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POTASSIUM-STIMULATED RESPIRATION AND INTRACELLULAR CALCIUM RELEASE IN FROG SKELETAL MUSCLE

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SUMMARY

1. In 1931 Fenn showed that the respiration of frog twitch muscles increases when $[K^+]_o$ is raised. The present paper is a further study of potassium-stimulated respiration. Stimulation depends on membrane potential, since respiration is also stimulated by elevated $[Rb_+]_o$ or $[Cs^+]_o$ in direct relation to their ability to depolarize.

2. When $[K^+]_o$ is elevated to 25 mM there is an increase in respiration which is sustained for hours. If $[K^+]_o$ is 30 mM or above, there is a transitory burst of stimulated respiration, followed by a decline back to the basal level.

3. If $[K^+]_o$ is raised in steps from 20 to 30 mM, there may never be a burst of increased oxygen consumption. Often a rise in $[K^+]_o$ from 20 to 24 mM decreases respiration.

4. The response to elevated $[K^+]_o$ can be blocked by divalent cations or by local anaesthetics; the blocking agents do not interfere with the depolarization of the membrane. The blocking agents act rapidly; they probably take effect soon after they contact the cell membrane.

5. Either extracellular Ca^{2+} or Sr^{2+} is important for the stimulation of respiration. The burst produced by 50 mM $[K^+]_o$ is transformed into a sustained rise in respiration when $[Ca^{2+}]_o$ or $[Sr^{2+}]_o$ are also raised. If a muscle is stimulated with 25 mM $[K^+]_o$ in the absence of extracellular calcium, respiration is elevated as usual, but now the rise is transitory unless Ca^{2+} or Sr^{2+} are added back to the extracellular solution.

6. Depolarization seems to stimulate respiration by causing the release of Ca^{2+} into the sarcoplasm. Since respiration is increased by a depolarization below the threshold for producing a contracture, respiration is more sensitive than contraction as an indicator of sarcoplasmic calcium concentration.

7. A model for the relation between sarcoplasmic calcium and membrane potential is proposed. The model accounts for the time course of the

stimulation of respiration and also for much of the available data on potassium contracture. In the model, Ca^{2+} is released into the sarcoplasm from a store in the cell. With depolarization, the release of Ca^{2+} from the store is increased, but the rate at which extracellular Ca^{2+} can replenish the store is decreased. The ability of the longitudinal reticulum to pump Ca^{2+} from the sarcoplasm does not vary with membrane potential.

INTRODUCTION

The trigger for muscular contraction is the depolarization of the cell membrane. Although not so widely recognized, there is also evidence that there is a relation between membrane potential and the rate of oxygen consumption. The first experiments pointing towards this conclusion were by Fenn (1931) and by Hegnauer, Fenn & Cobb (1934). They found that the respiration of the sartorius muscle of the frog is increased substantially when the potassium concentration of the Ringer solution ($[K^+]_o$) is raised. The total heat production by the muscle also rises, as was described by Solandt (1936). The increase in metabolism produced by elevated $[K^+]_o$ is often called the Solandt effect (Smith & Solandt, 1938; Hill & Howarth, 1957).

As the way in which $[K^+]_o$ influenced the respiration was still uncertain a systematic investigation was begun of the conditions in which respiration is stimulated and on the effects of other ions and compounds on K^+-stimulated respiration. The principal conclusions are that the respiratory rate begins to rise when the membrane potential falls to a critical value of about -55 mV. The fall in membrane potential seems to act by increasing the $[Ca^{2+}]$ in the sarcoplasm. The rise in sarcoplasmic calcium is the trigger for the increase in respiration. To account for the changes in respiratory rate following the initial stimulation, a compartment between the extracellular solution and the sarcoplasm is proposed. The rate constant for Ca^{2+} entry into this compartment appears to decrease when the fibre is depolarized. Therefore the stimulation of respiration that follows the potassium depolarization is often transitory, because the Ca^{2+} stored for release is used up.

When these experiments were under way, some of the same results and some additional data on $^{45}Ca^{2+}$ fluxes were reported by Novotný & Vyskočil (1966). They also conclude that a rise in the calcium in the sarcoplasm is the trigger for the increase in respiration.

METHODS

Solutions. The Ringer solution was a slight modification of one developed by Danforth & Helmrich (1964), based on their analysis of frog plasma. It contained 100 mM-NaCl, 2 mM-KCl, 2·5 mM-$CaCl_2$, 3 mM-$MgCl_2$ and 8 mM of tris buffer pH 7·4. Solutions were made from reagent grade salts and prepared in glass distilled water. Ringer solutions with an increased $[K^+]$, $[Ca^{2+}]$, or $[Mg^{2+}]$ were made by omitting equivalent amounts of Na^+. In experiments in which muscles were soaked for hours, 4000 u. of pencillin/ml. were added to the Ringer solution to inhibit bacterial growth.

Oxygen consumption. Grass frogs (*Rana pipiens*) were obtained from a dealer and kept in running tap water.

The sartorius muscles were carefully dissected from a pithed frog. The muscles usually weighed about 0·1 g. The muscles were weighed and placed in oxygenated Ringer solution for 1 hr. They were then transferred to Warburg flasks with two side arms. The centre well of the flask contained 0·2 ml. of 10% KOH and a piece of filter paper to absorb the carbon dioxide. Appropriate additions were made to the side arms and the flask was attached to one side of a differential manometer. The reference flask contained Ringer solution. The manometer was attached to the micrometer syringe; oxygen uptake was measured by a constant pressure method and readings were directly in μl. The flasks were placed in a water-bath at 23° C. Slices of brain, kidney, and liver were prepared using a Stadie–Riggs slicer.

Membrane potentials. Conventional glass micropipettes filled with 3 M-KCl were used for measuring membrane potentials. The electrodes used had tip potentials less than ± 5 mV and resistances of 10–20 MΩ. The preamplifier had a grid current less than 10^{-11} A.

RESULTS

Osmotic pressure and oxygen consumption. The design of the experiments would be greatly simplified if the respiration of the sartorius muscle was not changed markedly by an increase in the concentration of solutes in the bathing solution. In five experiments the respiration of a muscle in 2 ml. of Ringer solution was measured and then 0·85 ml. of 1 M sucrose solution was tipped in from the side arm. In Ringer solution the uptake was $4{\cdot}98 \pm 0{\cdot}91$ μmole O_2/g.hr; after the addition of the sucrose solution the rate was essentially unchanged at $4{\cdot}48 \pm 0{\cdot}87$ μmole O_2/g.hr. Therefore in subsequent experiments there was no hesitation in increasing the osmolality of the solution up to 450 m-osmole.

Extracellular potassium and the maximum rate of respiration. The relation between extracellular potassium, $[K^+]_o$, and the maximum rate of oxygen uptake after the tip-in of the additional KCl is summarized in Fig. 1. There is no significant rise in oxygen consumption until $[K^+]_o$ is between 10 and 20 mM. When $[K^+]_o$ is 20 mM the maximum respiration is increased threefold; at 30 mM the increase is sixfold. With a $[K^+]_o$ from 30 to 50 mM the maximum respiration reaches the same level, though with $[K^+]_o$ of more than 50 mM there is less stimulation of respiration.

The $[K^+]_o$ must reach a threshold level to stimulate respiration. In these experiments the threshold was between 10 and 20 mM. Other investigators

have found lower thresholds, ranging from 8 to 15 mM (Hegnauer *et al.* 1934; Hill & Howarth, 1957; Novotný & Vyskočil, 1966). One possible reason for the rather wide variation in thresholds found from one study to the next is the season of the year. In experiments done between 1 November 1965 and 15 February 1966, the maximum response to 20 mM ($[K^+]_0$ was 14·4 μmole O_2/g.hr (n = 19). In the experiments done between 15 February and 1 June 1966 the response was only 7·5 ± 3·9 μmole O_2/g.hr (n = 12). The threshold seems to be lower in winter frogs.

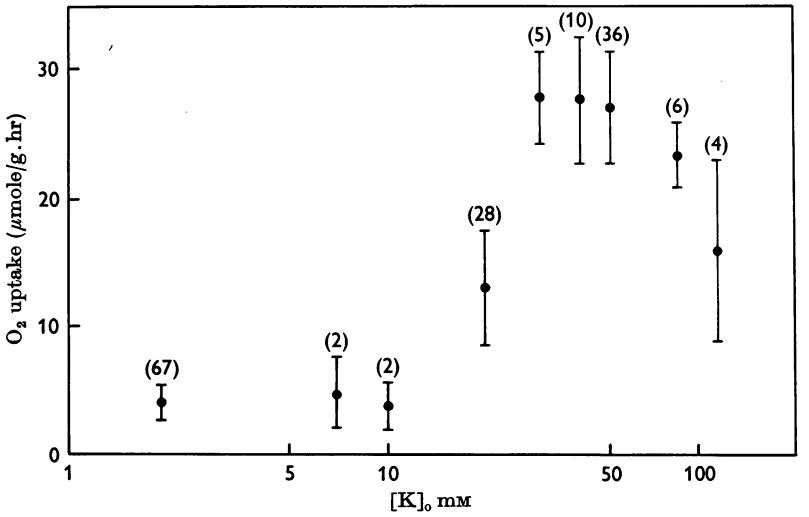

Fig. 1. The relation between $[K]_0$ and the maximum respiration measured from sartorius muscles. The bars indicate the S.D. of the measurements and in parentheses is the number of observations in each series.

The other reason for the variation in threshold is differences in the divalent cation content of the Ringer solution, which will be discussed later.

The respiration of the muscle increases at levels of $[K^+]_0$ which are below the threshold for eliciting any contracture. For example, in a solution containing a divalent cation concentration similar to that used in the respiration experiments (5 mM), single muscle fibres do not begin to exert tension at a $[K^+]_0$ less than 70 mM (Luttgau, 1963). The mechanism for producing an increase in respiration is more sensitive to changes in $[K^+]_0$ than the mechanism for excitation-contraction coupling.

Membrane potential and respiration. An obvious question is whether K^+ stimulates respiration by lowering the membrane potential. To try to give an answer, the effects of K^+, Cs^+ and Rb^+ were studied on both the membrane potential and respiration. The data on the lowering of the membrane potential produced by elevated extracellular concentrations

of the ions are summarized in Table 1. As expected, Rb^+ is a more powerful depolarizing agent than Cs^+. When increasing $[K^+]_o$ the threshold for eliciting a rise in respiration is reached when the membrane is depolarized

TABLE 1. Membrane potentials of muscles exposed to K^+, Rb^+, or Cs^+

Ion	Concentration (mM)	Number of fibres	Membrane potential (mV) (mean × S.D.)
K^+	2·5	33	87·9 ± 2·8
	25	46	51·9 ± 6·6
	50	31	41·0 ± 4·3
Rb^+	50	56	64·9 ± 7·8
	100	35	48·6 ± 8·0
Cs^+	50	77	67·6 ± 7·4
	100	53	58·0 ± 8·3

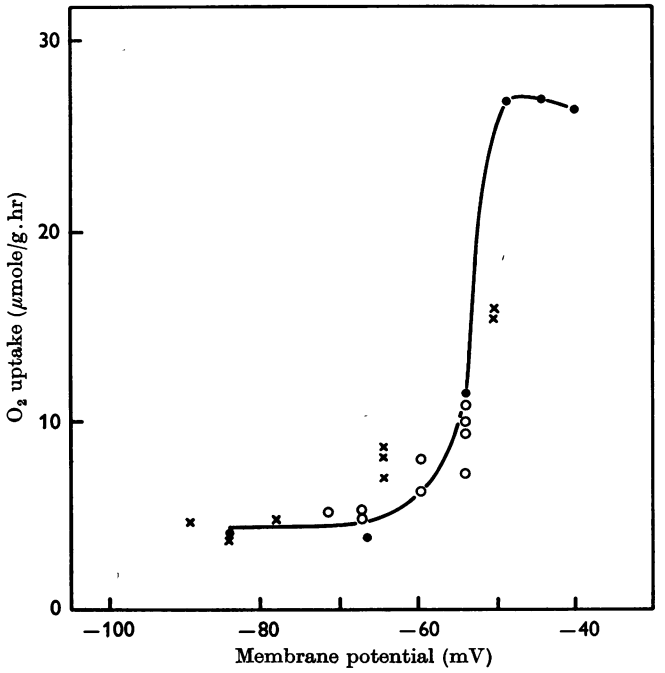

Fig. 2. The relation between membrane potential and the maximum rate of respiration measured after the muscle was depolarized. ● = K^+, ○ = Cs^+, × = Rb^+.

to about −55 mV. The same depolarization would be produced by $[Rb^+]_o$ of about 75 mM or, by extrapolation, $[Cs^+]_o$ of about 130 mM. The relation between membrane potential and the maximum respiratory rate is shown in Fig. 2. The oxygen consumption is a function of the membrane potential, regardless of whether the depolarization is produced by K^+, Rb^+, or Cs^+. A maximal stimulation of respiration is produced when the cell membrane is depolarized to about −48 mV.

Extracellular potassium and the long-term respiratory rate. So far only the maximum response to an increase $[K^+]_o$ has been described, but at higher levels of $[K^+]_o$ respiration rises to a maximum and then soon declines to a lower level (see Fig. 8). For ease in description, the initial transitory phase

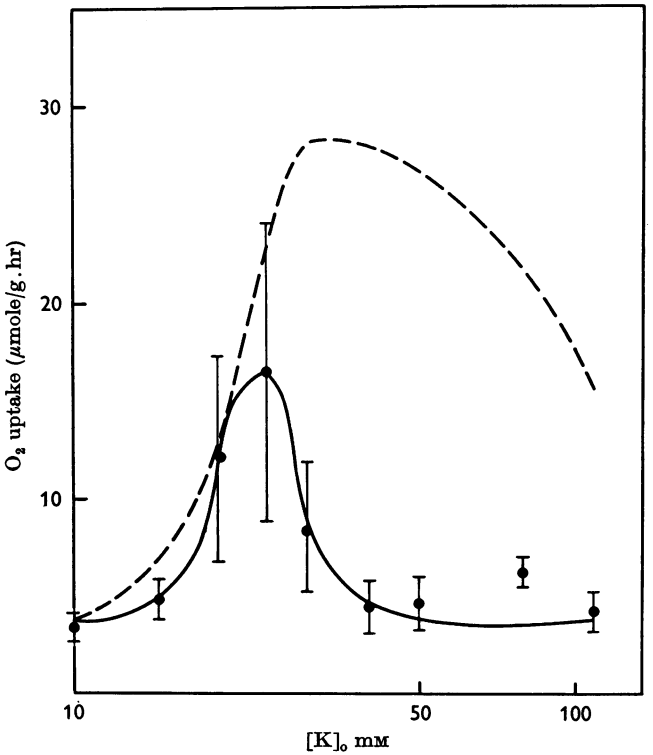

Fig. 3. The relation between $[K^+]_o$ and the respiration measured about one hour after the addition of the K^+ (———) for muscles in Ringer solution containing 2·5 mM-Ca^{2+}. The vertical bars indicate the S.D. of the measurements. The maximum respiration measured after the addition of the K^+ is also shown (— — — —), taken from Fig. 1. The number of observations is: 10 mM $[K^+]$ = 3, 15 mM $[K^+]$ = 4, 20 mM $[K^+]$ = 32, 25 mM $[K^+]$ = 13, 30 mM $[K^+]$ = 30, 40 mM $[K^+]$ = 40, 50 mM $[K^+]$ = 23, 80 mM $[K^+]$ = 3, 110 mM $[K^+]$ = 5.

of increased oxygen consumption will be called the 'burst'. Figure 3 compares the maximum rate of oxygen consumption measured during the burst with the rate measured 60–90 min later. When $[K^+]_o$ is raised to 25 mM the initial and 1 hr respirations are not significantly different—there is no burst. The increased respiratory rate is maintained for many hours; after 16 hr in 25 mM $[K^+]_o$ respiration is still above the level found in ordinary Ringer solution. However, when $[K^+]_o$ is raised to 30 mM or higher, the oxygen uptake first rises to a high level and then

declines to a steadier rate. The initial burst is about 7 times the level measured 60 min later. If $[K^+]_o$ is raised higher than 30 mM then after 60 min the respiration is no greater than that in normal Ringer solution.

There are several difficulties in being certain about the size and the time course of the burst. Because of changes in temperature it is rarely possible

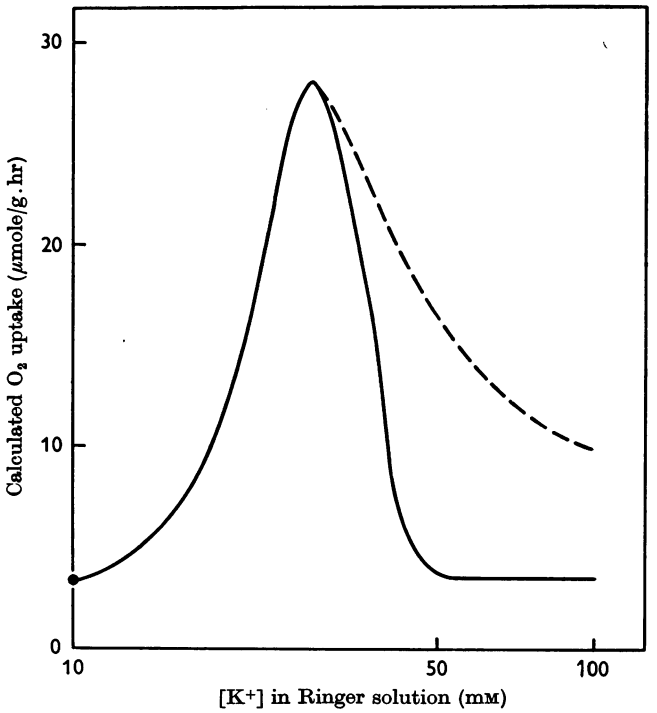

Fig. 4. An estimate of the effect of diffusion into the muscle mass on the stimulation of respiration by elevated $(K^+]_o$. The continuous line is a hypothetical relation between the maximum respiration of a single fibre exposed to an increased $[K^+]_o$. The interrupted line is a calculated curve for the maximum respiration of an entire muscle placed in elevated $[K^+]_o$. For further explanation see the text.

to obtain an accurate measurement of the oxygen uptake in the first minute or two after the tip-in of the additional K^+. With a manometric method the readings are only made every 2 or 3 min; so exceedingly fast changes in rate might be missed. Moreover, the elevated $[K^+]_o$ is diffusing into a rather thick sheet of muscle. This could lead to difficulty in interpretation. Suppose, for example, that 30 mM $[K^+]_o$ stimulates the muscle to produce a substantial increase in respiration but that 50 mM $[K^+]_o$ does not stimulate at all. Nevertheless, when a muscle is placed in a solution containing 50 mM $(K^+]_o$, the concentration in the inter-fibre spaces rises

gradually as K^+ diffuses into the muscle. During the period when the entire muscle is equilibrating with the external solution, at each moment some fibre is in the presence of 30 mM $[K^+]_o$, and the respiration of that fibre would be momentarily stimulated. The consequences of this line of thought are illustrated in Fig. 4. The muscle is assumed to be a flat sheet, 0·1 cm thick. The potassium diffusion coefficient was taken as $1{\cdot}6 \times 10^{-6}$ cm^2/sec. The additional assumption was made that the maximum rate of respiration is the function of $[K^+]_o$ drawn in the continuous line. The initial rising phase of the curve is taken from the data in Fig. 1, while the falling phase was drawn arbitrarily. The calculations were made by assuming that at time zero the potassium in the bathing solution was raised so that diffusion inward began. At 1 m intervals the $[K^+]_o$ at fifty equally spaced points across the muscle were calculated by a numerical solution of the diffusion equation (McCracken & Dorn, 1964). The respiratory rate for each point was then calculated from the continuous line in Fig. 4. By summing the respiratory rates at each of the fifty points, the value is obtained for the oxygen uptake of the entire sheet of muscle. The interrupted curve in Fig. 4 shows the maximum rate of respiration produced by the model. The maximum respiration at $[K^+]_o$ greater than 30 mM is substantially above the value which would be obtained for a single fibre, because of the relatively slow diffusion of potassium into the extracellular spaces. The true relation between $[K^+]_o$ and maximum stimulation of respiration could be measured only by working with single fibres or with small muscles with short diffusion pathways; it is likely that $[K^+]_o$ above 50 mM does not stimulate respiration to the extent that a first glance at Fig. 3 suggests.

Step-wise increments in external potassium concentrations. One way to avoid having a wide variation in $[K^+]_o$ at different points in the extracellular spaces is to raise the external potassium in small steps, allowing the muscle to equilibrate each time. Two examples of this type of experiment are shown in Fig. 5. In both experiments the muscle was first placed in 20 mM $[K^+]_o$. The muscle in Fig. 5*A* was not maximally stimulated by a $[K^+]_o$ of 20 mM. When $[K^+]_o$ was increased to 30 mM there was a burst of increased respiration. A further increase of $[K^+]_o$ to 40 mM produced, if anything, a further fall in respiration. Often in these experiments, a maximal rate was produced by 20 mM $[K^+]_o$ and there was no further stimulation with an increase to 30–40 mM. In Fig. 5*B* the results are shown for a muscle that had a high rate of respiration in 20 mM $[K^+]_o$. An increase to 24 mM $[K^+]_o$ produced only a slight increase followed by a marked fall in the respiration. The decline in oxygen consumption continued in spite of a further increase in $[K^+]_o$ to 28 mM. The experiment shows two points. First, the respiratory rate changes appreciably with a

variation in membrane potential of only 3 or 4 mV. Secondly, the condition of the muscle is altered when it is kept at an elevated $[K^+]_o$. The response depends on the previous exposure of the muscle as well as the absolute level of potassium in the medium at any given time. If the muscle is kept for a time at 20 mM $[K^+]_o$ a change occurs so that it will not be stimulated by a further depolarization.

Figure 5*B* also illustrates a feature of K^+ stimulated respiration that was described first by Hill & Howarth (1957). The Ringer solution used contained choline in place of sodium. This substitution produces no change

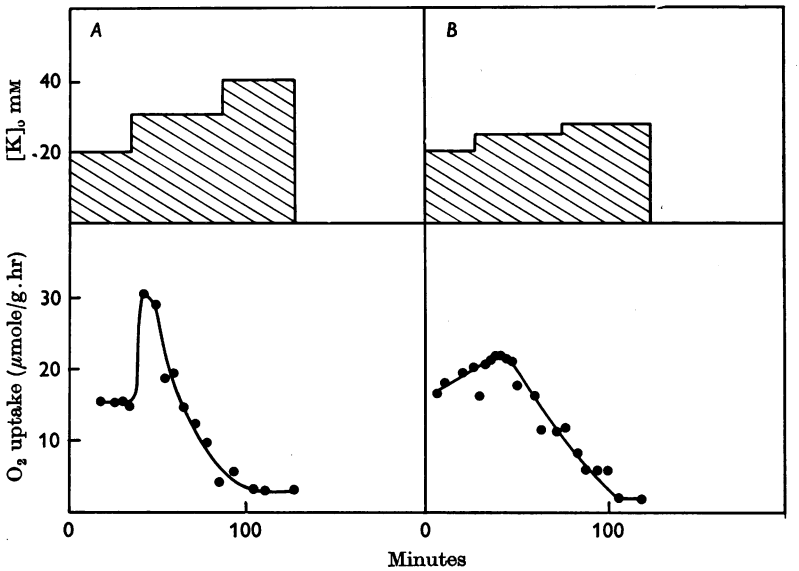

Fig. 5. Two examples of the effects of stepwise increase in $[K^+]_o$ on muscle respiration. For further explanation see text.

in respiratory rate or in the response to potassium. Therefore it is unlikely that respiratory rate increases in response to a rise in rate of sodium pumping from the muscle fibre.

Blocking the response to high potassium. Hill & Howarth (1957) found that the stimulation of heat production caused by a rise in $[K^+]_o$ could be counteracted or prevented by elevated concentrations of extracellular Ca^{2+} or Sr^{2+}. This effect is illustrated in Fig. 6. The muscle is first exposed to a $[K^+]_o$ of 20 mM which produced the usual increase in respiration. The addition of 10 mM calcium to the external solution brings respiration back down to the level in Ringer solution.

The curves drawn in Fig. 6 were calculated to give some idea of how the rate of diffusion into the extracellular space might effect the stimulation of respiration and the antagonism by added calcium. It was assumed that

the respiration at any point in the muscle in Ringer solution is 3 μmole O_2/g.hr. At any point in the muscle equilibrated with 20 mM $[K^+]_o$ the rate rises to 12·4 μmole O_2/g.hr. The muscle is assumed to be a flat sheet;

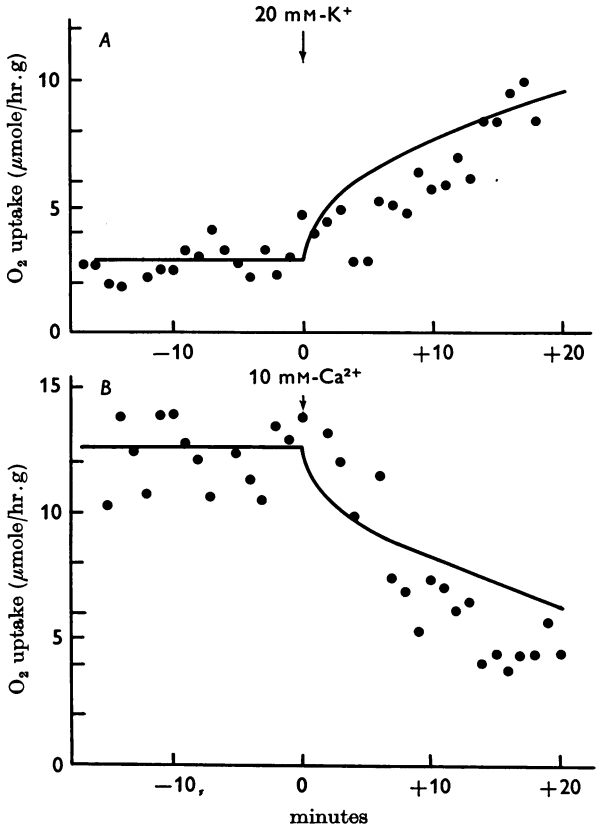

Fig. 6. *A*. The time course for the stimulation of muscle respiration by elevated $[K^+]_o$. The curve was calculated from a solution to the diffusion equation for a muscle shaped like an infinite sheet. *B*. The time course for the inhibition of respiration of the same muscle by increase $[Ca^{2+}]_o$. The curve was calculated, using the same dimensions for the muscle employed in *A*.

its fractional distance from equilibrium (C_{AV}/C_∞; average concentration at time t/concentration at infinite time) is given by:

$$\frac{C_{AV}}{C_\infty} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{\exp[-(2n+1)^2 \pi^2 Dt/4r^2]}{(2n+1)^2}, \qquad (1)$$

where the length of the diffusion path from one side of the muscle to the centre (r) is 0·22 cm and the diffusion constant for KCl (D) is $1{\cdot}6 \times 10^{-5}$ cm^2/sec. The curve in Fig. 6*B* was calculated by assuming that in the

fraction of the muscle equilibrated with 10 mM-$CaCl_2$, the respiration falls from 12·4 to 3 μmole O_2/g.hr, and that the diffusion coefficient for $CaCl_2$ is $1{\cdot}19 \times 10^{-6}$ cm^2/sec. The calculated curves for both the stimulation by K^+ and the antagonism by Ca^{2+} fit the data adequately. This suggests that K^+ and Ca^{2+} both act at a part of the cell that is equally accessible to ions diffusing in from the external medium. Since potassium acts by lowering the membrane potential, it seems likely that Ca^{2+} also acts on the cell membrane to antagonize the effects of K^+.

TABLE 2. The effect of divalent cations and procaine in blocking the increased respiration produced by 20 mM-K^+

Agent	Concentration (mM)	Respiratory rate in 20 mM-K^+ Ringer solution (percentage of rate in Ringer solution)
None	—	319 ± 112
Ca^{2+}	6	170, 142
	12	138, 122
	15	141, 131, 119, 103
	20	100, 104
Mg^{2+}	20	116, 126
	10	131, 95·2, 97·1, 135, 107
	6	128
	5	49·1, 291
Sr^{2+}	20	87·8, 88·0
	5	170, 128
Mn^{2+}	5	91·2, 108
Co^{2+}	5	121, 80
Procaine	5	188, 78
	1	152, 114, 99

Other divalent cations were also tested to see whether they antagonize K^+-stimulated respiration (Table 2). Strontium works as was reported by Hill & Howarth (1957) and Mg^{2+}, Mn^{2+} and Co^{2+} are also effective antagonists. Potassium-stimulated respiration is also blocked by procaine, as was first shown by Novotný, Vyskočil, Vyklický & Beránek (1962). In short, the effect of depolarization on respiration is blocked by a whole series of classical 'membrane stabilizers'.

As was pointed out before, the threshold $[K^+]_o$ needed to stimulate respiration varies substantially from experiment to experiment reported in the literature. There are also sizeable differences in the divalent cation concentrations in the Ringer solutions used by different investigators. Since divalent cations antagonize K^+-stimulated respiration, the Ringer solution would be expected to have a pronounced effect on the threshold. The Ringer solution used in the present experiments had the highest divalent cation concentration, and the threshold was also the highest.

The effect of divalent cations on membrane potential. Hill & Howarth (1957) suggest that Ca^{2+} blocks potassium-stimulated respiration by

reducing the depolarization of the membrane. This possibility was tested by measuring the membrane potentials of muscles in Ringer solution, after exposure to 20 mM $[K^+]_o$, or to 20 mM $[K^+]_o$ containing 12 mM $[Mg^{2+}]_o$.

The results, in Table 3, show that the membrane potential is, if anything, slightly lower in the muscles exposed to K^+ and Mg^{2+} than in muscles in

TABLE 3. Membrane potentials in sartorius muscles

Solution	Resting potential (mV mean ± S.E.M.)	Number of fibres
Ringer solution	88·4 ± 3·7	100
20 mM-K^+ Ringer solution	54·8 ± 6·9	132
20 mM-K^+, 12 mM-Mg^{2+} Ringer solution	48·9 ± 3·4	112

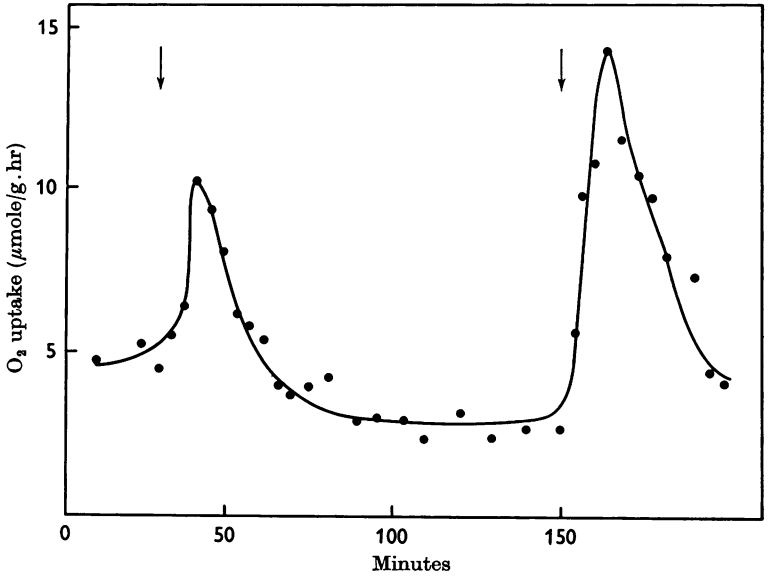

Fig. 7. The respiration of two muscles placed in the same Warburg flask. One muscle was in the centre well, the second was in a side arm. At the first arrow, K^+ was added to the centre well to raise the $[K^+]_o$ to 50 mM. At the second arrow, the other muscle was tipped from the side arm into the centre well. The experiment shows that the fall-off in respiration of muscles stimulated with 50 mM $[K^+]_o$ does not result from the accumulation of an inhibitor in the solution.

high K^+ alone. The stabilizing action of Mg^{2+}, and presumably the other effective divalent cations, is not produced by an action on the membrane potential. Novotný & Vyskočil (1966) reached the same conclusion.

Prolonging the burst. When $[K^+]_o$ is raised to levels about 30 mM there is a burst of increased respiration; a transitory increase followed by fall-off to the level found in Ringer solution. The cell remains depolarized, so there is no ready explanation for the decline. Hill & Howarth (1957) often

found a decrease in the heat production of muscles which were suspended in moist air; they suspected that the muscle might be releasing some substance which inhibited respiration. To check on this idea, a muscle was placed in a Warburg flask and a second muscle was inserted into the side arm. The second sidearm contained KCl solution. Both muscles were in Ringer solution. The respiration was measured. Then KCl was tipped in from the second side arm so that the muscle in the centre

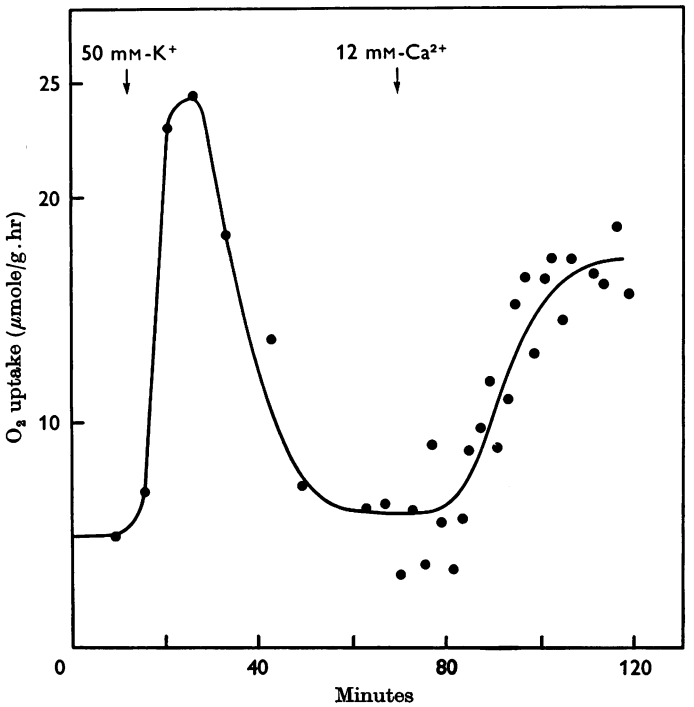

Fig. 8. The response of a muscle to 50 mM $[K^+]_o$ and the subsequent exposure to 12 mM $[Ca^{2+}]_o$. Notice the differences in the time course between the stimulation by K^+ and effect of the Ca^{2+}.

well was now in 50 mM $[K^+]_o$. As shown in Fig. 7 there is a typical burst in oxygen uptake. The time constant of the falling phase of the burst is about 16 min. Then the second muscle was tipped into the centre well from its side arm. Once again there was a burst of respiration. This shows that the respiration of the first muscle was not lowered by an accumulation of an inhibitor in the extracellular solution. In fact, the decrease in heat production of muscles suspended in moist air may be explained by the accumulation of K^+ in the extracellular solution; this would also lead to a decrease from maximal rates of heat production.

Another possibility is that the fall-off ending the burst occurs because the muscle is depleted of some essential metabolite. However, attempts to prolong the burst by adding glucose, lactate or pyruvate (5 mM) to the external solution was unsuccessful. The burst was also unaffected by 10^{-5} g/ml. acetylcholine, 0·1 mM epinephrine, 5 mM adenosine 5′-phosphate, or 0·4 u./ml. of insulin. Once the burst was over, 2,4-dinitrophenol was added to the external medium in a final concentration of 1×10^{-5} M, the muscle's respiration rose to 9·2 μmole O_2/g.hr, which was not significantly different from the rate produced by DPN alone. Even after the burst produced by 50 mM $[K^+]_o$ the muscle was still able to increase its respiration again in response to an effective stimulus.

TABLE 4. The effect of divalent cations and procaine on the respiration of muscles soaked in a Ringer solution containing 50 mM-K^+

Added substance	Concentration (mM)	Number	Respiration (μmoles/g.hr)
—	—	9	3·7 ± 0·9
Ca^{2+}	9	5	6·7 ± 3·7
	12	14	19·2 ± 4·0*
	15	4	22·0 ± 4·3*
Sr^{2+}	12	4	9·2 ± 1·8†
Mg^{2+}	12	2	4·1, 3·6
Procaine	5	2	3·7, 30

Statistical significance: * $P < 0.01$ † $P < 0.05$.

The transitory response to elevated $[K^+]_o$ can be changed into a sustained increase in respiration by simply raising $[Ca^{2+}]_o$ (Fig. 8). The figure also shows that, as usual, the stimulating effect of K^+ comes on rapidly, as quickly as the concentration in the extracellular space could be expected to rise. The stimulation by K^+ is virtually complete within 8 min. However, the onset of the Ca^{2+} effect is much slower, the time constant is about 25 min. It seems likely that Ca^{2+} must reach some site within the cell before respiration is stimulated.

The effect of Ca^{2+} in re-establishing a high rate of oxygen consumption after a burst, or in promoting a sustained rate of oxygen consumption if added along with K^+, is distinct from the effects of divalent cations in elevating the threshold for K^+ stimulation. One clear difference which was just described is in the speed of onset. Furthermore, the effects of calcium in prolonging the burst are not duplicated by Mg^{2+} or by procaine (Table 4). Strontium is somewhat effective as a replacement for Ca^{2+}. The importance of calcium in the potassium stimulation was also described by Novotný & Vyskočil (1966).

There is excellent evidence that muscle contraction is triggered by the release of calcium into the sarcoplasm. When the muscle fibre is exposed to 50 mM $[K^+]_o$ there is a brief contracture which is over within 5 sec

(Hodgkin & Horowicz, 1960; Luttgau, 1963). Presumably when the muscle fibre is depolarized the $[Ca^{2+}]$ in the sarcoplasm first rises and then falls again, despite the continuing depolarization of the membrane. Suppose that respiration also responds to the $[Ca^{2+}]$ in the sarcoplasm. Then the effect of an increased $[Ca^{2+}]_o$ in prolonging the burst, or in restoring a high level of respiration after the burst is over, would be caused by an increase in the concentration of calcium in the sarcoplasm itself. The question then is whether the rise in $[Ca^{2+}]_o$ causes a contraction as well as an increase in respiration. In six experiments sartorius muscles were arranged for tension recording. When the Ringer solution was replaced

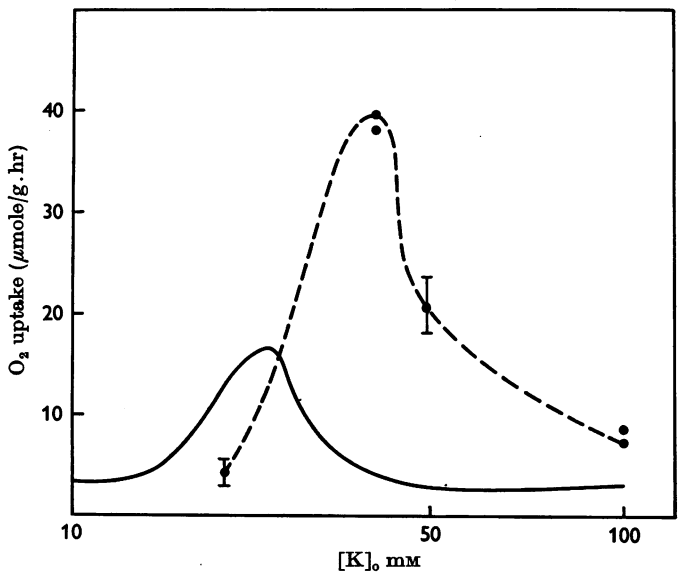

Fig. 9. Respiration rates measured about 1 hr following stimulation by elevated $[K^+]_o$. The continuous line is for muscles in 2·5 mM $[Ca^{2+}]_o$, taken from Fig. 3. The interrupted line is for muscles in 15 mM $[Ca^{2+}]_o$.

with a solution containing 50 mM-K^+, a series of rapid twitches was always recorded before the muscle became inactive. However, when the 50 mM-K^+ Ringer solution was then replaced by 50 mM potassium, 12 mM calcium solution there was no mechanical response. If the calcium in the sarcoplasm is the trigger for the increase in respiration, then the threshold for increasing oxygen uptake is below the threshold for contraction. This idea agrees with the observation that when the membrane potential is lowered, the respiration is stimulated at depolarizations less than the threshold for the development of tension.

The response of muscles in elevated Ca^{2+} to stimulation by different

levels of $[K^+]_o$ is summarized in Fig. 9. There are two notable points. First, the highest levels of respiration are found in $[K^+]_o$ above that in normal Ringer solution. Secondly, with a raised $[Ca^{2+}]_o$ the whole curve relating oxygen uptake to $[K^+]_o$ is shifted to the right.

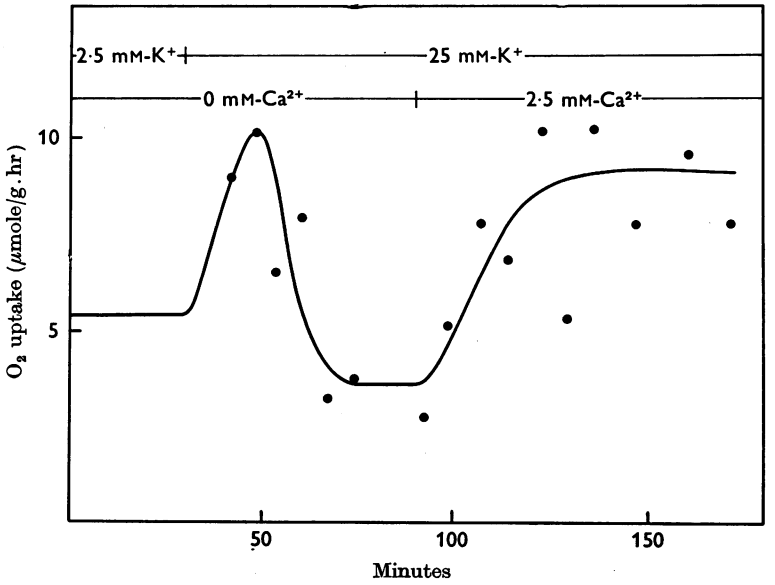

Fig. 10. The effect of elevated $[K^+]_o$ on a muscle in Ca^{2+}-free Ringer solution and the effect of subsequent raising of $[Ca^{2+}]_o$ to 2·5 mM.

TABLE 5. The respiration of muscles that were first soaked in 30 mM-K^+, 0 mM-Ca^{2+} Ringer solution and then transferred into 30 mM-K^+ Ringer solution containing divalent cations

Cation	Concentration (mM)	Number of muscles	μmoles O_2/g.hr (mean ± S.D.)
—	—	4	2·4 ± 0·22
Ca^{2+}	0·5	6	2·6 ± 1·09
	1	5	2·8 ± 2·0
	2	1	6·8
	3	4	13·4 ± 2·3*
	5	3	12·9 ± 1·6
	9	1	12·1
Sr^{2+}	1	1	2·8
	5	6	5·4 ± 1·3*
Mg^{2+}	5	4	2·3 ± 0·25

Statistical significance: * $P < 0·01$.

The importance of Ca^{2+} in potassium-stimulated respiration led to a series of experiments in which muscles were soaked in Ringer solution which did not include Ca^{2+}. The behaviour of a typical muscle is shown in Fig. 10. The elevation of $[K^+]_o$ to 25 mM produces, as usual, an increase in

respiration. However, when there is no Ca^{2+} in the extracellular solution the increase in respiration is transitory; there is a burst of oxygen consumption. Respiration can be raised back to the higher level by adding Ca^{2+} to the extracellular solution. The extent of the rise depends on the concentration of Ca^{2+} in the extracellular solution: less than 2 mM is usually ineffectual and a maximum is reached with about 3 mM (Table 5).

The results from experiments with zero Ca^{2+} Ringer solution suggest that Ca^{2+} is important for the stimulation of respiration by depolarization. However, a muscle soaked in low Ca^{2+} Ringer solution must retain a store of Ca^{2+} able to trigger a transitory increase in respiration when the muscle is stimulated by depolarization.

Potassium-stimulated respiration in other frog tissues. Eight experiments were run to see whether the respiration of slices of other tissues from the frog could be increased by raising $[K^+]_o$ to 25 mM. There was no detectable effect on slices of liver, kidney, or brain. Muscle seems to be the only tissue that shows potassium-stimulated respiration.

DISCUSSION

The respiration of frog twitch muscles can be increased by depolarizing the cell membranes with K^+, Rb^+, or with Cs^+; the effect of these ions on respiration is directly proportional to their ability to depolarize the fibres. The stimulation of respiration by depolarization also requires Ca^{2+}. Two types of experiments support this conclusion. First, muscles depolarized in 50 mM $[K^+]_o$ have a burst of increase oxygen consumption followed by a fall back to resting values. The decline from the burst may be prevented by increasing $[Ca^{2+}]$. Or if the burst is already over, respiration can be increased once again by raising $[Ca^{2+}]_o$. Secondly, the respiration of muscles in Ca^{2+}-free Ringer solution is increased when $[K^+]_o$ is raised to 25 mM. But the increase in respiration is transitory unless Ca^{2+} is also added back to the extracellular solution.

Depolarization apparently increases the concentration of Ca^{2+} in the sarcoplasm and this is the trigger for the increase in respiration. Respiration is stimulated by depolarizations below the threshold for producing a contracture. Consequently, respiration seems to be more sensitive as an indicator of sarcoplasmic Ca^{2+} than is contraction. The next step then is to try to fit the data on contracture and on respiration together into a single scheme which describes the relation between membrane potential and the concentration of Ca^{2+} in the sarcoplasm. A successful model must account for a number of different observations.

(1) A muscle fibre depolarized in high $[K^+]_o$ contracts for less than 10 sec, and then relaxes, even though the membrane remains depolarized.

The greater the depolarization, the shorter the contracture, and the greater the tension—at least until an upper limit on tension development is reached (Hodgkin & Horowicz, 1960).

(2) When a muscle fibre is placed in Ringer solution after it has spent some time in high $[K^+]_o$, there is an interval of somewhat less than a minute during which the fibre will not contract when stimulated (Hodgkin & Horowicz, 1960).

(3) Suppose a muscle fibre is depolarized with potassium to give a maximum contracture, and is then shifted to a lower $[K^+]_o$, at a level still capable of eliciting contracture. The relaxation from the maximum contracture then has two steps: an early phase, just after the shift to the reduced $[K^+]_o$, followed by a second, slower phase. The rate of relaxation during the second phase is proportional to $[K^+]_o$ (Foulks & Perry, 1966).

(4) Respiration is stimulated for long periods by depolarizations below the threshold for contracture. But depolarizations producing powerful contractures cause only a transitory increase in respiration.

(5) During the first minutes of depolarization the exchangeability of $^{45}Ca^{2+}$ is increased. Over long time periods depolarization does not produce detectable increases in $^{45}Ca^{2+}$ exchange (Novotný, 1965; Novotný & Vyskočil, 1966).

The inactivation hypothesis. One way of accounting for these points is by supposing that both respiration and contraction are stimulated by Ca^{2+} moving from the extracellular solution into the sarcoplasm. The movement of Ca^{2+} is presumed to increase when the membrane is depolarized—the greater the depolarization the greater the increase in permeability. There is an inactivation process for the increased calcium permeability, so with time Ca^{2+} permeability decreases, much like the inactivation of sodium permeability occurring in nerve and muscle (Hodgkin & Huxley, 1952). This is the model proposed by Novotný & Vyskočil (1966). The principal difficulty faced by this model is that the time constant for the inactivation process would have to vary over many orders of magnitude, depending on the membrane potential. When $[K^+]_o$ is 25 mM, the time constant for the decline of the stimulated respiration is at least 2 to 3 hr. When $[K^+]_o$ is 100 mM, the time constant for relaxation from a contracture—and presumably for the fall in the $[Ca^{2+}]$ in the sarcoplasm—is a few seconds.

Another difficulty with the idea that there is an inactivation mechanism for Ca^{2+} inflow is the behaviour of the muscles kept in Ca^{2+}-free Ringer solution. When $[K^+]_o$ is raised to 25 mM, these muscles have a burst of increased respiration, in place of the usual long-maintained response. When Ca^{2+} is added back to the extracellular solution, the respiration slowly rises back to the high level. Potassium-stimulation occurs when the muscles

are in Ca^{2+}-free solution, even though $[Ca^{2+}]_o$ is ultimately needed to keep up respiration. This suggests that muscles kept in Ca^{2+}-free solution retain a store of Ca^{2+}, which is released into the sarcoplasm when the fibre is depolarized, and that respiration falls off when the store is used up. Similarly, after the burst of increased respiration in response to 50 mM $[K^+]_o$ the respiration can be brought back to a high level by increasing $[Ca^{2+}]_o$. But again the time course of the action of calcium is quite slow, which is not explained by the inactivation hypothesis. The inactivation idea also does not readily account for the effects of step-wise increases in $[K^+]_o$, like those shown in Fig. 5.

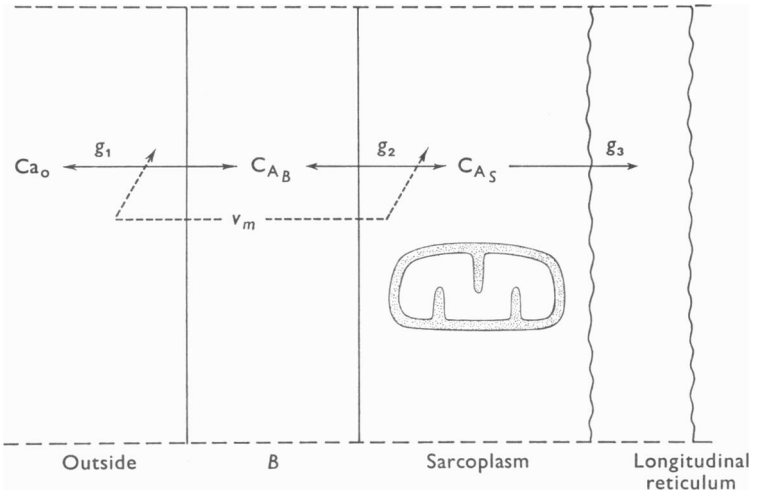

Fig. 11. A schematic diagram of a muscle with three compartments for Ca^{2+}. For further description see the text.

The most convincing evidence for the inactivation hypothesis is the $^{45}Ca^{2+}$ exchange data, which shows that exchange is stimulated above the control level by short depolarizations, but not by long depolarizations (Novotný & Vyskočil, 1966).

A three compartment hypothesis. A second possible model supposes that the muscle fibre is subdivided into three compartments (Fig. 11). Between the extracellular solution and the sarcoplasm, there is a calcium storing or binding compartment—compartment B in the illustration. Calcium can move down a gradient from the extracellular solution into compartment B, and from there into the sarcoplasm. Calcium is constantly taken from the sarcoplasm into the longitudinal sarcoplasmic reticulum. In this system

$$dC_{A_B}/dt = (g_1 Ca_o - g_1 C_{A_B} - g_2 C_{A_B} + g_2 C_{A_S})/V_B,$$
$$dC_{A_S}/dt = (g_2 C_{A_B} - g_2 C_{A_S} - g_3 C_{A_S})/V_S,$$

where V_B is the capacity of compartment B, and V_S is the capacity of the sarcoplasm. C_{A_B} is the calcium concentration in compartment B and C_{A_S} is the concentration in the sarcoplasm. The rate constants g_1, g_2, and g_3 are identified in Fig. 11. The first supposition is that g_2 increases when the cell membrane is depolarized, so calcium moves from compartment B into the sarcoplasm. Initially there would be a rapid flow of calcium from compartment B, and C_{A_S} would rise. But if the rate of calcium entry from the extracellular solution into compartment B does not match the flow of calcium from compartment B into the sarcoplasm, then C_{A_B} decreases and the rate of influx into the sarcoplasm declines. This model relies on the depletion of compartment B to account for the transitory changes in sarcoplasmic Ca^{2+} which occur when the membrane is depolarized sufficiently.

This model cannot account for all the observations. When a steady state is reached

$$C_{A_S} = Ca_o/g_3(1/g_1 + 1/g_2 + 1/g_3).$$

When muscles are placed in 25 mM ($[K^+]_o$ or in 50 mM $[K^+]_o$ with a 5-fold increase in $[Ca^{2+}]_o$, they have about the same elevated respiration and they presumably have the same elevated C_{A_S}. If g_2 is the only permeability constant that changes with depolarization, then g_2 must be smaller with 25 mM $[K^+]_o$ than it is with 50 mM $[K^+]_o$. Then the time constant for the system would be larger with 50 mM $[K^+]_o$ than it is with 25 mM $[K^+]_o$—which is contrary to all of the observations. To account for the results either g_1 or g_3 must also vary with membrane potential.

A model in which depolarization results in both an increase in the rate of Ca^{2+} inflow into the sarcoplasm and an increase in the rate at which Ca^{2+} is transported out of the sarcoplasm accounts for most of the features of muscle respiration and contracture. Foulks & Perry (1966) propose this idea to account for their data on contracture. Referring to Fig. 11, both g_2 and g_3 increase with depolarization. This model does not account for the experiment in which a muscle fibre is depolarized in high $[K^+]_o$ for a long period and is then replaced in normal Ringer solution. The muscle recovers excitability in a few seconds. This means that g_1 must be high so that calcium in compartment B is rapidly replenished. But if high values of g_1 are used, then the model works no longer, because C_{A_B} does not become depleted when the muscle is depolarized.

The other possible model along this line has a constant g_3—that is, the rate constant for the transport of calcium from the sarcoplasm does not change with membrane potential. But with depolarization, there is an increase in g_2 so more calcium enters the sarcoplasm from compartment B, and also a decrease in g_1 so less calcium moves from the extracellular

solution to replenish compartment *B*. By trial and error equations for g_1 and g_2 as a function of membrane potential were worked out to roughly fit the data. The point of the calculations at this stage is simply to see how well the model can duplicate the general properties of the muscle.

$$g_1 = (1 - 1/\exp((65 + V)/7))/10^5$$

$$g_2 = (1 + \exp((60 - V)/4))/10^5$$

where V is the membrane potential in mV. The equations are based on the distribution of charged particles across a membrane according to Bolzman's principle (see Hodgkin & Huxley, 1962), Graphs of g_2 and g_1 as a function of $[K^+]_o$ are seen in Fig. 12*A*. The other values used in the calculations were $Ca_o = 2{\cdot}5$, $V_B = 0{\cdot}01$, $V_S = 0{\cdot}001$, $g_3 = 1$.

Some aspects of the behaviour of the model are illustrated in Fig. 12.

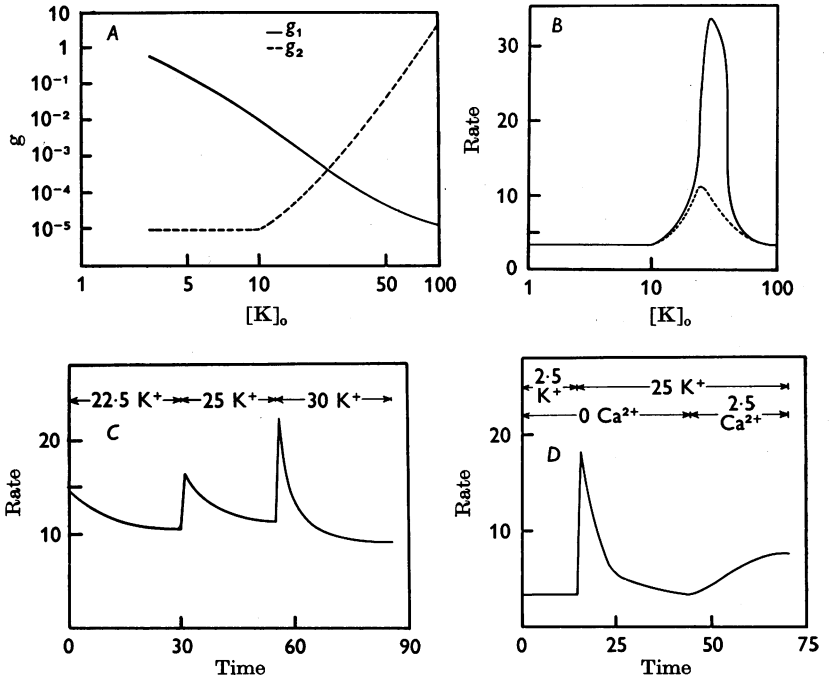

Fig. 12. Some features of the model developed to account for the effects of membrane depolarization on the stimulation of respiration. *A*, The variation of g_1 (———) and of g_2 (– – – –) with $[K^+]_o$. *B*. The rate of 'respiration' of the model when exposed to elevated '$[K^+]_o$'. The average response time 1–5 min after the increase in $[K^+]_o$ (———). The averaged response 56–60 min after the increase in $[K^+]_o$ (– – – –). *C*. The rate of respiration of the model in response to stepwise increases in $[K^+]_o$. *D*. The response of the model to 25 mM($[K^+]_o$ in the absence of extracellular Ca^{2+} and the effect of subsequently raising $[Ca^{2+}]$ to 2·5 mM.

The muscle is assumed to have a basal rate of 3·5 μmole O_2/g.hr, plus an increment equal to $C_{AS} \times 10^4$. There are no measurable increases in respiration at values of $[K^+]_o$ below 10 mM. Further elevations of $[K^+]_o$ produced an increase of the respiratory rate which reaches a maximum at a $[K^+]_o$ of about 30 mM (Fig. 12*B* should be compared with Figs. 2 and 3).

Step-wise increases in $[K^+]_o$ in the range of 20–30 mM produce only a small transient increase in rate, that would be averaged out in measurements of oxygen uptake in respirometers (compare with Fig. 5). If Ca_o is reduced to zero and $[K^+]_o$ is raised to 25 mM there is a burst of increased oxygen consumption and then respiration returns to the basal level (Fig. 12*D*); respiration can be stimulated by raising Ca_o to 2·5. There is a notable time lag for the action of the added Ca_o, since it takes a period for compartment *B* to refill with Ca^{2+} (compare with Fig. 8). The model—unlike the muscle—would soon become unresponsive if kept in Ca^{2+}-free Ringer solution. A better model would replace g_1 with two rate constants for calcium movement: a high rate constant for movement from the outside into compartment *B*, and a low rate constant for movement from compartment *B* to the outside. Calculations of the detailed properties of this model have not yet been made, but enough was done to show that a muscle with a fixed permeability for calcium movement between compartment *B* and the outside, but with the membrane potential favouring inward movement and opposing outward movement, cannot successfully account for the data.

The properties of the longitudinal sarcoplasmic reticulum assumed in the final model agree with the properties of vesicles isolated from muscle. The rate of calcium uptake by the vesicles formed from the lobster reticulum is proportional to the $[Ca^{2+}]$ in the solution outside of the vesicles (Van der Kloot, 1965). In the muscle Ca^{2+} probably leaks from the reticulum back into the sarcoplasm, but the rate is likely to be steady because in normal conditions the concentration of free Ca^{2+} within the reticulum is not likely to vary (Van der Kloot, 1966). One of the most important points in the model is that the Ca^{2+} released into the sarcoplasm by depolarization comes from compartment *B*, which is quite distinct from the longitudinal sarcoplasmic reticulum. Moreover, compartment *B* must be located close to the cell membrane, since release of Ca^{2+} from this store can be altered rapidly by changes in the divalent cation concentration of the extracellular solution, or by the addition of local anaesthetics to the extracellular solution. The logical place for the location of compartment *B* is in the cell membrane and the walls of the *T* (transverse) tubules of the sarcoplasmic reticulum.

Some of the predictions made by the model about sarcoplasmic Ca^{2+} concentrations during the first seconds after depolarization are shown in

Fig. 13. Depolarizations to levels below the threshold for contraction produce changes in C_{A_S} which are maintained for long time intervals. At higher concentrations of $[K^+]_0$ there is a greater rise in C_{A_S} but the time constant for the fall-off also shortens. With moderate depolarizations the muscle would undergo a partial contracture with a relatively slow relaxation; with high $[K^+]_0$s the muscle would undergo a powerful contracture

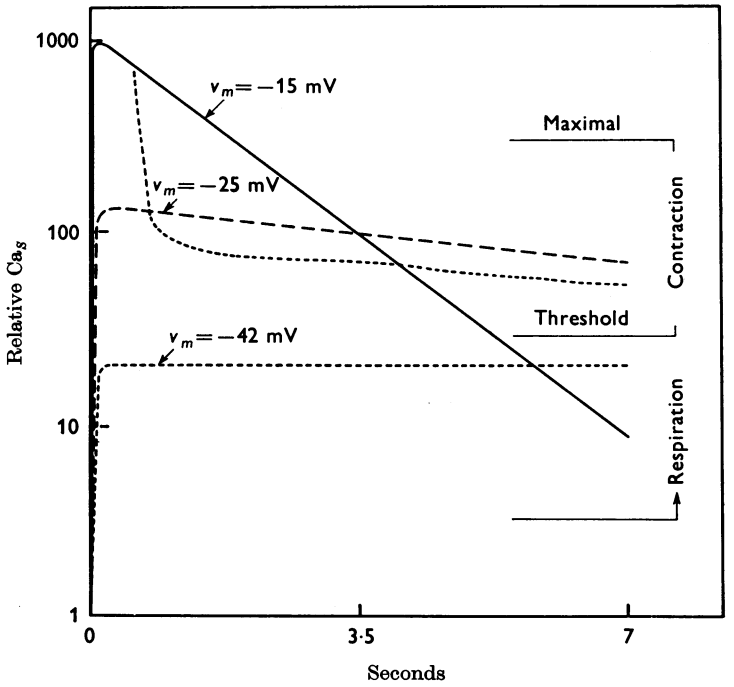

Fig. 13. The concentration of 'sarcoplasmic calcium' in the muscle model during the first seconds after depolarization. Levels are indicated at which respiration might be stimulated, at which contraction might begin, and at which a maximal contraction might be obtained. The principal points to notice are the differences in the time constant for the decline in Ca_s at different depolarizations. The effect of depolarizing a fibre to -15 mV, waiting until a maximum contraction is reached, and then at 0·5 sec shifting membrane potential to -25 mV is also shown (– – – –).

but with a faster relaxation. If the muscle is exposed to high $[K^+]_0$ until it reaches a maximum contracture and then if the $[K^+]_0$ is shifted to a lower value, still above the threshold for contracture, there will be an abrupt relaxation when the extracellular solution is first changed, followed by a slow second phase of relaxation. In these respects the model is a good mimic of the behaviour of muscle.

Tracer movements and the stimulation of respiration. One of the serious

defects of the model presented to account for the data on respiration is that it does not fit the available data on ^{45}Ca exchange. However, I do not regard this discrepancy as a decisive reason to reject the model. In the first place it is clear that much of the total calcium in the muscle is not involved directly in the systems described in the model, since they could account for only a small fraction of the total calcium measured in muscle. Moreover, the measurements on calcium exchangeability are routinely made by exposing muscles to radioactive calcium for the experimental period and then washing them for 90 min in tracer-free solutions. This treatment is used to wash the high tracer levels from the extracellular fluid, but may obscure many of the most important features of radioactive calcium movement.

The mechanism of respiratory stimulation. The results do little to show how a rise in sarcoplasmic Ca^{2+} or Sr^{2+} stimulates respiration. Both Ca^{2+} and Sr^{2+} are effective in frog muscle as activators of the actomyosin ATPase (Edwards, Lorkovic & Weber, 1966); Ca^{2+} and Sr^{2+} may promote the hydrolysis of ATP and the accumulation of ADP which then acts as a trigger for increasing respiration.

Mitochondrial respiration is stimulated by Ca^{2+} and by Sr^{2+} (Slater & Cleland, 1953; Siekevitz & Potter, 1953). The divalent cations are transported into the mitochondria and respiration is enhanced as long as transport is under way. The specificity of the system varies from species to species; beef heart sarcosomes will accumulate Ca^{2+}, Mg^{2+}, or Mn^{2+} (Rasmussen, 1966). Nothing is known about the abilities of frog muscle mitochondria to transport any of these ions. The differences between species and even between organs within a species means that we cannot rule out this mode of stimulation on the grounds that only Ca^{2+} and Sr^{2+} are effective. However, the usual concentrations of divalent cations required to stimulate mitochondrial respiration are high compared to the estimated intracellular concentrations in skeletal muscle, so it is somewhat unlikely that tissue respiration is stimulated in this way.

Potassium depolarization stimulates glycolysis in frog skeletal muscle, as long as extracellular calcium is present (Kaye & Mommaerts, 1960). Stimulation is accompanied by the appearance of phosphorylase a and increased activity of phosphofructokinase (Danforth & Helmreich, 1964; Özand & Narahara, 1964). It is possible that a rise in sarcoplasmic Ca^{2+} is a trigger for the increase in activity of a series of rate-limiting enzymes.

Further investigation is obviously needed to fit these diverse lines into a coherent view of the effects of depolarization on muscle metabolism. The major questions can be answered by available experimental techniques. Whatever its mode of action may be, sarcoplasmic $[Ca^{2+}]$ appears to influence many phases of muscle metabolism.

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