Sodium–potassium pump current in rabbit sino-atrial node cells

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1. The Na^+–K^+ pump current (I_p) was studied in sino-atrial (SA) node cells of rabbits using the whole-cell patch-clamp technique.

2. With 50 mM Na^+ in the pipette solution ($[Na^+]_{pip}$), changing the external K^+ concentration ($[K^+]_o$) from 0 to 5·4 mM caused the holding current to shift in an outward direction and reach a new steady state. The current–voltage relationships obtained by subtraction of current traces recorded at 0 mM K_o^+ from those recorded at 5·4 mM K_o^+ revealed time-independent and voltage-dependent characteristics. The external K^+-induced current was completely blocked by external application of 10 μM ouabain, indicating the existence of I_p in SA node cells of rabbit heart.

3. I_p increased as $[K^+]_o$ increased. With 30 mM Na^+_{pip}, I_p at 0 mV was activated by $[K^+]_o$ with non-linear least-squares fit parameters for the Hill equation of $K_{0.5}$ of 1·4 mM and a Hill coefficient (n_H) of 1·2 ($n = 7$).

4. The cation dependence of the K^+ site of the Na^+–K^+ pump was examined using various monovalent cations. The sequence was $K^+ \geqslant Rb^+ > Cs^+ >>> Li^+$.

5. I_p at 0 mV also increased as $[Na^+]_{pip}$ was increased from 10 to 150 mM at 5·4 mM K_o^+, with a $K_{0.5}$ value of 14 mM and a n_H of 1·3 ($n = 54$).

6. I_p at 0 mV was reduced by lowering the temperature from 37 to 25 °C with 30 mM Na^+_{pip} and 5·4 mM K_o^+. The temperature coefficient (Q_{10}) for I_p was 2·1 ($n = 27$).

7. With 10 mM Na^+_{pip} and 5·4 mM K_o^+, the half-activation voltage of I_p was -52 ± 16 mV and the current at this voltage was $22{\cdot}5 \pm 3{\cdot}5$ pA ($n = 10$), indicating that I_p contributes significantly to the background outward current during the normal pacemaker potential of SA node cells.

The pacemaker potential of the mammalian sino-atrial (SA) node cell has been investigated mainly with regard to time- and voltage-dependent ionic currents such as the delayed rectifier K^+ current (I_K), calcium current (I_{Ca}) and hyperpolarization-activated current (I_h or I_f) (for review see Irisawa, Brown & Giles, 1993). Recently, however, the presence of a time-independent background current has been reported and it has been shown that a Na^+-dependent inward current makes a significant contribution to pacemaker depolarization (Hagiwara, Irisawa, Kasanuki & Hosoda, 1992). This background current is non-selective for monovalent cations and has a reversal potential of −20 mV under normal ionic conditions. Thus, these observations indicate that a background inward current of substantial amplitude contributes to pacemaker depolarization.

Counteracting the depolarizing effect of this background inward current, there may be an outward current component either in the form of a Na^+–K^+ pump current (I_p) or a background outward current. The electrogenic nature of the Na^+–K^+ pump is well established: one forward cycle gives an outward current because it results in the extrusion of three Na^+ and the uptake of two K^+. The method for isolating I_p was developed in guinea-pig ventricular cells (Gadsby, Kimura & Noma, 1985; Gadsby & Nakao, 1989; Nakao & Gadsby, 1989). Although I_p has been investigated in multicellular preparations derived

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from the mammalian SA node (Noma & Irisawa, 1974, 1975) or atrio-ventricular (AV) node (Kurachi, Noma & Irisawa, 1981), its characteristics in single SA node cells have yet to be clarified. In the present study, we investigated both the characteristics of I_p and its contribution to the pacemaker potential of rabbit single SA node cells under controlled ionic conditions. We found that I_p contributes to the pacemaker potential as a time-independent background outward current that counteracts the background inward current in SA node cells.

METHODS

Cell isolation procedure

The methods of cell isolation and the enzymes used were the same as those described elsewhere (Hagiwara, Irisawa & Kameyama, 1988; Hagiwara *et al.* 1992). Briefly, an albino rabbit was fully anaesthetized with an intravenous injection of sodium pentobarbitone (40 mg kg^{-1}) with heparin (300 units kg^{-1}). After the heart had been dissected out, the SA node region was isolated, cut into pieces and incubated in warm Tyrode solution for 10 min. The strips were then transferred to a nominally Ca^{2+}-free Tyrode solution containing 350 units ml^{-1} collagenase (Yakult, Tokyo, Japan) with 0·5 mg ml^{-1} elastase (Type II-A; Sigma) for 60 min at 37 °C. The collagenase was then washed out by rinsing with high-K^+ low-Cl^- solution (Isenberg & Klöckner, 1982); the cells were then stored in the same solution.

Electrical recordings

The whole-cell voltage-clamp methods used were the same as those described previously (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Hagiwara *et al.* 1988). The resistance of the electrodes when filled with the pipette solution was 2–2·5 MΩ. Current and voltage signals were stored on a video recorder (SR-5050; Victor, Tokyo, Japan) using a PCM converter system (RP-880; NF Electronic Circuit Design, Tokyo, Japan) for computer analysis (PC 9801 RA; NEC, Tokyo, Japan). From a holding potential of −30 mV, ramp pulses of amplitude ±70 mV (0·93 V s^{-1}) or ±90 mV (1·2 V s^{-1}) were applied every 10–20 s. The amplifier employed was a whole-cell clamp system amplifier (TM-1000; ACT ME Laboratory, Tokyo, Japan); the feedback resistance was 100 MΩ and series resistance was partially compensated. Current signals were fed from the video recorder to the computer via a 2·5 kHz, 8-pole Bessel-type low-pass filter. For the illustrations, five to seven current–voltage (I–V) curves were averaged. To alter the Na^+ concentration of the patch pipette solution ($[Na^+]_{pip}$) during whole-cell recording, we used the perfusion technique described in detail by Soejima & Noma (1984).

Solutions

The standard 30 mM Na^+_{pip} solution contained (mM): CsOH, 90; NaOH, 30; CsCl, 20; aspartic acid, 100; EGTA, 10; $MgCl_2$, 2; MgATP, 5; potassium creatine phosphate, 5; and Hepes, 5 (pH = 7·4, adjusted with CsOH). $[Na^+]_{pip}$ was varied by equimolar substitution of Na^+ for Cs^+. The standard external solution contained (mM): NaCl, 145; Hepes, 5; $MgCl_2$, 0·5; $NiCl_2$, 2 (to block I_{Ca} and the Na^+–Ca^{2+} exchange current); CsCl, 2 (to block I_h/I_f); and $BaCl_2$, 1 (to block I_K); $CaCl_2$ was not included. In some experiments, CsCl was omitted from the standard external solution. The external K^+ concentration ($[K^+]_o$) was varied from 0·5 to 10 mM by addition of KCl. When the external cation dependence of I_p was to be examined, external KCl was omitted and replaced with equimolar RbCl, CsCl or LiCl (Nacalai tesque, Kyoto, Japan). External 150 mM Li^+ solution contained (mM): LiCl, 150; Hepes, 5; $NiCl_2$, 2; CsCl, 2; $BaCl_2$, 1; and $MgCl_2$, 0·5. When the strophanthidin-sensitive nature of I_p at 150 mM Li^+ was to be examined, CsCl was omitted from this solution. Either 10 μM ouabain or 0·5 mM strophanthidin (both obtained from Sigma) was added to block I_p. All experiments were performed at 37 ± 0·5 °C unless otherwise stated. Wherever possible, values are presented as means ± S.D., the number of experiments being denoted by n.

RESULTS

Existence of a time-independent outward Na^+–K^+ pump current in SA node cells

In 5·4 mM K^+_o standard external solution containing appropriate blockers and when $[Na^+]_{pip}$ was increased by loading 50 mM Na^+ via the patch pipette, a steady outward current was recorded at a holding potential of −40 mV (Fig. 1*A*). This outward current was completely blocked by application of 10 μM ouabain, indicating the existence of a Na^+–K^+ pump current (I_p) in SA node cells. Figure 1*B* shows current traces evoked by step pulses from a holding potential of −40 mV to +50 mV (i and iv), 0 mV (ii and v) or −90 mV (iii and vi), before (*a*) and during (*b*) exposure to 10 μM ouabain. At this holding potential, the amplitude of the outward current was 120 pA, but it was virtually abolished by the application of 10 μM ouabain. The ouabain-sensitive current was revealed by computer subtraction of current traces recorded in its absence (*c*). The traces in Fig. 1*Bc* show its time-independent characteristics. Figure 1*Ca* shows I–V relationships for the steady-state current levels. These were plotted using data at 200 ms from the experiment illustrated in Fig. 1*B* before (●) and during (○) exposure to 10 μM ouabain. Figure 1*Cb* also shows the I–V relationship obtained by subtracting these two currents. The ouabain-sensitive outward current saturated at around 0 mV and declined below −50 mV, indicating its voltage-dependent nature. As it showed time independence, for the rest of this study we measured I–V relationships using the ramp-clamp pulse method.

Comparison of the ouabain-sensitive current with the outward current activated by external K^+

Figure 2*A* shows a chart record of a current trace recorded at a holding potential of −30 mV, and illustrates the method of evaluation of I_p. The chart record reveals that the outward current activated by external K^+ with 5·4 mM K^+_o and 50 mM Na^+_{pip} was 120 pA and that it remained nearly constant for some 4 min.

Figure 2*B* shows I–V relationships obtained using ramp clamps, in 5·4 mM K^+_o (*a*), 0 mM K^+_o (*b*) and during exposure to 5·4 mM K^+_o with 10 μM ouabain (*c*), which completely blocked the outward current. Figure 2*Bc* shows

that ouabain reduced the current at 0 mV by 6 pA compared with that recorded in 0 mM K_o^+ (Fig. 2*B b*). Some monovalent cations including Cs^+ (Kurachi *et al.* 1981) have been reported to be activators of the K^+ site of the Na^+–K^+ pump. Since the external solutions used in this experiment contained 2 mM Cs^+, the small outward current that remained in the K^+-free solution was probably due to I_p activated by the external Cs^+. However, both *I–V* relationships obtained by subtraction, for the external K^+-induced current (Fig. 2*C*, *a* − *b*) and the ouabain-sensitive current (Fig. 2*D*, *a* − *c*), saturated at around 0 mV and decreased progressively between −50 and −100 mV. The voltage dependence of I_p was thus clearly demonstrated using the ramp-clamp method.

Since it was uncertain whether external K^+ evoked the same pump activity in the presence and absence of 2 mM Cs^+, we compared the current density and the voltage dependence of I_p under these two conditions. As shown in Fig. 3*A b*, superfusing the cell with 5·4 mM K^+ and 2 mM Cs^+ evoked an outward current which was deactivated by removing the external activator cations, K^+ and Cs^+. In addition, I_p was reversibly activated by external K^+ without Cs^+, this current being completely blocked by the application of 0·5 mM strophanthidin (Fig. 3*A c* and *d*). In the absence of 2 mM Cs^+, the inward current at negative potentials (Fig. 3*C c* and *d*) was increased, due to an activation of I_h/I_f, over and above that recorded in the presence of 2 mM Cs^+ (Fig. 3*B a* and *b*). *I–V* relationships

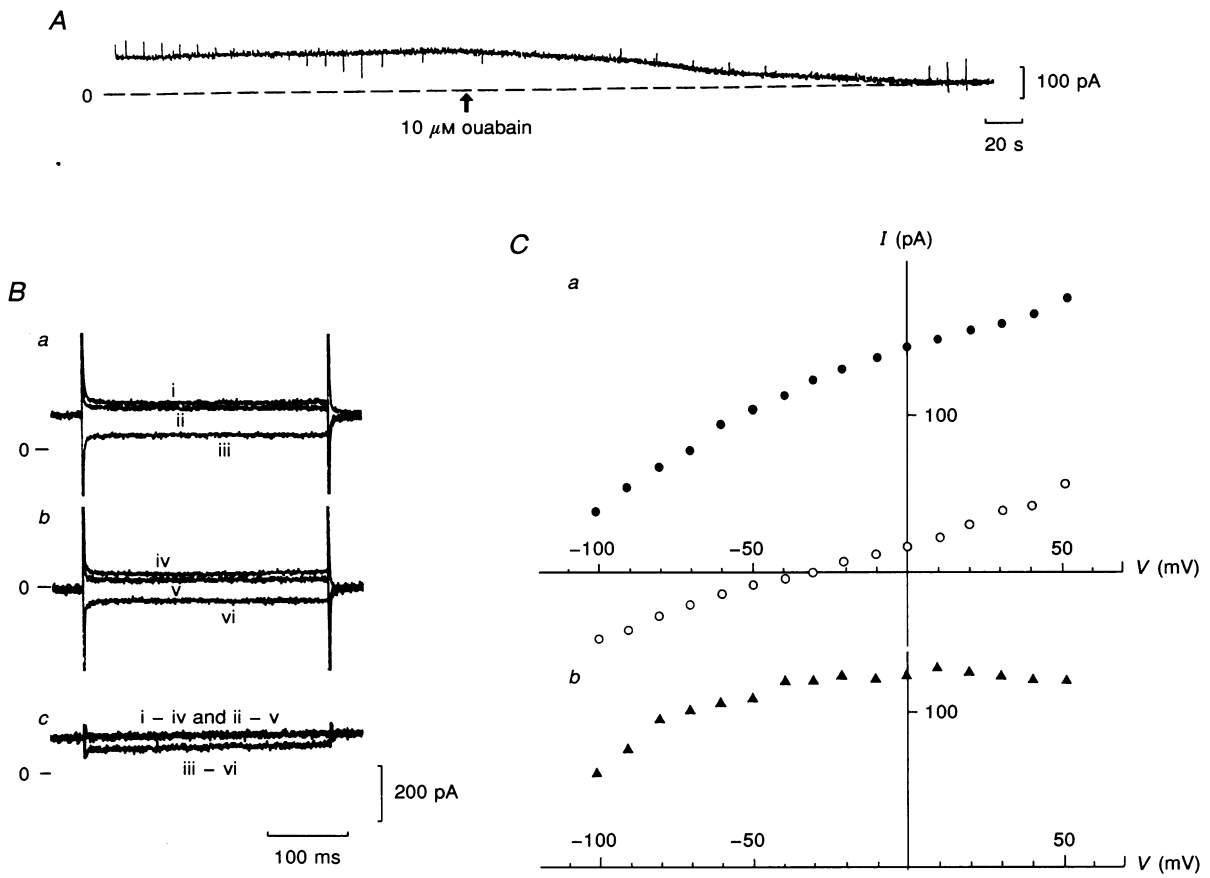

Figure 1. The presence of a Na^+–K^+ pump current in SA node cells

A, chart recording of the holding current with 50 mM Na^+_{pip} and 5·4 mM K^+ standard external solution. The dashed line below the current trace indicates the zero current level. Vertical excursions on the current trace indicate currents evoked by step pulses to various membrane potentials from a holding potential of −40 mV. Ouabain (10 μM) was added at the time indicated by the arrow. *B*, currents evoked by step pulses from a holding potential of −40 mV to +50 mV (i and iv), 0 mV (ii and v) and −90 mV (iii and vi) before (*a*) and during (*b*) exposure to 10 μM ouabain. *c*, difference currents (*a* − *b*), which represent ouabain-sensitive currents. *C*, *I–V* relationships for the steady-state current at 200 ms. *a*, before (●) and during (○) exposure to ouabain; *b*, the ouabain-sensitive current (▲).

were then obtained by subtraction: in the presence of 2 mM Cs^+, the current recorded with 0 mM K^+ (*a*) was subtracted from that recorded with 5·4 mM K^+ (*b*) and, in the absence of Cs^+, the current recorded with 5·4 mM K^+ plus 0·5 mM strophanthidin (*d*) was subtracted from that recorded with 5·4 mM K^+ alone (*c*). These two currents, the K^+-induced current ($b - a$) and the strophanthidin-sensitive current ($c - d$), were virtually superimposed on each other (Fig. 3*D*). This suggests that the current density and the voltage dependence of I_p were not changed by the presence of 2 mM Cs^+. In five experiments, the current density of the K_o^+-induced current at 0 mV was 1·50 ± 0·19 pA pF^{-1} and that of the strophanthidin-sensitive current was 1·49 ± 0·21 pA pF^{-1}, also at 0 mV. For this reason, the K_o^+-induced current in the presence of 2 mM Cs^+, and the strophanthidin-sensitive current in the absence of 2 mM Cs^+ were used interchangeably in our further analysis of I_p in SA node cells.

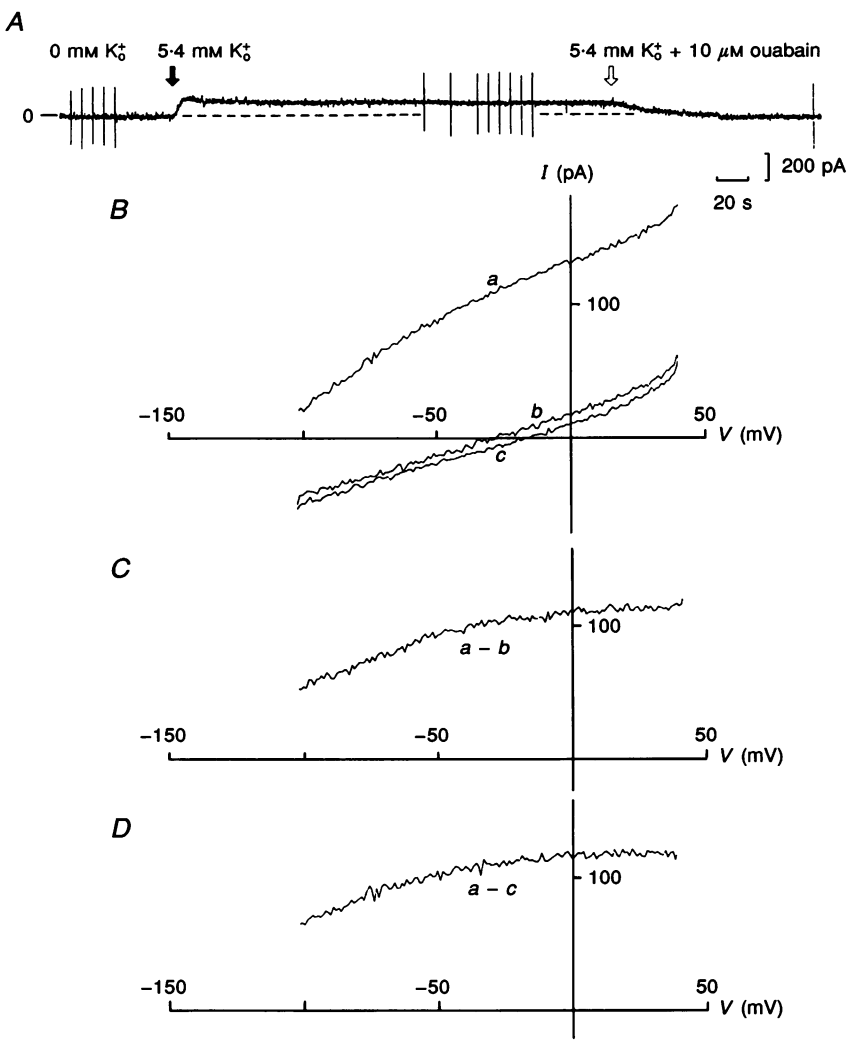

Figure 2. Na^+–K^+ pump current revealed by ramp-clamp pulses

$[Na^+]_{pip}$ was 50 mM and the standard external solution containing 2 mM Cs^+ was used. *A*, slow chart recording of current traces. The dashed line indicates the zero current level. Vertical excursions on the current trace indicate currents evoked by ramp pulses of ±70 mV from a holding potential of −30 mV. Activation of the Na^+–K^+ pump was initiated by superfusion of the cell with 5·4 mM K^+ standard external solution (filled arrow) after 5 min in a K^+-free standard external solution. Ouabain (10 μM) was added to the external solution at the time indicated by the open arrow. *B*, *I–V* relationships in 5·4 mM K^+ standard external solution (*a*), K^+-free standard external solution (*b*) and after application of 10 μM ouabain in 5·4 mM K_o^+ (*c*). *C*, external K^+-induced current in the presence of 2 mM Cs^+ (obtained by subtraction ($a - b$); 110 pA at 0 mV). *D*, ouabain-sensitive current in the presence of 2 mM Cs^+ (obtained by subtraction ($a - c$); 116 pA at 0 mV).

Effect of varying $[K^+]_o$ on I_p

The effect on the steady-state I–V relationship of varying $[K^+]_o$ between 0·5 and 10 mM was examined in cells equilibrated with 30 mM Na^+_{pip}. The chart recording in Fig. 4*A a* shows that the magnitude of the outward shift in the holding current at −30 mV was related to the value of $[K^+]_o$ that induced it. Figure 4*A b* shows representative I_p–V relationships for the strophanthidin-sensitive Na^+–K^+ pump current from this experiment. There was an outward shift of the I_p–V relationship, which corresponded to the increment in $[K^+]_o$. The graph (Fig. 4*B*) summarizes our data on the strophanthidin-sensitive current, the analysis being limited to I_p measured at 0 mV. The relative I_p for each level of $[K^+]_o$ was estimated by normalizing the magnitude of the I_p obtained at each test $[K^+]_o$ with respect to the mean I_p at 5 mM K^+_o. The data obtained from

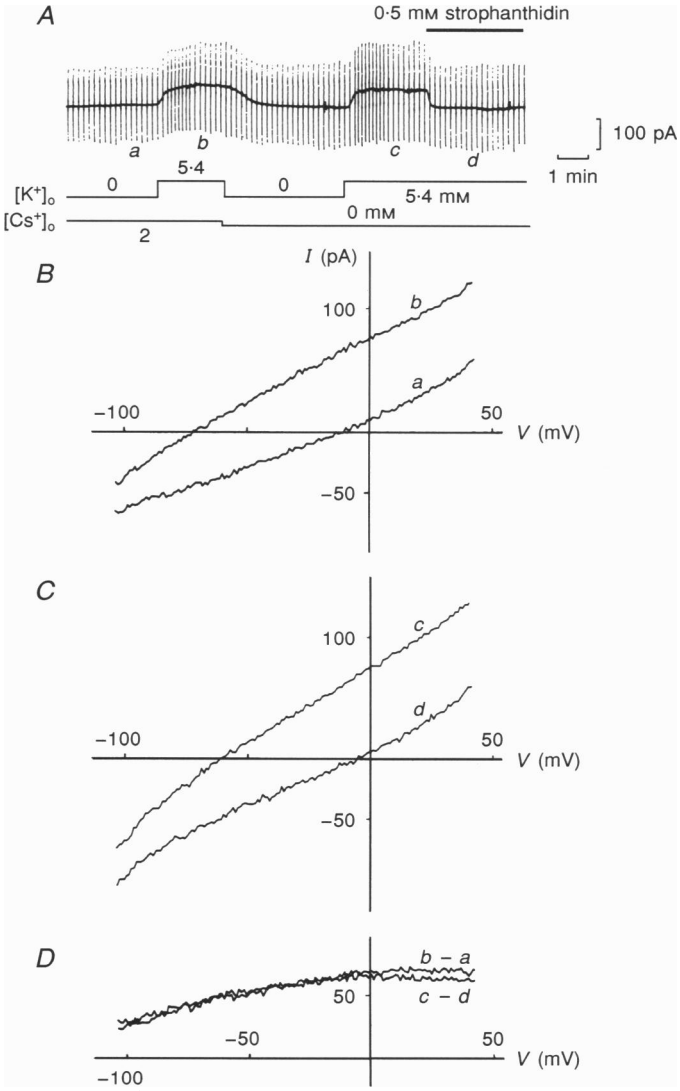

Figure 3. Comparison of external K^+-induced current in the presence of external Cs^+ with strophanthidin-sensitive current in its absence

A, chart recording of current traces with 30 mM Na^+_{pip}. Lines beneath the record indicate changes made in $[K^+]_o$ and external Cs^+ concentration. Vertical excursions on the current trace indicate currents evoked by ramp pulses of ±70 mV from a holding potential of −30 mV. The horizontal bar over the current trace marks the period of exposure to 0·5 mM strophanthidin. *B*, I–V relationships in the presence of 2 mM Cs^+ in 0 mM K^+_o (*a*) and 5·4 mM K^+_o (*b*). *C*, I–V relationships in the absence of Cs^+. *c*, in 5·4 mM K^+_o; *d*, after application of 0·5 mM strophanthidin in 5·4 mM K^+_o. *D*, graphs indicating external K^+-induced current (*b* − *a*) and strophanthidin-sensitive current (*c* − *d*).

seven cells were well fitted by the Hill equation:

$$\text{Relative current} = \frac{\text{Maximal relative current}}{1 + (K_{0.5}/[Na^+]_{pip})^{n_H}},$$

the values of $K_{0.5}$ and the Hill coefficient (n_H) being 1·4 mM and 1·2, respectively, and the maximal relative current at saturating $[K^+]_o$ being 1·8 times that at 5 mM K_o^+. We also checked the effects of $[K^+]_o$ on the K_o^+-induced current in the presence of 2 mM Cs^+ using the method shown in Fig. 3*D* (*b* − *a*). In this case also, the results from five cells were well described by the Hill equation and the parameters were similar to those obtained for the strophanthidin-sensitive current ($K_{0.5}$ = 1·3 mM; n_H = 1·1; results not illustrated).

Activation of I_p by external activator cations

To test whether other monovalent cations could activate the K^+ site of the Na^+–K^+ pump, as reported previously for the AV node (Kurachi *et al.* 1981), external K^+ was omitted and replaced with one of a number of monovalent cations, namely Cs^+, Rb^+ or Li^+, in the same cell using 30 mM Na^+_{pip}. Figure 5*A* shows a chart recording of the currents recorded in K^+-free solution (i; Control), in 5 mM Cs^+ (ii), in 5 mM K^+ (iii) and in 5 mM Rb^+ (iv). All solutions contained 2 mM Cs^+, except that containing 5 mM Cs^+. The outward current increased in the order: Cs^+, $K^+ = Rb^+$. Since the evoked outward current was completely blocked by either strophanthidin or ouabain, we concluded that both the Cs^+- and Rb^+-induced outward currents were

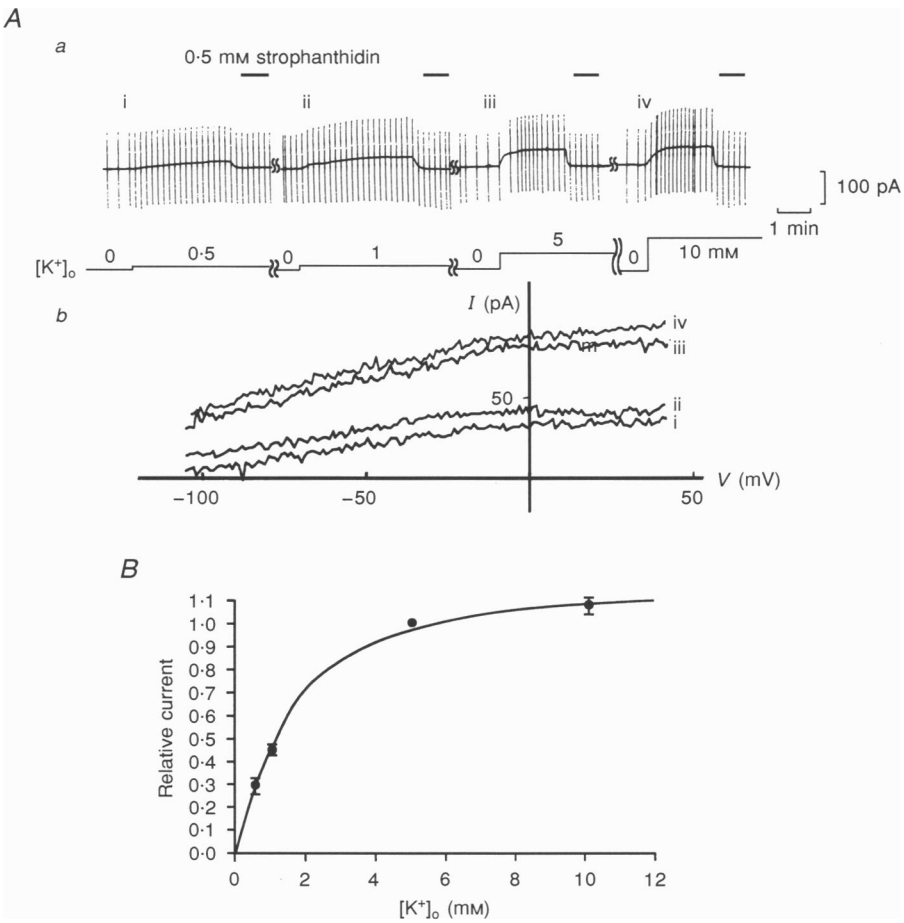

Figure 4. Effect of varying $[K^+]_o$ on strophanthidin-sensitive current

A a, chart recording of current traces with 30 mM Na^+_{pip}. The line beneath the trace indicates changes made in $[K^+]_o$. Cs^+ was omitted from all external solutions. Vertical excursions on the current trace indicate the currents evoked by ramp pulses of ±70 mV from a holding potential of −30 mV. The horizontal bars above the current trace mark periods of exposure to 0·5 mM strophanthidin. *A b*, *I–V* relationships at various $[K^+]_o$: 0·5 (i), 1 (ii), 5 (iii) and 10 (iv) mM K^+. At each $[K^+]_o$, the relationships were obtained by subtraction of currents recorded in the presence of strophanthidin from those in its absence. *B*, amplitudes of Na^+–K^+ pump currents obtained from 7 cells at 0 mV and various $[K^+]_o$ were normalized, the current density at 5 mM K_o^+ being taken as 1, and plotted against $[K^+]_o$. Values are means ± S.D. The Hill equation was then fitted to the data; maximal relative current was 1·18, $K_{0.5}$ was 1·4 mM and n_H was 1·2.

actually I_p. Figure 5*Ba* and *b* illustrate *I–V* relationships recorded under control conditions (i), and with K^+ (iii), Rb^+ (iv), Cs^+ (v) and Cs^+ with 0·5 mM strophanthidin (vi). Figure 5*Bc* shows representative I_p–*V* relationships for each monovalent cation, namely K^+ (iii – i), Cs^+ (v – vi) and Rb^+ (iv – i). I_p activated by external Cs^+ was identified as a strophanthidin-sensitive current. I_p activated by each of the other cations was assessed by subtraction of the current recorded in standard external solution from that recorded in the presence of the relevant monovalent cation. I_p values calculated on activation at 0 mV by 5 mM K^+, Rb^+ and Cs^+ were 1·47 ± 0·23, 1·38 ± 0·10 and 0·56 ± 0·16 pA pF^{-1}, respectively ($n = 7$). In Fig. 6*A*, the chart recording shows the outward current induced by external Li^+ in the presence of 2 mM Cs^+ at the holding potential of −30 mV. This outward current increased as the concentration of external Li^+ was increased from 5 to 150 mM. Figure 6*Bb* shows I_p–*V* relationships obtained by subtracting the currents in Li^+-free standard external solution from those in Li^+-containing solutions (5 mM Li^+ (ii – i); 150 mM Li^+ (iii – i)). The current density of I_p activated by 5 mM Li^+ at 0 mV was 0·10 ± 0·02 pA pF^{-1} ($n = 6$), whereas it was 0·90 ± 0·16 pA pF^{-1} ($n = 5$) in 150 mM Li^+. Since it was unclear whether the weak activator cation, Li^+, would cause the same activation of the Na^+–K^+ pump in media with or without 2 mM Cs^+, we also examined the affinity of Li^+ for the Na^+–K^+ pump in the absence of external Cs^+. Figure 6*C* illustrates I_p–*V* relationships for the strophanthidin-sensitive current with 5 mM Li^+ (lower trace) and 150 mM Li^+ (upper trace) without Cs^+. Although this experiment was on a different cell from that used to obtain the above data, the current

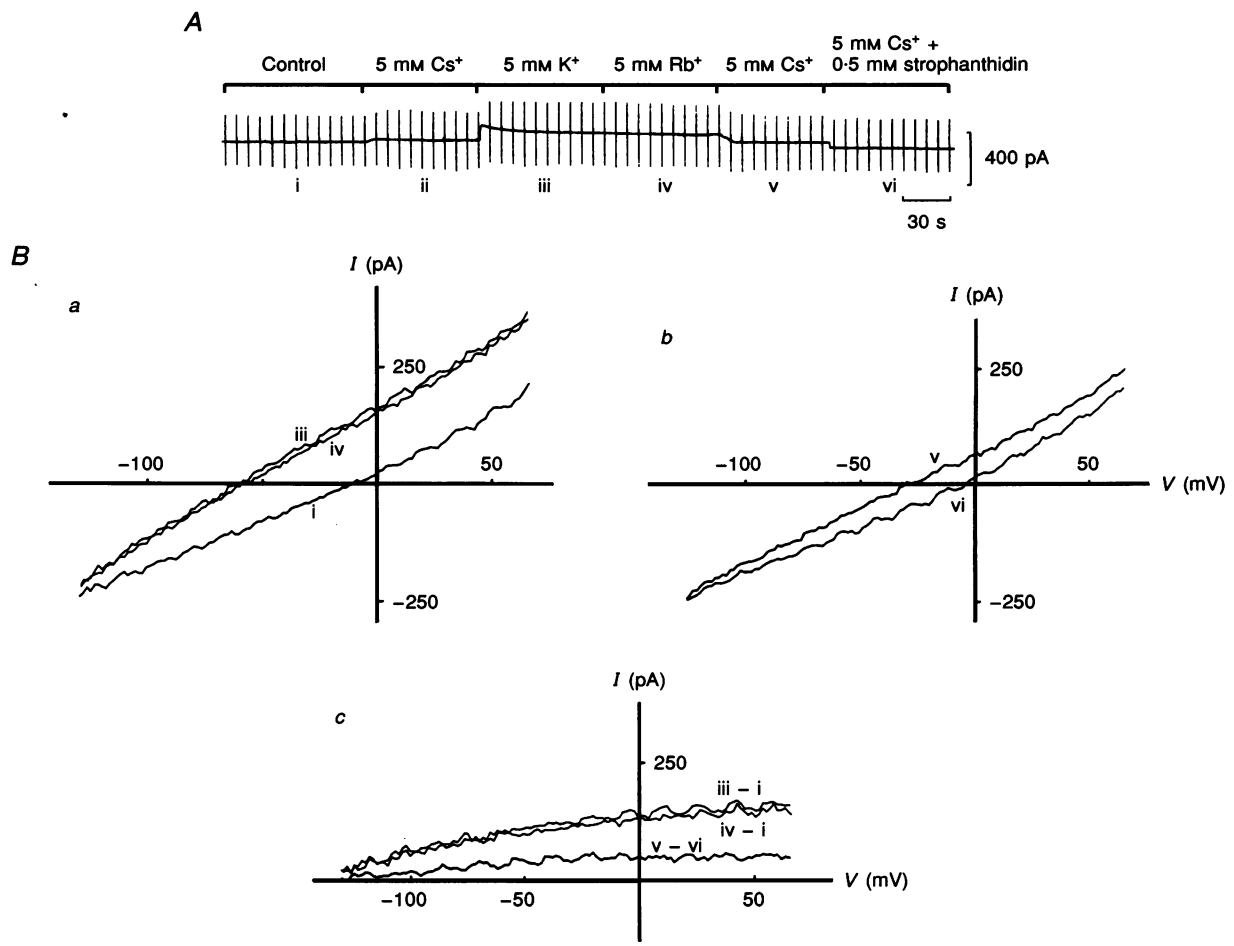

Figure 5. Cation dependence of the K_o^+ binding site of the Na^+–K^+ pump

A, chart recording revealing the activities of the Na^+–K^+ pump in the presence of various external cations: K^+ free (i; Control); 5 mM Cs^+ (ii and v); 5 mM K^+ (iii); 5 mM Rb^+ (iv); and 5 mM Cs^+ with 0·5 mM strophanthidin (vi); at 30 mM Na^+_{pip}. Vertical excursions on the current trace indicate currents evoked by ramp pulses of ±90 mV from a holding potential of −30 mV. *Ba* and *b*, *I–V* relationships obtained from *A*. *c*, K^+- (iii – i), Cs^+- (v – vi) and Rb^+- (iv – i) activated currents, taken to be the Na^+–K^+ pump current activated by each external cation.

density of I_p at 5 mM Li^+ without Cs^+ was similar (0·11 ± 0·03 pA pF^{-1}; $n = 10$).

Taking the current density of I_p in 5 mM K^+ as unity, the relative strength of the Na^+–K^+ pump activation by 5 mM external cations at 0 mV was determined to be: 1 : 0·94 : 0·38 : 0·07 for K^+, Rb^+, Cs^+ and Li^+, respectively. We obtained the same sequence of cation dependence when we analysed the strophanthidin-sensitive current without Cs^+ ($n = 5$; results not shown).

Influence of $[Na^+]_{pip}$ on amplitude of I_p

It has already been established in ventricular and Purkinje cells that I_p is dependent on both $[K^+]_o$ and $[Na^+]_{pip}$. Figure 7*A* shows *I–V* relationships obtained at various levels of $[Na^+]_{pip}$, namely 10 mM (*a*), 30 mM (*b*) and 50 mM (*c*). The upper panels illustrate results obtained in 5·4 mM K_o^+ (i) and in K^+-free standard external solution (ii). The lower panels illustrate I_p–*V* relationships determined by subtraction (i − ii). The amplitude of I_p at 0 mV increased as $[Na^+]_{pip}$ was increased. The current density of I_p at 0 mV with 10 mM Na^+_{pip} was 0·80 ± 0·24 pA pF^{-1} ($n = 10$), whereas with 30 mM Na^+_{pip} it was 1·49 ± 0·20 pA pF^{-1} ($n = 22$) and with 50 mM Na^+_{pip} it was 1·59 ± 0·38 pA pF^{-1} ($n = 9$). A non-linear least-squares fit of the Hill equation to the results obtained from fifty-four cells exposed to 5·4 mM K_o^+ solution yielded a maximal relative current of 1·2, a $K_{0.5}$ of 14·0 mM and an n_H of 1·3 (Fig. 7*B*). In 10 mM Na^+_{pip}, the half-activation voltage of I_p was −52 ± 16 mV and the current at this voltage was 22·5 ± 3·5 pA ($n = 10$).

Temperature sensitivity of I_p

In the experiments illustrated in Fig. 8, we examined the effect of temperature on I_p at 30 mM Na^+_{pip} by changing the temperature of the 5·4 mM K^+ standard external solution between 25 and 37 °C. The current trace in Fig. 8*A* shows that a 10 °C increase in temperature caused an outward shift of the holding current. Figure 8*B* shows the subtracted I_p–*V* relationships for this cell. I_p at 0 mV was 34 pA at 27 °C (*a* and *c*) and 72 pA at 37 °C (*b*). I_p at 0 mV was measured at each temperature and plotted semi-logarithmically against temperature (Fig. 8*C*). Using the data from these experiments, the temperature coefficient, Q_{10}, of I_p was calculated to be 2·1. Although Hepes buffer is

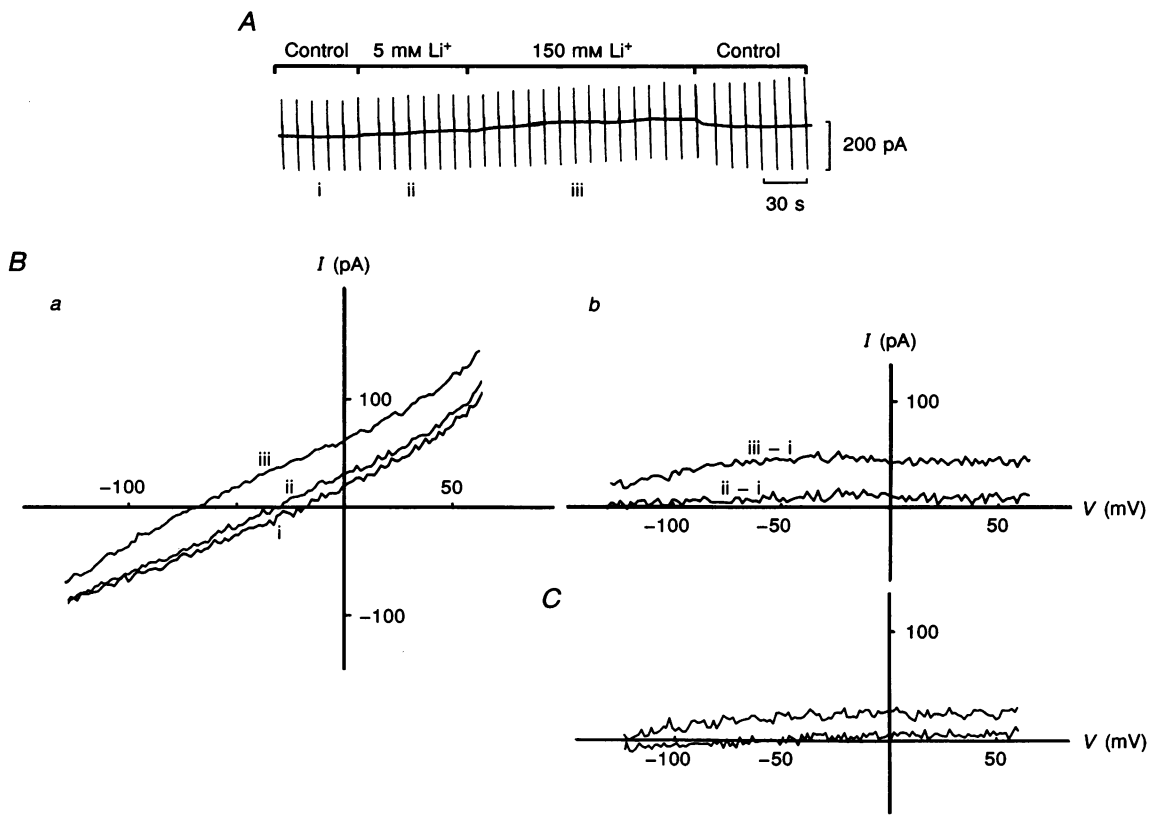

Figure 6. Affinity of Li^+ for the Na^+–K^+ pump

A, chart recording of current in standard external solution (i), and solutions containing 5 mM Li^+ (ii) and 150 mM Li^+ (iii) at 30 mM Na^+_{pip}. Vertical excursions on the current trace indicate currents evoked by ramp pulses of ±90 mV from a holding potential of −30 mV. *Ba*, *I–V* relationships obtained from *A*. *b*, subtracted currents (ii − i) and (iii − i), which indicate Na^+–K^+ pump currents in 5 and 150 mM Li^+, respectively. *C*, the strophanthidin-sensitive currents with 150 mM Li^+ (upper trace) and 5 mM Li^+ (lower trace) in the absence of Cs^+. $[Na^+]_{pip}$ was 30 mM. This experiment was performed on a different cell to that used in *A* and *B*.

temperature sensitive (the pH of the solution we used decreased from 7·43 to 7·32 on raising the temperature from 27 to 37 °C), I_p was only slightly affected by such a pH change under the conditions of the present study (results not illustrated).

DISCUSSION

Existence of a Na^+–K^+ pump in SA node cells

Since it is well established that not only time- and voltage-dependent ionic currents, but also time-independent currents, contribute to underlying electrical activity in SA node cells (Hagiwara *et al.* 1992), investigation of the properties of I_p is necessary in order to obtain a more complete understanding of normal pacemaker activity. In the present study, increasing $[Na^+]_{pip}$ by loading 50 mM Na^+ via the patch pipette elicited a steady outward current which was completely blocked by external application of 10 μM ouabain (Figs 1 and 2) or 0·5 mM strophanthidin. These results indicate that I_p is present in SA node cells.

To isolate I_p in SA node cells, it is necessary to block all time-dependent currents, such as I_K, I_{Ca} and I_h/I_f, using Ba^{2+}, Ni^{2+} and Cs^+. Although external Cs^+ is known to activate the Na^+–K^+ pump (Kurachi *et al.* 1981), our data revealed that the K_o^+-induced current in the presence of 2 mM Cs^+ was virtually identical to the strophanthidin-sensitive current in terms of its amplitude and voltage dependence (Figs 3–6). In an attempt to avoid the 'run-down' of I_p, which reflects the loss of a functional Na^+–K^+ pump (time constant = 20 min; Gadsby & Nakao, 1989), we usually identified I_p by its nature as a K_o^+-induced current, instead of as a strophanthidin- or ouabain-sensitive current. Using this method, we could repeatedly observe rapid Na^+–K^+ pump activation during short experimental periods without appreciable run-down.

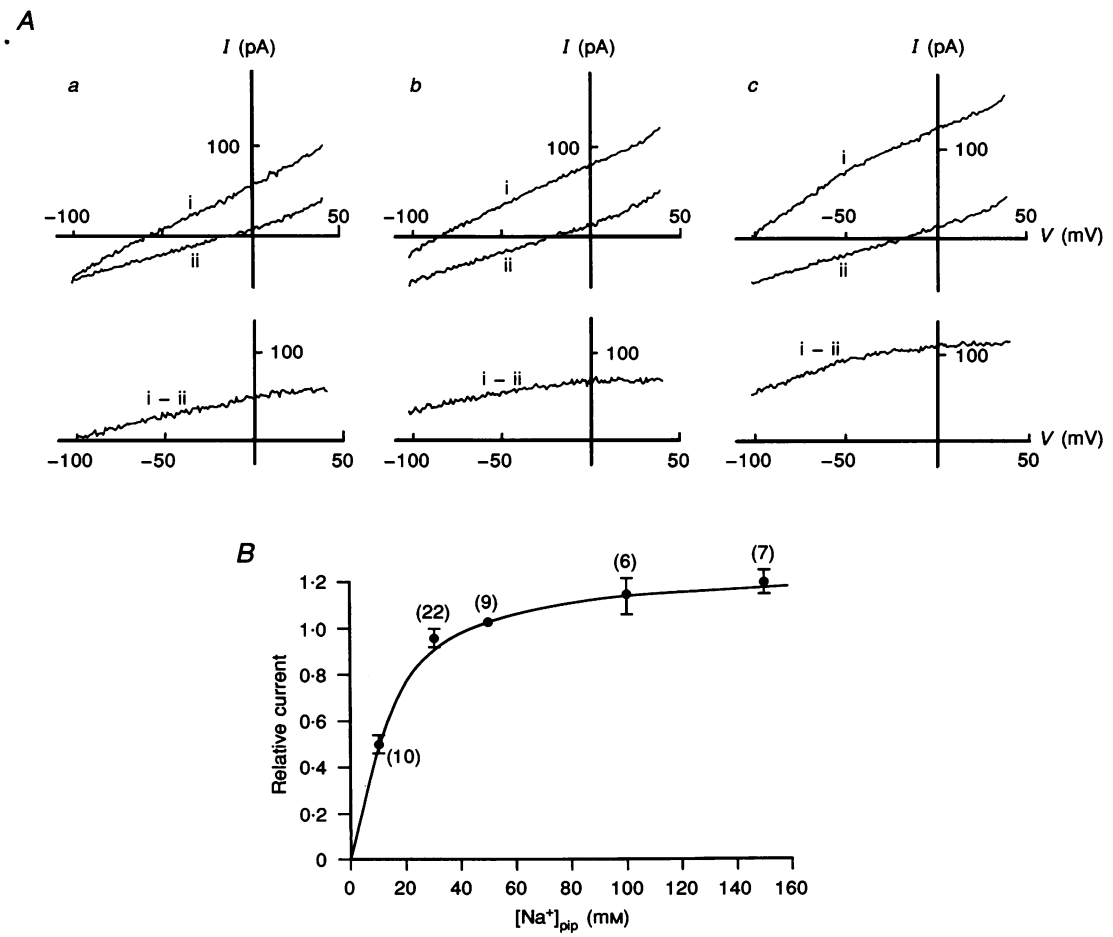

Figure 7. Na^+–K^+ pump current at various $[Na^+]_{pip}$

A, *I–V* relationships obtained from a single cell. $[Na^+]_{pip}$ was 10 mM (*a*), 30 mM (*b*) or 50 mM (*c*) in 5·4 mM K_o^+ (i) or K^+-free (ii) standard external solution. The lower panels show *I–V* relationships obtained by subtraction (i – ii). *B*, the Na^+–K^+ pump current at 0 mV was normalized, assuming that the current density in 50 mM Na^+_{pip} was unity, and plotted against $[Na^+]_{pip}$. The continuous curve drawn through the points obtained from 54 cells is the best fit of the Hill equation. This gave a maximal relative current of 1·2, a $K_{0.5}$ of 14·0 mM and an n_H of 1·3. Values are means ± S.D. Numbers in parentheses indicate the number of experiments.

The outward current appears to develop to a maximal level at potentials more positive than 0 mV. In the present experiments, using 50 mM Na^+_{pip} with 5·4 mM K^+_o, the current density of I_p was 1·59 ± 0·38 pA pF^{-1} ($n = 9$) at 0 mV. Gadsby & Nakao (1989) found that the mean I_p density at +40 mV was 1·1 ± 0·1 pA pF^{-1} at 50 mM Na^+_{pip} in guinea-pig ventricular cells. Glitsch, Krahn & Pusch (1989) found that I_p density at +20 mV with 10 mM Na^+_{pip} was 0·87 ± 0·35 $\mu A\ cm^{-2}$ in sheep Purkinje fibres. This value is in good accord with our data in SA node cells (0·80 ± 0·24 pA pF^{-1}). We also obtained similar values in single rabbit atrial cells (results not described) and came to the conclusion that the current density of I_p in SA node cells is similar to that in other cardiac myocytes.

Na^+–K^+ pump activation by extracellular K^+ and intracellular Na^+

I_p activation by $[K^+]_o$ was examined and a $K_{0.5}$ value of 1·4 mM determined in 30 mM Na^+_{pip} at 0 mV, using a holding potential of −30 mV. In previous studies using multicellular preparations the values reported were around 5–7 mM in canine Purkinje fibres and rabbit AV node (Eisner & Lederer, 1980; Kurachi *et al.* 1981). The higher values in multicellular preparations suggest that such experiments are complicated by the serious problems of K^+ accumulation and depletion in the extracellular spaces. Using single-cell preparations, it is possible to control both external and internal ionic conditions. Recent experiments using single cells have produced similar values of $K_{0.5}$ to

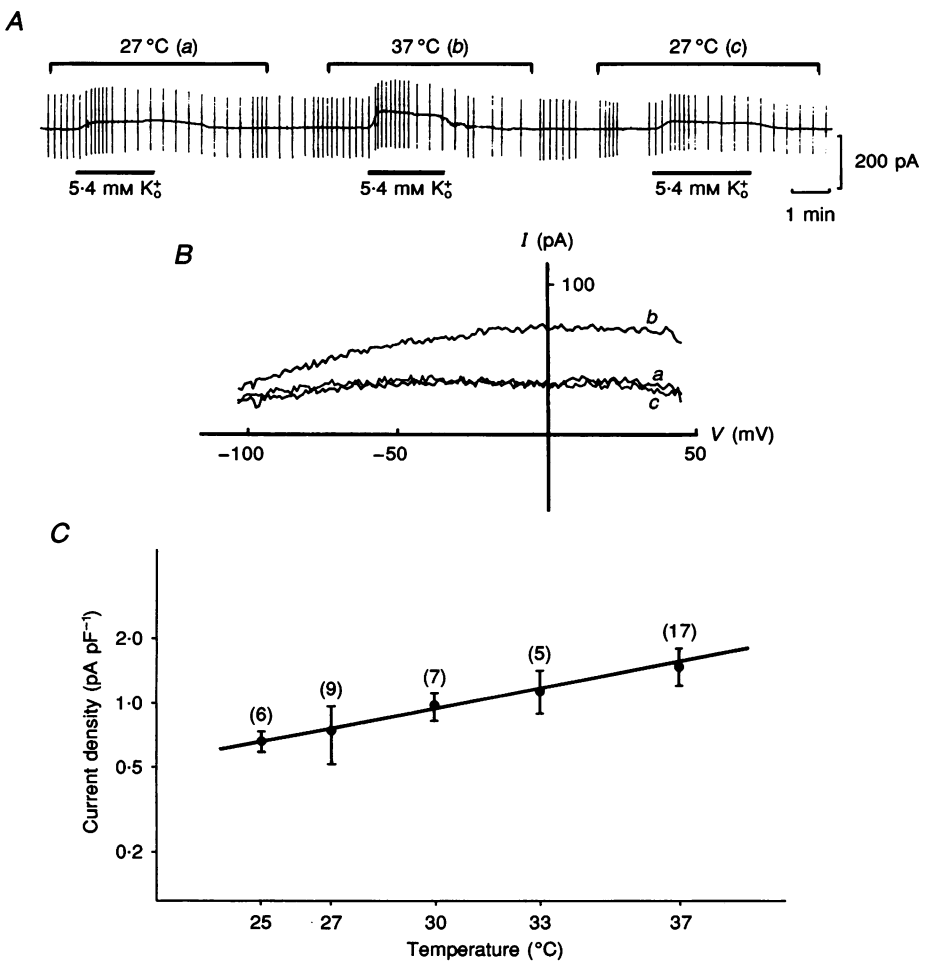

Figure 8. Temperature dependence of the Na^+–K^+ pump current

A, chart recording of current at 30 mM Na^+_{pip} and 5·4 mM K^+_o. Horizontal bars below the current trace indicate periods of exposure to 5·4 mM K^+ standard external solution at each temperature. Vertical excursions on the current trace indicate currents evoked by ramp pulses of ±70 mV from a holding potential of −30 mV. The temperature was gradually changed from *a* to *b*, and from *b* to *c*. The holding current shifted further in the outward direction during perfusion at 37 °C (*b*) than at 27 °C (*a* and *c*). *B*, subtracted currents at each temperature (obtained from *A*). The current change was fully reversed by decreasing the temperature from 37 °C (*b*) to the initial value of 27 °C (*c*). *C*, semilogarithmic plot of current densities for the Na^+–K^+ pump current at 0 mV against temperature. The straight line is the line of best fit obtained by linear regression (correlation coefficient (r) = 0·99) and indicates a Q_{10} value of 2·1. Values are means ± S.D. Numbers in parentheses indicate the number of cells examined at each temperature.

those obtained in the present experiments: 1·9 mM in cultured chick heart cells (Stimers, Shigeto & Lieberman, 1990*b*), 1·50 ± 0·32 mM in guinea-pig myocytes (Nakao & Gadsby, 1989) and 1·9 mM in rabbit Purkinje myocytes (Bielen, Glitsch & Verdonck, 1991). In a more recent study on rat ventricular myocytes, Kinard, Liu, Liu & Stimers (1994) derived the relationship between $K_{0.5}$ for K^+ binding and $[Na^+]_{pip}$. According to their results, $K_{0.5}$ increased from 1·8 to 2·4 mM as $[Na^+]_{pip}$ increased from 10 to 85 mM.

Our data for activation of I_p by $[Na^+]_{pip}$ ($K_{0.5} = 14$ mM; $n_H = 1.3$) also compare well with other reports. Previous authors have reported similar values, i.e. a $K_{0.5}$ of 11 mM ($n_H = 1.4$) in guinea-pig ventricular myocytes (Nakao & Gadsby, 1989), 9 mM in cultured sheep Purkinje cells (Glitsch *et al.* 1989) and 20 mM in sheep Purkinje fibres (Sejersted, Wasserstrom & Fozzard, 1988).

Na^+–K^+ pump activation by external monovalent cations

We have found that the K^+ sites of the Na^+–K^+ pump are also activated by other external monovalent cations and that the current amplitude decreases at identical concentrations in the order: K^+ (1) $\geqslant Rb^+$ (0·94) $> Cs^+$ (0·38) $>>> Li^+$ (0·07) (Figs 5 and 6). The same order of potency has been observed in bullfrog atrial muscles with K_m values of 2·7, 2·5, 9·1 and 30 mM for K^+, Rb^+, Cs^+ and Li^+, respectively (Hasuo & Koketsu, 1985). Various authors have also reported a similar order, namely: $Tl^+ > K^+ = Rb^+ > NH_4^+ \geqslant Cs^+ > Li^+$, for activation of the Na^+–K^+ pump in guinea-pig papillary muscles, sheep Purkinje fibres, multicellular AV node cells and rabbit single Purkinje cells (Eisner & Lederer, 1979; Kurachi *et al.* 1981; Bielen *et al.* 1991). These findings are in good agreement with the present findings, coincide with Eisenman's sequence 4 and are similar to that for the SA node background inward current (Hagiwara *et al.* 1992). Eisenman predicted eleven selectivity sequences for the five alkaline metal cations and sequence 4 was the order for free-solution mobilities and associated with a 'weak' site (Eisenman, 1962).

The effect of temperature on I_p

It is also well known that membrane currents are sensitive to temperature, so that heart rate is greatly influenced by temperature (Yamagishi & Sano, 1967). In our present study, the Q_{10} value calculated for I_p was 2·1. Nakao & Gadsby (1986) reported the effect of temperature on the Na^+ translocation of the Na^+–K^+ pump in guinea-pig ventricular cells. They showed that lowering the temperature slows the relaxation of the transient pump current at all voltages by a factor of three for each 10 °C fall, in 50 mM Na^+_{pip}. This value is slightly higher than that obtained in the present study. The precise value of Q_{10} may be dependent on ionic conditions, such as $[Na^+]_{pip}$. Glitsch & Pusch (1984) studied the temperature dependence of cardiac active Na^+ transport in voltage-clamped sheep Purkinje fibres by means of simultaneous measurements of membrane current and intracellular Na^+ activity. They concluded that the temperature dependence of the Na^+–K^+ pump was due to changes in the magnitude of maximal active Na^+ efflux, whereas the sensitivity of the Na^+–K^+ pump towards $[K^+]_o$ was little affected. They obtained a value of Q_{10} for the Na^+–K^+ pump that was similar to ours, viz. 2·5 ± 0·3 between 35 and 22 °C.

Functional significance of I_p in terms of pacemaker activity

Whether I_p simply acts as a time- and voltage-independent current or whether it changes rhythmically during the pacemaker potential has yet to be clarified. In the past, several reports suggested that I_p was voltage independent, but it has now been established that I_p actually has voltage-dependent characteristics (De Weer, Gadsby & Rakowski, 1988). Using the ramp-clamp mode of voltage clamp, we have clearly demonstrated the voltage dependence of I_p in SA node cells.

The voltage dependence of I_p is shifted in the positive direction when $[K^+]_o$ or $[Na^+]_{pip}$ is decreased to within the physiological range. Nakao & Gadsby (1989) described the voltage dependence of I_p in guinea-pig ventricular cells. The I–V relationship of I_p shifted only a little on reduction of $[Na^+]_{pip}$ from 50 to 25 mM, but a further reduction in $[Na^+]_{pip}$ to 3 mM caused a positive shift of as much as 55 mV. In SA node cells, I_p at 10 mM Na^+_{pip} with 5·4 mM K^+_o is steeply voltage dependent within the pacemaker range of potentials (half-activation voltage = −52 ± 16 mV; $n = 10$; Fig. 7). This change in the I_p–V relationship indicates that I_p fluctuates in accordance with the level of pacemaker depolarization.

In rabbit SA node cells, a transient hyperpolarization has been observed with Tyrode solution after pretreating the tissue in K^+-free Tyrode solution; this hyperpolarization was completely blocked by an application of 10^{-5} M strophanthidin (Noma & Irisawa, 1975). This suggests that I_p contributes to the maintenance of the membrane potential in SA node cells. The transient hyperpolarization was 14·0 ± 2·9 mV in 5 mM K^+_o (Noma & Irisawa, 1975). Since the membrane resistance of single SA node cells has been determined to be around 1–2 GΩ (Hagiwara *et al.* 1992; Irisawa *et al.* 1993), a 14 mV hyperpolarization of the resting membrane potential would give a net outward current through an electrogenic pump of 14 pA.

However, to determine the exact amount of I_p flowing during the pacemaker potential, we would need accurate information about the internal Na^+ concentration in SA node cells. Chemical measurements of the intracellular Na^+ concentration in the sinus venosus of toads were made by Danielson (1964) who reported a value of 16·7 mM, although this would have included both free and bound Na^+ because flame photometry was used. Microelectrode determination of Na^+ has not yet been used for this purpose, but, in ventricular cells, several reports have

indicated that the concentration of intracellular Na^+ in an intact quiescent preparation is 5·8–10·7 mM (Lee & Fozzard, 1975; Sheu & Fozzard, 1982; Stimers *et al.* 1990*b*; Stimers, Lobaugh, Liu, Shigeto & Lieberman, 1990*a*). If we can assume that the physiological intracellular Na^+ concentration in SA node cells is also around 10 mM, and we take the current density of I_p as 0·80 ± 0·24 pA pF^{-1} at 5·4 mM K_o^+ from the present study, this would give a total I_p of 30 pA (membrane capacitance = 36·8 ± 5·6 pF; $n = 51$) at voltages more positive than 0 mV. An outward pump current of around 20 pA would contribute to pacemaker depolarization in SA node cells (Fig. 7). This is consistent with the idea that I_p contributes to physiological pacemaker activity in SA node cells.

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