Journal of Physiology (1989), **409**, *pp.* 121–141
With 14 *text-figures*
Printed in Great Britain

MODULATION BY INTRACELLULAR Ca^{2+} OF THE HYPERPOLARIZATION-ACTIVATED INWARD CURRENT IN RABBIT SINGLE SINO-ATRIAL NODE CELLS

BY NOBUHISA HAGIWARA AND HIROSHI IRISAWA

From the National Institute for Physiological Sciences, Myodaiji, Okazaki 444, Japan

(*Received 30 March 1988*)

SUMMARY

1. The sensitivity to internal Ca^{2+} of the hyperpolarization-activated inward current (I_h or I_f) in rabbit single sino-atrial node cells was investigated by the whole-cell voltage-clamp method.

2. When the patch pipette contained an internal solution of pCa 10, the amplitude of I_f decreased by $74{\cdot}8 \pm 3{\cdot}3\%$ in 10 min ($n = 7$) after rupture of the patch membrane. When the pipette contained an internal solution of pCa 7, I_f increased by $43{\cdot}7 \pm 8{\cdot}7\%$ within 10 min ($n = 5$).

3. Increase of I_f by the higher Ca^{2+} internal solution was confirmed in the same cell using the cell dialysis method. Both I_f and its tail current were increased at every membrane potential. The amplitude of I_f increased most markedly between pCa 8 and 7.

4. The reversal potential and kinetics of I_f were unaffected by the internal Ca^{2+} concentration. Increase of I_f by the high internal Ca^{2+} concentration was sensitively blocked by Cs^+. These findings confirm that the increased current is indeed I_f and not a newly activated I_f-like current due to elevation of internal Ca^{2+}.

5. The activation curve of I_f shifted approximately 13 mV in a positive direction by elevating Ca^{2+} from pCa 10 to 7 ($n = 21$), indicating that the voltage dependence of I_f was modulated by internal Ca^{2+}.

6. β-Agonists also modulated I_f, but the underlying mechanisms of their effects on I_f differed from those of the internal Ca^{2+}. The former affected the I_f kinetics rather than its amplitude, whereas the latter acted on the I_f conductance rather than on its kinetics.

7. The increase in I_f by the internal Ca^{2+} was unaffected by protein kinase inhibitor or calmodulin inhibitor, suggesting that the internal Ca^{2+} directly modulates I_f.

8. When the patch pipette contained pCa 7 internal solution, the maximum diastolic potential shifted towards a positive potential but the heart rate remained almost constant.

INTRODUCTION

Since the hyperolarization-activated inward current (I_f or I_h) in mammalian sino-atrial node cells is activated when the membrane is hyperpolarized, I_f can depolarize the membrane during the pacemaker potential (Brown, DiFrancesco & Noble, 1979; Yanagihara & Irisawa, 1980). We have reported, however, that I_f may not contribute much to the cycle-to-cycle pacemaker potential, since the time course of activation of I_f is too slow to account for the high frequency of the primary pacemaker and the threshold of activation is more negative than the maximum diastolic potential (Yanagihara & Irisawa, 1980; Noma, Morad & Irisawa, 1983). Another finding which supports this concept is that Cs^+, a blocker of I_f, does not exert a strong effect on the spontaneous heart rate of the nodal cell (Brown, DiFrancesco, Kimura & Noble, 1981; Noma *et al.* 1983).

On the other hand, van Ginnecken & Giles (1985) found that the threshold potential of activation lies more positive than -60 mV. They also noted that the hyperpolarizing current on the pacemaker potential is much more effective in stopping the spontaneous activity when Cs^+ is present in the superfusate. DiFrancesco, Ferroni, Mazzanti & Tromba (1986) found that the threshold potential of activation ranged between -40 and -70 mV. These observations suggest that there are still unresolved questions regarding the contribution of I_f to the pacemaker depolarization of the sino-atrial node cell.

In the present study, we investigated the possible influence of internal Ca^{2+} concentration on single pacemaker cells isolated from sino-atrial node cells of the rabbit by the whole-cell patch-clamp method. The results indicated that both internal Ca^{2+} and β-agonist significantly modulate the size and the activation threshold of I_f with different mechanisms of action. A preliminary report of this work has been published previously (Hagiwara, Ho & Irisawa, 1987).

METHODS

Cell isolation and recording

Single cells were isolated from the sino-atrial node region of albino rabbits (1–1·5 kg) according to the chopping method of DiFrancesco, Ferroni, Mazzanti & Tromba (1986). The method of cell isolation was the same already described by Hagiwara, Irisawa & Kameyama (1988). We employed small round or spherical cells which continued to beat spontaneously in the Tyrode solution for at least 1 h, and stopped their spontaneous contraction following the administration of 0·1 μM-acetylcholine. The size of the nodel cells was approximately 15×20 μm and the membrane capacitance ranged from 27 to 40 pF.

The action potentials and membrane currents were measured using methods similar to those described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). The diameters of the glass pipettes were approximately 2–5 μm and the resistances were in the range of 2–5 MΩ. We corrected the liquid junction potential (-10 mV) between the pipette solution and the bath solution for all the data. In order to compare the effect of intracellular Ca^{2+} concentration on the I_f within one cell, the cell dialysis method as described by Soejima & Noma (1984) was employed. The bath solution was grounded through a 3 M-KCl–agar/Ag–AgCl bridge and the pipette potential was clamped at various levels. Current and voltage signals were stored on a video recorder (Victor, BR-6400) using a PCM converter system (NF Electronic Circuit Design, RP-880) for computer analysis (NEC 98 series). All statistical data were expressed as the mean $\pm$ S.D. All experiments were performed at temperatures of 36·5–37 °C.

Solutions

Superfusates. The Tyrode solution contained (in mM): NaCl, 136·9; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·33; glucose, 5; HEPES, 5; and the pH was adjusted to 7·4 with NaOH. When the tail current of I_f was measured, the bath solution contained 3 mM-$NiCl_2$ to block the calcium current and the Na^+–Ca^{2+} exchange current (Kimura, Miyamae & Noma, 1987) and 3 mM-$BaCl_2$ solution to block the delayed outward K^+ current.

Internal solutions. The pipette contained (in mM): KOH, 110; KCl, 20; $MgCl_2$ 1; ATP, 5; creatine phosphate, 5; aspartic acid, 90–100; EGTA, 10; HEPES, 5; and the pH was adjusted to 7·4. The concentration of Ca^{2+} in the pipette solution was buffered using 10 mM-EGTA according to the calculation used by Fabiato & Fabiato (1979) with the correction of Tsien & Rink (1980). Solution of pCa 7 was prepared by mixing 10 mM-EGTA and 6·25 mM-Ca^{2+}. The pCa values in this experiment were those of the pipette solution and might be shifted by other intracellular calcium buffers. Since the true intracellular pCa of the sino-atrial node cell is not known, we simply used the pCa of the pipette solution as a first approximation.

Drugs

Isoprenaline (Sigma) was dissolved in distilled water as a stock solution (1 mM) containing 1 mM-ascorbic acid. Forskolin (Sigma) was dissolved in ethanol as a 10 mM stock solution. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA, Sigma) was dissolved in dimethylsulphoxide as a 1 mM stock solution. 1-(5-Isoquinolylsulphonyl)-2-methylpiperazine dihydrochloride (H-7, Seikagaku Kogyo) was dissolved in distilled water as a 10 mM stock solution. 1-(bis(4-Chlorophenyl)-methyl)-3-(2-(2,4-dichlorophenyl))-2-((2,4-dichlorophenyl)-methoxyl)-ethyl-1H-imidazolium chloride (calmidazolium, compound R24571, Sigma) was dissolved in dimethylsulphoxide as a 1 mM stock solution. The stock solutions were diluted by the perfusate to a given concentration. Adenosine 3′,5′-cyclic monophosphate (cyclic AMP, Seikagaku Kogyo) was dissolved in the internal solution.

RESULTS

Dependence of the amplitude of hyperpolarization-induced inward current on the intracellular Ca^{2+} concentration

To change the intracellular Ca^{2+} concentration, we employed the following two different methods: (1) the simple diffusion method, which allows Ca^{2+} to diffuse into the cell interior from the patch pipette, after disruption of the patch membrane; and (2) the internal perfusion method, which perfuses the patch pipette continuously with various concentrations of Ca^{2+}. The latter method allows two or three different intrapipette Ca^{2+} concentrations to be analysed successively in one cell.

Figure 1*A*1 illustrates the time course of the decrease in hyperpolarization-induced inward current using the former method. The patch pipette in this example contained pCa 10 solution. At time zero, the whole-cell-clamp mode was established, and inward current was elicited by the hyperpolarization clamp pulse to −80 mV from a holding potential of −30 mV. The amplitude of inward current was 820 pA in this example, at the end of a 3 s pulse (control in Fig. 1*B*1). It decreased progressively by 73% of the control value (74·8 ± 3·3%, $n = 7$) as evaluated by the application of constant hyperpolarizing pulses, at 10 min after disruption of the membrane. Figure 1*B*1 gives six superimposed consecutive traces after the start of exchange of Ca^{2+} and buffer anions between pipette and cell. The time constant of the activation in the control (0·72 s) and that at 15 min after diffusion of the low-Ca^{2+} internal solution (0·73 s) was unchanged, suggesting that the kinetics of the current system was not significantly affected.

When we applied pCa 7 solution to the interior of the patch pipette, an opposite

effect was observed (Fig. 1*A*2). At time zero, the amplitude of inward current was also 820 pA in this cell, which increased progressively during the following 6 min to 1·2 nA (43·7 ± 8·7 %, $n = 5$). The time constant of the activation of inward current was unchanged (0·74 and 0·73 s). In three other examples with pCa 7 internal solution, we failed to observe such an increase in inward current. In these examples,

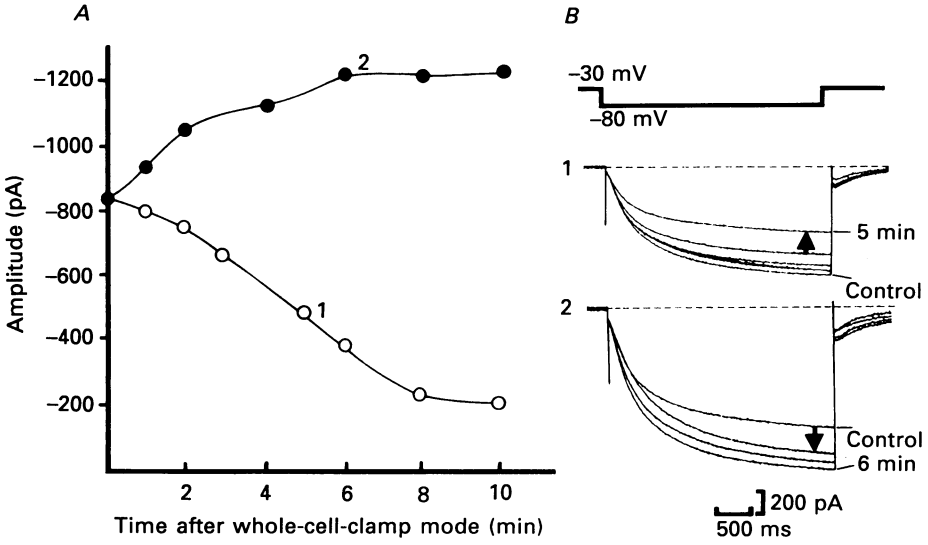

Fig. 1. Changes of I_f amplitude after the start of exchange of Ca^{2+} and buffer anions between pipette and cell. *A*1 and *B*1, the patch pipette contained pCa 10 solution. I_f was elicited by a 3 s pulse from a holding potential of −30 mV to −80 mV (*B*1, bottom trace) at every 30 s. At time zero, the amplitude of I_f was 820 pA and it decreased progressively during the following 10 min to 220 pA. *B*1 illustrates the current records at 0 (control), 1, 1·5, 2, 3 and 5 min. The interrupted line is the zero-current level. The arrows show the direction of the current change. *A*2 and *B*2, the patch pipette contained pCa 7 solution. The experimental protocol was the same as in *A*1 and *B*1, and the symbols also are as in *A*1 and *B*1. At time zero, the amplitude of I_f was 820 pA and it increased to 1·2 nA during the following 10 min. Current records at 0 (control), 2, 4, 6 min are shown in *B*2. 1 and 2 were taken from different cells.

the amplitude of tail current at −110 to 20 mV was 20·82 ± 2·31 pA/pF ($n = 3$), which was near to the maximum current (see Fig. 6*A*), suggesting that cell Ca^{2+} was very close to pipette Ca^{2+} when the membrane was ruptured.

The results shown in Fig. 1 suggested that the amplitude of hyperpolarization-induced inward current is dependent on the concentration of intracellular Ca^{2+}.

Properties of the hyperpolarization-activated current

Although the pattern of hyperpolarization-induced inward current shown above appeared to resemble those described previously by various authors, it is necessary to ascertain whether this current is the newly activated Ca^{2+}-sensitive current or a modulation of I_f by Ca^{2+}. To investigate this point, we compared the reversal potentials at pCa 10 and 7. Figure 2 shows five superimposed current traces, where

constant hyperpolarizing pulses to −110 mV from a holding potential of −30 mV were applied and clamped back to various test voltages between −30 and 0 mV. A marked difference in the amplitude of inward current at pCa 10 (Fig. 2*A*) and 7 (Fig. 2B) was observed, but the reversal potential in *A* and *B* was −15 mV, which was similar to those described in previous reports (−24 mV, van Ginnecken & Giles, 1985; −15 to −18 mV, DiFrancesco *et al.* 1986; −24 mV, Nathan, 1986).

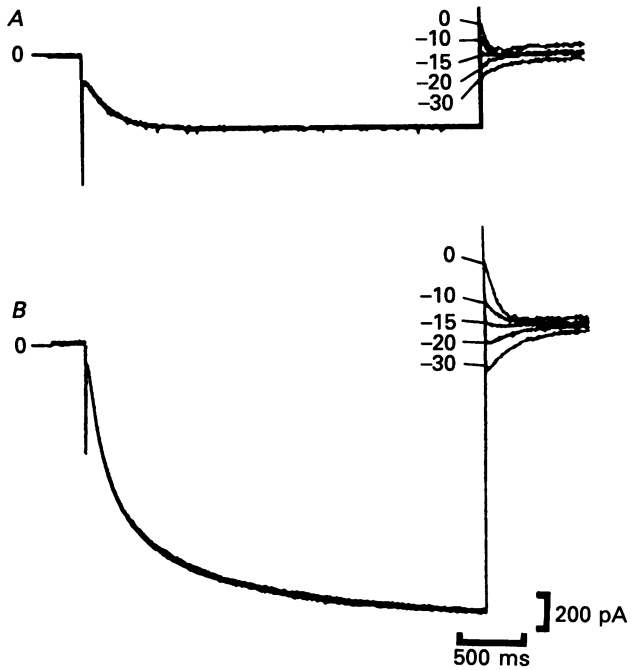

Fig. 2. Reversal potential of I_f measured at the low (*A*) and high (*B*) internal Ca^{2+} concentrations. Five superimposed current records of I_f are shown. The holding potential was −30 mV, and hyperpolarizing pulses to −110 mV were applied constantly and clamped back to five test pulses between −30 and 0 mV. The reversal potentials in both pCa 10 (*A*) and pCa 7 (*B*) are equal (−15 mV), indicating that the currents developed at pCa 10 and 7 are the same class of ionic current.

The time courses of activation of inward current and the envelope of deactivation of this current were fitted well by a single exponential. At −70 mV, the time constant of activation was 1·02 s, and that of deactivation was 1·00 s (Fig. 3*A*1). At −100 mV, the values were 0·46 and 0·42 s, respectively (Fig. 3*B* and *C*). These values are not much different from those given previously (Yanagihara & Irisawa, 1980; van Ginnecken & Giles, 1985).

We further examined whether the current activated at pCa 7 was inhibited by Cs^+, since Cs^+ is known to block I_f. Figure 4*A a* presents a family of control voltage-clamp records of inward current obtained under the conditions of pCa 7. After superfusing Cs^+ (Fig. 4*A* 2), inward current was blocked markedly.

The experiments described above showed that the reversal potential and kinetics

of the inward current recorded at pCa 7 were similar to those measured at pCa 10 and the sensitivity of Cs^+ was similar to the I_f described previously. The inward current described above is unlikely to be a current component of the kind shown by Colquhoun, Neher, Reuter & Stevens (1981) and Ehara, Noma & Ono (1988). Since we superfused 3 mM-Ni^{2+}, contribution of the Na^+–Ca^{2+} exchange current during

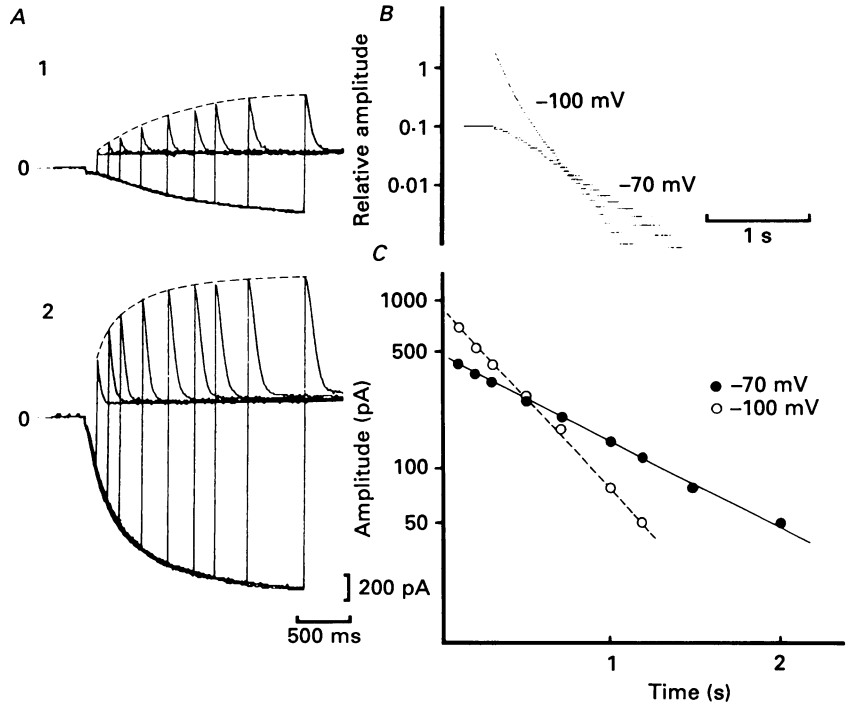

Fig. 3. Kinetic properties of I_f at the high internal Ca^{2+} concentration. *A*, I_f was activated by hyperpolarizing pulses from a holding potential of −30 mV to −70 mV (*A*1) and to −100 mV (*A*2), and was deactivated at the clamp pulse to 20 mV at various intervals. Nine traces are superimposed. The time course of activation and that of the envelope curves of deactivation (interrupted line) showed a close similarity. *B*, time courses of activation of I_f at −70 and −100 mV are fitted single-exponentially, with a time constant of 1·02 and 0·46 s, respectively. *C*, time courses of deactivation of I_f at −70 mV (●) and −100 mV (○), with a time constant of 1·00 and 0·42 s, respectively.

hyperpolarization which would result in an apparent increase in the amplitude of I_f is also unlikely. We conclude therefore that the current recorded at the high Ca^{2+} concentration was not a newly activated I_f-like current due to elevation of the intracellular Ca^{2+} concentration, but that I_f is modulated by alternation of the internal Ca^{2+} concentration.

Increased amplitude of I_f by elevation of the intracellular Ca^{2+} concentration

The above experiments were carried out on different cells and cell-to-cell variations must be taken into account. To evaluate the above results in one cell, we

dialysed the cell and measured the current change after exchanging the pipette solution with various Ca^{2+} solutions. The patch pipette was perfused at first with pCa 10 solution and the current–voltage relation of I_f was examined (Fig. 5*A*1). The pipette solution was then changed to pCa 7 solution, and 10 min later the current–voltage relations were again investigated (Fig. 5*A*2). The amplitude of I_f at

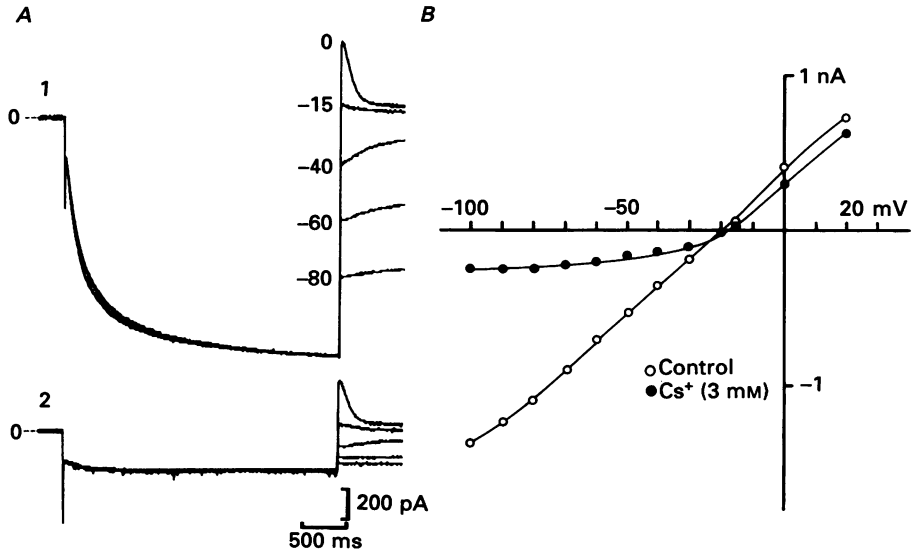

Fig. 4. Cs^+ blocks I_f at high internal Ca^{2+}. *A*1, I_f was fully activated by constant hyperpolarizing pulses to −110 mV for 3 s from a holding potential of −30 mV and deactivated to various potentials between −80 and 0 mV at pCa 7 in the pipette solution. *A*2, at 5 min after application of 3 mM-Cs^+ in the external solution, I_f was significantly inhibited, voltage dependently. The voltage-clamp protocol was the same as in *A*1. *B*, fully activated current–voltage relations in the control solution and after application of 3 mM-Cs^+. The initial amplitudes of the I_f tail currents were measured after depolarizing steps from −110 mV to various potentials between −100 and 20 mV, and were plotted against the membrane potentials, in the control solution (○) and in the presence of 3 mM-Cs^+ (●).

−110 mV increased by 84% and the tail current at 20 mV increased by 82%. The current–voltage relation indicated that I_f increased at every membrane potential (Fig. 5*B*). The threshold of activation of I_f at pCa 10 was −60 mV, but it was −40 mV at pCa 7 (Fig. 5*b*). The increase in the tail current was 102 ± 23% ($n = 3$) when measured at 20 mV.

All these experimental findings were consistent with the observation that the current I_f is modulated by a physiological concentration of internal Ca^{2+}.

Relation between the intracellular Ca^{2+} concentration and the amplitude of the I_f tail current

The dose–response relations between the amplitude of the tail current of I_f and pipette Ca^{2+} concentration measured in twenty-eight different cells are shown in

Fig. 6*A*. The increase in I_f between pCa 8 and 7 (99·7%) was much larger than that between pCa 10 and 8 (18·5%). The amplitude of I_f increased further when the pipette contained pCa 6. These data suggest that I_f is sensitively changed by the known physiological concentration ranges of internal Ca^{2+} in cardiac cells (Cobbold & Bourne, 1984; Marban, Kitakaze, Kusuoka, Porterfield, Yue & Chacko, 1987; Wier, Cannell, Berlin, Marban & Lederer, 1987).

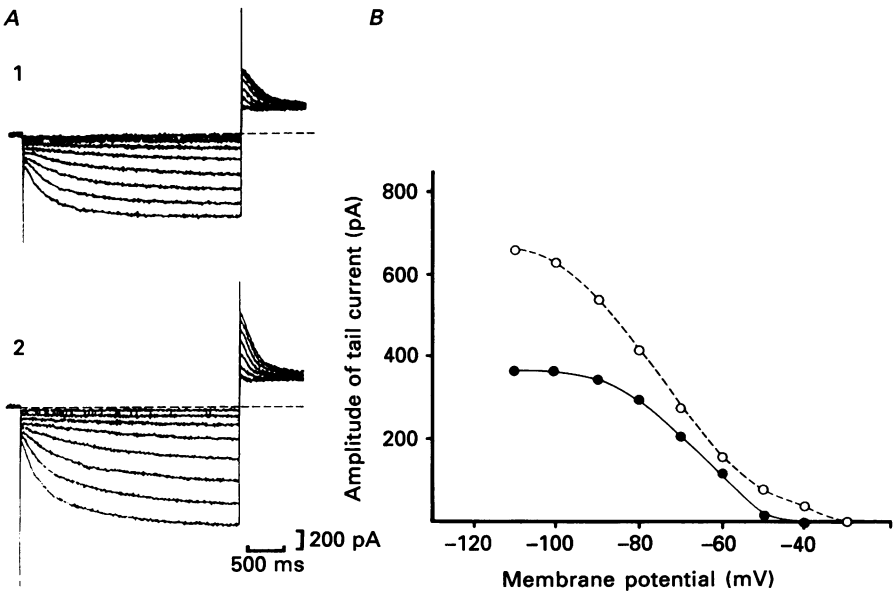

Fig. 5. Effect of increasing the Ca^{2+} concentration in the pipette from pCa 10 to 7 on I_f by using the internal perfusion method. *A*, I_f recorded with eight hyperpolarizing pulses between −40 and −110 mV for 3 s at 10 mV steps from a holding potential of −30 mV and clamped back to 20 mV. *A*1 was obtained at pCa 10 and *A*2 at pCa 7 in the pipette. The I_f at −110 mV and its tail current at 20 mV were increased by 84 and 82% and increased equally at every potential examined. The interrupted line indicates the zero-current level. *B*, amplitudes of the I_f tail current obtained at pCa 10 (continuous line) and at pCa 7 (interrupted line) at various membrane potentials. I_f increased at every membrane potential, and the threshold of activation at pCa 7 shifted positively by approximately 20 mV in comparison with that at pCa 10.

Figure 6*B* shows the activation curve of I_f at two different levels of internal Ca^{2+}. The amplitude of the tail current was normalized and plotted against the membrane potentials during hyperpolarization in twenty-one different cells. At pCa 10, I_f was activated at −60 mV as judged from the deactivation current, but at pCa 7 it was activated at −40 mV (inset to Fig. 6*B*), indicating that the threshold of activation of I_f was also affected by increase of the internal Ca^{2+}. The activation curves were fitted to the following equation:

$$q_\infty = \{1+\exp((V_m - V_{0.5})/s)\}^{-1}, \quad (1)$$

where q_∞ is the activation parameter, V_m is the membrane potential, $V_{0.5}$ is the potential to give a half-value and s is the slope factor which determines the steepness

of the curve. Under the conditions of pCa 10, s was 9·8 mV and $V_{0.5}$ was −75·1 mV, while for pCa 7, s was 10·6 mV and $V_{0.5}$ was −62·5 mV. The threshold of activation shifted approximately 13 mV towards a positive potential, suggesting that at the higher internal Ca^{2+} concentration, I_f could be activated in the physiological pacemaker potential range. The shift of the activation curve seems unlikely to be due to the surface charge on the internal side of the membrane, since reduction of the surface charge should shift the curve towards the negative direction.

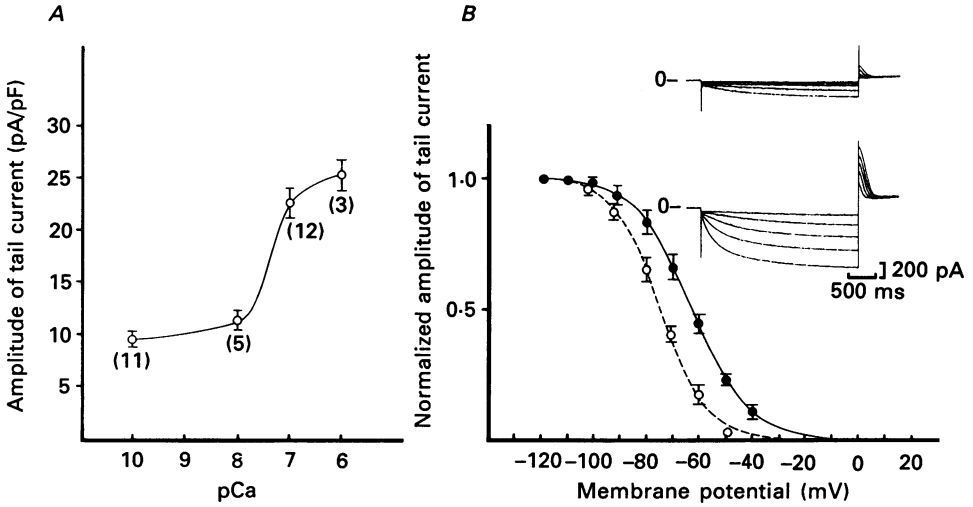

Fig. 6. Effect of internal Ca^{2+} on the amplitude and steady-state activation of I_f. *A*, dose–response relation of the amplitude of I_f. The tail amplitudes of I_f are plotted against four different Ca^{2+} concentrations within the pipette in twenty-eight different cells. I_f was fully activated at −110 mV and the tail current was recorded at 20 mV. The numerals in parentheses below the curve indicate the number of experiments. I_f increased 99·7% between pCa 8 and 7, but it increased only 18·5% between PCa 10 and 8. The curve was fitted by eye. *B*, effects of internal Ca^{2+} on the activation curve of I_f. The normalized amplitudes of the I_f tail current in twenty-one different cells are plotted against various membrane potentials. The interrupted (pCa 10) and continuous (pCa 7) curves were drawn according to eqn (1) in the text. The activation curve shifted approximately 13 mV in a positive direction at pCa 7. The inset shows the I_f recorded by hyperpolarizing pulses between −40 and −80 mV at pCa 10 (above) and pCa 7 (below) in the pipette solution.

Effect of β-agonist on I_f

It has been found that β-agonist increases the amplitude of I_f (Brown *et al.* 1979) and shifts the steady-state activation curve to a more positive potential (Tsien, 1974; Callewaert, Carmeliet & Vereecke, 1984). To evalute whether the mechanism of action of β-agonist is similar to that of internal Ca^{2+}, as described above, we investigated the effects of β-agonist on I_f, at both the low and high internal Ca^{2+} concentrations. The amplitude of I_f was compared before (Fig. 7*A*1) and after (Fig. 7*A*2) application of 1 μM-isoprenaline to the superfusate, using a patch pipette containing solution of pCa 10 (Fig. 7*A*) or pCa 7 (Fig. 7*B*). The isoprenaline

increased the amplitude of I_f under both pipette conditions (pCa 10, 130±5%, $n = 3$; pCa 7, 129±14%, $n = 5$).

Isoprenaline is known to activate adenylate cyclase and to enhance the transfer of ATP to cyclic AMP, which then activates the cyclic AMP-dependent protein kinase (PKA) to phosphorylate the calcium channel (Kameyama, Hofmann & Trautwein,

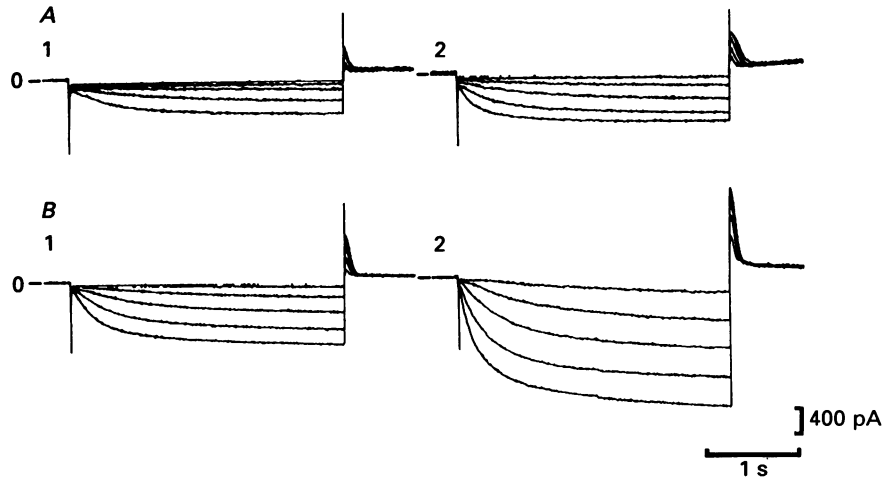

Fig. 7. Effect of isoprenaline on I_f. *A*, the patch pipette contained pCa 10, both in the control (*A*1) and in the presence of 1 μM-isoprenaline (*A*2). I_f was activated by hyperpolarizing pulses between −50 and −90 mV, and other aspects of the experimental protocol were the same as in Fig. 5. The amplitude of I_f increased at every membrane potential, and the time course of activation became faster in the presence of isoprenaline. *B*, the patch pipette contained pCa 7, both in the control (*B*1) and in the presence of 1 μM-isoprenaline (*B*2). Clearly, I_f increased similarly at both pCa 10 and 7 (131 and 125%, respectively) at −70 mV.

1985). To determine whether the effects of isoprenaline on I_f were also related to activation of adenylate cyclase, we superfused forskolin at either pCa 10 (Fig. 8*A*) or pCa 7 (Fig. 8*B*) in the pipette. Figure 8*A*1 shows the current trace at a slow sweep speed, while Fig. 8*A*2 shows those at a rapid sweep speed, before (*a*) and after (*b*) the application of 10 μM-forskolin. The amplitude of I_f upon hyperpolarization at −70 mV increased by 133% with pCa 10 solution in the pipette (128±11%, $n = 3$), and at pCa 7 I_f increased by 126±9% ($n = 3$). The amplitude of I_f recovered to the control level within 6–7 min after washing out the forskolin (Fig. 8*A* 3). The above results indicate that the effect of 10 μM-forskolin on I_f is similar to that of 1 μM-isoprenaline.

To obtain further confirmation of the contribution of second messengers to I_f activation, we measured I_f before and after intracellular application of cyclic AMP using the cell dialysis method. The patch pipette was perfused at first with Ca^{2+}-free, cyclic AMP-free solution (Fig. 9*A a*). Then when 50 μM-cyclic AMP-containing solution was perfused into the pipette, the amplitude of I_f increased progressively within 1 min, and remained unchanged during the following 10 min (Fig. 9*A b*). The

amplitude of I_f declined to its control level after washing out the cyclic AMP from the pipette (Fig. 9*A* c). We superfused 10 μM-forskolin to compare the effects of forskolin with those of cyclic AMP. The increase in I_f amplitude was broadly similar (Fig. 9*A b* and *d*). The current–voltage relationships were evaluated in the control (Fig. 9*B*1), during cell dialysis with cyclic AMP (Fig. 9*B*2), during recovery from

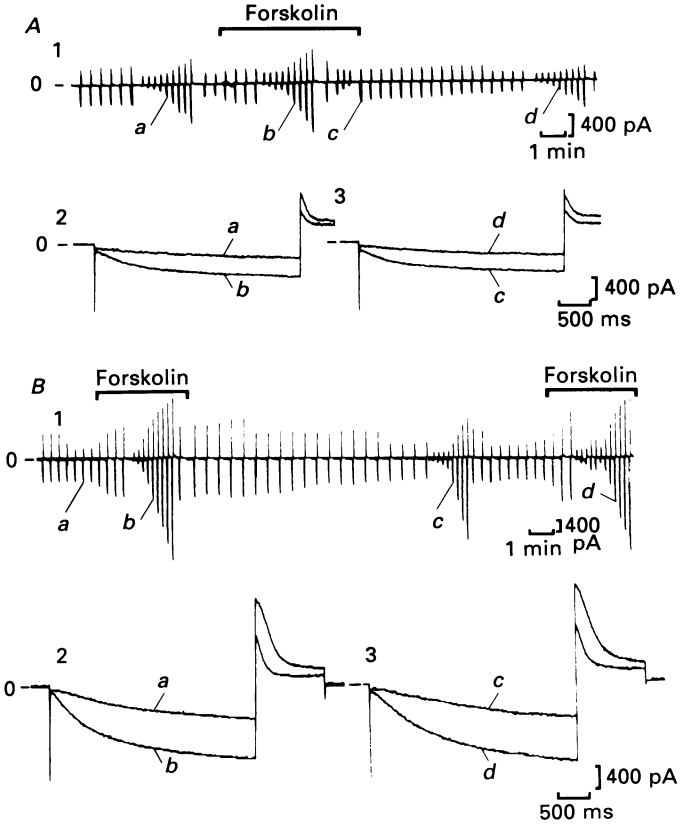

Fig. 8. Effect of forskolin on I_f at the two different internal Ca^{2+} concentrations. The pipette contained pCa 10 solution in *A*, and pCa 7 solution in *B*. *A*1 and *B*1 illustrate the current traces at a slow sweep speed, while those in *A*2 and *A*3 and *B*2 and *B*3 were at a rapid sweep speed, corresponding to *a–d* in *A*1 and *B*1, respectively. I_f was increased by 10 μM-forskolin similarly at both pCa 10 and pCa 7 (*A*2*b* and *B*2*b*). I_f returned to the control level within 6–7 min (*A*3*d* and *B*3*c*). After 15 min of recovery, application of 10 μM-forskolin yielded a similar response (*B*3*d*).

cyclic AMP application (Fig. 9*B*3*c*) and following the administration of 10 μM-forskolin (Fig. 9*B* 3*d*). In the example shown in Fig. 9*B*, the amplitude of I_f increased by 141 % at −70 mV (134 ± 6 %, $n = 3$). On the other hand, I_f was unchanged when we applied 50 μM-cyclic AMP to the pipette under the influence of 10 μM-forskolin (Fig. 9*Bd* and *e*), suggesting that I_f was almost saturated in response to those two agents. It can be concluded that β-agonist increased the amplitude of I_f through

activation of adenylate cyclase which resulted in an increase in the intracellular cyclic AMP concentration independent of the internal Ca^{2+} concentration.

Since internal Ca^{2+} shifted the steady-state activation curve to a more positive potential (Fig. 6*B*), we examined the effect of isoprenaline on the steady-state activation curve (Fig. 10*A*). The normalized amplitude of the I_f tail current against

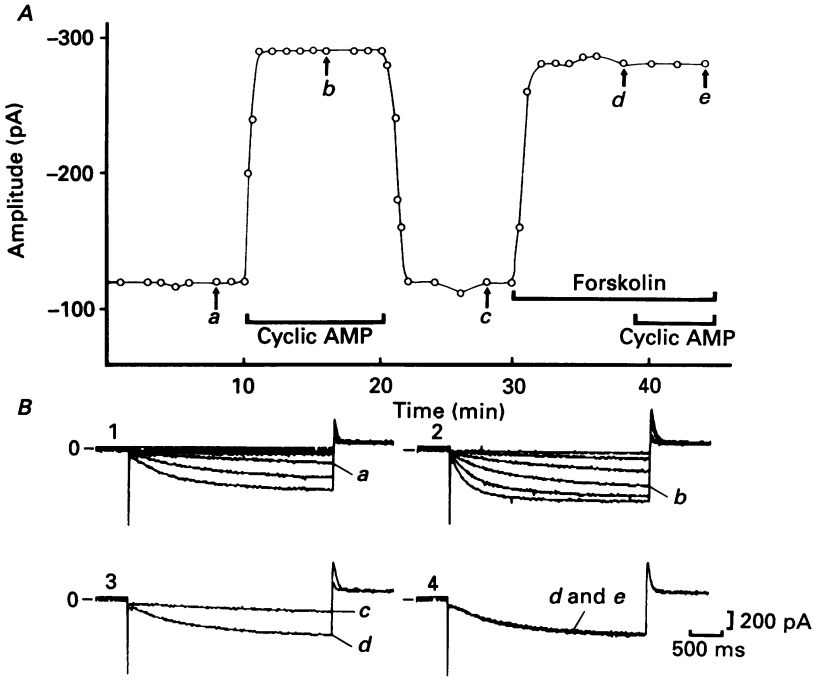

Fig. 9. Effect of cyclic AMP and forskolin on I_f. *A*, the amplitude of I_f was measured at −70 mV and plotted against time. In the control, the pipette contained Ca^{2+}-free, cyclic AMP-free solution. Cyclic AMP (50 μM) applied intracellulary through the patch electrode as indicated by the horizontal bars from 10 to 20 min, and 38 to 45 min, respectively. Forskolin was superfused from 30 to 45 min, as indicated in the figure. I_f increased from −120 pA (*a*) to −290 pA (*b*) within 1 min. At 2 min after washing out the cyclic AMP from the pipette, I_f declined to the control current level (*c*). Application of 10 μM-forskolin increased I_f to −280 pA (*d*), which was approximately the same level as that measured under the influence of cyclic AMP. Further dialysis of cyclic AMP into the cell was not effective (*e*). *B*, superimposed voltage-clamp records of I_f at various hyperpolarization pulses between −40 and −90 mV were given in control (*B*1) and during the application of 50 μM-cyclic AMP (*B*2). *B*3 illustrates the case after washing out the cyclic AMP (*B*3*c*) and that 8 min after superfusion of forskolin (*B*3*d*). *B*4 shows the superimposed traces at *d* and *e* in *A*. Hyperpolarization voltages at −70 mV were applied in *B*3 and *B*4.

the membrane potential during hyperpolarization in five different cells fitted well with eqn (1), with an s of 11·2 mV and $V_{0.5}$ of −61·9 mV in the control. In the presence of 1 μM-isoprenaline, s was 7·9 mV and $V_{0.5}$ was −47·1 mV. The activation curve shifted approximately 15 mV towards a positive potential in the presence of 1 μM-isoprenaline, so resembling that in the presence of pCa 7 internal Ca^{2+} solution. However, the activation curve was steeper under the influence of isoprenaline than at the high internal Ca^{2+} concentration, suggesting a difference in kinetics between

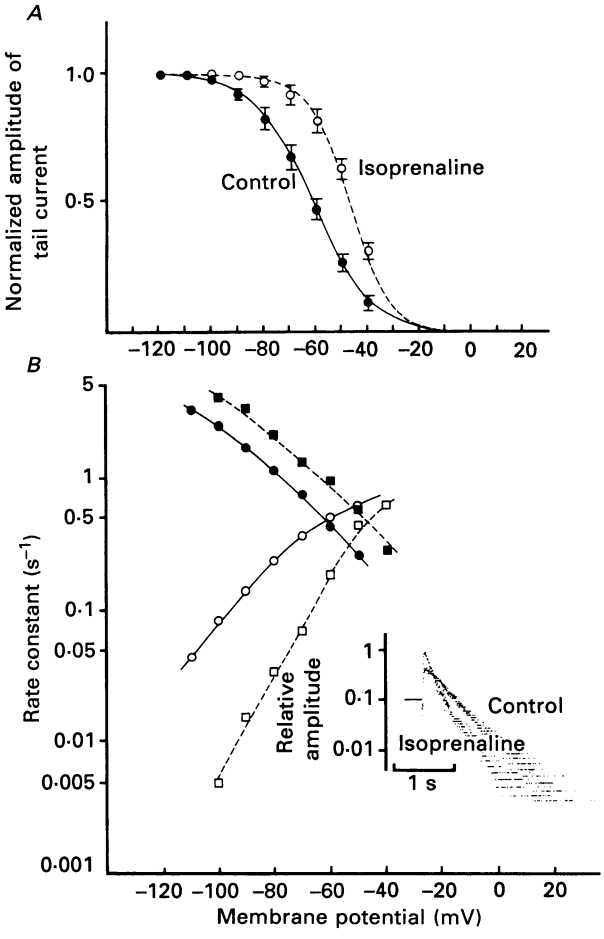

Fig. 10. Effect of isoprenaline on the kinetics of I_f. *A*, the normalized amplitudes of the I_f tail current in five different cells are plotted against the membrane potentials, in the control (continuous curve) and in the presence of 1 μM-isoprenaline (interrupted curve). These curves were drawn according to eqn (1) in the text. The activation curve shifted approximately 15 mV in a positive direction and the curve was steepened in the presence of isoprenaline. *B*, the rate constants are plotted against the membrane potentials on a semilogarithmic graph, in the control (continuous line) and in the presence of 1 μM-isoprenaline. α (filled symbols) and β (open symbols) shifted to a more positive potential and the voltage dependence of β was steepened in the presence of isoprenaline. Activation time courses at −70 mV are shown in the inset, in the control and in the presence of isoprenaline, with time constants of 0·99 and 0·73 s, respectively.

the two conditions. To test this difference, we analysed the kinetics of I_f under the conditions of high internal Ca^{2+} and in the presence of isoprenaline. The conductance of I_f (g_f) can be approximated by the following equation:

$$g_f = \bar{g}_f q_\infty, \tag{2}$$

where $\bar{g}_f$ is the maximum value of g_f. The value of $\bar{g}_f$ is increased by internal Ca^{2+} and β-agonist.

To explain the activation kinetics of I_f, we assumed a simple model as follows:

$$\text{Closed} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{Open},$$

where α is the forward rate constant of activation and β is the backward rate constant. The time constant (τ) and the activation parameter (q_∞) can be expressed by the following equations:

$$q_\infty = \frac{\alpha}{(\alpha+\beta)}, \tag{3}$$

$$\tau = \frac{1}{(\alpha+\beta)}, \tag{4}$$

and α and β were calculated as follows:

$$\alpha = q_\infty/\tau, \tag{5}$$

$$\beta = (1-q_\infty)/\tau. \tag{6}$$

α and β are plotted against the membrane potentials on a semilogarithmic graph in the control (continuous line) and in the presence of 1 μM-isoprenaline (interrupted line) in five cells (Fig. 10*B*). Isoprenaline shifted both α and β to a more positive potential compared to those in the absence of isoprenaline, and increased the steepness of the voltage dependence of β, without having much influence on the voltage dependence of α.

The effects of internal Ca^{2+} on the kinetics of I_f are illustrated in Fig. 11, where α and β are plotted against the membrane potentials at pCa 10 (interrupted line) and pCa 7 (continuous line) in the pipette. The high internal Ca^{2+} concentration also shifted α and β in a similar manner to isoprelanine, but it caused no appreciable change in the steepness of the voltage dependence of α and β.

Effects of modulators of protein kinase C and calmodulin on I_f

To determine whether internal Ca^{2+} directly affects I_f or exerts an indirect influence through intracellular Ca^{2+}-dependent factors, such as activation of protein kinase C (PKC) or calmodulin, we designed several experiments as follows. Since PKC activity is also Ca^{2+} sensitive, we applied tetradecanoylphorbol-13-acetate (TPA), a well-known activator of PKC (Nishizuka, 1984). When the patch pipette contained pCa 7, superfusion of 10^{-8} M-TPA caused no appreciable change in I_f for more than 15 min (Fig. 12*B*1 and *B*2). We obtained similar results in three examples. At a concentration higher than 10^{-7} M, however, the amplitude of I_f was decreased, most probably due to the toxic effect of TPA. Thus, internal Ca^{2+} appears not to affect I_f through PKC, but more probably acts directly on I_f. To confirm this view, we superfused 20 μM-H-7, which is known to block protein kinases (Hidaka, Inagaki, Kawamoto & Sasaki, 1984). The enhanced effect of 1 μM-forskolin on I_f was blocked by H-7 (data not shown), but application of pCa 7 in the pipette could elicit a further increase in the amplitude of I_f (Fig. 13).

The high internal Ca^{2+} also increased the amplitude of I_f in the presence of 1 μM-calmidazolium, a known blocker of the effect of calmodulin (data not shown). The above findings support the idea that internal Ca^{2+} directly affects I_f, without acting through protein kinases (PKC or PKA) or calmodulin.

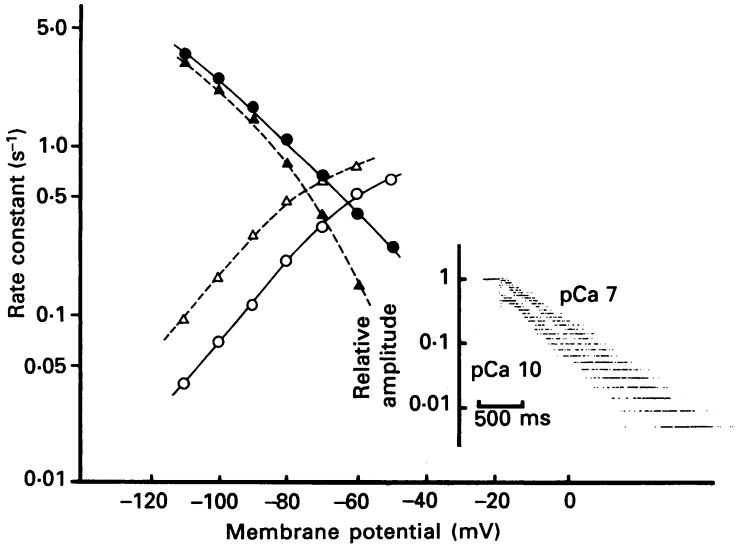

Fig. 11. Effect of internal Ca^{2+} on the rate constants of I_f. The rate constants of I_f at pCa 10 (interrupted line) and pCa 7 (continuous line) in the pipette solution are plotted against the membrane potential. The high internal Ca^{2+} concentration shifted both α (filled symbols) and β (open symbols) to a positive potential, without exerting much effect on the voltage dependence of α and β. The activation time courses at −70 mV are shown in the inset, at pCa 10 and pCa 7, with time constants of 1·07 and 1·03 s, respectively.

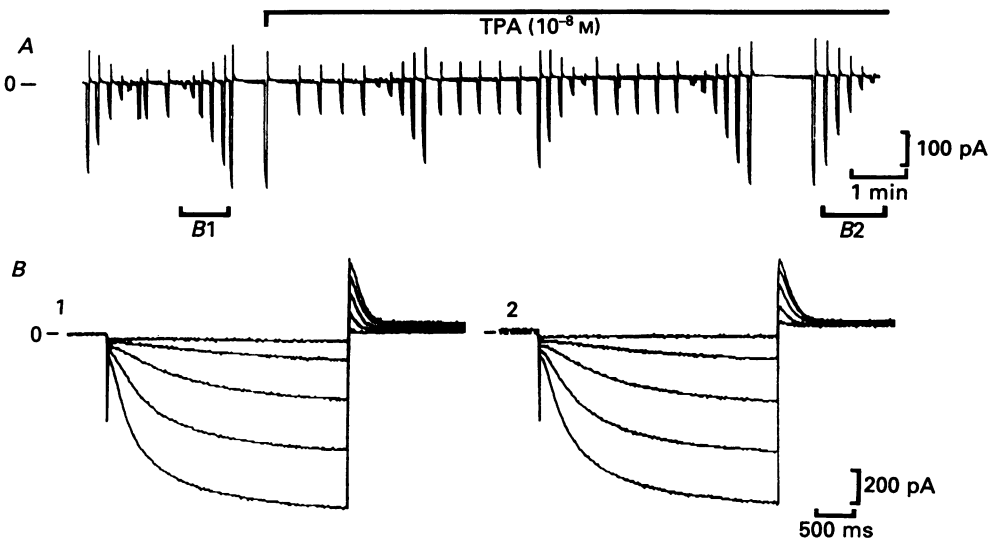

Fig. 12. Effect of protein kinase C activator (TPA) on I_f. Current traces at a slow sweep speed (A) and at a rapid sweep speed (B) were recorded. The currents shown in A correspond to $B1$ and $B2$. I_f was activated to hyperpolarizing pulses between −40 and −80 mV in the control ($B1$) and after the application of 10^{-8} M-TPA for 10 min ($B2$). The experimental protocol was the same as in Fig. 5. TPA caused no appreciable change in the amplitude of I_f.

Effect of internal Ca^{2+} concentration on the action potential pattern

Since a physiological concentrations of internal Ca^{2+} markedly modulates I_f, we examined the question of whether an increase of the internal Ca^{2+} causes any change in the action potential pattern. While recording the action potentials, Ca^{2+} (pCa 7) was allowed to diffuse into the cell from the recording electrode. Neither the action

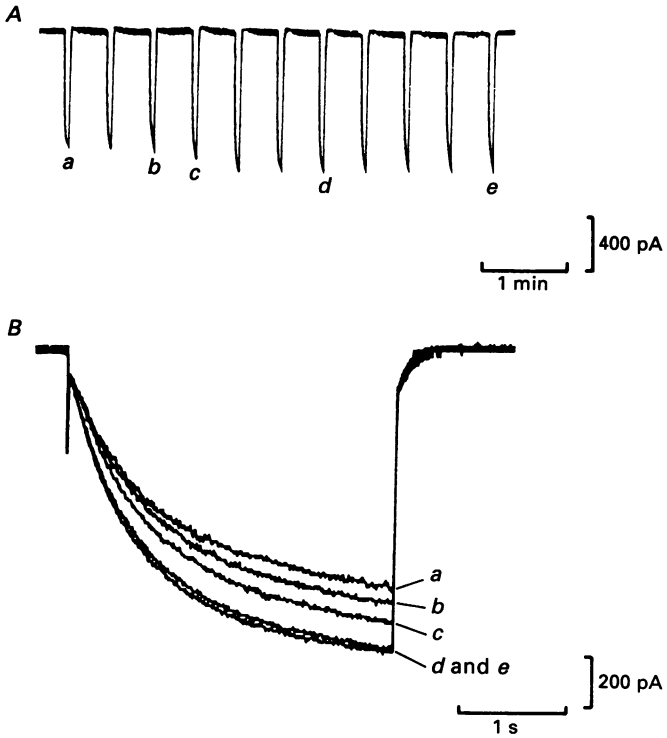

Fig. 13. Modulation of the amplitude of I_f by internal Ca^{2+} in the presence of protein kinase inhibitor (H-7). The pipette contained pCa 7 internal solution. After pre-incubation with 20 μM-H-7 in the control solution for 30 min, the membrane was ruptured to diffuse Ca^{2+} into the cell. The experimental protocol was the same as in Fig. 1. *A* illustrates the current traces at a slow sweep speed, while those in *B* were at a rapid sweep speed, corresponding to *a–e* in *A*, respectively. At 5 min after disruption of the membrane, I_f was increased 38% in the presence of protein kinase inhibitor (*a–e*).

potential pattern nor the heart rate changed much, at the beginning (Fig. 14*Aa*) and at 10 min after the diffusion of Ca^{2+} took place (Fig. 14*Ab*). In another example (Fig. 14*Bb*), the maximum diastolic potential shifted positively by 8 mV and the action potential amplitude was reduced by 13 mV, but the heart rate was unaffected. Similar findings were observed in two other cells. If the increase in intracellular Ca^{2+} causes an enhancement of I_f, as shown in the above experiments, the membrane should be depolarized and the heart rate might become increased. This discrepancy between the predicted and experimental results will be discussed later.

When the amplitude of I_f is increased by introducing the high-internal-Ca^{2+}

solution, the effect of Cs^+ on the action potential should be much larger than that observed in the low-internal-Ca^{2+} solution. Contrary to our expectation, the spontaneous pacing rate decreased only slightly ($4{\cdot}33 \pm 0{\cdot}76\%$, $n = 3$) even though I_f was strongly depressed at potentials negative to -40 mV in the presence of Cs^+.

DISCUSSION

Modulation of I_f by the internal Ca^{2+} concentration

We have shown that I_f is increased by elevating internal Ca^{2+}. Findings which support this view are as follows. (1) The reversal potential of I_f was unaffected by a

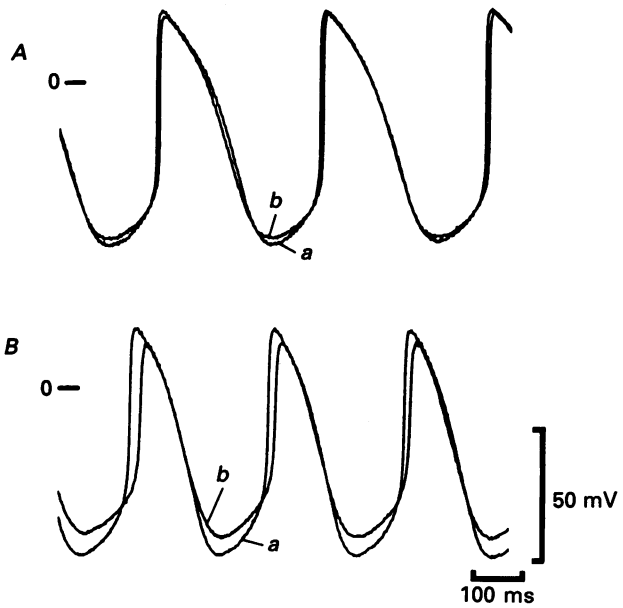

Fig. 14. Effect of internal Ca^{2+} concentration on the action potential pattern. Two different cells (*A* and *B*). Traces *Aa* and *Ba* were obtained immediately after establishing the seal with cells of an unknown pCa; traces *Ab* and *Bb* were obtained 10 min later when the pipette solution of pCa 7 had equilibrated with the inside of the cells. The maximum diastolic potential was shifted in a positive direction by 3 mV in *A* and 8 mV in *B*. The amplitude of the action potential was reduced by 13 mV in *B*. In both examples, the heart rate remained almost constant. 0 is the zero membrane potential in both *A* and *B*.

change in internal Ca^{2+}. (2) The time courses of activation and deactivation of I_f were broadly similar to those reported by other authors. (3) I_f was blocked by external application of Cs^+ at both the low and high internal Ca^{2+} concentration. While the interventions described in the present paper thus bear on a modulation by internal Ca^{2+} of I_f, we do not wish to exclude the possibility that currents other than I_f are fully independent of internal Ca^{2+} (see later). It is possible that the increased internal Ca^{2+} could give rise to the inward Na^+–Ca^{2+} exchange current during hyperpolarization which would result in an apparent increase in I_f amplitude. This complication was ruled out by the fact that the reversal potential was unchanged and

that 3 mM-Ni^{2+} was superfused (Kimura *et al.* 1987). The possibility of activation of a Ca^{2+}-activated non-specific cation channel seems unlikely, because of the lack of a voltage and time dependence (Colquhoun *et al.* 1981). The increased amplitude of I_f during hyperpolarization could possibly elevate the internal Na^+ level (Glitsch, Pusch & Verdonck, 1986), which in turn would tend to activate the outward Na^+–K^+ pump current. We superfused 20 μM-ouabain to avoid this possibility, but the tail current of I_f was not affected (data not shown), suggesting that the Na^+–K^+ pump current did not contribute significantly under our experimental conditions. We conclude therefore that the increased current represents a modulation of I_f and not a newly activated current system which is sensitive to an increase in the internal Ca^{2+}.

In mammalian sino-atrial node, the hyperpolarization-activated inward current (I_h or I_f) has been clearly demonstrated in multicellular preparations (Brown *et al.* 1979; Yanagihara & Irisawa, 1980). In the single sino-atrial node cell, however, this current displays a large variety of sizes (Nakayama, Kurachi, Noma & Irisawa, 1984; DiFrancesco *et al.* 1986). Nakayama *et al.* (1984) observed an absence of I_f in four out of seven cells. DiFrancesco *et al.* (1986) found that in some cells I_f was not activated even at voltages more negative than -100 mV, and that the I_f amplitude varied from cell to cell. On the other hand, van Ginnecken & Giles (1985) observed I_f consistently and its variation in size was small in rabbit sino-atrial node cells. In the present experiments, we found that the amplitude of I_f was smaller when the patch pipette contained the low Ca^{2+} concentration than when it contained the high Ca^{2+} concentration. These findings suggest that the variation in the amplitude of I_f among different authors could be due partly at least to variations in the intracellular Ca^{2+} concentration. This phenomenon is consistent with previous results for Purkinje fibres, in which intracellular injection of Ca^{2+} increased the amplitude of the pacemaker current (I_{K2}; Isenberg, 1977).

The threshold of activation of I_f varied from worker to worker. The values ranged between -40 and -70 mV (Nakayama *et al.* 1984; DiFrancesco *et al.* 1986), although van Ginnecken & Giles (1985) reported that it was approximately -50 mV with little cell-to-cell variability. These differences may in part reflect differences in the experimental conditions. Nakayama *et al.* (1984) and DiFrancesco *et al.* (1986) used pipette solutions which contained EGTA to buffer the internal Ca^{2+}, and the diameter of the electrode ranged from 2 to 3 μm (electrode resistances, 2–4 MΩ). On the other hand, van Ginnecken & Giles (1985) employed an internal solution which had no EGTA, and the electrode resistances ranged between 5 and 20 MΩ. In the present experiments, we found that when the concentration of intracellular Ca^{2+} was high, the threshold of activation of I_f shifted by 13 mV in a positive direction. It seems probable therefore that the large variety in size and voltage dependence of I_f might be due partly to variations in the internal Ca^{2+}.

Modulation of I_f by internal Ca^{2+} differs from that by β-agonist

The amplitude of I_f is known to increase under the influence of β-agonist (Brown *et al.* 1979; Noma, Kotake & Irisawa, 1980; DiFrancesco *et al.* 1986). Tsien (1974) found that adrenaline acts on the current kinetics rather than on the amplitude of I_{K2} in the Purkinje fibre. Adrenaline shifted both the activation rate constant of I_{K2}

(k_1) and the deactivation rate constant (k_{-1}) to a more positive potential, and increased the steepness of the voltage dependence of k_1. As a result, the steady-state activation curve steepened in the presence of adrenaline. Since I_{K2} has been reinterpreted as I_f (DiFrancesco, 1981), k_1 may represent the deactivation rate constant of I_f (β) and k_{-1} may correspond to the activation rate constant (α). Taking this into consideration, our results for the effects of β-agonist on the kinetics of I_f were similar as those observed in cardiac Purkinje fibres (Tsien, 1974). In the presence of β-agonist, α and β shifted to a more positive potential and the voltage dependence of β became steeper than in the control in the absence of β-agonist.

The effect of internal Ca^{2+} on the kinetics of I_f differed from that of β-agonist. The high internal Ca^{2+} also shifted both α and β in a similar manner to that observed for the effect of β-agonist, but it caused no appreciable change in the slope of the voltage dependence of α and β, suggesting that the internal Ca^{2+} may affect the conductance rather than the kinetics of I_f.

Site of action of internal Ca^{2+} on I_f

The increase in I_f caused by the high internal Ca^{2+} concentration was not dependent on cyclic AMP-dependent protein kinase, since β-agonist and internal Ca^{2+} exerted different effects on the kinetics of I_f The Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) has been biochemically demonstrated in the myocardium (Wise, Raynor & Kuo, 1982), and activation of PKC is known to enhance the delayed rectifier K^+ current of the guinea-pig ventricular cell (Tohse, Kameyama & Irisawa, 1987). However, no appreciable effect of TPA on I_f was observed at a concentration of 10^{-9}–10^{-8} M (Fig. 12). Furthermore, the high internal Ca^{2+} concentration increased the amplitude of I_f even in the presence of a protein kinase inhibitor (H-7) (Fig. 13).

Activation of Ca^{2+}-calmodulin-dependent protein kinase results in the phosphorylation of several proteins (for review see Cohen, 1982), and calmodulin modulates Ca^{2+}-activated K^+ channels in mouse fibroblasts (Okada, Yada, Ohno & Oiki, 1987). Since the inhibitor of calmodulin had no appreciable effect on I_f, we ruled out the possibility that increase of I_f caused by the internal Ca^{2+} was due to activation of calmodulin. These results are consistent with the idea that internal Ca^{2+} may directly modulate I_f without acting through protein kinases or calmodulin.

Contribution of I_f to the pacemaker activity

Concerning the contribution of I_f to the pacemaker potential, we suggested previously that the current I_f may not be essential for the pacemaker depolarization since the current is activated at a more negative potential than the maximum diastolic potential and its activation kinetics are slow (Yanagihara & Irisawa, 1980). Application of Cs^+ exerted only a slight bradycardiac effect on the spontaneous activity (Brown *et al.* 1981; Noma *et al.* 1983). van Ginnecken & Giles (1985), however, reported that after application of Cs^+, a constant hyperpolarizing current produced more obvious bradycardia than in the absence of Cs^+. We found that the change in the spontaneous activity was small in the presence of 2 mM-Cs^+, even in the presence of the higher internal Ca^{2+}, suggesting that the contribution of I_f to the spontaneous activity was small.

As shown in Fig. 14, the cardiac rate was maintained unchanged during the experiment by introducing the higher Ca^{2+} into the pipette. A preliminary experiment showed that the delayed rectifier K^+ current (I_K) and the calcium current (I_{Ca}) are also under the control of internal Ca^{2+}, suggesting that both I_K and I_{Ca} are also affected by the increased internal Ca^{2+}. In intact heart the true pacemaker cells in the sino-atrial node are electrically coupled with a large number of cells that have a negative potential with respect to the maximal diastolic potential, thus tending to arrest pacing of the sino-atrial node. A slight activation of I_f at the level of maximal diastolic potential might thus protect the pacemaker cells against electrotonic arrest. Since the time constant of activation of I_f became faster and the amplitude of I_f increased under the effect of β-agonist, the inward flow of I_f may partially contribute to the positive chronotrophic effect of β-agonist.

Dr M. Kameyama and Dr J. Kimura made many helpful suggestions during the course of this work. N.H. is affiliated to the Department of Cardiology, The Heart Institute Japan, Tokyo Women's Medical College, Shinjuku-ku, Tokyo. He wishes to thank Professor K. Hirosawa and Dr H. Kasanuki for providing him with the opportunity of conducting joint research at NIPS. The technical assistance of Mr M. Ohara and Mr O. Nagata is greatly appreciated. This study was supported by a research grant from the Ministry of Education, Science and Culture of Japan.

REFERENCES

BROWN, H. F., DIFRANCESCO, D. & NOBLE, S. J. (1979). How does adrenaline accelerate the heart? *Nature* **280**, 235–236.

BROWN, H. F., DIFRANCESCO, D., KIMURA, J. & NOBLE, S. (1981). Caecium: a useful tool for investigating sino-atrial (S–A) nodes pacemaking. *Journal of Physiology* **317**, 54*P*.

CALLEWAERT, G., CARMELIET, E. & VEREECKE, J. (1984). Single cardiac Purkinje cells: general electrophysiology and voltage clamp analysis of the pace-maker current. *Journal of Physiology* **349**, 643–661.

COBBOLD, P. H. & BOURNE, P. K. (1984). Aequorin measurements of free calcium in single heart cells. *Nature* **312**, 444–446.

COHEN, P. (1982). The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296**, 613–620.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C. S. (1981). Inward current channels activated by intracellular Ca in cultured heart cells. *Nature* **294**, 752–754.

DIFRANCESCO, D. (1981). A new interpretation of the pace-maker current in calf Purkinje fibres. *Journal of Physiology* **314**, 359–376.

DIFRANCESCO, D., FERRONI, A., MAZZANTI, M. & TROMBA, C. (1986). Properties of the hyperpolarizing-activated current (i_f) in cells isolated from the rabbit sino-atrial node. *Journal of Physiology* **377**, 61–88.

EHARA, T., NOMA, A. & ONO, K. (1988). Calcium-activated non-selective cation channel in ventricular cells isolated from adult guinea-pig hearts. *Journal of Physiology* **403**, 117–133.

FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de physiologie* **75**, 463–505.

GLITSCH, H. G., PUSCH, H. & VERDONCK, F. (1986). The contribution of Na and K ions to the pacemaker current in sheep cardiac Purkinje fibres. *Plügers Archiv* **406**, 464–471.

HAGIWARA, N., HO, W. K. & IRISAWA, H. (1987). Hyperpolarization-activated current is regulated by an intracellular Ca in rabbit sino-atrial node cells. *Journal of Physiology* **394**, 30*P*.

HAGIWARA, N., IRISAWA, H. & KAMEYAMA, M. (1988). Contribution of two types of calcium currents to the pacemaker potentials of rabbit sinoatrial node cells. *Journal of Physiology* **395**, 233–253.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Plügers Archiv* **391**, 85–100.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.

ISENBERG, G. (1977). Cardiac Purkinje fibres: $[Ca^{2+}]_i$ controls the potassium permiability via the conductance components g_{K1} and $\bar{g}_{K2}$. *Pflügers Archiv* **371**, 77–85.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a KB medium. *Pflügers Archiv* **395**, 6–18.

KAMEYAMA, M., HOFMANN, F., & TRAUTWEIN, W. (1985). On the mechanism of β-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflügers Archiv* **405**, 285–293.

KIMURA, J., MIYAMAE, S. & NOMA, A. (1987). Identification of sodium–calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology* **384**, 199–222.

MARBAN, E., KITAKAZE, M., KUSUOKA, H., PORTERFIELD, J. K., YUE, D. T. & CHACKO, V. P. (1987). Intracellular free calcium concentration measured with ^{19}F NMR spectroscopy in intact ferret hearts. *Proceedings of the National Academy of Sciences of the USA* **84**, 6005–6009.

NAKAYAMA, T., KURACHI, Y., NOMA, A. & IRISAWA, H. (1984). Action potential and membrane currents of single pacemaker cells of the rabbit heart. *Pflügers Archiv* **402**, 248–257.

NATHAN, R. D. (1986). Two electrophysiologically distinct types of cultured pacemaker cells from rabbit sinoatrial node. *American Journal of Physiology* **250**, H325–329.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693–698.

NOMA, A., KOTAKE, H. & IRISAWA, H. (1980). Slow inward current and its role mediating the chronotrophic effect of epinephrine in the rabbit sinoatrial node. *Pflügers Archiv* **388**, 1–9.

NOMA, A., MORAD, M. & IRISAWA, H. (1983). Does the 'pacemaker current' generate the diastolic depolarization in rabbit SA node cells? *Pflügers Archiv* **397**, 190–194.

OKADA, Y., YADA, T., OHNO, T. & OIKI, S. (1987). Evidence for the involvement of Calmodulin in the operation of Ca-activated K channels in mouse fibroblasts. *Journal of Membrane Biology* **96**, 121–128.

SOEJIMA, M. & NOMA, A. (1984). Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Archiv* **400**, 424–431.

TOHSE, N., KAMEYAMA, M. & IRISAWA, H. (1987). Intracellular Ca^{2+} and protein kinase C modulate K^+ current in guinea pig heart cells. *American Journal of Physiology* **253**, H1321–1324.

TSIEN, R. W. (1974). Effects of epinephrine on the pacemaker potassium current of cardiac Purkinje fibers. *Journal of General Physiology* **64**, 293–319.

TSIEN, R. Y. & RINK, T. J (1980). Neutral carrier ion-selective microelectrodes for measurements of intracellular free calcium. *Biochimica et biophysica acta* **599**, 623–638.

VAN GINNEKEN, A. C. G. & GILES, W. R. (1985). I_f in isolated cells from the rabbit S-A node. *Biophysical Journal* **47**, 496a.

WIER, W. G., CANNELL, M. B., BERLIN, J. R., MARBAN, E. & LEDERER, W. J. (1987). Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by Fura-2. *Science* **235**, 325–328.

WISE, B. C., RAYNOR, R. L. & KUO, J. F. (1982). Phospholipid-sensitive Ca^{2+} dependent protein kinase from heart: Purification and general properties. *Journal of Biological Chemistry* **257**, 8481–8488.

YANAGIHARA, K. & IRISAWA, H. (1980). Inward current activated during hyperpolarization in rabbit sinoatrial node cell. *Pflügers Archiv* **385**, 11–19.