J. Physiol. (1973), **231**, *pp.* 233–249
With 8 text-figures
Printed in Great Britain

THE EFFECTS OF TEMPERATURE AND METABOLIC INHIBITORS ON THE SPONTANEOUS RELAXATION OF THE POTASSIUM CONTRACTURE OF THE HEART OF THE FROG *RANA PIPIENS*

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(*Received 12 July 1972*)

SUMMARY

1. The spontaneous relaxation of the potassium contracture and the relaxation induced by the removal of extracellular calcium or the restoration of the original potassium concentration, in the frog heart, show a strong dependence on temperature.

2. The energy of activation of the later exponential phase of the spontaneous relaxation is 10·43 kcal mole^{-1}, a value close to that reported for the binding of calcium ions by isolated sarcoplasmic reticulum, but larger than that for the calcium efflux from mammalian heart.

3. The use of metabolic inhibitors shows that relaxation can be sustained when glycolysis is poisoned, but the disruption of oxidative phosphorylation slows relaxation.

4. Poisoning of both glycolysis and oxidative phosphorylation blocks all but the small initial part of the spontaneous relaxation of the potassium contracture and also interferes with relaxation induced by other means.

5. The results are considered to favour the existence, in frog heart, of an active intracellular relaxing system which uses ATP as its substrate to lower the sarcoplasmic calcium concentration. This system is likely to be the sarcoplasmic reticulum but the mitochondria could also be involved.

INTRODUCTION

In the previous paper it has been shown that atrial trabecules isolated from the heart of the frog are able to relax even when the muscle is continuously exposed to solutions containing an elevated potassium concentration (Chapman, 1973). This spontaneous relaxation was accounted for by assuming that a relaxing system inside frog cardiac muscle cells exists,

similar to that found in skeletal muscle cells. Relaxing systems of this type have to remove activator calcium from the sarcoplasm against a concentration gradient and will, therefore, presumably have to use metabolic energy to do so. In the present paper, the effects of temperature on the spontaneous relaxation of contractures induced by high-potassium solutions have been studied, and a very marked temperature dependence has been found. The spontaneous relaxation rates have been measured following the application of a variety of metabolic inhibitors to see if either glycolysis and/or oxidative phosphorylation is providing the energy used to reduce the sarcoplasmic calcium concentration and thereby bring about relaxation. Certain metabolic inhibitors have highly specific effects on the calcium-binding of isolated sarcoplasmic reticulum and mitochondria, and experiments using these chemicals might reveal which of these two intracellular structures is directly responsible for the relaxation of frog myocardium.

METHODS

The method of isolation and mounting of the preparation was as described previously (Chapman & Tunstall, 1971), while the experimental solutions, and the general way in which the results were analysed have been listed in the preceding paper (Chapman, 1973). The temperature of the perfusing solutions was altered by passing them through Pyrex coils immersed in water baths, whose temperature could be controlled to 0·1° C over the range 0–40° C. The temperature of the perfusing fluid was measured using either a small mercury bulb thermometer placed in the main drain of the experimental chamber or a bead thermistor mounted directly in the experimental channel.

A cloudy white precipitate, seen when cyanide was added to phosphate-buffered solutions containing calcium, was not chemically identified. This precipitate was not formed in solutions buffered with Tris-HCl, and so when cyanide was used in part of the experiment all the solutions were buffered with Tris-HCl.

Oligomycin, Antimycin A (obtained from Sigma Chemicals) and carbonyl cyanide *m*-chlorophenyl hydrazine (Calbiochemicals) dissolved in ethanol, were added to the Ringer. An equivalent amount of ethanol was added to the other solutions in these experiments, its concentration never exceeding 0·1 %. The various metabolic inhibitors take some time to act, in all cases exposure was for at least 6 min before a potassium contracture was elicited. In the case of oligomycin or antimycin A this time was much longer.

RESULTS

The effect of temperature on the high-potassium contracture. The heart, stimulated at 4 min^{-1}, was exposed to various temperatures in the range 4–35° C for between 10 and 15 min in normal Ringer before stimulation was stopped and contracture fluid at the same temperature was applied to the muscle and the contracture allowed to develop. In normal Ringer the amplitude and duration of the twitches increased as the temperature was lowered, the slowing of the relaxation phase of the twitch being greater

than that of the rising phase, so that the time to peak invariably increased as the temperature was lowered, as originally found by Heintzen, Kraft & Wiegmann (1956). When the amplitude of the twitches had stabilized at each experimental temperature the contracture solution (in all cases 100 mM potassium) was applied to the muscle. In the potassium contracture fluid the muscle developed contractures whose strength showed a U-shaped dependence on the temperature, with a fairly insensitive region between 15 and 25° C while temperatures above and below this range resulted in an increase in the amplitude of the contracture (Fig. 1). The increased tension generated following prolonged perfusion by normal Ringer at low temperatures is probably the after effect of the long duration twitches that occur at these temperatures, which could build up the intracellular stores of activator calcium in much the same way as that described during the staircase response (Chapman & Niedergerke, 1970). Lowering the temperature during a potassium contracture results in a reduction of the tension the muscle is generating. The rate of spontaneous relaxation during perfusion with the contracture fluid is also temperature dependent; the exponential phase of the spontaneous relaxation was slowed as the temperature was reduced having a Q_{10} of 2·09 ± S.D. 0·22 between 6 and 16° C. This means that the duration of the contracture also changed, becoming very long at low temperatures and very short at high ones (Fig. 2). The duration of the period before the onset of the exponential phase of spontaneous relaxation was also increased when the temperature was reduced. The relaxation occurring when the $[K]_0$ or the $[Ca]_0$ is reduced, also shows a strong dependence on temperature and has a Q_{10} of 2·00 ± S.D. 0·74 (between 6 and 16° C). This value, only slightly less than that for the spontaneous relaxation, suggests that this type of relaxation is not simply dependent upon some diffusion process, but probably depends on the same processes as the spontaneous relaxation.

An estimate of the energy of activation of the process responsible for the spontaneous relaxation can be obtained by means of an Arrhenius plot (Fig. 3). Estimated in this way, the energy of activation was found to have a mean value of 10·43 kcal mole^{-1} (S.D. 1·28 kcal mole^{-1}), a value close to that obtained for the binding of calcium by sarcoplasmic reticulum isolated from mammalian heart (Harigaya & Schwartz, 1969). It is smaller than that found for microsomes isolated from rabbit skeletal muscle (Inesi & Watanabe, 1967), and much smaller than that found for the spontaneous relaxation of potassium contractures in skeletal muscle fibres (Caputo, 1972).

Prolonged periods of perfusion at temperatures above 30° C sometimes had irreversible effects on the preparations, the rate of spontaneous relaxation becoming slower as the temperature was raised further. By alternating exposure to high and

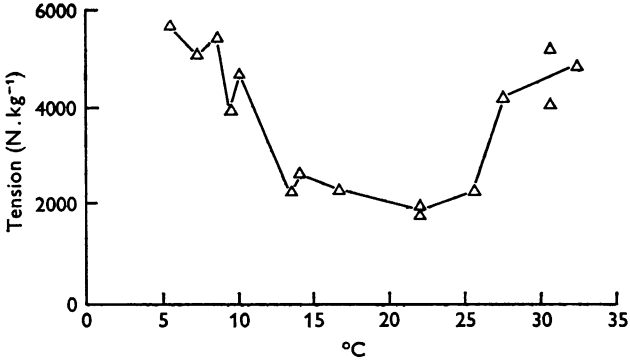

Fig. 1. The amplitude of 100 mM potassium contractures shows some dependence on temperature. In each case the temperature was maintained for between 10 and 20 min, while the muscle was electrically stimulated at 4 min^{-1} before the potassium contracture, at the same temperature, was evoked. High and low temperatures were alternated so as to avoid the adverse effects of long periods at high temperatures. The $[Ca]_o$ was 1 mM.

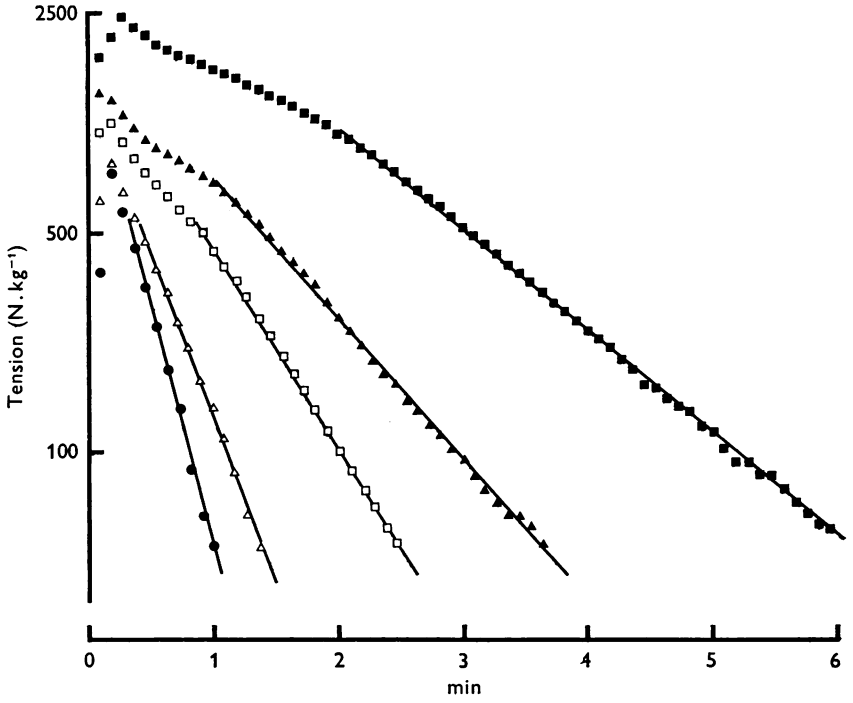

Fig. 2. The semilogarithmic plots of the spontaneous relaxation of the contractures of various temperatures. The solid lines drawn through the later part of the relaxation are obtained by regression analysis. The temperatures at which the contractures were elicited are as follows: 8·0° C, ■—■; 10·0° C, ▲—▲; 12·0° C, □—□; 17·0° C, △—△; and 22·5° C. ●—●.

low temperatures, i.e. by avoiding prolonged exposure to high temperatures, this irreversible effect was avoided.

The influence of chemicals that interfere with the process of oxidative phosphorylation on relaxation. Niedergerke (1956) found that relaxation of potassium contractures was slowed in frog ventricle strips deprived of oxygen. This is not observed with frog auricle strips, using the present experimental set-up, probably because sufficient oxygen is present in the solutions that perfuse the muscle to support respiration, even after they

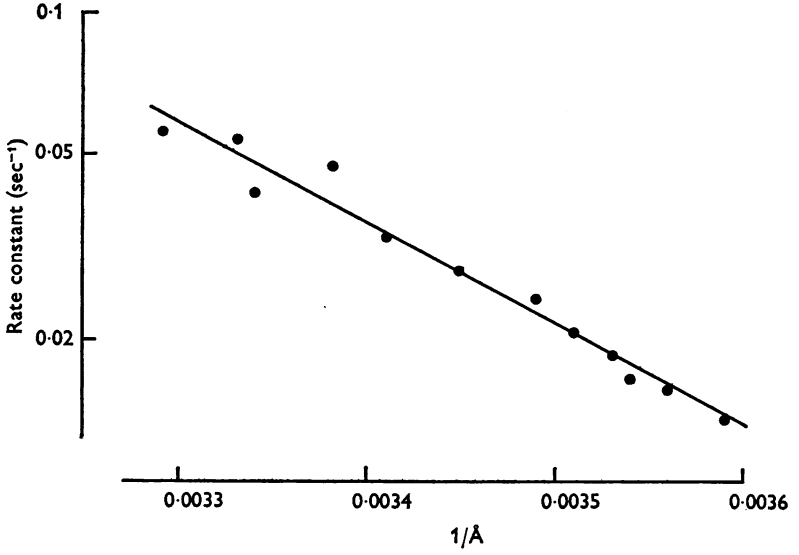

Fig. 3. An Arrhenius plot of the logarithm of the rate constant of the exponential phase of the spontaneous relaxation against the reciprocal of the absolute temperature. The continuous line was obtained by regression analysis and has a coefficient of correlation of 0·98, the energy of activation calculated from the slope of this line was 9·98 kcal mol^{-1}. The potassium contractures were evoked by 100 mM potassium contracture fluids containing 1 mM calcium.

have been gassed with nitrogen. Measurements with an oxygen electrode show that oxygen can pass through the walls of the silicone rubber supply tubes into experimental solutions as they flow from their reservoirs into the experimental chamber.

Experiments in which chemical inhibitors of oxidative phosphorylation, sodium cyanide, 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenyl hydrazine (CCmP), antimycin A and oligomycin at optimal concentrations, were applied to the muscle caused a marked reduction in the rate of the spontaneous relaxation of the contracture. There was consider-

able variation in the time to maximum effect of, and the recovery from, each drug, as well as considerable variation in the sensitivity of the heart to each particular chemical. This variation was one of the most remarkable features of this work; it would seem that the reserves of energy must vary considerably from one heart to another.

2,4-Dinitrophenol and carbonyl cyanide m-chlorophenyl hydrazine. Both chemicals act at the same site on the oxidative phosphorylation chain to uncouple the phosphorylation of ADP to ATP. Each caused a drastic slowing of the final rate of spontaneous relaxation of the high-potassium contracture and the relaxation on lowering the $[K]_0$ but the early phases of relaxation were unchanged (Fig. 4*A*). In all cases DNP was required at a larger concentration than CCmP to induce maximal slowing of the exponential phase of the spontaneous relaxation, 0·1 mM being a typical concentration for DNP and 1 μM being typical for CCmP. This difference would be anticipated from the difference between the dose response relationship found for these to inhibitors on mitochondrial oxidative phosphorylation (Heytler & Prichard, 1962). In normal Ringer these compounds took between 4 and 10 min to reach a maximal effect on the relaxation of a potassium contracture. During this time the twitches became steadily smaller and very short, probably due to the shortening of the action potential which occurs when frog heart is exposed to these chemicals (R. A. Chapman & C. H. Fry, unpublished work).

Oligomycin is a powerful blocking agent of mitochondrial-oxidative phosphorylation, but its action on calcium transport by isolated sarcoplasmic reticulum occurs only at high concentrations (Carsten & Mommaerts, 1964). In the heart the application of oligomycin at concentrations of between 10^{-6} and 10^{-5} g ml.$^{-1}$ results in a slowing of the spontaneous relaxation of the potassium contracture only after at least 20 min exposure to the chemical. This delay may be due either to its large molecular weight which might limit penetration into the sarcoplasm, or that the relaxing system itself is not very sensitive to the antibiotic (Fig. 4*B*). Alternatively, the relaxing system within the muscle cells may derive some energy, presumably ATP, from other sources, such as glycolysis, or via creatine phosphate.

Antimycin A, which blocks mitochondrial respiration based phosphorylation by interfering with the cytochrome system, potentiates calcium ion uptake in cardiac sarcoplasmic microsomes (Carsten & Mommaerts, 1964). When applied to frog atrial trabecules at a concentration of 3·0 μg ml.$^{-1}$ this inhibitor takes some 60 min to reach its full effectiveness, again perhaps due to its large size and hence slow penetration into the muscle cells. After this time the high-potassium contracture had an amplitude similar to the controls but the relaxation was slowed. The contractility of the muscle was impaired, so that reduction of the calcium or restoration of the normal

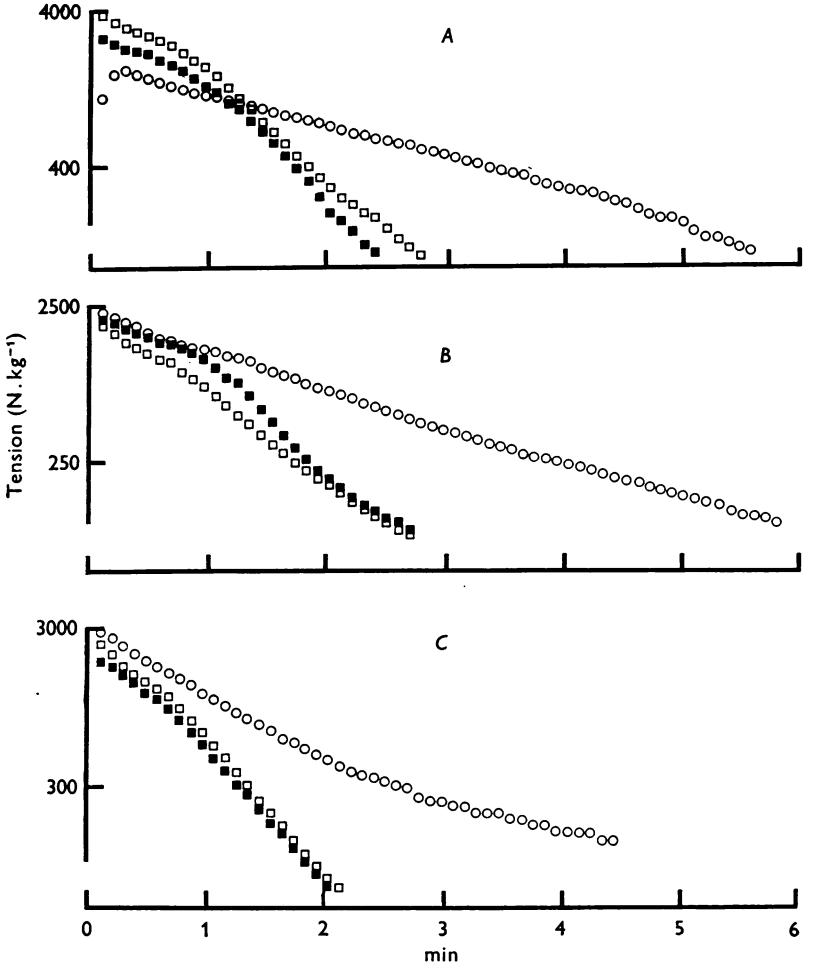

Fig. 4. Semilogarithmic plots of the spontaneous relaxation of 100 mM-potassium contractures before (□—□) and after (■—■) the application of inhibitors of oxidative phosphorylation are compared to the relaxation that occurs in the presence of these compounds (○—○). *A*, $9{\cdot}5 \times 10^{-5}$ M-DNP when applied in the perfusing medium for 8 min before the high-potassium contracture causes a pronounced slowing in the rate of spontaneous relaxation. $[Ca]_o$ 1 mM, 20° C. *B*, 5 $\mu g.ml.^{-1}$ oligomycin applied for 20 min also slows the rate of spontaneous relaxation of the 100 mM-potassium contracture evoked in the presence of 1 mM-calcium at 15° C. *C*, 1 mM cyanide applied for 6 min has a similar effect on the rate of spontaneous relaxation of the potassium contracture. $[Ca]_o$ 1 mM, 17° C. The recovery from each inhibitor is seen to be quite complete with the rate of spontaneous relaxation becoming close to that of the previous control contractures.

$[K]_o$ failed to induce a further fall in the tension level. It would appear that the effects of antimycin A are not readily reversible.

Cyanide was the major inhibitor used in the present work for two reasons, (*a*) it had a pronounced effect on the rate of relaxation, and (*b*) unlike DNP, CCmP and oligomycin it was rapidly reversible, so that as little as 10 min after a period in cyanide the heart behaved normally in all ways (Fig. 4*C*).

The slowing of spontaneous relaxation, following a 5–10 min exposure to cyanide, exhibits a strong dose dependency over the range from 10^{-5} to 5×10^{-3} M. At concentrations below 10^{-5} M cyanide had no noticeable effects; up to 10^{-4} M and sometimes as high as 10^{-3} M, the spontaneous relaxation of the potassium contracture was slowed and at the higher

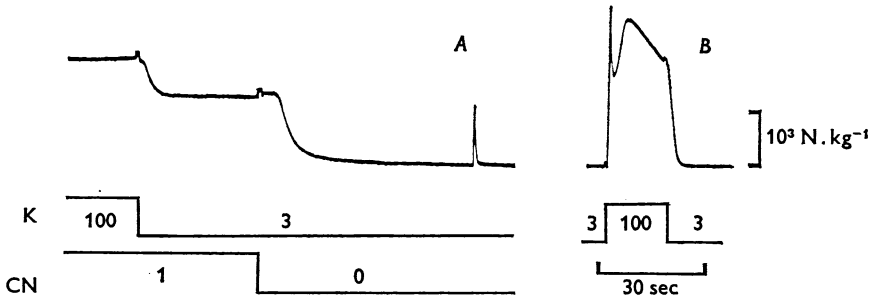

Fig. 5. *A*, after 6 min in 1 mM cyanide Ringer solution the contracture elicited by raising the $[K]_o$ to 100 mM only partly relaxes when the $[K]_o$ is returned to 3 mM, if the cyanide is still present in the bathing medium. Removal of the cyanide initiates a further relaxation that starts after a delay of several seconds and within a minute an electrically induced twitch can be obtained. *B*, in the absence of cyanide the relaxation of an otherwise similar potassium contracture is more rapid and complete in the same preparation. $[Ca]_o$ was 1 mM and the temperature 16° C.

concentrations was sometimes incomplete, so that there was a sustained tension developed by the muscle. In this situation, however, the over-all rate of relaxation can increase, probably due to a large reduction in the proportion of the relaxation that is brought about by the slower (temperature sensitive) phase of relaxation, so that the early faster phase now constitutes the bulk of the relaxation. Restoration of the low $[K]_o$ generally resulted in a slow relaxation, which in extreme cases was incomplete. If the cyanide was removed, however, after a short delay of 2–5 sec, the muscle relaxed fully and became electrically excitable within a minute (Fig. 5*A*).

The degree to which a given concentration of cyanide affects the relaxa-

tion of a high-potassium contracture depends on the stimulus frequency and calcium concentration during the period of pretreatment by the inhibitor. Increasing the number and strength of the contractions that the trabecule undergoes enhances the degree to which cyanide slows, or reduces, the extent of spontaneous relaxation (Fig. 6). This probably means that the energy supplies of the heart are depleted more rapidly when the contractions and relaxations are larger or occur more often.

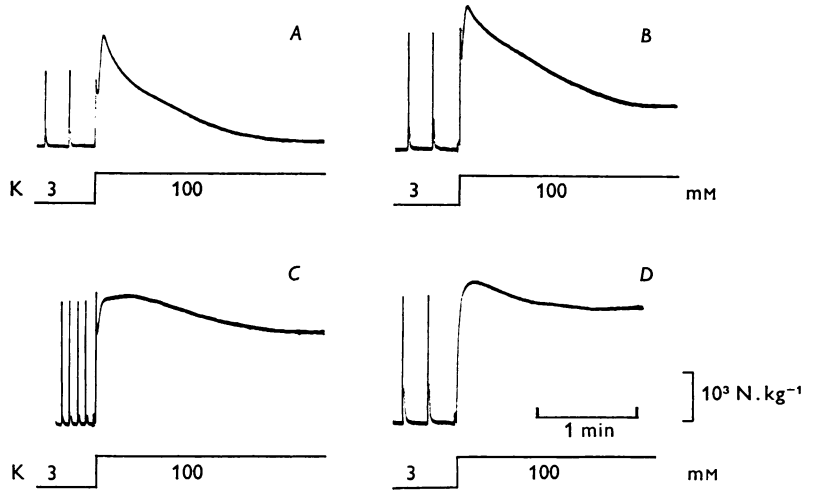

Fig. 6. The degree to which cyanide interferes with the spontaneous relaxation of the high-potassium contracture depends on the $[Ca]_o$ and the frequency of the preceding twitches. *A*, control contracture evoked by raising the $[K]_o$ from 3 to 100 mM in the presence of 1 mM-calcium after a period during which the muscle was stimulated at 4 min^{-1}. *B*, with 1 mM cyanide in the bathing Ringer solution, before the initiation of a potassium contracture, spontaneous relaxation becomes incomplete. *C*, after a similar period in 1 mM cyanide when the muscle was stimulated at 12 min^{-1} the spontaneous relaxation of the potassium contracture is slowed and incomplete. *D*, raising the $[Ca]_o$ during the cyanide application to 5 mM results in an even more severe disruption of the spontaneous relaxation. The cyanide was applied for 9 min in the low potassium Ringer before the contracture was evoked in each trace. 16° C.

Cyanide is then more able to interfere with the supply of energy to the relaxing system, rather than the relaxation process becoming directly dependent on the $[Ca]_o$.

In a few preparations that showed the highest sensitivity to cyanide, a small contracture developed after a few minutes in Ringer containing cyanide. This was apparently due to a slowing of the final phase of the relaxation of the twitch; on exposure to cyanide, however, most prepara-

tions showed an initial shortening and reduction of the twitch amplitude accompanied by an increase in the maximum rate of tension development. With longer exposure, the twitch amplitude slowly increased again for several minutes with the time to peak still shortened.

The experiments which have investigated the consequences of interfering with oxidative phosphorylation have shown that although uncoupling or blocking of this pathway slows the rate of spontaneous relaxation of potassium contractures in frog heart, this relaxation still develops. A few hearts showed a more profound change in the relaxation, but even in these preparations a significant part of the spontaneous and other types of relaxation persisted. This would suggest that part of the energy that is used to bring about relaxation is derived from the respiratory process while part must come from elsewhere. A source that will be unaffected in heart muscle is the glycolytic break-down of glycogen (electron micrographs reveal large amounts of glycogen in such hearts).

The effects of glycolytic inhibitors on the relaxation. In very poor preparations the presence of glucose is necessary for spontaneous relaxation of the high-potassium contracture to take place (Chapman, 1973). However, in preparations taken from healthy frogs, omission of glucose from the bathing solutions fails to have any effect on the responses of the heart in experiments lasting several hours, so a variety of glycolytic inhibitors were applied to the heart.

Phloridzin has been found to inhibit the transport of glucose into cells and to block the phosphorylation of glycogen by muscle homogenates; at concentrations as high as 1 mM and for periods as long as 30 min in the absence of external glucose it had no effect on the twitches or the potassium contractures. This may be due to the failure of this substance to enter the muscle cells but Webb (1950) found evidence that it entered rabbit atrial cells.

Fluoride, although used extensively as an inhibitor, is not always satisfactory because calcium fluoride is quite insoluble, and addition of fluoride to the experimental solutions therefore results in the appearance of a white cloudy precipitate when calcium was present. On exposure to a Ringer solution of this type the twitch tension fell to a value close to that found in a fluoride-free Ringer containing the same amounts of calcium (the free calcium concentration in the fluoride solutions was determined by atomic absorption spectroscopy of the supernatant fluid, after mild centrifugation). Prolonged exposure to fluoride-containing solutions was therefore attempted in only a few experiments, and resulted in a reduction of the high-potassium contracture, presumably as a consequence of the reduction of the $[Ca]_o$. The rate of spontaneous relaxation was not changed.

2-Deoxy-D-glucose, being an analogue of glucose that is able to enter the glycolytic chain of reactions for only part of the way, is therefore, a highly specific inhibitor of glycolysis. Its ability to totally block glucose utilization in some tissues, notably rat liver, has been doubted (Walker & Rao, 1963). The total replacement of glucose in the experimental solutions by 2-deoxy-D-glucose was found to have no noticeable effect on the twitch or contracture responses of frog atrial muscle even after 30 min perfusion.

Iodoacetate is a powerful but not truly specific inhibitor of glycolysis, inhibiting other reactions by combining with sulphydryl groups. It is also an irreversible poison, being able to induce rigor in rabbit heart (Webb, 1950). When applied to frog atrial muscle, iodoacetate eventually abolishes spontaneous relaxation of the potassium contracture, and also disrupts other forms of relaxation, so that a sustained contracture may develop. Inclusion of pyruvate in the perfusing medium reverses the development of this contracture, and more or less normal twitches can be evoked. However, the spontaneous relaxation of the potassium contracture never fully recovers.

The effect of the inhibition of glycolysis and oxidative phosphorylation on the potassium contracture. In all the preparations in which cyanide was applied and the glucose in the perfusing solutions was replaced by 2-deoxy-D-glucose spontaneous relaxation was abolished and the relaxation on return to the normal $[K]_o$ was progressively slowed as the period of exposure was increased (Fig. 7). These results show that when all the means of synthesizing ATP apart from the break-down of creatine phosphate and the activity of myokinase are blocked, then the relaxation, but not the contraction, of the muscle is impaired. Therefore ATP provided by both oxidative phosphorylation and glycolysis is required to bring about relaxation of the muscle. When both sources are poisoned, repolarizing the membrane or removing much of the calcium from the bathing medium cannot bring about complete relaxation. When the inhibitors are removed, even in the presence of an elevated $[K]_o$, spontaneous relaxation commences and is completed achieving a final rate comparable to that seen in an unpoisoned muscle (Fig. 7*D*). After a prolonged period of exposure to cyanide and 2-deoxy-D-glucose, the contracture shows a secondary rise in tension following the small initial relaxation.

The action of caffeine on the poisoned atrial muscle. A further transient development of tension following the spontaneous relaxation of a potassium contracture in frog heart is initiated by caffeine (Chapman & Miller, 1971; Chapman, 1973). This observation forms the basis of an argument to support the candidature of the sarcoplasmic reticulum as the intracellular relaxing system in this muscle. This also occurs when caffeine is added in presence of agents that block or uncouple oxidative phosphorylation

(Fig. 8). In this situation the contracture is smaller and shows little readiness to relax even when the caffeine is removed (Fig. 8). If all but the initial phase of relaxation of the contracture has been blocked by cyanide and 2-deoxy-D-glucose, caffeine fails to evoke the development of a second contracture.

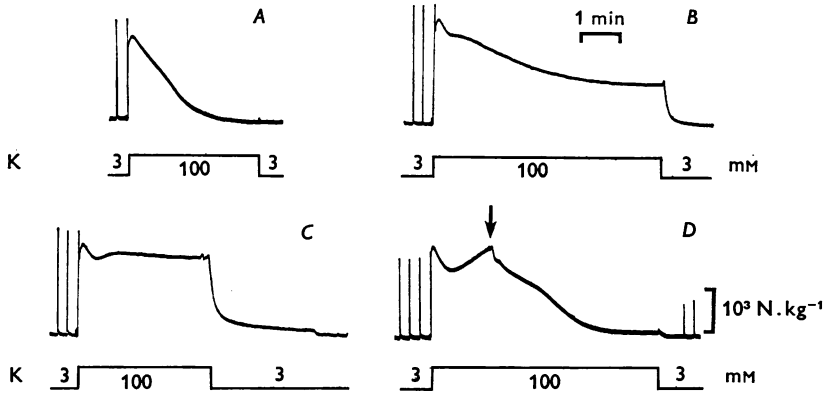

Fig. 7. The spontaneous relaxation of the tension developed by a trabecule on exposure to potassium-rich media is almost totally abolished when the muscle is subjected to a mixture of inhibitors which would be expected to block both oxidative phosphorylation and glycolysis. The effect of replacing the glucose by 5 mM-2-deoxy-D-glucose and of adding 2 mM cyanide, on the spontaneous relaxation of a 100 mM potassium contracture elicited in the presence of 3 mM calcium. *A*, control contracture, no inhibitors. *B*, contracture evoked after 4 min treatment by 2 mM cyanide. *C*, the effects of 8 min perfusion by Ringer containing 2 mM cyanide and 5 mM-2-deoxy-D-glucose instead of glucose. *D*, simultaneous removal of the cyanide and 2-deoxy-D-glucose during a contracture elicited by high potassium, at the arrow, results in the onset of spontaneous relaxation which reaches a rate comparable with that achieved in the muscle before exposure to these agents. 16·5° C.

DISCUSSION

The relaxation of a contracture of frog cardiac muscle, which occurs when the $[K]_o$ is lowered, the $[Ca]_o$ reduced, or that develops spontaneously during perfusion with elevated potassium concentrations and the relaxation of the twitch response, all have a marked dependence on temperature, and are slowed and even abolished in the presence of metabolic inhibitors. If it is assumed that each type of relaxation is brought about by the same active process that reduces the concentration of calcium ions in the sarcoplasm, then the experimental results are consistent with the theory that the energy for this relaxation is derived from the break-down of ATP. In healthy muscles sufficient energy for normal relaxation is provided by

oxidative phosphorylation, but glycolysis alone, as shown by the reduced rate of relaxation, is unable to supply this energy.

Certain features of the physical nature of the relaxing system can be derived from these experiments. As the activation energy for the spontaneous relaxation is much larger than that reported for the efflux of calcium from mammalian heart (Reuter & Seitz, 1968), then an active extrusion of calcium ions from the cells is not the process that brings about

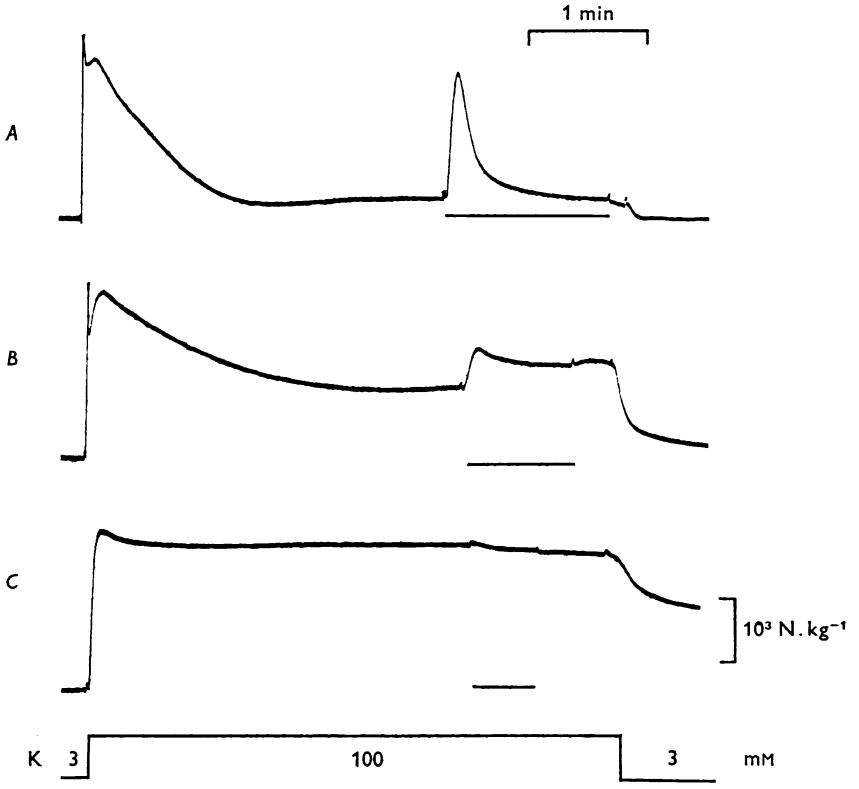

Fig. 8. The ability of 5 mM caffeine to induce a redevelopment of tension after a high-potassium contracture has undergone spontaneous relaxation is impaired by interfering with the spontaneous relaxation with metabolic inhibitors. *A*, control potassium contracture followed by addition of 5 mM caffeine as indicated by the horizontal bar. *B*, a potassium contracture evoked in the presence of 1 mM cyanide in which the spontaneous relaxation is slowed and incomplete; caffeine yields a weak contracture which does not relax when the caffeine is removed. *C*, when the spontaneous relaxation of the potassium contracture is fully abolished by a mixture of 1 mM cyanide and replacement of the glucose by 2-deoxy-D-glucose, then caffeine fails to induce any tension when it is added to the perfusing contracture fluid. The horizontal bar in each record indicates the period of perfusion by solutions containing 5 mM caffeine. $[Ca]_o$ 3 mM, 17·5° C.

relaxation. The absence of data relating to the energy of activation of calcium transport in mitochondria, however, makes it difficult to assess the importance of the similarity between the activation energies of spontaneous relaxation and those reported for the calcium binding by isolated cardiac and skeletal muscle sarcoplasmic reticulum (Inesi & Watanabe, 1967; Harigaya & Schwartz, 1969).

The effect of intracellular ATP depletion on the activity of the sodium pump must also be taken into account. Should the activity of this pump be severely altered, then sodium will accumulate inside the muscle cells, and might alter the rate at which the intracellular relaxing system can take up calcium, or the calcium efflux from the cells, both of which could interfere with the relaxation of the muscle. This problem might not be important if exposure to the metabolic poison is not too long, because McDonald & MacLeod (1972) have found that there is little change in the intracellular sodium content during the first 30 min of exposure of rat heart to DNP, although there is a large loss of potassium during this time.

Webb (1950, 1966) showed that the twitch responses of rabbit atrium were depressed by the glycolytic inhibitors, phloridzin, fluoride, iodoacetate and others, as well as by inhibitors of oxidative phosphorylation, cyanide and azide, and that the time required for these changes to occur was between 2 and 25 min depending on the particular chemical. This short time, when compared to results presented here, may be due to the lower energy requirement of the frog preparations used, for apart from the higher temperature of Webb's experiments, the frequency at which the heart was beating was typically between 72 and 144 beats min^{-1}, while in the present experiments it never exceeded 12 min^{-1} and was generally 4 min^{-1}. Alternatively, the mammalian heart might depend more directly on the energy derived from glycolysis than frog heart. In the presence of inhibitors of oxidative phosphorylation, the contraction of the muscle may not be impaired and may even be enhanced while the relaxation is much slowed. Relaxation, particularly the spontaneous relaxation of the potassium contracture, is therefore more sensitive than the contractile process to the availability of energy supplies derived from these sources in the frog heart. This suggests that either the intracellular relaxing structures are remote from the energy supplied by substrate level phosphorylation and because glycolysis takes place in the cytoplasm the mitochondria would appear to be the more likely intracellular relaxing structure, or the system that regenerates the ATP used in contraction from creatine phosphate is remote from the structures concerned with relaxation.

Webb (1950) found that the effects of iodoacetate were only slightly alleviated by pyruvate, and he concluded that this agent was probably interfering with other reactions inside the cells, possibly fatty acid break-

down. This idea is consistent with the results obtained on frog heart except that the remedial action of pyruvate, although not complete, is certainly greater than reported for rabbit atria.

A direct comparison of the results on living frog cardiac muscle and isolated mammalian cardiac sarcoplasmic reticulum (Carsten & Mommaerts, 1964) is not very informative. In both preparations, the low sensitivity to oligomycin may not be meaningful because this may be due to the slow penetration of oligomycin into the muscle cells. The response to antimycin A might be interpreted as evidence that mitochondria are directly involved in bringing about the relaxation of the frog heart, while the similarity of action of all inhibitors of oxidative phosphorylation suggest that antimycin A acts by simply blocking the production of ATP by the mitochondria. The same conclusion was reached by Haugaard and his co-workers, who used metabolic inhibitors in an attempt to understand the mechanism by which adrenaline potentiates the heart beat. They found it difficult to decide between the mitochondria and the sarcoplasmic reticulum as the site of the cardiac relaxing system (Horn, Levin & Haugaard, 1968; Horn, Aronson, Hess & Haugaard, 1967). This confusion has been partly cleared by the use of inhibitors that have little ambiguity in the site of their action; caffeine has been shown to be a specific inhibitor of calcium uptake by isolated sarcoplasmic reticulum. A variety of other compounds of this type have been described by Balzar, Makinose & Hasselbach (1968); Carafoli, Patriarca & Rossi (1969), and Duggan (1971), and it would be hoped that use of these compounds might help in ending this dilemma. When the spontaneous relaxation of a potassium contracture has been blocked by a mixture of cyanide and 2-deoxy-D-glucose the failure of caffeine to initiate further contraction suggests that the calcium removed from the sarcoplasm during spontaneous relaxation is deposited in a store from where it can be released by caffeine. When the heart is beating in normal Ringer there cannot be a large amount of calcium in this store, because caffeine does not then induce a contracture (Chapman & Miller, 1971). It would seem likely that calcium is in turn removed from this store, and passes out into the bathing medium; the possibility of this type of scheme has been discussed in the previous paper (Chapman, 1973).

The initial rapid phase of relaxation of the potassium contracture which persists when all other types of relaxation have been blocked and even after long exposure to inhibitors, cannot be energy dependent. The persistence of this relaxation could mean that the sites which bind calcium are still able to do so and therefore it is the transport of the calcium that requires the supply of energy.

I would like to thank Mrs J. M. Blackett for her excellent technical help. This work was supported by grants from the British Heart Foundation and the Royal Society.

REFERENCES

BALZER, H., MAKINOSE, M. & HASSELBACH, W. (1968). The inhibition of the sarcoplasmic calcium pump by prenylamine, reserpine, chlorpromazine and imipramine. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **260**, 444–455.

CAPUTO, C. (1972). The effect of low temperature on the excitation-contraction coupling phenomena of frog single muscle fibres. *J. Physiol.* **223**, 461–482.

CARAFOLI, E., PATRIARCA, P. & ROSSI, C. R. (1969). A comparative study of the role of mitochondria and the sarcoplasmic reticulum in the uptake and release of Ca^{++} by rat diaphragm. *J. cell Physiol.* **74**, 17–29.

CARSTEN, M. E. & MOMMAERTS, M. F. H. M. (1964). The accumulation of calcium ions by sacrotubular vesicles. *J. gen. Physiol.* **48**, 183–192.

CHAPMAN, R. A. (1973). The ionic dependence of the strength and spontaneous relaxation of the potassium contracture induced in the heart of the frog *Rana pipiens*. *J. Physiol.* **231**, 209–232.

CHAPMAN, R. A. & MILLER, D. J. (1971). The action of caffeine on frog myocardial contractility. *J. Physiol.* **217**, 64–66*P*.

CHAPMAN, R. A. & NIEDERGERKE, R. (1970). Interaction between heart rate and calcium concentration in control of contractile strength of frog heart. *J. Physiol.* **211**, 423–443.

CHAPMAN, R. A. & TUNSTALL, J. (1971). The dependence of the contractile force generated by frog auricular trabeculae on the external calcium concentration. *J. Physiol.* **215**, 139–162.

DUGGAN, P. F. (1971). Stimulation of calcium uptake of muscle microsomes by phenothiazines and barbiturates. *Eur. J. Pharmac.* **13**, 381–386.

HARIGAYA, S. & SCHWARTZ, A. (1969). Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circulation Res.* XXV, 781–794.

HEINTZEN, P., KRAFT, H. C. & WIEGMANN, O. (1956). Uber die elektrische und mechanische Tätigkeit des Herztreifenpräparates vom Frosch in Abhängigkeit von der Temperatur. *Z. Biol.* **108**, 401–411.

HEYTLER, P. G. & PRICHARD, W. W. (1962). A new class of uncoupling agents – carbonyl cyanide phenylhydrazines. *Biochem. biophys. Res. Commun.* **7**, 272–275.

HORN, R. A., ARONSON, C. E., HESS, M. E. & HAUGAARD, N. (1967). The effect of metabolic inhibitors on the response of the perfused rat heart to epinephrine. *Biochem. Pharmac.* **16**, 2109–2116.

HORN, R. S., LEVIN, R. & HAUGAARD, N. (1968). The influence of oligomycin on the actions of epinephrine and theophylline upon the perfused rat heart. *Biochem. Pharmac.* **18**, 503–509.

INESI, G. & WATANABE, S. (1967). Temperature dependence of ATP hydrolysis and calcium uptake by fragmented sarcoplasmic membranes. *Archs Biochem. Biophys.* **121**, 665–671.

MCDONALD, T. F. & MACLEOD, P. D. (1972). The effect of 2,4-dinitrophenol on electrical and mechanical activity, metabolism and ion movements in guinea-pig ventricular muscle. *Br. J. Pharmac.* **44**, 711–722.

NIEDERGERKE, R. (1956). The potassium chloride contracture of the heart and its modification by calcium. *J. Physiol.* **134**, 584–599.

REUTER, H. & SEITZ, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ionic composition. *J. Physiol.* **195**, 451–470.

WALKER, D. G. & RAO, S. (1963). The effect of glucose analogues on the hepatic glucose-phosphorylating enzymes. *Biochim. biophys. Acta* **77**, 662–665.

WEBB, L. J. (1950). The actions of metabolic substrates and inhibitors on the rabbit auricle. *Br. J. Pharmac.* **5**, 87–117.

WEBB, J. L. (1966). *Enzyme and Metabolic Inhibitors*, vols. II and III. New York: Academic Press.