The Role of Calcium Ions in the Acceleration of Resting Muscle Glycolysis by Extracellular Potassium

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ABSTRACT The activation of the glycolysis of resting muscle by increased extracellular potassium is dependent upon the simultaneous presence of calcium, but not of sodium ions. This regulation of metabolism by a membrane characteristic seems to act upon an early link in the glycolytic enzyme chain.

The classical investigations of Overton (1902, 1904) have shown that, while an isolated muscle maintains its irritability in balanced ion media, replacement of the sodium by a non-electrolyte, or partial replacement by potassium, leads to a loss in excitability. Later studies, emphasizing especially the potassium effect (*cf.* Dulière and Horton (1929); Horton (1930)) have furthermore demonstrated that in such media there is a significant increase in resting metabolism (Fenn (1930); Hegnauer, Fenn, and Cobb (1934); Tipton (1936); Solandt (1936*a*, *b*); Smith and Solandt (1938); Hill and Howarth (1957)). The transfer of a muscle into a high K medium may also lead to initial twitching or contracture (*cf.* Hodgkin and Horowicz (1957)), but the metabolic effects are of considerable magnitude and of much longer duration, and are held to be independent of any small initial contractile activity (*e.g.* Hill and Howarth (1957)). It has become accepted to ascribe this metabolic activation to a partial depolarization of the membrane, too small, or too slowly established, to elicit contraction. Clearly, this regulation of metabolic intensity by a membrane property is of considerable interest for the understanding of the link between excitation and response.

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In the present study, we have examined an additional parameter of possible significance, namely the calcium ion, in view of its presumed role in the mechanism of excitation-contraction coupling (*e.g.* Wilbrandt and Koller (1948); Weidmann (1959); Lüttgau and Niedergerke (1958); Niedergerke (1959); Bianchi and Shanes (1959)). The literature quoted indicates that different metabolic activities may be affected differentially by the changed K concentration. This work deals with anaerobic glycolysis only.

METHODS

All the experiments described were performed with isolated sartorius muscles of *Rana pipiens*, mostly of about 100 mg. weight. These were tied with cotton threads on the tendons at both ends, and allowed to recover in oxygenated Ringer's solution at 0°C. for 30 minutes before the experiment. They were then placed in the chamber (Fig. 1) so that the length was somewhat below resting length *in situ*, and placed in 5 ml. of Ringer's solution (96 mM NaCl; 2.5 mM KCl; 1 mM $MgSO_4$; 20 mM $NaHCO_3$; 1 mM $CaCl_2$) with a flow of helium containing 5 per cent CO_2 at 25°C. The apparatus is so constructed that the bathing fluid can be drained, and replaced by a fresh solution which had already been deaerated with He–CO_2. Usually, one such chamber contained a control muscle in normal Ringer, another the contralateral muscle in a medium of altered composition.

As a rule, the bathing solution was exchanged every hour, and the drained fluids kept for analysis. At the termination of the experiment, the muscles were removed from the apparatus, frozen by immersion into isopentane at −180°C., powdered, and extracted for analysis (Mommaerts, 1955). It was found that practically all lactate formed diffused into the suspension fluid, and that only minor amounts were retained in the muscle (which are not included in the graphs of Figs. 3 to 4). A number of experiments were done in different fashion (*e.g.* by hourly incubation in media with stepwise increase in K content), and others were performed on bullfrog muscles. These variations will not be separately described, except for the statement that they fall into the same general picture.

For the lactate determinations, the protein-free filtrates or solutions were precipitated with copper-lime, and their filtrates analyzed according to Barker and Summerson (1941).[1]

EXPERIMENTS

Mechanical Responses in High Potassium Media In separately conducted experiments, in which the main chamber (Fig. 1) was not closed, but in which a holder was inserted permitting the muscle to be connected to an isotonic lever (the variable capacitance gauge according to Schilling (1960)) under a slight load (100 gm. per cm.^{2}), we have investigated whether the immersion

[1] We are indebted to Mr. Krikor Seraydarian for his participation in the elaboration of this part of the methodology.

into a high potassium medium (20 mM) would give rise to persistent activity of the muscle. In some muscles, there was indeed a transient period of moderate twitching, starting 1 to 2 minutes after the change of medium and persisting at a declining level for about that same time. In others, there was no me-

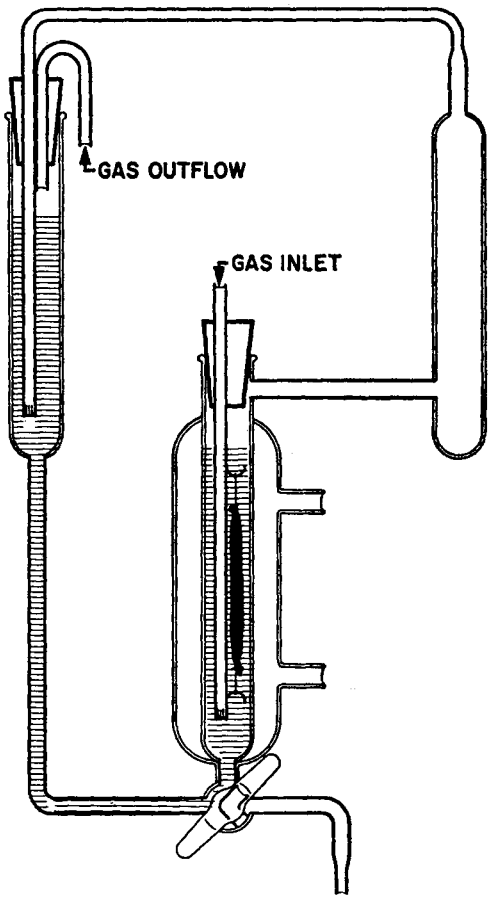

FIGURE 1. Apparatus for the anaerobic incubation of muscle, for the drainage of suspension fluid, and for replacement of the latter by deoxygenated bathing solution. The muscle chamber (lower right) is surrounded by a temperature-controlled water jacket.

chanical activity at all, neither directly after transfer, nor at any later moment up to 4 hours. According to visual observations in the majority of the metabolism experiments, absence of mechanical response was the rule, apparently because the high potassium medium reaches even the outer muscle fibers gradually.

In other experiments, we have conducted long time observations, after the first minutes in the new medium, with intracellular microelectrodes,[2] and

[2] We are indebted to Dr. Allan J. Brady for his participation in these experiments.

have never observed any sign of spontaneously occurring action potentials, although in one such experimental series about one-third of the fibers in the surface layer were penetrated, and observed over extended periods of time.

The Metabolic Effect of Increased Potassium Levels As is illustrated in Fig. 2, addition of K^+ to otherwise normal Ringer solution resulted in increased rates

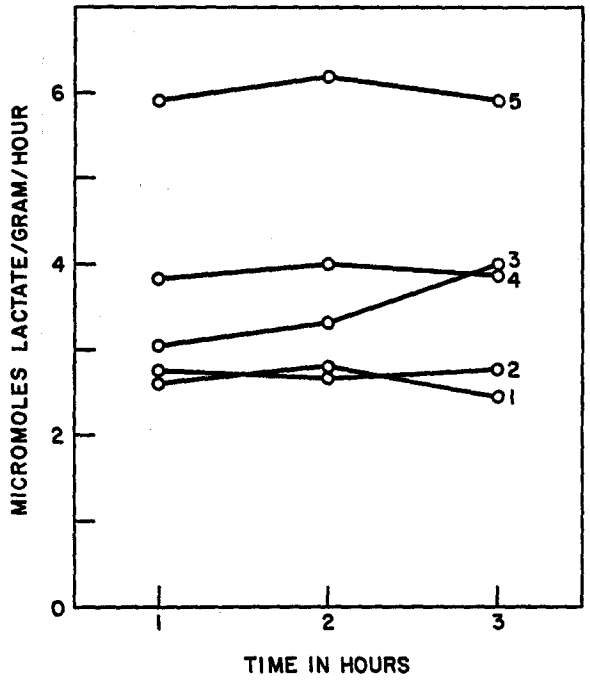

FIGURE 2. Lactic acid production (micromoles of lactate per gram muscle per hour) in different media. The points are averages obtained from two to four individual experiments, which usually varied within 20 per cent. Curves 1 to 5, results obtained by incubation in normal Ringer (2.5 mM KCl) and in Ringer with 8, 12, 16, and 20 mM KCl, respectively. Ca concentration 1 mM throughout.

of glycolysis, in keeping with the relevant literature (Hegnauer, Fenn, and Cobb (1934); Tipton (1936)). The glycolytic rate may change in the course of time, but no special interpretation suggests itself for this.

The Influence of Calcium upon the Potassium Effect Experiments represented in Fig. 3 show that the stimulation of glycolysis by an elevated K^+ concentration is dependent on the Ca^{++} present. For the "no calcium" experiments, the muscle was first soaked in Ca-free Ringer, sometimes containing 0.002 M versene, whereupon it was placed in Ca^{++}-free Ringer with the selected K^+

content. It is seen that increased Ca^{++} greatly accentuates the K-activated glycolysis, whereas in the absence of Ca^{++}, it is reduced to the normal level. The addition of 5 mM Ca^{++} to normal Ringer has no significant effect.

Substitution of Sodium Ions by Choline It was observed (Fig. 4) that replacement of Na^{+} by choline in otherwise normal Ringer has no influence upon the resting metabolism, but that with high K^{+} and Ca^{++} this substitution causes a significant exaggeration of the described activation effects.

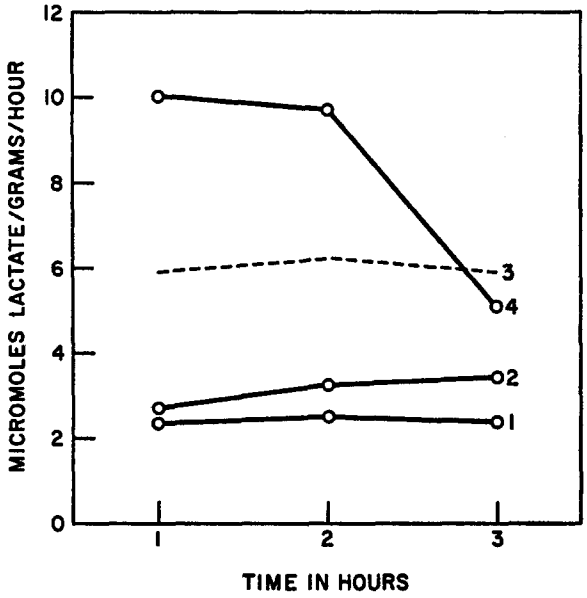

FIGURE 3. Lactic acid production, as in Fig. 2. Curves 1 to 4, solutions with 2.5 mM KCl and 5 mM $CaCl_2$, with 20 mM KCl but without Ca, 20 mM KCl with 1 mM $CaCl_2$ (from Fig. 2), and 20 mM KCl with 5 mM $CaCl_2$, respectively.

The experiments in choline media happened to show considerably greater variability than the others, whether due to the nature of the experiment or to a more heterogeneous group of animals at the time is not known. We state, therefore, that in the crucial curves 3 and 4 in Fig. 4, the points represent the averages of six experiments each, with the following means and standard deviations; for curve 3, 27.4 ± 7.0, 13.7 ± 3.0, and 8.2 ± 2.2; for curve 4, 32.0 ± 9.8, 30.2 ± 7.4, and 20.6 ± 3.8.

Experiments in a Carbon Dioxide Atmosphere Our observations in this regard were suggested by the report of Kerly and Ronzoni (1933) that in a pure CO_2 gas phase glycolysis is inhibited, which, however, was not found in our material. We do not consider this as a contradiction to those authors' findings, since an over-all result like this depends on the total kinetical characteristics of a multienzyme system, which may well depend on species differences and

other factors. We did arrive at the tentative conclusion that this form of acidification primarily acts upon the velocity of the phosphofructokinase reaction, which in our material was apparently present in sufficient abundance to still allow a sizable rate of glycolysis.

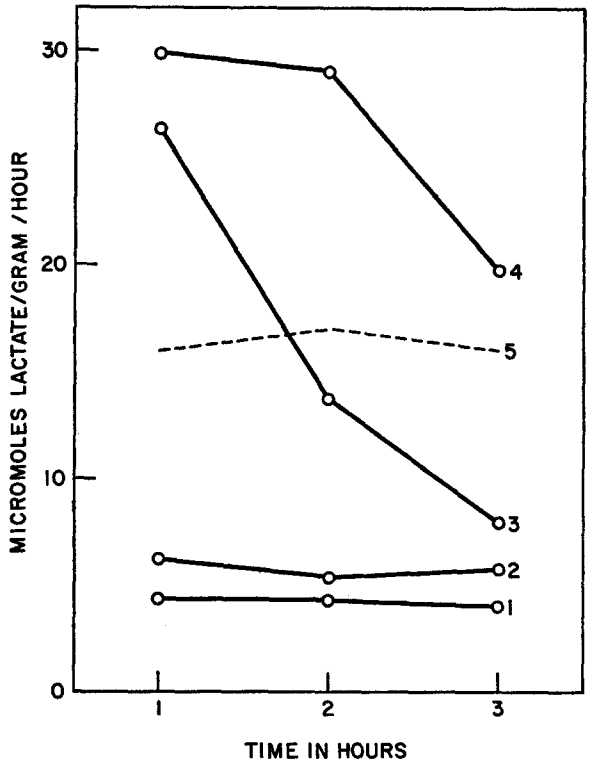

FIGURE 4. Lactic acid production in choline media, as in Fig. 2. Curves 1 to 4, normal Ringer, choline Ringer with 2.5 mM KCl and 1 mM $CaCl_2$, with 20 mM KCl and 1 mM $CaCl_2$, and with 20 mM KCl and 5 mM $CaCl_2$, respectively. This experiment was done on a different group of frogs in which the stimulation of metabolism was somewhat greater than in those of Figs. 2 and 3, and the variations between individual experiments of curves 3 and 4 were greater. Results corresponding to curve 4 obtained on the frogs of Figs. 2 and 3 are indicated by the broken line, curve 5.

In such experiments, it was established with the aid of the methods of Slater ((1953); see Seraydarian, Mommaerts, and Wallner, (1960)) for the determination of hexosephosphates that after 3 hours in a CO_2 atmosphere in 20 mM KCl-Ringer of normal Ca content, an increase of 3.5 to 4.2 μmoles of hexosemonophosphates per gram had occurred, besides a formation of 6.5 to 8.5 μmoles of lactate per gram during the same interval. This total glycolysis of 16 microequivalents of lactate is closely comparable to the 18 μmoles normally formed in 3 hours (Fig. 2, curve 5) in this medium, but the in-

creased hexosemonophosphate content as compared to the normal range below 1.0 μmoles (Seraydarian, Mommaerts, and Wallner (1960)) indicates that a much higher degree of saturation of phosphofructokinase is required to maintain this metabolic rate. Clearly, in material with a lesser amount of this enzyme, this reaction step may become limiting to such an extent as to lead to the Kerly and Ronzoni findings. With respect to the immediate subject of this present paper, the result indicates that the K-Ca effect acts upon an early step in the glycolytic pathway, presumably upon the phosphorylase reaction.

DISCUSSION

At a time when the first experimental efforts are being made to penetrate into the problem of the link between excitation and mechanical response, findings on the coupling between membrane effects and metabolic activation may assume a significance of similar scope. Experiments such as these represent an uncoupling of chemical reactions from mechanical responses, and a demonstration that not only contractility but also metabolism is subjected to some measure of control by the cell membrane (*cf.* Hill and Howarth (1957)).

It should first be discussed to what extent the described metabolic activation might be due to mechanical activity evoked by the high K medium. Immediately upon transfer, there sometimes is a transient phase of irregular twitching. Even when such activity occurs, the observed effects cannot be regarded as the recovery metabolism associated with this initial activity from the observation (Hill and Howarth (1957)) that upon return to normal Ringer solution the increased metabolism is immediately reduced to normal. Nor can the described phenomena be due to continuous asynchronous twitching throughout the experimental period, since the results described in the experimental part clearly show that no such activity occurs.

In one respect, our results differ from those of Solandt (1936) and of Hill and Howarth (1957), namely in so far as in their work Ca^{++} was found to counteract the K^+ effect while in ours it is a prerequisite for it. Apart from a technical difference (long duration of our experiments, *versus* short lasting observations by Hill and Howarth), this will mainly be related to the different types of metabolism studied: respiration (measured myothermally) *versus* anaerobic glycolysis. These two processes may well be regulated differently, as they also have different threshold concentrations for K^+ (Hegnauer, Fenn, and Cobb (1934); Tipton (1936)). Undoubtedly, the velocities of these two metabolic patterns are determined by entirely different rate-limiting enzymes, affected independently by membrane effects and other influences. This difference also implies that the magnitude of the Pasteur effect is modified by the external ionic medium, an insight not hitherto encountered in bio-

chemical considerations, although the sensitivity of the Pasteur effect in certain tissues toward unnatural media has been noted (Warburg (1925)), and there have been references to the influence of potassium ions (Ashford and Dixon (1935)) and of absence of calcium (Dickens and Greville (1935)) upon the Pasteur effect in brain slices.

Our work, then, indicates that this membrane-controlled activation of glycolysis is contingent upon the presence of extracellular Ca^{++}, and not upon Na^{+} ions, which view coincides well with the current tendency to ascribe to Ca^{++} a major role in the membrane excitation process. We notably wish to give recognition to the important findings by Bianchi and Shanes (1959) that the uptake of Ca^{++} is a fundamental feature of stimulation as well as of the influence of increased extracellular K^{+}, and that the magnitude of the Ca^{++} exchange per stimulus is correlated with the duration of the active state set up by each stimulation.

The fact that in choline media with increased K the activation effects in the presence of Ca are even higher than in Na media must be connected with the view that, in the case of cardiac muscle, the Na ion acts as a competitor for Ca entry (Niedergerke (1959)). Hence, there is a complete parallelism between our findings and the current views on the role of Ca in excitation, and we are led to the surmise that in the described activation of glycolysis, enhanced Ca entry is the determining factor, since Bianchi and Shanes (1959) and Niedergerke (1959) have also demonstrated that depolarization by K leads to increased Ca entry.

The experiments on the K-Ca activation of glycolysis in a CO_2 atmosphere have been interpreted as indicating that the metabolic activation affects primarily an early step in the glycolytic reaction chain. A further discussion of this problem must await the termination of our current work on the rate-limiting steps in glycolysis (Guillory and Mommaerts, and Seraydarian, Guillory, Wallner, and Mommaerts, data to be published).

We wish to express our indebtedness to Mrs. Dorothy Gellermann for her invaluable assistance in the later stages of this investigation.

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