Cytosolic calcium in the ischemic rabbit heart: assessment by pH- and temperature-adjusted rhod-2 spectrofluorometry

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Received 2 April 2003; received in revised form 22 May 2003; accepted 28 May 2003

Abstract

\textbf{Objectives:} Cytosolic calcium ([Ca\textsuperscript{2+}]) mediates ischemia–reperfusion (I/R) injury, but magnitude and time course of I/R-induced [Ca\textsuperscript{2+}] overload remain unclear. Fluorescent indicators are commonly used to measure [Ca\textsuperscript{2+}], and have a dissociation constant (K\textsubscript{i}) that depends on pH and temperature. We hypothesized that changes of K\textsubscript{i} during I/R lead to misleading interpretations of [Ca\textsuperscript{2+}] recordings. \textbf{Methods:} (1) In isolated rabbit hearts (n=4 each), intracellular pH was measured during I/R at 37 °C, 20 °C, and 4 °C with and without cardioplegic arrest by \textsuperscript{31}P-NMR-spectroscopy. (2) K\textsubscript{i} for rhod-2 and calcium was determined at varying pH and temperature in in vitro experiments. (3) Isolated rabbit hearts were subjected to I/R, and [Ca\textsuperscript{2+}] was recorded by surface rhod-2 spectrofluorometry. Finally, [Ca\textsuperscript{2+}] was computed using either the conventional K\textsubscript{i}, or the pH- and temperature-adjusted K\textsubscript{d}. \textbf{Results:} K\textsubscript{d}([Ca\textsuperscript{2+}Rhod-2]) remained stable between pH 7.1 and 6.8, but increased exponentially with lower pH and/or temperature. Calculations using a static K\textsubscript{d} indicated that [Ca\textsuperscript{2+}] rose only mildly during warm ischemia and did not rise during cardioplegic arrest, while a large Ca\textsuperscript{2+} influx appeared to occur during early reperfusion. When the pH and temperature-adjusted K\textsubscript{d} was used for calculation, [Ca\textsuperscript{2+}] rose significantly during ischemia (431±37% during 20 min 37 °C ischemia, and 78±19% during 20 min cardioplegic arrest at 20 °C). During early reperfusion, [Ca\textsuperscript{2+}], decreased rapidly, without significant further [Ca\textsuperscript{2+}] elevation. \textbf{Conclusions:} In contrast to previous reports, [Ca\textsuperscript{2+}] accumulation occurs during unprotected ischemia as well as hypothermic ischemia with cardioplegic arrest, without further net Ca\textsuperscript{2+} influx on reperfusion. This finding has important implications for timing of protective strategies during myocardial ischemia.

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Keywords: Calcium (cellular); Ischemia; Cardioplegia; Cardiovascular surgery

1. Introduction

It is generally acknowledged that myocardial ischemia–reperfusion injury provokes cytosolic free calcium ([Ca\textsuperscript{2+}]) accumulation [1,2]. During ischemia, several mechanisms result in failure of intracellular Ca\textsuperscript{2+} compartmentalization and extrusion from the cell against high concentration gradients. At the onset of reperfusion, intracellular Ca\textsuperscript{2+} overload is thought to be further ex-

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myocardial ischemia and reperfusion can be experimental-
ly performed only in the intact perfused organ, in which
intracellular Ca\(^{2+}\) is much more difficult to measure. The
complex methodology problems include light absorbance
by chromatic molecules (i.e. myoglobin, hemoglobin),
tissue autofluorescence (i.e. NAD\(^{+}\)), motion artefacts of
the beating heart, and the impact of temperature and pH on
Ca\(^{2+}\)-induced signal emission. Signal quality greatly
depends on the fluorescence indicator used, and each in-
dicator has particular advantages and disadvantages. We
have recently introduced the long-wavelength Ca\(^{2+}\) in-
dicator rhod-2 for recording of beat-to-beat Ca\(^{2+}\) trans-
ients, because rhod-2 possesses several advantages over
other fluorescence indicators that are of particular impor-
tance in the heart. However, the signal of any cation-
sensitive indicator is affected by changes in intracellular
pH as well as temperature, both of which can vary greatly in
clinically relevant models of myocardial ischemia and reperfusion [3]. The extent of pH- or temperature-induced
signal changes depends on the physical properties and
binding affinity for Ca\(^{2+}\) and protons, and is unknown for
most of the indicators used today.

In order to achieve a better understanding of intracellu-
lar calcium during ischemia in the intact heart, we
measured the change in intracellular pH during several is-
chemia/reperfusion protocols that mimic clinically and
experimentally relevant situations, determined the disso-
ciation constant \(K_a\) for rhod-2 and Ca\(^{2+}\) at the respective pH
and temperature, and determined cytосolic calcium by
spectrofluorometry in whole hearts, using the appropriate
\(K_a\).

2. Methods

Our approach to obtain pH and temperature-corrected
measurements of intracellular calcium using rhod-2 in-
cluded the following steps:

1. Measurement of intracellular pH in isolated rabbit
hearts subjected to ischemia at 37 °C, 20 °C, and 4 °C
without cardioplegic arrest, and at 20 °C with high-
kaliuim cardioplegic arrest, using 31P nuclear mag-
netic resonance.

2. Determination of the specific \(K_a\) for rhod-2 and Ca\(^{2+}\) in
series of in vitro experiments at pH 7.1–6.2 in incre-
ments of 0.1, each at the above described temperatures.

3. Recording of intracellular Ca\(^{2+}\) signals in intact per-
fused rabbit hearts using rhod-2 spectrofluorometry,
during ischemia at 37 °C without cardioplegic arrest and
at 20 °C with cardioplegic arrest: two commonly used
experimental protocols that are of particular clinical
relevance.

4. Computation of the intracellular free Ca\(^{2+}\) concen-
tration using the specific pH and temperature-corrected
\(K_a\) for a given protocol and time point.

2.1. Isolated heart perfusion

The investigation conforms with the Guide for the Care
and Use of Laboratory Animals published by the US
National Institutes of Health (NIH Publication No. 85-23,
revised 1996). New Zealand white rabbits (2–2.5 kg) were
euthanized by intravenous injection of ketamine (100 mg/
kg), and heparin (500 U/kg). Hearts were rapidly excised
and placed in 4 °C cold buffer solution. After cannulation
of the aorta, hearts were perfused in the Langendorff mode
at 80 mmHg constant perfusion pressure with modified
Krebs–Henseleit (KH) buffer (115 mmol/l NaCl, 26
mmol/l NaHCO\(_3\), 11 mmol/l glucose, 1.8 mmol/l 31
KgSO\(_4\), 1.8 mmol/l KH\(_2\)PO\(_4\), 4.7 mmol/l KCl, 1.25
mmol/l CaCl\(_2\), and 10 U/l insulin), that had been equili-
brated with a 95% O\(_2\)/5% CO\(_2\) gas mixture and passed
through a 0.2 micrometer nylon filter. The final buffer pH
was 7.35–7.45, PO\(_2\) was 550–600 mmHg, and PCO\(_2\) was
30–40 mmHg. A fluid-filled latex balloon connected to a
micromanometry catheter (Millar Instruments, Houston,
TX) was placed in the left ventricle via the left atrium.
After 30 min stabilization, the hearts were placed in the
respective perfusion chamber for 31P NMR spectroscopy
or rhod-2 spectrofluorometry. Global ischemia was in-
duced by occlusion of the aortic cannula. Cardiac tempera-
ture was monitored throughout the experiment, including
the period of ischemia, using a temperature probe inserted
in the right ventricle. During stabilization and reperfusion
myocardial temperature was adjusted to 37 °C. During
the period of ischemia, temperature was maintained at 37 °C or
lowered to 20 °C by adjusting the ambient temperature in
the perfusion chamber.

2.2. 31P NMR spectroscopy

Hearts were perfused with phosphate-free modified KH
buffer using a customized perfusion system. The isolated
heart in its perfusion chamber was positioned within a
20-mm solenoid radiofrequency coil. NMR spectra were
acquired in an 8.45 Tesla vertical bore Bruker spectrometer
(Bruker Instruments, Billerica, MA). Spectra were ob-
tained by signal averaging 120 scans with a 2-s delay,
resulting in a time resolution of 4.5 min. Intracellular pH
was calculated from the shift of the inorganic phosphate
(P\(_i\)) peak using the following equation [4]:

\[
\text{pH} = 6.9 + \log \left[ \frac{(x - 3.28)}{(5.7 - x)} \right]
\]

where \(x\) is the position of the P\(_i\) peak with respect to the
PCr peak in p.p.m.

Following a stabilization period of 20–30 min, hearts
were subjected to 45 min ischemia at 37 °C \((n=4)\), at
20 °C \((n=4)\), and at 4 °C \((n=4)\). In another group of
hearts, cardioplegic arrest was induced by injection of
perfusion buffer containing 20 mM K\(^+\) \((n=4)\).
2.3. Dissociation constants for rhod-2 and Ca$^{2+}$

To determine the $K_d$ for rhod-2 and Ca$^{2+}$ at changing pH and temperature, fluorescence measurements were made in 11 samples of varying Ca$^{2+}$ solutions containing 10 µM rhod-2, which were prepared for each combination of pH and temperature. The solution contained 100 mM KCl, 1 mM Mg$^{2+}$, and 0.5 mM myoglobin in order to mimic ionic strength, autofluorescence, and absorption properties of the cardiomyocyte cytosol. The various Ca$^{2+}$ concentrations were prepared from two stock solutions of known Ca$^{2+}$ concentration (calcium calibration buffer kit #1 with Mg$^{2+}$, Molecular Probes) using the method by Tsien and Pozzan [8]. This method is based on the principle that when the concentrations of Ca$^{2+}$ and EGTA are very close to each other, the only free Ca$^{2+}$ available is that which is in equilibrium with EGTA. Thus, $[Ca^{2+}]_{\text{free}}$ is a function of the dissociation constant of Ca$^{2+}$EGTA, $K_d(Ca^{2+}EGTA)$, which by itself is dependent on ionic strength, pH, and temperature. Therefore, the effective free Ca$^{2+}$ concentration of each calibration solution has to be determined based on the specific dissociation constants of EGTA for Ca$^{2+}$ for each given pH and temperature. Both $K_d(Ca^{2+}EGTA)$ and the free Ca$^{2+}$ concentration in the undiluted Ca$^{2+}$EGTA buffer solution at a given temperature and pH were derived using the web-based computer program Webmaxclite v1.00 (Stanford University, http://www.stanford.edu/~cpatton/maxclite.htm). The free Ca$^{2+}$ concentration in the Ca$^{2+}$ dilution series obtained by mixing Ca$^{2+}$EGTA and K$^{+}$EGTA was then computed according to the equation:

$$[Ca^{2+}]_{\text{free}} = K_d(Ca^{2+}EGTA) \times ([Ca^{2+}EGTA]/[K^+_EGTA])$$

Once the actual free Ca$^{2+}$ concentration in each sample was known, the $K_d$ for rhod-2 and Ca$^{2+}$ was determined. Samples were prepared in a 3 ml quartz cuvette, which was placed in a water-jacketed temperature controlled cuvette holder in the SLM 8100 spectrofluorometer, and all solutions were constantly stirred during fluorescence measurements. Excitation light of 524 nm was used, and emission was recorded between 540 and 620 nm in 1 nm increments with an integration time of 0.5 s (Fig. 1). Fluorescence intensity at 589 nm was then used to construct the rhod-2 calibration curves. The $K_d$ for rhod-2 and Ca$^{2+}$ was computed based on the actual free Ca$^{2+}$ concentration in each sample and the resulting fluorescence using automated software supplied by the vendor (Dissociation Constant Calculator, http://www.probes.com/), based on the equation:

$$[Ca^{2+}]_{\text{free}} = K_d(Ca^{2+}Rhod-2) \times (F - F_{\text{min}})/(F_{\text{max}} - F)$$

Principally, data are plotted as the log of the $[Ca^{2+}]_{\text{free}}$ (x-axis) versus the log $[(F - F_{\text{min}})/(F_{\text{max}} - F)]$ (y-axis).

![Fig. 1. Intracellular pH measured by 31P NMR spectroscopy in whole hearts subjected to a 45 min period of ischemia at different temperatures with or without high-potassium cardioplegic arrest. Indicated are the mean values of four experiments each and the respective standard deviation.](https://example.com/fig1.png)

This double-log plot gives an x-intercept that is the log of the $K_d$ for rhod-2 under the given conditions.

2.4. Rhod-2 spectrofluorometry

Measurement of beat-to-beat intracellular Ca$^{2+}$ transients was performed as we have previously described and validated in detail [5]. During the 30 min stabilization period, hearts were loaded with the Ca$^{2+}$-sensitive dye rhod-2 AM (Molecular probes, Eugene, OR) by perfusion with the cell-permeable acetoxymethylester (Rhod-2 AM; 0.5 mg/0.25 ml DMSO infused over 2 min without recirculation). Dye-loading was followed by a 15 min washout to remove any extracellular dye. A modified spectrofluorometer (SLM-Amino, Springfield, IL) was set to provide excitation light at 524 nm and recorded emission light at 589 nm. Recordings were performed with a time-resolution of 2 ms for analysis of single Ca$^{2+}$ transients (recordings over 5 s) and 40 ms for observance of changes in Ca$^{2+}$ levels over longer time periods. Since rhod-2 has no spectral shift after Ca binding, it is necessary to account for differences in dye loading or changes in tissue dye concentration over time (leakage or photobleaching). Therefore, tissue absorbance was quantified using the ratio of scattered excitation light at 524 nm (peak rhod-2 absorbance in myocardial tissue) and 589 nm (isosbestic point for rhod-2 in myocardium). The change in absorbance over time was then used to normalize emission light intensity by calculating fluorescence/absorbance (F/A) for each time point. In order to quantify $[Ca^{2+}]$, the maximum Ca$^{2+}$-induced rhod-2 fluorescence ($F_{\text{max}}$) must be determined at the end of each experiment because the fluorescence intensity is proportional to the amount of dye loaded. Therefore, $F_{\text{max}}$ was determined at the end of the experiment as follows: To induce Ca$^{2+}$ release from the sarcoplasmic reticulum, 2,2'-dithiodipyridine (100 µM)
was infused over a period of 2 min, immediately followed by calcium ionophore A23187 (calcimycin) in 10 ml 10% Ca\textsuperscript{2+} solution to maximize Ca\textsuperscript{2+} entry from the extracellular space. Fluorescence was recorded with a time resolution of 40 ms during the infusion, and maximum fluorescence was determined to calculate systolic and diastolic [Ca\textsuperscript{2+}], using the following equation:

\[
[\text{Ca}^{2+}] = K_d(\text{Ca}^{2+}\text{Rhod-2}) \times \frac{(F_i - F_o)}{(A_i/A_{max})(F_{max} - F_o) - (F_i - F_o)}
\]

where [Ca\textsuperscript{2+}] is the free intracellular Ca\textsuperscript{2+} concentration, \(K_d\) is the dissociation constant for rhod-2 with Ca\textsuperscript{2+}, \(F_i\) is fluorescence at a specific time point, \(F_o\) is background fluorescence measured before dye loading, \(A_i\) and \(A_{max}\) are tissue light absorbance at the specific time point or at the end of the experiment, respectively.

[Ca\textsuperscript{2+}] was recorded during the following ischemia/reperfusion protocols: 20 min unprotected ischemia at 37°C without cardioplegic arrest followed by 10 min reperfusion (n=6); 20 min ischemia at 20°C with cardioplegic arrest (20 mM K\textsuperscript+), followed by 10 min reperfusion. The fluorescence signal was then used to calculate [Ca\textsuperscript{2+}] in 5 min intervals, using the \(K_d(\text{Ca}^{2+}\text{Rhod-2})\) of 710 nM for every time point. This is the \(K_d(\text{Ca}^{2+}\text{Rhod-2})\) in a simulated intracellular myocardial environment that was determined by del Nido et al. when they first described rhod-2 spectrofluorometry for measurement of [Ca\textsuperscript{2+}] in isolated rabbit hearts [5]. Then, the calculation was repeated, but now the adjusted \(K_d\) for a given pH (derived from the NMR experiments) and temperature (according to the experimental protocol) was used for each time point.

2.5. Statistics

Linear and nonlinear regression analysis was utilized to determine the best fitting models for describing the relationship between pH and \(K_d\) at a given temperature. The Levenberg–Marquardt method was applied to derive nonlinear models. The coefficient of determination (\(R^2\)) was used to assess the proportion variability in \(K_d\) accounted for by pH in each regression model with goodness-of-fit evaluated by the F-test. SPSS statistical package (version 11.0) was used.

3. Results

3.1. Intracellular pH

As depicted in Fig. 1, pH decreased during ischemia. The extent of intracellular acidification depended largely on the temperature, reaching pH 6.05 after 45 min ischemia at 37°C, pH 6.4 at 20°C, and pH 6.6 at 4°C. Ischemia at 20°C with cardioplegic arrest resulted in mild acidification, reaching pH 6.8 after 45 min. Irrespective of temperature or cardioplegic arrest, pH increased rapidly during early reperfusion, reaching near-normal levels between pH 7.0 and pH 7.1 at 5 min reperfusion in all groups.

3.2. \(K_d\) for rhod-2 and Ca\textsuperscript{2+}

Fig. 2 depicts a typical series of in vitro rhod-2 fluorescence emission scans, recorded at increasing free Ca\textsuperscript{2+} concentrations (here: pH 7.1, 20°C). As expected, increasing Ca\textsuperscript{2+} in the solution results in an increase in fluorescence intensity. The impact of temperature on Ca\textsuperscript{2+}-induced rhod-2 fluorescence is demonstrated in Fig. 3A. Lowering temperature from 37°C to 20°C resulted in a marked rightward shift of the Ca\textsuperscript{2+}-fluorescence relationship, indicating that less rhod-2 fluorescence is induced at a given Ca\textsuperscript{2+} concentration. Further reducing temperature to 4°C increased the rightward shift moderately. Hence, it should be noted that lowering temperature from 37°C to room temperature, as easily happens in experimental settings studying ischemic and thus non-perfused hearts, has a significant effect on intensity of the fluorescence signal. The relationship between pH and Ca\textsuperscript{2+}-induced rhod-2 fluorescence at 37°C is shown in Fig. 3B. It is evident that lowering pH from pH 7.1 to pH 6.8 had no significant effect on Ca\textsuperscript{2+}-induced rhod-2 fluorescence, both curves are virtually congruent. At pH 6.5, however, a marked rightward shift was apparent, again indicating a
the relationship between $K_d$ ($Ca^{2+}$Rhod-2) and pH (Fig. 4). At 37 °C and pH 7.1, $K_d$ was calculated as 480 nM, and remained more or less stable up to pH 6.8 ($K_d=484$ nM). With further lowered pH, $K_d$ rose steeply, was determined to equal 2459 nM at pH 6.2. A quadratic model had the highest $R^2$ among the regression models tested ($R^2=0.996$) and represented the most accurate description of the relationship between pH and $K_d$ ($F$-test=961.5, $P<0.0001$). It is described by the following equation:

$$K_d(Ca^{2+}\text{Rhod-2})_{37°C} = 190.62 - (54.98 \times \text{pH}) + (3.97 \times \text{pH}^2)$$

Fig. 3. Fluorescence intensity measured at increasing calcium concentrations in a simulated intracellular environment at varying temperature (A) and at different pH (B). The y-axis depicts fluorescence emission at 589 nm during excitation at 524 nm. pH or temperature-corrected free calcium concentrations of the EGTA-buffered calibration solution were used on the x-axis. Note that a lowering temperature from 37 °C to 20 °C results in a significant rightward shift of the curve, indicating a decrease in fluorescence emitted at a given calcium concentration (A). The emission signal at pH 7.1 and pH 6.8 are virtually congruent, whereas a rightward shift is apparent at pH 6.5 and pH 6.2 (B). The depicted data were obtained in single preparation. To ensure reproducibility, the $K_d$ measurements were repeated twice and gave virtually identical values.

Once the actual $K_d$ for rhod-2 and $Ca^{2+}$ was determined for each pH increment at a given temperature, we plotted the relationship between $K_d$ ($Ca^{2+}$Rhod-2) and pH (Fig. 4). At 37 °C and pH 7.1, $K_d$ was calculated as 480 nM, and remained more or less stable up to pH 6.8 ($K_d=484$ nM). With further lowered pH, $K_d$ rose steeply, was determined to equal 2459 nM at pH 6.2. A quadratic model had the highest $R^2$ among the regression models tested ($R^2=0.996$) and represented the most accurate description of the relationship between pH and $K_d$ ($F$-test=961.5, $P<0.0001$). It is described by the following equation:

$$K_d(Ca^{2+}\text{Rhod-2})_{37°C} = 190.62 - (54.98 \times \text{pH}) + (3.97 \times \text{pH}^2)$$

Fig. 4. Relationship between the dissociation constant for rhod-2 and calcium and intracellular pH in whole hearts at 37 °C (A) and at 20 °C (B). Depicted are measured values (open circles) and the quadratic regression (see Results section for model characteristics). Note that $K_d$ remains relatively stable with acidifying pH up to pH 6.6. When intracellular pH drops further, $K_d$ rises, indicating a decreasing affinity of rhod-2 for calcium. The depicted data were obtained in single preparation. To ensure reproducibility, the $K_d$ measurements were repeated twice and were virtually identical.
At 20°C and pH 7.1, $K_d$ was 935 nM, and again remained stable up to pH 6.8 ($K_d$ = 877 nM). Then, the curve rose parallel to that at 37°C, reaching 4.8 μM at pH 6.2. Again, a quadratic model best described the relationship between pH and $K_d$ ($R^2 = 0.995$, $F$-test = 711.9, $P < 0.0001$):

$$K_d[Ca^{2+}\text{Rhod-2}]_{20^\circ C} = 395.75 - (114.53 \times \text{pH}) + (8.31 \times \text{pH}^2)$$

At 4°C and pH 7.1, however, $K_d$ was calculated as 10-fold higher than at 37°C (4354 nM), remained at that level up to pH 6.8, and then increased even more. Because the high $K_d$ does not permit accurate estimation of $[Ca^{2+}]$, at 4°C the data were not further analyzed.

3.3. $[Ca^{2+}]$ measurements

Fig. 5A depicts the rhod-2 fluorescence recorded from the LV surface of an intact perfused rabbit heart at 589 nm during 20 min ischemia at 37°C, followed by 10 min reperfusion. Signal intensity rises by approximately 100%

![Graph A]

![Graph B]

![Graph C]

Fig. 5. (A) Rhod-2 fluorescence at 589 nm recorded from the left ventricular surface of a Langendorff-perfused rabbit heart subjected to 20 min ischemia at 37°C (interrupted lines). (B) Cytosolic calcium concentration was calculated using the conventional dissociation constant for rhod-2 and calcium in an intracellular environment, 710 nM. Note that the maximum rise in $Ca^{2+}$ appears to occur during early reperfusion. (C) Here, cytosolic calcium concentration was computed using the pH-adjusted dissociation constant for rhod-2 and calcium at 37°C. Note that here, cytosolic calcium increases markedly during the period of ischemia, and decreases during early reperfusion.
during the ischemic period. On reperfusion, there is a rapid
and pronounced increase in fluorescence, followed by a
rather rapid decrease over the next 5 min. When \([\text{Ca}^{2+}]_i\) is
calculated after determining \(F_{\text{max}}\) using a constant \(K_d\)
(here: 710 nM) (Fig. 5B), mean \([\text{Ca}^{2+}]_i\) appears to
increase only mildly during ischemia, followed by an
immediate increase on early reperfusion. However, when
the pH-adjusted \(K_d\) is used to quantify \([\text{Ca}^{2+}]_i\) at each time
point (Fig. 5C), it becomes evident that \([\text{Ca}^{2+}]_i\) rises by
almost 400% throughout the period of ischemia
(431±37% in four experiments), followed by a rapid
decrease during early reperfusion, without reaching pre-
ischemic levels during the first 10 min of reperfusion.
Beat-to-beat \(\text{Ca}^{2+}\) transients during 15 min unprotected
ischemia are shown in Fig. 6. As mean \([\text{Ca}^{2+}]_i\) increases,
the amplitude of the \(\text{Ca}^{2+}\) transient decreases. After 20
min ischemia, no rhythmic \(\text{Ca}^{2+}\) transients were record-
able. In order to mimic the cardiac surgical situation of
cardioplegic arrest at lowered temperature, we recorded
cytosolic \(\text{Ca}^{2+}\) in rabbit hearts subjected to 20 min
ischemia at room temperature with high-potassium cardiac
arrest. As shown in Fig. 7A, injection of the cardioplegic
solution effectively abolished cytosolic \(\text{Ca}^{2+}\) transients.
Over the following period of ischemia at 20 °C, the rhod-2
fluorescence signal increased only by approximately 10%.
On reperfusion, there was again a rapid increase in signal
intensity, followed by a decline over the next 5 min. When
\([\text{Ca}^{2+}]_i\) was then calculated using a fixed \(K_d\) of 710 nM,
the plot depicting mean \([\text{Ca}^{2+}]_i\), (Fig. 7B) follows the raw
fluorescence signal in parallel. However, when the pH-
adjusted \(K_d\) is used in to determine the actual cytosolic
\(\text{Ca}^{2+}\) concentration, it becomes clear that \([\text{Ca}^{2+}]_i\) increases
by approximately 80% during ischemia (78±19% in four
experiments), and decreases immediately on reperfusion
(Fig. 7C).

4. Discussion

As expected, based on the physicochemical properties of
a \(\text{Ca}^{2+}\) indicator dye such as rhod-2, we found the
dissociation constant \(K_d\) for rhod-2 and \(\text{Ca}^{2+}\) to be
dependent on pH and temperature. At lower pH, ac-
cumulating protons compete with \(\text{Ca}^{2+}\) ions at the binding
sites resulting in a net decrease of \(\text{Ca}^{2+}\) affinity of the dye,
while lower temperature decreases the quantum efficiency
of the dye, i.e. blunting the fluorescence intensity emitted
when a \(\text{Ca}^{2+}\) ion binds to rhod-2. Both mechanisms

![Fig. 6. Calcium transients recorded from the LV surface of a heart subjected to 15 min normothermic ischemia. (A) The cytosolic calcium concentration was calculated at each time point using the pH-adjusted dissociation constant (\(K_d\)) for rhod-2 and calcium. (B) \([\text{Ca}^{2+}]_i\), as calculated using a constant \(K_d\) (here: 710 nM).](image-url)
Fig. 7. (A) Rhod-2 fluorescence at 589 nm recorded from the left ventricular surface of a Langendorff-perfused rabbit heart subjected to 20 min ischemia with high-potassium cardioplegic arrest at 20 °C (interrupted lines). (B) Cytosolic calcium concentration was calculated using the conventional dissociation constant for rhod-2 and calcium in an intracellular environment, 710 nM. Note that [Ca\(^{2+}\)], does not seem to rise during ischemia, but a large increase in [Ca\(^{2+}\)], appears to occur during early reperfusion. (C) Here, the cytosolic calcium concentration was computed using the pH-adjusted dissociation constant for rhod-2 and calcium at 37 °C. Note that cytosolic calcium increases mildly but steadily throughout the period of ischemia, and decreases during early reperfusion.

Inevitably increase the effective $K_d$ for rhod-2 and Ca\(^{2+}\). Hence, if the fluorescence signal appears not to change while intracellular pH and/or temperature drop, although [Ca\(^{2+}\)] does in fact rise. Or, in other words, the actual increase in [Ca\(^{2+}\)] during ischemia is systematically underestimated if $K_d$ is not adjusted according to the changes in pH or temperature. The pathophysiologic relevance is that, in contrast to previous reports based on Ca\(^{2+}\) recordings quantified using a static $K_d$, [Ca\(^{2+}\)], mainly rises during the period of ischemia and not during early reperfusion. Based on our findings, the increase in signal intensity on reperfusion is caused by the rapidly normalizing intracellular pH (and thus decreasing $K_d$). The clinical implication is that strategies aiming at inhibiting the rise in [Ca\(^{2+}\)] during ischemia may be more effective than those that focus on the early reperfusion period.

Various techniques to assess the [Ca\(^{2+}\)] have been developed. NMR measurements using BAPTA-based Ca\(^{2+}\)-sensitive indicators first allowed for observation of changes in [Ca\(^{2+}\)] over time [6], and beat-to-beat recordings of Ca\(^{2+}\) transients became possible using fluorescent or luminescent indicators. Measurements of intracellular
Ca\(^{2+}\) handling during whole-organ ischemia and reperfusion have been reported since the late 1980s using fluorescent indicators such as Indo-1, Fura-2, Fluo-3, or the visible light-emitting protein aequorin. Although we focused on rhod-2, it is important to acknowledge that the principles of pH- and temperature depending \(K_{d}\) apply to all ion-sensitive indicator molecules. Following Lattanzio and Pressman’s recordings of intracellular Ca\(^{2+}\) transients using the first-generation indicator Quin-2 [7,8], Lee et al. established the measurement of \([\text{Ca}^{2+}]_{i}\) in intact rabbit hearts using Indo-1 [9]. They described a rapid increase of systolic and diastolic \([\text{Ca}^{2+}]_{i}\), during the first 30 s of global normothermic ischemia that reached a plateau after 90 s. Subsequently, the same group described the changes in \([\text{Ca}^{2+}]_{i}\), during 2–3 min ischemia in greater detail [10]. Again, they observed a rapid increase of the Indo-1 fluorescence ratio that appeared to reach a plateau in less than 60 s. However, they did not attempt to quantify \([\text{Ca}^{2+}]_{i}\), using the dissociation constant, assuming that the raw fluorescence signal correlates closely with \([\text{Ca}^{2+}]_{i}\). Since all BAPTA-based indicators are to some extent pH sensitive, the investigators performed a series of experiments lowering pH in a mixture of Ca\(^{2+}\) and Indo-1, and no change in fluorescence intensity was obvious. In response, Lattanzio systematically studied the pH dependency of the \(K_{d}\) for Indo-1 and Ca\(^{2+}\) in the setting of myocardial acidosis [11,12]. By loading isolated rat hearts with Indo-1 and the pH sensitive indicator BCECF, he demonstrated that a rise of mean \([\text{Ca}^{2+}]_{i}\), from 170 nM to 390 nM (230%) during 8 min global ischemia was not reflected by the Indo-1 fluorescence ratio, because the simultaneous drop in pH decreased the affinity of Indo-1 for Ca\(^{2+}\). Jimenez et al. described the redistribution of Ca\(^{2+}\) between the membranous and cytosolic compartment during 60 min global ischemia using fura-2, and did not observe a significant rise of \([\text{Ca}^{2+}]_{i}\), at 20 min [13]. However, these measurements were not corrected for pH-induced changes in \(K_{d}\) either, and hence the actual increase in \([\text{Ca}^{2+}]_{i}\), was probably underestimated. More recently, Morgan and coworkers studied the intracellular Ca\(^{2+}\) homeostasis during ischemia and reperfusion using the bioluminescent protein aequorin, which is injected subepicardially and emits photons as visible light upon binding to Ca\(^{2+}\). Using this technique, Kihara et al. recorded \([\text{Ca}^{2+}]_{i}\), in isolated ferret hearts during 3 min global normothermic ischemia, and observed only a mild increase in \([\text{Ca}^{2+}]_{i}\), [14]. Later, the technique was adapted to rat hearts, and they observed an initial increase in Ca\(^{2+}\) transient amplitude during 10 min global ischemia, followed by a very mild increase in diastolic \([\text{Ca}^{2+}]_{i}\), [15]. During reperfusion, however, a rapid increase in \([\text{Ca}^{2+}]_{i}\), was described and interpreted as Ca\(^{2+}\) influx from the extracellular space. Subsequently, the aequorin technique was also used in isolated mice hearts [16]. Again, only a mild increase in \([\text{Ca}^{2+}]_{i}\), during the ischemic period of 15 min was described, followed by a significant increase in signal intensity on early reperfusion. The physicochemical properties of the protein aequorin are of course not comparable with those of the BAPTA-based fluorescence indicators, and a systematic investigation of pH and temperature dependency of the dissociation constant for aequorin and Ca\(^{2+}\) has not been reported. However, it can be assumed that the affinity of aequorin for Ca\(^{2+}\) is also pH-sensitive, and that its quantum efficiency is a function of the ambient temperature. If this is indeed the case, one can infer that the true increase of \([\text{Ca}^{2+}]_{i}\), in these studies was also systematically underestimated, while the apparent rapid \([\text{Ca}^{2+}]_{i}\), increase on reperfusion is in fact a function of the normalizing intracellular pH.

In the majority of the studies investigating post-ischemic Ca\(^{2+}\) homeostasis, unprotected normothermic ischemia is utilized to simulate clinical myocardial ischemia. However, various cardioprotective measures are routinely used during cardiac surgical procedures, but little is known about the effects of cardioplegic arrest and hypothermic ischemia on intracellular Ca\(^{2+}\) handling. When we measured \([\text{Ca}^{2+}]_{i}\), during cardioplegic arrest at 20°C, only a very mild increase was noted when a constant \(K_{d}\) was used for calculation. By using the appropriate pH and temperature corrected \(K_{d}\) at a given time point, it became evident that, in fact, there was an 80% increase in \([\text{Ca}^{2+}]_{i}\). This indicates that even routinely used methods of cardioprotection are associated with a significant intracellular Ca\(^{2+}\) overload. Determination of \([\text{Ca}^{2+}]_{i}\), at low temperature, however, is not possible with current methods. The exceedingly high \(K_{d}\) for rhod-2 and Ca\(^{2+}\) is probably due to the low quantum efficiency of the dye at low temperatures, but perhaps also due to altered binding kinetics for Ca\(^{2+}\) at the binding site in the rhod-2 molecule that decreases the affinity of the dye for Ca\(^{2+}\). Whatever the mechanism, the effective \(K_{d}\) is too high to allow for an assessment of \([\text{Ca}^{2+}]_{i}\), with reasonable accuracy.

4.1. Rhod-2 for measurement of \([\text{Ca}^{2+}]_{i}\). Rhod-2, which was first introduced in 1989, is a typical modern long-wavelength indicator that is valuable for experiments in cells and tissues with high levels of autofluorescence [17]. Rhod-2 fluorescence intensity increases >100-fold upon binding Ca\(^{2+}\), and a good signal-to-noise ratio can be achieved with very low intracellular rhod-2 concentrations. The estimated intracellular rhod-2 concentration in our model in less than 2 μM, compared with millimolar concentrations of older indicators such as Quin-2, or typically 20–30 μM with fura-2 [18]. The superior signal quality of rhod-2 allows for real-time recordings with extremely high spatial and/or temporal resolution in tissue [19,20]. For example, Qian et al. recorded high-quality Ca\(^{2+}\) transients with an imaging frequency of 300 frames/s in blood-perfused rabbit hearts and were able to show marked spatial heterogeneity of the Ca\(^{2+}\) transient during the early phase of ischemia [21]. A fluorescent Ca\(^{2+}\) indicator should have an appropriate Ca\(^{2+}\) affinity, ideally somewhat higher than the basal
4.2. Limitations of the study

There are several limitations to our study that are important to acknowledge. Intracellular pH and $[Ca^{2+}]$ were not measured simultaneously but in separate sets of experiments using different methods ($^{31}P$ NMR spectroscopy and rhod-2 spectrofluorometry). However, the change in intracellular pH is usually very uniform in a given ischemia-reperfusion protocol, and the inference made should at least allow for proof-of-principle. The cytosolic concentration of other intracellular cations such as Mg$^{2+}$ or Na$^+$ also change significantly during myocardial ischemia. While a changing Na$^+$ concentration in a biological system is unlikely to influence the affinity of indicator for Ca$^{2+}$, the bivalent cations Mg$^{2+}$ probably competes significantly with Ca$^{2+}$ at the binding site. The cytosolic Mg$^{2+}$ concentration is known to increase markedly during ischemia, and we did not adjust for this change in our experiments. Furthermore, oxygen free radicals, which are produced in abundance during ischemia and reperfusion, may also interfere with the binding affinity of a fluorescent indicator, but the magnitude of this effect remains unclear.

5. Conclusions

Observation of $[Ca^{2+}]$, during myocardial ischemia and reperfusion using fluorescent indicators such as rhod-2 is feasible, but care must be taken to account for changes in the $K_d$ for rhod-2 and Ca$^{2+}$ that are induced by changing pH and temperature. If $K_d$ is not adjusted accordingly, $[Ca^{2+}]$, is systematically underestimated, and this should apply to other indicators as well. Since experimental settings vary greatly, the $K_d$ values presented here should not be considered absolute, but $K_d$ should be re-evaluated whenever a new experimental protocol requires determination of $[Ca^{2+}]$.

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