Mapping of capillary flow, cellular redox state, and resting membrane potential in hypoperfused rat myocardium

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Brasch, Frank, Marion Neckel, Rolf Volkman, Gerhard Schmidt, Gerhard Hellige, and Friedrich Vetterlein. Mapping of capillary flow, cellular redox state, and resting membrane potential in hypoperfused rat myocardium. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2050–H2064, 1999.—The influence on myocardial viability of ischemia-induced changes in capillary perfusion was studied in the hearts of anesthetized rats subjected to partial occlusion of the left coronary artery for 45 min. Timed plasma labeling was applied to determine perfusion patterns. Changes in the fluorescence of preloaded potential-sensitive dyes [tetramethylrhodamine methyl ester (TMRM) and bis-oxonol], of trypan blue, and of endogenous NADH were utilized in characterizing myocyte viability in histological sections of the heart. Within the hypoperfused zone, localized areas appeared vascularity nonperfused for periods of at least 10 min. Within these areas a reduction in TMRM fluorescence occurred in 82.5% of the tissue, signaling a reduced resting membrane potential. In the same areas 37.7% of the myocytes revealed an NADH fluorescence lower than that regularly found in anoxic tissues. This correlated with an especially low level of TMRM, with increased fluorescence bis-oxonol and with an accumulation of trypan blue. In conclusion, in localized hypoperfusion-induced areas lacking capillary flow, an inhomogeneous pattern of reductions in myocyte viability develops, which appears to be relevant in ischemia-induced arrhythmias.

heart; low-flow ischemia; timed plasma labeling; bis-oxonol; tetramethylrhodamine methyl ester; reduced nicotinamide adenine dinucleotide; trypan blue

MYOCARDIAL ISCHEMIA due to critical narrowing of a coronary artery induces conspicuous heterogeneities of supply in the area affected. This effect has been most impressively demonstrated by exciting the fluorescence of NADH on the surface of the heart. The blue autofluorescence of the tissue rises in an irregular, spotlike pattern during conditions of low-flow perfusion (3, 15, 33). It is known that the level of NADH fluorescence may be taken as an indicator of the myocardial redox state; the signal increases when myocytes are exposed to severe degrees of oxygen deficiency (7).

With respect to the implication of this phenomenon, it has been discussed whether such regional hypoxic states, if persisting for long periods of time, might cause focal necrosis in the myocardium because anaerobic metabolism may not compensate for the lack of oxygen over longer periods of time (18, 33). There is quite a different and perhaps even more important aspect concerning supply heterogeneity, which has not yet been discussed within this context. A spatially irregular pattern of metabolically disturbed cardiac cells can be considered a risk with regard to the development of arrhythmias. An increase in the dispersion of conduction times and repolarization periods resulting from the occurrence of normal and altered cells that coexist are considered an important factor in arrhythmogenesis and especially in reentrant arrhythmias (12).

The observation of an inhomogeneous distribution of hypoxic zones in the hypoperfused heart and the known arrhythmogenic potential of an irregular pattern of disturbances in the metabolic state of the myocytes (e.g., Ref. 4) give rise to the question as to whether the generation of localized increases in NADH fluorescence might in any way be indicative of the generation of disturbances in cardiac excitability. Hypoxia per se is known to cause a decrease in action potential duration (29). When capillary flow is also lacking and the metabolic waste products, hydrogen and potassium ions, accumulate in the extracellular space, disturbances in membrane function become much greater (12). A decrease in the upstroke velocity of the action potential, a reduction in refractory period, and a decrease in resting membrane potential are known to result.

To conclude that increases in NADH fluorescence lead to the described electrophysiological changes is, however, impossible because increases of this signal in the hypoperfused myocardium may arise from all transitional stages between pure hypoxia and complete ischemia. In a recent study on the effects of coronary hypoperfusion on blood flow distribution, biopsies of the ventricular wall were snap-frozen after intravascular dyes were circulating for certain periods of time (37). In areas showing elevated NADH fluorescence, part of the tissue was found to be completely perfused within a few seconds, whereas in others capillary perfusion was lacking within a period exceeding 1 min. In other words, the rise in NADH per se did not allow us to conclude whether cells had been affected by disturbances typical of hypoxia or ischemia. Because both states differ greatly in their potency to alter membrane functions (e.g., Ref. 27), the relevance of the heterogeneously distributed fields of increased NADH fluorescence is hard to assess with respect to their potential of disturbing cardiac excitability.

For this reason, the present investigation is aimed at introducing additional markers of cellular function...
that might help elucidate the significance of hypoperfusion-induced supply heterogeneities. The resting membrane potential is a parameter relevant to cellular excitability. Decreases in this value, which result in slowed impulse propagation (12), have been reported to occur to a much greater extent during ischemic rather than pure hypoxic conditions (20, 26, 38).

In numerous in vitro studies the resting membrane potential has been measured by detecting the changes in the distribution of potential-sensitive Nernstian dyes (22). In the present investigation the attempt was made to apply such indicators in vivo to test whether a comparison with cellular NADH fluorescence and intravascular dye labeling might be possible by studying the tissue in a maplike fashion as performed in the study cited (37). The dyes tetramethylrhodamine methyl ester, perchlorate (TMRM) (6) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol (bis-oxonol) (16) fulfilled the necessary requirements and were applied in experiments on anesthetized rats.

MATERIALS AND METHODS

General Experimental Procedure

The experiments were performed on male Wistar rats (200–220 g body wt) that were anesthetized with thiobutabarbital (120 mg/kg ip) and a continuous inhalation of a gas (200–220 g body wt) that were anesthetized with thiobutabarbital (120 mg/kg ip) and a continuous inhalation of a gas mixture containing 70% N₂O–30% O₂. Catheters were placed into the left femoral artery for registration of blood pressure (Statham P23 Db and SP 1200) and heart rate (beat-to-beat counter) and into the left femoral vein for drug applications. The trachea was carefully laid free, and a tube was inserted for artificial ventilation (Harvard respirator, type 681). The tidal volume and respiration rate were set to a value of 3.5 ml and 40 breaths/min, respectively. The thoracic cavity was opened by transecting the clavicula and the upper six ribs. The thymus gland was removed, a catheter for monitoring left atrial pressure (SP 1200, Statham P23 Db) was inserted into the left atrium via the left atrial appendage, and both carotid arteries were made accessible.

An anastomosis was inserted between the right femoral artery and the distal trunk of the transected left carotid artery to avoid pressure reflexes that might arise from interrupting flow through both carotid arteries. For determination of flow and perfusion pressure in the left coronary artery, a second anastomosis was placed between the proximal trunk of the left carotid artery and the left coronary artery. Blood flow was determined by an electromagnetic flow probe (Cardina) and coronary perfusion pressure via a side branch of the system (Statham P23 Db, SP 1200). A hydraulic occluder was placed around the feeding trunk of the carotid artery. Details of the latter procedures have been described in a preceding paper (37).

Systemic arterial pressure, left atrial pressure, and coronary blood flow were continuously recorded on a Gould Brush direct-writing system; heart rate and coronary perfusion pressure, indicated on the instruments, were protocolled every 5 min.

Special Substances Used

Membrane-sensitive dyes. To make up the TMRM (Molecular Probes, Eugene, OR) solution, a stock solution containing 1.0 mg/100 µl DMSO was prepared, stored at 4°C, and used within a maximum of 1 wk. Immediately before use 10 µl of the solution were diluted in 1 ml of Ringer solution, and 1 ml/kg body wt was injected intravenously, corresponding to a dose of 0.1 mg/kg.

A dose of 7.5 mg/kg of bis-oxonol (Molecular Probes) was injected intraperitoneally. The quantity actually needed in the experiment was dissolved in 60 µl of DMSO immediately before use, to which 840 µl of prewarmed Ringer solution and 100 µl of pluronic acid (10% aqueous solution; Molecular Probes) were added.

Intravascular labels. FITC, 8% solution (0.5 ml/kg body wt) and sulforhodamine (RB200) albumin, 8% solution (0.5 ml/kg body wt), were prepared as described in Ref. 37.

Dye for testing pathological cellular permeability. Trypan blue (50 mg/ml) was used in doses of 250 mg/kg iv.

General Course of Experiments

To attain a steady-state distribution of the membrane-sensitive dyes, the substances were injected in the early phase of the general preparation (shortly after insertion of peripheral catheters). After the general preparations were finished, an equilibration period of at least 20 min was maintained. During this period the blood gases were controlled (AVL 990, automatic blood bas system) and, if necessary, the respiration volume was readjusted. Reducing the coronary perfusion pressure was then begun. To take into account the compressive forces of the left ventricular wall, which impede coronary flow especially during diastole, the left atrial pressure was added to the 30-mmHg perfusion pressure value; this level was then adjusted manually by using the hydraulic occluder. Coronary hypoperfusion was maintained for 45 min. FITC and RB200 were intravenously injected 10 min and, in part of the experiments, 1 min before the end of this period. A cylindrical, transmural biopsy of the affected left ventricular myocardium was then obtained with a special biopsy drill (37). With the use of a vacuum, this instrument made it possible to pull a transmural, inferoposterior sample of 2.2 mm in diameter into an attached vessel filled with isopentane precooled to −150°C. The entire heart was then excised, and a slice of ~4-mm thickness was prepared from the largest diameter of the organ and also frozen in precooled isopentane.

Special Procedures Applied in Different Experimental Groups

Group 1: Study of temporal stability of the capillary filling pattern (n = 6). In the first group the question studied was whether the capillary perfusion pattern changes in long-term rhythms. The intravascular indicator RB200 was allowed to circulate for 1 min, and FITC circulated for 10 min. If the localization of ischemic areas changes during such periods of time, differences in the distribution of the dyes should become evident with this approach.

In this experimental group only the transmural biopsies were evaluated. Frozen sections of 5-µm thickness were cut, freeze-substituted in melting alcohol, and embedded in an artificial medium (Histokit). For evaluation the sections were systematically scanned in a fluorescent microscope using a filter set of 460/510/528 nm for observation of FITC and 546/580/590 nm for the study of RB200 fluorescence. The microscopic stage was equipped with a motor drive (Mährhäuser, Wetzlar, Germany) controlled by a computer program, which allowed a systematical, step-by-step (distance 250 µm) scanning of the tissue. Provided the capillaries were found rectangularly sectioned, the distance from the center point of the microscopic field to the next dye-labeled capillary (FITC and RB200, respectively) was determined according to Loats et al. (21). The process was facilitated by projecting a
system of concentric rings of equal distance to each other into the viewing field through a camera lucida. Frequency histograms of groups of equal distance were calculated for both labels.

Group II: Study of capillary filling pattern and cellular NADH and TMRM fluorescence (n = 6). In the second experimental group, FITC was used as the indicator for perfusion, NADH as an endogeneous marker for cellular redox state and viability, and TMRM as an indicator for resting membrane potential. FITC was allowed to circulate for 10 min during the terminal period of coronary hypotension. TMRM was injected about 1 h before induction of coronary occlusion. These histological sections were handled somewhat differently from those described in Group I. The unprocessed frozen sections were placed on object holders that were still in a cold (≈25°C) environment of cryostat microtome for a period of at least 1 h. Within that time the tissue's water had sublimated; the sections were covered with a cover glass and placed between two metallic plates attached to the branches of a wooden pair of tongs. The latter instrument was not precooled; therefore, it rapidly raised the section's temperature without allowing water to condense in the tissue during transition to room temperature. This procedure made it possible to study not only the natural distribution of FITC but also that of NADH (as described in Ref. 37) and of TMRM as well. NADH was observed by using a 365/395/420-nm filter combination; for TMRM the 546/580/590-nm filter combination was applied.

In the first step of evaluation the complete transection of the heart was evaluated to obtain an overview of the regional changes in dye distributions. The tissue was again scanned step by step with the aid of a computer-controlled motor drive at a distance of 250 µm, producing about 500 measuring points. In each microscopic field it was noted 1) whether a central, circular field of 100-µm diameter contained the intravascular label, 2) whether the myocyte located within the center point of the viewing field (or in the case of extracellular localization of the midpoint, the cell closest to it) had lost NADH fluorescence, and/or 3) whether it had lost TMRM fluorescence. Such a semiquantitative evaluation was facilitated by the fact that decreases in cellular NADH fluorescence were accompanied not only by a reduced intensity but also by a change in the color of emitted light (blue vs. green); evaluation of reductions in TMRM fluorescence was facilitated by the fact that areas of reduced fluorescence were surrounded by a margin of cells showing an especially bright fluorescence.

In the next step, a more detailed evaluation of the dyes' distributions was performed in the biopsies. In 2–3 sections of each sample, 8–10 fields were selected that contained vascularly labeled and nonlabeled areas to about the same extent. These areas, exposed to the three different modes of fluorescence excitation, were recorded with a 12-bit charge-coupled camera (Photometrics, Tucson, AZ); the TIFF-formatted fluorescent images were stored in an Apple computer.

By use of an image-analyzing system (SigmaScan/Image, Jandel Scientific), the parallel images were analyzed. Great care was taken to correct the viewing fields for slight shifts that had unavoidably occurred due to changing the optical filter system. With the use of the FITC label, areas in the first step were defined as being vascularly labeled and nonlabeled. As a rule the minimal distance of the respective zone to the border of vascular labeling was longer than 60 µm. Within the two types of fields a measuring diaphragm of 6.0 µm in diameter was placed on each myocyte, and the level of light intensity was determined for NADH and TMRM.

For evaluation of the changes that occurred especially in the transitional area between vascularly labeled and nonlabeled tissue, scanning lines were used. They were placed perpendicular to the border of the latter fields. In each transected myocyte the light intensities of TMRM and NADH were determined by using a procedure analogous to that described above. The distance of the respective cell to the next dye-labeled capillary as well as the absolute number of labeled capillaries within a circle of 120 µm in diameter were also determined.

To make a comparison among sections from different experiments possible, the following mode of calibration was applied: Use was made of the fact that arterioles and small arteries in the vascularly labeled zones were surrounded by a halo of fully oxygenated cells even though the tissue as a whole had been hypoperfused (37). For each section the mean fluorescence intensities of NADH and TMRM, respectively, were determined in the cells and taken as relative unit 1.0.

Group III: Study of the distribution of TMRM and bis-oxonol (n = 6). To validate the observation that spatial differences in TMRM distribution were induced by effects on the resting membrane potential, the intensity of its fluorescence was compared with that of the anionic dye bis-oxonol. To test the viability of those myocytes that showed a reduction in NADH despite hypoxic conditions, trypan blue was injected intravenously 60 min and intraperitoneally 120 min, respectively, before the induction of hypoperfusion. Their distributions were studied as described above. The fluorescence of bis-oxonol was observed by using the same optical filters as in the case of FITC. To evaluate the fluorescence intensities of TMRM and bis-oxonol, the analysis in vascularly labeled and nonlabeled fields, the method of line scans, and the determination of the reference values were applied just as they were for Group II, using sections of biopsies.

Group IV: Special controls. The experiments on this group were performed to provide answers to questions pertaining to individual details (each subgroup n = 4). CONTROL A: TESTING OF MYOCYTE VIABILITY BY TRYPAN BLUE (HYPOPERFUSION 45 MIN). To test the viability of those myocytes that showed a reduction in NADH despite hypoxic conditions, trypan blue was injected 1 min before obtaining a myocardial biopsy and freezing the remaining myocardium. Sections were prepared from the samples as described above. By use of the CCD camera 8–10 fields were stored. The myocytes in the images were systematically scanned and analyzed for fluorescence intensities of NADH and trypan blue; these were then correlated with each other. The latter dye was visible due to its red fluorescence in vivo, which was observed using the 546/580/590-nm system of filters. CONTROL B: STUDY OF TMRM AND NADH IN 10-MIN HYPOPERFUSION EXPERIMENTS. The same procedure was applied as described for Group II except that hypoperfusion was maintained for only 10 min. Images were recorded by the camera; the areas were randomly selected within vascularly labeled and nonlabeled zones. These were scanned cell by cell for the intensity of TMRM and NADH fluorescence. CONTROL C: STUDY OF TMRM, BIS-OXONOL, AND NADH IN EXPERIMENTS WITHOUT HYPOPERFUSION. A nonoccluded control group was studied to determine the basic fluorescence attainable through the present technique of tissue sampling. The animals were thoracotomized, and a catheter was inserted into the left atrium but no anastomosis was inserted. TMRM and bis-oxonol were applied in the same doses as in Group III. After a 15-min period of stabilization, a myocardial biopsy was withdrawn and studied for the fluorescence of TMRM, bis-oxonol, and NADH.

For comparison of the data the Mann and Whitney U-test was used. Values are given as means ± SD.
RESULTS

Group I: Study of Temporal Stability of Capillary Filling Pattern

Reduction of coronary perfusion pressure to a value exceeding the left atrial pressure by 30 mmHg (in the mean a pressure of 38 mmHg) induced a significant decrease in systemic arterial blood pressure, an increase in left atrial pressure, and a decrease in coronary blood flow (Table 1). In the course of coronary hypotension, only moderate changes in these general hemodynamic values were observed.

The histological sections of the myocardial biopsies taken from the hypoperfused left ventricle at the end of the low flow period showed an inhomogeneous pattern of capillary plasma filling. Large areas lacking capillary labeling occurred adjacent to zones of homogeneous capillary staining. When the closest-neighbor method was applied, regular short as well as long distances (\(>50\) µm) were found somewhat more often in the latter lengths were found; the latter lengths were dominant in the extended areas that lacked capillary staining (Fig. 1).

To estimate the temporal stability of this pattern, the distributions of the two indicator dyes were compared. When RB200 (the 60-s label) was considered, a labeled capillary was found within a distance of 10 µm in 38.3 ± 7.3% of measuring points in the tissue. This fraction of shortest distances slightly increased (33.8 ± 7.1%) when the FITC-labeled capillaries were studied (circulation of dye for 10 min). Correspondingly, longer distances (\(>50\) µm) were found somewhat more often in the evaluation of RB200 compared with that of FITC.

Both differences, however, did not attain statistical significance.

Group II: Study of Capillary Filling Pattern and Cellular NADH and TMRM Fluorescence

In the second experimental group the general hemodynamic conditions were similar to Group I (Table 1).

The general pattern of tissue fluorescence was first studied in the histological sections obtained from the entire hearts and from the biopsies on a qualitative basis (Fig. 2). Vascular labeling displayed an irregular pattern of perfused capillaries similar to that observed in the preceding group during reduction in coronary perfusion pressure.

NADH fluorescence in zones lacking capillary labeling was expected to have become elevated throughout that area. This conclusion did, however, prove to be nonvalid. Within the nonvascularly labeled zones, circumscribed areas appeared showing quite a low blue autofluorescence. These areas usually differed from the circumscribed oxygenated zones found in the perfused tissue. When occurring within perfused regions, areas of low NADH fluorescence were separated from the surrounding highly fluorescent cells by a soft margin, which covered about one or two cellular layers and which induced a gradual transition of the light intensity to the neighboring tissue; in contrast, those of the ischemic zones were sharply bordered, their margins ran exactly along cellular boundaries, and no intermediate states of NADH fluorescence were discernable across the border to the adjacent cells (Fig. 2). The speciality of the latter state became even more evident.

Table 1. General hemodynamic data of three experimental groups of anesthetized rats subjected to coronary hypoperfusion

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control period</td>
<td>143 ± 11/93 ± 12</td>
<td>141 ± 15/94 ± 11</td>
<td>140 ± 18/89 ± 9</td>
</tr>
<tr>
<td>Hypoperfusion</td>
<td>121 ± 13/81 ± 11</td>
<td>114 ± 11/76 ± 14</td>
<td>118 ± 18/81 ± 12</td>
</tr>
<tr>
<td>5 min</td>
<td>124 ± 6/82 ± 11</td>
<td>113 ± 9/75 ± 14</td>
<td>118 ± 20/78 ± 16</td>
</tr>
<tr>
<td>45 min</td>
<td>361 ± 29</td>
<td>373 ± 23</td>
<td>373 ± 15</td>
</tr>
<tr>
<td>Heart rate, l/min</td>
<td>373 ± 20</td>
<td>377 ± 26</td>
<td>377 ± 17</td>
</tr>
<tr>
<td>Control period</td>
<td>359 ± 19</td>
<td>364 ± 22</td>
<td>364 ± 16</td>
</tr>
<tr>
<td>Hypoperfusion</td>
<td>4.5 ± 1.9</td>
<td>5.7 ± 3.0</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>5 min</td>
<td>9.6 ± 4.2</td>
<td>11.7 ± 3.8</td>
<td>7.9 ± 2.2</td>
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<tr>
<td>45 min</td>
<td>8.7 ± 2.8</td>
<td>9.3 ± 1.4</td>
<td>7.1 ± 1.7</td>
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<tr>
<td>Left atrial pressure, mmHg</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control period</td>
<td>1.1 ± 0.4</td>
<td>0.81 ± 0.18</td>
<td>1.2 ± 0.34</td>
</tr>
<tr>
<td>Hypoperfusion</td>
<td>0.48 ± 0.18</td>
<td>0.23 ± 0.13</td>
<td>0.47 ± 0.17</td>
</tr>
<tr>
<td>5 min</td>
<td>0.40 ± 0.08</td>
<td>0.28 ± 0.12</td>
<td>0.46 ± 0.10</td>
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<tr>
<td>45 min</td>
<td>100 ± 12; 36 ± 2</td>
<td>107 ± 15; 33 ± 4</td>
<td>106 ± 9; 38 ± 2</td>
</tr>
<tr>
<td>Blood gases: PO2, mmHg; PCO2, mmHg; pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control period</td>
<td>7.37 ± 0.04</td>
<td>7.41 ± 0.05</td>
<td>7.36 ± 0.04</td>
</tr>
<tr>
<td>Hypoperfusion (40 min)</td>
<td>97 ± 10; 35 ± 2</td>
<td>110 ± 9; 28 ± 5</td>
<td>102 ± 6; 35 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 rats for each group. Arterial blood pressure values reflect systolic/diastolic pressures.
when the sections of the entire heart were studied, the tissue of which had passed a certain period of hypoxia due to delayed freezing after excision. In these samples low intensities were not found in the zones reached by the vascular label; the localized reductions in NADH fluorescence in the nonvascularly labeled areas appeared, however, to be entirely unaltered.

With respect to changes in the intensity of TMRM fluorescence, a generally high level was found in the vascularly labeled zones irrespective of a low or high level of cellular NADH fluorescence. In the zones lacking vascular staining, however, its fluorescence was found to be significantly reduced. Enclosed by a small margin of highly increased TMRM fluorescence, large areas appeared showing a distinct reduction in the intensity of this dye. The extension of such fields was closely correlated with the zones lacking capillary labeling (Fig. 2).

The semiquantitative evaluation of the sections of the entire organ produced the following results. In 24.6 ± 6.8% of the transectional area of the heart, vascular labeling was lacking. In 82.5 ± 3.5% of these vascularly nonstained zones, the TMRM fluorescence was reduced; in contrast, a corresponding reduction was observed in only 14.4 ± 3.1% of the vascularly labeled zones. Reductions in NADH fluorescence, as described above, were observed only in areas lacking FITC-staining where this phenomenon was observed in 37.7 ± 9.1% of the tissue.

More differentiated evaluations were performed in the sections of the myocardial biopsies.

In the first step, the intensities of NADH and TMRM fluorescence were compared with each other on a cell-to-cell level and then separately for vascularly labeled and nonlabeled areas. In both zones the intensity of NADH fluorescence scattered widely. Intensities reached 6 relative units (rel.U) in the vascularly labeled and up to 8 rel.U in the vascularly nonlabeled zones. Comparison of these data with those of TMRM made clear that these changes in NADH fluorescence had to be interpreted differently depending on the state of perfusion. In the vascularly labeled zones, the differences in NADH were paralleled by only slight variations in TMRM fluorescence, which were scattered around 1 rel.U (Fig. 3). This observation indicates that resting membrane potential had not changed significantly in these zones. The only exception concerned the lowest fraction of NADH (0–0.5 rel.U) in which case a few cells were found also showing a rather low level of TMRM. This effect probably resulted from the fact that some TMRM/NADH-depleted cells, characteristic of the vascular nonlabeled zones (see below), had extended in some areas into the vascularly labeled regions.

In the nonvascularly labeled areas in which large differences in NADH fluorescence intensity were found, a different state of myocytes must be concluded. Especially in the myocytes showing no increase in NADH at all, the intensity of TMRM had become significantly lower than that detected in the vascularly labeled zones. A direct relationship was found: the lower the intensity of NADH fluorescence, the lower the level of TMRM fluorescence (Fig. 3).

Next, the tissue was analyzed by line scans, focusing on areas of transition from vascularly labeled to nonlabeled myocardium (Fig. 4). On the border to the nonvascularly labeled areas, the fluorescence of NADH increased slightly; this rise was statistically significant despite the increased scattering of the data. Greater changes became evident with respect to the TMRM distribution. As long as the mean distance from the measuring point on the line to a labeled capillary was < 10 μm and the number of surrounding labeled capillaries above 1,750/mm², the cellular TMRM fluorescence still scattered around the reference level in oxygenated cells. However, when the distance to a dye-containing capillary became > 10 μm and the density of FITC marked capillaries fell below 1,750/mm², a sharp drop in the TMRM intensity followed after passing through a small border of cells of greatly increased TMRM fluorescence. When this highly characteristic borderline of increased TMRM fluorescence
was used as a marking point, this border proved to separate cells of significantly differing intensity of TMRM fluorescence ($P < 0.001$, U-test, Fig. 4).

Group III: Study on Distribution of TMRM and Bis-Oxonol

To improve the interpretation of the present data, a third experimental group was established in which the distribution of the anionic potential-sensitive dye bis-oxonol was compared with that of TMRM. On theoretical grounds this dye should have distributed complementarily to the cationic TMRM. Bis-oxonol proved to be tolerable under in vivo conditions in the same way as TMRM. No changes in arterial blood pressure or heart rate were observed following the intraperitoneal injection of the dye. The general hemodynamic data of this experimental group are included in Table 1.

Comparison of both fluorochromes on a cellular level revealed the following results (Fig. 5). In the vascularly labeled regions, the majority of values of both dyes scattered between 0.5 and 2.5 rel.U and showed a slightly positive correlation between the two parameters.
Quite a different interrelation of the dyes' fluorescence intensities was found in the nonvascularly labeled areas. Similar to the vascularly labeled regions, in a certain fraction of the myocytes a TMRM fluorescence $>1\text{ rel. U}$ was detected (34.3% of myocytes studied); these cells also showed a generally high level of NADH fluorescence ($4.37 \pm 1.62\text{ rel. U}$). In this group of myocytes, the fluorescence of bis-oxonol lay only slightly...
above the level measured in the vascularly labeled zones (2.56 ± 1.2 vs. 1.68 ± 0.51 rel.U). As expected, a further decrease in TMRM (<1 rel.U) paralleled a reduction in NADH fluorescence. When the TMRM fluorescence intensity amounted to 1.0–0.5 and <0.5 rel.U, the corresponding values of NADH were 2.58 ± 1.20 rel.U and 1.64 ± 0.84 rel.U. Myocytes showing a fluorescence of NADH <1 rel.U occurred almost exclusively in the latter class (Fig. 5).

Whenever myocytes attained such a low level of <1 rel.U TMRM fluorescence, a broad scattering of bis-oxonol intensities became evident. In part of the cells (40.3% of all cells studied), fluorescence lay below 3 rel.U; a significant fraction, however, had values of up to 10 rel.U (25.4% of all cells studied). This was a condition that could not be found in any vascularly labeled areas.

Evaluation of the line scans, placed in the sections of the myocardial biopsies as described above, revealed that the bis-oxonol fluorescence had changed its intensities in exactly the opposite mode as observed for TMRM (Fig. 6). As soon as the fluorescence of TMRM began to decrease, the intensity of bis-oxonol rose and significantly surpassed the mean level found in the vascularly labeled zone. The mean intensity of oxonol was significantly higher in the latter zones than in the former (P < 0.001, U-test).

Group IV: Special Controls

Control A: Testing of myocyte viability by trypan blue. When trypan blue was injected before occlusion, the dye was able to distribute within the extracellular space of the entire myocardium and remained confined to this compartment during regular perfusion. Under low flow conditions, however, it was observed that a certain fraction of myocytes had taken up this indicator. The distribution of such cells correlated with changes in NADH fluorescence (Fig. 7). Those myocytes that, despite an hypoxic condition, revealed an NADH fluorescence equal or even lower than that found in normoxic cells, displayed an accumulation of this dye. In myocytes showing NADH fluorescence >1 rel.U, the fluorescence of trypan blue produced a value of 0.63 ± 0.03 rel.U. When NADH fluorescence was lower than 1 rel.U, the intensity of trypan blue in most cells was found to be highly increased (3.6 ± 0.5 rel.U). The data gathered in these experiments are presented in Fig. 8.

Control B: Hypoperfusion for 10 min. When the rats had been exposed to hypoperfusion for 10 min only, nonsignificantly lower values of TMRM fluorescence were observed in the vascularly nonlabeled areas compared with the vascularly labeled zones (1.0 ± 0.52 vs. 1.35 ± 0.72 rel.U). These data did not differ significantly from the corresponding values of the 45-min...
experiments (0.81 ± 0.52 vs. 1.12 ± 0.41 rel.U). In contrast to the 45-min hypoperfusion experiments, however, no data were collected showing both, NADH <1 rel.U and TMRM <0.5 rel.U. This observation supports the view that such a low level of both fluorochromes had indeed developed during periods of zero capillary flow over periods >10 min.

Control C: Control conditions without hypoperfusion. In the nonoccluded control group a generally low fluorescence of NADH was found in the tissue within the same range as those cells localized at the natural boundaries (subepicardium or subendocardium of which cells remain constantly oxygenated from the environmental air and left ventricular blood, respectively, independently of changes in capillary flow): NADH: 0.77 ± 0.17 rel.U. Correspondingly, the fluorescence of TMRM and bis-oxonol also scattered only moderately: TMRM, 1.02 ± 0.27 rel.U; bis-oxonol, 1.07 ± 0.39 rel.U.

DISCUSSION

Discussion of Methods

Inhomogeneities in capillary filling patterns studied in the present investigation have been characterized by the closest-neighbor method of Loats et al. (21). This procedure is based on the assumption that each point in the tissue is supplied by the surrounding perfused capillaries and that the vessel located at the shortest distance is the most relevant one. The influence of the more distant vessels decreases exponentially. Although somewhat simplifying the actual condition, this method makes it possible to characterize heterogeneous patterns of capillary supply by showing the frequency distribution of minimal distances of tissue points to any perfused vessel.

A quantification of the oxygen-carrying capacity of the labeled vessel cannot be given because dye enclosure only signals that there was contact with the marker during the rather long period of dye exposure. As demonstrated in the preceding study (37), the oxygen delivery especially along the venous section of capillaries under low flow conditions may be insufficient.

A certain limitation of the present histological methods results from the fact that such an analysis can only be two-dimensional. Even when capillaries are sectioned rectangularly, oxygen-carrying arterioles may still be present beneath the sectional plane and can thus influence the results. This must therefore be considered when using such methods.

In parallel with the intravascular dyes intracellular, potential-sensitive dyes have been applied in the present study. A basic precondition of this approach is the lack of toxicity of these indicators in vivo. Several studies have proven that TMRM (8, 11) and bis-oxonol (16) do not damage isolated cells and tissues, respectively; nevertheless, it was not possible to simply conclude an in vivo tolerability from such in vitro data. In pilot experiments an indicator dye used in isolated living cells (mitochondrion stain, MitoTracker, Molecular Probes) caused the instantaneous death of the experimental animal.

A second precondition that had to be subsequently fulfilled was the maintenance of the dyes' natural

Fig. 6. Measurement of cellular TMRM and bis-oxonol fluorescence intensities determined along scanning lines as described in Fig. 4. Data obtained at positions below and above 0 µm on position line, respectively, were compared by use of U-test: P < 0.001: a vs. b, c vs. d, and b vs. d.
Localization in the tissue while the specimen was being processed. The indicator substances, including NADH, occur unbounded in the tissue. Aqueous media lead to the extraction of NADH and the intravascular indicator proteins and, to a minor extent, to the extraction of TMRM and oxonol as well; apolar, organic solvents cause an even greater extraction of the latter dyes. To circumvent this problem, exposure of the samples to fluid was completely avoided. Freeze-drying the frozen sections caused the water to evaporate during its frozen state and prevented any free diffusion of solutes in the tissue as described in a preceding paper (37). No hints of diffusion artifacts were found with this technique even in those samples obtained from the entire heart, which took longer to freeze than the biopsies. The local differences in the fluorescences of NADH, TMRM, and

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**Fig. 7. Photomicrograph of myocardium from a heart subjected to coronary hypoperfusion for 45 min (hypoxic conditions before freezing).**

A: blue autofluorescence of cellular NADH, in reproduction appearing as light areas. (Note circumscribed regions of myoctes showing reduction of this signal like one indicated by arrow.) B: same field as shown in A, revealing red fluorescence of previously intravenously injected trypan blue. (Note that trypan blue fluorescence is confined to extracellular space in most areas where cells show high NADH fluorescence; however, it is discernable in intracellular space of those cells having developed a decrease in NADH fluorescence.)
bis-oxonol correlated with cellular structures (Fig. 2); the dyes never appeared as light halos, the typical sign for diffusion artifacts.

The latter aspect addressed the problem of displacements of the dyes' natural distribution that might have occurred in the tissue due to diffusion during processing. There is quite a different aspect with regard to dye diffusion. Attaining the steady-state distribution in vivo according to the electrical potentials of the different compartments necessitates the free accessibility of the potential-sensitive dye from the extracellular fluid. This precondition is clearly fulfilled in experiments on isolated cells in which the capacity of the extracellular space, the culture medium, is practically unlimited. This does not, however, apply to intact tissues with their limited extracellular space, especially when drainage of the latter compartment is disturbed as a consequence of capillary flow stagnation. From this point of view it was surprising to note that ischemic areas of even a few hundred micrometers in diameter were found to have lost the indicator dye TMRM. The fact that no gradient of concentration could be detected toward the center of these areas and the observation of an inverse behavior of the anionic dye bis-oxonol, which was found to concentrate in the ischemic areas, support the view that diffusion could not have been a limiting factor of dye distribution within flow-restricted myocardium.

With respect to the exchange of this dye in accord with the polarity of the different intracellular compartments, TMRM, which behaves like a Nernstian dye, is considered to be one of the best indicators (22). This substance, synthetized by Loew (22), has been found not only to be freely membrane permeable but also to possess a low capacity of membrane binding and to lack the tendency to aggregate. For these reasons the fluorescence of TMRM has been considered to be a reliable measure of its local concentration, largely governed by the gradients of electrical potentials across the membranes of mitochondria and sarcolemma (8, 11, 22).

Because of the much greater negativity of the mitochondrial matrix space, TMRM preferentially concentrates in these cellular organelles and, when the cell is regularly polarized, it is taken in within a few minutes (8, 11). Vice versa, on depolarization of the mitochondrial and plasma membranes, the cationic dye leaves the cells. Bis-oxonol is also highly cell permeant; however, its intensity of fluorescence is greatly affected by protein binding (5, 9, 16). On depolarization there is a voltage-dependent increase in cytosolic fluorescence of the dye; still, a specific accumulation of the dye in mitochondria of even severely depolarized cells has not been reported.

With the present approach, membrane potentials could not be given in absolute terms because it is impossible to determine the concentration of the dyes in the extracellular space. The extracellular extravascular space is too small in the heart, and an estimation in the extracellular intravascular space is impeded by the "fluffy" structure of the blood plasma in the histological sections induced by drying. For this reason the evaluations were restricted to comparisons among different areas of tissue.

Fig. 8. Correlation of fluorescence intensities of cellular NADH and trypan blue in vascularly nonlabeled zones of myocardium hypoperfused for 45 min. Each graph represents results of one experiment; each point refers to an individual myocyte studied in histological section. Myocardium passed through a phase of hypoxia before freezing.
In addition to the possibility of producing diffusion artifacts as discussed above, the detection of NADH in the tissue is critical because changes in NAD/NADH ratios might have occurred during tissue sampling. The time lag between cutting and freezing the sample was estimated to be 0.7 s. This value is based on a video recording of the drill used during sampling (time lag between the onset of cutting and the immersion of the sample in the freezing medium: 0.4 s) and the data of Allard et al. (1). These authors determined a freezing time of 0.3 s for a biopsy of the same size as in the present investigation (2 mm diameter). The homogeneously low NADH fluorescence in the nonoccluded controls also demonstrates that the rapidity of tissue sampling using the biopsy drill is indeed sufficient. No difference in NADH fluorescence was detected when comparing the fluorescence in the natural outer or inner layer of the myocardial wall with that of the tissue laying in between. As an additional parameter of cellular viability the distribution of trypan blue has been studied. Trypan blue, generally used as a life-dead stain in isolated cells (32), was found to be tolerable in the anesthetized animal and was detectable through red fluorescence of the protein-dye complex in the myocardial sections. The fluorescent complex is first formed under in vivo conditions in an evidently reversible process. This conclusion was drawn from the observation that FITC-albumin, a covalently bound conjugate, which, even when located in direct contact with a cell having accumulated trypan blue, did not diffuse into such myocytes. In other words, only trypan blue, not albumin, was able to penetrate the cell’s plasma membrane.

Discussion of Results

It has been discussed that the cessation of capillary flow might change with time and space under low flow conditions, thus avoiding irreversible damage of the myocytes under such conditions (31). Previous findings (37) and present results do not support this idea. Losses of NADH fluorescence in the hypoxic myocardium were not observed when hypoperfusion was run for 10 min but were in the 45-min experiments. Apparently, in nonvascularly labeled zones of hearts subjected to the longer durations of low flow conditions, regional failure of capillary flow does indeed develop for periods >10 min.

In addition, the differences in the density of capillaries accessible to a plasma label during periods varying between 1 and 10 min were modest and did not attain statistical significance. The labeling method is based on the observation that capillaries once perfused with dye-containing blood maintain the indicator even when flow stops later during the period of observation (36). If capillaries had been perfused in the initial phase of labeling only and if they were to lose the labeled plasma through squeezing, a certain fraction of vessels in the histological sections would be expected to be stained by the short-time label but to be unstained by the long-time marker. Such a constellation has never been observed. In preliminary experiments increases in tissue pressure were found to squeeze blood out of veins and larger arteries but to be unable to remove the plasma from microvessels in a muscle. Because of an apparently stable distribution of capillary perfusion during low flow conditions, the changes observed in the functional state of the myocytes are discussed separately for the various conditions of blood supply.

Areas of sustained capillary flow. As previously shown (37) and as confirmed in the present study, cellular fluorescence of NADH still increases during maintained capillary flow under low flow conditions. Because a rise of this signal indicates a state of severe hypoxia in myocytes (7), the conclusion may be drawn that capillary flow was slowed to such a degree that supply of surrounding cells became insufficient.

When myocardial tissue or isolated myocytes are exposed to hypoxia/anoxia during sustained perfusion, and the exchange of metabolic waste products and ions is thus maintained, the cardiac action potential typically shortens, whereas the resting transmembrane potential is only slightly, if at all, reduced (12, 27). This finding is supported by the present observation of no significant correlation of TMRM versus NADH fluorescence in the perfused areas. This means that the vast majority of myocytes that have become hypoxic during maintained capillary flow have not yet developed a significant reduction in resting membrane potential. A small fraction of cells that did not follow this general trend (TMRM < 0.5 rel.U, NADH < 1.0 rel.U) are assumed to have been located in a region of nearly stagnant flow, sufficient for vascular labeling but insufficient for maintaining vital functions, and thus belonging functionally to the zones described in the following sections.

A slight positive correlation of TMRM versus bis-oxonol was, however, observed. The intracellular concentration of oxonol is known to increase on depolarization of the plasma membrane (5, 9). The fluorescence intensity of TMRM was also found to increase during the initial phase of mitochondrial depolarization. Under regular conditions of cellular polarization, TMRM is concentrated in the mitochondria to such a degree that self-quenching gains importance. This aspect becomes evident when agents are applied that specifically depolarize cellular membranes (valinomycin and potassium); in this case a transient increase in overall cellular fluorescence of TMRM occurs (11). In parallel, in the late phase of chemical hypoxia a similar rise in the cellular fluorescence has been described by Chacon et al. (Fig. 7 in Ref. 6) in isolated myocytes. Thus the present data may be explained by assuming slight variations in cellular polarity in the vascularity labeled tissue.

Border zones between vascularity labeled and nonlabeled areas. In the zone halfway between the vascularity labeled and nonlabeled tissue, a small layer of myocytes was consistently found, which displayed a conspicuously high fluorescence of TMRM but not yet an overall rise in bis-oxonol fluorescence. The most likely explanation of this phenomenon is an intensified unload-
ing of TMRM from mitochondria due to hypoxic conditions in a state when the transmembrane potential has not yet declined to a corresponding level, thus resulting in the greatly increased optical discernability of TMRM but not in a significant increase in the bis-oxonol fluorescence as discussed in the preceding paragraph.

Ischemic nonvascularly labeled areas. In the nonvascula-

rly labeled areas an overall increase in NADH fluorescence and a significant reduction in the intensity of TMRM was observed. From the above considerations, the conclusion may be drawn that the latter effect was due to mitochondrial depolarization as well as a loss of plasma membrane polarity, resulting in reduced intracellular negativity and thus the extrusion of TMRM from the cytosolic space and a significant entrance of bis-oxonol into the latter compartment, respectively.

In the nonvascularly labeled zones an additional, noteworthy observation was that despite lacking perfusion in part of these areas, NADH fluorescence was not increased but rather reduced to basal levels. This observation appears to be of great significance with respect to an interpretation of the irregular pattern of NADH fluorescence found in ischemic myocardium (e.g., 2, 17, 24). The special microscopic appearance of these fields (see Fig. 2), the exceptionally low fluorescence of TMRM in these cells alone, and the fact that even prolonged hypoxia did not cause a rise in the blue autofluorescence, support the view that the disturbances in cellular function were fundamentally different from those described above.

In principle, a low level of cellular NADH may result from an accelerated consumption of electrons via the respiratory chain or from a decreased production of electron-supplying substrates like NADH via citric acid cycle and/or β-oxidation (25). An increased electron flux in the respiratory chain is rather unlikely because oxygen, the final electron acceptor, is lacking. Although glycolysis is slowed in ischemic cells due to the accumulation of lactate and protons (28), an inhibition of glycolysis by 2-deoxyglucose in cultured myocytes did not alter the level of cellular NADH fluorescence (10).

In the latter study (10), however, further conditions were also studied, which appear to much more readily simulate the present findings and which offer an explanation of the present results. Isolated myocytes were exposed to metabolic inhibition not only by 2-deoxyglucose but also by cyanide; these caused an instantaneous increase in cellular NADH fluorescence, an effect still enhanced by elevating potassium and lactate in the superfusion fluid. When such a simulation of ischemia was extended beyond a period of 10 min, NADH decreased in one cell after the other and was soon followed by the development of a hypercontracture. The authors attribute the latter effect to an intracellular overload of free calcium. This conclusion concurs with the present observations in that the loss of NADH fluorescence in the nonvascularly labeled areas was only found in those cells that revealed greatest reductions in TMRM fluorescence. A close temporal association of a decrease in TMRM fluorescence, calcium overload, and contracture has been described by Chacon et al. (6). In that investigation calcium accumulation and contracture were even found to precede the loss in mitochondrial TMRM fluorescence. A correlation of mitochondrial membrane depolarization, calcium overload, hypercontracture, and severe cellular injury have been observed in many other studies (for a review see Ref. 31) and lead to the conclusion that those cells that were evidently unable to develop an increase in NADH fluorescence during hypoxic conditions had lost their vitality.

By applying trypan blue, it was found that those myocytes showing low NADH fluorescence despite hypoxic conditions had accumulated this dye. This provides an additional argument in favor of the view that these myocytes had developed pathological sarcolemmal permeability, a sign that these cells had indeed lost viability.

With respect to the mechanism that may be responsible for the link between a reduction in cellular NADH content, membrane depolarization, and calcium overload, the following considerations may be of importance. In the ischemic cell the transmembrane conductance of potassium ions is, on the one hand, greatly increased (12). On the other hand, the lack of exchange of interstitial fluid due to ischemia causes potassium to accumulate in the extracellular space and eliminates the driving force of the resting diffusional potential, thereby causing a reduction in the transmembrane potential. In addition, energy depletion induces intracellular acidosis and, via H+/Na+ and Na+/Ca2+ exchange, leads to an enhanced entrance of sodium and calcium ions into the cell (e.g., Ref. 30). Calcium overload has been found to favor the opening of large, nonspecific hydrophilic channels in the inner mitochondrial membrane, which is permeable to solutes with a molecular mass of up to 1,200 Da, called the mitochondrial permeability transition pore (see Ref. 13). A disturbance of the barrier function of the inner mitochondrial membrane impedes the process of charge separation; this causes mitochondrial depolarization, evident from TMRM redistribution, as observed by Chacon et al. (6) and in the present study. In addition, formation of the mitochondrial permeability transition pore is expected to cause the release of mitochondrial NADH into the cytosol, where metabolic degradation of the oxidized species via glycohydrolase may occur (23). This enzyme, known to be present in heart muscle (14), has been reported to be involved in the depletion of pyridine nucleotides, which in turn characterize the transition to myocardial necrosis (19). The latter process appears to be the most plausible explanation for the loss of NADH fluorescence detected in circumscribed groups of myocytes in the ischemic myocardial tissue.

It is only possible to speculate why just a certain fraction of cells in the affected area displayed the changes described above. Regional differences in the sensitivity to ischemic stress may result from spacial inhomogeneities in glycogen content as have been detected in myocardial tissues (35) and/or from a
differing energy demand due a variable amounts of catecholamines released locally (12).

The present results point to the existence of not only a mixture of oxygenated and hypoxic, perfused and nonperfused myocytes (37) but also polarized and depolarized myocytes, including vital and avital myocytes that occur when a mixed pattern of low and high levels of NADH fluorescence arises during hypoperfusion, as described by Steenbergen et al. (33) in isolated hearts. On the basis of the arrhythmogenic potential of dispersions in conduction times and repolarization periods (12) that are closely linked to the changes described above, our results suggest that the effects presented are highly relevant in the generation of cardiac arrhythmias.

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