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DEPENDENCE OF Na^+ PUMP CURRENT ON EXTERNAL MONOVALENT CATIONS AND MEMBRANE POTENTIAL IN RABBIT CARDIAC PURKINJE CELLS

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SUMMARY

1. The effect of membrane potential and various extracellular monovalent cations on the Na^+ pump current (I_p) was studied on isolated, single Purkinje cells of the rabbit heart by means of whole-cell recording.

2. I_p was identified as current activated by external K^+ or its congeners NH_4^+ and Tl^+. The current was blocked by dihydroouabain ($1–5 \times 10^{-4}$ M) over the whole range of membrane potentials tested.

3. In Na^+-containing solution half-maximum I_p activation ($K_{0.5}$) occurred at 0·4 mM-Tl^+, 1·9 mM-K^+ and 5·7 mM-NH_4^+ (holding potential, −20 mV).

4. The pump current (I_p)–voltage (V) relationship of the cells in Na^+-containing media with K^+ or its congeners at the tested concentrations $> K_{0.5}$ displayed a steep positive slope at negative membrane potentials between −120 and −20 mV. Little voltage dependence of I_p was observed at more positive potentials up to +40 mV. At even more positive potentials I_p measured at 2 and 5·4 mM-K^+ decreased.

5. Lowering the concentration of K^+ or its congeners below the $K_{0.5}$ value in Na^+-containing solution induced a region of negative slope of the I_p–V curve at membrane potentials positive to −20 mV.

6. The shape of the I_p–V relationship remained unchanged when the K^+ concentration (5·4 mM) of the Na^+-containing medium was replaced by NH_4^+ or Tl^+ concentrations of similar potency to activate I_p (20 mM-NH_4^+ or 2 mM-Tl^+).

7. In Na^+-free, choline-containing solution half-maximum I_p activation occurred at 0·13 mM-K^+ (holding potential, −20 mV).

8. At negative membrane potentials the positive slope of the I_p–V curve was flatter in Na^+-free than in Na^+-containing media. A reduced voltage dependence of I_p persisted, regardless of whether choline ions or Li^+ were used as a Na^+ substitute.

9. Lowering the K^+ concentration of the Na^+-free, choline-containing solution to 0·05 mM evoked an extended region of negative slope in the I_p–V relationship at membrane potentials between −40 and +60 mV.

10. It is concluded that the apparent affinity of the Na^+–K^+ pump towards K^+ in cardiac Purkinje cells depends on both the membrane potential and the extracellular Na^+ concentration.

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11. The region of negative slope of the I_p–V curve observed in cells which were superfused with media containing low concentrations of K^+ or its congeners strongly suggests the existence of at least two voltage-sensitive steps in the cardiac Na^+–K^+ pump cycle. It is generally accepted that one step is linked to the active Na^+ efflux. A second voltage-sensitive step seems to be related to the binding of external K^+ or its congeners to the pump molecule.

INTRODUCTION

The active Na^+–K^+ transport of animal cell membranes generates a current, the Na^+ pump current (I_p). Neither the mechanism of current generation nor the factors governing the amplitude and the voltage dependence of I_p are completely understood. Recent papers present evidence that changes of the extracellular Na^+ and/or K^+ concentration affect I_p in cardiac ventricular myocytes (Gadsby & Nakao, 1989; Nakao & Gadsby, 1989). Lowering the extracellular K^+ concentration mainly scales down the I_p amplitude without major effects on the voltage dependence of I_p, whereas a reduction in the external Na^+ concentration increases I_p at negative membrane potentials without marked changes in I_p at positive potentials. Similarly, in depolarized single cardiac Purkinje cells a decrease of the extracellular K^+ concentration diminishes the I_p amplitude (Cohen, Datyner, Gintant, Mulrine & Pennefather, 1987; Glitsch, Krahn & Verdonck, 1989*b*). External Rb and Cs ions act as K^+ congeners (Glitsch, Krahn & Pusch, 1989*a*). The effect of substituting extracellular Na^+ on amplitude and voltage dependence of I_p in Purkinje cells is unknown. The pump current–voltage relationships (I_p–V relationships) published so far strongly suggest the existence of only *one* voltage-sensitive step within the ion translocation performed by the cardiac Na^+–K^+ pump (Gadsby, Kimura & Noma, 1985; Gadsby & Nakao, 1989; Glitsch *et al.* 1989*a*; Nakao & Gadsby, 1989). In contrast to these findings Schwarz and his colleagues (Lafaire & Schwarz, 1986; Rakowski, Vasilets & Schwarz, 1990; Schwarz & Vasilets, 1990) have repeatedly reported a distinct maximum of the I_p–V curve in *Xenopus* oocytes indicating at least *two* voltage-dependent partial reactions within the pump cycle. The aim of the present work is to study in some detail the effect of membrane potential and various types of external monovalent cations on the Na^+ pump current of cardiac Purkinje cells in order to obtain further insights into the mechanism of the pump's electrogenicity.

METHODS

Preparation of single cells

Single Purkinje cells were obtained from rabbit hearts by means of an enzymatic procedure similar to that described by Scamps & Carmeliet (1989). The animals were killed by a blow on the neck. The aorta was quickly cannulated. The excised heart was attached to a Langendorff apparatus and continuously perfused with various oxygenated Tyrode solutions via the coronary arteries at 37 °C. First, the heart was perfused with a Ca^{2+}-free medium for 10 min. Afterwards the perfusion was continued for 12 min with a solution containing 25 μM-Ca^{2+}, 0·4 mg ml^{-1} collagenase (WAKO, Tokyo, Japan) and 0·15 mg ml^{-1} hyaluronidase (Type I-S; Sigma, St Louis, MO, USA). For the last 3 min 0·1 mg ml^{-1} protease (Type XIV, 25% calcium acetate; Sigma) was added to the enzyme solution. The heart was then disconnected from the apparatus and transferred to a large Petri dish filled with the last enzymatic solution. Free-running Purkinje fibres were dissected from

both ventricles and gently stirred by a small magnetic stirrer for 20 min to 1 h in a small culture dish (diameter 36 mm; Falcon, Becton Dickinson, Lincoln Park, NJ, USA) containing the former solution. The fibres were transferred about every 15 min into a new dish. By this treatment single cells were dissociated from the fibres. The length and the width of the cells measured were 141·2 ± 3 μm and 16·1 ± 0·5 μm (means ± S.E.M.; $n = 72$), respectively. The dishes containing the isolated cells were fixed on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, Germany), where the whole-cell recording was carried out. An annular plastic device was pressed down to the bottom of the dishes in order to decrease the volume of the dishes to about 0·3 ml and to superfuse the cells with the pre-warmed bathing solution at ≃ 2 ml min^{-1}. All experiments were performed at 32–34 °C.

Solutions

The standard superfusion medium contained (mM): 144 NaCl; 5·4 KCl; 0·5 $MgCl_2$; 1·8 $CaCl_2$; 10 glucose; 10 HEPES (adjusted by NaOH to pH 7·4 at 32–34 °C). For some experiments NaCl was completely replaced by LiCl or choline chloride (plus 5×10^{-6} M-atropine sulphate). The pH of these media was adjusted to pH 7·4 by LiOH. For other measurements KCl was replaced by TlCl or NH_4Cl. The concentration of KCl (TlCl, NH_4Cl) was varied without osmotic compensation. All of these modified superfusion media contained 2 mM-$BaCl_2$in order to diminish the K^+ conductance of the sarcolemma. In some experiments dihydroouabain (DHO; 0·1–0·5 mM), a specific blocker of the Na^+–K^+ pump, and/or 5 mM-$NiCl_2$, an inhibitor of Na^+–Ca^{2+} exchange, were added from aqueous stock solutions. The test superfusion media were applied to the cell under study via two multibarelled pipettes (tip diameter ≃ 100–200 μm) nearby. The release of solutions under gravitational force was regulated by a command valve unit. Solution change at the cell surface was complete within 250 ms (verified by means of a pH-sensitive microelectrode and media of different pH values). The temperature of the pre-heated test solutions in the multibarrelled pipettes was not directly controlled. About 2·5 mm of the tips of the pipettes were immersed in the pre-warmed standard bathing medium. Switching from bath-applied to pipette-applied solutions of identical ionic composition did not change the membrane current of a voltage-clamped cell. The standard solution within the patch pipettes (patch pipette solution) contained (mM): 80 caesium aspartate; 20 CsOH; 5 NaOH; 10 EGTA; 40 HEPES; 5 $MgCl_2$; 5 glucose; 5 Mg-ATP; 5 sodium creatine phosphate (pH 7·2). The NaCl concentration was occasionally increased to 30 mM by replacing 15 CsOH with NaOH.

Electrical measurements

Whole-cell recording was performed according to Hamill, Marty, Neher, Sakmann & Sigworth (1981). For this purpose a 'Giga seal' was established between a wide-tipped, fire-polished glass pipette and the sarcolemma. The initial resistance of the patch pipettes, filled with one of the patch pipette solutions, was measured to be between 1 and 4 MΩ. The pipette was positioned at the cell surface by means of a micromanipulator (Leitz, Wetzlar, Germany). The image of the cell was displayed on a TV-monitor (Philips, Eindhoven, Netherlands) via a video camera (Expt-1000 C Elbex, Tokyo, Japan). The cell membrane potential was clamped at pre-set voltages and the resulting membrane current was measured by means of a voltage clamp amplifier (Axoclamp 2A, Axon Instruments, Burlingame, CA, USA). Starting from a holding potential of −20 mV (in order to inactivate the Na^+ and Ca^{2+} conductances of the sarcolemma) current (I)–voltage (V) relationships were obtained either by step-like changes of the command potential generally for 4–10 s or by triangular voltage ramps at 30 mV s^{-1}. The clamp potential (V_c) and the corresponding membrane current (I) were recorded on a pen-recorder (Watanabe Multicorder, Tokyo, Japan) and occasionally on an analog tape-recorder (Racal 4DS, Hardley, Southampton).

Statistical evaluation of the data

Whenever possible data are presented as means ± S.E.M. The symbol n denotes the number of cells studied. Differences between means were checked for significance by Student's paired or unpaired t test. They were deemed significant if $P < 0·05$.

RESULTS

Estimation of the Na^+ pump current

The Na^+ pump current (I_p) was measured as K^+ (Tl^+, NH_4^+)-activated current. This is illustrated in Fig. 1. Following an equilibration period of 10–30 min in the standard superfusion medium, a K^+-free solution containing 2 mM-$BaCl_2$ was applied to the Purkinje cell under study via a multibarrelled pipette. When the membrane current at a holding potential of −20 mV had reached a constant value, short pulses

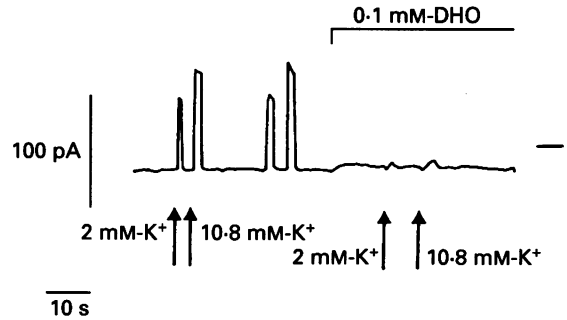

Fig. 1. I_p identified as K^+-activated current. At the arrows K^+-containing solution is applied to a Purkinje cell for ≈ 2 s. The K^+-evoked outward current is blocked by dihydroouabain (DHO). Clamped membrane potential (V_c), −20 mV. Horizontal bar represents zero current level.

of K^+ (Tl^+, NH_4^+)-containing media were repeatedly delivered to the cell via different barrels of the pipette until the amplitude of the evoked currents remained constant. For example, in Fig. 1, solutions containing 2 or 10·8 mM-KCl are successively used for 1–2 s as superfusion media. The amplitude of the outward currents elicited by the solutions depends on their K^+ concentration. If the procedure is repeated with pipette solutions containing in addition 0·1 mM-DHO (applied via the second multibarrelled pipette) the outward currents are blocked. The holding current in the K^+-free medium is essentially unchanged. Corresponding results were obtained at each membrane potential tested, regardless of whether the solutions applied contained K^+, Tl^+ or NH_4^+. Since the evoked outward current was almost completely inhibited by DHO, a specific blocker of the Na^+–K^+ pump, we conclude that the K^+ (Tl^+, NH_4^+)-induced outward current is the Na^+ pump current I_p. The slight decay of I_p during the application of solutions containing activator cations (e.g. Figs 1 and 3*A*) is probably due to an, at least, transient decrease in the subsarcolemmal Na^+ concentration (Bielen, Glitsch & Verdonck, 1990). In order to avoid any uncertainty about the actual subsarcolemmal Na^+ concentration I_p was estimated as the *initial* outward current. During each experiment I_p at −20 mV was repeatedly measured in order to make sure that no appreciable 'run-down' of I_p (Gadsby & Nakao, 1989) occurred. Occasionally small changes of the holding current were noticed upon application of DHO-containing superfusion media with pump activator cations (Figs 3*B* and 9*A*). They occurred at any clamp potential and never exceeded 10% of the outward current evoked by the activator cations (K^+; Tl^+; NH_4^+).

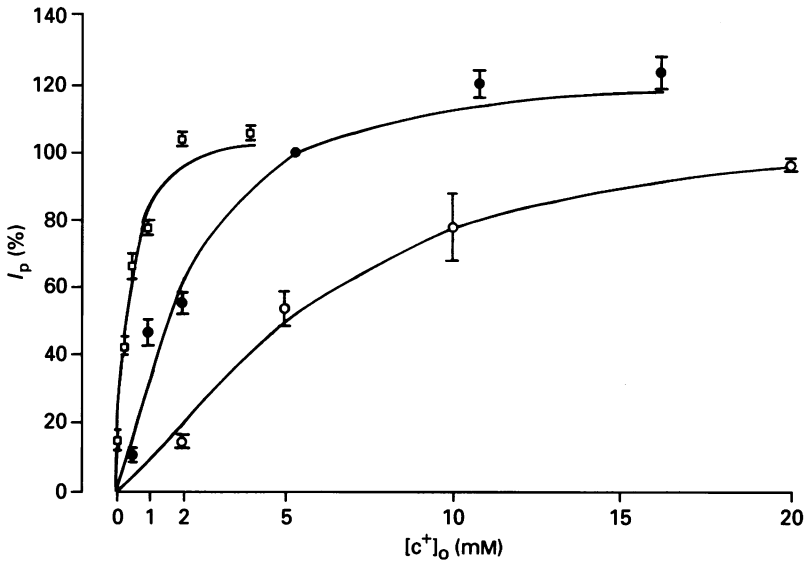

Fig. 2. I_p amplitudes as a function of the external concentration of various monovalent cations $[c^+]_o$ (Tl^+, □; K^+, ●; NH_4^+, ○). I_p amplitudes are normalized to the I_p amplitude at 5·4 mM $[K^+]_o$ which is arbitrarily set to 100%. V_c, −20 mV. The curves obey the equation:

$$I_p = \frac{I_{p,max}[c^+]_o^h}{K_{0.5}^h + [c^+]_o^h}.$$

Activation of I_p by external activator cations

The effect of various concentrations of the different activator cations (K^+, Tl^+ or NH_4^+) on the amplitude of I_p was examined at a holding potential of −20 mV in experiments similar to that illustrated in Fig. 1. However, in each experiment the I_p activation by 5·4 mM-K^+ was measured and used to normalize the activation strength of different cations and/or concentrations applied during the same experiment (cf. Figs 3 and 7*A*). The I_p amplitude of cells superfused with the medium containing 5·4 mM-KCl was measured at −20 mV to be 98·6±7·5 pA ($n = 71$; standard patch pipette solution). The potency of various external K^+, Tl^+ and NH_4^+ concentrations for I_p activation is shown in Fig. 2. Obviously Tl^+ is a more potent, and NH_4^+ a less potent activator cation than K^+. I_p activation by the monovalent cations tends to saturate. The curves fitted to the data by least squares non-linear regression obey the equation displayed under the figure. In deriving the appropriate Hill coefficients (h) and half-maximum I_p activation ($K_{0.5}$) values it was assumed that (a) I_p at the highest K^+ or Tl^+ concentrations represents the maximum pump current ($I_{p,max}$) and (b) no I_p can be activated at a very low K^+ or Tl^+ concentration (10^{-4} mM). The fitting procedure yielded h values of 1·47 or 1·45 and $K_{0.5}$ values of 0·4 or 1·9 mM for Tl^+ or K^+, respectively. As to the NH_4^+ data it is not clear from Fig. 2 whether I_p is maximal at the highest NH_4^+ concentration (20 mM). Therefore, an h value of 1·46 (the mean of the Hill coefficients estimated above) and no I_p activation at a low NH_4^+ concentration (10^{-4} mM) were assumed in order to fit the curve to the data. The procedure resulted in a $K_{0.5}$ value of 5·7 mM-NH_4^+ and an $I_{p,max}$ of 112·4%.

I_p–V relationships of cells superfused with K^+-containing media

The I_p–V curve of the Purkinje cells was studied at various external K^+ concentrations. Figure 3*A* displays the experimental procedure. Starting from a holding potential of −20 mV, the cell membrane is clamped to various potentials

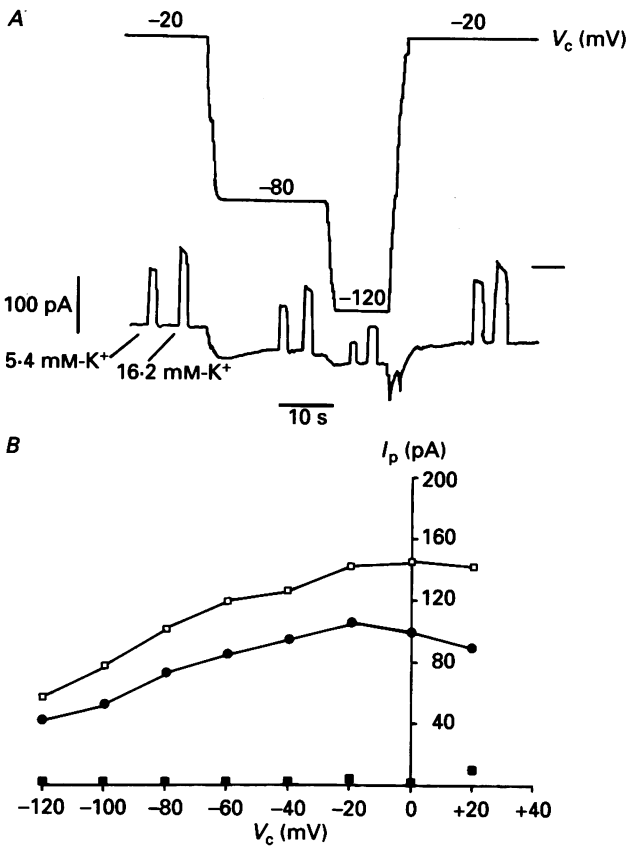

Fig. 3. Dependence of I_p on $[K^+]_o$ and membrane potential. *A*, upper trace shows clamp potential (V_c), lower trace shows membrane current. Horizontal bar marks zero current level. At each clamp potential I_p was successively induced by 5·4 and 16·2 mM-K^+. *B*, I_p–V relationships measured during the experiment illustrated in *A*. DHO, a specific blocker of active Na^+–K^+ transport, almost completely inhibits I_p at 16·2 mM-K^+ over the whole potential range studied. □, 16·2 mM-K^+; ●, 5·4 mM-K^+; ■, 16·2 mM-K^+ plus 0·2 mM-DHO.

(upper trace). At each clamp potential the K^+-free superfusion fluid is replaced at the cell surface by K^+-containing media (e.g. 5·4 and 16·2 mM-K^+) during short pulses of positive pressure applied to the multibarelled pipette. The K^+-evoked outward (pump) currents are recorded at each potential (lower trace). It is clear from Fig. 3*A* that the amplitude of I_p varies not only with the external K^+ concentration ($[K^+]_o$) but also with the membrane potential. The complete I_p–V curves of the cell at 5·4 and 16·2 mM-K^+ are depicted in Fig. 3*B*. At each potential tested I_p is larger at 16·2 than at 5·4 mM-K^+. The I_p–V curve obtained at the higher K^+ concentration tends to

saturate with increasingly more positive membrane potentials, whereas the curve measured at 5·4 mM-K^+ displays a shallow maximum at −20 mV. The figure also demonstrates that very little K^+-induced outward current is observed following blockade of the Na^+–K^+ pump by DHO. Similarly, in different experiments, the

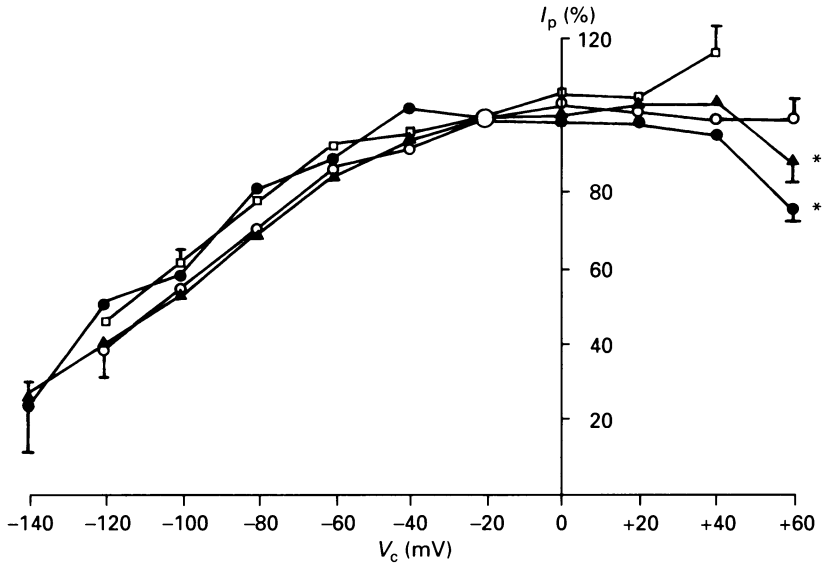

Fig. 4. Normalized mean I_p–V curves at various $[K^+]_o$. I_p amplitudes are normalized to the corresponding I_p amplitudes at −20 mV, which are arbitrarily set to 100%. * indicate I_p amplitudes smaller at positive membrane potentials than at 0 mV ($P < 0·05$). □, 16·2 mM-K^+ (n = 3–9); ○, 10·8 mM-K^+ (n = 4–8); ▲, 5·4 mM-K^+ (n = 4–19); ●, 2·0 mM-K^+ (n = 2–5). S.E.M. is shown for only two I_p amplitudes in each curve for clarity.

current evoked by 10·8 mM-K^+ in five cells treated with 0·5 mM-DHO varied between $-5 \pm 1·5$ pA at −100 mV and $+4 \pm 2$ pA at +60 mV.

Mean I_p–V curves at various external K^+ concentrations are depicted in Fig. 4. In order to facilitate comparison, the I_p values are normalized to their corresponding amplitudes measured at −20 mV. The general shape of the I_p–V relationships remains nearly unaffected if the extracellular K^+ concentration varies between 2·0 and 16·2 mM. The curves display a positive slope within the potential range −140 to −20 mV and almost constant I_p amplitudes between −20 and +40 mV. (The I_p amplitude observed at +40 mV in the bathing solution containing 16·2 mM-K^+ does *not* differ significantly either from the corresponding value at 0 mV or from the I_p amplitudes measured at +40 mV in solutions containing different K^+ concentrations). However, a closer inspection of the data reveals that the I_p values at +60 mV measured in cells that were superfused with media containing 2 or 5·4 mM-K^+ are significantly smaller than the corresponding numbers at 0 mV.

Possible differences of the I_p–V relationships at high and low external K^+ concentrations were also studied in cells where the I_p amplitudes evoked by 1 and 16·2 mM-K^+ were *successively* measured at each clamp potential. The results of these

paired measurements are presented in Fig. 5. Again the I_p amplitudes at each voltage step are normalized to the corresponding I_p values at -20 mV. In the K^+-rich solution the I_p amplitude rises with increasingly more positive clamp potentials between -100 and about -20 mV and remains constant at more positive voltages.

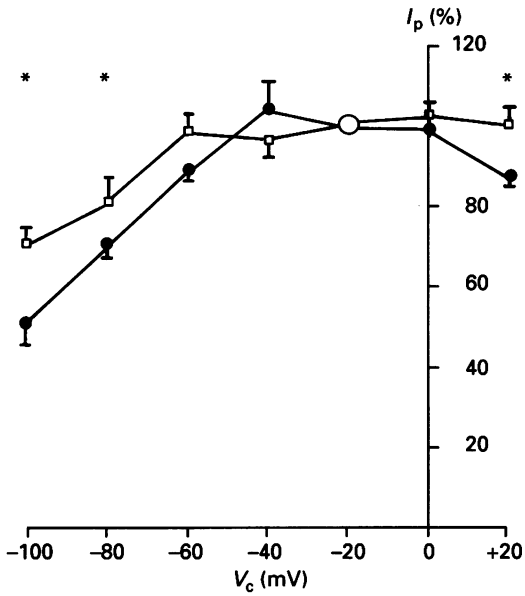

Fig. 5. Normalized mean I_p–V relationships of cells in solutions containing 1 (●) or 16·2 (□) mM-K^+. I_p values normalized to the I_p amplitudes at -20 mV. I_p was successively measured at both K^+ concentrations at each membrane potential. * mark significantly different I_p amplitudes at corresponding membrane potentials ($P < 0.05$). $n = 4$–6. S.E.M. is shown where it is larger than the size of the symbols.

If the cells are bathed in the medium containing 1 mM-K^+ the I_p–V relationship displays a shallow maximum at -40 to -20 mV and declines at more positive potentials. The normalized I_p amplitude at $+20$ mV is significantly smaller at the low external K^+ concentration. Furthermore, there is a steeper slope of the I_p–V curve at membrane potentials negative to -40 mV as revealed by the significantly lower relative I_p amplitude of cells superfused with the solution containing 1 mM-K^+ at -80 and -100 mV. Essentially the same results were obtained by comparing the mean I_p–V relationship (normalized to I_p at -20 mV) of all cells superfused with a solution containing 16·2 mM-K^+ to the mean I_p–V curve of all cells in a medium containing 1 mM-K^+. The normalized I_p amplitudes of the latter cells were significantly smaller at -60 to -100 mV and at $+20$ to $+40$ mV (not illustrated).

I_p–V relationships of cells superfused with NH_4^+-containing media

The effect of NH_4^+ on the I_p–V curve of cardiac Purkinje cells was investigated as shown in Fig. 6*A*. The membrane potential is clamped to various levels from a holding potential of -20 mV (upper trace). At each potential the outward current evoked by various external NH_4^+ concentrations is measured (lower trace). The

outward current evoked in three cells by a solution containing 20 mM-NH_4^+ plus 0·5 mM-DHO varied between 0 pA at −100 mV and +3 pA at +60 mV and never exceeded 4% of the corresponding I_p amplitude. The amplitude of I_p depends on the extracellular NH_4^+ concentration and on the membrane potential. A decrease in the

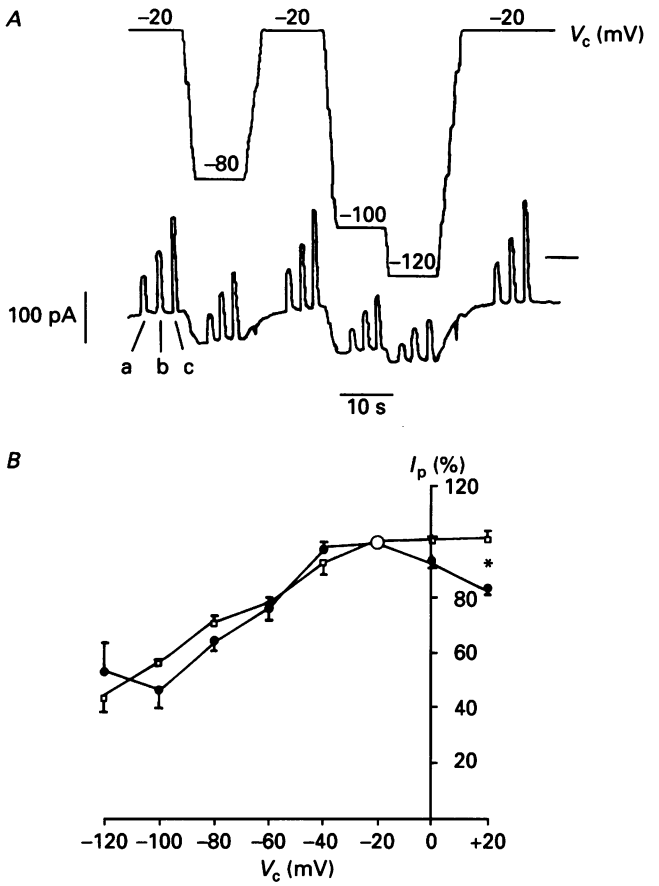

Fig. 6. *A*, dependence of I_p on $[NH_4^+]_o$ and membrane potential. Upper trace, clamped membrane potential (V_c). Lower trace, membrane current. Horizontal bar indicates zero current level. At each membrane potential I_p was successively evoked by 5(a), 10(b) and 20(c) mM-NH_4^+. *B*, normalized mean I_p–V curves of cells in solution containing 5(●) or 20(□) mM-NH_4^+. I_p amplitudes are normalized to the corresponding I_p amplitudes at −20 mV, arbitrarily set to 100%. At each membrane potential I_p was successively measured at both NH_4^+ concentrations. * marks significantly different I_p amplitudes ($P < 0·05$) at +20 mV. $n = 2$ (at −120 mV) –7.

NH_4^+ concentration and an increasingly more negative membrane potential diminish the current.

Figure 6*B* shows mean I_p–V curves of cells where the pump current was *successively* measured at 20 and 5 mM-NH_4^+ at each clamp potential. The current amplitudes are normalized to the corresponding I_p values at −20 mV. The figure reveals little difference in the shape of the I_p–V relationships at membrane potentials negative to

-20 mV. However, the I_p amplitude observed in a solution containing 5 mM-NH_4^+ decreases at more positive potentials, while the current amplitude at 20 mM-NH_4^+ remains constant. The currents differ significantly from each other at $+20$ mV. Thus a negative slope of the I_p–V curve is observed in the range of positive membrane potentials not only at lower external K^+ but also at lower extracellular NH_4^+ concentrations.

I_p–V relationships of cells superfused with Tl^+-containing solutions

It has been known for many years, that Tl ions are potent external activator cations of the Na^+–K^+ pump (for references see Schuurmans Stekhoven & Bonting, 1981). We studied the effect of extracellular Tl^+ on the I_p–V relationship of cardiac Purkinje cells as shown in Fig. 7*A*. The membrane potential is clamped to various levels from a holding potential of -20 mV (upper trace). The corresponding membrane currents are displayed in the lower trace. The cell is superfused throughout the measurements by a solution without any activator cations of the Na^+–K^+ pump except for short pulses with media containing either Tl^+ (0·5 or 2 mM) or K^+ (5·4 mM). The pulses evoke an outward current of different amplitudes. The current amplitudes elicited by 2 mM-Tl^+ or 5·4 mM-K^+ are almost the same, whereas the amplitude measured at 0·5 mM-Tl^+ is smaller. Irrespective of the ion species and concentrations used, the current amplitudes decline at negative membrane potentials. The 4 mM-Tl^+-activated currents of three cells were completely abolished by 0·5 mM-DHO within the range of membrane potentials between -100 and $+60$ mV. Figure 7*B* depicts mean I_p–V relationships of cells superfused with solutions containing four different Tl^+ concentrations. The I_p amplitudes are normalized to the corresponding I_p values at -20 mV arbitrarily set to 100%. The curves display a positive slope at membrane potentials between -120 and -20 mV. At more positive potentials the I_p amplitudes of cells superfused with media containing 2 or 4 mM-Tl^+ increase slightly or remain constant, whereas the I_p amplitudes at the two lower Tl^+ concentrations decrease. A statistical analysis reveals that the reduction of I_p at 0·1 mM-Tl^+ in the range of positive membrane potentials is the only significant deviation from the I_p amplitude measured at 4 mM-Tl^+.

Specific modification of the I_p–V curve by the species of the activator cation?

We studied whether or not the species of the cations used for the extracellular activation of the Na^+ pump exerts a specific effect on the shape of the I_p–V relationship in cardiac Purkinje cells. For this purpose we compared the I_p–V curves of cells superfused with media containing different species of activator cations but in equipotent concentrations (5·4 mM-K^+, 2 mM-Tl^+, 20 mM-NH_4^+; cf. Fig. 2). There was no ion species-dependent modification of the general shape of the I_p–V relationship at the stated concentrations of the activator cations.

I_p–V relationships of cardiac Purkinje cells in Na^+-free media

The effect of substitution for extracellular Na^+ on the shape of the I_p–V curve was studied in two series of experiments. In one series the organic cation choline served as a substitute for Na^+, whereas the smaller anorganic cation Li^+ was used in the

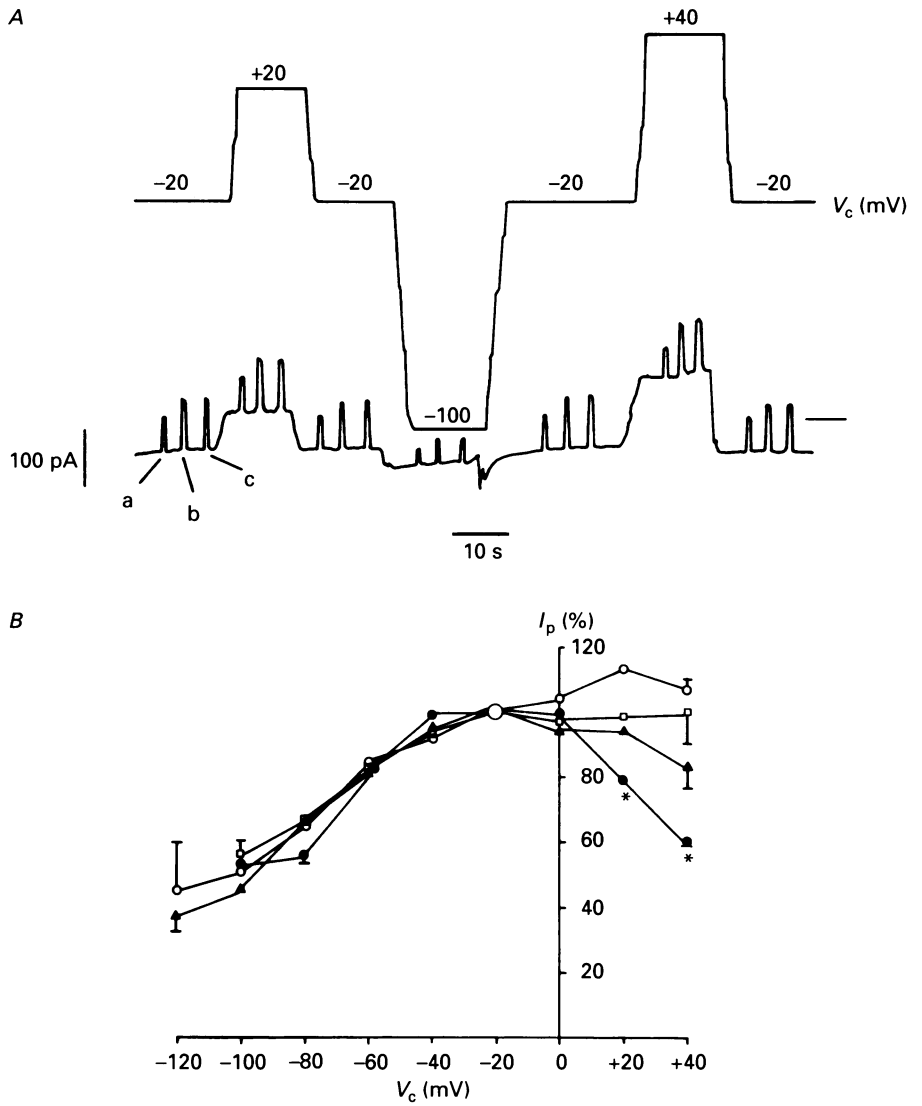

Fig. 7. *A*, dependence of I_p on $[Tl^+]_o$ and membrane potential. Upper trace, clamped membrane potential (V_c). Lower trace, membrane current. Horizontal bar indicates zero current level. At each membrane potential I_p was successively measured at 0·5 mM-Tl^+(a), 2 mM-Tl^+(b) and 5·4 mM-K^+(c). *B*, normalized I_p–V curves of cells at various $[Tl^+]_o$. The I_p amplitudes are normalized to the corresponding amplitudes at −20 mV, arbitrarily set to 100%. □, 4 mM-Tl^+ (n = 3–5); ○, 2 mM-Tl^+ (n = 2–3); ▲, 0·5 mM-Tl^+ (n = 2–3); ●, 0·1 mM-Tl^+ (n = 2–7). * indicate significantly different I_p amplitudes at 0·1 and 4 mM-Tl^+. S.E.M. is shown only for two means of each curve for clarity.

second set of experiments. The Na^+ concentration of the patch pipette solution was increased to 30 mM in both series in order to minimize inhibition of the Na^+–K^+ pump due to a possible decrease of the intracellular Na^+ concentration during

superfusion with the Na^+-free media. In a further attempt to prevent a possible decrease of internal Na^+, the I–V curves of some cells were measured in a short period of time by application of voltage ramps (≈ 30 mV s^{-1}). Between two runs in Na^+-free solution all cells were superfused with K^+-free, Na^+-containing solution until short pulses with a medium containing 5·4 mM-K^+ indicated no further increase in the K^+-induced outward current (I_p) (cf. Bielen *et al.* 1990). All superfusion media contained 5 mM-Ni^{2+} in order to block the Na^+–Ca^{2+} exchange (Kimura, Miyamae & Noma, 1987) and thus Ca^{2+} uptake and Na^+ extrusion via the exchange in Na^+-free solution. By itself 5 mM-Ni^{2+} did not affect I_p.

I_p–V curve of cells in choline-containing solution

Variations of the I_p–V relationship of cardiac Purkinje cells following replacement of the extracellular Na^+ by choline ions were measured either by voltage ramps or by stepwise changes of the holding potential. The latter procedure, used in nine out of eleven cells, is displayed in Fig. 8*A*. The upper trace in Fig. 8*A* depicts the clamp potential which is manually set to various levels starting from the holding potential of -20 mV. The lower trace shows the membrane current measured at the various clamp potentials. Since choline ions have no activating effect on the cardiac Na^+–K^+-ATPase (Portius & Repke, 1967) I_p was identified as K^+-activated current in both the Na^+-containing and the choline ion-containing solution. At the holding potential of -20 mV, I_p amounted to $90·2 \pm 7·4$ pA in the Na^+-containing solution and to $99·8 \pm 11·3$ pA in the choline-containing bathing fluid ($n = 9$; $P > 0·05$). Switching to the Na^+-free medium without K^+ slightly shifts the membrane current in the outward direction. This is probably due to the fact that the permeability of the sarcolemma towards choline ions is lower than towards Na^+. The K^+-activated (pump) current, successively measured at each clamp potential in Na^+-containing and in Na^+-free solution, decreases with increasingly more negative potentials. Clearly, the potential dependence of I_p is less pronounced in the choline-containing medium.

Mean I_p–V curves of the cells superfused with Na^+- or choline-containing solution are depicted in Fig. 8*B*. The mean values include I_p–V relationships of two cells measured in ramp experiments. The data were pooled because no major difference was found in one cell between I_p–V curves produced either by stepwise changes of the membrane potential or by application of a voltage ramp. The I_p values shown are normalized to the corresponding I_p amplitudes at -20 mV. At membrane potentials negative to -60 mV the decline of the normalized I_p amplitudes is significantly smaller if the cells are superfused with the choline-containing medium. However, a voltage dependence of I_p is clearly present also in the Na^+-free bathing fluid. No difference is found with regard to the I_p–V curve of the cells in Na^+-containing solution at more positive clamp potentials.

I_p–V curve of cells in Li^+-containing solution

The effect of the replacement of external Na^+ by Li^+ on the I_p–V relationship was studied in eight cells. The membrane potential was changed by means of a voltage ramp in five cells and by means of the procedure illustrated in Fig. 8*A* in the

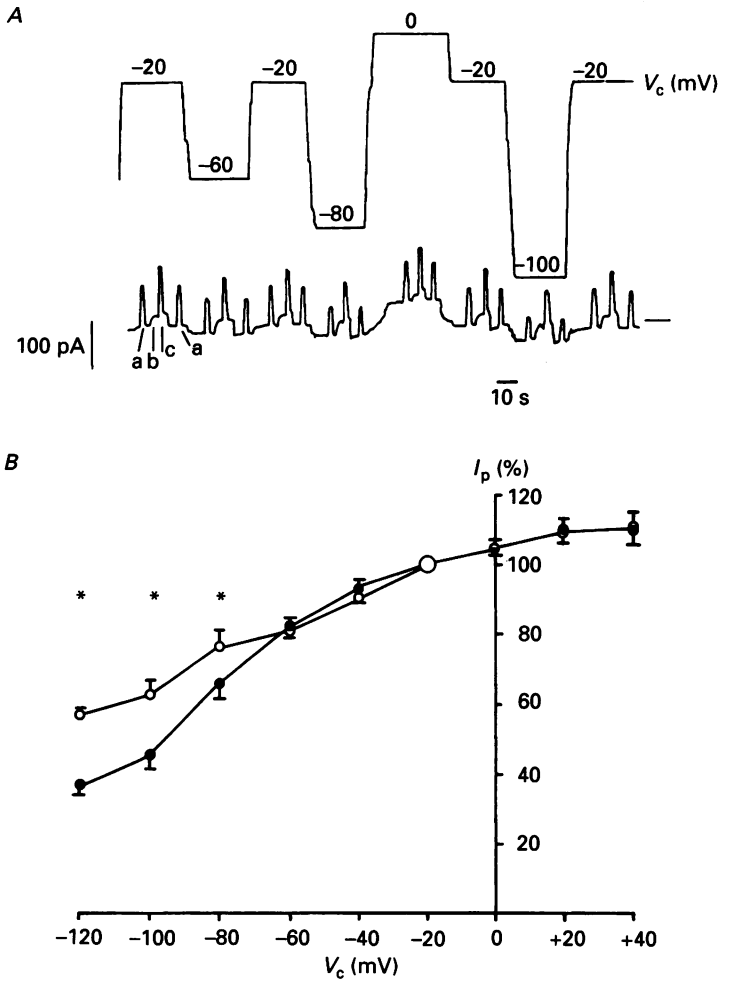

Fig. 8. *A*, voltage dependence of I_p in a Purkinje cell superfused with Na^+- or choline-containing solutions. All bathing fluids contained 5 mM-Ni^{2+}. I_p was activated by 5·4 mM-K^+. Upper trace, clamped membrane potential (V_c). Lower trace, membrane current. Horizontal bar indicates zero current level. At each membrane potential I_p was successively measured in Na^+-containing, in choline-containing and again in Na^+-containing solution. a, 150 mM-Na^+ + 5·4 mM-K^+; b, 150 mM-choline + 0 mM-K^+; c, 150 mM-choline + 5·4 mM-K^+. Note the outward shift of the membrane current in choline-containing K^+-free medium. *B*, normalized mean I_p–V curves of cells in Na^+ (●)- or choline (○)-containing solutions. I_p was estimated as current activated by 5·4 mM-K^+. I_p amplitudes are normalized to the corresponding I_p amplitudes at −20 mV. Means include data from two ramp experiments and nine experiments with stepwise changes of the clamp potential. * mark significantly different I_p amplitudes at respective membrane potentials ($P < 0.05$). $n = 6$–11.

remaining three. Li^+ ions are known to be weak external activators of the cardiac Na^+–K^+ pump (Portius & Repke, 1967). Thus the K^+-activated current does not represent the whole I_p of cells superfused with Li^+-containing media but the major

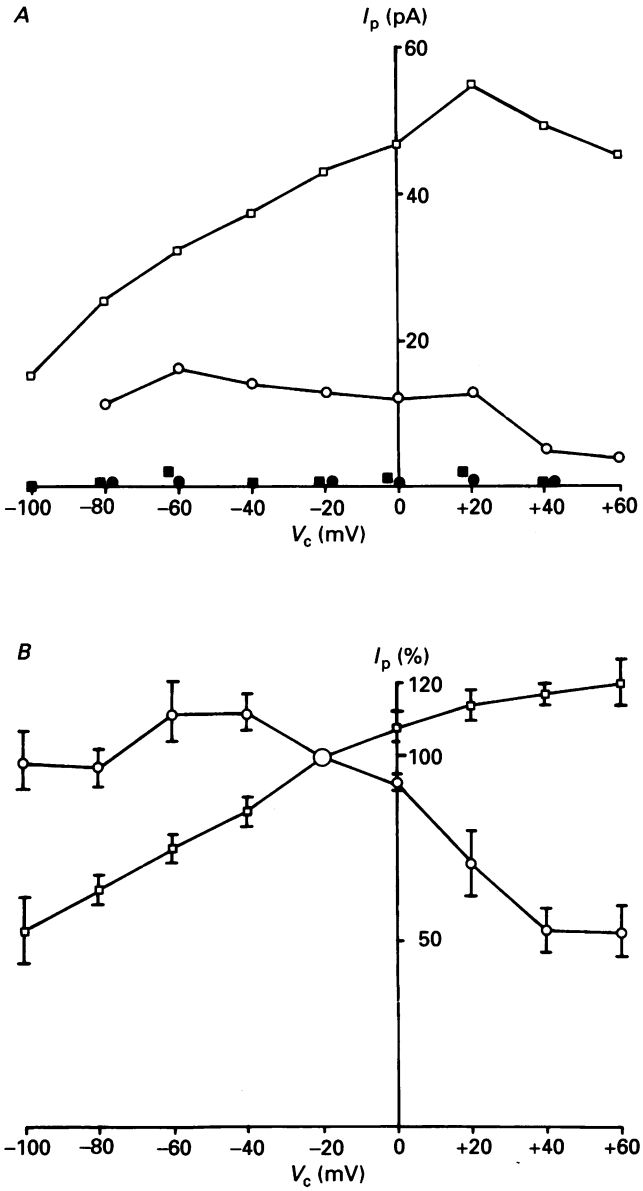

Fig. 9. I_p–V relationships of Purkinje cells in Na^+-free, choline-containing media with different K^+ concentrations. *A*, mean I_p–V curves of cells superfused with solutions containing 0·05 mM-K^+ with (●) or without (○) 0·2 mM-DHO or 10·8 mM-K^+ with (■) or without (□) 0·2 mM-DHO. I_p in pA ($n = 3$). *B*, mean I_p–V relationships of cells in bathing fluids containing either 0·05 (○) or 10·8 (□) mM-K^+. I_p normalized to the corresponding I_p amplitudes at −20 mV ($n = 5$–9).

fraction. In order to estimate reliably I_p under these conditions measurements of the DHO-sensitive current are required. I_p at −20 mV amounted to 145 ± 11·7 pA in the Na^+-containing solution and to 158·3 ± 15·3 pA in the Li^+-containing medium (n =

3. $P > 0.05$). The I_p–V curve of cells in the Li^+-containing solution was measured to be quite similar to the I_p–V relationship observed in choline-containing medium (Fig. 8*B*). However, the normalized I_p amplitudes of the cells in Li^+-containing solution exceeded the controls only at membrane potentials negative to −80 mV.

An extended region of negative slope of the I_p–V relationship

The I_p–V curve of Purkinje cells was also studied in Na^+-free, choline-containing medium with various K^+ concentrations by means of a procedure very similar to that depicted in Fig. 8*A*. A large increase in the apparent K^+ affinity of the Na^+ pump was observed in the Na^+-free medium as already described by Nakao & Gadsby (1989) for ventricular cells. Half-maximum activation of I_p occurred at 0·13 mM-K^+ ($n = 9$) at a holding potential of −20 mV as compared to 1·9 mM-K^+ in Na^+-containing solution (see Fig. 2). Figure 9 demonstrates that lowering the K^+ concentration of the Na^+-free medium caused marked changes in the shape of the I_p–V relationship. Figure 9*A* displays mean I_p–V curves of three cells superfused with Na^+-free solution containing 0·05 or 10·8 mM-K^+ with and without 0·2 mM-DHO, respectively. The I_p–V relationship of the cells at 10·8 mM-K^+ exhibits the conventional shape described in connection with Fig. 8*B*. If the K^+ concentration of the choline-containing medium is decreased to 0·05 mM the amplitude of I_p is diminished at all membrane potentials tested. Interestingly, the I_p–V curve displays a shallow maximum at −60 mV and an extended region of negative slope at more positive potentials. Little K^+-activated current is measured in DHO-containing solutions between −100 and +40 mV. Figure 9*B* shows mean I_p–V relationships of nine cells in choline-containing solution with 0·05 or 10·8 mM-K^+. I_p amplitudes are normalized to the corresponding I_p values at −20 mV. Clearly, the I_p–V curve measured at the low K^+ concentration exhibits a positive slope only between −100 and −60 mV and a large region of negative slope at potentials positive to −40 mV, whereas the I_p–V relationship at 10·8 mM-K^+ displays saturation kinetics with a steep positive slope in the whole range of negative membrane potentials.

DISCUSSION

The current activated by K^+ or its congeners represents I_p

As shown in Fig. 1 the specific Na^+ pump inhibitor DHO completely blocks the outward current induced in cardiac Purkinje cells by a solution containing either 2 or 10·8 mM-K^+. This finding strongly suggests that the K^+-activated current is a reliable measure of the Na^+ pump current I_p. This is true not only at the holding potential of −20 mV (Fig. 1) but over the whole potential range tested (Fig. 3*B*, see also Fig. 9*A*) and also for media containing NH_4^+ or Tl^+ instead of K^+. Furthermore, the fact that there is nearly no change in the I–V curves of the cells measured either at 0 mM-K^+ or at 16·2 mM-K^+ plus 0·2 mM-DHO (Fig. 3*B*) indicates that no other current than I_p is affected by a change in the external K^+ concentration or by application of the cardiac steroid under our experimental conditions. Corresponding observations were reported from measurements on guinea-pig ventricular myocytes (Gadsby *et al.* 1985; Gadsby & Nakao, 1989; Mogul, Rasmussen, Singer & Ten Eick, 1989; Nakao & Gadsby, 1989), squid giant axons (Rakowski, Gadsby & De Weer,

1989) and *Xenopus laevis* oocytes (Lafaire & Schwarz, 1986; Schweigert, Lafaire & Schwarz, 1988).

Affinity of the cardiac Na^+–K^+ pump towards K^+ and its congeners

The experiments illustrated in Fig. 2 reveal the following relative potency of external cations to activate I_p: $Tl^+ > K^+ > NH_4^+$. The same order of potency is observed for the activation of the isolated Mg^{2+}-dependent Na^+–K^+-ATPase, the molecular basis of the Na^+–K^+ pump (cf. Schuurmans Stekhoven & Bonting, 1981). Furthermore, Eisner & Lederer (1979) deduced from measurements on voltage-clamped guinea-pig papillary muscles and sheep Purkinje fibres that the potency of external monovalent cations to activate cardiac active Na^+ transport obeys the sequence $Tl^+ > K^+ = Rb^+ > NH_4^+ > Cs^+ > Li^+$. All these findings agree with the present observations. The K^+ concentration of Na^+-containing media for half-maximum I_p activation ($K_{0.5}$ value) was derived to be 1·9 mM. Earlier estimations resulted in similar numbers. According to Nakao & Gadsby (1989) the $K_{0.5}$ value amounts to 1·5 mM-K^+ in guinea-pig ventricular myocytes. Cohen *et al.* (1987) reported a value of 0·8 mM-K^+ from measurements on canine Purkinje cells and Stimers, Shigeto & Lieberman (1990) calculated a $K_{0.5}$ value of 1·9 mM-K^+ for the Na^+ pump of cultured embryonic chick cardiac myocytes.

Does the coupling ratio change?

It is generally accepted that the Na^+–K^+ pump transports three Na and two K ions per ATP molecule split. For the interpretation of the data described above, it is of fundamental importance to know whether the coupling ratio between active Na^+ and K^+ transport varies as a function of the external concentration of monovalent cations and/or the membrane potential. Earlier investigations indicated that variations in the extracellular K^+, Rb^+ or Cs^+ concentration do not change the coupling ratio of the cardiac Na^+–K^+ pump (see Gadsby, 1984). Furthermore, the quantitative agreement between the $K_{0.5}$ values for Tl^+ and NH_4^+ derived from the present I_p measurements with data obtained in studies of the Na^+–K^+-ATPase (cf. Portius & Repke, 1967; Schuurmans Stekhoven & Bonting, 1981) strongly suggests a constant coupling ratio at various concentrations of these external activator cations. The effect of changes in the extracellular Na^+ concentration or in membrane potential on the coupling ratio of cardiac cells is unknown. In recent studies carried out on squid giant axons (Rakowski *et al.* 1989) and *Xenopus laevis* oocytes (Schwarz & Gu, 1988; Schwarz & Vasilets, 1991) no effect of external Na^+ or membrane potential (0 to −90 or −100 mV) on the coupling ratio was found. Thus for the interpretation of the present data it is assumed that the coupling ratio of rabbit cardiac Purkinje cells remains constant following changes of the concentration of external monovalent cations or of the membrane potential. Variations in I_p are considered to reflect changes of the unidirectional Na^+ and K^+ fluxes mediated by the Na^+–K^+ pump.

I_p–V curves of cells in Na^+-containing media

Figure 4 shows for the first time I_p–V relationships of cardiac Purkinje cells at various external K^+ concentrations. The general shape of the curves is quite similar

to those measured by Gadsby and co-workers (Gadsby *et al.* 1985; Bahinski, Nakao & Gadsby, 1988; Gadsby & Nakao, 1989; Nakao & Gadsby, 1989) on guinea-pig ventricular myocytes but differs from the corresponding curve obtained by Mogul *et al.* (1989) from guinea-pig cardiomyocytes. For unknown reasons the latter authors were unable to detect any voltage dependence of I_p. Nakao & Gadsby (1989) noticed a shift of the normalized I_p–V curve towards more positive potentials at 1 mM-K^+. This shift, which indicates a decrease in the apparent affinity of the Na^+ pump towards K^+ at low external K^+ concentrations and negative membrane potentials, was also observed in the present experiments (Fig. 5). The mechanism of the shift is not yet understood but it seems to be K^+ specific. Low external NH_4^+ or Tl^+ concentrations did not cause a comparable shift (Figs 6 and 7). In contrast to the report by Nakao & Gadsby (1989) that low extracellular K^+ concentrations exert little effect on the shape of the normalized I_p–V curve at positive membrane potentials we found consistently a decline of I_p within this potential range upon a decrease of the concentration of the external activator cation to $\leqslant K_{0.5}$ (Figs 4, 5, 6 and 7). This point will be discussed below in some detail.

The negative slope of the I_p–V relationship at low concentrations of external activator cations

Hitherto little experimental evidence was available for a region of negative slope in the I_p–V curve of cardiac cells. Gadsby & Nakao (1989) and Nakao & Gadsby (1989) observed regularly in guinea-pig cardiac myocytes a positive slope of the I_p–V relationship at negative membrane potentials but an approximately constant I_p at positive potentials over a wide range of concentrations of intra- and extracellular activator cations and membrane potentials. In line with their results these authors interpreted the shape of the I_p–V curve to mean that only a *single* voltage-sensitive partial reaction exists in the (unbranched) Na^+–K^+ pump cycle (cf. De Weer, 1984; Reynolds, Johnson & Tanford, 1985). Several lines of evidence suggest that the Na^+-extruding part of the pump cycle includes the voltage-sensitive step in question (Fendler, Grell, Haubs & Bamberg, 1985; Nakao & Gadsby, 1986; Rephaeli, Richards & Karlish, 1986; Apell, Borlinghaus & Länger, 1987). More specifically, the deocclusion and release of Na^+ to the extracellular solution are thought to represent this step (see Apell, 1989), which in turn controls the concentration of an intermediate that enters the rate-limiting partial reaction (probably the K^+ translocation; see De Weer, Gadsby & Rakowski, 1988). The translocation of K^+ or its congeners into the cell is electroneutral (Goldshleger, Karlish, Rephaeli & Stein, 1987; Bahinski *et al.* 1988). Thus the characteristics of the I_p–V relationship of guinea-pig ventricular myocytes were explained by the hypothesis that depolarization of the sarcolemma enhances the voltage-sensitive step of the Na^+ export to the extent where K^+ translocation becomes rate limiting for the overall Na^+–K^+ pump cycle (Bahinski *et al.* 1988). In contrast to this interpretation Schwarz and his co-workers (Lafaire & Schwarz, 1986; Schweigert *et al.* 1988; Schwarz & Gu, 1988) pointed out that the Na^+–K^+ translocation carried out by the Na^+ pump of both *Xenopus laevis* oocytes and *Torpedo californica* electroplax (expressed in *Xenopus* oocytes) may include (at least) *two* voltage-sensitive steps since a region of negative slope of the I_p–V curve at positive membrane potentials was measured under a variety of conditions. In a

recent paper (Rakowski, Vasilets, La Tona & Schwarz, 1991) the authors reported on observations which suggest that binding of external K^+ to the Na^+–K^+ pump might be a second voltage-dependent step in the pump cycle. The suggestion is supported by fluorescence changes in adenosine triphosphatase (ATPase)-rich membrane fragments labelled with an electrochromic styryl dye which reveals K^+ binding (and occlusion) as a voltage-sensitive step in Na^+–K^+ pumping (Stürmer, Bühler, Apell & Läuger, 1990, 1991). In accordance with these results a negative slope of the I_p–V curve was also found in the present experiments in Na^+-containing media particularly at low concentrations ($\leqslant K_{0.5}$) of the external activator cations (Figs 4, 5, 6 and 7). As discussed by both groups of authors a conceivable mechanism underlying the voltage-dependent K^+ binding might be that the binding site of the pump is accessible only via a narrow channel from the extracellular space. A K ion travelling through this channel will be affected by part of the electrical field across the membrane. A positive membrane potential will inhibit, a negative potential will facilitate, the migration of the K ion. K^+ binding may become rate determining for the whole Na^+–K^+ pump cycle at low external K^+ concentrations and positive membrane potentials. Thus our measurements are completely in line with the hypothesis that K^+ binding (and occlusion) is a voltage-sensitive step of the pump cycle also in cardiac Purkinje cells. At high concentrations of the external activator cations the I_p–V curve did not display a negative slope (Figs 3–8). According to Stürmer *et al.* (1991) K^+ binding is no longer rate limiting at high external K^+ concentration and the same seems to be valid for the binding of its congeners.

I_p–V curve of cells in Na^+-free solutions

Gadsby & Nakao (1987, 1989) and Nakao & Gadsby (1989) reported a strong decrease of the voltage dependence of I_p in guinea-pig ventricular myocytes superfused with Na^+-free bathing fluids. Similar alterations of the I_p–V relationship were observed in *Xenopus laevis* oocytes (Rakowski *et al.* 1991), on *Torpedo californica* electroplax pumps expressed in *Xenopus* oocytes (Schwarz & Valisets, 1991) and in squid giant axons (Rakowski *et al.* 1989). The data presented in Fig. 8 indicate that a corresponding effect exists in cardiac Purkinje cells superfused with Na^+-free media. The effect seems to be largely independent of the cation species used as a Na^+ substitute (choline ions, Li^+). However, compared to the I_p–V curves measured in Na^+-free solutions by Gadsby & Nakao (1989) and Nakao & Gadsby (1989), a stronger voltage dependence of I_p persisted in the present experiments. As already mentioned above, there is agreement that Na^+ deocclusion and release represent one voltage-sensitive step of the Na^+–K^+ pump cycle (P-E_2 $(Na_3) \rightarrow$ P-$E_2 + 3Na^+$, in terms of the Post–Albers scheme). It follows from the law of mass action that the step is enhanced in Na^+-free solution. In view of the altered voltage dependence of I_p in Na^+-free medium (Fig. 8*B*) an additional effect on a voltage-sensitive mechanism has to be assumed, too. It does not seem unreasonable to propose that a narrow channel connects the release site of Na^+, just as it does the K^+ binding site, with the external solution. The released Na^+ has to migrate in this channel within a part of the electrical field across the cell membrane. A negative membrane potential and external Na^+ inhibit, a positive potential and Na^+-free solution facilitate the migration. This hypothesis is in line with the recent findings by

Stürmer *et al.* (1991) that the K^+ binding and the Na^+ release sites are hidden in the membrane dielectric.

The shape of the I_p–V relationship of Purkinje cells superfused with a Na^+-free, choline-containing solution with only 0·05 M-K^+ was quite different from that observed at 5·4 or 10·8 mM-K^+ (Fig. 9; see also Fig. 8*B*). The former I_p–V curve displayed a shallow maximum at −40 to −60 mV and a region of negative slope over the potential range from −40 to +60 mV (Fig. 9). The shape indicates that at very low external K^+ the K^+ binding is sufficiently slow to become the rate-determining step of Na^+–K^+ pumping even at membrane potentials around −60 mV. The I_p–V relationship is similar to that observed by Rakowski *et al.* (1991) and Schwarz & Vasilets (1991) in *Xenopus* oocytes with respect to the extended region of negative slope at negative and positive membrane potentials. However, in the oocytes this region reaches up to −150 mV. The I_p–V curve of Purkinje cells in Na^+-free solution containing 0·05 mM-K^+ differs from that found in guinea-pig ventricular cells under comparable conditions (Na^+ free, 0·1 mM-K^+), where I_p displayed little voltage dependence at membrane potentials between −120 and +60 mV (Nakao & Gadsby, 1989).

The causes of this and the other discrepancies between the observations of Nakao & Gadsby (1989) and the present findings are unknown and demand further work. However, it seems conceivable that relatively small differences in the dimensions of the access channels to the Na^+ and K^+ binding sites of the pump can account, at least partly, for the different shapes of the I_p–V curves measured in cardiac cells.

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