JOURNAL OF MAGNETIC RESONANCE **124,** 42–50 (1997)
ARTICLE NO. MN961026

Continuous Monitoring of Intracellular Volumes in Isolated Rat Hearts during Normothermic Perfusion and Ischemia

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Received June 10, 1996; revised October 3, 1996

The present study describes an experimental setup that enables continuous measurement of cellular volumes in isolated organs. The procedure is a modification of a recently reported method that uses multinuclear NMR measured by ^{59}Co NMR of cobalticyanide and ^{1}H NMR of water in isolated rat hearts at normothermia. The new apparatus contains a background flow which is shown to improve the rate of exchange of the marker between the interstitium and the external solution and allows detection of cellular shrinkage during no-flow ischemia. A series of experiments of marker loading and wash-out were performed to validate the method. In the Langendorff preparation, intracellular volumes (in units of milliliters per gram dry weight) of hearts perfused with Krebs–Henseleit solution oscillated around a mean value of 2.50 ± 0.06 ml/gdw. During 30 min of ischemia the cells swelled to 2.88 ± 0.08 ml/gdw and residual edema was observed after 30 min of reperfusion (2.62 ± 0.08 ml/gdw). A hypoosmotic shock was used to assess changes in membrane permeability at different time points of ischemia and reperfusion. Water influx induced by the hypoosmotic shock at the end of ischemia was similar to that elicited in perfused hearts. After 15 and 30 min of reperfusion, the magnitude of the response to hypoosmolarity decreased by 9 and 37%, respectively, indicating a gradual permeabilization of the membranes, presumably to ions. The experimental setup was also used to monitor intracellular volumes as a function of time in anisoosmotic conditions. Cellular swelling/shrinkage were delayed for periods of 5 and 8 min at osmolarities of ±50 and ±100 mosmol/liter, suggesting a limited capability of the heart to absorb an anisoosmotic shock. The variation in cellular volumes was proportional to the deviation of the conditions from isoosmolarity, and activation of volume-regulatory mechanisms was demonstrated. The noninvasive technique presented in this study is capable of providing quantitative evidence of changes in cellular volumes in isolated hearts at a temporal resolution of 1 min and a spatial resolution of 4% (of cellular volume). As demonstrated in the cases of global ischemia and anisoosmolar conditions, the technique is expected to provide new insights into the mechanism of cellular-volume regulation.

INTRODUCTION

The working hypothesis of many studies that investigate the irreversibility of ischemic insult is that cellular swelling is one of the factors which promote the transition to this state (*1, 2*). This hypothesis was based on the morphological description of tissue slices, showing the accumulation of water in both the intra- and the extracellular spaces. Water follows changes in the osmotic gradient of metabolites and ions, as the membrane permeability to water is much higher relative to the osmolites (*3*). Thus, the basic cause of water accumulation in the injured heart is an increase in tissue osmolarity, and volume adjustment is to be considered a passive process. In conditions of dynamic modulation of multiple systems which affect osmolarity, it is difficult to predict the accompanying volume changes.

Cell-volume regulation in the cardiac muscle has been studied in isolated cardiomyocytes (*4, 5*). This system benefits from the known and constant composition of the extracellular space; however, the intrinsic conditions within the heart are practically impossible to simulate. For this reason, tissue slices were investigated in parallel, improving the accuracy of the measurements at the expense of precision (*6–8*). In these studies, the most important parameter was the width of the slice, which must be thin enough to allow diffusion of the substrates, and still be thick enough in order to preserve an interstitial space. These experimental limitations led to a search for methods capable of monitoring compartment volumes in isolated organs and *in vivo.* One of the approaches to the evaluation of intracellular volumes was based on the introduction of labeled isotopes as extracellular markers, such as inulin (*9*), ^{58}CoEDTA (*10*), ascorbic acid (*11*), and [^{35}S]sulfate (*12*). The noninvasive character of NMR further advanced this approach and permitted measurements of cellular volumes in suspensions with the aid of ^{1}H spectroscopy of mannitol (*13*), ^{59}Co of cobalticyanide (*14*), and ^{31}P of phosphonates (*15*).

In parallel to spectroscopy, several MR imaging techniques have been proposed for the assessment of tissue-water distribution. Taking advantage of the differences in the relaxation parameters of protons in various tissue compartments, several attempts were made to determine intra- and extracellular fractions (*16, 17*). Such an analysis was, however, limited by the exchange of water between compart-

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1090-7807/97 $25.00

ments, resulting in relatively large experimental errors (*16, 18*). This limitation was reduced by the introduction of contrast agents, allowing *in vivo* determination of cellular fraction at a temporal resolution of minutes and an error of ~10% (*19*). This technique was shown to be optimally applied in tissues with low vascular volumes and low capillary permeabilities; however, since it depends on equilibration of the contrast agent it cannot be applied to the brain. Based on the observation that cellular swelling is associated with early cerebral ischemia (*20*), diffusion-weighted MR was used to observe changes in water contents of brain tumors *in vivo* (*21*). An alternative technique proposed ^{23}Na imaging, and thus indirect detection of edema; however, its application is limited by the poor temporal and spatial resolution of the sodium signals (*22*). Development of these methods is expected to lead to an improved capability of monitoring edema *in vivo* and is promising in the sense of potential clinical applications. Nevertheless, characterization of the cellular-volume regulatory mechanisms requires sensitive quantitative methods which are able to operate with a high temporal and spatial resolution.

Application of multinuclear NMR spectroscopy improved the precision of measurements of volumes in cell suspensions: ^{1}H and ^{23}Na NMR (*23*), ^{1}H or ^{1}H and ^{59}Co NMR (*15*), and ^{2}H and ^{35}Cl NMR (*24*). Concomitant measurement of several nuclei also allowed the transition to measurements in tissues and organs. A combination of ^{1}H and ^{23}Na NMR was used for measurements in the brain (*25*) while ^{1}H, ^{23}Na, ^{35}Cl, and ^{59}Co NMR were combined to estimate cellular volumes in the perfused heart (*26*). Recently, two NMR methods have been developed, aiming to improve the spatial and temporal resolution of intra- and extracellular spaces in the intact isolated organ (*27–31*). One method employed two phosphonates characterized by different membrane permeabilities, which could be measured in one ^{31}P NMR spectrum at a relatively slow temporal resolution (*27–29*), while the other used ^{1}H of water and ^{59}Co of cobalticyanide or ^{13}C of mannitol, allowing intermittent measurement of the absolute cell volumes (*30, 31*). The present study describes a modification of the latter technique, for continuous measurement of volumes in the isolated rat heart.

MATERIALS AND METHODS

Principles of the Method

Our previous NMR method for measurement of intracellular volumes in isolated organs (*31*) was modified to allow continuous monitoring of perfused hearts at a temporal resolution of 30–90 s. In the new apparatus (see Fig. 1), the hearts are introduced in an experimental chamber built inside a 15 mm NMR tube, composed of two Teflon pieces. The upper Teflon part served as the inlet for: (a) a cannula providing the aortic perfusion (Langendorff preparation); (b) a cannula providing a background perfusion of 5–10 ml/min; (c) pacing electrodes for external stimulation; (d) an intraventricular balloon for measurement of the contraction force; and (e) a suction cannula for elimination of excess solution (Fig. 1). Filling of the experimental chamber to a constant level was ensured by the background perfusion. Continuous circulation of solution in the experimental chamber maintained a constant concentration of cobalticyanide in the outer bath, improving the precision of the volume measurements. Equilibration of the cobalticyanide concentration in the bathing solution during no-flow ischemia allowed detection of cellular shrinkage during no-flow ischemia (determined from the increase in cobalticyanide contents of the chamber). The constant fluid level was maintained by diversion of the excess solution to a series of channels engraved in the gadget, the solution being sucked with a peristaltic pump (Sarns Inc.). The bottom of this part was connected to the channels engraved on its walls to ensure exclusion of the air bubbles entrapped in the chamber. The lower Teflon device had the shape of the heart apex and contains the external reference.

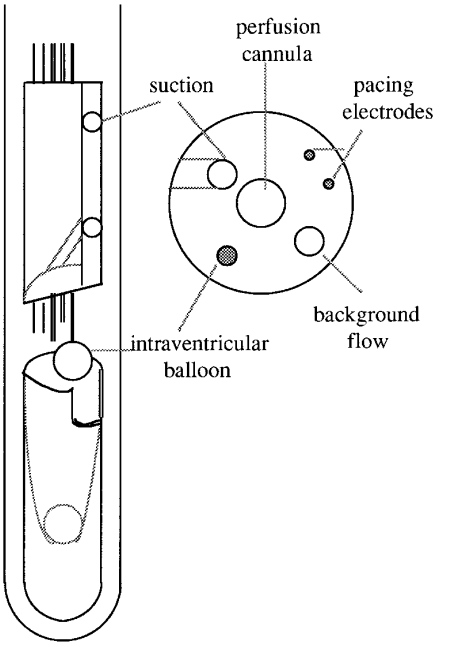

FIG. 1. The experimental chamber used for cell-volume measurements in the perfused rat heart. The upper part of the experimental chamber is detailed. The overall volume of the chamber built inside a 15 mm NMR tube was 1.7 ml.

The technique is based on the selective distribution of a marker between the tissue compartments: cobalticyanide is an agent which spreads in the interstitial space and neither penetrates nor is incorporated in cellular membranes (*14, 31*). The rate of equilibration of $Co(CN)_6^{3-}$ between the outer solution and the interstitial space was measured in our previous (*31*) as well as in our present work, and was found to be fast relative to the measured changes in cell volumes.

Our model of heart compartmentalization includes three spaces: V_{in}, the intracellular compartment; V_o, the nonaqueous volume; and V_{ex}, the extracellular space. The intracellular aqueous volume (V_{in}) is bound by membranes which are freely permeable to water but do not permit penetration of cobalticyanide. V_o, the nonaqueous component of the heart, includes lipid and protein structures and cellular membranes and sutures. V_{ex} includes the solution surrounding the heart, in the heart chambers, in the vascular system, and in the interstitial space.

Determination of the various tissue compartments is based on the differences between the ^{59}Co signals of the experimental chamber filled with solution (I_c) and the chamber containing the heart (I_h) (Fig. 1). These differences are proportional to the space which does not contain the marker, i.e., the intracellular (V_{in}) and the nonaqueous (V_o) spaces:

$$V_{in} + V_o = k^{Co}(I_c^{Co} - I_h^{Co}). \quad [1]$$

The nonaqueous volume of the tissue (V_o) is measured by ^{1}H NMR spectroscopy:

$$V_o = k^H(I_c^H - I_h^H). \quad [2]$$

The constants k^{Co} and k^H were calibrated against glass bulbs of known volume containing a cobalt-free saline solution in D_2O (144 m*M* NaCl). The bulbs were introduced into the experimental chamber filled with the cobalt solution instead of the hearts, and NMR spectra were recorded. The difference between the signal of the chamber containing a glass bulb from that of the chamber filled with solution ($I_c - I_h$) is proportional to the volume of the bulb. The constants k^H and k^{Co} relate between the volume of the bulbs as measured by either ^{59}Co or ^{1}H NMR and their known volumes. These constants transformed the relative spectral data of the hearts to absolute values. The values of the constants vary in each specific system, according to the concentrations of the markers and according to the relative position of the chamber components within the sensitive volume of the NMR receiver. A small external reference was placed at the bottom of the NMR tube and was continuously measured, to avoid any deviations due to changes in the tuning of the probe. The reference contained $Co(CN)_6^{3-}$:$LaCl_3$ at a ratio of 1:10, resulting in an upfield shift of the cobalt resonance of about 10 ppm. (Fig. 2).

Animal Preparation

Male Sprague–Dawley rats (200–230 g) were heparinized (ip heparin sulfate, 500 u/kg) and anesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The hearts were excised through a bilateral thoracotomy and cooled at 4°C, and the aorta was cannulated. The hearts were initially perfused at 37°C for 20 minutes in a Langendorff preparation (perfusion pressure of 95 cm H_2O). Temperature was continuously measured in the perfusion solution, above the heart and inside the magnet bore. The hearts were paced at 5 Hz (Harvard Student Stimulator), using copper wires and gold electrodes. Heart rate and contraction force were measured with a latex balloon (filled with water) introduced into the left ventricle. Signals were converted with a Gould pressure transducer and were recorded on a personal computer. In the perfused heart, the diastolic pressure was set to 5 mm Hg. Onset of the ischemic contracture was considered as an increase of 5 mm Hg in the diastolic pressure.

Solutions and Materials

The basic Krebs–Henseleit (KH) solution contained (in m*M*) 121 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.75 $CaCl_2$, 23 $NaHCO_3$, and 11 glucose. Solutions were bubbled with a mixture of 95% oxygen and 5% carbon dioxide, to give a buffered pH measured at 7.4. For NMR spectroscopy, the solution was modified by substitution of KCl with 2 m*M* $K_3Co(CN)_6$ (KH–Co). All materials were purchased from Sigma Chemicals Co. (St. Louis, Missouri).

NMR Spectroscopy

Spectra were recorded on a Bruker AMX-360 WB spectrometer, equipped with a 20 mm broadband probe operating at 360.13 MHz for ^{1}H and 85.446 MHz for ^{59}Co. ^{1}H spectra were collections of 2 transients of 26° pulses, with an acquisition time of 0.254 s and a relaxation delay of 4 s. ^{59}Co spectra were collections of 128 transients of 50° pulses with an acquisition time of 0.205 s and a relaxation delay of 0.2 s. No correction for partial saturation was needed since the T_1 of ^{59}Co in cobalticyanide is 0.14 s in saline and shorter in body fluids (H. Shinar and G. Navon, unpublished results). Since both ^{1}H and ^{59}Co spectra are practically fully relaxed, no effect of flow was expected on their signal intensities. The FIDs were multiplied with a 30 Hz Lorentzian window. The ^{59}Co resonance frequencies are known to be very sensitive to temperature. As a result, the lineshapes of ^{59}Co are distorted due to minute temperature gradients within the chamber. Therefore peak integrals and not signal intensities were quantified. ^{59}Co and ^{1}H spectra were collected during perfusion of the chamber, before and following introduction of each heart, and the mean value of 10 spectra was used for determination of I_c^{Co} and I_c^H, respectively.

Data Analysis

Intracellular and tissue volumes were determined from the changes in ^{59}Co and ^{1}H signal intensities of the experimental chamber filled with solution and containing a heart ($I_c - I_h$). At constant values of I_c and k^{Co}, a decrease in ^{59}Co signal intensity (I_h) was proportional to cellular swelling (Eq. [1]). Areas of the peaks were integrated using a Lorentzian–

Gaussian simulation subroutine. The area of the external reference was used to calibrate the ^{59}Co signal integrals. Analysis of variance was applied to evaluate differences within the experimental groups. A post hoc Scheffe *t* test was used to examine differences between the groups, considering significance at $p < 0.05$.

RESULTS

The Experimental Procedure

The stability and precision of the measurements performed with the new experimental chamber were tested in two sets of experiments: (a) Sequential ^{59}Co spectra were collected during perfusion of the chamber with KH−Co at a flow rate of 25 ml/min. Although the lineshape was variable as a result of minor temperature fluctuations (especially during switch between the perfusion solutions), the integrals were practically constant: the standard deviation of 20 consecutive measurements was 2%. The same variability was found for 20 measurements of the external reference. (b) Expansion of the marker-free space was simulated by graded injection of known amounts (50 μl increments) of cobalt-free saline into the latex balloon. During these measurements the chamber was perfused with KH−Co. The volumes of the balloon calculated according to Eq. [1] agreed with the known amounts of added cobalt-free solution.

Measurement of the intracellular space using an extracellular marker requires experimental evidence concerning the distribution of the marker between the external solution and the interstitial space of the heart. The transport of the marker between these spaces was evaluated in two sets of experiments: (a) $Co(CN)_6^{3-}$ loading and washout profiles in the perfused heart; (b) equilibration by diffusion during ischemia. Marker loading was evaluated by continuous measurement of I_h^{Co} (acquisition time 1 min) when marker-free hearts ($n = 5$) were perfused with KH containing 2 m*M* $Co(CN)_6^{3-}$. The measurements demonstrated a fast increase in integrals due to marker penetration into the interstitium; the saturation curve reached its half-maximum in 1.5 min and near steady state after 4 min of perfusion. The overall increase in ^{59}Co peak integral was 10%. The reciprocal experiment, i.e., perfusion of marker-loaded hearts ($n = 5$) with a cobalt-free KH solution, showed a similar initial fast decrease in ^{59}Co spectrum, with disappearance of the cobalt signal after 4 min of perfusion. During no-flow ischemia, the exchange of $Co(CN)_6^{3-}$ between the interstitium and the surrounding solution was measured as a function of time. In the present experimental setup, a background flow was used at a rate of 5 ml/min. At this rate, the solution in the experimental chamber containing a heart is exchanged approximately every 18 ± 3 s. After 30 min of ischemia, marker-loaded hearts ($n = 5$) superfused with a cobalt-free KH solution lost half of their cobalticyanide contents in 2.5 min. At the end of the wash-out period, cobalticyanide was not detectable in the spectrum, indicating that it was not entrapped to a significant extent in the cells.

In the present experimental setup, the marker-free space of a heart ($V_{in} + V_o$), which is expressed by ($I_c^{Co} - I_h^{Co}$), was 25−33% of the signal area measured with the chamber being filled with cobalt solution (I_c^{Co}). The non-aqueous space (V_o) was found to be stable during these short-term experiments; thus, the value for an individual heart was calculated using all the ^{1}H spectra recorded. The absolute value of V_o was 1.1 ± 0.2 ml/gdw. It should be mentioned that this value includes the sutures, but not all the other devices within the chamber, such as the tip of the cannula, electrodes, the latex balloon, and the external reference, which were present during the measurements with and without the heart (I_h and I_c). The nonaqueous space was proportional to the difference between two large ^{1}H integrals, giving a relatively large experimental error. The fact that all the ^{1}H spectra collected for each heart were used to determine V_o reduced this error.

Continuous Monitoring of Cell Volumes

Hearts were initially perfused at 37°C for 15−20 min for stabilization of the functional metabolic parameters in the Langendorff preparation. Most of the hearts presented a trend of cell-volume reduction during this period, which was statistically nonsignificant (Fig. 2). It was attributed to cell-volume changes following cold arrest of the excised hearts and initiation of perfusion with crystalloid solution. In steady-state perfusion conditions of 36 hearts, with an average of five measurements for each heart ($n = 180$), the mean cell volume was 2.50 ± 0.06 ml/gdw, at a pacing rate of 300 beats per min (bpm). During 90 min of continuous perfusion, the intracellular volume for individual hearts oscillated with a standard deviation of ±4%. The mean cell volume of perfused hearts was slightly dependent on the pacing rate: 2.42 ± 0.08 and 2.59 ± 0.09 ml/gdw at 500 and 100 bpm, respectively.

Cellular volumes were measured at a temporal resolution of 1 min. During no-flow ischemia, the volumes were stable for the first 5 min ($n = 6$), followed by cellular swelling to an end-ischemic value of 2.88 ± 0.08 ml/gdw ($p < 0.001$ vs pre-ischemic volume). Cellular-volume time course did not follow a linear function throughout ischemia, presenting a higher rate of water influx at 12 min (Fig. 3). The steep increase in cellular water contents after 12 min of ischemia coincided in time with the onset of ischemic contracture (determined as an increase of 5 mm Hg in the diastolic tonus). The maximal amplitude of contracture at 30 min of ischemia was 39 ± 6 mm Hg. Cell volumes stabilized at 20 ± 3 min of ischemia, presenting a slower rate of swelling.

Reperfusion is associated with a reduction in cell volumes, as previously reported in hearts submitted to 30 min of ischemia (*31*). In the experimental setup used in this study, it

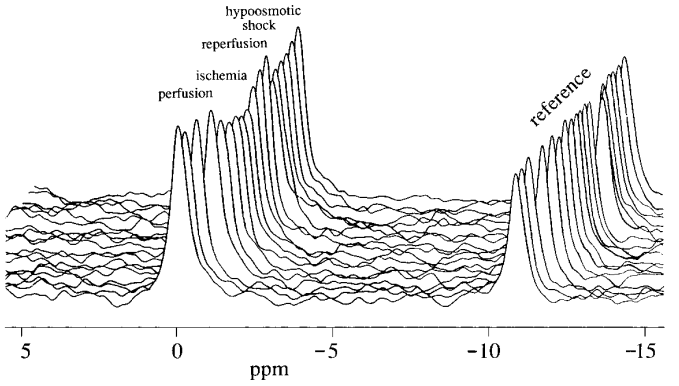

FIG. 2. Sequential ^{59}Co NMR spectra of the experimental chamber containing a heart, during perfusion, ischemia, reperfusion, and hypoosmotic shock (210 mosm/liter). The cobalt resonance in the reference was shifted by $LaCl_3$. The shift and the broadening of the cobalt signals of the chamber and the reference during exchange of solutions were caused by temperature fluctuations. The areas of the peaks were not altered significantly by this change in lineshape.

was possible to follow cell volumes continuously during reperfusion (Fig. 3). Hearts reperfused after 12 min of no-flow ischemia (prior to onset of ischemic contracture) recovered their preischemic volumes (not shown). Residual swelling was observed during reperfusion after 30 min of ischemia. The cells shrank during the first 6 ± 2 min of reperfusion and remained stable during the following period. Cellular volumes stabilized at a value of 2.62 ± 0.08 ml/gdw which is higher by 6% than the initial volumes ($p < 0.05$ vs preischemic volume). The developed left ventricular pressures at 30 min of reperfusion following 12 and 30 min of ischemia were 91 ± 5 and 41 ± 4% of the preischemic values, respectively.

The reliability of cell-volume measurements at reperfusion depends on the extracellular location of cobalticyanide, in other words, impermeability of the sarcolemmal membranes to the marker. This was tested by application of a hypoosmotic shock at 210 mosm/liter ([NaCl] = 71 m*M*) during perfusion, following ischemia, or after 15 or after 30 min of reperfusion ($n = 4$). In all these cases, cellular swelling was measured after 5–8 min (Fig. 2). The amplitude of the volume response to the hypoosmotic shock can be considered an indicator of membrane impermeability to cobalticyanide. If the sarcolemma was permeable to the marker, its influx into the cells would be expressed as a lack of swelling. The response to application of the hypoosmotic shock following ischemia was comparable to that applied in perfusion conditions, while it decreased by 9 and 37% after 15 and 30 min of reperfusion, respectively. This implies an increased permeability of the membranes, either due to an enhanced efflux of intracellular components such as potassium or penetration of cobalticyanide.

Responses to Anisoosmolarity

Hearts were perfused for a stabilization period of 20 min, followed by switch of the solution to hypo- and hyperosmotic solutions. The changes in cellular volumes are shown in Fig. 4. In all the four experimental groups ($n = 6$) the hearts were paced at 5 Hz, and ventricular contraction was sustained during 60 min of anisoosmotic perfusion. First, it is worth noting that the responses to hypo- and hyperosmolarity were symmetrical, at both ±50 and ±100 mosm/liter. The cells remained stable for periods of 8 and 5 min of perfusion at ±50 and ±100 mosm/liter, respectively. Following these periods, the cells rapidly swelled (3 min) to values of 2.87 ± 0.10 and 3.33 ± 0.11 ml/gdw at 265 and 215 mosm/liter, respectively ($p < 0.001$ vs isoosmotic cell volume). These volumes remained high, followed by a gradual decrease toward baseline values. The same time evolution of the changes in cellular volumes was observed during perfusion with hyperosmotic solutions.

DISCUSSION

Methodological Considerations

In the present study we present a modified apparatus for cell-volume measurements in the intact isolated heart (*31*). Both experimental setups are based on the introduction of an extracellular marker into the interstitial space, and indirect calculation of the absolute cell volumes (*14*). As for any other *ex vivo* system, exposure to a crystalloid solution may alter the intrinsic parameters of the organ, and the extrapolation of the data to the physiological state should be made considering this limitation. The experimental setup employed benefits from two advantages: (a) the cells are structurally organized in their native organ arrangement; (b) the

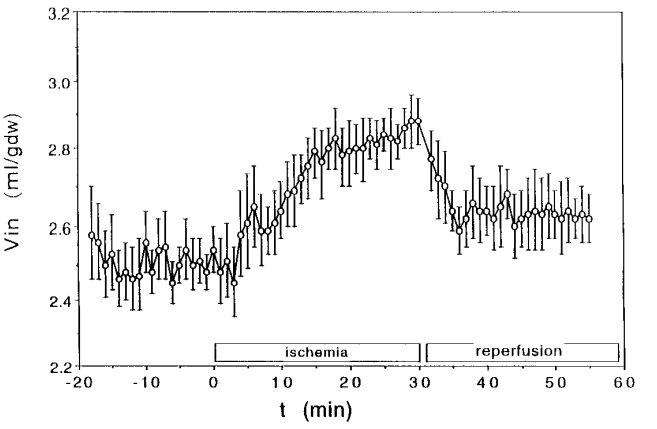

FIG. 3. Means ± SD of cellular volumes of the group of hearts ($n = 6$) at a temporal resolution of 1 min. Onset of perfusion following cold arrest was accompanied by reduction of the volumes and their stabilization around a value of 2.50 ml/gdw. Ischemic cellular swelling did not subside completely at reperfusion.

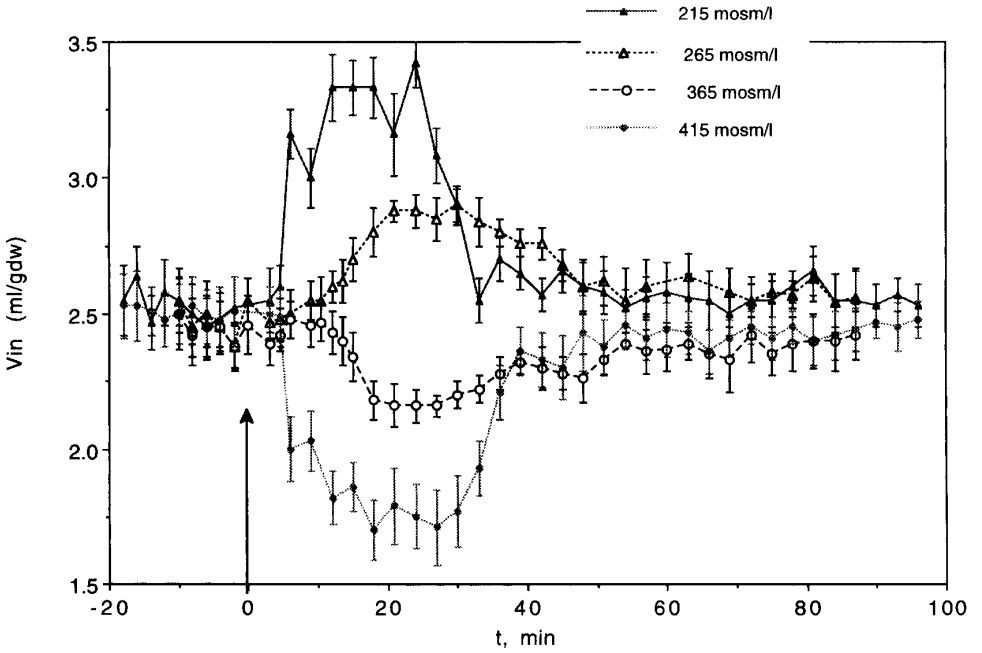

FIG. 4. Means ± SD of cellular volumes of hearts (n = 6) perfused at 215–415 mosm/liter. The arrow indicates onset of osmotic stress. Osmolarity of the solutions was performed by modulation of the NaCl concentration.

composition of the interstitium is influenced by cellular metabolism in contrast to measurements performed on myocytes, where the composition of the external solution is controlled artificially.

The main advantage of the methodological development presented in this study is continuous monitoring of the cell-volume dynamics in the perfused as well as in the ischemic organ, at an improved temporal resolution. As a consequence, the mechanism of cellular-volume regulation can be analyzed in a variety of pathophysiological states with respect to the individual heart, which is first assessed in baseline perfusion conditions and then during application of the desired intervention. Using the modified method of cell-volume measurement, one should no longer be concerned with the location of the experimental chamber relative to the receiver coil, since it maintains its fixed position during the entire experiment for each heart. The method described, however, has the disadvantages of an isolated organ preparation: (a) there is no discrimination between different cell lines in the heart, assuming that myocytes are the major component; (b) there is no differentiation between the responses of atrial and ventricular cardyocytes to ischemia or anisoosmotic conditions. In addition to this compositional heterogeneity of the heart which is not evident in this preparation, the functional heterogeneous state is also hidden. One cannot exclude the presence of cells which are extremely swollen or become permeable to the extracellular marker during the experiments. The above-mentioned features are not unique to the experimental setup described, but should be considered limiting factors for the interpretation of NMR studies of isolated organs.

A detailed validation of the experimental conditions for measurement of cell volumes in the rat heart with cobalticyanide and mannitol as extracellular markers was recently reported (*31*). In that work, we estimated an intracellular volume of perfused hearts to be 2.45 ± 0.13 ml/gdw using a linear extrapolation of the ischemic volumes. Direct measurements performed in the present work in the perfused heart indicate that in this preparation the intracellular volume is 2.50 ± 0.06 ml/gdw. Although this difference is within the experimental error, it can be accounted for by the fact that cellular volumes are stable during the first 5 min of ischemia; therefore, the linear extrapolation was not justified. Introduction of a background flow in the present experimental setup improved the rate of exchange of cobalticyanide between the interstitial space and the outer bath, and ensures a constant marker concentration in the chamber. This is expected to improve the accuracy of the measurements, and also allows monitoring of cellular shrinkage.

Our method is closely related to the method that uses ^{31}P NMR of permeable and impermeable phosphonate compounds (*27–29*). The advantages of the present method are: (a) the higher intrinsic sensitivity of the ^{59}Co NMR and its shorter relaxation times (0.14 s as compared to 14.1 s for dimethyl methylphosphonate in saline solution), allowing a fast repetition rate and performance of measurements which are independent of the relaxation time and the flow of the perfusion solution; (b) the estimation of the water space

using ^{1}H NMR which ensures equal distribution of the marker in the intra-, and extracellular spaces, and does not depend on the diffusion rate of the permeable marker; (c) the small volume of our apparatus reduces the external volume, thereby increasing the relative changes due to cellular swelling, with the consequent improved precision of its measurement. Another advantage of the small volume is its location well within the detection volume of the receiver coil, and the results are not sensitive to minor changes in its position.

Physiological Considerations

Continuous monitoring of the individual hearts revealed a cellular volume oscillation in the range of 2.35–2.65 ml/gdw, with a standard deviation of ±4%. These values exceed the experimental error of volume measurements of the external reference and the chamber in the absence of the heart; thus, it is possible that cell-volume oscillation is a true tissue behavior. In addition, volumes were dependent on the stimulation rate, suggesting that brady arrhythmias (1.66 Hz) are associated with an increase in water contents. Although the extent of swelling was not statistically significant, it complements the observation of cellular shrinkage at high stimulation rates (8.33 Hz). These experimental observations were recorded during perfusion of a crystalloid solution, while the presence of oncotic agents reduced the amplitude of volume changes in the perfused and ischemic heart and in anisoosmotic conditions (unpublished data). It is possible that the crystalloid solution amplified the changes in volumes, allowing for their experimental detection. This is a valuable experimental tool when minor perturbations of a tightly regulated parameter such as cellular volume is studied. However, this also implies that, in physiological conditions, cell volume oscillations are blunted by the oncotic pressure of the blood.

During 30 min of ischemia, a certain population of cells (ischemia-sensitive) become irreversibly injured (*1*). Since the permeability of the membranes for water is significantly higher than for other ions and molecules (*3*), changes in water distribution precede other pathological processes, such as nonselective membrane permeabilization, disintegration, and rupture under ischemic conditions (*32, 33*). Considering the ischemic injury to be a continuous process, as far as membrane permeabilities are concerned (*32–34*), the irreversibly injured cells may excessively swell at first, leading to detection of cellular edema in the whole-organ measurements. When membrane integrity is lost, the extracellular marker would have penetrated the cellular membranes, leading to underestimation of cellular swelling. The procedure used in the present study cannot differentiate between cardiac cell populations which present various degrees of sensitivity to an ischemic injury. However, the fact that cobalticyanide was efficiently washed out of the heart after 30 min of ischemia indicates that the extracellular marker was not retained inside the cells to a significant extent. This is also supported by the amplitude of the response to hypoosmotic shock during the early stages of reperfusion, indicating that the membranes were impermeable to the extracellular marker. For periods of prolonged ischemia, when irreversible ischemic injury is accompanied by membrane disruption, methods which assess cellular volumes using extracellular markers are incompetent. Nevertheless, given that introduction and elimination of cobalticyanide in the extracellular space is on the order of minutes, the experimental setup may be further exploited for detection in changes in membrane permeability and cellular viability following ischemia (*35*).

Prolongation of the ischemic period from 12 to 30 min resulted in incomplete functional and cellular-volume recovery at reperfusion. The parallel occurrence of ischemic contracture, considered one of the parameters related to irreversible injury (*1, 2*), and the steep rate of water accumulation raises the question of a possible causal relationship between these parameters. This question has been addressed by attenuation of the onset of contracture during inhibition of sodium ion transporters (*36*) and attenuation of energy metabolism (*35, 36*). Indeed, ischemic contracture was accompanied by an increase in intracellular water contents; however, it did not cause it. It was also shown that cellular-volume expansion was not the result of a change in cardiac shape, as during metabolic inhibition, contracture was associated with cellular shrinkage (*36*). The complexity of the mechanism of cellular-volume regulation imposes experimental difficulties and careful interpretation of the results. The capability of monitoring changes in cellular water contents in isolated organs (*28–31*) and *in vivo* (*19–22*) is expected to provide new insights into the mechanism of volume regulation and its pathophysiological relevance.

The improved temporal resolution allows the introduction of the hypoosmotic shock as a test to evaluate the integrity of the membranes. Impermeability of the sarcolemma to cobalticyanide is essential for the reliability of the measurements, especially at reperfusion, since marker penetration would result in a false cellular-volume reduction. The steep volume increase in response to the hypoosmotic environment depends on the ratio between the fast water transients and the relative membranal permeability to intra- and extracellular compounds. The decrease in the amplitude of cellular swelling after 15 and 30 min of reperfusion is caused by transport of osmolites across the membrane. The gradual deterioration of the response to the hypoosmotic shock as a function of the reperfusion period may be viewed as evidence of the development of reperfusion injury. Although we cannot present experimental evidence for the identity of the osmolites to which membranes become permeable, it is likely that transport of sodium and potassium will precede the influx of cobalticyanide. An increased permeability of sodium during an extensive period of ischemia has been observed at both low temperatures (*34*) and normothermia (*32*).

Cellular Volumes in Anisoosmotic Conditions

The present study demonstrates one of the numerous possible applications of the procedure used for cell-volume measurement: behavior of cell volumes in anisoosmotic conditions. It was possible to follow the changes in cell volumes as a function of time and to appreciate quantitatively the changes in water contents at different osmolarities. The time course of the volumes shown in Fig. 4 presents several interesting features. First, the onset of cellular-volume swelling/shrinkage is delayed by 8 and 5 min in conditions of ±50 and ±100 mosm/liter, respectively. The straight forward interpretation of this phenomenon is based on the ability of the tissue to absorb an osmotic stress for a limited period of time. This behavior was characteristic for sodium chloride hyperosmotic solutions, in variance from the immediate response achieved when mannitol was used to increase osmolarity (unpublished data).

When an anisoosmotic stress is applied, the interstitial space undergoes a series of transient states before the cells reach a new steady state. We shall analyze the various possible tissue responses to a hyperosmotic solution: (a) If the interstitial space shrinks initially, concentrating its contents of cobalticyanide, the NMR measurement would show a false decrease in cellular volumes, due to the increase in cobalticyanide contents in the chamber. This was not observed in the experiments, indicating that the exchange of both water and cobalticyanide between the interstitial space and the perfusion solution is relatively fast. (b) If the cells shrink, the only possibility for obtaining a constant ^{59}Co signal intensity is by swelling of the interstitium with a concomitant dilution of its cobalticyanide contents. This is an unlikely situation since it requires penetration of sodium into the interstitium without a concomitant efflux of water to the outer solution. Thus we can conclude that the delay in the changes of the measured volume is the result of the delay in the response of the intracellular volume to anisoosmolarity, and not due to the slow equilibration of cobalticyanide. This is in line with our measured rate of equilibration of this marker. Apparently, during the period of tissue resistance to anisoosmolarity, the volume of the interstitial space is stable as it equilibrates with the perfusion solution.

Following the delay period, a fast response of the tissue was observed. This fast tissue response is attributed to the water flux across the sarcolemma according to the osmotic gradient. The magnitude of the response was proportional to the deviation of the solutions from the isoosmolar condition, and occurred in all cases in less than 3 min (the interval between two successive volume measurements). Considering an intracellular osmolarity of 315 mosm/liter and a volume of 2.50 ml/gdw, the expected changes in volumes at ±50 and ±100 mosm/liter would be 0.40 and 0.79 ml/gdw, respectively. These calculated data are in good agreement with the measured values of 0.37 and 0.83 ml/gdw, compatible with the notion of free water permeability across the membrane. The new osmotic steady state reached by the cells was sustained for a mean period of 18 min, and interestingly the regulatory volume correction was steeper in hearts perfused with solutions ±100 mosm/liter. The description of cell-volume time courses presented in this study are characteristic for modulation of the sodium chloride solution contents, and cannot be extrapolated to other conditions. The temporal pattern of cell volumes and the amplitudes of responses were significantly different when osmolarity was attenuated by mannitol or urea (unpublished results).

CONCLUDING REMARKS

The experimental setup presented in this study enables measurement of cellular volumes in isolated organs during perfusion, ischemia, and reperfusion. The measurement is continuous, giving a time course of volume changes, and allows the description of transient events. It is demonstrated that cardiac tissue is able to resist a hypo- and hyperosmotic shock for a limited period of time, and that cells can transiently swell or shrink up to 33% of their initial volumes, without any evidence of functional deterioration. Application of noninvasive monitoring techniques for determination of compartment volumes in intact organs is expected to provide new insight into the mechanisms of cell-volume regulation.

ACKNOWLEDGMENTS

Support was obtained from the Israeli Ministry of Health. The Foulkes and the Emerico Letay Foundations are gratefully acknowledged. The excellent technical assistance of Ruth Panigel, Nachum Lavy, Beny Agam, and Arie Ben-Amram is gratefully acknowledged.

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