Kasetsart J. (Nat. Sci.) 44 : 1137 - 1143 (2010)

Measurement of Relative Coronary Blood Flow in a Rabbit Model of Acute Myocardial Ischemia by the Optical Mapping Method with RH-237

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ABSTRACT

A method was developed to produce acute regional ischemia and reperfusion in a rabbit heart that was used in conjunction with optical voltage recordings and the method was validated for imaging relative myocardial perfusion. The left anterior descending branch of the coronary artery in an isolated Langendorff perfused rabbit heart was ligated using a snare to allow acute blockage and reperfusion of an apical region of the myocardium. The voltage-sensitive dye, RH237, was used to map electrical activity. RH237 is virtually non-fluorescent in free solution, but becomes fluorescent when partitioned into the cardiac membrane. Partitioning is very rapid (less than 1s) and so measuring the rate and extent of rise of fluorescence in the whole heart could be used to quantify local perfusion rates. The use of 0.33 μmole RH237 per gram tissue of the heart resulted in 50% maximal binding and this technique revealed the extent and shape of the ischemic region in the isolated perfused rabbit heart, in conjunction with electrical activity and using a single fluorescent dye.

Keywords: acute myocardial ischemia, RH237, rabbit heart

INTRODUCTION

Coronary artery disease (CAD) is a common precursor of sudden cardiac death worldwide (Davies, 1981). Animal heart models have been used for many years to gain insights into disturbance of the normal mechanical, physiological and biochemical functions in the human heart. The rabbit myocardium shows important similarities to the functions of the human heart, such as the predominance of β-myosin heavy chain isoforms and excitation-contraction coupling processes (Hasenfuss *et al.*, 1991; Bers, 2001). Previous studies have described a reliable chronic heart failure model in rabbits (Hasenfuss, 1998). However, so far few studies have looked specifically at the acute stage. The electrophysiological effects during the acute stage of ischemia using an isolated-perfused rabbit heart were largely unexplored prior to the current study. Observing abnormalities of regional myocardial perfusion is a cornerstone in the diagnosis of coronary artery disease and determination of myocardial viability. Many approaches have been

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Received date : 04/05/10 Accepted date : 12/07/10

used in an attempt to provide precise measurements of regional myocardial blood flow, including the inert gas wash out technique and positron emission tomography (PET), while the injection of radio-labelled microspheres or ultrasound contrast agents have been used to map the route of the vessels through the tissue (Page, 2004). The non-invasive PET technique can be used to quantify regional myocardial flow in vivo, but the technique is not widely available because it requires special, costly equipment. Myocardial contrast echocardiography (MCE) is an emerging technique that is able to assess rapidly myocardial perfusion at the capillary level in many different clinical settings (Lichten and Engel, 1979; Kamiya *et al*., 2002; Kaufmann and Camici, 2005). The thermal diffusion method allows real-time, continuous, tissue blood-flow measurement based on the relationship between the blood flow and changes in the temperature gradient using a thermal probe. This technique can be used in beating hearts and other organs, such as the liver and brain, during surgery (Kamiya *et al*., 2002). While several techniques are available to assess the coronary flow, there has been no study to date that has looked specifically at the correlation between the regional myocardial flow and electrophysiological properties during reductions in coronary perfusion. The objective of this study was to validate a new method to assess relative myocardial blood flow using the RH237 dye. Experimental measurement of the relative flow in acute myocardial ischemia would provide a novel procedure for the measurement of regional myocardial flow.

MATERIALS AND METHODS

Isolation of rabbit ventricular cardiomyocytes

Hearts from ten adult male New Zealand White rabbits were removed and set up in Langendorff perfusion as described above. They were then perfused with Krebs-Henseleit (HERPES-buffer) solution to which was added 1.4mg/mL collagenase (Type 1, Worthington Chemical Co.) and 0.1 mg/mL protease (Type XIV, Sigma Chemical Co.). Calcium (50μM) was added to activate the enzymes. After 6 min, the heart was perfused with 0.1% bovine serum albumin to stop digestion by providing substrate for any superfluous enzyme. The tissue was subsequently finely chopped in this solution. Cardiac myocytes were dissociated by trituration. Maintaining low extracellular Ca^{2+} subsequently ensured a high yield of rod-shaped myocytes, based on the protocols conducted by the Glasgow group for a number of years under Professor GL Smith, at the University of Glasgow.

Measurement of relative affinity of RH237 for cardiac cell membranes

Myocytes were loaded with RH237 by applying 0.3-30 μL of 1mM RH237 to 1.5mL cell suspension. The time course and concentration dependence of the RH237 binding were measured using a Perkin-Elmer fluorescence spectrophotometer (model LS55). A software application (Timedrive, The LS-55 fluorescence Spectrometers product Literature) was used to measure the change in fluorescence at 1 sample/second. An excitation wavelength of 475nm and an emission wavelength of 675 nm were used with the excitation and emission slit widths set at 20nm.

Measurement of the relative flow

Regional myocardial flow was assessed by measuring the rate of rise of the regional fluorescence signal following the rapid injection of a bolus of RH237 into the coronary circulation at pre-occlusion, after 15 min of occlusion and after 15 min of reperfusion. The principle of this technique is that binding of RH237 to cardiac membrane is both very fast (less than 1s) and irreversible. Thus, the local time course of the rise in fluorescence on injection of RH237 should reflect the extent of local coronary perfusion. The

study investigated whether the amplitude of the rise in fluorescence on injection of RH237 could be used to assess coronary flow. Signals were recorded simultaneously from 256 photodiodes at the rate of 100 Hz over 100 s. The optical action potential signals were transferred to MATLAB 7.0 and the relative perfusion rate was calculated as the ratio between the amplitude of the increase in fluorescence on injection of RH237 under control conditions and the amplitude of the signal obtained on injection of the same amount of RH237 during occlusion or low-flow ischemia.

Langendorff perfusion and left coronary artery occlusion

Thirty-four adult male New Zealand White rabbits (2.5-3.5 kg) were killed humanely by a procedure that conformed to 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) and the Animals (Scientific Procedures) Act 1986 of the United Kingdom. The hearts were isolated and transferred from chilled saline to the surgical rig and perfused at a rate of 20 mL/min with standard Tyrode's solution, as illustrated in Figure 1A. The pattern of coronary artery branching was recorded. The left posterior division of the coronary artery was ligated with 4-0 polyester (Ethibond™) and the ends of the ligature were threaded into a 2mm piece of polyethylene tubing to form a snare. Then, the heart was transferred rapidly to the optical rig and perfused in Langendorff mode using a Gilson Minipulse 3 peristaltic pump. The heart was perfused with Tyrode solution composed of (units shown in mM): Na^+, 134.5; K^+, 5.0; Ca^{2+}, 1.9; Mg^{2+},1.0; Cl^- , 101.8; SO_4^{2-}, 1.0; HPO_4^-, 0.7; HCO_3^-, 20; acetate (CH_3COO^-), 25; and glucose, 25. The solution was bubbled continuously with 95% O_2 and 5% CO_2 to maintain a constant pH of 7.4. The temperature was maintained at 37°C. The snare tubing was passed through a hole in the wall of the Plexiglass™ chamber and the ends of the ligature were connected to an external, calibrated spring device (Figure 1B).

Optical mapping

The heart was restrained inside the chamber in order to limit gross movement. In addition, motion artefact was reduced by perfusing the heart with 2, 3- butane-dione monoxime (BDM 15 mM) or blebblistatin (5 μM) (Molecular Probe, Inc). The heart was loaded with 100 μL of 2 mM solution of a voltage sensitive dye (RH237) in dimethyl sulfoxide (DMSO). The heart was illuminated using an array of light-emitting diodes with peak wavelength 525nm. The emitted light passed through a 645 nm long-pass filter and focused on a 16×16pixel Hamamatsu photodiode array. Optical action potentials were recorded from an area of approximately 13 mm × 13mm of epicardium on the anterior surface of the heart.

RESULTS AND DISCUSSION

Measurement of the capacity and relative affinity of cardiomyocytes for RH237

Figure 2 illustrates the rapid time course of the fluorescence increase on addition of RH237 to a cell suspension (1×10^5 cells/mL). The rise of fluorescence had a time course that was comparable to that achieved by simply adding cells loaded previously with fluorescent dye. These data suggested that the RH237 binds at least as fast as the mixing of the solution (less than 1s).

Correlation between amount of saturating [RH237] binding to rabbit heart tissue

Figure 3 shows the relationship between the amount of cardiac tissue in gram wet weight, the maximum fluorescence at saturation with RH237 (B_{max}) and amount of RH237 required for 50% binding ($K_{1/2}$). The tissue weight was calculated using conversion factors of 1.0×10^{-6} cell = 1.5 mg protein and 1.5mg protein= 0.015 g wet weight. These values were based on previous

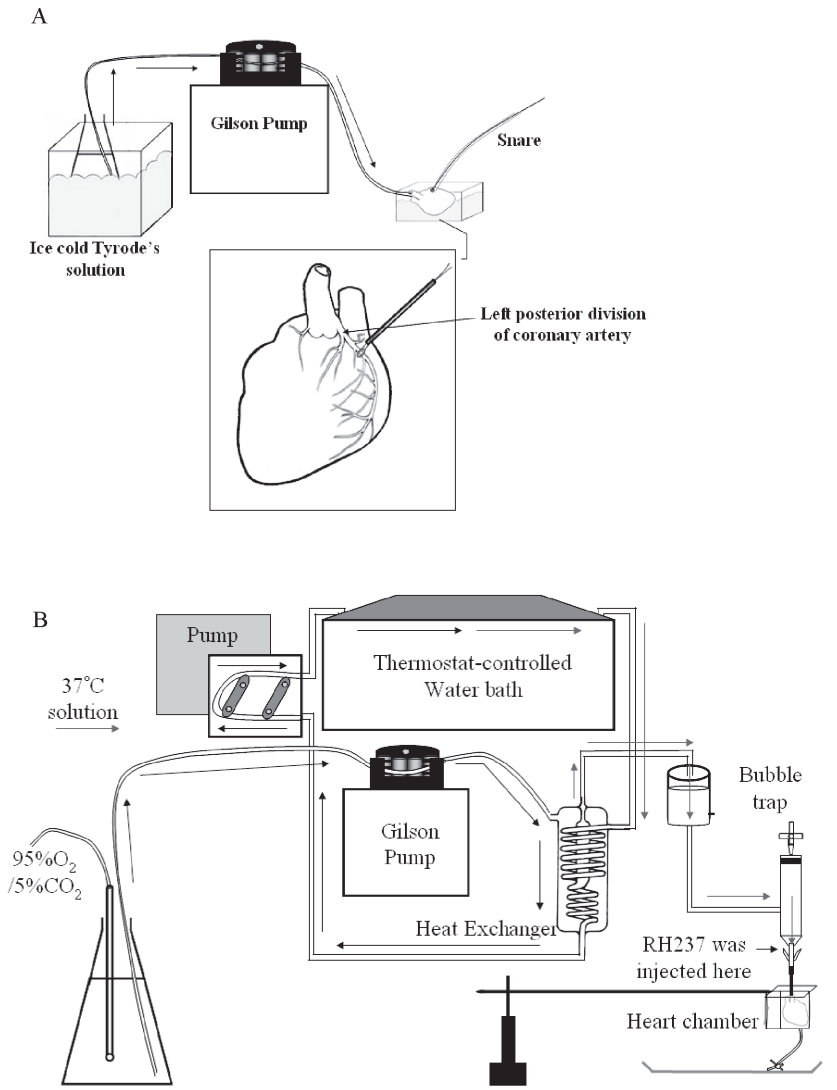

Figure 1 Surgical rig to attach snare (A) and Langendorff apparatus (B).

measurements of the total protein content of cells and the conversion of total protein to tissue weight (Duncan *et al.*, 2001). These values can be used to estimate the extent of binding of RH237 to the whole heart, when an aliquot of RH237 is injected as a bolus into the coronary circulation. Normally a bolus of 50 μL of 2.2mM RH237 is injected to load the heart. This represents approximately 0.1 μmole of RH237 injected into approximately 5 gram of wet weight of heart, or 0.02 μmole RH237 per gram tissue (wet weight). From the relationship shown in Figure 3, 0.33 μmole RH237 per gram tissue (wet weight) provides 50% of maximal binding. This suggested that the normal loading of the RH237 into a rabbit heart results in less than half the maximal loading of the cardiac membranes with RH237.

Relative perfusion rate during acute regional myocardial ischemia

Myocardial blood flow in humans can be assessed by several techniques that were described

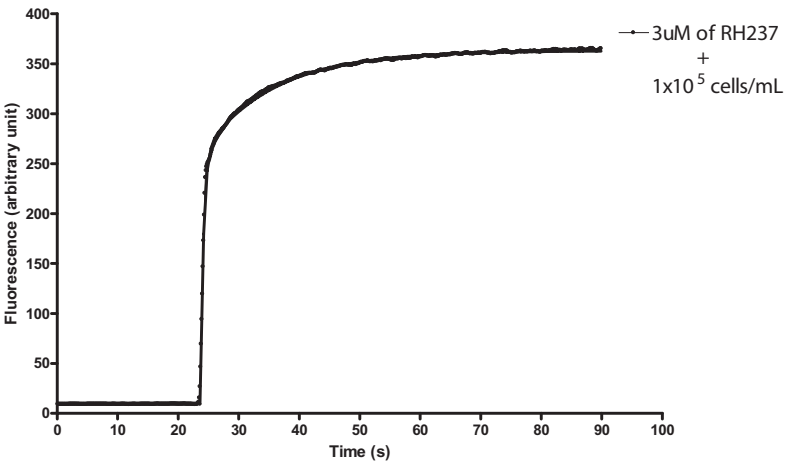

Figure 2 Kinetics of binding between RH237 and ventricular myocytes.

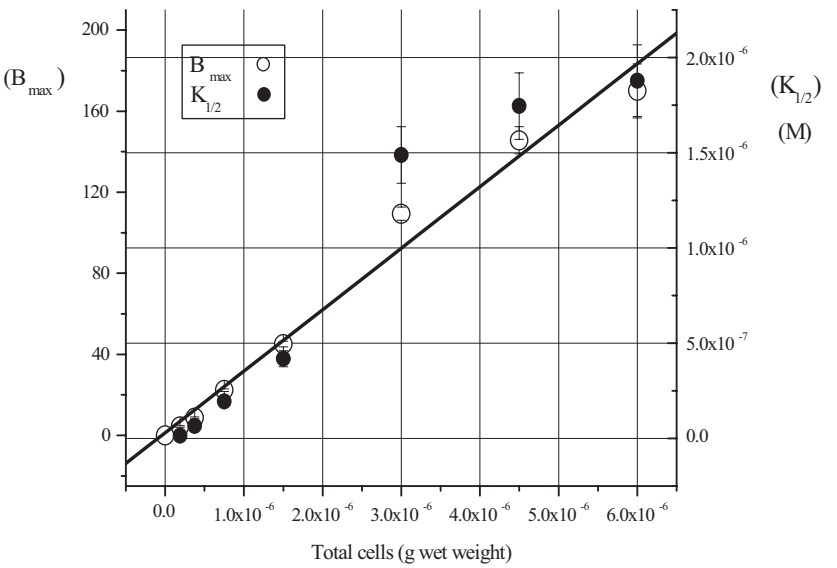

Figure 3 Relationship between total cells in gram wet weight, the fluorescence achieved at saturation with RH237 (B_{max}) and the concentration of RH237 required to produce half maximal fluorescence ($K_{1/2}$) The vertical bars show the standard error of the mean.

above. Although several methods are available to measure regional flow (Kamiya *et al*., 2002), to date, there has been no study that has looked specifically at the correlation between the regional myocardial flow and the electrophysiological properties during reductions in coronary perfusion. Figures 4A(1) and 4B(1) represent the colour maps of the relative flow at pre-occlusion and during occlusion, respectively. The relative perfusion rate as shown in Figure 4B(1) was calculated as the ratio between the RH237 loading profile under control conditions (30mL/min) and the signal during occlusion. The amplitude of the rise in fluorescence on injection of RH237 in zone (a) and zone (b) at pre occlusion and during occlusion are shown in Figures 4A(2) and B(2), respectively.

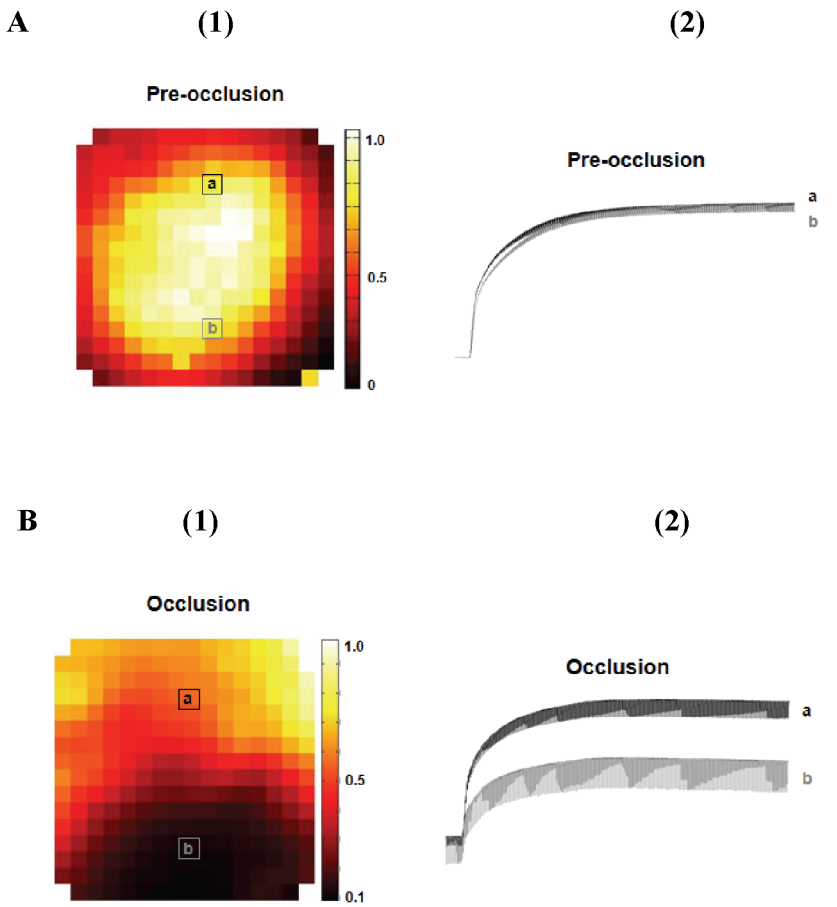

Figure 4 Relative flow, A(1) and B(1), representing the colour maps of the relative flow at pre-occlusion and during occlusion. The comparison between the amplitude of the rise in fluorescence on loading of RH237 in an apical area of the heart at zone (a) and zone (b) are shown in A(2) and B(2).

Figure 4B(2) shows the shortening of the amplitude of the rise in fluorescence on loading RH237 during occlusion. This result suggested that this simple and convenient approach by loading RH237 dye can be used to give an approximate indication of relative (not absolute) flow. However, this method is limited by the number of the injections, because the membranes become saturated after a few injections, suggesting that the injections must be performed at key stages in the experimental protocol, as described in the Methods section.

CONCLUSION

In the current study, normal loading of RH237 into a rabbit heart was used to assess relative myocardial perfusion. This was a simple technique to reveal the pattern of the regional perfusion during local ischemia, in conjunction with electrical activity, using a single fluorescent dye. However, it is not applicable in vivo, due to the unknown toxicity of RH237 dyes. In addition, a further refinement of this technique is required to make the signal a better indicator of the absolute flow.

ACKNOWLEDGEMENTS

This study was supported by a scholarship from the Ministry of Science and Technology, Thailand. The author would like to thank Mrs Aileen Rankin and Mrs June Irvine, from the Faculty of Biomedical and Life Sciences, University of Glasgow, UK for arranging and preparing the laboratory animals and the isolated myocytes.

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