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EXPERIMENTAL ALTERATION OF THE RELATIONSHIP BETWEEN THE EXTERNAL CALCIUM CONCENTRATION AND THE CONTRACTILE FORCE GENERATED BY AURICULAR TRABECULAE ISOLATED FROM THE HEART OF THE FROG, *RANA PIPIENS*

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SUMMARY

1. The contractile strength generated by isolated frog auricular trabeculae has been determined by perfusion with high-K Ringer over a range of $[Ca]_o$.

2. Experiments are described in which the cubic relationship between the contracture tension and $[Ca]_o$ has been changed to a square or a linear relationship.

3. These results have been interpreted by proposing that three Ca compounds, whose concentrations are proportional to $[Ca]_o$, act co-operatively at some stage of the process leading to the generation of tension.

4. The change in contractile strength, determined by regular electrically evoked twitches, has been investigated at different temperatures and the results have been explained by assuming that the concentrations of the three hypothetical activating compounds vary at different rates when $[Ca]_o$ is altered.

5. The staircase response is supposed to develop as the consequence of an increase in the concentrations of the two activating Ca compounds with the slowest time constants.

6. The possible physical representations of the hypothetical activating compounds are discussed.

INTRODUCTION

The time courses of the changes of contractile strength brought about by alteration of the Ca concentration in the medium bathing frog ventricles has been explained by assuming that contraction is brought about by the co-operative action of two Ca compounds somewhere within the heart cells.

The concentrations of these Ca compounds are believed to change at different rates when the external Ca concentration is varied (Chapman & Niedergerke, 1970*a*). However, the contracture tension induced by perfusion of frog auricular trabeculae by solutions containing an elevated K concentration has been shown to be proportional to the cube of the external Ca concentration (Chapman & Tunstall, 1971). This observation has been interpreted by proposing that three Ca ions or compounds acted co-operatively at some stage in the process leading to the generation of tension. A coherent explanation would be achieved if one of the compounds proposed by Chapman & Niedergerke (1970*a*) could be shown to be produced by a second order reaction with the ionic Ca in the bathing medium.

The proposition of Chapman & Niedergerke (1970*a*) is taken as a starting point, so that the strength of contraction is supposed to depend on the concentrations of two hypothetical Ca compounds Ca_1 and Ca_2. The concentrations of these compounds are considered to vary exponentially when the $[Ca]_o$ is altered with markedly different time constants which can be determined from the time courses of the twitch tension changes. The large difference between the time constants of the changes of $[Ca_1]$ and $[Ca_2]$ enables the contribution of these two 'reactions' to be separately determined. To illustrate how this separation is achieved, let us consider a typical preparation in which the half time of the fast exponential phase Ca_1 is 5 sec and that of the slower exponential phase Ca_2 is 300 sec. 15 sec after $[Ca]_o$ is altered the concentration of Ca_1 will have changed to within 12·5 % of its final value, while $[Ca_2]$ will have changed by less than 5 %. After 30 sec $[Ca_1]$ will be within 3 % of its final value while $[Ca_2]$ will have fallen by only 9·5 %. The relationship between either $[Ca_1]$ or $[Ca_2]$ and the contracture tension can be determined by either increasing or decreasing $[Ca]_o$ for only a brief period before a high-K contracture is evoked to measure the contractile strength. The tension evoked by high-K solutions has been preferred to the twitch tensions in these experiments for the reasons already stated in Chapman & Tunstall (1971) and also so that these results can be compared with the 'equilibrated' condition, i.e. when the $[Ca_1]$ and $[Ca_2]$ are both considered to be proportional to the $[Ca]_o$.

The changes of contractile strength, as determined from the peak twitch tension or the maximum rate of twitch tension development, when the frequency of stimulation or the $[Ca]_o$ is reduced at different temperatures, have been analysed because these results lead to a modified interpretation of the action of Ca in controlling contraction of frog heart.

METHODS

The preparation, experimental set up and procedure were as described previously (Chapman & Tunstall, 1971), except that these experiments have been performed on isolated single trabeculae with no cut side branches which can be found running through the atrial cavity from the central septum to the wall of one of the auricles. There are generally two such trabeculae in each auricle and they are strap-like in form being between 20 and 40 $\mu \times 100$–$200\ \mu$ and up to 2 mm long.

The Ringer solution contained 117 mM-NaCl, 3 mM-KCl, 0·8 mM-Na_2HPO_4, and 0·2 mM-NaH_2PO_4, while the contracture fluid (high K) contained an additional 97 mM-KCl added as solid to the normal Ringer solution, the pH of both solutions was 7·3. The Ca was added as 1 M-$CaCl_2$ solution.

Preparations were first allowed to develop hypodynamia as described by Chapman & Niedergerke (1970*a*) and by Chapman & Tunstall (1971), after which the $[Ca]_o$ was reduced and the peak twitch tension (P_{max}) and the maximum rate of twitch tension development ($[dP/dt]_{max}$) were displayed, measured and assessed to determine the time constants of the fast $[Ca_1]$ and slow $[Ca_2]$ exponential components of the tension changes by means of the method described by Chapman & Niedergerke (1970*a*). Preparations in which the time constants of fast and slow phases of tension change differed by less than a factor of 15 were not used in the contracture experiments. These time constants were checked in the middle and at the end of the experiment. Experiments in which changes in the time constants of greater than 10 % occurred were rejected.

Thirty-one contracture experiments were attempted but due to the stringent requirements for a successful experiment only ten were completely satisfactory, although a further seven experiments were partly so, ten more were unsatisfactory. Another four preparations failed to develop strong hypodynamia and the results from these will also be reported.

In other experiments the temperature of the perfusing medium was varied, and the changes of the twitch response associated with alteration of $[Ca]_o$ or the stimulus frequency were determined at different temperatures. Alteration of the temperature was achieved by passing the solutions through Pyrex glass coils immersed in a water-bath the temperature of which was controlled to $\pm$ 1/10th of a degree within the range +15 to +30° C. The temperature of the perfusing solution was measured with a small mercury bulb thermometer placed in the main drain of the experimental chamber. In all experiments where the P_{max} or $[dP/dt]_{max}$ have been used as a measure of the contractile strength of the muscle, analysis has been confined to preparations in which the time to the peak of the twitch response, at each particular temperature, changed by less than 10 % during the responses to alterations of the $[Ca]_o$ or the stimulus frequency. Parallel changes of the peak tension and the maximum rate of twitch tension development have also been used as a criterion for accepting results for analysis (for discussion see Chapman & Niedergerke, 1970*a*).

Hearts from healthy large frogs of the species *Rana pipiens* were used for these experiments.

RESULTS

The dependence of C_{max} *on* $[Ca_1]$

The relationship between $[Ca_1]$ and the high-K contracture tension (C_{max}) was determined by making a series of paired observations. The high-K contractures were evoked over a range of $[Ca]_o$. At each $[Ca]_o$ two contractures were elicited.

The procedure was as follows; the preparation was perfused with a Ringer containing a [Ca] made standard for that particular experiment (generally 1 mM). When the values P_{max} and $[dP/dt]_{max}$ were steady, the heart was considered to have come into 'equilibrium' with the bathing medium, i.e. $[Ca_1]$ and $[Ca_2]$ were steady and proportional to $[Ca]_o$. The $[Ca]_o$ was then reduced and 15 or 30 sec later a contracture was evoked by a brief perfusion with high-K solution containing the lower [Ca]. The muscle was then allowed to 'equilibrate' at the lower $[Ca]_o$ when a second high-K contracture was evoked. The strip was then returned to the standard high [Ca]-Ringer and the experiment was repeated for another lower $[Ca]_o$.

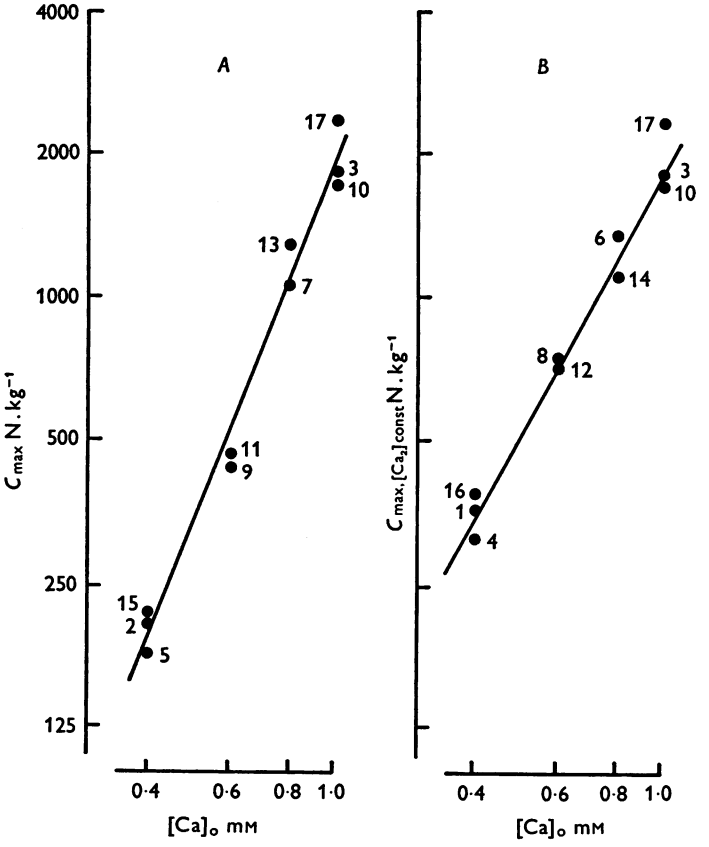

Fig. 1*A*, *B*. For legend see opposite page.

The 'equilibrated' contractures were, in terms of the interpretation of Chapman & Niedergerke (1970*a*), evoked when both $[Ca_1]$ and $[Ca_2]$ were proportional to the $[Ca]_o$. In the case of a contracture evoked shortly after a reduction in $[Ca]_o$, $[Ca_1]$ can be assumed to be proportional to the new $[Ca]_o$ as in eqn. (1), while the $[Ca_2]$ will be proportional to the previous higher $[Ca]_o$, and is therefore constant in these experiments.

$$[Ca_1]_{[Ca_2]\,const} = K_1[Ca]_o^n, \qquad (1)$$

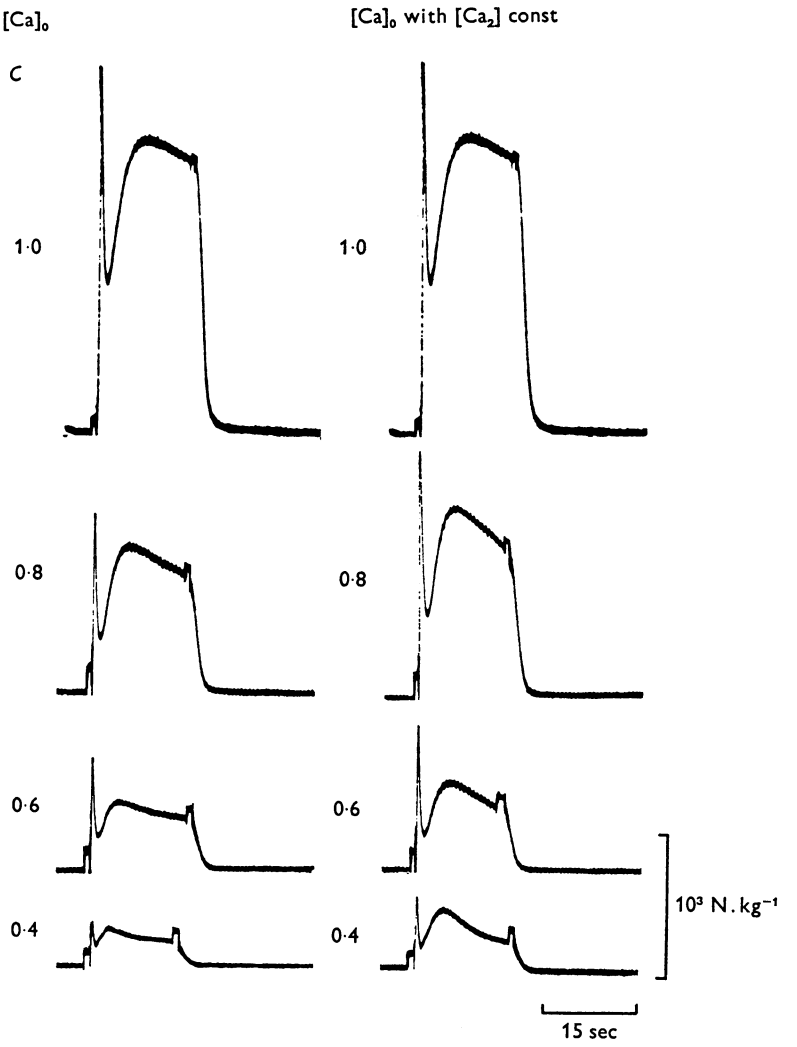

Fig. 1. *A*. The log contracture tension evoked by Ringer + 97 mM-KCl is related to the log $[Ca]_o$, when the preparation has become 'equilibrated', ($[Ca_2]$ and $[Ca_1]$ proportional to $[Ca]_o$) by a straight line of slope + 2·51, coefficient of correlation is 0·99.

B. The log contracture tension, when the $[Ca_2]$ is maintained constant, is related to the log $[Ca]_o$ by a straight line of slope + 1·84, coefficient of correlation 0·99. The $[Ca_1]$ is proportional to the recently established $[Ca]_o$ when $[Ca_2]$ is unchanged as in eqn. (1).

The numbers in parts *A* and *B* indicate the sequence in which the contractures were evoked.

C. Experimental records of contractures evoked when the heart is 'equilibrated' to a range of $[Ca]_o$ and when $[Ca]_o$ is varied at constant $[Ca_2]$. 18° C. Time constants of change in P_{max} for this experiment were $\tau_1 = 5{\cdot}05$ sec, $\tau_2 = 153$ sec.

where K_1 is a constant, and n is the number of ions forming the Ca_1 complex.

Fig. 1 *A* shows the relationship between the log $[Ca]_o$ and the log C_{max} when both $[Ca_1]$ and $[Ca_2]$ are proportional to the $[Ca]_o$. Under these conditions, log C_{max} is related to log $[Ca]_o$ by a straight line of slope +2·52, a result that has already led Chapman & Tunstall (1971) to conclude that the contracture tension was proportional to the $[Ca]_o^3$ when this concentration was below 1 mM, i.e. if α is a constant

$$C_{max,\,[Ca]_o \leqslant 1\,mM} = \alpha[Ca]_o^3 \tag{2}$$

Part *B* of Fig. 1 shows that when $[Ca_2]$ is constant the log contracture tension is related to the log of the recently established $[Ca]_o$ by a straight line of slope +1·84 suggesting the relationship expressed in eqn. (3).

$$C_{max,\,[Ca_2]\,const} = P_1[Ca]_o^2, \tag{3}$$

where P_1 is a constant and $C_{max,\,[Ca_2]\,const}$ is the contracture tension evoked when only $[Ca_1]$ is varied. Substituting eqn. (1) into eqn. (3) yields eqn. (4) (when $n = 2$), so that two Ca ions involved in the generation of tension are derived from each hypothetical Ca_1 compound.

$$C_{max,\,[Ca_2]\,const} = P_1 \frac{[Ca_1]}{K_1}. \tag{4}$$

The abscissa of part *B* in Fig. 1 has, according to eqn. (4), also the dimension of log $[Ca_1]/K_1$. The mean experimental values for the slope of the relationship, log C_{max} against log $[Ca]_o$, was +2·58 (S.D. 0·36) and that for log $C_{max,\,[Ca_2]const}$ against log $[Ca]_o$ or log $[Ca_1]/K_1$ was +1·78 (S.D. 0·35).

A correction could be made to allow for the fact that the $[Ca_1]$ has not quite reached its final value when the high-K contracture is evoked. However, the $[Ca_2]$ will have also changed and correction for this is more difficult to estimate. For this reason and since the two corrections will be opposite to each other, they have not been taken into account in the present work.

A few preparations failed to develop hypodynamia, such preparations lacked a definite slow phase of twitch tension change when $[Ca]_o$ was altered (i.e. one that could be associated with the variation of $[Ca_2]$) and the contracture tension developed in high-K solution was proportional to the $[Ca]_o^2$. Preparations of this type yielded a slope relating log C_{max} to log $[Ca]_o$ that was not significantly different from the line relating to the log $[Ca_1]/K_1$ to log $C_{max,\,[Ca_2]\,const}$ and in fact the actual C_{max} tensions at each $[Ca]_o$ under the two experimental conditions hardly differed at all.

The dependence of C_{max} on $[Ca_2]$

The relationship between the $[Ca]_o$ and the contracture tension was determined in the normal way, while at each $[Ca]_o$ (where $[Ca_1]$ and $[Ca_2]$ are proportional to $[Ca]_o$) a further contracture was evoked 15–30 sec after the $[Ca]_o$ had been increased to an upper value made standard for each particular experiment (generally 1 mM), by a high-K fluid containing the same standard $[Ca]_o$. Under these conditions the $[Ca_1]$ would be the same for each contracture due to the small time constant for the change of $[Ca_1]$, while the $[Ca_2]$ would be proportional to the previous lower $[Ca]_o$ ($[Ca']_o$), as represented by eqn. (5).

$$[Ca_2]_{[Ca_1]const} = K_2[Ca']_o^n, \quad (5)$$

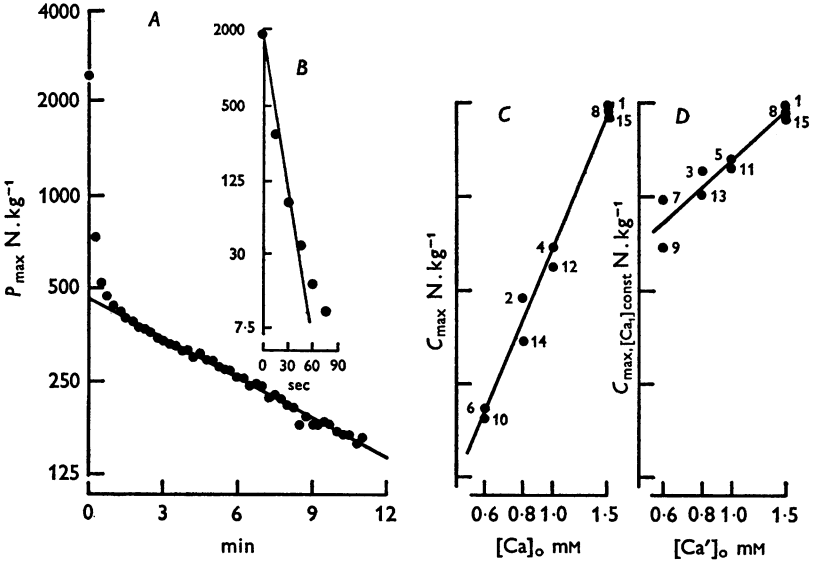

Fig. 2. *A*. Semilogarithmic plot of the decline of P_{max} (at constant heart rate of 4 min^{-1}) when the $[Ca]_o$ is reduced from 1·5 to 0·6 mM. The regression line provides the time constant for the fall of $[Ca_2]$ and is 530 sec.

B. Semilogarithmic plot of the rapid phase of decline of P_{max} obtained by subtraction of the slow phase extrapolated back to zero time from the experimental values. Regression line provides the time constant for the fall of $[Ca_1]$ and is 6·5 sec.

C. The log contracture tension (evoked by Ringer + 97 mM-KCl) is related to the log $[Ca]_o$ (to which the heart has been 'equilibrated') by a straight line of slope + 2·50, coefficient of correlation 0·99.

D. The log $C_{max,[Ca_1]\,const}$ is related to log of the previous $[Ca]_o$ ($[Ca']_o$) by a straight line of slope + 0·92, coefficient of correlation 0·97. The $[Ca_2]$ is proportional to the $[Ca]_o$ before it was raised to a standard upper value (1·5 mM in this experiment) for 15 sec, as in eqn. (6). 15° C.

The numbers in *C* and *D* indicate the sequence in which the contractures were evoked.

where K_2 is a constant, and n is the number of Ca ions in the Ca_2 complex.

An experiment of this type is illustrated in Fig. 2 together with the contracture tension developed by the preparation when it had been perfused in the lower $[Ca]_o$ Ringer for over 10 min and when the contracture fluid contained the same lower calcium concentration. It is seen that $\log C_{max}$ is related to $\log[Ca]_o$ by a straight line of slope $+2{\cdot}50$, while $[Ca']_o$ or $\log[Ca_2]/K_2$ is related to the $\log C_{max,\,[Ca_1]\,const}$ by a straight line of log slope $+0{\cdot}92$, indicating a linear relationship which can be formalized as eqn. (6).

$$C_{max,\,[Ca_1]\,const} = P_2[Ca_2], \tag{6}$$

where $C_{max,\,[Ca_1]\,const}$ is the contracture tension evoked when only $[Ca_2]$ is varied and P_2 is a constant, so equation 6 becomes

$$[Ca_2]_{[Ca_1]\,const} = K_2[Ca']_o. \tag{7}$$

The mean experimental values of the slopes of the regression lines relating $\log C_{max}$ to $\log[Ca]_o$ was $+2{\cdot}48$ (S.D. $0{\cdot}52$) and $\log C_{max,\,[Ca_1]\,const}$ to $\log[Ca_2]/K_2$ or $\log[Ca']_o$ was $+0{\cdot}77$ (S.D. $0{\cdot}32$).

When the heart is 'equilibrated' the contractile strength will depend on $[Ca_1]$ and $[Ca_2]$ so that substituting eqns. (6) and (8) into eqn. (2) we obtain

$$C_{max,\,[Ca]_o \leqslant 1\,mM} = \alpha\left\{\frac{[Ca_1]}{K_1}.\frac{[Ca_2]}{K_2}\right\} \tag{8}$$

where $\alpha = P_1.P_2$.

In hearts which failed to develop hypodynamia, and in which C_{max} was proportional to the square of $[Ca]_o$, the line relating $\log C_{max,\,[Ca_1]\,const}$ to $\log[Ca']_o$ was found to have a slope very near to zero. This observation supports the suggestion made by Chapman & Tunstall (1971) that in these preparations the $[Ca_2]$ is not altered by varying the $[Ca]_o$ within the range used in these experiments.

The reaction involving the formation of the hypothetical compound Ca_1 has been shown to involve a second order reaction with Ca^{2+} in the bathing medium. This being so, the time constants originally derived by Chapman & Niedergerke (1970*a*) and those used so far in the present paper have been underestimated. If two Ca ions are required to form a Ca_1 complex then the time constant for the change in $[Ca_1]$ should be twice the one used, i.e. between 10 and 25 sec and not 5–12·5 sec. On the other hand, the rapid phases of tension change could be due to two separate Ca-activated processes with similar time constants. This proposition is supported by the evidence of a slower phase of tension change when the $[Ca]_o$ is reduced (see Fig. 2) and by the fact that the time constant of the slow phase of tension build up in increased $[Ca]_o$ is smaller when the heart has been exposed to

a lower $[Ca]_o$ for only a short time (cf. Chapman & Niedergerke, 1970*a*, Fig. 14 and relevant discussion). It is clear that the change in $[Ca_1]$ cannot be solely attributed to the change of [Ca] in the extracellular spaces because the change in membrane potential associated with altering $[K]_o$ has been shown to have a half-time of 3 sec in frog auricular trabeculae (Chapman, 1971; Chapman & Tunstall, 1971).

The effect of temperature on the twitch tension changes associated with reduction of $[Ca]_o$ *or the stimulus rate*

These experiments follow up some unpublished findings of R. A. Chapman & R. Niedergerke on frog ventricle. The decline of P_{max} and of $[dP/dt]_{max}$ were followed when the $[Ca]_o$ or the stimulus frequency was reduced while the temperature of the bathing solution was maintained at one of several temperatures within the range +15 to 30° C (at temperatures below 15° C the changes in twitch tension become complicated, R. A. Chapman & D. J. Miller, unpublished). In the present experiments the temperature, $[Ca]_o$ and stimulus rates were adjusted in an attempt to demonstrate the existence of three exponential phases in the fall in contractility associated with reduction of the $[Ca]_o$, and to identify one with the fast phase of the staircase response.

The graphs *A* and *B* in Fig. 3 are obtained by plotting the logarithm of the difference between P_{max} and that value to which P_{max} eventually subsides, against the time since the stimulus frequency was reduced. The composite nature of the staircase responses are clearly seen. The slower phase (corresponding to the fall of $[Ca_2]$) is fitted by a regression line and the values of the slow phase extrapolated back to zero time (dashed lines) are subtracted from the remaining points to obtain the fast phase of tension decline (Fig. 3*C*). This fast exponential tension change is shown to have a very marked temperature dependence, the time constant at 20·5° C being 22·8 sec while at 24° C it has fallen to 12·8 sec. The Q_{10} for this change of rate constant from several experiments was 3·15 (S.D. 0·49). The slower phase (Ca_2) is also slowed by reducing the temperature and has an average Q_{10} of 1·52 (S.D. 0·29). The decline of P_{max} associated with a reduction of $[Ca]_o$ at 20·5 and 24° C are shown in Fig. 3*D* and *E*. Again the composite form of the response is observed with the time constant of the slowest (Ca_2) phase being slightly increased by reducing the temperature. The fast phases of tension decline obtained by subtraction of the extrapolated slow phase are compared in Fig. 3, part *F*. There is a clear difference between the time courses at the two temperatures. When regression lines are fitted to the last few points, the lines are found to have a time constant very similar to that of the faster phase of the staircase response at each temperature. Subtraction of these intermediate regression lines from the early tension

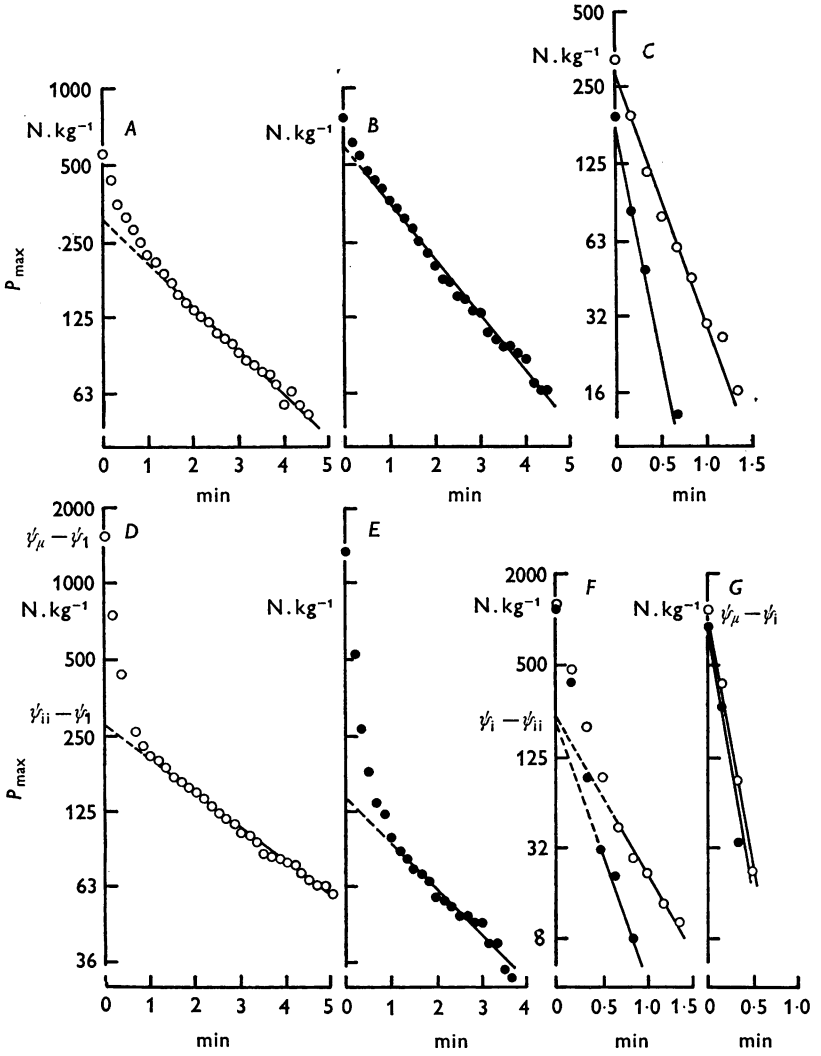

Fig. 3. All graphs are semilogarithmic plots of the decline of P_{max} associated with either a reduction of the $[Ca]_o$ from 1 to 0·5 mM at a constant heart rate of 5 min^{-1} *D*, *E*, *F* and *G*; or a reduction of the heart rate from a high level 12 or 20 min^{-1} to 5 min^{-1} at a constant $[Ca]_o$ of 0·5 mM *A*, *B* and *C*; at two temperatures 20·5° C (open circles), 24° C (filled circles). *A*, *B*, *D* and *E* shows the composite form of the tension changes resulting from a reduction of the stimulus rate (*A* and *B*) or a halving of the $[Ca]_o$ (*D* and *E*). *C* shows the effect of temperature upon the more rapid phase of P_{max} decline associated with a reduction of the heart rate obtained by subtraction of the extrapolated slow phase from the experimental results. *F* shows the composite nature of the more rapid phases of the decline of P_{max} at both experimental temperatures. *G* the fast phase of P_{max} decline associated with reduction of $[Ca]_o$. The correlation coefficients of all the continuous lines in this Figure are better than 0·98. For decline of P_{max} at 20·5° C (when $[Ca]_o$ is reduced (open circles parts *D*, *F* and *G*)) the tension levels and intercepts used to derive eqn. (13) are shown.

values yields Fig. 3*G* where the final faster exponential phases have time constants of 3·8 sec and only a small temperature dependence, the average Q_{10} for all the experiments being 1·02 (S.D. 0·05).

The results of experiments of this type show that the fast phase of twitch tension change previously assigned to a single phase and the change in concentration of a single compound $[Ca_1]$ can be divided into two exponential phases, the slower of which shows similar features to the faster phase of the staircase response, while the other has a time constant very similar to that estimated for the clearance time of the extracellular spaces within the heart trabeculae. If it is assumed that the fast phase of change of P_{max} is due to the variation of the concentration of two calcium compounds which act co-operatively with a further compound Ca_2 in controlling the contractile strength of the muscle, then eqn. (8) can be rewritten as

$$C_{max} = \alpha \left\{ \frac{[Ca_I]}{K_I} \frac{[Ca_{II}]}{K_{II}} \frac{[Ca_2]}{K_2} \right\}, \tag{9}$$

where Ca_I is the most rapidly changing Ca compound with

$$[Ca_I]_{[Ca]_o \leqslant 1\,mM} = K_I[Ca]_o \tag{10}$$

and Ca_{II} is the calcium compound with the intermediate time constant and large temperature dependence, and represented by Chapman & Niedergerke (1970*b*) as an intermediary compound I with

$$[Ca_{II}]_{[Ca]_o \leqslant 1\,mM} = K_{II}[Ca]_o \tag{11}$$

while Ca_2 is as before the compound with the slowest time course, so that

$$\frac{[Ca_1]}{K_1} = \frac{[Ca_I][Ca_{II}]}{K_I K_{II}}. \tag{12}$$

The time constant of the fast phase of the staircase response shows a marked variation from one preparation to another at a given experimental temperature, although the temperature dependence of the time constant was very consistent. The variation shows some seasonal trend although this has not been thoroughly investigated. The experiment used for illustration in Fig. 3 was from a preparation in which the time constant for the fast phase of the staircase response was larger than the average value obtained at these temperatures.

If the rapidly changing phase of the change of contractile strength (P_{max} or $[dP/dt]_{max}$) which follows an alteration of $[Ca]_o$ involves two reactions, reconsideration of the contracture experiments becomes necessary, because these experiments were devised by assuming a single reaction. Taking the experiment illustrated in Fig. 2 the half-times of the two rapid phases of tension change, assumed to be due to the change of two hypothetical Ca compounds, can be determined from Part *B* of this

Figure and are 3 sec (Ca_I) and 10 sec (Ca_{II}). In this experiment, 15 sec after the $[Ca]_o$ is altered $[Ca_I]$ will have risen to 97 % of its final value, while $[Ca_{II}]$ will only be 69 % of its final value, so that the activating $[Ca_{II}]$ would contribute less than the anticipated amount to the tension generated during the following high-K contracture. If both $[Ca_I]$ and $[Ca_{II}]$ had risen to their final values within the time of exposure to the elevated $[Ca]_o$, the slope of the $\log C_{\max,\,[Ca]_i\ \text{const}}$ by $\log [Ca']_o$ relationship should have been close to +0·54 (i.e. 2·54–2, assuming that $[Ca_2]$ had not altered significantly), whereas the experimental slope was +0·92. This increased slope is presumably the consequence of the reduced $[Ca_{II}]$. Applying a similar argument to the results in Fig. 2 the slope of $\log C_{\max,\,[Ca_2]\ \text{const}}$ against $\log [Ca]_o$ should be +1·5 instead of the experimental value of +1·84. This difference could be partly accounted for by the additional contribution of the residual Ca_{II} molecules that are remaining 15 sec after the $[Ca]_o$ is reduced.

Chapman & Niedergerke (1970*a*) analysed the tension changes associated with alterations of the $[Ca]_o$, and a similar analysis is possible with the present results which can be described by eqn. (13) for a reduction of $[Ca]_o$ when the $[Ca]_o$ is always less than 1 mM.

$$\psi_u - \psi_1 = (\psi_u - \psi_i)\exp\frac{-t}{\tau_I} + (\psi_i - \psi_{ii})\exp\frac{-t}{\tau_{II}} + (\psi_{ii} - \psi_1)\exp\frac{-t}{\tau_2} \quad (13)$$

where ψ_u is the steady P_{max} or $[dP/dt]_{max}$ developed at the upper $[Ca]_o$, ψ_1 is the steady contractile strength generated at the lower $[Ca]_o$, ψ_i is the intercept of the intermediate half-timed fraction at zero time, ψ_{ii} is likewise the intercept of the slowest fraction (see Fig. 3), τ_I, τ_{II} and τ_2 are the time constants of these tension changes which correspond, in the present hypothesis, to the fall of the $[Ca_I]$, $[Ca_{II}]$ and $[Ca_2]$, and t is the time since the $[Ca]_o$ was lowered.

A feature of this model is revealed when the $[Ca]_o$ is reduced by half whereupon the concentrations of the three hypothetical activating chemicals should also fall to one half of their initial value with differing time constants. If it is assumed that each Ca compound has the same effect in controlling the contractile strength then the relative contribution of each phase to the total tension change should be as follows:

if $\psi_u - \psi_1 = 100$ then $\psi_u - \psi_i = 57{\cdot}2$; $\psi_i - \psi_{ii} = 28{\cdot}6$; $\psi_i - \psi_1 = 14{\cdot}3$.

From six experiments where several analyses of the fall in P_{max} for a halving of $[Ca]_o$ have been possible and a staircase response enables an estimate of τ_{II} to be made, the percentage values for $\psi_u - \psi_i$ was 60·4 (S.D. 5·7), for $\psi_i - \psi_{ii}$ was 23·2 (S.D. 6·8) and for $\psi_{ii} - \psi_1$ was 16·5 (S.D. 5·1) which are reasonably close to the predicted values, when the errors inherent

in the analysis for estimating the intercepts by fitting lines by regression are taken into account (Atkins, 1969).

DISCUSSION

The rapidly changing phase of contractility, associated with alteration of $[Ca]_o$, appears to involve a co-operative action of two processes, while the slowly changing component involves a first-order reaction with $[Ca]_o$. These results have been interpreted by proposing that three Ca compounds (Ca_I, Ca_{II} and Ca_2) act co-operatively to control the contractile strength of the muscle. The concentrations of these hypothetical activating compounds are proportional to $[Ca]_o$ below 1 mM and these concentrations vary at different rates when the outside $[Ca]_o$ is altered. This variation in concentration is considered to be responsible for the changes of P_{max} and $[dP/dt]_{max}$ that are observed after the sudden change of the bathing Ca concentration. A reasonable correspondence is achieved between the experimental results and the relative contributions of each phase of tension change when $[Ca]_o$ is halved so long as the $[Ca]_o$ is not more than 1 mM. Above 1 mM-$[Ca]_o$, i.e. where the contractile strength is less steeply related to $[Ca]_o$, the contribution of the slowest $[Ca_2]$ phase falls below the predicted value, while at still higher $[Ca]_o$ halving the concentration may not lead to a significant reduction of contractile strength. This suggests that either the maximum activation of the contractile apparatus may be responsible for the flattening of the dose–action curve in high $[Ca]_o$, or that there is an upper limit to the concentrations of at least one of the hypothetical activating compounds.

The similarity between the time constants for slowest phase of tension change observed when either $[Ca]_o$ or the stimulus frequency is altered has already led Chapman & Niedergerke (1970*b*) to conclude that the change is associated with a common process (i.e. a change in $[Ca_2]$). Moreover, the fast phase of tension change observed during the staircase response and the intermediate phase observed when the $[Ca]_o$ is reduced may well be due to another common process (i.e. a change in $[Ca_{II}]$), because of the similarity of the time constants which show the same marked dependence on temperature. The fastest phase of tension change present only when the $[Ca]_o$ is varied has a time constant which corresponds closely to the time constant estimated for the change of the extracellular spaces within the trabeculae (Chapman, 1971; Chapman & Tunstall, 1971). The presence of two phases in the staircase response and three phases when the $[Ca]_o$ is varied makes a proposal of Chapman & Tunstall (1971), that three Ca ions or compounds are required per tension generating site on the contractile apparatus unlikely, while favouring the hypothesis that the activation of

the contractile proteins requires one Ca at the unit level, and that the Ca concentration established in the sarcoplasm during depolarization of the sarcolemma varies with the $[Ca]_o^3$. This proposal is in agreement with the Ca sensitivity of extracted muscle proteins (Ebashi, Endo & Ohtsuki, 1969; Fuchs & Briggs, 1968), and the relationship found between the total Ca content of frog ventricles and the tension they develop (Sands & Winegrad, 1970). A simple interpretation involving a polyanionic carrier molecule within the muscle membrane which bears three identical sites at which Ca can be bound is not consistent with the widely differing time constants and temperature dependencies of the various phases of tension change, the composite nature of the staircase response, or the known relationship between $[Ca]_o$ and Ca influx (Niedergerke, 1963).

The tension changes believed to be associated with changes in $[Ca_I]$ having a similar time constant to the change of the extracellular fluid and occurring only when $[Ca]_o$ is altered, could correspond to a change in the quantity of Ca ions entering the cells during depolarization of the surface membrane. A significant slow inward current which is dependent upon $[Ca]_o$ has been shown to exist in frog heart muscle (Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969). During the staircase response the twitch tension changes suggest that the contribution of Ca_I remains unaltered, i.e. the influx of Ca^{2+} per action potential is constant. However, the Ca influx per unit time, and hence the intracellular [Ca], depends on the frequency of beating (Niedergerke, Page & Talbot, 1969; Niedergerke & Orkand, 1966; Sands & Winegrad, 1970). If sarcoplasmic calcium is taken up by some intracellular structures and is slowly inactivated or lost from the cells, further changes in twitch tension would result, should this Ca be released during a second stimulation of the muscle. This hypothesis requires that Ca_I is a messenger that initiates Ca release from the other sites. In this scheme a sequential release of activating Ca from the Ca_{II} and Ca_2 sites would be triggered by the Ca^{2+} entering during depolarization of the sarcolemma, in much the same way as proposed by Endo, Tanaka & Ogawa (1970) to account for the contraction of skinned skeletal muscle fibres. A sequential release of this type could be obtained if the Ca_I, Ca_{II} and Ca_2 'compartments' were in series, and the amount of Ca released (or if all the Ca is released then the amount contained there) is proportional to $[Ca]_o$. A possible series arrangement would be with Ca_I sites on the outer surface of the muscle membrane, with Ca_{II} sites on the inner surface of the muscle membrane, and the Ca_2 sites in the sarcoplasm perhaps the unorganized sarcoplasmic reticulum or possibly the mitochondria. To account for the staircase responses then either the Ca_{II} or the Ca_2 compartment must lose Ca^{2+} to the extracellular fluid via a pathway which does not involve the Ca_I compartment. This model implies that

relaxation is brought about by binding of sarcoplasmic Ca^{2+} at Ca_{II} and Ca_2 sites, an implication that is supported by the high temperature dependence of relaxation and of the fast phase of the staircase response (Ca_{II}).

These discussions are, of course, highly speculative and must remain so until more is known about the quantity of Ca entering heart cells at each action potential and the ability of the sparse sarcoplasmic reticulum to take up Ca, as well as the Ca sensitivity of the contractile proteins.

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