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CALCIUM CONDUCTANCE IN RELATION TO CONTRACTILITY IN FROG MYOCARDIUM

BY MAGDA HORACKOVA AND GUY VASSORT*

From the Department of Physiology and Biophysics, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada, and Laboratoire de Physiologie Comparée, et de Physiologie cellulaire associé au CNRS, Université Paris Sud, Centre d'Orsay, France

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SUMMARY

1. Ca inward current and the corresponding phasic component of tension were measured in frog atrial muscle under voltage-clamp conditions in Na-free (Li) Ringer solution with tetrodotoxin (TTX) added.

2. The quantity of Ca ions entering the cell upon depolarization, $\Delta[Ca]_i$, was linearly related to peak phasic tension.

3. The voltage dependence of the steady-state inactivation of the Ca-carrying system, f_∞, against voltage yielded similar relationships whether determined directly from variations of Ca inward current or peak phasic tension. The Ca system was almost fully available at potentials more negative than -45 mV and almost fully inactivated at potentials more positive than $+10$ mV.

4. It was established that the time- and voltage-dependence of Ca current and of phasic tension are directly related. The time constants of Ca inactivation, τ_f, were comparable in the range of membrane potential investigated (-20 to $+25$ mV), whether determined directly from the decay of Ca current or indirectly from peak phasic tension.

5. It was concluded that the Ca current, I_{Ca}, directly activates phasic contraction and that either parameter can be used as an indicator of the kinetics of the Ca-carrying system. Peak phasic tension was used to determine τ_f further in the membrane potential range in which interference by other membrane currents renders direct analysis of Ca current difficult.

6. The τ_f against voltage relationship determined from phasic tension

* Reprint address: Dr G. Vassort, Laboratoire de Physiologie Comparée, Université Paris Sud, 91405 Orsay, France.

showed that the inactivation process of the Ca-carrying system is slowest at membrane potentials around -13 mV ($\tau_f = 55$ msec) and that the rate of inactivation increases with both increasing and decreasing depolarizations.

7. It is suggested that normal repolarization in frog myocardium depends mainly on the decay of Ca inward current rather than on an increase of outward current.

INTRODUCTION

The existence of Ca conductance in cardiac muscle, first reported by Rougier, Vassort, Garnier, Gargouil & Coraboeuf (1969) in frog and by Beeler & Reuter (1970*a*) in mammalian heart, is now well supported by experimental evidence obtained by several other groups; for a review see Reuter (1973). However, the characteristics of this conductance have not been precisely determined, especially the voltage- and time-dependence of the activation and inactivation processes. Voltage-clamp data of the inactivation process of Ca conductance vary considerably, depending on the type of analysis, direct analysis of the decay of inward Ca current (New & Trautwein, 1972) or analysis of the Ca tail current (Beeler & Reuter, 1970*a*). As these analytical studies are based on the assumption of a single ionic current in the range of potential and duration studied, their validity is restricted to a range in which other ionic conductances are negligible.

In the present study we determined the voltage- and time-dependence of Ca inactivation, by direct analysis of ionic currents and indirect analysis of mechanical response. The latter method is based on the finding that mechanical activity in frog heart has two components: phasic, which is linked to Ca inward current; and tonic, which is not so linked and is completely suppressed in Na-free, Li–Ringer solution (Vassort, 1973*a*). The work described here, which extends previous studies (Vassort & Rougier, 1972; Vassort, 1973*a*, *b*), provides detailed information of voltage- and time-dependence of the phasic tension. As preliminary experiments showed that the phasic component is directly related to the activation and inactivation of the Ca channel, we used this tension to determine the kinetics of the Ca conductance (g_{Ca}), thus eliminating possible errors due to the interference of other conductances during the direct analysis of Ca current.

METHODS

The experiments were performed on frog atrial trabeculae 70–150 μm in diameter, by means of the voltage-clamp method with simultaneous recording of mechanical activity (Vassort & Rougier, 1972). Electrical and mechanical responses were measured simultaneously under current-clamp or voltage-clamp conditions. Experiments were performed at room temperature ($20 \pm 2°$ C) in Li-Ringer solution which contained: LiCl, 112 mM; $CaCl_2$, 1·8 mM; $KHCO_3$, 2·4 mM; tetrodotoxin (TTX), 10^{-6} g/ml.

Atrial bundles were dissected in normal Ringer solution and immersed in Li-Ringer solution for 20 min (after application of Na-free solution, the muscle bundle immediately develops a marked contracture, then relaxes almost completely within 20 min (Vassort, 1973*a*)). The preparation was then placed in the experimental bath, and suppression of the tonic mechanical component (dependent on the intracellular Na concentration) was tested. No sustained mechanical response was recorded when a depolarizing step of high amplitude (>100 mV) and long duration (3 sec) was imposed, confirming that the tonic component had been eliminated and that only phasic tension would be measured.

Nomenclature

Variation in membrane potential, V (mV), is imposed from the resting potential (E_r; estimated to equal -70 mV) and is expressed as the value of membrane potential (E_m). Thus E_m (mV) $= E_r + V$.

Ca current (I_{Ca}) for a given depolarization is the product of a time-varying conductance and a driving-force; $I_{Ca} = g_{Ca}(E_m - E_{Ca})$, where E_{Ca} represents the Ca equilibrium potential. Ca conductance (g_{Ca}), as used by Bassingthwaighte & Reuter (1972), is given as the product of maximal conductance ($\bar{g}_{Ca}$) with two dimensionless variables (d and f) which vary with voltage and time from 0 to 1. Thus, $g_{Ca} = \bar{g}_{Ca} df$, where d and f are activation and inactivation variables respectively.

τ_f is the time constant of the inactivation process during depolarization and τ_c the time constant of the decline in tail current at the end of depolarization. T is the increase in tension from its resting value, depending on the duration and amplitude of the imposed voltage, and is measured as peak amplitude of the phasic tension.

RESULTS

Changes in action potential configuration and in contraction induced by current application

To determine whether the level of membrane potential during the first third (150 msec) of an action potential governs the rate of tension development in amphibian heart, as in mammalian heart (Sumbera 1970), we recorded action potential and contraction simultaneously under current-clamp conditions in Na-free Li–Ringer solution. Increasing the action potential amplitude by applying outward current after the onset of the action potential markedly decreased the amplitude of tension and its rate of rise (Fig. 1*A*). When the action potential amplitude was altered by the same current pulse 20 msec later (i.e. after the peak of the action potential), tension development was much less affected (Fig. 1*B*). Since the tension under these experimental conditions consists of the phasic component only, this result implies a fast rate of inactivation of Ca conductance in the range of $E_m + 20$ to $+40$ mV.

Ca inward current and its relation to contractile activity

To determine the time-dependence of inactivation of the Ca-carrying system at various membrane potentials we analysed Ca currents and/or

contractile responses elicited by depolarizing steps. To validate this approach we first established direct relationship between electrical and mechanical events, using the following mathematical formulation based on Hodgkin–Huxley-type kinetics (1952).

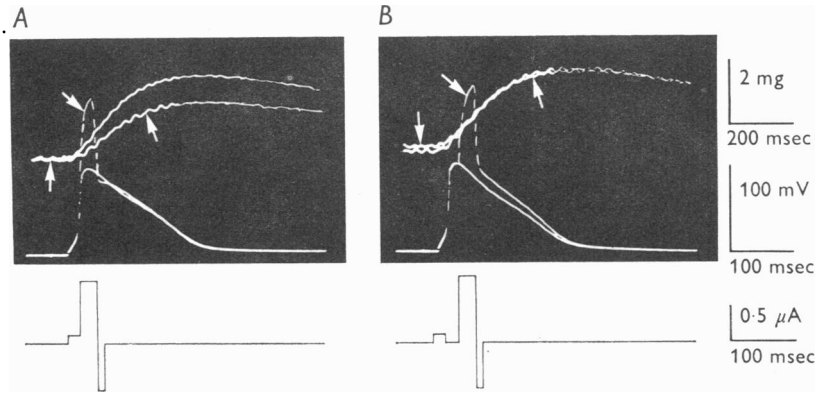

Fig. 1. Effect of a depolarizing current pulse applied at various periods during the action potential. Superimposed action potentials (middle traces) and tension (upper traces) recorded simultaneously in Li–Ringer solution under current-clamp conditions. The current pulse (25 msec) was applied at the end (*A*) and 20 msec after the end (*B*) of a driving stimulus lasting 20 msec (lower traces); in both cases the depolarizing current was followed by short (10 msec) hyperpolarizing current, to restrict modification of the action potential to 25 msec. The arrows indicate changes in action potential (traces re-touched) and corresponding variation in mechanical activity.

Theoretical aspect: mathematical formulation of calcium influx elicited by depolarization. For depolarizations of given duration exceeding the threshold, the quantity of Ca ions entering the cell, $\Delta[Ca]_i$, is assumed to be proportional to the total amount of Ca inward current, I_{Ca}, and is given by

$$\Delta[Ca]_i = \frac{1}{2F} \int I_{Ca}\, dt. \tag{1}$$

A schematic representation of Ca inward current is given in Fig. 2. Upon depolarization of a given duration, t_D, lasting longer than the time to full activation of g_{Ca}, t_1, the total area S could be schematically divided according to the duration of depolarization into three compartments (A, B, C) as follows.

Area A (for $t_D \leqslant t_1$) is constant for $t_D > t_1$. Since activation of the Ca system is complete at t_1, the subsequent shape of the current trace is determined solely by the rate of inactivation.

Area B (for $t_D > t_1$) depends on τ_f (the time constant of Ca inactivation). To describe the voltage- and time-dependence of the Ca current, the following assumptions and symbols were used:

$$I_{Ca} = a\alpha. \tag{2}$$

If α_0 is the value of the current at $t_D = 0$, assuming an instantaneous depolarization and repolarisation and an instantaneous maximal activitation of g_{Ca} at a given membrane potential (i.e. $g_{Ca} = \bar{g}_{Ca}$), then

$$\alpha_0 = (1/a)\ \bar{g}_{Ca}(E_m - E_{Ca}),$$

where $\alpha_0 = 1$, at $E_m = E_r$ and a = constant = $(E_r - E_{Ca})\ \bar{g}_{Ca}$. Thus for various E_m α_0 is the ratio of driving force during the imposed pulse to that at the resting potential, i.e.

$$\alpha_0 = (E_m - E_{Ca})/(E_r - E_{Ca}).$$

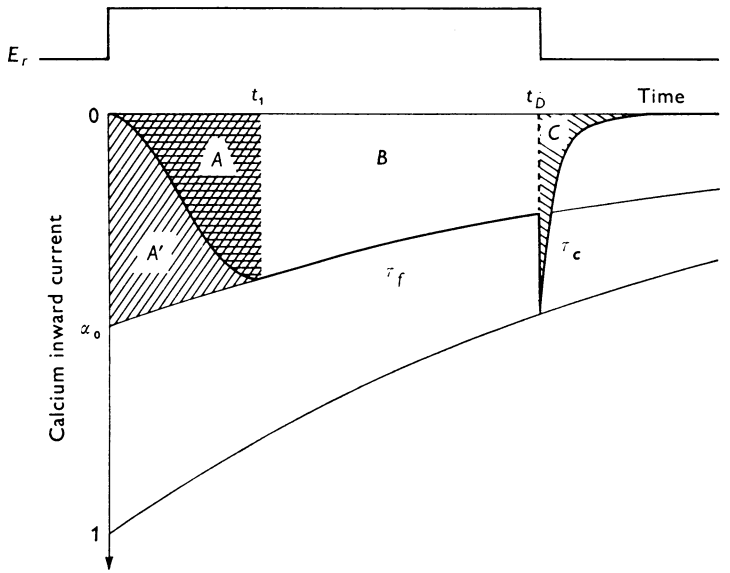

Fig. 2. Schematic diagram of the Ca inward current and Ca tail-current (heavy line) elicited by a depolarizing step (upper trace). τ_f is the time constant of Ca inactivation and τ_c, of Ca deactivation; t_D is the duration of depolarizing step, t_1 the time needed to reach full activation of Ca conductance. $A + B + C$ represents total Ca current elicited by a depolarizing step of a given amplitude and duration; the total shaded area on the left, $A' = \alpha_0 \tau_f (1 - e^{-t_1/\tau_f})$ and is a constant for $t_D > t_1$; $\alpha_0 = (E_m - E_{Ca})/(E_r - E_{Ca})$.

Activation of the Ca system is not, however, instantaneous but is maximal at t_1.

Area B can be expressed by

$$B = \alpha_0 \tau_f (1 - e^{-t_D/\tau_f}) - \alpha_0 \tau_f (1 - e^{-t_1/\tau_f}),$$

where $\alpha_0 \tau_f (1 - e^{-t_1/\tau_f})$ is a constant for $t_D > t_1$ and is later referred to as area A' (area A' comprises the whole of the shaded area on the left of Fig. 2).

Area C is related to the Ca inward tail-current elicited by repolarization at the end of the depolarizing step; this current declines with a time constant(τ_c). Area C is given by

$$C = \tau_c \alpha_0 e^{-t_D/\tau_f},$$

with $\alpha_0 = 1$, since $E_m = E_r$ during the tail current

and thus

$$C = \tau_c \, e^{-t_D/\tau_f}.$$

Finally, the total *area* S is given by

$$S = \alpha_0 \tau_f (1 - e^{-t_D/\tau_f}) + \tau_c \, e^{-t_D/\tau_f} - (A' - A)$$

$$= \alpha_0 \tau_f + (\tau_c - \alpha_0 \tau_f) e^{-t_D/\tau} - (A' - A)$$

and can be written

$$S = K + k \, e^{-t_D/\tau_f} \tag{3}$$

where

$$K = \alpha_0 \tau_f - (A' - A) \quad \text{and} \quad k = \tau_c - \alpha_0 \tau_f,$$

with a maximal value of $S = S_\infty = \alpha_0 \tau_f$ for instantaneous activation and $t_D = \infty$; K and k are constant at a given potential.

Since $S = \int \alpha \, dt$, from this and eqn. (2) it follows that

$$aS = \int I_{Ca} \, dt \tag{4}$$

then eqns. (1), (3) and (4) give

$$\Delta[Ca]_i = \frac{a}{2F} (K + k \, e^{-t_D/\tau_f}). \tag{5}$$

As is apparent from eqns. (3) and (5), the value of S and thus of $\Delta[Ca]_i$ is modified by the duration of depolarization (t_D); the direction of change (increase or decrease) is determined by k, which could be negative, zero, or positive, according to the membrane potential and the corresponding time constant of inactivation (τ_f, while τ_c is constant). Thus in experimental situations the following three alternatives occur.

(i) When $E_m < E_{Ca}$, area S will increase with increasing duration of depolarization for $\alpha_0 > \tau_c/\tau_f$ and thus $k < 0$.

(ii) When E_m is close or equal to E_{Ca}, area S will decrease with increasing duration of depolarization for $\alpha_0 < \tau_c/\tau_f$ and thus $k > 0$. As K is now approaching zero, area S depends mainly on the Ca tail-current and therefore is given by area C.

(iii) in the special case of a given E_m being close to E_{Ca}, area S will not be affected by the duration of depolarization, for $\alpha_0 = \tau_c/\tau_f$ and thus $k = 0$. The constant value of S will be given by K, i.e. any increase in Ca inward current due to depolarization will be compensated for by equal decrease in Ca tail-current elicited by repolarization.

Relationship between $\Delta[Ca]_i$ *and phasic tension.* The first step in the experimental analysis was to establish the relation between the quantity of Ca ions entering the cell upon excitation ($\Delta[Ca]_i$) and phasic tension (Fig. 3). $\Delta[Ca]_i$ was estimated as the graphical integration of the area under the current trace and was related to the corresponding peak tension for a given depolarization. Both current and tension were modified by various predepolarizing steps, the test step remaining constant (Fig. 3*A*). Peak tension yielded a linear relationship, at least in the range of 1–6 mg, when plotted against the respective value of $\Delta[Ca]_i$ (Fig. 3*B*), and could be expressed by

$$T = b \Delta[Ca]_i + T_0, \tag{6}$$

where b and T_0 are constants.

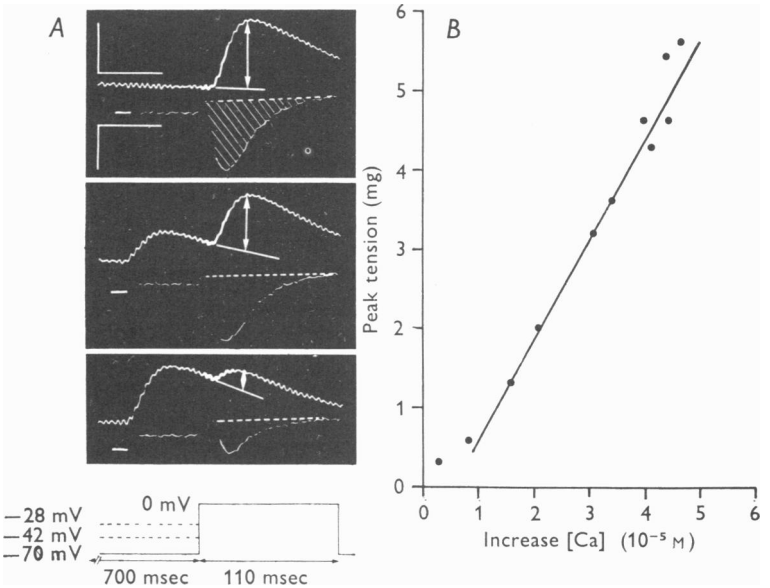

Fig. 3. Relationship between $\Delta[Ca]_i$ and peak tension in Li–Ringer solution with TTX added. *A*, current (middle traces) and tension (upper traces) elicited by a 70 mV depolarizing (test) step preceded by a 700 msec depolarizing pre-step of varied amplitude (shown diagrammatically below frame 3). Note that the inward current corresponds to the test step only. Frame 1: no pre-depolarizing step was used. Frames 2 and 3: pre-pulse amplitude, +28 mV (E_m = −42 mV) and +42 mV (E_m = −28 mV) respectively; the first peak tension was elicited by this pre-step; only the last 50 msec of corresponding current is shown. I_0 is the current level at resting potential (E_r). *B*, values of peak tension (measured as indicated by arrows) plotted against $\Delta[Ca]_i$ values obtained by graphical integration of the hatched area under the current trace (as shown in *A*).

Steady-state Ca inactivation and phasic tension

Experiments similar to those shown in Fig. 3*A* revealed the steady-state availability of Ca current and phasic tension (f_∞, f'_∞). Fig. 4 illustrates the dependence of Ca current (*A*) and peak tension (*B*), elicited by the 90 mV depolarizing test step (E_m = +20 mV), on the amplitude of the pre-depolarizing step. The amplitude of both Ca current and tension decreased with increasing amplitude of the pre-depolarizing step; at membrane potentials more positive than +10 mV, f_∞ and f'_∞ approached zero, indicating almost complete inactivation of the Ca-carrying system. A direct relationship between both is apparent: half-inactivation of current and tension occurs at membrane potentials of −19 mV (slope factor = −4·7 mV) and −21 mV (slope factor = −3·7 mV) respectively; the Ca

system is fully available, and phasic tension is maximal, when the holding potential preceding depolarization is more negative than or equal to -45 mV, at which potentials f_∞ and f'_∞ are nearly 1.

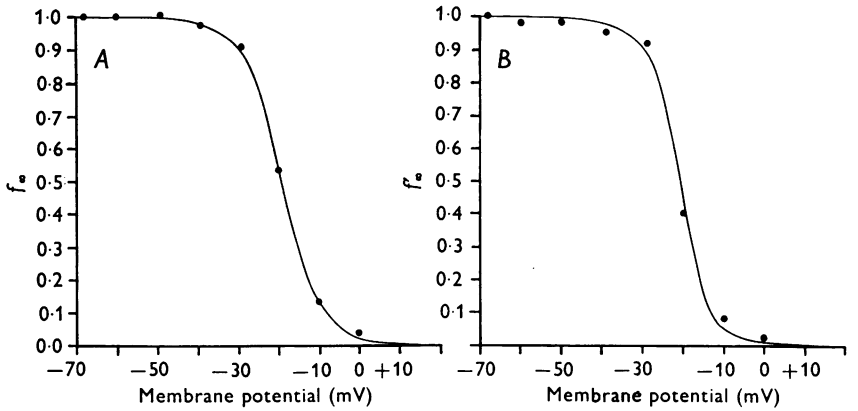

Fig. 4. Voltage-dependence of steady-state Ca inactivation (f_∞; in A) and phasic tension (f'_∞ in B) in Li–Ringer solution with TTX added. Abscissa: membrane potential during the conditioning pre-step. Ordinate: peak amplitude of Ca current (A) and tension (B) during the test step ($E_m = +20$ mV) relative to Ca current or tension in the unconditioned test step. Using minimization of least squares the curves were fitted by equations: $f_\infty = 1/1+e^{(E_m+19)/4\cdot7}$ and $f'_\infty = 1/1+e^{(E_m+21)/3\cdot7}$.

Similar results were obtained in three other experiments. The average values of f_∞ and $f'_\infty = 0{\cdot}5$ (after individual fitting of the inactivation curves by minimization of least squares) were obtained at membrane potentials of -22 ± 3 mV and -24 ± 4 mV (slope factor $= -5{\cdot}6 \pm 0{\cdot}8$ mV and $-4{\cdot}9 \pm 0{\cdot}4$ mV) from Ca current and peak phasic tension respectively (due to the slight variation in resting membrane potential between individual preparations the best fit method used for averaging all four experiments gave the same membrane potential at which f_∞ and $f'_\infty = 0{\cdot}5$ but the apparent slope factor was then $-7{\cdot}3$ and $-7{\cdot}4$ mV).

Time- and voltage-dependence of phasic tension

Peak tension elicited by a 61 mV depolarizing step ($E_m = -9$ mV) reached approximately 25 % of its maximal value at a step lasting 10 msec and 50 % at a 50 msec step (Fig. 5A). When a 122 mV depolarizing pulse ($E_m = +52$ mV) was applied, peak tension was already maximal at a 20 msec step (Fig. 5B). The difference between time courses for reaching maximal peak tension at these two membrane potentials suggests noticeable voltage dependence of the Ca kinetics.

Having established a direct relation between tension and quantity of Ca ions entering the cell upon depolarization (eqn. 6), we can use the variation in peak tension against duration as an indicator of Ca kinetics. From eqns. (5) and (6) it follows that

$$T = \frac{ab}{2F}(K + k\,e^{-t_D/\tau_f}) + T_0. \tag{7}$$

Thus we can determine τ_f at different membrane potentials by measuring the peak phasic tension (T) elicited by various depolarizing steps of varied duration (for $t_D \geqslant t_1$).

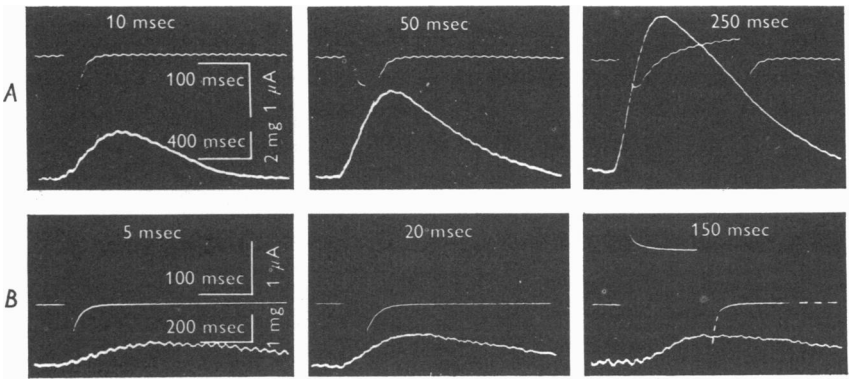

Fig. 5. Variation in phasic tension (lower traces) with duration of the depolarizing step and corresponding current (upper traces), recorded in Li–Ringer solution with TTX added. Duration of the depolarizing step is indicated at the top of each frame. Amplitude of step: *A*, 61 mV ($E_m = -9$ mV); *B*, 122 mV ($E_m = +52$ mV). (The current trace in *B* (right frame) was re-touched.)

Time- and voltage-dependence of Ca inactivation

To validate the above approach, we tried to compare τ_f determined from tension with τ_f determined from Ca current directly at $E_m = -5$ mV, at which calcium current flow is large and considered predominant. Fig. 6*A* shows the slow inward current recorded simultaneously with tension for this 65 mV depolarizing step, and the semilogarithmic plot of the net Ca current ($I_\infty - I$) against time, the time constant of decline of the Ca inward current (τ_f) was 41 msec. The relation of peak tension (T) and of rate of rise of tension (dT/dt) to the depolarizing step duration (Fig. 6 *B*) can be fitted by

$$T = T_\infty[1 - e^{(t_0 - t_D)/\tau_f}]. \tag{8}$$

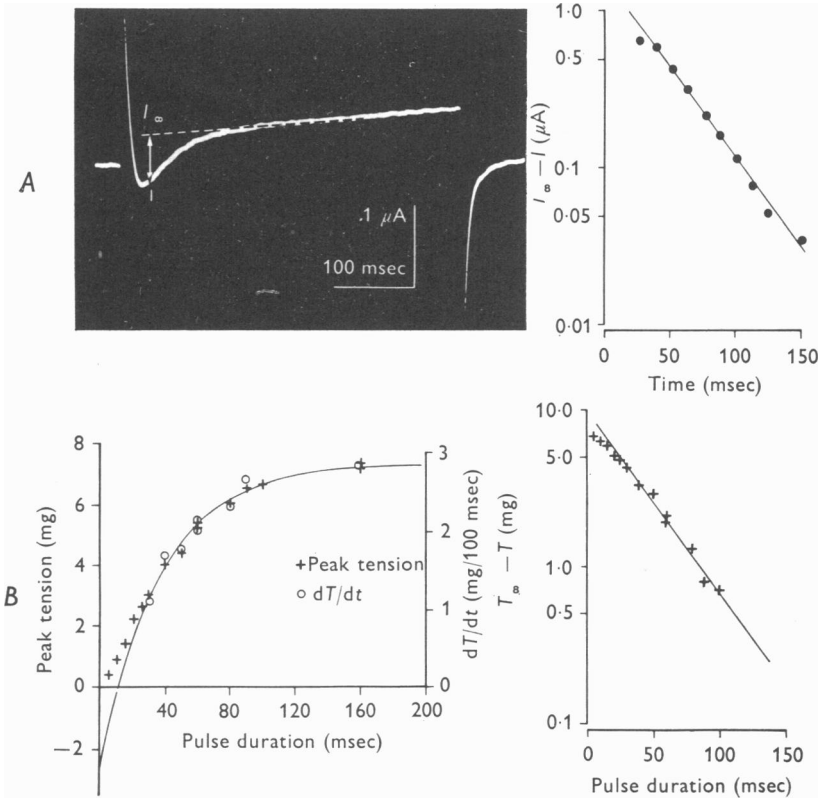

Fig. 6. Determination of time constant of Ca inactivation (τ_f) from current and peak tension in Li–Ringer solution with TTX added at a depolarization of 65 mV ($E_m = -5$ mV). *A*: *left*, subtraction of Ca current from total membrane current; the arrow between I_∞ and I indicates the method of measuring the decline of Ca inward current ($I_\infty - I$), where I_∞ is the extrapolated value of Ca current at infinite duration of depolarization. *Right* – the semilogarithmic plot of these values of ($I_\infty - I$) against time during the depolarizing step; the line was drawn by eye. *B*: *left*, peak tension and maximal rate of rise of tension (dT/dt) plotted against various durations of depolarizing step using the minimization of least squares. The curve was fitted by the equation $T = T_\infty [1 - e^{(t_0 - t)/\tau_f}]$, with $T_\infty = 7{\cdot}3$, $t_0 = 11{\cdot}5$, and $\tau_f = 37$. *Right*, the semilogarithmic plot of values $T_\infty - T$ against duration of the depolarizing step; T is the peak amplitude of tension with a maximal value (T_∞) elicited by a depolarizing step of 160 msec. The maximal theoretical value of tension, $T'_\infty = 9{\cdot}9$; the line was drawn by eye.

Eqn. (8) is derived from eqn. (7) as follows. Assuming instantaneous activation, $T_0 = 0$ and $t_D = \infty$ the theoretical maximal value of tension (T'_∞) for a given membrane potential is given by

$$T'_\infty = \frac{ab}{2F}\alpha_0 \tau_f.$$

For actual, non-instantaneous activation, however, the maximal experimental value of tension (T_∞) is given by

$$T_\infty = T'_\infty - \frac{ab}{2F}(A' - A).$$

$$= \frac{ab}{2F}(\alpha_0\tau_f - (A' - A) = \frac{ab}{2F}K.$$

Eqn. (7) can be expressed using T_∞:

$$T = T_\infty + \frac{ab}{2F}k\, e^{-t_D/\tau_f} + T_0$$

$$= (T_\infty + T_0)\left(1 - \frac{(ab/2F)(-k)}{T_\infty + T_0} e^{-t_D/\tau_f}\right). \qquad (7^1)$$

Let $t_0 = \tau_f \ln(y)$ with

$$y = \frac{(ab/2F)(-k)}{T_\infty + T_0} = e^{-t_0/\tau_f} \quad \text{for} \quad k < 0,$$

thus

$$T = T_\infty [-e^{(t_0 - t_D)/\tau_f}]. \qquad (8)$$

Using eqn. (8) for fitting the curve in Fig. 6*B*, $\tau_f = 37$ msec. Thus, both peak tension and dT/dt can be fitted by the same equation; as T can be measured more accurately (especially for short pulse durations), we used this parameter to obtain τ_f. This time constant was determined at various other membrane potentials, by plotting semilogarithmically ($T_\infty - T$) against pulse duration. This plot for a given E_m (-5 mV) shows that the relationship is linear for pulse durations < 30 msec (Fig. 6*B*); graphical determination gives $\tau_f = 38$ msec. (The non-linearity of tension values corresponding to pulse durations < 30 msec, is due to not-yet-completed Ca activation). This τ_f value obtained from tension almost equals that obtained directly from the current. τ_f values determined by these two methods were comparable at other membrane potentials, also, in the range -24 to $+20$ mV (Table 1).

Fig. 7 gives a more complete picture of the voltage-dependence of τ_f; these values were obtained from tension measurements as described above. The computed curve of the relationship τ_f^{-1} against potential shows that the decay of Ca conductance was slowest around $E_m = -13$ mV with $\tau_f = 55$ msec, and that the rate of Ca inactivation was faster at more-negative and more-positive potentials.

TABLE 1. Values of τ_f obtained from current and tension at various membrane potentials as shown in Fig. 6

V (mV)	E_m (mV)	τ_f (current) (msec)	τ_f (tension) (msec)
46	−24	64	60
47	−23	54	58
50	−20	35	34
61	−9	53	54
65	−5	41	38
69	−1	53	54
78	+8	40	44
90	+20	22	21

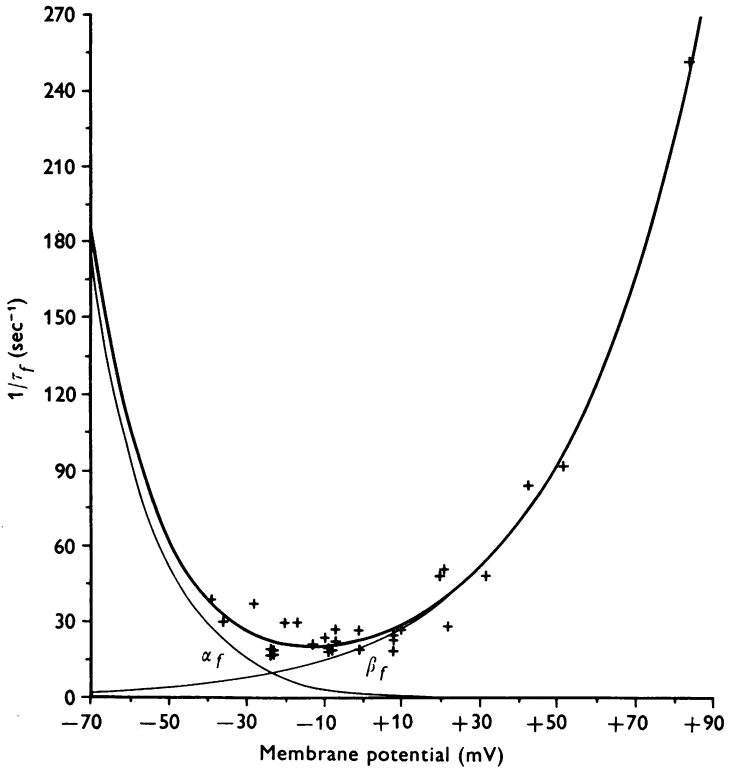

Fig. 7. Relationship between τ_f^{-1} and membrane potential. In seven experiments in Li–Ringer solution with TTX added, τ_f values were determined from tension measurements at various potentials as shown in Fig. 6*B*. The continuous curve was fitted using minimization of least squares by the equation $\tau_f^{-1} = \alpha_f + \beta_f$, where $\alpha_f = 185e^{-(E_m+70)/17\cdot5}$ and $\beta_f = 1930/1 + e^{(145-E_m)/32}$ (The eqn. is not appreciably affected by the τ_f corresponding to $E_m = +80$ mV.)

We further validated our assumption of direct proportionality between tension and the amount of calcium entering the cell as stated in eqn. (7). When E_m is clamped close to E_{Ca}, the quantity of Ca ions entering the cell (due mainly to tail current following depolarization) decreases with increasing duration of depolarization (see alternative (ii), p. 602). Thus the tension, also, elicited by such depolarization should decrease with prolongation of the pulse.

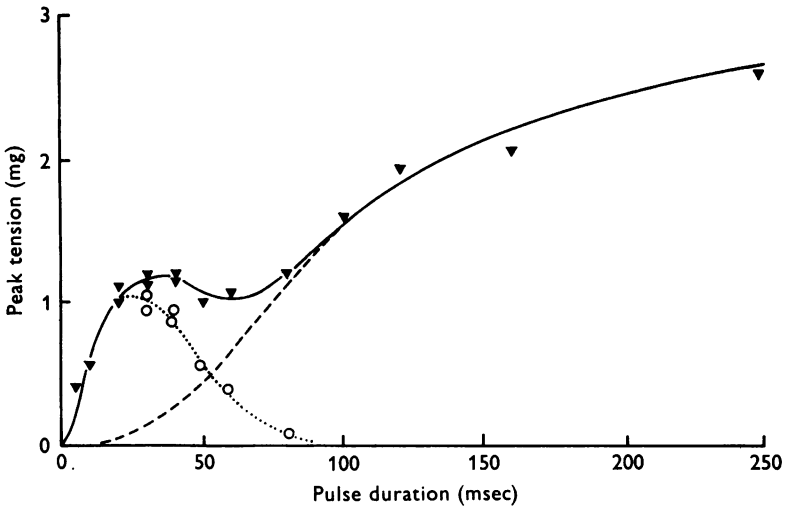

Fig. 8. Dependence of peak tension (▼) on duration of a 96 mV depolarizing step ($E_m = +26$ mV) immediately after application of Na-free, Li–TTX Ringer solution. The continuous line was drawn by eye. The interrupted line is the expected tonic tension (i.e. elicited by a 96 mV depolarizing step in the absence of Ca inward current; Vassort & Rougier, 1972). Subtraction of the extrapolated values (– – –) from the experimental values (▼) gives the phasic tension (○).

This prediction was tested in the following experimental situation. Instead of stepping E_m close to the normal E_{Ca} ($\sim +100$ mV), E_{Ca} was lowered experimentally to $\sim +30$ mV, to avoid the possibility of cellular damage due to the high depolarization ($+170$ mV) and because at this membrane potential the kinetics of Ca conductance are slower and therefore more easily analysed. E_{Ca} was decreased by exposing the preparation briefly to Na-free (Li–Ringer) solution, causing a transient large increase in $[Ca]_i$: it has been estimated (Vassort, 1973*a*) that during the first few minutes of contracture in such a Na-free solution E_{Ca} falls to between $+20$ and $+40$ mV. Tension dependence on the duration of depolarization (96 mV) was measured immediately after the introduction

of Li–Ringer solution, i.e. during contracture (Fig. 8). (Note that, unlike previous experiments, the preparation was not equilibrated in a Na-free solution.) The relationship showed a maximum, due to phasic tension, at very short pulse duration; after an interim decrease it increased further at depolarizations lasting >80 msec. The latter increase was due to the development of tonic tension, still present under these conditions (exposure to the Na-free solution was brief). Phasic tension was determined by subtracting the extrapolated values of tonic tension from total tension at short durations (<80 msec) of depolarization. The relationship between phasic tension and pulse duration was maximal at 30 msec, and then decreased (as expected from the τ_f values determined earlier) with a time constant of 20 msec. At this E_m ($+26$ mV), which is close to E_{Ca}, phasic tension is due predominantly to Ca tail-current, both diminishing with increasing duration of depolarization.

DISCUSSION

The considerable variations in published data on the time- and voltage-dependence of Ca inactivation (Beeler & Reuter, 1970*a*; New & Trautwein, 1972; Besseau, 1972; Kohlhardt, Krause, Kubler & Herdey, 1975) could be accounted for by the use of different methods for estimating pure Ca current, and the difficulty of separating this from the other membrane currents. In this study our indirect approach using phasic tension measurements as an indicator of Ca kinetics avoided such difficulties and its validity is supported by the close relation between calcium current and phasic tension.

Ca current and activation of phasic tension

According to theoretical analysis, the activation of phasic tension (peak amplitude, T) depends directly on the amount of Ca ions entering the cell as the Ca inward current (eqn. 7), which in turn depends mainly on the Ca inactivation process as long as this is much slower than the activation (eqn. 5).

A first point relevant to the relation between the Ca current and the activation of phasic tension is the observation of no delay in onset of tension activation. Depolarizing pulses even as short as 5–10 msec elicited measurable tension on the first depolarization in these experiments with Li–Ringer solution (Fig. 5) as well as in normal Na-containing Ringer. This finding agrees with Beeler & Reuter's data (1970*b*) obtained from mammalian heart under similar experimental conditions (in Na-free solution) and Leoty & Raymond's data (1972) from frog heart in normal Ringer solution. By contrast, Morad & Orkand (1971) reported that tension was not elicited by the first depolarizing step (e.g. 80 or 100 mV) when the

pulse duration was < 80 msec, and that a measurable mechanical response was elicited by the second step only when this was applied shortly after the first (see their Fig. 8). Part of the difference is certainly due to the relatively low Ca concentration in their Ringer solution (0·2–1·0 mM) which greatly diminishes the tension elicited by short depolarizations, because phasic tension is the major component of the mechanical response at short clamp durations in normal Na-containing Ringer solution. In fact, Morad & Goldman later stated (1973) that unpublished observations showed an appreciably shortened time of tension activation when the Ca concentration was increased above 1·0 mM.

Our theoretical model was validated by establishing a relation between Ca current (that is, the quantity of Ca ions entering the cell during excitation) and phasic tension. When the current amplitude elicited by a clamp step was varied by an imposed pre-step, the amplitude of phasic tension was correspondingly altered. The relationship between $\Delta[Ca]_i$ and peak tension was linear in most of the range investigated (Fig. 3). Several previous investigations in mammalian heart also reported a relationship between Ca current and tension (Fozzard & Hellam, 1968; Beeler & Reuter, 1970*b*; Gibbons & Fozzard, 1971; Ochi & Trautwein, 1971; Bassingthwaighte & Reuter, 1972; New & Trautwein, 1972; Trautwein, McDonald & Tripathi, 1975); these data indicated that inflowing Ca ions serve mainly to fill intracellular stores and to stimulate a regenerative release of activator Ca. However, in both frog heart (Einwächter, Haas & Kern, 1972; Goto, Kimoto & Suetsugi, 1972; Leoty & Raymond, 1972; Vassort & Rougier, 1972) and mammalian heart during Na-free perfusion (Beeler & Reuter, 1970*b*) phasic tension appears to be more directly dependent on Ca current. The quantity of charges flowing into the cell during depolarization can account for an increase of 10^{-5} M in the intracellular Ca concentration (Beeler & Reuter, 1970*b*, Vassort & Rougier, 1972): even if this amount is overestimated, due to limitations of the voltage-clamp technique (see McGuigan & Tsien, in McGuigan, 1974), and only one-sixth of this amount remains ionized and is available for activation of the contractile proteins (Katz, 1970), one might expect the enhancement of free Ca (due to Ca current) to be sufficient by itself to trigger mechanical activity. It was recently shown by Solaro, Wise, Shiner & Briggs (1974) that tension development is half-maximal at 2×10^{-6} M free-Ca concentration and is already noticeable at Ca concentrations lower than 10^{-7} M. A further point for consideration is the possibility of triggered release of activator Ca. Several of our data and observations indicate that if Ca release in atrial muscle is Ca-induced, the amplifying factor is constant, i.e. a given amount of trigger Ca induces proportional release of activator Ca. Thus, tension elicited by a given depolarization reached

maximal amplitude upon the first step, the voltage dependence of Ca current and peak phasic tension were similar, whether the conditioning potential was changed before each test pulse or irregularly, and finally, the quantity of Ca ions entering a cell was linearly related to the developed tension.

Our finding is in agreement with that of Ashley & Ridgway (1970), who used aequorin to measure Ca-dependent light emission. They showed that the force developed by a single barnacle-muscle fibre depends directly upon the increase in internal free Ca elicited by depolarization, and concluded that the linear relation in a given range of force is part of an S-shaped relationship. A similar situation is presumed with our preparation of frog atrial muscle, in which the linear relationship between $\Delta[Ca]_i$ and phasic tension was determined only in a certain middle range of these two variables.

The above experimental approach was also validated by investigating steady-state inactivation of the Ca-carrying system. The availability of the Ca conductance–voltage relationship was practically the same whether obtained from Ca current or peak-tension measurements (Fig. 4), again indicating a linear relationship between Ca current and activation of phasic tension. Our values of steady-state Ca inactivation are comparable with data from mammalian heart although our absolute value of slope factor is smaller. The average value of membrane potential for half-inactivation was -22 ± 3 mV (slope factor $-5{\cdot}6 \pm 0{\cdot}8$ mV) from Ca current and -24 ± 4 mV (slope factor $-4{\cdot}9 \pm 0{\cdot}4$ mV) from phasic-tension measurements, compared with reported values of -27 mV (Bassingthwaighte & Reuter, 1972) and -28 mV (Trautwein *et al.* 1975) with a slope factor of $-8{\cdot}3$ mV. The results indicate almost full availability of the Ca-carrying system at membrane potentials more negative than -45 mV and almost complete inactivation at membrane potentials more positive than $+10$ mV. In contrast to our results and those of Lenfant, Mironneau & Aka (1972) are Besseau's results (1972), which showed on the same tissue (frog atria) half-inactivation of Ca conductance at membrane potentials 30–40 mV more negative (i.e. in the same voltage range as Na conductance). In our experiments (not shown), although conditioning depolarizations of 0–20 mV markedly decreased fast inward current, they modified neither slow inward current nor phasic tension, demonstrating that the Ca-availability curve is shifted higher than the Na-availability curve towards depolarization.

Finally, the linear relationship between the Ca current and the phasic tension (and thus the validity of our theoretical and experimental approach) was confirmed by the similarity of the time constants of Ca inactivation in a certain range of membrane potential (-20 to $+25$ mV), whether

determined directly from the Ca current or indirectly from tension, i.e. its rate of rise or the peak values (Fig. 6; Table 1). Furthermore, with membrane potentials close to E_{Ca}, the dependence of phasic tension on pulse duration with the tonic component present (Fig. 8) showed a time constant of inactivation almost identical to that obtained in experiments with only the phasic component present. This finding confirms that phasic tension and its relation to Ca current is not affected by the specific experimental procedure (LiCl, which suppresses tonic tension) and that the linear relationship is valid even when the tension is due mainly to Ca tail-current.

Kinetics of calcium inactivation

The above results, mostly obtained indirectly by analysis of peak phasic tension, showed that the time constants for the inactivation process of Ca conductance were short (50–60 msec) in the potential range -20 to 0 mV and decreased further at both more negative and more positive membrane potentials.

Interpretation of our results is based on an assumption of complete inactivation of Ca conductance at all membrane potentials studied. As shown by Trautwein *et al.* (1975) in cat heart, this is probably not true for the range -35 to 0 mV (assuming in frog heart a similar relative position of d_∞ and f_∞ curves). However, the data indicate that the largest steady-state current would not exceed 0·17 of the maximal current; thus it would only slightly modify maximal peak tension (T_∞), since the expected increase in tension would result from subsequent addition of small quantities of Ca ions.

Our results are in general agreement with those obtained by Besseau (1972) except that the relationship of time constant of inactivation against voltage described by him was shifted 30–40 mV towards more negative potentials. However, the same shift occurred along the voltage axis in his determination of the Ca steady-state inactivation curve, which also conflicts with our results and other published data (as discussed earlier). Our results contrast with earlier reports (Beeler & Reuter, 1970*a*; New & Trautwein, 1972 and Kohlhardt *et al.* 1975) in that our values of time constant showed a decrease with increasing positivity of the membrane potential (above -10 mV). Beeler & Reuter (1970*a*) by analysis of Ca current obtained time constants of several hundred milliseconds in the plateau range of potential and the values further increased at more positive membrane potentials. Thus, our values of the rate of Ca inactivation were generally smaller than the values previously reported and even when they appear to be rather similar in a certain potential range (-20 to 0 mV; see New & Trautwein, 1972) they are actually smaller, if we take into account the temperature difference (we worked at 18–20° C instead of 37° C).

A slow inactivation of Ca conductance suggests that maintenance of

E_m during the beginning of repolarization in the plateau region requires a large outward current. This contrasts with the weak delayed outward current described by Giebisch & Weidmann (1971) and Vitek & Trautwein (1971) and the recovery of high membrane resistance during the action potential plateau reported earlier by Weidmann (1951). A slowly inactivating Ca system also contradicts the short time constant of inactivation (50 msec) at $E_m = 0$ mV attributed to Ca conductance for reconstruction of a computed cardiac action potential in mammalian heart (E. McAllister, 1975, personal communication).

Of pertinence here are Sumbera's data (1970) from experiments in which the cardiac action potential was temporarily modified by imposed current pulses, indicating that the value of the membrane potential during the first third of the action potential determined the development of tension. After about 150 msec following the onset of the action potential, the current pulse had almost no effect on tension amplitude and rate of rise, i.e. modification of the driving force for Ca ions was no longer effective, suggesting full inactivation of the process controlling tension development with a time constant in the range of 30–50 msec. We obtained similar results in preliminary experiments with frog heart in normal Ringer solution under the same conditions, though the effects were partly masked and interpretation was hindered by the effect of tonic tension, which is also potential-dependent. When we used Li–Ringer solution (which suppressed tonic tension), phasic tension was greatly modified by changing membrane potential during the first 50 msec of the action potential but not when the pulse was applied later (Fig. 1); this, also, supports our finding of fast inactivation of Ca conductance in the range of membrane potentials between +20 and +40 mV. In another type of experiment concerned with the effect of action potential duration on contraction in mammalian heart Morad & Trautwein (1968) also concluded that shortening the action potential can alter the time course and amplitude of contraction only within the first 200 msec of the action potential and that most of the tension develops within the first 50 msec of depolarization.

Contrary to our finding of a fast Ca-inactivating process at high depolarizations would be the demonstration by McGuigan (1968) and Beeler & Reuter (1970*b*) of a second contraction elicited by repolarization after a clamp step lasting 1 sec in voltage-clamp experiments with mammalian heart. This has not been observed with frog heart preparations, for which the double sucrose-gap technique has been used (Goto, Kimoto & Kato, 1971; Einwächter, Haas & Kern, 1972; Vassort & Rougier, 1972), suggesting the possibility that contractions elicited by repolarization reflect secondary activation in other parts of the preparation rather than very slow inactivation of Ca conductance.

On the basis of our results we conclude that the normal repolarization of action potential in frog atrium depends mainly on the time-dependent decay of slow inward current rather than on a time-dependent increase in outward currents, whose time constants are one or two orders of magnitude larger (Brown & Noble, 1969; Ojeda & Rougier, 1974). The situation is probably similar in mammalian myocardial action potential, it having been shown that the outward current is small (Giebisch & Weidmann, 1971) and increases too slowly to play a significant role in repolarization (Vitek & Trautwein, 1971).

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