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## Measurement of extracellular pH, $K^+$ , and lactate in ischemic heart<sup>☆</sup>

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### Abstract

Simultaneous and continuous measurements of extracellular pH, potassium ( $K^+$ ), and lactate ( $L^-$ ) in ischemic rabbit papillary muscle are presented for the first time. Potentiometric pH and  $K^+$  sensors and an amperometric lactate biosensor were used. These miniature electrodes were previously developed and individually tested for this purpose. The pH sensor was based on an iridium oxide layer electrode deposited on a planar platinum electrode fabricated on a flexible substrate. The potentiometric  $K^+$  sensor was based on a polymeric membrane and valinomycin ionophore. The  $L^-$  biosensor was based on lactate oxidase and an organic conducting salt polarized at 0.15 V vs Ag/AgCl reference electrode. The utility of this novel analytical system to cardiovascular research was demonstrated by using the system to study the interrelationship of cellular  $K^+$  and lactate loss in ischemic myocardium, and the role of extracellular pH and buffer capacity on this relationship. The results indicated: (i) sequential brief episodes of ischemia produced reproducible trends of  $L^-$ , pH, and  $K^+$  changes during the first three episodes, (ii) extracellular  $L^-$  increased with increasing buffer capacity of extracellular compartment, (iii) the patterns of extracellular  $L^-$  and  $K^+$  changes were not related directly, and (iv)  $L^-$  transport and lactic acid diffusion were not the primary cause of extracellular acidosis during ischemia. © 2002 Elsevier Science (USA). All rights reserved.

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Myocardial ischemia is characterized by the inhibition of oxidative phosphorylation and the onset of anaerobic glycolysis with attendant lactic acid production, intracellular acidification, and cellular  $K^+$  loss. In the absence of extracellular washout, the accumulation of extracellular  $K^+$  accounts for much of the early electrophysiological changes measured in ischemic heart muscle [1]. These electrophysiological changes constitute an electrical substrate necessary for the initiation of ventricular arrhythmias accounting for the high mortality rate associated with myocardial ischemia [2]. As such, the mechanism explaining the

cellular  $K^+$  loss is clinically important. A close temporal relationship between extracellular lactate ( $L^-$ ) and  $K^+$  loss exists in ischemic myocardium [3,4]; yet, a definitive mechanism explaining cellular  $K^+$  loss has not been confirmed. Several hypotheses have been proposed to explain cellular  $K^+$  loss. Cellular  $K^+$  loss may occur in ischemic tissue to balance the charge caused by electrogenic lactate transport [5–7]. This concept is supported by a pH dependence of cellular  $K^+$  loss in ischemic heart [7,8] and skeletal muscle [9]. Alternatively, the opening of  $K^+$  channels or passive  $K^+$  movement in response to cellular loading of  $Na^+$  may facilitate the loss of cellular  $K^+$ .

To address the quantitative relationship between cellular  $L^-$ ,  $H^+$ , and  $K^+$  loss, two sensors having the appropriate miniature size and operational characteristics for measurements at the surface of an isolated arterially perfused rabbit papillary muscle were specifically developed [10,11]. These sensors include the  $L^-$  biosensor based on an organic conducting salt,

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TTF-TCNQ<sup>2</sup> [12], and the pH sensor based on anodic electrodeposited iridium oxide film [4]. These two sensors, when combined with the previously developed miniature K<sup>+</sup> potentiometric sensor [13] based on polymeric membrane and valinomycin as K<sup>+</sup> ionophore, can provide a powerful analytical research tool [4]. Therefore, once the L<sup>-</sup>, pH, and K<sup>+</sup> sensors became available with adequate size and operational characteristics, we proceeded to the main goal, i.e., to realize the simultaneous and continuous measurements of extracellular L<sup>-</sup>, pH, and K<sup>+</sup> during ischemia.

To demonstrate the general utility of the developed technology for the simultaneous measurements we conducted a series of experiments in two stages. The first stage assessed the reproducibility of changes in the extracellular concentration–time profiles for L<sup>-</sup>, H<sup>+</sup>, and K<sup>+</sup> during three successive 10-min ischemic episodes using the same papillary muscle and a single set of experimental conditions. In the second stage, studies were designed to address a controversial question: “Does L<sup>-</sup> loss from the intracellular compartment play any role in K<sup>+</sup> efflux from intra- to extracellular compartments in ischemic myocardium?” To address this question, a series of experimental protocols were designed to either increase or decrease extracellular L<sup>-</sup> accumulation or decrease intracellular Na<sup>+</sup> loading and to monitor simultaneously the attendant changes in K<sup>+</sup> efflux. The outcomes of these experiments are discussed.

## Materials and methods

Lyophilized lactate oxidase, EC 1.1.3.2., from *Pediococcus* sp., 34 U/mg, L-lactic acid (lithium salt), bovine serum albumin (fraction V), and glutaraldehyde were obtained from Sigma Chemical (St. Louis, MO). TTF and TCNQ were obtained from Aldrich Chemical (Milwaukee, WI). Iridium tetrachloride of 99.95% purity was obtained from Alfa (Ward Hill, MA). All other chemicals were of the analytical reagent grade. All solutions were prepared with water from a Barnstead Nanopure II system.

A Princeton Applied Research Model 363 Potentiostat/Galvanostat was utilized for amperometric L<sup>-</sup> measurements. An Orion pH/mV meter (Model 720 A) was used for pH measurements. Potassium measurements were accomplished using a custom-made differential amplifier with high input impedance. A miniature Ag/AgCl reference electrode, AgCl-saturated 3 M KCl,

with a flexible barrel (Cypress System, Lawrence, KS), was used as a common reference for the three sensors.

The pH sensors were based on AEIROF as a pH-sensing layer [4]. Sputtered platinum on flexible Kapton films were used as planar-electrode substrates. The preparation and characterization of the pH sensors were previously described [4]. The K<sup>+</sup> biosensors were miniature polymeric ion-selective electrodes, based on valinomycin as ionophore and prepared as previously described [13]. The L<sup>-</sup> biosensors were based on cross-linked lactate oxidase embedded in organic conducting salt, TTF-TCNQ. This biosensor was developed to function properly under oxygen depletion present in ischemic tissue. The active part of the sensor (top) was reduced by 40% (1.8 × 1.5 mm vs 3 × 1.5 mm) compared to that previously described [12]. This reduction was made to accommodate smaller papillary muscles having diameters of 1 mm and lengths of 4 mm, thus maximizing the available space on the surface of the muscle for the pH and K<sup>+</sup> sensors (see Fig. 1). The cavity containing the conducting salt and the enzyme was reduced also by 60%. The response time was optimized by avoiding the formation of a thick enzyme layer by proportionally reducing the amount of the enzyme mixture solution. The performance characteristics of the miniaturized L<sup>-</sup> biosensor were almost the same as those reported previously [4].

The experimental technique for simultaneous measurements of extracellular L<sup>-</sup>, pH, and K<sup>+</sup> in ischemic rabbit papillary muscle was described previously [12]. New Zealand white rabbits (*n* = 30) were anticoagulated and anesthetized. In each case the heart was rap-

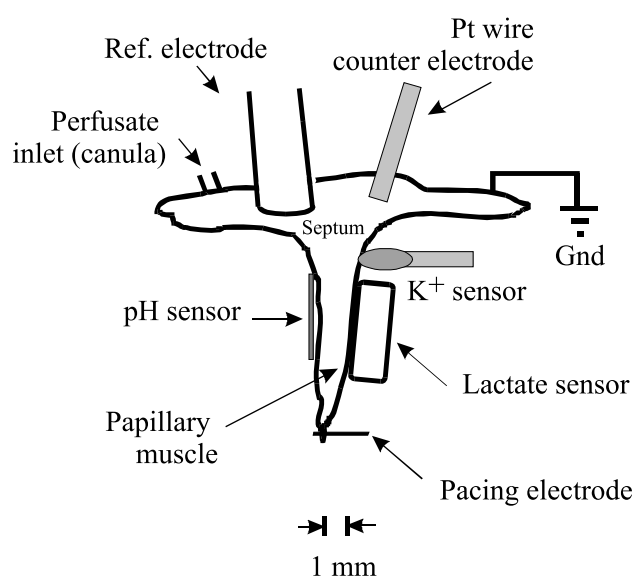


Fig. 1. Top view of the isolated arterially perfused rabbit papillary muscle preparation with lactate, pH, and K<sup>+</sup> biosensors positioned at the cylindrical part and the reference and counter electrodes positioned at the septum.

<sup>2</sup> Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); TTF, tetrathiafulvalene; Tris, tris(hydroxymethyl)aminoethane; BT, bis(2-hydroxymethyl)iminotris(hydroxymethyl)methane; TCNQ, 7,7,8,8-tetracyanoquinodimethane; AEIROF, anodic electrodeposited iridium oxide film; EIPA, ethyl isopropyl amiloride.

idly excised and arrested in cold Tyrode's solution. The atria and left ventricular free wall were removed and the left ventricular septal surface of the tissue was pinned to a wax plate. The septal artery was cannulated with a small polyethylene catheter and perfused with a modified Tyrode's perfusate. The perfusate was pumped through a custom-made membrane gas exchanger affording control of the partial pressures of  $O_2$ ,  $N_2$ , and  $CO_2$  of the perfusate. The relative amount of  $CO_2$  was adjusted in the membrane gas exchanger to yield a pH of 7.40. When bicarbonate was not used as buffer in the perfusate,  $CO_2$  was omitted and pH of the perfusate stock solution was adjusted by addition of NaOH and HCl. The longest muscle was chosen, attached by its tendon to a piezoresistive element and stimulated at 2 Hz through a platinum wire attached at the tendonous end. The chamber was closed and a humidified atmosphere surrounded the preparation. At 45 s prior to the arrest of flow, the atmosphere in the recording chamber was changed from a mixture of  $CO_2$ ,  $N_2$ , and  $O_2$  to a mixture of  $CO_2$  and  $N_2$ .

$L^-$ , pH, and  $K^+$  biosensors were positioned against the surface of the cylindrical part of the muscle (Fig. 1). The miniature Ag/AgCl electrode was positioned at the base of the muscle and used as a common reference for all the three sensors. The  $L^-$  biosensor was polarized at 0.15 V relative to the Ag/AgCl electrode. While the muscle was perfused, the three sensors were allowed to reach a stable baseline. The signal output of each electrode was measured using a separate chart recorder. Ischemia was induced by arresting flow and decreasing the  $O_2$  tension inside the chamber to 3–6 mm Hg. Ischemia was terminated by restoring the  $O_2$  to the surrounding atmosphere and reperfusing the muscle, unless otherwise stated. The muscle recovered for at least 30 min before another ischemic episode. Up to three or four ischemic episodes can be studied using the same muscle within a period of 4–5 h. The electrodes were calibrated in situ, at the end of the experiment, by perfusing the muscle with two standard solutions containing two concentrations of  $L^-$ , pH, and  $K^+$  prepared in the same perfusate solution. The standard concentrations were 8.0 and 16.0 mM  $K^+$ , 3.0 and 6.0 mM  $L^-$ , and pH 6.9 and 6.4, for standards I and II, respectively.

## Results

### Concentration–time profiles of $L^-$ , pH, and $K^+$ changes during ischemia

Fig. 2 shows the typical profiles of extracellular  $L^-$ , pH, and  $K^+$  obtained during ischemia using perfusate solution buffered with 10 mM Hepes. The  $L^-$  and pH profiles were similar, in that there was a small steady increase in the measured extracellular  $L^-$  and acidosis

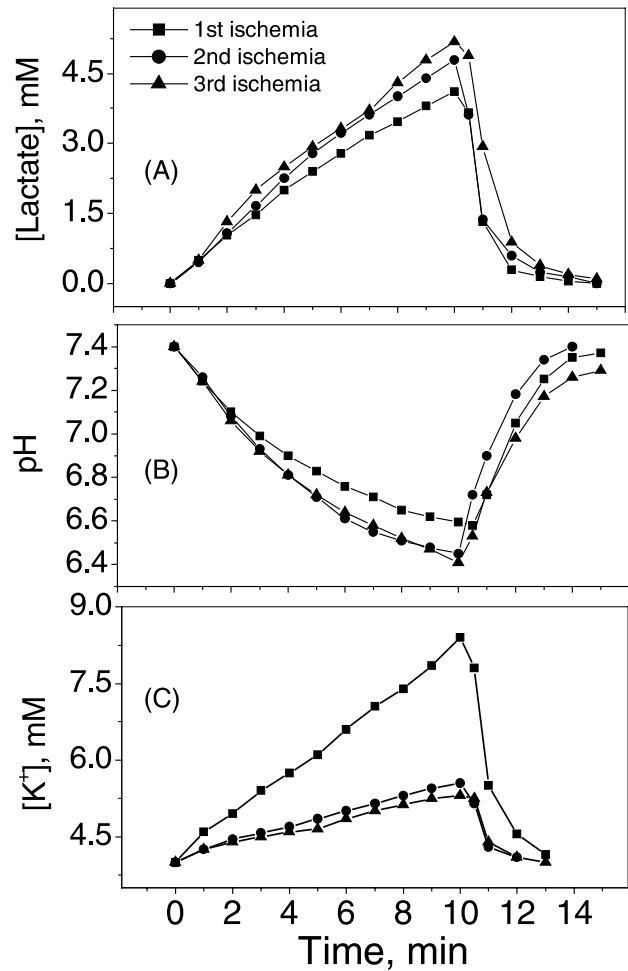


Fig. 2. Typical profiles of extracellular  $L^-$ , pH, and  $K^+$  changes during the first, second, and third 10-min ischemic episodes are shown. Perfusate solution buffered with 10 mM Hepes. The time zero refers to the onset of ischemia by arresting the flow. During reperfusion, the accumulated species are washed out and the sensors return to their respective baselines.

with each successive ischemic event. The  $K^+$  profile was significantly different from those of the other two chemical species. The highest extracellular  $K^+$  was observed in the first ischemic episode. In the subsequent episodes, ischemia-induced  $K^+$  accumulation reached only about half the first value measured. Similar trends were observed using other biological buffers, as will be discussed.

Profiles obtained with the bicarbonate buffer-based perfusate (data not shown) showed the same trends. However, in the remainder of the work, bicarbonate-based perfusates were excluded to simplify the experimental conditions by eliminating the requirement to control  $PCO_2$  and to eliminate the contribution of  $HCO_3^-$ -dependent cellular  $Na^+$  loading. Other advantages are offered by Hepes and the other biological buffers used, i.e., Tris and BT. These include the ease of manipulation of the perfusate pH, buffer capacity, and

the possibility of using a mixture of buffers to assess the role of initial extracellular pH on  $L^-$  and  $K^+$  loss at a comparable extracellular buffer capacity.

#### Effect of buffer capacity

Initially the effect of buffer capacity for extracellular  $L^-$  and  $K^+$  accumulation was assessed using three HEPES-based perfusate solutions at pH 7.4 having different HEPES concentrations, i.e., 10, 20, and 5 mM during the first, second, and third ischemic episodes, respectively. The relative extracellular  $L^-$  measured by the end of each 10-min ischemia was 1:1.08:0.79. To derive more significant changes in  $L^-$ , pH, and  $K^+$  profiles from the standard profiles shown in Fig. 2 and from the above-mentioned results, two perfusate solutions of widely different buffer concentrations were utilized. The selected buffers were 10 mM HEPES and a mixture of 35 mM Tris ( $pK_a = 8.1$ ) and 65 mM HEPES ( $pK_a = 7.35$ ), abbreviated as HT, adjusted to pH 7.4.

Results indicating the effect of perfusate buffer capacity are shown in Fig. 3. The first ischemic episode was performed with 10 mM HEPES. Note that the extracellular  $L^-$  concentrations were in the order of 3.8 mM after 10 min of ischemia similar to that in Fig. 2A. After reperfusing the preparation and restoring the background levels of the three sensors, the perfusate was switched to the HT-based perfusate having a higher buffer capacity for at least 20 min. Then, arresting the flow produced a second ischemic event. Under these conditions extracellular  $L^-$  almost doubled (7.3 mM after 10 min of ischemia). Acidosis of the extracellular compartment was correspondingly diminished as expected with high buffer capacity in comparison with the lower buffer capacity perfusate (10 mM HEPES) as shown in Fig. 3B. The lactate and pH profiles collected from several different ischemic episodes and six different muscles are presented in Fig. 4 to show the effect of buffer capacity on both extracellular  $L^-$  accumulation and acidosis.

$K^+$  profiles shown in Fig. 3C show a maximum for extracellular  $K^+$  observed at the first ischemia (the expected trend), whereas in the second and the third episodes net cellular  $K^+$  loss did not follow the increased extracellular  $L^-$ . Instead, it was decreased during the second and third episodes.

Another perfusate was prepared and used during 10-min ischemia using 10 mM HEPES plus 90 mM glycine matching the high ionic strength of the HT perfusate. Fig. 5 shows the results of titrations of different perfusate solutions with HCl. As shown in this figure the buffer capacity of 10 mM HEPES perfusate and 10 mM HEPES–90 mM glycine perfusate would be the same within the physiological pH range (i.e., 7.4–6.4). This was concluded from the trivial buffer capacity produced from 90 mM glycine.

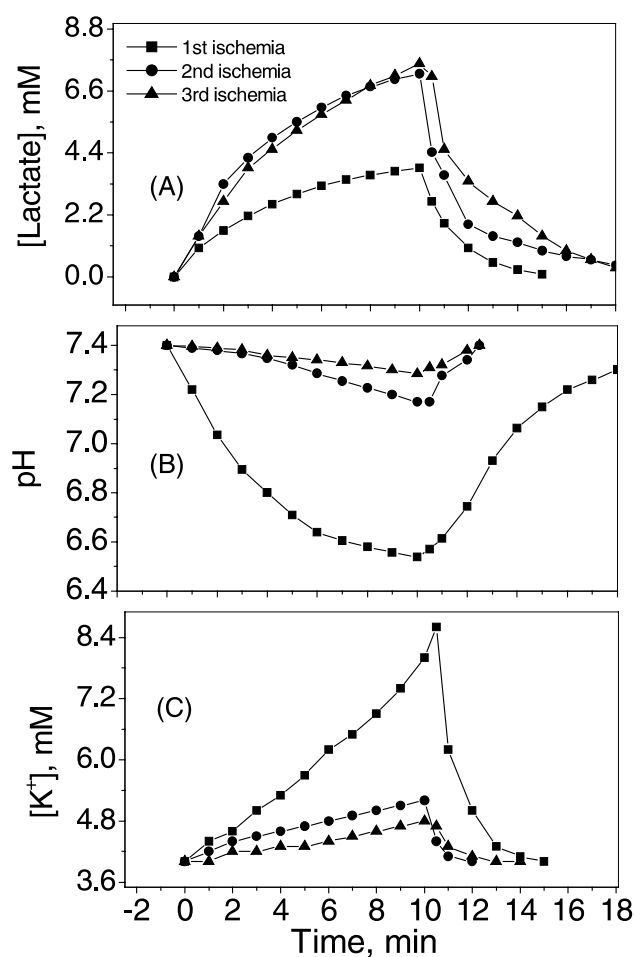


Fig. 3. Effect of the perfusate buffer capacity on the extracellular  $L^-$ , pH, and  $K^+$  changes. The first ischemia is based on perfusate buffered with 10 mM HEPES. The second and third ischemic episodes are based on HT-based perfusate.

#### Effect of the initial pH

Perfusate buffered with 100 mM BT of lower initial pH, i.e., pH 6.8 was utilized in the second 35-min ischemia. The obtained results (Fig. 6) showed 10–15% lower extracellular  $L^-$  accumulation in comparison with the first ischemia performed at regular 100 mM HT buffer. The magnitude of the pH drop was lower also from pH 6.8 to 6.65.

#### Effect of sodium channels blockers

The important association between  $Na^+$  influx and  $K^+$  efflux during hypoxia was established in a similar isolated rabbit heart preparation by using a combination of pharmacological agents to fully inhibit  $Na^+$  influx and while showing that cellular  $K^+$  loss was abolished [14]. Likewise, in our studies we used tetrodotoxin (13  $\mu$ M), verapamil (10  $\mu$ M), furosemide (1 mM), and EIPA (5  $\mu$ M) to inhibit  $Na^+$  influx pathways in the

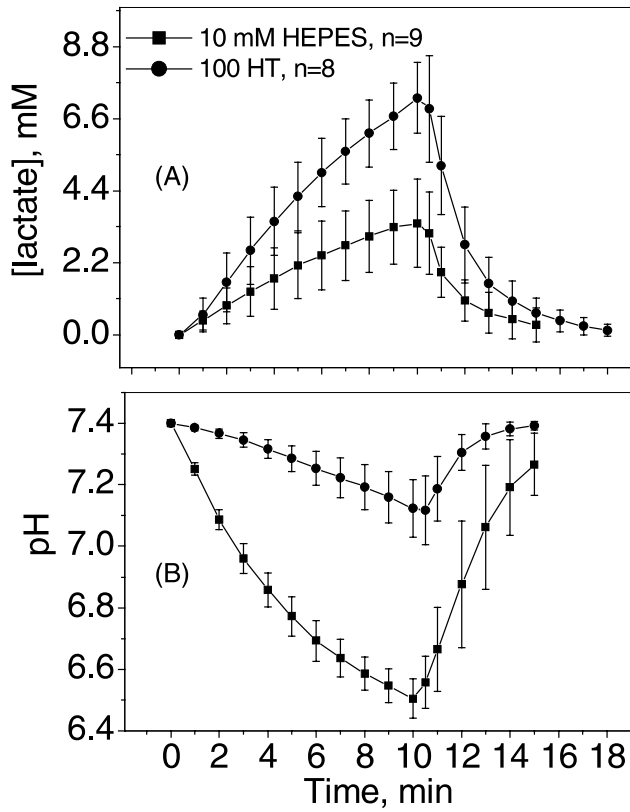


Fig. 4. Repeated measurements of extracellular lactate and pH using different muscle preparations, ischemic episodes, and perfusate solutions buffered with either 10 mM HEPES or 100 mM HT.

ischemic myocardium while simultaneously measuring extracellular  $L^-$  accumulation and the change in pH. The results presented in Fig. 7 show that the use of the drug combination reduced the  $L^-$  and  $K^+$  efflux profiles slightly, but did not abolish extracellular  $K^+$  accumulation.

*Long ischemic episodes*

Finally, the reliability of simultaneous measurements of greater  $L^-$ ,  $K^+$ , and pH changes was tested during two longer, yet reversible, ischemic episodes over 35 min. The results of the first experiment were already presented in Fig. 6. The results of the second experiment, presented in Fig. 8, showed that the same trend of  $L^-$ , pH, and  $K^+$  profiles remained, but the magnitude of the change was significantly larger.

Importantly, the magnitude of the net cellular  $K^+$  loss and extracellular acidification was markedly attenuated by the HT perfusate while extracellular  $L^-$  was markedly increased. These differences were present during the first 10 min of ischemia as previously shown; however, the differences increased further during the subsequent 25 min of ischemia. These results provide further evidence of a lack of evidence for a cotransport of  $K^+$  and  $L^-$  in ischemic myocardium or a

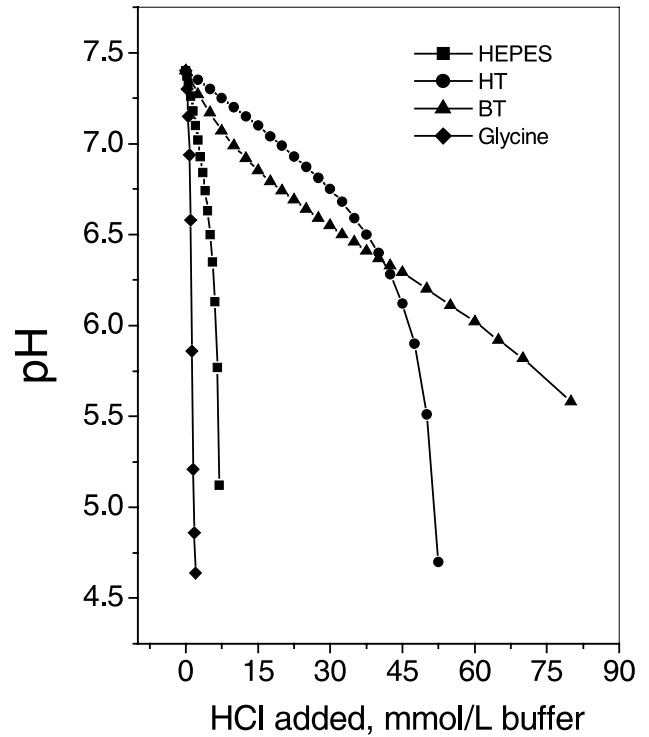


Fig. 5. Titration curves of perfusate solutions containing different buffers, which give direct comparison between their buffer capacities. HEPES (10 mM HEPES), glycine (90 mM glycine). Buffer capacity at pH 7.4: HT > BT > HEPES > glycine; at pH 6.8: HT ~ BT > HEPES > glycine. Buffer capacity of a given perfusate at a certain pH equals 1/slope at the specified point.

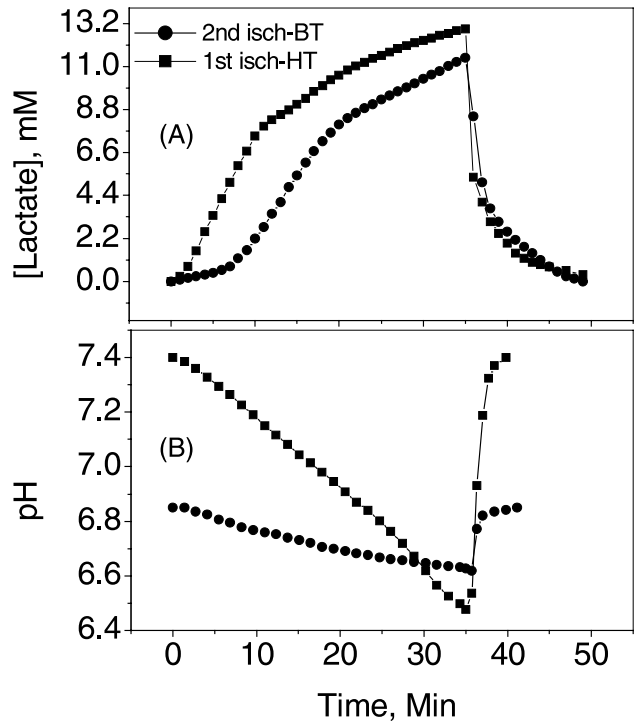


Fig. 6. The effect of perfusate initial pH on the profiles of lactate and pH changes during 35 min of ischemia.

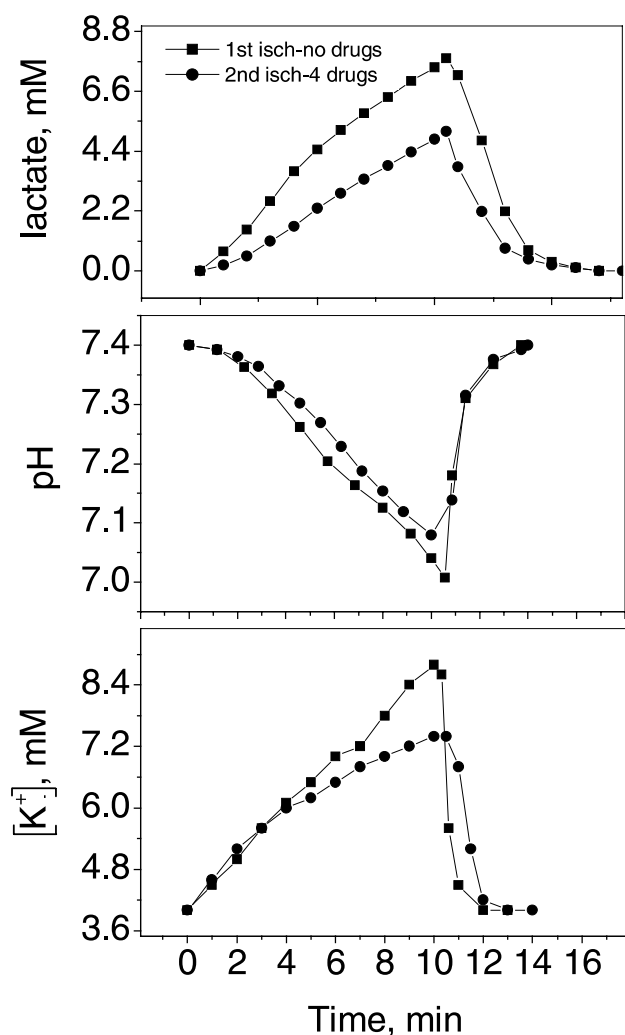


Fig. 7. Effect of sodium channels blockers on the profiles of lactate, pH, and  $K^+$  changes. First ischemia, HT-based perfusate only; second ischemia, HT-based perfusate containing the sodium channels blockers.

mechanism of  $L^-$  transport that requires  $K^+$  as an integral participant.

## Discussion

Cellular  $K^+$  loss is characteristic feature of myocardial ischemia and contributes directly to the electrophysiological substrate accounting for arrhythmias accompanying coronary artery occlusion. As such the mechanism of cellular  $K^+$  loss has clinical and physiological importance. Several mechanisms are postulated to explain cellular  $K^+$  loss in ischemic myocardium. In brief, a decreased  $K^+$  influx, an increased  $K^+$  efflux, or both might cause net cellular  $K^+$  loss. Decreased  $K^+$  influx secondary to inhibition of the  $Na^+/K^+$  pump is unlikely to be a significant factor during the first 10 min of ischemia. Increased  $K^+$  efflux secondary to the

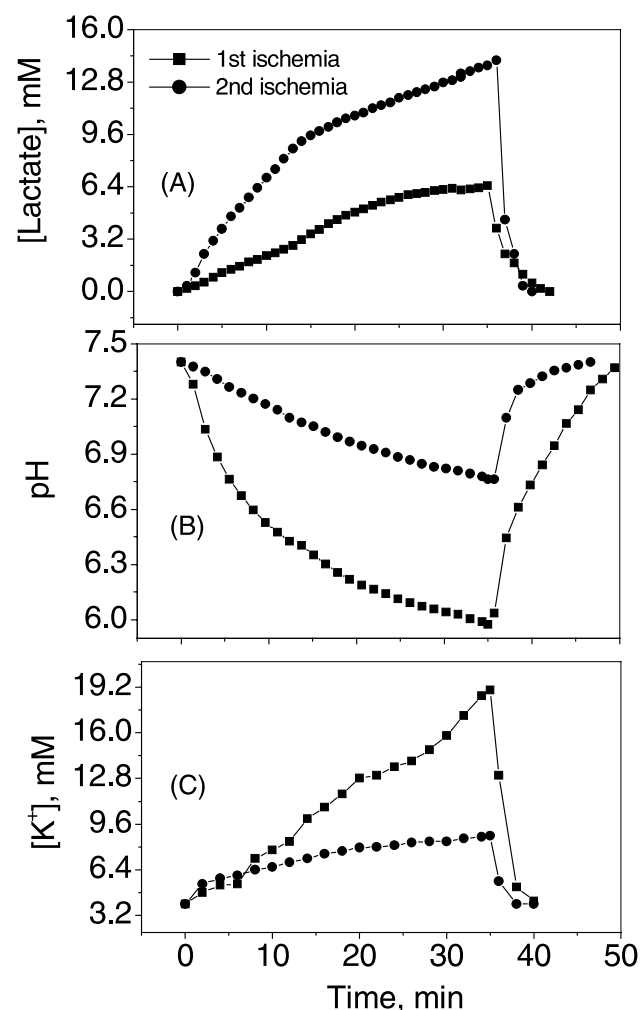


Fig. 8. Lactate, pH, and  $K^+$  profiles during 35-min ischemic episodes. First ischemia, 10 mM HEPES perfusate; second ischemia, HT perfusate.

opening of the  $K_{ATP}$  channel [14] or other  $K^+$  channels is unlikely to be a major contributor to  $K^+$  efflux during this early period [15]. An increased inward current may balance  $K^+$  efflux and thereby maintain electroneutrality. An inward  $Ca^{2+}$  and an outward  $Cl^-$  are unlikely to compensate for  $K^+$  efflux, whereas an inward  $Na^+$  current and lactate transport could compensate for the  $K^+$  efflux and are more likely.

### Concentration–time profiles of $L^-$ , pH, and $K^+$ changes during ischemia

In the first stage of experiments, the aim was to establish the concentration–time profiles of  $L^-$ , pH, and  $K^+$  during repetitive ischemic episodes under the same set of conditions such as the same (i) muscle preparation, (ii) type of the perfusate buffer, and (iii) perfusate buffer capacity. The established profiles, when compared with other profiles measured with one experimental variable being changed, in the second or third ischemia,

gave a reasonable estimation of the size of the effect of the studied variable, e.g., buffer capacity, extracellular pH, or inhibitors of  $\text{Na}^+$  influx.

In any subsequent experiment, the first ischemic event was conducted under standard conditions, i.e., without introduction of any new variable, to confirm that all the elements of the experimental system (e.g., muscle preparation, perfusion, cannulation, pacing and grounding of the muscle, the three sensors, spatial positioning of all sensors to the surface of the muscle and the reference electrode, etc.) were functioning and/or made properly. This was accomplished by comparing the profiles obtained from the first ischemia with the standard profiles collected from three preliminary experiments using different muscle preparations and different sensors. Between experiments, variations of up to 30–40% in the magnitude of the measured extracellular concentrations were observed. However, the features of the profiles were the same regardless of the magnitude of the change. The large variations between experiments were attributed, primarily, to the variations of the diameter and/or the nature of the papillary muscles [16].

Previous studies assessing the reproducibility of extracellular  $\text{K}^+$  accumulation in ischemic myocardium indicated that extracellular  $\text{K}^+$  accumulation was lower in the first episode relative to subsequent episodes of ischemia [17]. The change in the absolute magnitude of extracellular  $\text{K}^+$  accumulation occurs in combination with a progressive decrease in the associated extracellular acidification from one ischemic episode to another. Those results were interpreted to indicate that net cellular  $\text{K}^+$  loss depends more on glycolytic activity during the first occlusion than on subsequent ischemic events. However,  $\text{L}^-$  was not measured in those studies. In our study we found that extracellular  $\text{K}^+$  accumulation during the first ischemic event was increased relative to subsequent events, however, the magnitude of pH change and lactate production were essentially the same from one event to the next. Thus, in our preparation, the increased net cellular  $\text{K}^+$  loss during the first ischemic episode cannot be ascribed to enhanced glycolytic activity and greater lactate production. Because brief periods of ischemia can increase the permeability of the microvasculature, resulting in interstitial edema during reperfusion, one possible mechanism accounting for the decrease in extracellular  $\text{K}^+$  accumulation during subsequent ischemic events is dilution of the  $\text{K}^+$  by an expanded extracellular compartment [18]. Nevertheless, the pattern of changes of extracellular  $\text{L}^-$ , pH, and  $\text{K}^+$  from one ischemic episode to the next was reproducible.

#### *Is extracellular $\text{L}^-$ and $\text{K}^+$ accumulation interdependent during ischemia?*

In the second stage, a series of experiments was designed to induce changes in extracellular  $\text{L}^-$  concen-

tration to either lesser or greater extents and to monitor, simultaneously, the accompanying changes in the extracellular  $\text{K}^+$ . The assumption was made that if a certain condition induced, for example, higher extracellular  $\text{L}^-$  accumulation during ischemia and at the same time a higher extracellular  $\text{K}^+$  was driven in the same direction, we could conclude that extracellular  $\text{L}^-$  and  $\text{K}^+$  were influenced by the changes that occurred to the cellular lactate loss process. In other words, the two processes were somehow interdependent. On the other hand, if extracellular  $\text{K}^+$  did not follow the changes occurring to the extracellular  $\text{L}^-$ , we could conclude that the mechanisms that determine  $\text{K}^+$  efflux to the extracellular space were independent of the parallel process that was responsible for extrusion of  $\text{L}^-$  to the extracellular compartment. The variables that could induce such discrimination were (i) the buffer capacity of the extracellular solution, (ii) the initial pH of the perfusate solution, and (iii) the inhibition of the  $\text{Na}^+$  influx.

#### *Effect of buffer capacity*

The interest to study the effect of buffer capacity originated from the hypothesis that lower perfusate buffer capacity would lead to higher degrees of acidosis caused by cellular  $\text{H}^+$  loss to the extracellular fluid [19]. Such lower pH values would inhibit glycolysis, i.e., lower LH production and/or diminish the diffusion and/or transport of undissociated LH to the more acidic extracellular compartment. Either one or both of these two effects should lead to lower measured extracellular  $\text{L}^-$  concentration. An opposite effect was expected when perfusate solution with high buffer capacity was utilized. In either case, we could derive changes in the typical  $\text{L}^-$  profiles and observe parallel changes measured in extracellular  $\text{K}^+$  accumulation. The role of pH monitoring in such experiments was to confirm the degree of acidosis of the extracellular compartment.

Data obtained with perfusates based on Hepes concentrations of 10, 20, and 5 mM indicated that the least  $\text{L}^-$  production, observed in the third ischemia, was explained only on the basis of the low buffer capacity because, from the standard profiles (shown in Fig. 2), one expected the highest  $\text{L}^-$  production when the buffer capacity remained constant. One can conclude from the third ischemic episode that the observed decrease in extracellular  $\text{L}^-$  attributed to the lower buffer capacity was greater than the increase in extracellular  $\text{L}^-$  expected in the third ischemia when performed using the same buffer capacity.

Perfusate solution with significantly higher buffer capacity based on 100 mM Hepes was not suitable for the present purpose. Hepes ( $\text{pK}_a = 7.35$ ) solution was acidic. Consequently, large amounts of NaOH were required to adjust the pH to 7.4 using NaOH, resulting in high concentrations of Na ions in the concentrated

Hepes solution compared to the 10 mM Hepes-based perfusate. Such a higher  $\text{Na}^+$  concentration in the perfusate could complicate the interpretation of the results by its physiological impact on osmolarity and inward  $\text{Na}^+$  current,  $\text{Na}^+/\text{K}^+$  pump,  $\text{Na}^+/\text{H}^+$  exchanger,  $\text{Na}^+/\text{HCO}_3^-$  cotransporter, and  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. To overcome this limitation, a perfusate solution buffered with a mixture of 35 mM Tris ( $\text{p}K_a = 8.1$ ) and 65 mM Hepes ( $\text{p}K_a = 7.35$ ) was utilized. This mixture has pH value of 7.2 and was adjusted to pH 7.4 without the need for excessive amounts of NaOH.

The obtained data indicated that the molar ratio of  $\text{L}^-$  to  $\text{K}^+$  loss was approximately 1.2:1 after 10 min of ischemia in the first ischemic episode, 3:1 in subsequent ischemic episodes, and increased to approximately 8:1 in the presence of solutions with high buffer capacity. Data presented in Figs. 3 and 4 show that the extracellular  $\text{L}^-$  accumulation was almost doubled as a result of the eightfold increased buffer capacity of the extracellular compartment. This result, in addition to the extracellular  $\text{K}^+$  data shown in Fig. 3C, lead to a preliminary conclusion that changes in the extracellular  $\text{K}^+$  and  $\text{L}^-$  were not coupled when conditions favored the diffusion or transport of lactic acid. This finding supports the conclusion that  $\text{K}^+$  is not cotransported with  $\text{L}^-$  by the monocarboxylic acid transporter [20] and supports the possibility that as LH diffusion and/or transport is favored, electrogenic  $\text{L}^-$  transport, if it occurs, may decrease proportionally.

The effects observed with increased buffer capacity might be influenced by the different ionic strengths of the perfusate solutions. Such a possibility has not been considered in previous studies, although increased osmolarity affects  $\text{Na}^+/\text{H}^+$  exchange in hypoxic tissue [21]. When perfusate solution based on 10 mM Hepes plus 90 mM glycine was used during a second 10-min ischemia, extracellular  $\text{L}^-$  accumulation decreased by 9% and a similar smaller extent of acidosis rather than the expected small increase during the second ischemia as compared to reference curves. At least it can be concluded that the increased ionic strength itself was not responsible for the observed large increase in  $\text{L}^-$  production observed when concentrated HT buffer was used. Therefore, it is reasonable to assume that the increased  $\text{L}^-$  is attributed to the increased buffer capacity.

#### *Effect of the initial pH*

To confirm our previous conclusion that  $\text{K}^+$  efflux did not follow the induced increase in extracellular  $\text{L}^-$  caused by using higher buffer capacity, we examined an experimental parameter that would lead to lower extracellular  $\text{L}^-$ . It was assumed that a lower pH, e.g., 6.8, would be expected to lower  $\text{L}^-$  production due to the inhibition of the glycolysis at lower pH values and a decreased diffusion and/or transport of LH. The HT

combination buffer used before was not suitable for this experiment because it had a lower buffer capacity at lower pH values. On the other hand, BT had a lower  $\text{p}K_a$  value ( $\text{p}K_a = 6.5$ ) and had buffer capacity at pH 6.8 comparable to that of HT at pH 7.4.

The first conclusion from this result was that LH transport to the extracellular fluid was not the primary cause for extracellular acidosis. This conclusion is inferred from the observation that  $\text{L}^-$  production was diminished only by about 10% whereas the pH fall was decreased by about 75% (compared to the first ischemia at comparable buffer capacity) during the same period of 35-min ischemia. Because the magnitude of the decrease of  $\text{L}^-$  production and the extent of acidosis were not comparable, one can conclude that diffusion and transport of LH and transport of  $\text{L}^-$  to the extracellular fluid was not the only cause of acidosis. Within the same experiment the  $\text{K}^+$  efflux did not show any significant change in response to the changed starting extracellular pH.

#### *Effect of sodium channels blockers*

Recently, Shivkumar et al. [14] showed that  $\text{K}^+$  efflux in myocardium perfused with hypoxic solutions was linked to  $\text{Na}^+$  influx and independent of anion-coupled efflux [22]. Yet, in our study of ischemic rabbit myocardium, inhibition of  $\text{Na}^+$  influx pathways with a combination of drugs previously shown to fully block the efflux of  $\text{K}^+$  from the hypoxic rabbit heart did not abolish  $\text{K}^+$  efflux in the ischemic heart under no-flow conditions. With regard to the lactate production and extracellular pH, the inhibition of  $\text{Na}^+$  influx decreased extracellular lactate only slightly, and because of the high buffer capacity of the HT perfusate, the change in extracellular pH during ischemia was not affected. These results differ substantially from those of Shivkumar and colleagues in hypoxic tissue, suggesting that  $\text{K}^+$  efflux in hypoxic heart is more dependent on  $\text{Na}^+$  influx compared to no-flow ischemia. The difference might be related to greater inhibition of  $\text{Na}^+/\text{H}^+$  exchange,  $\text{Na}^+/\text{Ca}^{2+}$  exchange, and inward  $\text{Na}^+$  current in no-flow ischemia compared to hypoxia. Further studies will be necessary to define these mechanisms.

Overall, these results showed the reliability of the presented analytical methodology for stable and accurate recording of such important chemical species to the field of cardiovascular research.

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