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PARAMETERS AFFECTING THE SLOW INWARD CHANNEL REPRIMING PROCESS IN FROG ATRIUM

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SUMMARY

1. The time of recovery (from inactivation) of the slow inward current was studied in the frog atrium, using the double sucrose gap voltage clamp technique.

2. The 'repriming' process was found to be distinct from the current inactivation, and to depend on experimental protocol: double pulses given at low frequencies (at 'rest') gave a faster recovery time when compared to recovery during constant stimulation, with interposed stimuli monitoring the recovery. Longer durations of the clamp pulses led to a faster recovery process.

3. Changing the holding potential of the membrane (with double pulses to the same absolute membrane potential monitoring the recovery process) greatly affect the repriming with depolarized levels slowing down the process.

4. The recovery time was fastest following clamp pulses to intermediate membrane potentials (in the plateau range). This was determined by double pulses, from a constant holding potential, to different levels.

5. Decreasing extracellular Ca prolonged, and increasing Ca enhanced the recovery process.

6. The recovery process was markedly slowed down in Na or in K-free solutions.

7. The recovery process was enhanced in solutions with a raised concentration of Mg or H ions (lower pH). In higher Mg solutions, the inactivaton of the slow inward current was slower.

8. It is proposed that the recovery process is sensitive to alterations in intracellular Ca ions and to variations in extracellular surface charges. The possible implications are discussed.

INTRODUCTION

The membrane of cardiac muscle fibres contain ionic channels which conduct primarily Ca ions (Reuter, 1967; Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969; Reuter & Scholz, 1977). During activity, the calcium influx is thought to activate the contractile process, either indirectly, by triggering release from intracellular stores, as suggested for the mammalian heart by Beeler & Reuter (1970), New & Trautwein (1972), Gibbons & Fozzard (1975), or more directly, as proposed for the frog heart (Einwächter, Haas & Kern, 1972; Horackova & Vassort, 1976).

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The Ca current (termed also the slow inward current, $i_{s,i}$) also affects the configuration of the action potential and thus takes part in determining the duration of the contraction (Morad & Trautwein, 1968).

The Ca channel has been shown to display Hodgkin-Huxley-type kinetics, with well defined activation and inactivation parameters (Besseau, 1972; Reuter, 1973; Horackova & Vassort, 1976; Trautwein, McDonald & Tripathi, 1975). Since this channel undergoes inactivation after each action potential (or after a voltage clamp pulse which elicits the current), the process of reactivation or repriming of the channel is relevant to both normal and abnormal activity. The rapidity of repriming is of crucial importance at high rates of activity (Boyett & Jewell, 1980), where incomplete reactivation of the channel can become a limiting factor in the supply of calcium for the production of tension. At the higher rates, shorter duration action potentials and contractions are necessary to ensure a sufficient time for diastolic filling of the heart. Action potential shortening can result from incomplete decay of outward K currents (Hauswirth, Nole & Tsien, 1972) or from incomplete recovery of the inward current (Gettes & Reuter, 1974; Giles, Hume & Noble, 1980).

In nodal tissue, where the slow inward current is the predominant (or sole) inward current during the rapid upstroke of the action potential (Noma, Yanagihara & Irisawa (1977); Noma, Irisawa, Kokobun, Kotake, Nishimura & Watanabe (1980)), the repriming of the 'slow' channel determines the rate at which excitation can occur. Incomplete reactivation will affect the action potential upstroke and thus the conduction velocity. The availability of this current system might also be crucially important in partially depolarized atrial or ventricular tissue, in which the fast Na system could be inactivated, as e.g. in anoxic or ischemic regions (Cranefield, 1975).

The present work attempts to clarify some parameters affecting the ionic channel repriming process in frog atrial muscle, in which the Ca influx and contraction are intimately linked. Previous work (Noble & Shimoni, 1981*a*, *b*) has shown a frequency and voltage-dependent increase in the Ca current. This augmentation was limited in the upper frequency range by intervals which were too short to enable complete recovery from inactivation.

Conversely, in attempting to quantify the rate of repriming, the underlying facilitatory mechanism could be an influencing factor. This would result from the effects of a 'conditioning' depolarization on a following 'test' pulse (Noble & Shimoni, 1981*b*). Some evidence for such an effect on the current reactivation process is presented (Results, section *C*).

METHODS

The experiments were performed on atrial trabeculae of the bullfrog *Rana catesbeiana*, using a double sucrose gap voltage clamp technique. The set up is described in detail in Brown, Clark & Noble (1976*a*). The normal Ringer solution contained (mM) NaCl 110, KCl 2·0, $CaCl_2$ 1·1, $MgCl_2$ 1·0, Tris 0·5, glucose 5·5. The pH was 7·6–7·9. The end chambers were separated from the central, test, gap by an isotonic solution of either sucrose or mannitol. TTX (2×10^{-6} g/ml., 6·3 μM) was routinely used to inactivate the fast Na current. This dose has been found to block the fast Na current in this preparation effectively (Rougier *et al.* 1969; Connor, Barr & Jakobsson, 1975; Giles, 1974).

Consideration was given to the possibility that the experimental protocol for determining the repriming time of the slow inward current could affect the results. Thus, for example Weingart, Kass & Tsien (1978) and Trautwein *et al.* (1975) use constant stimulation with test pulses given

at varying intervals after a train of pulses. Gibbons & Fozzard (1975) give twin pulses with varying intervals to a preparation which is otherwise at rest.

The method employed in this work was mainly the latter, with at least 15 sec rest between each pair of pulses. In several experiments, both methods were compared (see Results, section *B*). It must also be pointed out that another difference exists in the manner of determining the repriming time, which may affect the results. Trautwein *et al.* (1975) and Gettes & Reuter (1974) obtain a slow inward current by holding the preparation at a very positive membrane potential (e.g. 0 mV), which inactivates the channel. Repolarizing steps are given for various durations, at the end of which the return to the holding potential elicits the current. Kohlhardt, Krause, Kübler & Herdey (1975) and Ehara & Kaufmann (personal communication to D. Noble) give twin pulses from a negative holding potential.

In experiments on frog, it is impossible to hold the membrane at positive potentials for long periods due to massive extracellular accumulation of K ions which leads to a continuing 'creep' of the holding current (Noble, 1976). The rate of recovery was therefore determined by twin pulses given from a holding potential, which was equal to the 'resting', or gap potential of the preparation.

Possible sources of error

The measurement of slow inward currents entails an underlying problem, in the assessment of whether, and to what extent does the pulse activating the inward current also activate outward currents. In determining the repriming time of $i_{s,i}$ the relevant issue is how much *extra* outward current is still present at the end of the interval between two pulses, rather than how much outward current is activated by a given pulse, in addition to $i_{s,i}$. The 'basic' amount of 'contamination' of $i_{s,i}$ by outward currents should be the same, for identical pulses.

Extensive studies of the outward current systems in this preparation have been done by Brown, Clark & Noble (1976*a*, *b*); Giles (1974), Brown, DiFrancesco, Noble & Noble, 1980) and in other species of frog by Ojeda & Rougier (1974), de Hemptinne (1971) and others. On the basis of these studies one can try to assess the magnitude of outward current tails after depolarizations within the range of amplitudes and durations used in the present study. The standard pulse duration was 300 msec, with steps given from the holding potential (usually close to −80 mV), to around −10 to +10 mV.

On taking the 'worst' possible case, steps to +15 mV, activating an outward current with a time constant of 1 sec (Brown *et al.* 1976*b*, Fig. 4), should produce an outward tail of about 1 μA after 300 msec (the sigmoid onset would produce a smaller value, in fact). On the basis of their Figs 12 and 13, at a holding potential of −80 mV, the time constant for tail decay would be 195 or 120 msec. Thus, assuming a monoexponential decay, there would be 36 or 18 nA of outward current remaining after 200 msec, or 21 and 8 nA after 300 msec, which would be 'extra' outward current countering inward currents activated by identical pulses. As the estimates of the tail decay time constants vary, as did the actual steps and holding potentials used, Table 1 outlines the range of extra outward currents expected at different inter-pulse intervals for conditions similar to those used in this study.

Taking Fig. 4 of Brown *et al.* (1976*b*), we note as *a*, a pulse to +15 mV, giving 100 nA after 300 msec; *b*, a pulse to +5 mV giving (with a time constant of 1·25 sec) 70 nA after 300 msec; *c*, a pulse to −5 mV (time constant for tail activation = 1·85 sec), giving an initial tail amplitude of 38 nA, at the end of the first pulse. These three tails will decay from their individual initial magnitude (I_{t_0}) as follows (all current values in nA): (*a*) $I_{t_0} = 100$ nA; (*b*) $I_{t_0} = 70$ nA; (*c*) $I_{t_0} = 38$ nA.

These examples can given an idea of the magnitudes of extra outward currents at different interpulse intervals. With typical peak inward currents in this preparation and apparatus being of the order of 0·2 to 1·0 μA, and full recovery of $i_{s,i}$ occurring usually not before an interval of 300–400 msec between two pulses, the conclusion is that the extra outward currents *can* distort the time course of $i_{s,i}$ recovery. However, the main effect will be at short intervals. At 300–400 msec intervals, the worst conditions will give an outward current which is 10–15 % of the peak inward current. More typically, as was in fact observed experimentally, at the time of complete recovery all, or most, of the extra outward current is negligible.

It must be emphasized, however, that the actual amount of outward current which is activated can vary considerably from fibre to fibre. Giles (1974), for example illustrates a 300 msec pulse to +30 from −70 mV, producing a tail of 9 nA, whereas another pulse from −70 to 0 mV (for 360 msec) produces a tail of 300 nA. Maughan (1973) illustrates pulses to −10 mV for 2 sec giving tails of 5 nA of magnitude, or to +10 mV for 0·5 sec giving a tail of less than 10 nA.

TABLE 1. Examples of variation in outward current (taken from the data of Brown *et al.* (1976*b*), the inter-pulse intervals being similar to those studied in this paper

	Holding potential (mV)	Decay time constant (msec)		Time (msec) 100	200	300	400
Fig. 8	−70	400	*a*	78	61	47	36
			b	54·5	42	33	25
			c	30	23	18	14
	−80	285	*a*	70	50	35	24·5
			b	49	35	24	17
			c	27	19	13	9
Fig. 12	−80	194	*a*	60	36	21	13
			b	42	25	15	9
			c	23	13·5	8	5
Fig. 13	−70	149	*a*	51	26	13·5	7
			b	35	18	7	5
			c	20	10	5	3
	−80	118	*a*	43	18	8	3
			b	28	13	5·5	2
			c	16	7	3	1
	−85	100	*a*	36	13·5	5	2
			b	26	9·5	3·5	

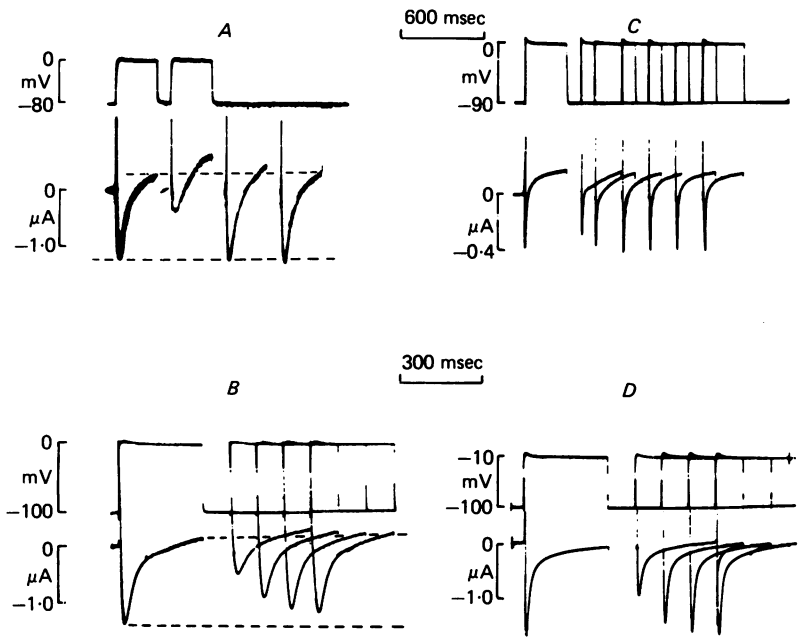

Fig. 1. Examples of the repriming process illustrating different amounts of 'extra' outward current, activated by the second of two pulses, given at different intervals. The dashed lines in *A* and *B* are at the level of the peak inward current and the amount of outward current at the end of the first pulse. *A*: an extreme example in which very much extra outward current was activated. The slow inward current was fully recovered at an interval of 500 msec in which there was still some extra outward current. *B*: the extra outward current has decayed completely at an interval of 600 msec, before the complete reactivation of the slow inward current. In *C* and *D* there is negligble or no extra outward current.

This variability was apparent in this study. Fig. 1 shows different patterns of recovery, with and without extra outward current. Thus, the extra current can in extreme cases (Fig. 1*A*) still be evident by the time the two inward currents are equal. It continues to decay after the full recovery of $i_{s,1}$ at longer intervals. In other cases, the additional outward current decays before the completion of $i_{s,1}$ recovery (1*B*). In 1*C* and *D* there is negligible or no extra outward current during the test pulses.

An additional possibility to be considered is the accumulation of potassium ions in restricted extracellular clefts. Such accumulation, if produced by the first pulse, and still present at the time of the second pulse, will have the effect of producing a larger instantaneous outward 'jump', due to the 'crossover' effect (Brown *et al.* 1980). This effect will lead to more outward (instantaneous) current at the higher cleft K^+ level, in the relevant voltage range (−15 to +15 mV). This could, in turn, reduce the magnitude of $i_{s,1}$ (during the second pulse) to some extent.

In view of the possible distortions of the initial time course of recovery by extra outward currents, and possibly by some accumulation, the parameter chosen to describe the full recovery was that interval at which the amplitudes of the two net inward currents were equal. The slow inward current was always measured as the difference between the peak amplitude and the base line, 'holding' current.

The effects to be described are all of large magnitude, so that possible distortions were probably swamped by the major effect on repriming. The possible effects of increased outward current activation or accumulation will be pointed out in the relevant sections.

The influence of non-uniformity on analysis

Since the experiments were done in a closed double sucrose gap, there was no way for a direct measurement of voltage non-uniformities, with a micro-electrode. However, Giles & Noble (1976) in their Appendix, have calculated the amount of distortion to be expected from this preparation in the identical experimental setup. This was done for various assumed values of maximum inward current conductance.

In two preceding papers, there are examples of typical current–voltage relationships obtained in an earlier part of this work (Noble & Shimoni, 1981*a*, Fig. 13, and Noble & Shimoni, 1981*b*, Fig. 2). The ratios between maximum inward to maximum outward currents (obtained in the range −80 to −40 mV) in the illustrated cases were 8·3 and 5·6, respectively.

On the basis of the computations in the above mentioned Appendix, these values would fit an increase of the resting conductance by a factor of about 2·5 (see Giles & Noble, Fig. 8). This would lead to a maximum degree of non-uniformity of 4 mV.

In all but one of the treatments described in this paper, the basic current magnitude did not change by much. In the case of elevated extacelluar Ca levels, larger currents might have increased the axial non-uniformity to some extent. In all the cases used, the membrane voltage appeared stable, without sagging during the clamp pulse. All the current records used for analysis appeared smooth (without 'notches').

RESULTS

It is important to stress that the recovery process had quite a variable time course, which depended on a large number of factors, as described below. However, even with identical conditions and the same experimental protocol the full recovery time of the slow inward current could range from 300 to 2000 msec in different preparations. One reason for this could be the different amounts of extra outward current activated during the first pulse, or the different amount of K accumulation.

Another likely reason for this, which is supported by the results described in this paper, is a large variability in the levels of intracellular Ca. This might arise from a different metabolic state of dissected preparation (or animal), or from different degrees of healing over of the trabeculae after dissection.

The large spread of 'control' repriming times led inevitably to a large spread of the shifts in recovery time due to a change of experimental conditions. It was thus

felt that an indication of the average shifts, with the range of change in each of the treatments, was an appropriate way of expressing the results.

It should be stressed that in most cases (Figs. 3, 4, 5, 9, 10, 11) the recovery is more than 100%. This is the effect described in an earlier paper (Noble & Shimoni, 1981*b*), whereby two depolarizing pulses produce an augmentation of the second elicitied $i_{s,i}$, at a certain interval range. This potentiation becomes apparent at intervals sufficiently long to overcome incomplete reactivation of the channel.

(*a*) *A comparison of the* $i_{s,i}$ *inactivation to the repriming process*

In order to compare the time constant of $i_{s,i}$ inactivation to that of repriming, one must measure the two processes at the same membrane potential. However, as shown in a following section (*e*) the magnitude of the activating voltage (V_1) affects the repriming process occurring at the holding potential (HP) (the membrane potential between the pulses). Therefore, a simple shift of the holding potential to V_1 (and giving a series of pulses to a more depolarized leve, V_2) to compare the repriming at V_1 to the inactivation time constant of pulses to V_1 (from HP) is invalid, since the new activating voltage (V_2) will alter the repriming at V_1. (This method was used by Kohlhardt *et al.* 1975).

Unfortunately, the only valid method, used by Gettes & Reuter (1974) and Trautwein *et al.* (1975) is impossible to employ in this preparation (see Methods section).

By comparing the time course of inactivation of the slow inward current, τ_f with that of the channel repriming, in the data of New & Trautwein (1972), Trautwein *et al.* (1975) and Kohlhardt *et al.* (1975) it is clear that inactivation is much faster than repriming, at all membrane potentials. At -40 mV, for example, τ_f is around 50–60 msec, whereas the time constant for repriming is close to 200 msec. Gettes & Reuter (1974) obtain similar time constants for the two processes, but Reuter & Scholz (1977) find that the repriming is slower.

In the frog atrium, where it is impossible to obtain a precise comparison of the two processes, an indication of the separation of the two processes is given by examining the data for τ_f of Besseau (1972) and Horackova & Vassort (1976) in relation to the repriming times as described in this paper (also in Giles, Hume & Noble, 1980). The slowest τ_f values given are around 50–55 msec, whereas the time constants for repriming are several hundreds of msec. A relevant observation (see below, section (*i*)) is that elevating the Mg levels enhanced the repriming while retarding inactivation, for a given stimulation protocol. This clearly points to a dissociation of the two processes (see Discussion). Interestingly, elevated Ca levels enhance both processes, while the addition of Ba ions slows down both processes (Noble & Shimoni, 1981*a*).

To summarize this point: there is strong evidence that the $i_{s,i}$ inactivation is a separate process from the channel reactivation, or repriming, in this preparation. In any case, in a simulation of the 'physiological' action potential, with a holding potential of -80 mV, and pulses to $+10$ mV, lasting 300 msec, the repriming time constant is considerably slower than the inactivation time constant (compared at membrane potentials of -80 and $+10$ mV, respectively), as is shown in Fig. 2.

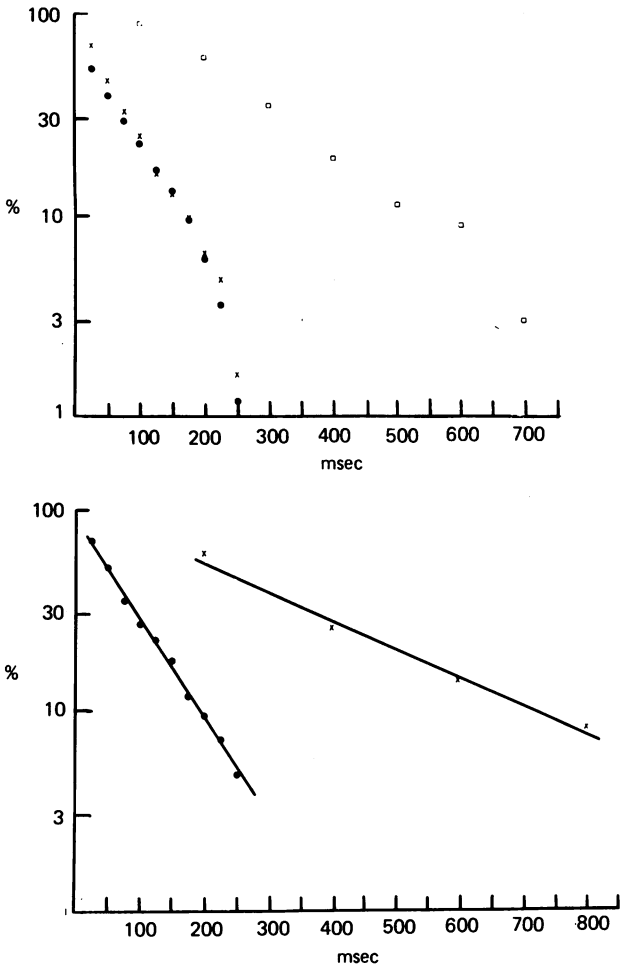

Fig. 2. A comparison of the recovery (reactivation) and inactivation time course. The reactivation was plotted on a logarithmic scale, as the decay (in %) of the difference between the first and second current amplitudes, in a two-pulse protocol, at different intervals. This difference (open squares) decreases as recovery proceeds. The decay of the slow inward current is plotted (from its peak amplitude) as filled circles and crosses, for the first and second of two pulses, respectively. The deviation from linearity occurs sometimes, and is an indication of some outward current activation. The pulses in this experiment were from -80 to $+10$ mV. The time constant for repriming was 200 msec whereas the inactivation time constant is 87 msec, for the first pulse and 72 msec for the second pulse (taken as the one at an interval of 300 msec from the first one, in the case). On the bottom is another example, with the repriming time constant of 312 msec and the inactivation time constant of 90 msec. In this example only the first pulse inactivation is plotted out, showing a simple exponential decay. The lines were fitted by eye.

(b) The effect of experimental protocol on the channel repriming process

The majority of results presented in this paper relate to recovery time as determined by twin pulses (with a variable interval between them), given to a preparation which was close to 'rest' (i.e. stimulated at a low frequency of 3–4 times a minute, as in Gibbons & Fozzard (1975). In seven experiments, the alternative method (as e.g. in Weingart *et al.* 1978) was used. Repetitive stimulation, at rates

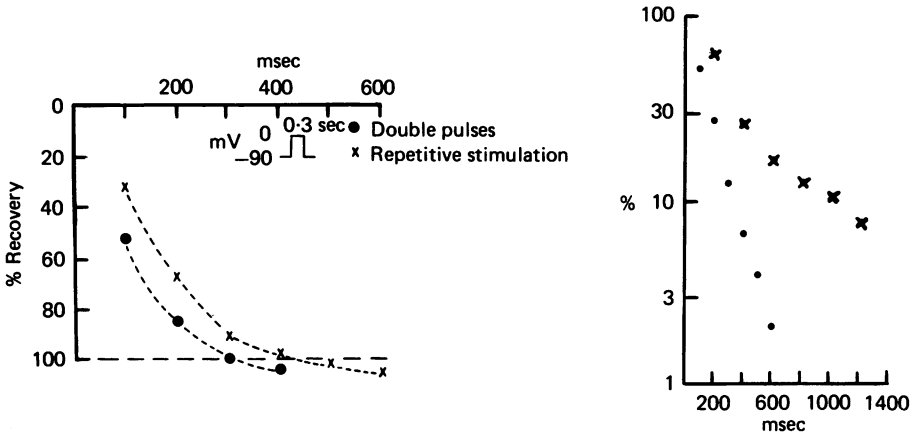

Fig. 3. The effect of stimulation frequency on the reactivation time course. The % recovery is plotted against the inter-pulse interval for double pulses, given every 20 sec (circles) and for repetitive stimulation (30/min), where the test pulses were interposed at different intervals (crosses). On the right hand of the Figure is the plot of the semilogarithmic decay of the difference between the first and the second of two pulses, for the intervals shown, demonstrating the slower recovery at the higher rate of stimulation.

comparable to the normal frog heart rate (30–40/min) was initiated. Interposing test stimuli at different intervals enabled the reconstruction of the time course of repriming. As Fig. 3 shows, this process has a slower time course when determined at a higher rate of stimulation. This retardation of the recovery process, found in five out of seven preparations (the other two showing no significant change), might be caused by a higher level of potassium accumulation which would accompany a higher rate of stimulation (as shown by Kline & Morad, 1978; Kunze, 1977). However, the amounts of extra outward currents activated during repetitive stimulation, in comparison to those observed during double pulses, given at a low stimulation frequency, are probably not sufficient to explain the larger time constants. An alternative hypothesis, which is further explored in the following sections, is that at the higher rate of stimulation the level of Ca ions on the inner side of the channel is elevated, leading to a slowing of repriming. Other procedures, known to affect internal calcium in a similar manner, also produce such retardation.

(c) The effects of changes in pulse duration

According to earlier work (Noble & Shimoni, 1981*b*), the slow inward current which flows in response to a second of two clamp pulses of sufficient amplitude and duration, is larger than the first. This potentiation was found to depend on the pulse duration.

One prediction of this finding was that the repriming process would also be affected by the duration of the pulse, since an additional augmenting factor is present with the longer pulses. This facilitatory effect produces a faster channel recovery when the twin pulses are of longer duration, as is shown in Fig. 4. Such an effect was seen in eight fibres (with three other preparations showing no effect of changing the pulse duration).

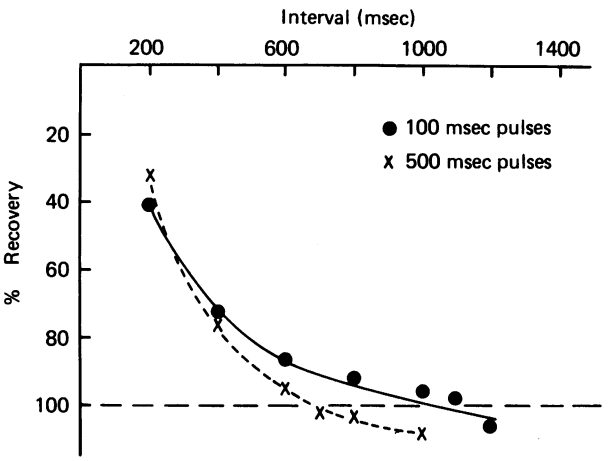

Fig. 4. The dependence of the slow inward channel recovery time on the duration of the monitoring pulses. Double pulses were given (at 4/min) from −85 mV to −5 mV. The shorter pulses (100 msec), shown as circles, produce a slower reactivation than the 500 msec pulses (crosses).

These results are particularly interesting since the prolongation of the pulse is expected to, and does in most cases, increase the amount of outward currents activated (Brown *et al.* 1976*b*; Giles, 1974). The amount of accumulation is also expected to be larger (Noble, 1976). Both of these effects should apparently slow down the repriming process (see Methods), whereas in fact the opposite is observed.

(*d*) *The effects of changing the holding potential*

A much more dramatic relation was found between the recovery process and the potential at which the membrane was held. Twin pulses, given to the same absolute membrane potential, but originating from different holding potentials showed a considerable slowing of repriming as the holding potential was made more positive (depolarized). Such a slowing, as demonstrated in Fig. 5, was found in seven fibres, in which a 20 mV depolarizing shift of the holding potential slowed the full recovery time by an average of 320 msec, ranging from 100 to 500 msec. A similar dependence was described in the mammalian heart by Gettes & Reuter (1974) and by Kohlhardt *et al.* (1975). These results are consistent with changes in intracellular Ca, which would accompany more depolarized membrane potentials (see Discussion).

These results are not compatible with the alternative mechanisms mentioned previously. Shifting the holding potential to a more depolarized level could entail some activation of outward currents. However, a step to the same absolute membrane potential (as from the more hyperpolarized holding potential) would lead to a smaller

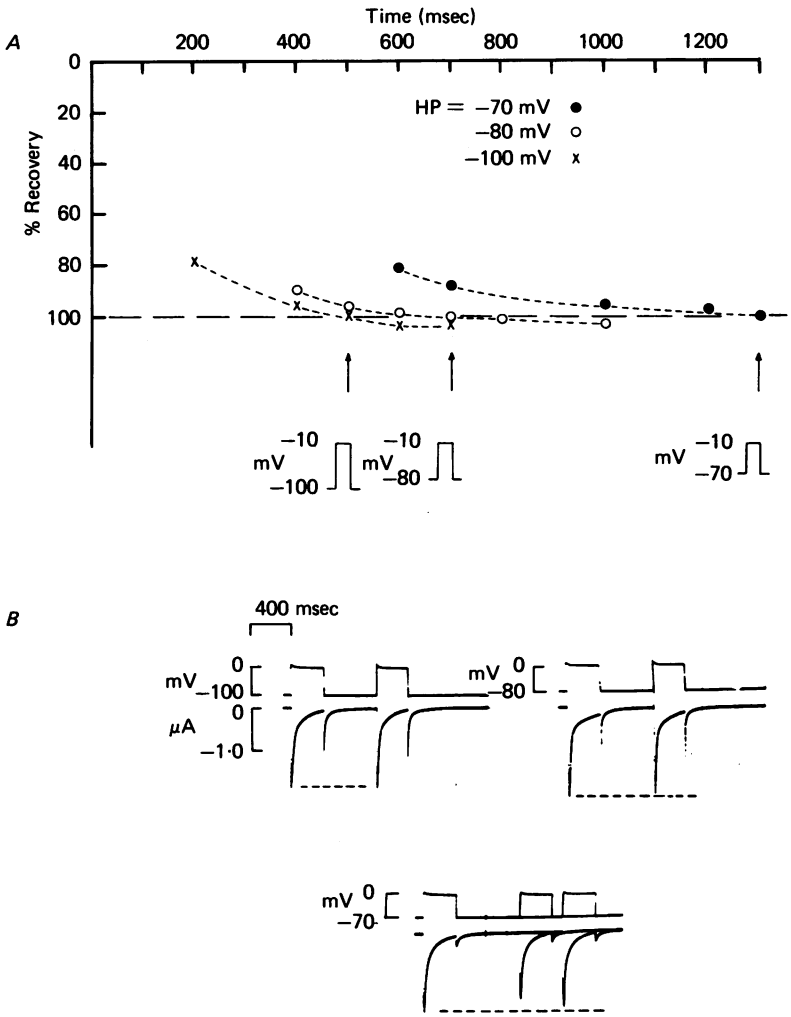

Fig. 5. The dependence of $i_{s,i}$ recovery on the holding potential. *A*: double pulses were delivered at a rate of 3–4/min. The recovery time was plotted as the percentage of the second current relative to the first, against the interval. The pulses were all of fixed duration (300 msec) to a constant membrane potential. The holding potentials were −100 mV (×), −80 mV (○), and −70 mV (●). *B*: examples of the records at the three holding potentials. Dashed lines were drawn at the level of the peak amplitude of the first current. With a 500 msec interval, the two currents are equal, when the holding potential is −100 mV (left). The second current is slightly smaller than the first at this interval when the membrane potential was held at −80 mV (right). When the membrane was held at −70 mV (bottom), the second current is smaller even at intervals of 600 and 700 msec.

outward tail on repolarization, and thus to less possible interference with $i_{s,i}$. Furthermore, if one assumes some K accumulation during the first of two pulses, then, at the holding potentials used (Fig. 5), steps to around 0 mV would lead to a more negative tail on repolarization, when the holding potential is more positive, as long as it is to the left of the crossover potential. This is at around −60 mV (Brown *et al.* 1980), for K levels of 2 (the bulk concentration) and 4 mM. For smaller amounts of accumulation the crossover point would be more negative but the effect on outward tails would then be negligible, and insufficient to produce the considerable slowing of repriming.

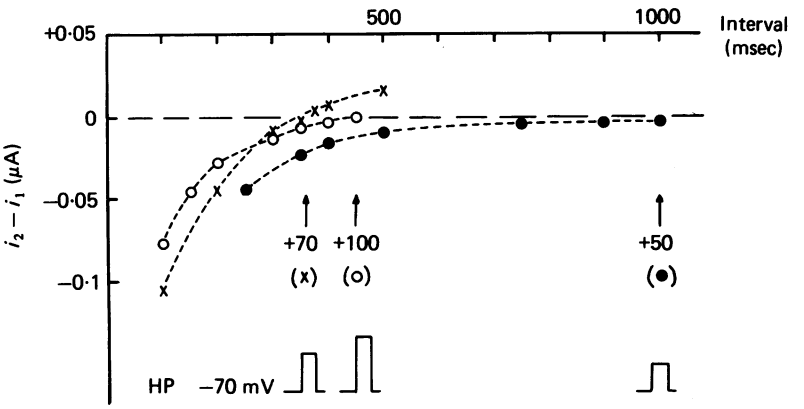

Fig. 6. The dependence of $i_{s,i}$ recovery time on the activating voltage. Twin pulses were given every 15 sec, from a holding potential of −70 mV, with a pulse duration of 300 msec. The difference between the second and the first current amplitude is plotted against the interval, for clamp pulses to three membrane potentials: −20 mV (50 mV pulse), ●; 0 mV (70 mV pulse), ×; +30 mV (100 mV pulses), ○. The intermediate pulse amplitude led to the fastest recovery time.

(e) The dependence of channel recovery on the activating voltage (constant holding potential)

In earlier work (Noble & Shimoni, 1981*b*) it was reported that very small prepulses, to membrane potentials below the threshold for activation of the Ca current, could augment subsequent currents flowing in response to test pulses. On the other hand, during repetitive stimulation with clamp pulses to moderately depolarized membrane potentials, which did activate the calcium current, a decrease in current amplitude was often observed (in contrast to the 'positive' staircase at larger depolarizations). One possible explanation for this apparent discrepancy was that the repriming process was dependent on the clamp pulse amplitude in a manner which would produce this effect.

This dependence was tested in this work, in a series of experiments which examined the recovery process when pulsing to different membrane potentials, from a constant holding potential. In eight preparations, there was a slow recovery time at the smallest and largest membrane potentials (all above the threshold of $i_{s,i}$), with the fastest recovery time occurring at the intermediate range, close to the plateau range of the action potential. Fig. 6 shows an example of this behaviour. The membrane

was held at −70 mV, and the recovery time was plotted as a function of the interval between two pulses, for different clamp amplitudes. In this Figure, the recovery was plotted as the difference between the second and the first current amplitude. At the intermediate potential (0 mV) the recovery was fastest. Fig. 7 (top) shows the time to full recovery as a function of the membrane potential (from a different preparation).

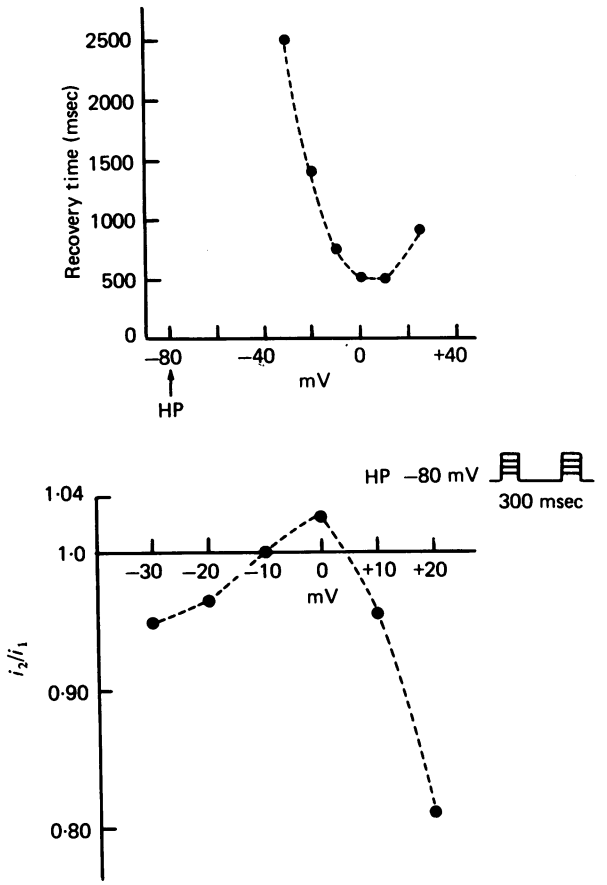

Fig. 7. Top: the full recovery time, at which the second of two current magnitudes is equal to the first, is plotted against the amplitude of the clamp pulse. The holding potential was constant, at −80 mV, as were the pulse duration (300 msec) and the frequency of stimulation (3–4/min). Bottom: for a fixed interpulse interval of 300 msec, the ratio of the second to the first current magnitude is plotted, as a function of the membrane potential during the activating pulses. Only the intermediate range of potentials shows a ratio of over 1, which indicates complete recovery. The holding potential was −80 mV.

The fastest repriming times were, as in Fig. 6, at the intermediate levels, with slower recovery for the small and very large depolarizations. Some fibres (three), however, did not show this U-shaped dependence, and only showed a gradual increase of recovery time with larger depolarization. This could be due to a higher resting level of intracellular Ca (see below and Discussion). Another way of demonstrating the

voltage dependence of the recovery process is to show the relationship between the second and first currents, for one constant interpulse interval, at different membrane potentials. Fig. 7 (bottom) shows that the recovery process is fastest for intermediate levels of depolarization. The dependence of repriming on the activating voltage in the more positive range could be due to increasing activation of outward currents and increasing accumulation, both of which could produce a similar retardation (see Methods). The enhancement of repriming at moderate depolarizing pulses could explain why the $i_{s,i}$ current 'staircase' can be negative for small pulses, if the interpulse interval is shorter than the time for complete recovery. Larger pulses with the same interval could produce positive 'staircases' due to the faster reactivation time.

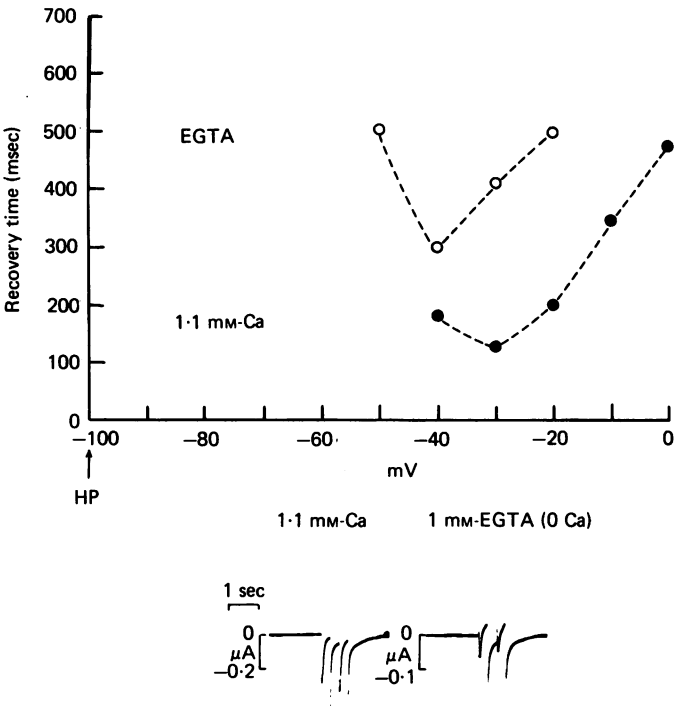

Fig. 8. The activating-voltage dependence of the $i_{s,i}$ recovery time as a function of extracellular Ca. The recovery time is plotted for different clamp pulse amplitudes (from a holding potential of −100 mV), in a normal Ringer solution, with 1·1 mM-Ca (●) and for a 0 Ca, 1 mM-EGTA Ringer solution (○). On the bottom are two pairs of current traces (from another fibre) obtained in response to two identical pulses, in the presence and absence of Ca. In 1·1 mM-Ca at this interpulse interval (200 msec) there is complete recovery (and augmentation), whereas in EGTA there is incomplete recovery.

(*f*) *The effects of altering extracellular Ca*

Since different degrees of depolarization alter the amount of Ca influx it could be argued that the current magnitude determines the repriming time. In earlier work (Noble & Shimoni, 1981*b*), the facilitatory process was shown to be voltage dependent in addition to and independently of the Ca influx. This dissociation is also true for

the channel repriming time. In the absence of extracellular Ca ions the same dependence of the recovery process on the activating voltage is maintained, as shown in Fig. 8. However, there is an upward shift of the curve relating to membrane potential, indicating a slowing of repriming on removal of Ca.

The converse is also true. The effect of increasing the Ca concentration was to produce an enhancement of the recovery time (this was also found in the mammalian heart by Kohlhardt *et al.* 1975). Such an effect can be seen in Fig. 9 which shows one out of ten such cases, in which the average enhancement was by 470 msec, ranging from 150 to 1000 msec. However, increases of the Ca level by more than moderate amounts, or prolonged exposure to moderate increases (such as 3 or 4 mM-Ca) tended to reduce or reverse the enhancement of recovery time.

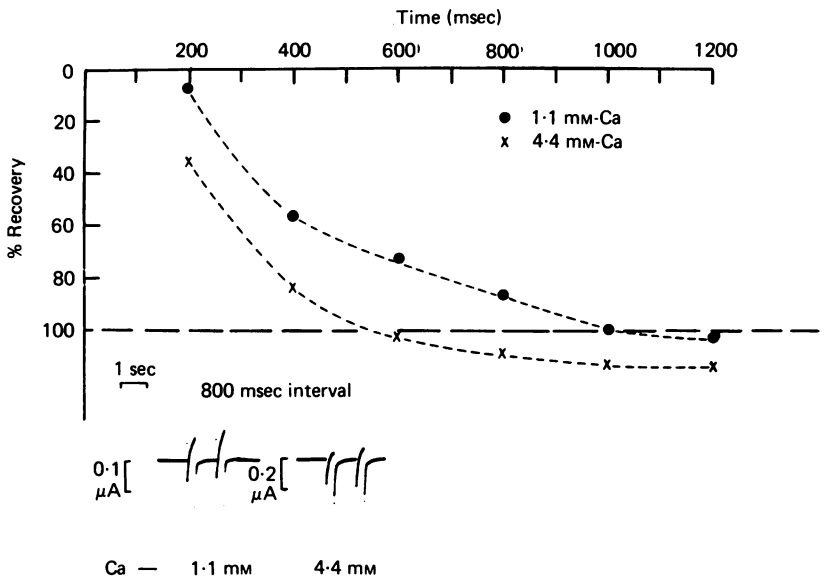

Fig. 9. The dependence of the slow channel recovery time on the level of extracellular Ca. The % of recovery of the second of two pulses (given 3–4 times a minute) is plotted against the inter-pulse interval. ● at 1·1 mM-Ca, × at 4·4 mM-Ca. At the bottom are current traces obtained during twin pulses (from another experiment), at one interval (800 msec). At 1·1 mM-Ca there is incomplete recovery, whereas at 4·4 mM-Ca the recovery is complete (and the net effect is an augmentation of the second current).

This supported the hypothesis that the repriming process might be sensitive to alterations in the intracellular medium (such as the accumulation of intracellular Ca which would be the result of a prolonged exposure to the higher Ca levels), in addition to extracellular factors, as shown by the effect of changing or removing Ca in the bathing medium.

This hypothesis was tested in two ways which are demonstrated in the remainder of this paper.

(*g*) *Na-free solutions*

Eight experiments were done in Na-free solutions, based on the fact that Na removal blocks the Na–Ca exchange mechanism, and leads to a build-up of intracellular Ca (Reuter & Seitz, 1968; Chapman, 1974; Mullins, 1979). Upon the replacement of most (75 or 90 %) or all of the Na ions by Li ions, the repriming process became considerably longer, with an average shift of the recovery time by 1115 msec (five

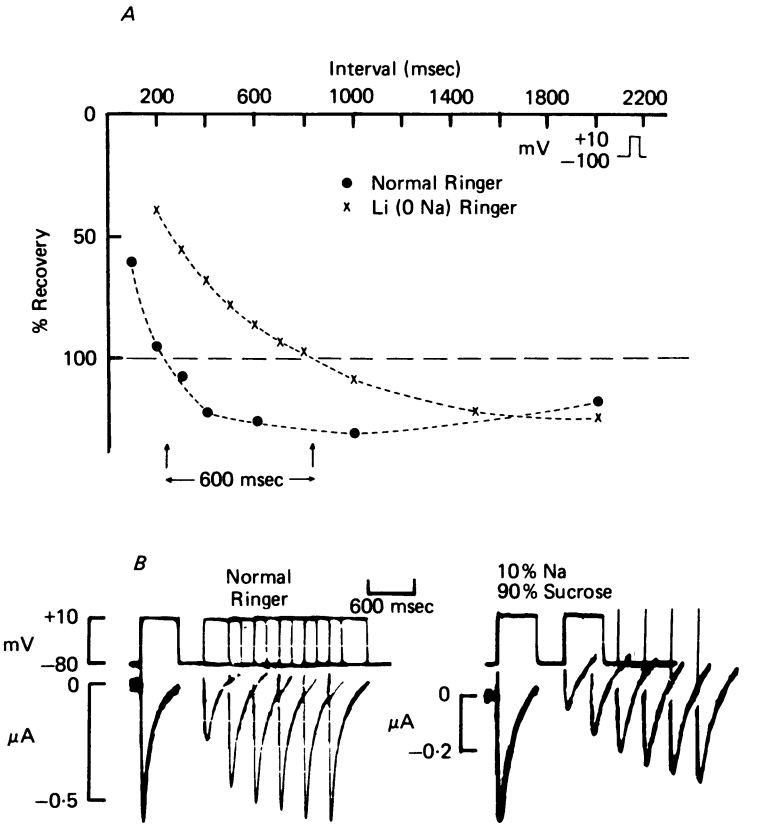

Fig. 10. The effect of Na removal on the recovery of the slow inward channel. *A:* double pulses were given at a rate of 4/min from a holding potential of −100 to −10 mV in normal, Na containing Ringer (○) and in a Na-free Ringer, containing 110 mM-LiCl (×). Plotted is the % of recovery of the second current amplitude, relative to the first, against the interval between the pulses. Na removal slowed the full recovery time by 600 msec in this preparation. *B:* an illustration of the effects of Na replacement by sucrose on the repriming of the slow inward current. The full recovery time in the low-sodium solution was over 3 sec.

experiments). Such an effect is seen in Fig 10 (top). In order to exclude a specific effect of Li ions, or the possibility that Ca is extruded in exchange for Li, three other substitution experiments were done, using sucrose instead of Li. Fig. 10 (bottom) shows that a Na-free, sucrose-Ringer solution produces a slowing of repriming, which is more marked than in Li-Ringer. The recovery time was longer than 3 sec in all three

cases. The common effect of Na removal, i.e. a raised intracellular Ca level, points to the crucial role of this parameter on the channel repriming process.

The next step was to see whether any other method of increasing intracellular Ca would have the same result.

(h) K-free solutions

Removal of extracellular K inhibits the Na-K pump in cardiac muscle (.e.g Goto, Tsuda & Yatani, 1977; Eisner & Lederer, 1979), and indirectly leads to the accumulation of Ca ions in the fibres. This indirect method of altering intracellular Ca levels was chosen also for the additional information provided as to the relation between Na–K pump blockage and the recovery process of the slow inward current.

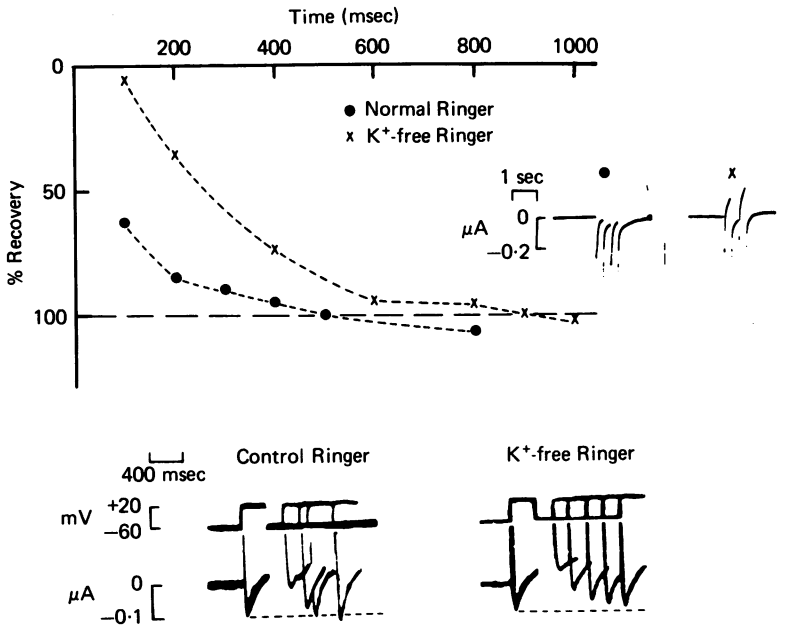

Fig. 11. The effects of K removal on the repriming of the slow inward channel. Top: a plot of the % of recovery of the second current amplitude in relation to the first, against the inter-pulse interval, in normal Ringer (○) and in K^+-free Ringer solution (×). The inset shows two double current traces, from another preparation, at an interval of 300 msec, in which full recovery was attained in the control solution, but not in the K^+ free one. Bottom: an illustration, from another preparation of the retardation of reactivation of the slow inward current in the absence of K ions.

The exposure of the preparation to K-free solutions led to a marked slowing of the recovery process, as illustrated in Fig. 11. In eleven fibres, the average slowing of the time to full recovery was 630 msec, ranging from 300 to 1500 msec (only one fibre showed no change). The plotting out of such a shift is shown in Fig. 11.

This retardation could be due in part to the activation of a larger amount of extra outward currents (larger tails) which would occur in K-free solutions. A larger accumulation would have two effects. On the one hand there would be a more negative (or less positive) tail at the end of the first 'conditioning' pulse which would counteract

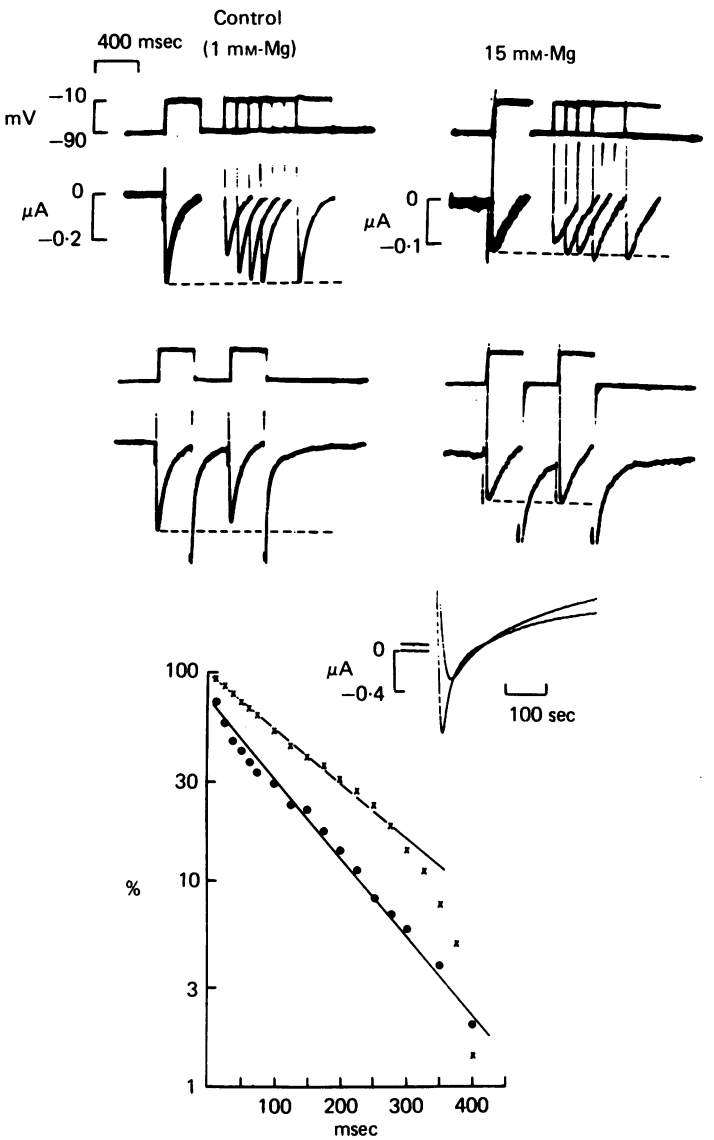

Fig. 12. The effects of increasing the Mg concentration on the repriming and inactivation of the slow inward current. *A*: the top panel shows a series of double pulses, from −90 to −10 mV, of 300 msec duration, given at varying intervals, in 1 mM-Mg Ringer (left) and in 15 mM-Mg Ringer (right). The higher Mg level speeds up the reactivation process, while at the same time slowing the inactivation (the time constants of inactivation in this case were 108 msec in the lower, and 142 msec in the higher Mg concentrations). The bottom panel shows two pairs of pulses separated by 300 msec. At this interval there is complete recovery in the 15 mM solution, but incomplete recovery in 1 mM Mg. *B*: the decay of $i_{s,i}$ from its peak amplitude in 1 mM (●) and 5 mM (×) Mg Ringer solutions (from another fibre). The pulses were longer in this case (500 msec), from −75 mV to +15 mV. The time constants of inactivation were 106 msec in 1 mM and 200 msec in 5 mM-Mg. The inset shows the superimposed current traces, with the smaller current being in the higher Mg level. There was a slight outward shift in the holding current level at 5 mM-Mg, as well as more activation of an outward current, as shown by the deviation from the simple exponential decay in higher Mg. In spite of this there was a faster reactivation in higher Mg in this fibre as well.

outward tails produced by activated outward currents. However, at the second voltage step, a larger amount of accumulation could produce a larger instantaneous outward 'jump', and thus a smaller measured $i_{s,1}$, as described in the Methods section.

As the elevation of extracellular Ca leads to the effect of speeding up the recovery process, the final set of experiments was addressed to the possibility that alterations of membrane surface charges could also affect the repriming process.

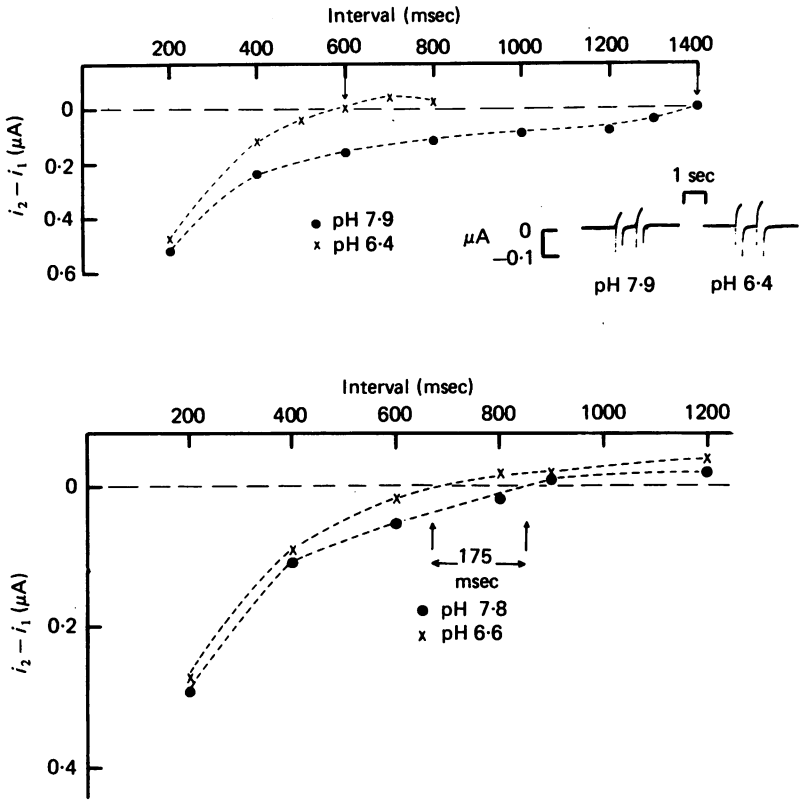

Fig. 13. The effects of lowering the pH on the $i_{s,1}$ recovery process. Double pulses of 300 msec duration were given every 15 sec, with varying intervals. The holding potential was −75 mV and the pulses were to −5 mV. The difference between the second and the first current amplitudes is plotted out as a function of the interval between the two pulses, for normal Ringer, at pH 7·9 and for a Ringer solution which had been titrated with HCl to a pH of 6·4 (crosses). The recovery is speeded up at the lower pH. On the bottom right are two double current traces from this experiment, with an interval of 600 msec. At the lower pH there is complete recovery, whereas at pH 7·9, the reactivation is not yet complete at this interval. On the bottom is another example from a different fibre.

(*i*) *The effects of changing magnesium and pH*

It was found that increasing the concentration of Mg ions in the bathing medium had the same effect as raising Ca, namely the enhancement of the recovery process. This is illustrated in Fig. 12, which shows one of seven such cases.

As also demonstrated in this Figure, increasing the level of Mg ions in the medium slows down the inactivation of the slow inward current. This is relevant to the

dissociation of inactivation of $i_{s,i}$ from its reactivation. A detailed study of the effects of elevated Mg on the f inactivation parameter of the channel was not done. However, whatever the mechanism by which f is affected, whether due to a shift in the activation curve, or to a direct effect on its magnitude, the dissociation must hold, since at a given membrane potential, inactivation is retarded and the reactivation is speeded up. The elevation of Ca ion concentration has the same effect on the two processes: both inactivation and the reactivation are speeded up (such an effect on the inactivation parameter has been reported by Kohlhardt *et al.* (1975) for the mammalian heart).

Reducing the pH of the bathing medium had similar effects on the reactivation process. This is presumed to result from a blocking effect of the additional protons on negative surface charges connected with the repriming of the slow inward channel, since the effect was very fast in onset. An example of such an enhancement of the the reactivation process is given in Fig. 13. Such an effect was seen in fifteen fibres, with different amounts of pH reduction. In lumping together nine of these preparations, in which the pH was reduced by either 1·5 or 2 pH units, the average enhancement of the recovery time was 280 msec, ranging from 50 to 900 msec. In several fibres the effects of reducing the pH were transient, with an initial enhancement followed by a retardation (transient effects of a reduced pH are also described by Brown & Noble, 1978). Obviously, changes in pH will have a variety of other effects (e.g. Deitmer & Ellis, 1980). In this case, the initial effect on repriming serves to illustrate the changes in repriming which are due (presumably) to changes in negative surface charges.

DISCUSSION

The results presented in this paper demonstrate the great sensitivity of the repriming process of the slow inward channel to a variety of experimental conditions. The precise method of monitoring this process can therefore greatly determine its time course, as was shown in Fig. 2. The fact that repetitive stimulation delays full recovery can be interpreted as resulting from an intracellular accumulation of Ca ions in the vicinity of the channel (which was also the mechanism proposed for explaining the current 'staircase effect' in Noble & Shimoni, 1981*a*). Such accumulation is probably responsible for the retardation of the recovery process in a variety of other experimental conditions, all of which have in common the alteration of intracellular Ca, e.g. Na removal or the blocking of the Na–K pump by potassium removal. The marked effect of changing the holding potential can also be interpreted along the same line of reasoning, in light of considerable evidence supporting the existence, in this preparation, of a powerful Na–Ca exchange mechanism (Horackova & Vassort, 1979; Chapman & Tunstall, 1980), which is voltage dependent. Thus, a depolarization of the holding potential would reduce this electrogenic exchange and lead to a rise in intracellular Ca (see also Mullins, 1979). This in turn would slow down the reactivation process.

The slowing of repriming in K-free solutions could also be due to an increased amount of accumulation, or increased outward current tails due to a larger activation of outward currents. However, in view of all the other evidence presented, the likely effect on repriming is thought to be due to the changes in the internal Ca level.

The secondary, late retardation of reactivation after a prolonged exposure to high-Ca Ringer solutions also supports the role of intracellular Ca. The initial effect of these solutions, together with the effect of elevated magnesium and a reduced pH, points to some effect due to a blocking of negative surface charges in the vicinity of the channel. Preliminary experiments with salicylate ions, which are presumed to increase the free surface charges on the membrane (Cohen, Noble, Ohba & Ojeda, 1979), showed a retardation of the recovery process, as opposed to the enhancing reported here by agents which block surface charges.

The implications of these results are that any factor which would depolarize the membrane or change the intracellular Ca level (or block surface charges) would affect the 'responsiveness' of the Ca channel. This could affect the tension produced by atrial or ventricular tissue, and also the refractoriness and conduction velocity of nodal tissue. A slower channel recovery time in nodal tissue (or depolarized ventricle) would entail a lower frequency of activity. Above a certain frequency of activity, insufficient channel recovery would reduce the Ca influx, and thus the action potential upstroke. The conduction time would thus also be affected.

The changes described in channel recovery time could thus be contributory to arrhythmic disorders, by either limiting the rate of activity or by changing the rate of spread of activation. Such changes could then also be one perspective of assessing anti-arrhythmic drugs (e.g. as suggested in Ehara & Kaufmann, 1978, and in personal communication to D. Noble).

Consideration was given to recent evidence (e.g. Tillotson, 1979) pointing to a Ca entry-dependent inactivation of the Ca conductance, rather than a voltage dependent inactivation of the channel. If an increased current also increasingly inactivates the channel, one could argue that such a mechanism also underlies the changes in channel repriming.

However, several of the findings presented here seem to rule out such a mechanism in the frog atrium. Changing the holding potential (Fig. 5) produced a marked slowing of recovery, for the *same* magnitude of $i_{s,i}$. The dependence on the activating voltages (Figs 6 and 7) is also the opposite of that described by Tillotson, 1979 (see his Fig. 2*A*). In this preparation, for larger currents (at intermediate membrane potentials) the repriming is faster, so that for a given, short interval, there would be more current in response to the second pulse following the *larger* initial currents, and less current available at the time of the second pulse, for the smallest and largest voltage steps (where the initial influx was smaller).

Furthermore, increasing Ca or Mg levels augments and depresses, respectively, the slow inward current, yet both procedures speed up the channel recovery time.

Repetitive stimulation should increase the calcium level near the channel, inside the membrane, and on Tillotson's model, give the same effect as an increase in the calcium level, by enhancing inactivation of the channel. However, in this preparation, repetitive stimulation has the opposite (slowing) effect on channel repriming to the enhancing effect of increasing Ca.

Finally, the large variability of recovery times at 'normal' conditions might result from different amounts of Ca loading of the trabeculae. Different degrees of Ca overload have also been implicated in describing other effects, such as the inotropic

effects of glycosides in Purkinje fibres (Earm, Hart, Noble & Shimoni, 1980). Different degrees of Ca overload could explain why some fibres might show only a slowing of the recovery process with increasing depolarizing clamp pulses. If some fibres have a high 'resting' level, the recovery process would be slow at all membrane potentials, and the enhancement seen at intermediate potentials (Figs 6 and 7) might be masked.

Since it is suggested that changes in intracellular Ca levels are of crucial importance in determining the repriming time it cannot be excluded that using a double sucrose gap, which leads to a Ca ion build up in the sucrose regions, could affect the level of Ca in the test region by lateral diffusion. This would be an additional reason for the large scatter of 'control' repriming times. The effects reported here, however, are of quite a considerable magnitude, and are present at both short or long 'basic' repriming times. It is thus assumed that all the interfering influences such as outward current tails and K levels have only some quantitative modifying effect (the order of magnitude being 10–15%, as described in the Methods section) but do not change the basic findings.

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