LVP

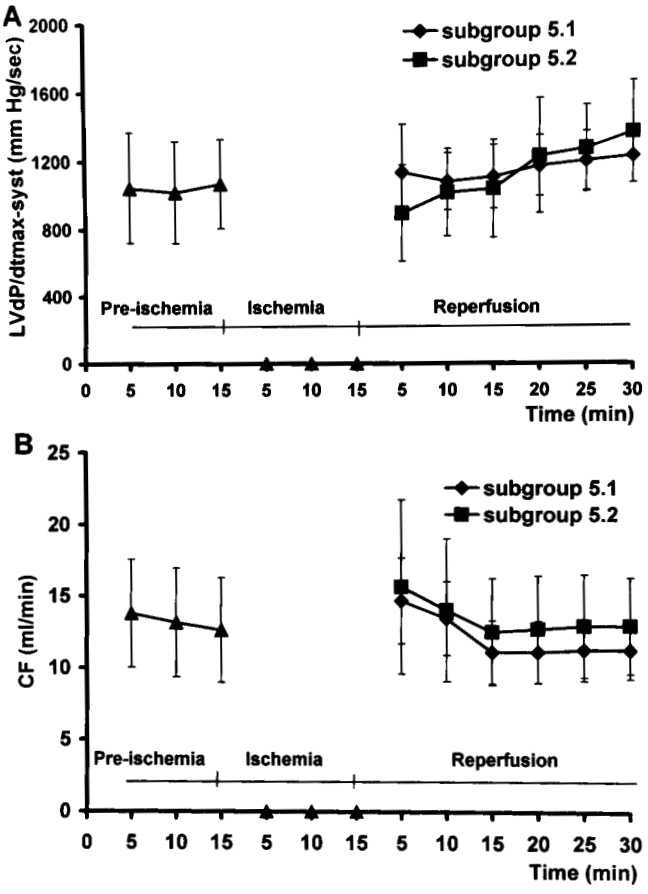

Fig. 4. Hemodynamic recovery during reperfusion in Group 5 (Ischemia and reperfusion). Hemodynamic data belonging to subgroups 5.1 (n = 8) and 5.2 (n = 5) separated according to the type of change of fluorescence ratio during reperfusion is shown. Pre-ischemic hemodynamic values (%) did not differ among the two subgroups, hence, these data are pooled.

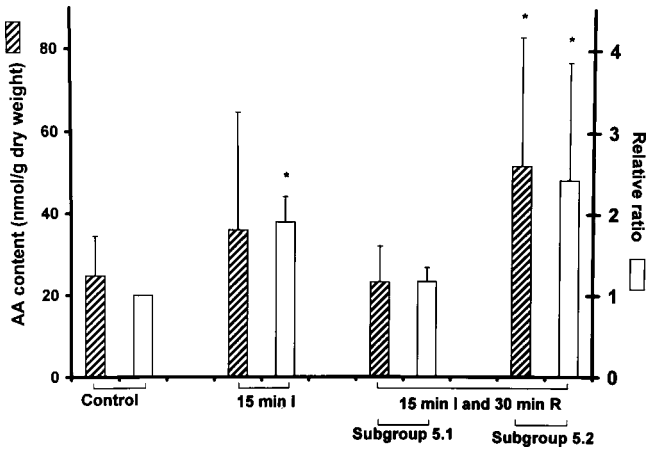

Fig. 5. Relationship between fluorescence ratio and arachidonic acid (AA) accumulation in the different experimental groups. Left ordinate indicates levels of AA (hatched bar), right ordinate shows relative fluorescence ratio values (open bar). Control hearts (Group 3, n = 7) had 24.7 ± 9.7 nmol/g dry weight AA content after 120 min normal perfusion. Both AA and fluorescence ratio increased during 15 min ischemia (I) (n = 8). Reperfused hearts were allotted to two different subgroups based upon the type of change of fluorescence ratio during reperfusion. *($p < 0.05$) indicates values significantly different from control.

was 67 ± 12 mmHg, which changed to 70 ± 13 mmHg in the subgroup 5.1 and 74 ± 18 mmHg in subgroup 5.2 by the end of the reperfusion period. Heart rate was also not significantly different between the two subgroups (pre-ischemic control: 241 ± 46 beats/min; subgroup 5.1 and 5.2 at the end of the reperfusion period: 263 ± 49 and 241 ± 21 beats/min, respectively).

Arachidonic acid content

The results of tissue lipid analysis are presented in Fig. 5. The control arachidonic acid (AA) content was determined in seven hearts after 120 min normal perfusion (Group 3) and amounted to 24.8 ± 9.8 nmol/g dry weight. During this time the hearts were hemodynamically stable. Levels of AA in the Control dye-leakage group (Group 2) were not different from that of this group, indicating that Indo-1 had no apparent effect on PLA_2 activity. Hearts, which were freeze-clamped after 15 min ischemia without reperfusion had 36.1 ± 28.9 nmol/g dry weight AA concentration (n = 8). The mean AA content displayed a significant inter-individual variation at the end of 30 min reperfusion in hearts subjected to a complete ischemia/reperfusion protocol. The AA content did not differ from control levels in the hearts, where the fluorescence ratio returned to normal values in the reperfusion period (23.5 ± 8.9 nmol/g dry weight, subgroup 5.1). In contrast, AA increased substantially ($p < 0.05$) in the hearts, which displayed a significant rise of the fluorescence ratio level in the second

half of the reperfusion period (51.9 ± 31.5 nmol/g dry weight, subgroup 5.2).

Discussion

Methodological considerations

The Indo-1 ratiometric approach is a widely used method to obtain a measure of Ca^{2+}_i in the isolated, perfused heart [21–23]. Under our experimental conditions dye-leakage did occur without affecting the ratio calculations since all of the fluorescence measurements were carried out in a relatively short time frame (15 min ischemia followed by 30 min reperfusion). In this time period the myocytes still retained the necessary amount of dye to ensure adequate signal to noise ratio. Interestingly, during ischemia and early reperfusion leakage of Indo-1 came almost to a halt, again indicating an active pump activity removing the dye from the cell.

Ischemia induces a shift in the $NAD(P)^+$/NAD(P)H redox state leading to increased tissue autofluorescence [18]. This increase would result in the overestimation of the 506 nm signal by 35%, leading to incorrect estimation of Ca^{2+}_i. Therefore, special attention was paid to correct the 506 nm signal prior to ratio calculation. No corrections were necessary of the fluorescence signal measured at 400 nm, because the influence of shifts of $NAD(P)^+$/NAD(P)H redox state will be negligible at this wavelength as predicted by the emission spectrum of NAD(P)H. In this study loading with Indo-1 AM resulted in a 3.9-fold and 2.4-fold increase of fluorescence measured at 400 and 506 nm, respectively, compared to autofluorescence. A possible way to minimize the effect of autofluorescence would be to increase levels of Indo-1 in the cytosol by at least an order of magnitude to minimize contributions of NAD(P)H fluorescence to the 506 nm fluorescence signal. This procedure, however, should be avoided, because high cytosolic Indo-1 levels would – amongst other adverse effects – buffer Ca^{2+}_i changes.

It should be emphasized that precise values of intracellular Ca^{2+} concentration cannot be derived from the Indo-1 fluorescent ratio due to the lack of a calibration curve appropriate for the intact heart. However, earlier studies [18, 24] indicated a quasi-linear relationship between fluorescence ratio and free Ca^{2+} concentrations at Ca^{2+} concentrations lower that 800 nM in the conditions of our experimental setup.

Although Indo-1 AM has been frequently used to estimate Ca^{2+}_i in isolated perfused organ studies, there is still a great deal of controversy as to the origin of the fluorescence signals. A number of previous studies [16, 24–26] suggested that part of the Indo-1 fluorescence signal originates from non-cytosolic origin. For example, Schreur *et al*. [16], based on indirect evidence, claimed that about 50% of Indo-1 related fluorescence stems from mitochondria instead of the cytoplasmic compartment of the heart. In our study fluorescence of the mitochondria was directly measured. No evidence of sequestration of Indo-1 by the mitochondria could be obtained as opposed to Schreur's indirect observations, hence, the epifluorescence signals from the heart most likely reflect the concentration of free Ca^{2+} in the cytosol, not mitochondrial matrix. Others [17, 25, 27] showed that significant amounts of Indo-1 were taken up by the endothelial cells instead of cardiomyocytes. According to our observation the dye was evenly distributed in the heart, no evidence of local 'hot spots' was found. These findings strongly suggest that ratio values in this study represent levels of Ca^{2+}_i in the myocytes, with minor contributions from non-contractile elements.

Pioneering studies of Chance *et al.* [28] and of Stewart [29] indicated that the penetration depth of light in biological material, as used in our experimental setup, is between approximately 0.3 and 1 mm, which corresponds with about one-third to two-thirds of the left ventricular wall of the isolated rat heart. Since the temperature in all regions of the heart was kept constant by immersing the heart in temperature-controlled fluid throughout the experiment, and cessation of flow was complete in all layers of the heart during the ischemic episode, the fluorescence signals derived from the subepicardial layers are most likely representative for all layers of the left ventricle.

During the ischemic period the Ca^{2+}_i-related fluorescence ratio increased significantly. Caution must be taken when estimating Ca^{2+}_i from the fluorescence ratio in states of ischemic acidosis as this elevation in Ca^{2+}_i may be underestimated because acidosis increases the apparent K_d of Indo-1 [30].

Indo-1 fluorescence ratio during ischemia and/or reperfusion

During the ischemic period the Indo-1 fluorescence ratio significantly increased, reflecting an increase in the free cytoplasmic Ca^{2+} concentration of the flow-deprived heart. Upon reperfusion, the mean of the fluorescence ratio remained elevated while the standard deviation of the mean substantially increased. Critical analysis of the findings of individual hearts revealed that in 8 out of 13 hearts studied the fluorescence ratio returned to pre-ischemic values upon reperfusion. These hearts, in which the fluorescence ratio returned to pre-ischemic levels, were allotted to subgroup 5.1. In contrast, the ratio of 5 out of 13 reperfused hearts showed a significantly further increase after reinstallation of flow through the coronary arteries. This pronounced increase in ratio points towards elevated cytoplasmic Ca^{2+} levels in the reperfused hearts (subgroup 5.2). At present, one may only speculate about the reasons for the two dif-

ferent responses of cytoplasmic Ca^{2+} following reperfusion of the heart. The deviation may reflect differences in susceptibility towards ischemia. This notion is supported by the finding that hearts showing a significant rise in fluorescence ratio during the reperfusion phase already tended to an elevated ratio during the ischemic period.

It is of interest to note that in both subgroups of hearts post-ischemic haemodynamic recovery was identical and values of developed pressure and coronary flow were not significantly different from pre-ischemic values. These findings suggest that the sensitivity of the contractile machinery towards intracellular calcium might be decreased in hearts belonging to subgroup 5.2.

Tissue AA in the reperfused heart

Upon reperfusion, those hearts which showed a substantial increase in fluorescence ratio (subgroup 5.2) had a concomitant significant increase in arachidonic acid. In contrast, subgroup 5.1, consisting of reperfused hearts with fluorescence ratios returning to pre-ischemic values, the tissue content of AA was not significantly different from normoxic, control levels. Since increased fluorescence ratio reflects elevated cytoplasmic Ca^{2+} levels, to the best of our knowledge, the present study is the first to show a positive relationship between accumulation of AA and elevated cytoplasmic Ca^{2+} in the intact, reperfused heart.

Relationship between cytoplasmic Ca^{2+} and membrane phospholipid degradation

Since cardiac AA is predominantly incorporated in the membrane phospholipid fraction, elevated tissue AA levels indicate that the rate of deacylation of phospholipids exceeds the rate of reacylation [15]. The present findings may suggest a causative relationship between elevated cytoplasmic Ca^{2+} and increased tissue AA. The precise nature of this relationship is, however, open to speculation. First, hearts which were able to restore near control Ca^{2+}_i levels during reperfusion were most likely able to normalize the deacylation-reacylation cycle of membrane phospholipids, whereas a relatively high post-ischemic fluorescence ratio most likely promotes phospholipid degradation. These observations support the hypothesis that membrane phospholipid degradation associated with ischemia/reperfusion injury is – at least partly – mediated by a Ca^{2+}-dependent PLA_2 enzyme. The relatively higher level of AA during reperfusion as compared to ischemia may also indicate a multifactorial process of membrane degradation. For example, during reperfusion production of oxygen free radicals may be induced contributing to phospholipid catabolism. It is of interest to note that in this protocol we did not observe irreversible functional damage, since all hearts recovered hemodynamically by the end of reperfusion. This indicates that hearts showing a precipitous fluorescence ratio elevation during the reperfusion period were also able to tolerate the enhanced PLA_2 activity in terms of hemodynamics, at least during 30 min of reperfusion. The observed quantitative changes in AA levels show that approximately 1–2% of membrane phospholipids is affected by ischemia/reperfusion [7], which indicates that no massive loss of membrane integrity occurred during 15 min ischemia and the following 30 min reperfusion period. This notion is in line with the observation that functional recovery of the heart was almost complete in the present experimental protocol.

As indicated above, hydrolysis of membrane phospholipids is catalyzed, among others, by PLA_2 [31]. At least three types of PLA_2 have been identified in myocytes, i.e. a Ca^{2+}-insensitive PLA_2 with high specificity for arachidonic acid-containing plasmalogens [32], a second type of PLA_2 that translocates from the cytoplasm to the sarcolemma when Ca^{2+}_i is elevated in the (sub)micromolar range, and a third type of PLA_2, the activity of which is highly dependent on calcium ions in the millimolar concentration range but with low specificity for the fatty acyl chain present at the Sn-2 position of the phospholipid molecule [15]. At present, it is still unclear, which Ca^{2+}-dependent type(s), if any, is (are) involved in the degradation of membrane phospholipids during ischemia/reperfusion injury. On basis of the alterations in fluorescence signals, it can be concluded that the maximal increase of Ca^{2+}_i during reperfusion was not more than fourfold, leaving Ca^{2+}_i still in the micromolar range. From this it follows that a most likely candidate responsible for the accelerated phospholipid degradation is the cytosolic PLA_2, which is probably translocated from the cytosol to the cell membrane by increases in cytosolic Ca^{2+} in the physiological range [15].

Alternatively, enhanced activity of a Ca^{2+}-insensitive phospholipase A_2 may disturb the deacylation-reacylation cycle resulting in a net accumulation of AA in the reperfused heart. Since in rat myocardium the content of the target of this hydrolytic enzyme, i.e. plasmalogen, is relatively low as compared with rabbit and human heart [32], we consider the Ca^{2+}-insensitive PLA_2 a less likely candidate for catalyzing phospholipid degradation in the present experimental model. Finally, it cannot be excluded that elevated AA levels, in turn, may evoke an increase in the cytoplasmic Ca^{2+} levels. This notion is based on earlier studies providing circumstantial evidence that increased myocytal AA levels cause a significant rise in the cellular Ca^{2+} concentration [33, 34].

Conclusion

In conclusion, the present results indicate that 15 min ischemia is most likely a critical time duration to affect normal Ca^{2+}_i handling upon restoration of flow in the perfused rat heart. This intervention leads to the development of inter-individual differences indicated by either complete restoration of the ability to resume normal calcium handling or by increased levels of post-ischemic Ca^{2+}_i associated with a disturbed deacylation-reacylation cycle of membrane phospholipids. Although cause and effect relationships are difficult to investigate in a multifactorial process, our results strongly suggest a functional relationship between Ca^{2+}_i and AA accumulation in the reperfused myocardium. The exact mechanisms underlying the changes in Ca^{2+}_i and phospholipid homeostasis during ischemia/reperfusion are subject to further investigations.

Acknowledgements

The authors are grateful to Drs Kopper and Paku for their help in preparing the myocardial slides. This work was supported by grants from OTKA (T026477 and T026144, Hungary) and NWO (N028769, the Netherlands).

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