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ON THE MECHANISM BY WHICH CHANGES IN EXTRACELLULAR pH AFFECT THE ELECTRICAL ACTIVITY OF THE RABBIT SINO-ATRIAL NODE

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SUMMARY

1. The effect of altering the extracellular pH (pH_o) on the electrical activity of the isolated rabbit sino-atrial (s.a.) node was studied using the two-micro-electrode voltage-clamp technique.

2. Alkalinization of the perfusate increased the maximum rate of rise and amplitude of the nodal action potential, and also the frequency of spontaneous beating of the s.a. node; acidification produced opposite effects.

3. A change in the extracellular pH from a value of 6·5 to 8·5 caused increases of the maximal conductance for both the slow inward current and the steady-state outward current systems without affecting the gating processes of the channels.

4. H^+ could modify the electrical activity of s.a. nodal cells, not through altering the membrane surface potential but through the protonation of the ionic channels themselves. From the titration curves, the apparent pK_a values for the slow inward current and the steady-state outward current were estimated to be 6·4 and 6·0, respectively.

INTRODUCTION

Since Mines (1913) and Andrus & Carter (1926) described the chemosensitive properties of cardiac muscle, there have been numerous reports to the effect that cardiac tissues, such as the rabbit atrium (Vaughan Williams & Whyte, 1967), frog atrium (Wada & Goto, 1975; Chesnais, Coraboeuf, Sauviat & Vassas, 1975), cat papillary muscle (Kohlhardt, Haap & Figulla, 1976), chick embryo heart (Vogel & Sperelakis, 1977), and dog sino-atrial (s.a.) node cells (Satoh & Hashimoto, 1983), are fairly responsive to acidification of the extracellular medium in such a way that the slow inward current and maximum rate of rise of the action potential are decreased as the pH is decreased. It has been suggested that a change in extracellular pH (pH_o) could alter the electrical activity of the excitable membrane either by modifying the surface potential or by protonating the channel protein (Woodhull, 1973; Campbell,

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1982). Because of the central role of s.a. node cells in the electrical activity of the heart, it seemed desirable to investigate which of these mechanisms might be responsible for the changes in the electrical activity of s.a. node cells in response to alterations in pH_o.

A preliminary report of this work has been published elsewhere (Satoh, Hashimoto & Seyama, 1982).

METHODS

Rabbits of either sex weighing 1·5–2·0 kg were killed by a blow to the neck and exsanguinated. The method employed for making small preparations of s.a. node cells and that for the two-micro-electrode voltage clamp are essentially the same as previously described (Noma & Irisawa, 1976; Seyama, 1979). The composition of the Tyrode solution (mM) was: NaCl, 134; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 0·5 and HEPES, 5·0. HEPES was used to buffer solutions having a pH between 6·5 and 8·5. Acid solutions with a pH lower than 6·5 were prepared using 2-(*N*-morpholino)ethanesulphonic acid, monohydrate (MES) as the buffer, and alkaline solutions with a pH greater than 8·5, using Tris as the buffer. Solutions were perfused at a rate of 3 ml/min. Since the Tyrode solution in the bath could be completely exchanged for a solution having a different pH within 3 min, data were obtained 10 min after changing to the new solution. The temperature of the perfusing solution was kept constant at 36 ± 1 °C. Data are presented as mean ± 1 S.E. of mean (number of observations) unless otherwise stated.

RESULTS

Action potential experiments

The spontaneous beating of s.a. node cells was markedly reduced when a perfusing solution having a normal pH was switched to one having a pH of 6·5 (Fig. 1, lower records). By contrast, when the pH of the perfusing solution was increased to 8·5, the electrical activity of the s.a. node was enhanced (Fig. 1, upper records). The action potential parameters of spontaneously beating s.a. node cells at three different pH values are summarized in Table 1. In quiescent s.a. node cells, the resting membrane potential had a mean value of $-33{\cdot}3 \pm 0{\cdot}5$ mV ($n = 5$) at pH 6·5, $-40{\cdot}5 \pm 0{\cdot}8$ mV ($n = 5$) at pH 7·4 and $-46{\cdot}0 \pm 0{\cdot}8$ mV ($n = 5$) at pH 8·5.

Voltage-clamp experiments

Slow inward current. The membrane was held at -50 mV and stepped to various test potentials. This activated the slow inward current, which was followed by an outward current (Figs. 2 and 3). Peak values of the slow inward current were measured by the method developed by McDonald & Trautwein (1978) and were plotted against the membrane potential of the test pulses. In altering pH_o, the most striking change in the *I–V* curve for the slow inward current is that the slow current increased with alkalinization of the perfusing solution (Fig. 2) and decreased with acidification (Fig. 3). The membrane potential at which the maximum peak inward current was observed (about -10 mV) (Figs. 2 and 3) was essentially identical at pH 6·5, 7·4 and 8·5. Moreover, the membrane potential ($V_{\frac{1}{2}}$) at which the peak inward current attained one-half of its maximum value remained nearly constant between pH 6·5 and 8·5 (*I–V* curves in Figs. 2 and 3, arrows). A change in $V_{\frac{1}{2}}$ can be regarded as a good indication of a difference in surface potential (Gilbert, 1971; Ohmori & Yoshii, 1977). Consequently, these findings suggest that the contribution of a change

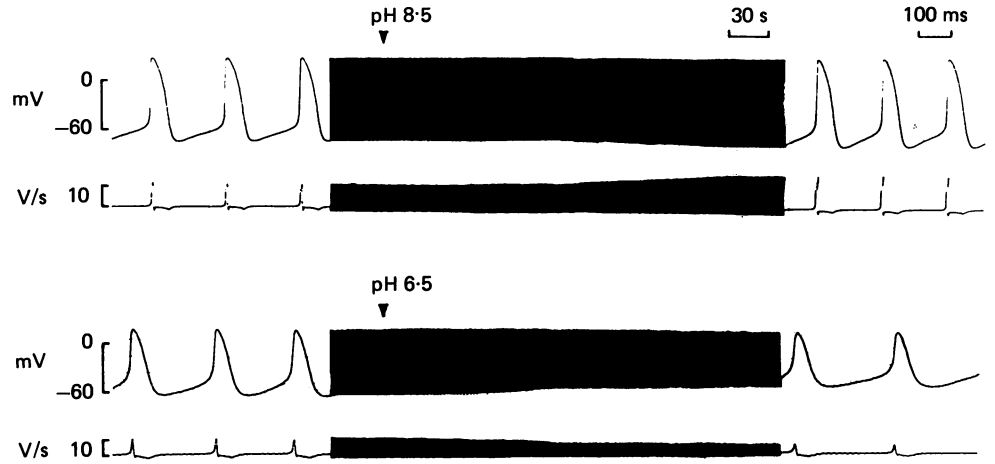

Fig. 1. Effect of changes in pH_o on the action potential of s.a. node cells shown at two different time scales. The upper set of two rows shows changes in the action potentials caused by alkalinization of the perfusing solution relative to normal (pH 7·4); the lower set of two rows shows effects of acidification. The arrow in each set of records indicates time of solution change.

TABLE 1

	pH 6·5	pH 7·4	pH 8·5
n	12	17	11
Amplitude (mV)	65·0±11·0	82·0±9·2	90·1±8·5
Max. diastolic potential (mV)	−51·6±16·4	−61·8±13·0	−68·5±12·6
Duration (ms)	60·5±42·2	60·9±42·8	59·8±43·4
Max. rate of rise (V/s)	5·5±2·3	10·1±2·4	13·5±4·5
Cycle length (ms)	293·5±75·0	273·9±57·4	248·8±46·6

in the surface potential to the observed changes in membrane currents should be negligible. This conclusion is further strengthened by the finding that the steady-state inactivation curve for the slow inward current, as shown later in Fig. 4, did not shift along the membrane potential axis.

A plausible reason for the changes in the magnitude of the slow inward current with changes of pH_o would be an increase in the maximum conductance of the slow inward current system with alkalinization of the perfusing solution and a decrease with acidification. Although Fig. 2 suggests that the reversal potential was shifted toward a more positive potential at pH 8·5, it is difficult to determine whether or not this is a spurious finding for the following reasons: (1) the increase in the capacitative surge that accompanies the larger step depolarizations tends to obscure the early phase of the slow inward current; (2) intracellular accumulation of the ions entering the cell during the flow of the slow inward current could occur; and (3) the current carried by the Na^+–Ca^{2+} exchange mechanism could change the intracellular concentration of Ca^{2+} (Mullins, 1979). (Recently, experiments using whole-cell clamp of guinea-pig ventricular cells (Kimura, Noma & Irisawa, 1986) and patch clamp of

atrial cardio-balls (Mechmann & Pott, 1986) have confirmed the existence of an electrogenic Na^+–Ca^{2+} exchange mechanism in cardiac cells.)

Thus, the chord conductance is not an appropriate measure of the slow-channel conductance, because of the uncertainty in determining the reversal potential for the slow inward current. For this reason an attempt was made to estimate the

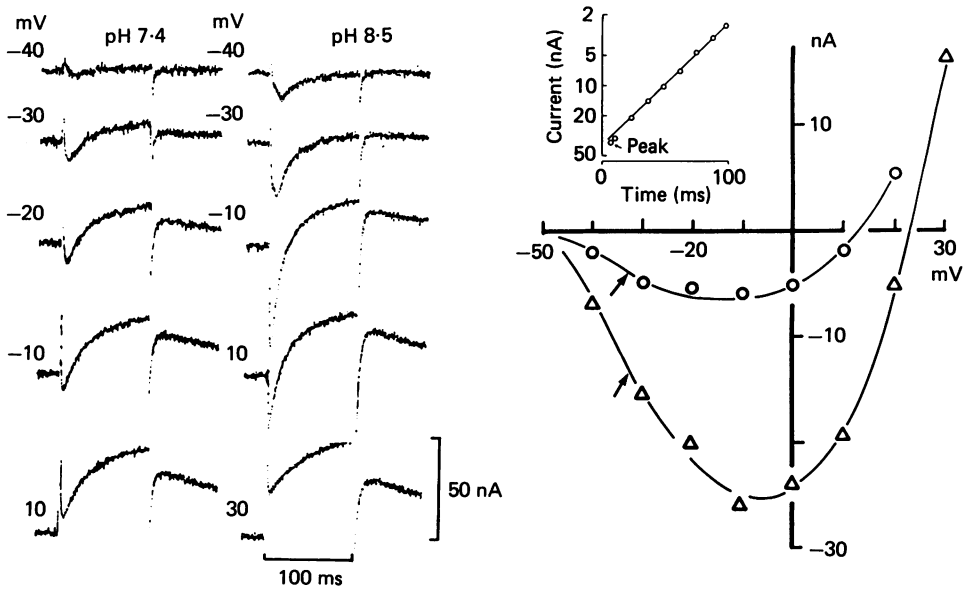

Fig. 2. Changes in the slow inward current in response to alkalinization of the perfusing medium. Columns at the left show original records of the slow inward currents at pH 7·4 and 8·5. The membrane potential of the clamp step is indicated to the left of each trace; holding potential in all the cases was −50 mV. Curve shown to the right is the current–voltage (I–V) relationship for the slow inward currents at pH 7·4 (○) and pH 8·5 (△); arrows point to $V_{\frac{1}{2}}$ (see text), which was unaffected by pH change. Clamp pulses were 100 ms in duration. The inset graph indicates the method for evaluating the slow inward current, with the current record of 10 mV in pH 7·4 taken as an example. Semilogarithmic plot was made of the current record and the line was fitted by the least-squares method. The inward current was determined as a difference between the peak current and the value obtained from the least-squares fit when the peak current was observed.

slow-channel conductance by measuring the 'slope' conductance of the I–V curve between 0 and 10 mV. The relative value of the slope conductance at pH 6·5 referred to that at pH 7·4 was estimated to be $0{\cdot}79 \pm 0{\cdot}03$ ($n = 6$) and that at pH 8·5 was $1{\cdot}13 \pm 0{\cdot}03$ ($n = 12$).

To examine the effect of change in pH_0 on the steady-state inactivation of the slow inward current, 300 ms conditioning pulses of various amplitudes were applied prior to a standard test pulse to 0 mV (see Fig. 4). Slow inward currents during the test pulses were measured as above. The sigmoidal curves were drawn through the data points in Fig. 4 according to the equation developed by Hodgkin & Huxley (1952), and, as can be seen, they adequately fit the experimental data. The membrane potential (V_h) at which half-inactivation occurred at pH 6·5, 7·4 and

8·5 was calculated to be $-20{\cdot}0 \pm 1{\cdot}4$ mV ($n = 3$), $-20{\cdot}5 \pm 1{\cdot}3$ mV ($n = 3$) and $-22{\cdot}5 \pm 2{\cdot}1$ mV ($n = 3$), respectively. The slope factors at pH 6·5, 7·4 and 8·5 were $7{\cdot}8 \pm 0{\cdot}1$, $7{\cdot}7 \pm 0{\cdot}5$ and $7{\cdot}1 \pm 1{\cdot}1$, respectively. The differences in the value of V_h and slope factor under these conditions were not statistically significant ($P > 0{\cdot}1$). Thus, it is concluded that the changes in pH do not affect the voltage dependence of inactivation of the slow current. Another noteworthy point is that the maximal slow

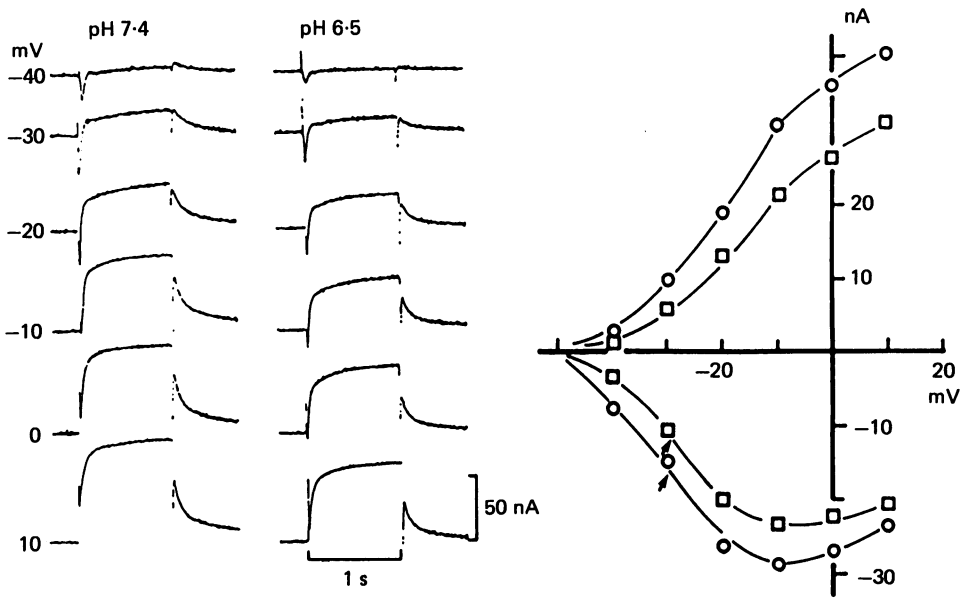

Fig. 3. Influence of acid pH on the slow inward current and the outward current. Columns at left show original records of membrane currents at pH 7·4 and 6·5; the clamp potentials listed apply to both columns. Curves to the right are *I–V* relationships both for the peak slow inward current and for the outward current as measured at the end of the 1 s step depolarizations; values of the current are shown at pH 7·4 (○) and 6·5 (□). Holding potential was −50 mV. Arrows indicate V_i for the slow inward current (see text).

inward current (i.e. following conditioning pulses more negative than −40 mV) decreased as pH was decreased and increased as pH was increased. Since inactivation of the slow inward current is completely removed with such conditioning pulses, this finding again supports the notion that alterations in pH_o affect the maximal conductance of the slow inward current system but do not produce a voltage shift of its activation curve.

Outward current. Fig. 5 (left column) shows records of membrane currents during depolarizing or hyperpolarizing clamp steps from the holding potential (−50 mV). The 'steady-state' currents at the end of the 1 s pulses have been plotted against the membrane potential in Fig. 5*A*. As pH_o was increased from 6·5 to 8·5, the amount of outward current at a given potential became larger (see also Fig. 3); the percentage increase in outward current was uniform over the potential range explored (−40 to +10 mV). In other words there was no shift in the *I–V* curve for outward currents along the membrane potential axis. A similar tendency was recognized as regards the

inward current system activated by hyperpolarizing the membrane from −50 mV. These observations were confirmed by determining the activation curve for the outward current (Fig. 5*B*). This curve was obtained by plotting the peak magnitude of the tail current (obtained on repolarizing the membrane to −50 mV) against the membrane potential of the preceding clamp step. Because of the sizeable capacitative

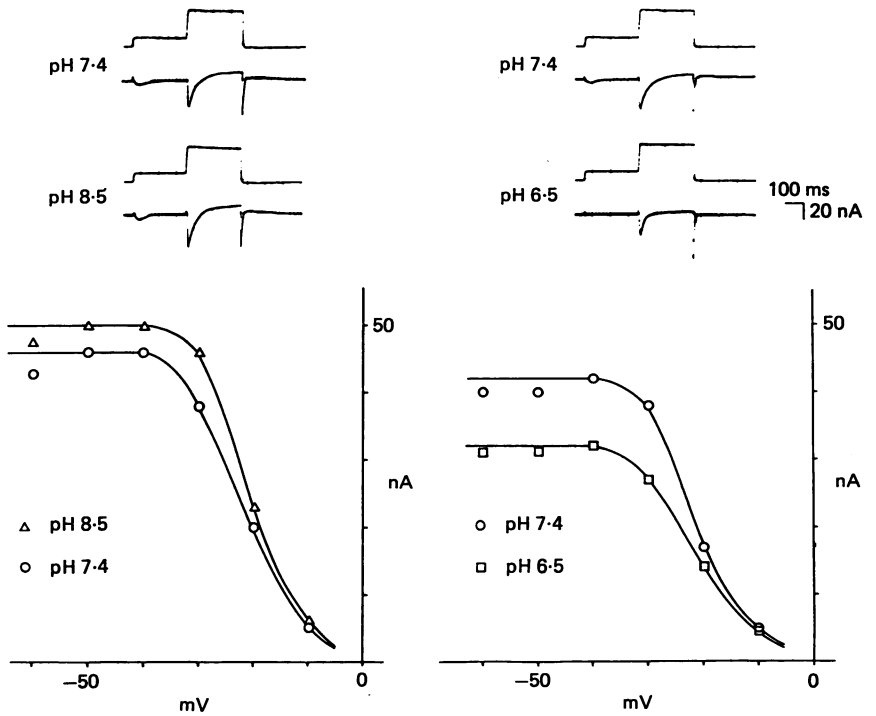

Fig. 4. Influence of changes in pH_o on steady-state inactivation of the slow inward current. The protocol used to determine steady-state inactivation consists of a 300 ms conditioning pulse to a potential between −60 and −10 mV followed by a 300 ms test pulse to 0 mV. The amplitude of currents elicited by the test pulse has been plotted against the potential of the conditioning pulse to give the steady-state inactivation *vs.* potential curves shown. The curves for pH 8·5 (△, left graph) and pH 6·5 (□, right graph), are presented in comparison to curves for pH 7·4 (○, both graphs). The lines through the data points were drawn according to the empirical equations of Hodgkin & Huxley (1952) (see text for further details). Holding potential was −40 mV. Above each graph are original records of membrane potential and the associated membrane currents at the pH values indicated. Conditioning potential in all cases was −30 mV; test potential, 0 mV. Current and time calibrations adjacent to lower right records apply throughout.

surge associated with a step change of the membrane potential, the peak value of the tail was estimated by extrapolating the tail current record to the point in time at which the step was made. The line drawn through the points in Fig. 5*B* was calculated by the empirical equation developed by Hodgkin & Huxley (1952). The slope factor (dimensionless) and the membrane potential (V'_h) at which half-maximal activation of the outward current occurred were estimated to be: 6·9±1·9 and

$-19{\cdot}9 \pm 2{\cdot}7$ mV ($n = 8$) at pH 6·5; $7{\cdot}8 \pm 0{\cdot}6$ and $-23{\cdot}1 \pm 1{\cdot}4$ mV ($n = 15$) at pH 7·4; and $8{\cdot}9 \pm 0{\cdot}7$ and $-25{\cdot}2 \pm 2{\cdot}2$ mV ($n = 11$) at pH 8·5, respectively. The differences among these values were not statistically significant ($P > 0{\cdot}05$). Thus, it is concluded that changes in pH_o alter the maximum conductance of the outward current system.

Titration curves for the slow inward and outward current systems. The results obtained thus far indicate that changing pH_o from 6·5 to 8·5 causes an increase in

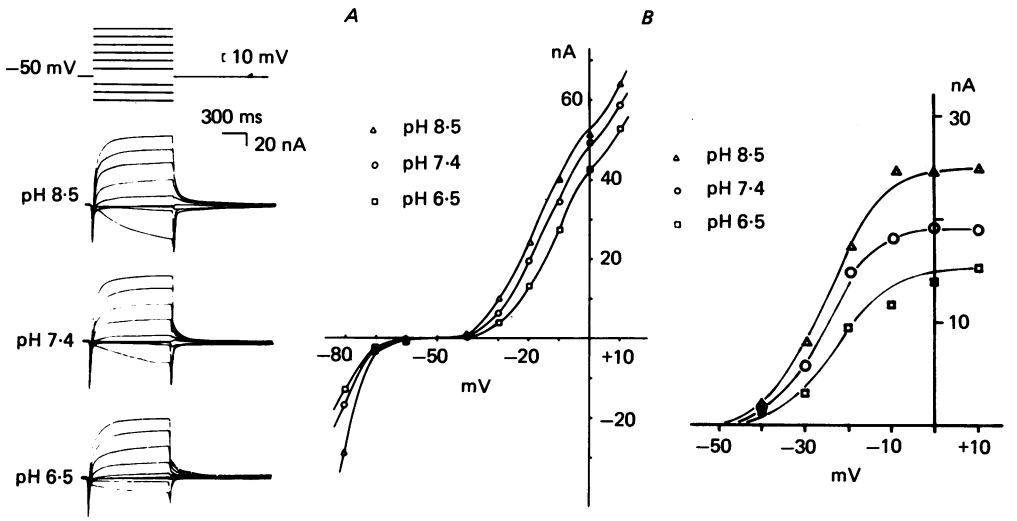

Fig. 5. Effect of changes in pH_o on the steady-state outward current and the associated tail current. At left are families of membrane currents obtained at the pH levels indicated; clamp steps, which are displayed over the current records, were varied between −80 and +10 mV in 10 mV increments. *A*, relationship between the current measured at the end of the 1 s test pulses and the membrane potential during the pulses. *B*, activation curve for outward current. Amplitude of the tail currents (obtained on repolarizing the membrane to −50 mV) has been plotted against the potential of the preceding clamp pulse. The lines drawn through the data points were obtained by adjusting the maximum value of tail current, V'_h and slope factor so as to minimize the variance between the data and the empirical equation. The obtained values for the maximum value, V'_h and the slope factor are 15·2 nA, −22·5 mV and 6·01 at pH 6·5, 19·0 nA, −25·8 mV and 5·37 at pH 7·4 and 25·0 nA, −25·3 mV and 5·65 at pH 8·5, respectively.

the maximum conductance of both the slow inward and outward current systems without a shift in the I–V relationship for either current along the membrane potential axis. This finding strongly suggests that the effect of pH_o changes in mammalian s.a. node is to bind protons to specific membrane receptors which can themselves alter the 'slow inward' and 'delayed outward' conductances rather than interfering with the conductance systems via more generalized changes in surface charge (see Discussion). The pK_a values for receptor binding were obtained by plotting the relative values of the maximal conductance against pH_o and constructing titration curves for the slow channel conductance (g_s) (Fig. 6, upper graph) and for the conductance of the outward current system (g_K) (Fig. 6, lower graph). In each plot, the value obtained in pH 8·5 was abitrarily taken as the maximum value of g_s

or g_K for purposes of normalization. (It could not be determined whether the values in pH 8·5 were truly maximal, because perfusion with media having a pH of 9·0 or greater usually caused irreversible damage to the s.a. node preparation.) As shown in Fig. 6, the apparent dissociation constants (pK'_a) were estimated to be 6·4 for the slow inward current, and 6·0 for the outward current system, respectively.

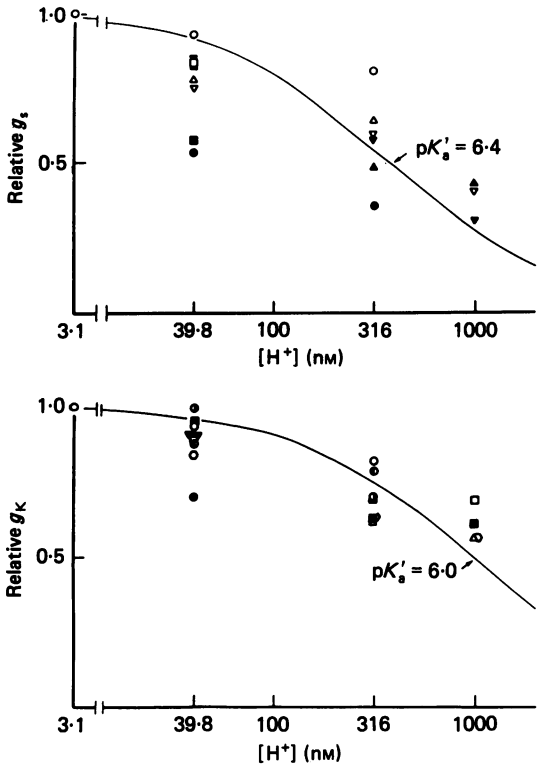

Fig. 6. Titration curves for the peak slow inward current and the 'steady-state' outward current systems. The relative slope conductance for the slow inward current and the relative maximum conductance for the steady-state current have been plotted against the H^+ concentration in the upper and lower graph, respectively (see text for further explanation). The theoretical lines drawn through the data points were calculated from

$$\text{Relative conductance} = 1/(1+[H^+]/K_D),$$

using the value of K_D that minimized the variance between the data and the theoretical line. The above equation assumes a one-to-one stoicheiometry between protons and a binding site.

DISCUSSION

In rabbit s.a. nodal cells, the main effect of increasing pH_0 in the range between 6·5 and 8·5 is to increase both the slow inward and outward currents without causing a shift in the $I-V$ relationship for either current. There is no evidence to suggest that the changes in these currents are due to a modification of the surface potential. In support of this view is the finding that changes in pH_0 did not significantly affect $V_{\frac{1}{2}}$; in addition, neither the steady-state inactivation curve for the slow inward

current nor the activation curve for the outward current were shifted along the membrane potential axis. Thus, these findings lead us to the conclusion that changes in pH_o only affect the maximum conductance of the two current systems.

This experiment has shown that an increase in hyperpolarizing activated current (referred to as either I_f or I_h) with alkalinization of the perfusate accompanies an enhancement of the electrical activity of s.a. node cells (see Figs. 1 and 5). It seems that I_h is actively involved in the generation of pace-maker potential. The relevance of the function of I_h in the generation of pace-maker depolarizing phase is still controversial (reviewed by DiFrancesco, 1985). Brown, DiFrancesco & Noble (1979) have observed that addition of adrenaline causes a substantial increase in this current which occurs at the membrane potential range of the pace-maker potential. On the contrary, Yanagihara & Irisawa (1980) have calculated the extent of participation of I_h to the pace-maker potential on the basis of kinetic analysis and reached a conclusion that I_h plays a smaller role during normal action potential. Since the rest of the ionic currents besides I_h also increase, and a remarkable activation of I_h occurs at a membrane potential which is more hyperpolarized than that of the pace-maker potential in alkaline medium, I_h appears to play an insignificant role in the activity of s.a. node. In order to determine how much I_h is implicated in the response of s.a. node cells to change in pH_o, further in-depth studies are suggested.

Two major mechanisms can be proposed to explain the effects of altering pH_o on the ionic currents of the cardiac membrane: (1) an alteration in the surface potential of the membrane, and (2) a change in conductance due to protonation of the channels. Brown & Noble (1978) and Yatani & Goto (1983) have shown in myocardial cells that acid pH causes the kinetics of the Na^+ and Ca^{2+} currents to shift in their voltage dependence to more positive potentials; this would indicate that a prominent effect of changing pH_o is an alteration of the surface potential, involving either the screening or binding of negatively charged groups associated with the membrane. Their results are well in accord with previous findings in the frog node of Ranvier (Hille, 1968), frog skeletal muscles (Campbell & Hille, 1976), tunicate egg cells (Ohmori & Yoshii, 1977), *Myxicola* axons (Schauf & Davis, 1976) and crayfish giant axons (Schrager, 1974). However, some evidence provides support for the second mechanism mentioned above. Kohlhardt *et al.* (1976), for example, showed in cat papillary muscles that lowering pH_o suppresses the slow inward current without causing shifts in either the *I–V* or steady-state inactivation curves. The present results obtained on rabbit s.a. node cells are clearly in harmony with these observations.

Since the present study has demonstrated that the suppression of g_s and g_K by acid pH is potential independent, it appears plausible to suggest that the gating systems for these channels must be electrically isolated from the influence of surface charge, and that the binding site at which protons block the channels must be located outside the transmembrane field. One interpretation, for example, could be that the binding site actually protrudes from the membrane surface. This notion is in accord with Campbell's (1982) proposal that, in Na^+ channels, the proton binding site is located at or near the mouth of the channel at the external face of the membrane.

Kurachi (1982) was able to change the intracellular H^+ concentration of guinea-pig ventricular cells using the method of pressure injection; the changes in membrane

currents that were observed resemble those found in the present study with s.a. node cells. The findings of Kurachi (1982) suggest that there is a proton binding site located on the membrane's intracellular surface.

The observation of Ellis & Thomas (1976) that the intracellular pH stays relatively constant when pH_o is changed from a value of 7·4 to 6·4 strongly suggests that the intracellular medium has a high buffering capacity. There is also evidence for additional mechanisms present in heart cells that contribute to the regulation of the intracellular H^+ concentration. These include most notably the Na^+–H^+ exchange mechanism (for which there is now convincing evidence in Purkinje fibres (Ellis & MacLeod, 1985) and in cultured chick heart cells (Piwnica-Worms, Jacob, Horres & Lieberman, 1985)), and the high permeability of the membrane to H^+. Despite the similar effects on heart cells of intracellular and extracellular pH changes, it is possible that there are distinct binding sites for internal and external H^+. In order to reach a firm conclusion regarding the site(s) of action of H^+ on the membrane surface, further extensive investigation will be necessary.

During the preparation of our paper, Dr Susan Noble kindly conducted a computer simulation using the computer model, Oxsoft Heart (see Noble & Noble, 1984; DiFrancesco & Noble, 1985). The incorporation of the experimental data into this model successfully reproduces the electrical behaviour of the s.a. node in response to the pH changes described here. Simulation based on the model of Yanagihara, Noma & Irisawa (1980) also gave satisfactory results. We would like to sincerely thank Dr S. Vogel (University of Illinois) for reading the manuscript. Grant support was provided by the Ministry of Health and Welfare of Japan.

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