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Na^+ PUMP CURRENT–VOLTAGE RELATIONSHIPS OF RABBIT CARDIAC PURKINJE CELLS IN Na^+-FREE SOLUTION

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SUMMARY

1. The Na^+ pump current (I_p) of isolated, single rabbit cardiac Purkinje cells in Na^+-free solution was measured at 32–34 °C by means of whole-cell recording.

2. The I_p amplitude was studied as a function of clamp potential (V_c) and external concentration of various monovalent cations known to activate the Na^+–K^+ pump.

3. Under conditions which strongly activated I_p the I_p–V_c curve of the cells displayed a positive slope at membrane potentials negative to −20 mV and little variation at more positive potentials.

4. The I_p–V_c relationship showed an extended region of negative slope at positive and negative potentials in solutions containing low concentrations of activator cations which caused little I_p activation. A positive slope of the I_p–V_c curve was occasionally observed at clamp potentials negative to −60 mV under these conditions.

5. The shape of the I_p–V_c relation was independent of the cation species used as external I_p activator.

6. At zero membrane potential half-maximum I_p activation ($K_{0.5(V_c=0\,mV)}$) occurred at 0·05 mM Tl^+, 0·08 mM K^+, 0·4 mM NH_4^+ and 1·5 mM Cs^+. The Hill coefficient derived amounted to 0·9 for Tl^+, 1·2 for K^+, 1·04 for NH_4^+ and 1·5 for Cs^+.

7. The concentrations of external activator cations required for half-maximum I_p activation increased with depolarization. The voltage dependence of the $K_{0.5}$ values could be described by a single exponential function for clamp potentials positive to −40 mV.

8. The steepness of the function is determined by a factor α, indicating the apparent fraction of an elementary charge which moves in the electrical field across the sarcolemma when external monovalent cations bind to the Na^+–K^+ pump.

9. The α values were calculated to be 0·32 for Tl^+, 0·24 for K^+, 0·29 for NH_4^+ and 0·18 for Cs^+. Possible interpretations of the α values are considered.

10. It is suggested that binding of external monovalent activator cations to the Na^+–K^+ pump (or a process related to the binding) is voltage dependent. This potential-dependent process determines mainly the shape of the I_p–V_c curve in cardiac Purkinje cells superfused with Na^+-free media containing low concentrations ($< K_{0.5(V_c=0\,mV)}$) of K^+ or its congeners.

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INTRODUCTION

The active Na^+–K^+ exchange across the cell membrane of animal cells is electrogenic. The cation transport carried out by the Na^+–K^+ pump generates an outward current of positive charges since three Na^+ ions are expelled from the cell but only two K^+ ions are taken up per ATP molecule hydrolysed. It is anticipated that the Na^+–K^+ pump current I_p should vary with the cell membrane potential (V_m). The first studies of cardiac I_p–V_m curves in Na^+-containing media revealed a monotonic increase of I_p with depolarization in the potential range between -160 and -20 mV and little voltage dependence of I_p at more positive membrane potentials (Gadsby, Kimura & Noma, 1985; Gadsby & Nakao, 1989; Nakao & Gadsby, 1989; Glitsch, Krahn & Pusch, 1989). The monotonic increase of I_p with depolarization at negative potentials is a common characteristic of many different cells (De Weer, Gadsby & Rakowski, 1988) and is generally considered to be related to the Na^+ deocclusion and/or Na^+ release in the Na^+–K^+ pump cycle (review: Apell, 1989). An I_p–V_m relationship displaying a negative slope at positive membrane potentials under physiological conditions was first reported for endogenous Na^+–K^+ pumps of *Xenopus* oocytes (Lafaire & Schwarz, 1986) and for pump molecules of *Torpedo* electroplax expressed in the oocytes (Schwarz & Gu, 1988). The negative slope of the I_p–V_m curve indicates a second voltage-dependent step in the pump cycle. Several observations suggest that binding of external K^+ or other activator cations to the Na^+–K^+ pump is the step involved (Rakowski, Vasilets, La Tona & Schwarz, 1991; Omay & Schwarz, 1992; Vasilets & Schwarz, 1992). In Na^+-containing solution the cardiac I_p–V_m relationship exhibits a negative slope at positive potentials only if the activation of the Na^+–K^+ pump by extracellular activator cations is less than half-maximal. However, in Na^+-free media, a region of negative slope is also present at negative membrane potentials (Bielen, Glitsch & Verdonck, 1991*a*, 1992) and might be adequately studied under these conditions. The aim of the present work is to explore the region of negative slope of the cardiac I_p–V_m curve in some detail and to obtain thereby new insights into the voltage dependence of the binding of external K^+ and its congeners to the Na^+–K^+ pump in heart cells.

METHODS

The methods used have been described previously in detail (Bielen *et al.* 1991*a*). Therefore, only a brief account of the procedure is presented here.

Preparation of single cells

The rabbits were killed by a blow on the head. Single Purkinje cells were isolated from the hearts by an enzymatic treatment. The treatment included a Langendorff perfusion of the heart with Ca^{2+}-poor media containing collagenase, hyaluronidase and, for the last 3 min, protease. Purkinje fibres were dissected from either ventricle and gently stirred by a magnetic stirrer for up to 1 h in the latter solution. The dissociated cells were about 140 μm in length and 16 μm in width. A culture dish containing isolated cells was fixed on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, Germany). The volume of the dish was reduced to about 0·3 ml by means of a plastic ring, which was pressed to the bottom of the dish. The cells were superfused with pre-warmed bathing fluid at 2 ml/min and 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 to pH 7·4 at 32–34 °C with NaOH). In Na^+-free solutions NaCl was

replaced by choline chloride (plus 5×10^{-6} M atropine sulphate; pH adjusted with LiOH). Na^+-free media containing KCl, TlCl, CsCl or NH_4Cl were used to evoke the Na^+–K^+ pump current I_p of the cells. All solutions except the standard medium contained 2 mM $BaCl_2$ and 5 mM $NiCl_2$ in order to suppress the K^+ conductance and the Na^+–Ca^{2+} exchange of the sarcolemma, respectively. Control experiments by means of atomic absorption spectrometry revealed a K^+ concentration of $< 2\ \mu M$ in nominally K^+-free solutions. Dihydroouabain (DHO; 0·2–0·5 mM), a specific inhibitor of the Na^+–K^+ pump, was occasionally added to the superfusion media from an aqueous stock solution. The Na^+-free (test) solutions were applied to the cell under investigation via two multibarrelled glass pipettes nearby. The medium 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).

Electrical measurements

I_p was measured by means of whole-cell recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The patch pipette (initial resistance, 2–4 MΩ after filling with patch pipette solution) was positioned at the sarcolemma by means of a micromanipulator (Leitz, Wetzlar, Germany) under visual control on a TV monitor (Philips, Eindhoven, Netherlands). Starting from a holding potential of −20 mV the cell membrane potential was clamped to preset values by step-like variations of the command potential. The clamp potential (V_c) and the resulting membrane current were measured by means of a voltage clamp amplifier (Axoclamp 2A; Axon Instruments, Burlinghame, CA, USA) and recorded on a pen recorder (Watanabe Multicorder, Tokyo, Japan).

Statistics

Whenever possible data are presented as means ± S.E.M; n indicates the number of cells studied. The significance of differences between means was checked by Student's one-sample, one-tailed t test. A difference was deemed significant if $P \leqslant 0{\cdot}05$.

RESULTS

Measurement of I_p–V_c curves

Figure 1*A* illustrates the measurement of I_p in an isolated rabbit cardiac Purkinje cell superfused with Na^+-free, choline-containing solution at three external K^+ concentrations ($[K^+]_o$). The top trace shows the clamp potential which was manually set to the voltages indicated starting from the holding potential of −20 mV. The bottom trace represents the membrane current. At the beginning of the record the bathing fluid is a Na^+-containing, K^+-free solution. Switching to a Na^+- and K^+-free, choline-containing medium shifts the current by about 17 pA in the outward direction, probably because the sarcolemma is less permeable to choline than for Na^+. Short applications (1–2 s) of Na^+-free solutions containing various K^+ concentrations evoke an additional outward current. The amplitude of the additional current depends on the K^+ concentration applied. Previous studies on cardiac cells have extensively documented that this additional outward current, which can be activated by external K^+ and other pump activator cations in Na^+-containing or Na^+-free media, represents the Na^+–K^+ pump current, I_p. It can be blocked by cardioactive steroids, specific inhibitors of the Na^+–K^+ pump (e.g. Gadsby & Nakao, 1989; Nakao & Gadsby, 1989; Bielen *et al.* 1991*a*). Correspondingly, in the present study I_p is defined and measured as the cardiac steroid-sensitive outward current induced by external K^+ or its congeners. The *initial* I_p amplitude was used for plotting the I_p–V_c relationships because the subsarcolemmal Na^+ concentration and thereby I_p might decline even during very short I_p activations (Bielen, Glitsch & Verdonck, 1991*b*). I_p activation at various clamp potentials alternated with a corresponding activation at −20 mV. If required, I_p at a distinct clamp potential

was normalized to the mean value of I_p derived from measurements at −20 mV before and after the clamp pulse. Between two runs in Na^+-free solution the cells were superfused with K^+-free, Na^+-containing medium.

Figure 1*B* shows I_p–V_c curves obtained from the sample record illustrated in Fig. 1*A*. As mentioned above, the I_p amplitude depends on $[K^+]_o$ at every clamp potential

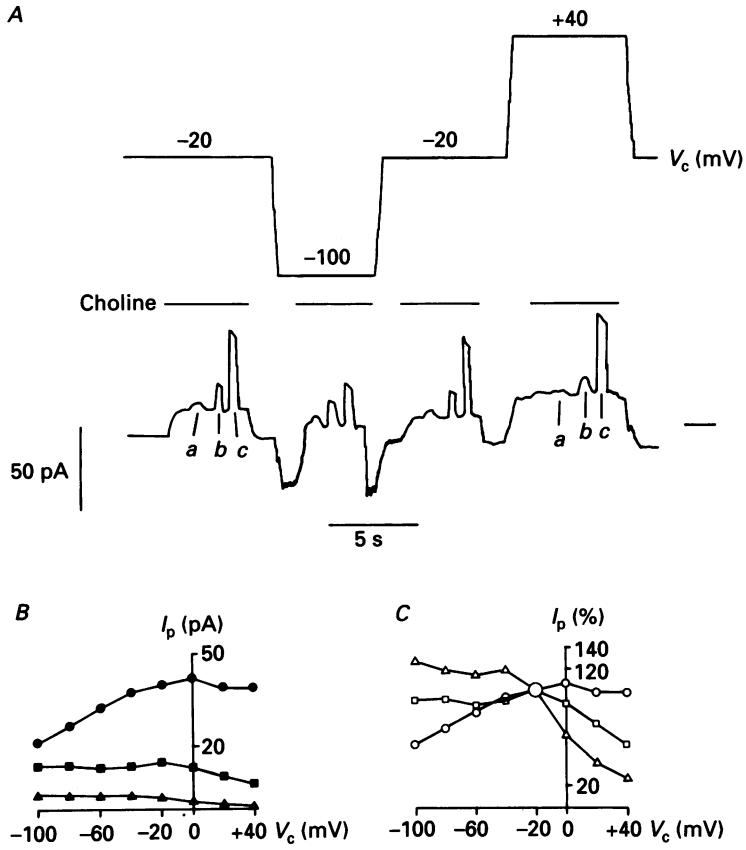

Fig. 1. Dependence of I_p on $[K^+]_o$ and membrane potential in a Purkinje cell superfused with Na^+-free solution. *A*, top trace shows clamp potential (V_c); bottom trace represents membrane current; middle traces signify presence of Na^+-free, choline-containing solution. Horizontal bar to the right of the bottom trace indicates zero current level. At each clamp potential I_p was successively activated by 0·02 mM K^+ (*a*), 0·08 mM K^+ (*b*) and 5·4 mM K^+ (*c*). *B*, I_p–V_c curves measured in the experiment illustrated in *A*. ▲, 0·02 mM K^+; ■, 0·08 mM K^+; ●, 5·4 mM K^+. *C*, normalized I_p–V_c relationships (from *A*). I_p amplitudes are normalized to the corresponding I_p amplitudes at −20 mV, which are arbitrarily set to 100%. △, 0·02 mM K^+; □, 0·08 mM K^+; ○, 5·4 mM K^+.

tested. I_p can be activated by micromolar K^+ concentrations in Na^+-free solution in accordance with earlier reports (Nakao & Gadsby, 1989; Bielen *et al.* 1991*a*). Furthermore, the voltage dependence of I_p varies with the external K^+ concentration. At 0·02 mM K^+ the I_p–V_c relationship of this cell displays a negative slope over almost the whole range of clamp potentials between −100 and +40 mV. Moreover, a

negative slope of the I_p–V_c curve is also observed between −20 and +40 mV at 0·08 mM K^+. The I_p–V_c relation exhibits a more conventional shape at 5·4 mM K^+, i.e. a positive slope between −100 and −20 mV and little further increase of I_p at more positive voltages. The different shapes of the I_p–V_c relationship are even more distinct if the I_p amplitudes at the various clamp potentials are normalized to the corresponding I_p values at the holding potential of −20 mV. Figure 1*C* presents the normalized I_p–V_c curves and emphasizes the negative slope of the I_p–V_c relationships obtained from the cell at low external K^+ concentrations.

Mean I_p–V_c curves in Na^+-free solutions containing K^+

Mean I_p–V_c relationships of cells superfused with Na^+-free media containing 0·05, 0·08 or 5·4 mM K^+ are shown in Fig. 2*A*. The I_p amplitudes are presented in picoamps. The I_p amplitudes decrease with decreasing $[K^+]_o$. At 0 mV the I_p amplitude in 0·08 mM K^+ is half that recorded in 5·4 mM external K^+, suggesting that the latter K^+ concentration causes maximum I_p activation under these conditions. However, the difference between the amplitudes becomes smaller with hyperpolarization. Little difference is seen at −100 mV.

The phenomenon is due to the different shapes of the I_p–V_c curves. The I_p–V_c relationship of cells superfused with solution containing 5·4 mM K^+ exhibits a positive slope between −100 and −20 mV and no *significant* change of the I_p amplitude at more positive membrane potentials. A positive slope of the I_p–V_c curve at potentials negative to −20 mV is also observed in 0·08 mM external K^+. The I_p–V_c curve displays a region of negative slope between −40 and +60 mV if the cells are superfused with a bathing fluid containing only 0·05 mM K^+.

The corresponding normalized I_p–V_c curves are presented in Fig. 2*B* which stresses the different voltage dependences of the I_p activation at various $[K^+]_o$. Compared to the maximal I_p activation at 5·4 mM K^+ low external K^+ concentrations are relatively less effective at positive membrane potentials and equally or more efficient at voltages negative to −20 mV. On the one hand the decrease of the S.E.M. values at the two higher $[K^+]_o$ in Fig. 2*B* suggests that the larger corresponding errors in Fig. 2*A* are due to different I_p amplitudes rather than to different voltage dependences of I_p in various cells. On the other hand the increase of the errors at 0·05 mM K^+ probably reflects the problems of measuring correctly small I_p amplitudes. I_p–V_c relationships corresponding to those depicted in Fig. 2*A* and *B* were also measured at 0·1 mM K^+ ($n = 2$), 0·15 mM K^+ ($n = 3$) and 0·3 mM external K^+ ($n = 2$) (not illustrated).

I_p–V_c curves of cells in media containing Tl^+

Thallium ions are known to be strong activators of the Na^+–K^+ pump. According to an earlier report I_p is maximally activated by 2 mM Tl^+ in a Na^+-free, choline$^+$-containing solution (Bielen *et al.* 1992). Under these conditions I_p amounted to 50 ± 6 pA ($n = 7$) at −20 mV in the present study. The pump current decreased to 23·5 ± 6 pA ($n = 2$) at 0·01 mM Tl^+ and −20 mV. Figure 3*A* depicts mean I_p–V_c relationships measured at 2 mM ($n = 5$–7), 0·02 mM ($n = 5$) and 0·01 mM Tl^+ ($n = 2$). The I_p amplitudes are normalized to their corresponding values at the holding potential of −20 mV. The voltage dependence of the fully activated I_p (at 2 mM Tl^+)

is conventional. The I_p–V_c curve displays a positive slope at negative membrane potentials but little unequivocal variation at positive voltages. Lowering the external Tl^+ concentration ($[Tl^+]_o$) to 0·02 or 0·01 mM causes a dramatic change in the shape of the I_p–V_c relationship. A negative slope of the I_p–V_c curve now becomes

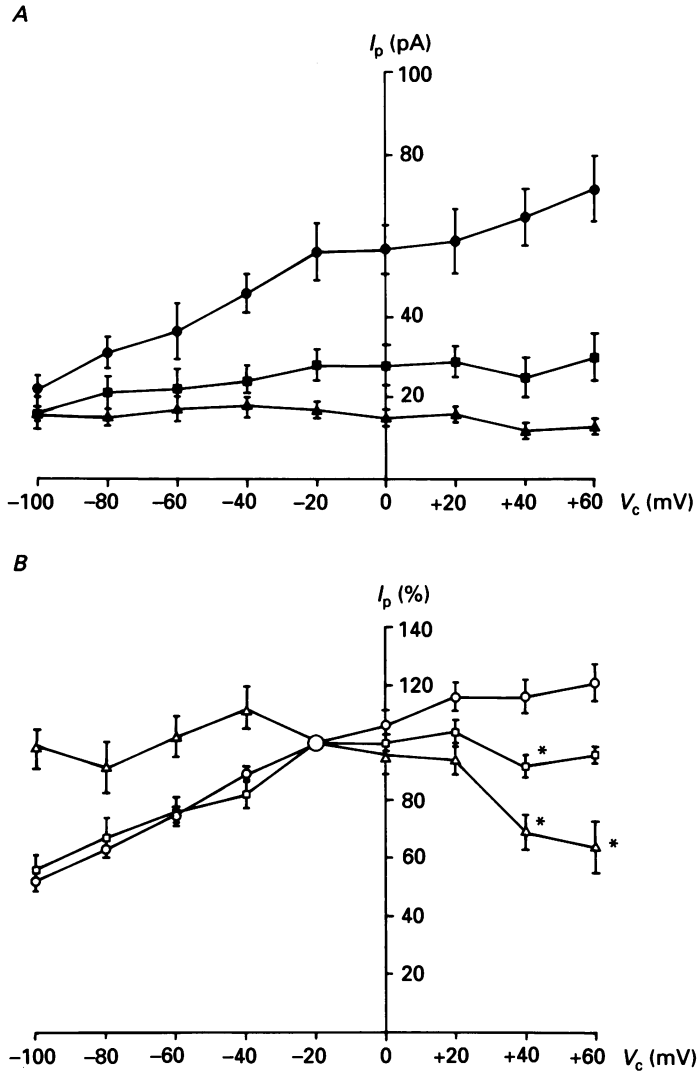

Fig. 2. Mean I_p–V_c curves in Na^+-free media containing various K^+ concentrations. *A*, I_p given in pA. ▲, 0·05 mM K^+ (n = 9–18); ■, 0·08 mM K^+ (n = 4–7); ●, 5·4 mM K^+ (n = 6–25). *B*, normalized mean I_p–V_c relationships. I_p amplitudes are normalized to the corresponding I_p amplitudes at the holding potential (−20 mV), which are arbitrarily set to 100%. Asterisks indicate I_p amplitudes smaller at more positive clamp potentials than at −20 mV ($P < 0·05$). △, 0·05 mM K^+; □, 0·08 mM K^+; ○, 5·4 mM K^+.

apparent over a wide range of membrane potentials positive to −60 mV. However, the curve still exhibits a positive slope at voltages negative to −60 mV. At the two lower Tl^+ concentrations the I_p–V_c relationships are nearly identical. It is clear from

Fig. 3*A* that depolarization from −60 mV decreases the I_p activation by low $[Tl^+]_o$ relative to the activation by the maximally activating concentration of 2 mM Tl^+. In addition an I_p–V_c curve was obtained from cells in a Na^+-free medium containing 0·05 mM Tl^+. At −20 mV I_p was measured to be 40·5 ± 0·7 pA (n = 2). The normalized

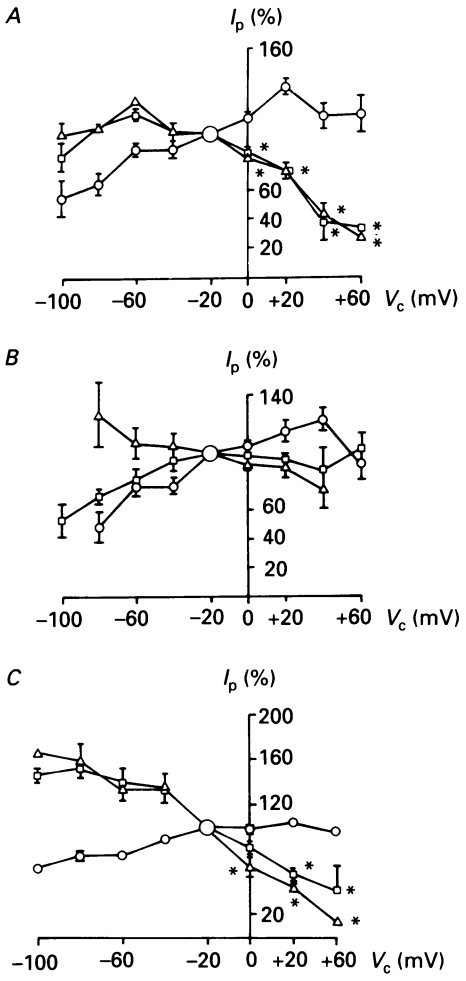

Fig. 3. I_p–V_c curves of Purkinje cells in Na^+-free solution containing various concentrations of different pump activator cations. The I_p amplitudes are normalized to the corresponding I_p amplitudes at −20 mV, arbitrarily set to 100%. Asterisks indicate I_p amplitudes smaller at more positive clamp potentials than at −20 mV ($P < 0·05$). In this and the following figures error bars are shown only if they exceed the size of the symbols. *A*, normalized mean I_p–V_c relationships at various $[Tl^+]_o$. △, 0·01 mM Tl^+ (n = 2); □, 0·02 mM Tl^+ (n = 5); ○, 2 mM Tl^+ (n = 5–7). *B*, normalized mean I_p–V_c curves at various $[Cs^+]_o$. △, 0·75 mM Cs^+ (n = 3–4); □, 1·0 mM Cs^+ (n = 5–12); ○, 20 mM Cs^+ (n = 2–7). *C*, normalized mean I_p–V_c relationships at various $[NH_4^+]_o$. △, 0·16 mM NH_4^+ (n = 2–4); □, 0·32 mM NH_4^+ (n = 3); ○, 5 mM NH_4^+ (n = 8–11).

I_p–V_c relation was situated between the curves observed at 2 mM Tl^+ and the two lower Tl^+ concentrations (data not shown).

I_p–V_c relationships of cells in solutions containing Cs^+

Compared to external Tl^+, caesium ions are rather ineffective as Na^+–K^+ pump activators in sheep cardiac Purkinje fibres (Eisner & Lederer, 1979). In the present experiments the I_p of isolated Purkinje cells was measured at the holding potential to be 42 ± 7 pA ($n = 7$) in a Na^+-free medium containing 20 mM Cs^+. The pump current decreased to 24 ± 5 pA ($n = 12$) at 1 mM Cs^+ suggesting about half-maximal I_p activation at this concentration. I_p amounted to 6 ± 1 pA ($n = 4$) at -20 mV in a solution containing only 0·75 mM Cs^+. During the same set of experiments I_p was found to be 68 ± 11 pA ($n = 11$) at 5·4 mM K^+. Thus the pump current was significantly larger than at 20 mM Cs^+ although the Cs^+ concentration was probably high enough for maximal I_p activation. Over the range of clamp potentials tested the I_p amplitudes observed at 20 mM Cs^+ reached *ca* 70% of the corresponding I_p values recorded at 5·4 mM K^+.

Mean I_p–V_c curves obtained at three external Cs^+ concentrations ($[Cs^+]_o$) are plotted in Fig. 3*B*. The I_p data are normalized to the appropriate amplitudes measured at the holding potential of -20 mV. The I_p–V_c relationships of cells in solutions containing 20 mM or 1 mM Cs^+ display a region of positive slope at membrane potentials negative to -20 mV and little consistent variation at more positive voltages. Purkinje cells in media containing only 0·75 mM Cs^+ exhibit a negative slope of the I_p–V_c relationship over the whole range of membrane potentials tested. I_p–V_c curves were also obtained from cells superfused with Na^+-free solution containing 4 or 10 mM Cs^+. I_p amounted to 41 ± 8 pA ($n = 7$) and $44 \pm 18{\cdot}5$ pA ($n = 3$), respectively, at the holding potential. The normalized mean I_p–V_c relationship of the three cells at 10 mM Cs^+ was identical to the curve measured at 20 mM Cs^+, except for the I_p amplitude at $+60$ mV (105 ± 9%), whereas the normalized I_p data obtained at 4 mM Cs^+ were consistently smaller at positive membrane potentials (not illustrated for clarity in Fig. 3*B*).

I_p–V_c curves of cells in NH_4^+-containing solution

The effect of NH_4^+ as an external pump activator cation on the I_p–V_c relationship of single rabbit Purkinje cells in Na^+-free media was studied at various external NH_4^+ concentrations ($[NH_4^+]_o$). At the holding potential I_p of cells superfused with a bathing fluid containing 5 mM NH_4^+ was measured to be $47{\cdot}8 \pm 13{\cdot}5$ pA ($n = 11$) or 97·7% of the I_p amplitude found at 5·4 mM K^+ during the same set of experiments. The I_p amplitude was clearly smaller at 0·16 mM NH_4^+ ($7{\cdot}7 \pm 1{\cdot}9$ pA; $n = 4$), 0·32 mM NH_4^+ (17 ± 5 pA; $n = 3$), 0·5 mM NH_4^+ ($27 \pm 5{\cdot}9$ pA; $n = 4$) and 1 mM NH_4 ($38{\cdot}5 \pm 8{\cdot}4$ pA; $n = 4$). Judging from the dependence of the I_p amplitude on $[NH_4^+]_o$ I_p was half-maximally activated somewhat below 0·5 mM NH_4^+ at -20 mV.

Normalized mean I_p–V_c relations of cells in NH_4^+-containing media are shown in Fig. 3*C*. A positive slope of the I_p–V_c curve at membrane potentials negative to -20 mV and little further increase at positive voltages are observed at 5 mM NH_4^+, a concentration causing full I_p activation. At the intermediate concentration of

1 mM NH_4^+ the I_p–V_c relation exhibits a small positive slope at negative potentials and a small negative slope at positive membrane potentials (not shown). A distinct negative slope over the whole voltage range is present at 0·16 mM and 0·32 mM NH_4^+. The I_p–V_c curve of cells ($n = 4$) superfused with a solution containing 0·5 mM NH_4^+

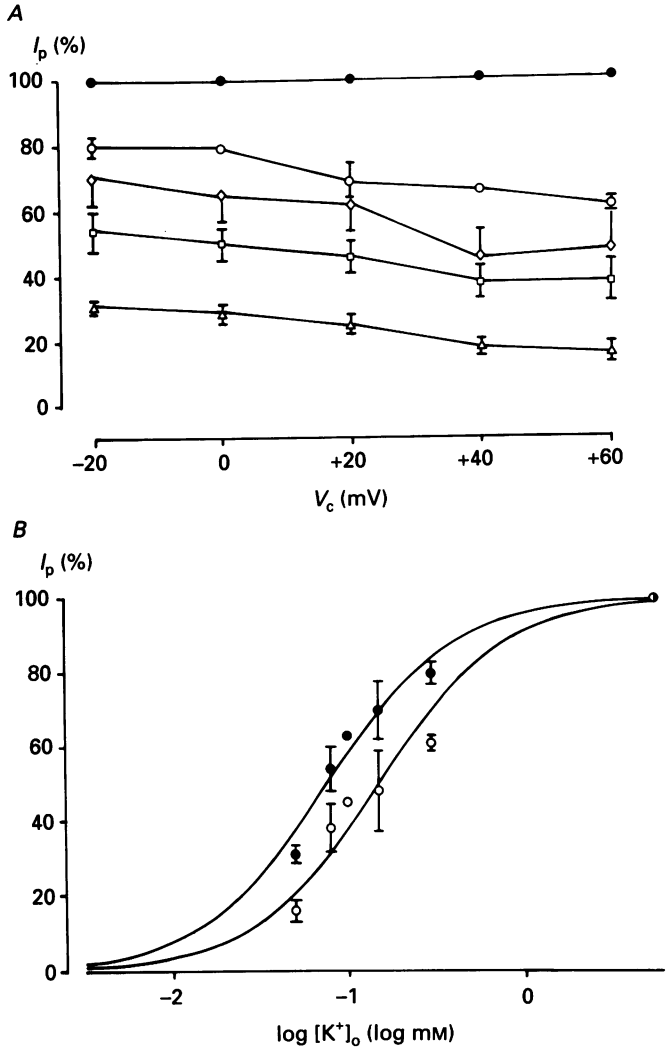

Fig. 4. I_p activation at various K^+ concentrations and clamp potentials. *A*, normalized mean I_p amplitudes at various $[K^+]_o$ and membrane potentials. The I_p amplitude at 5·4 mM K^+ is thought to represent maximum I_p at all potentials and is set to 100%. The corresponding I_p amplitudes at the various $[K^+]_o$ are normalized to the maximum I_p amplitude. The potency of $[K^+]_o \leqslant 0{\cdot}3$ mM for I_p activation decreases with depolarization. △, 0·05 mM K^+ ($n = 12$–18); □, 0·08 mM K^+ ($n = 7$); ◇, 0·15 mM K^+ ($n = 3$); ○, 0·3 mM K^+ ($n = 2$); ●, 5·4 mM K^+ ($n = 6$–25). *B*, semilogarithmic plot of normalized mean I_p amplitudes as a function of $[K^+]_o$ at two clamp potentials. ●, −20 mV; ○, +60 mV. The curves fitted to the data obey eqn (1) with $h = 1{\cdot}2$ and $K_{0{\cdot}5} = 0{\cdot}8$ mM K^+ (●) or $K_{0{\cdot}5} = 0{\cdot}15$ mM K^+ (○). $r^2 = 0{\cdot}99$ (●); $r^2 = 0{\cdot}97$ (○).

displayed a negative slope at all potentials positive to −80 mV and a small positive slope between −100 and −120 mV (not illustrated).

Does the species of the activator cation modify the I_p–V_c relationship?

Possible effects of the cation species on the shape of the I_p–V_c relationship were studied at two concentrations. Normalized I_p–V_c curves of cells in media containing a fully I_p-activating concentration of a cation (5·4 mM K^+, 20 mM Cs^+, 2 mM Tl^+ or 5 mM NH_4^+) displayed very much the same shape. The curves exhibited a similar positive slope at membrane potentials negative to −20 mV and little variation at more positive voltages. Likewise, the normalized I_p–V_c relations of cells in solutions containing a low concentration of an activator cation with a comparable potency for I_p activation (0·045 mM K^+, 0·02 mM Tl^+, 0·75 mM Cs^+ or 0·2 mM NH_4^+) displayed a similar negative slope between −60 and +40 mV. Thus little effect of the cation species on the shape of the I_p–V_c curve was observed in Na^+-free solution if the cation concentrations chosen evoked similar I_p amplitudes (in pA) at a distinct membrane potential.

Estimation of $K_{0.5}$ as a function of membrane potential for K^+ and its congeners

As mentioned above at least two voltage-dependent partial reactions exist in the Na^+–K^+ pump cycle. In solutions containing high or intermediate concentrations of a pump activator cation Na^+ deocclusion and/or release are rate determining at membrane potentials negative to −20 mV, whereas a different step, probably the binding of the external activator cation to the pump, determines the pumping rate and therefore the I_p amplitude at more positive voltages. In order to study the latter mechanism in more quantitative detail the I_p amplitudes measured at the various concentrations of the different activator cations were normalized to the I_p value obtained at the corresponding fully activating cation concentration (5·4 mM K^+, 2 mM Tl^+, 20 mM Cs^+ or 5 mM NH_4^+) for each clamp potential tested positive to −40 mV. The procedure is illustrated in Fig. 4*A*. The I_p amplitude recorded at 5·4 mM K^+ between −20 and +60 mV (abscissa) is arbitrarily set to 100% (ordinate) and the other current amplitudes are plotted relative to the current at 5·4 mM K^+. The plot emphasizes that I_p activation by low external K^+ concentrations decreases with depolarization. Figure 4*B* shows normalized I_p amplitudes (ordinate) as a function of the external K^+ concentration (mM; note logarithmic scale on abscissa) for two clamp potentials (●, −20 mV; ○, +60 mV). It includes I_p values measured at 0·1 mM K^+ ($n = 2$) which were omitted from Fig. 4*A* for clarity. The curves fitted to the data by least-squares non-linear regression (computer program GraphPad InPlot Version 4.0) obey the following equation:

$$I_p = A + \frac{B-A}{1+(K_{0.5}/[K^+]_o)^h}. \quad (1)$$

In order to derive adequate $K_{0.5}$ values it was assumed that maximum I_p (measured at 5·4 mM K^+) represents the top value B (100%) and no I_p activation (bottom value $A = 0\%$) occurs at 10^{-4} mM K^+. A Hill coefficient (h) of 1·2 was used for all clamp potentials. The h value resulted from a least-squares non-linear regression fit to the I_p amplitudes measured as a function of the external K^+ concentration ($[K^+]_o$) at zero

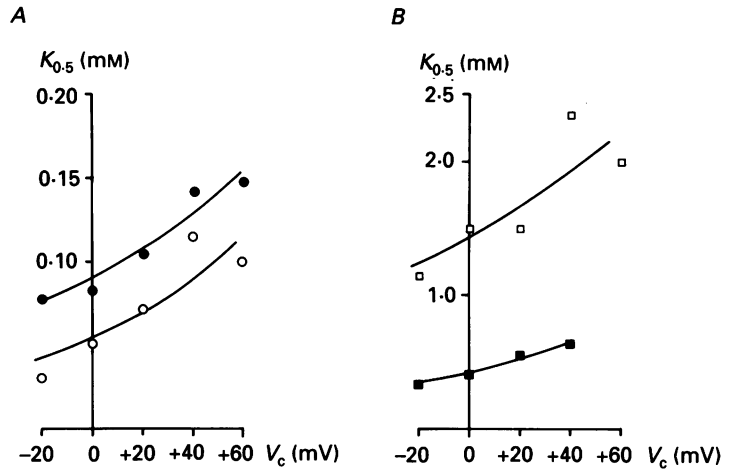

Fig. 5. $K_{0.5}$ values for I_p activation by external cations at various clamp potentials. The curves fitted to the data obey eqn (2) with parameters listed in Table 1. *A*: ●, K^+; r^2 = 0·93. ○, Tl^+; r^2 = 0·79. *B*: ■, NH_4^+; r^2 = 0·98. □, Cs^+; r^2 = 0·77. Note the different scales of the ordinates in *A* and *B*.

TABLE 1. $K_{0.5(V_c=0mV)}$ and α values for I_p activation by external cations

	Tl^+	K^+	NH_4^+	Cs^+
$K_{0.5(V_c=0mV)}$ (mM)	0·05	0·08	0·4	1·5
α	0·32	0·24	0·29	0·18

membrane potential. The fitting procedure yielded $K_{0.5}$ values of 0·08 mM and 0·15 mM K^+ at −20 and +60 mV, respectively. The normalized I_p amplitudes of cells superfused with media containing the different K^+ congeners were evaluated by the same procedure. The Hill coefficients derived from the corresponding I_p data at 0 mV amounted to 0·9 (Tl^+), 1·04 (NH_4^+), and 1·5 (Cs^+). The calculated $K_{0.5}$ values for K^+ and its congeners at 0 mV ($K_{0.5(V_c=0mV)}$) are listed in Table 1.

α values

According to Rakowski *et al.* (1991) and Omay & Schwarz (1992) the voltage dependence of the $K_{0.5}$ values for I_p activation by external K^+ or its congeners in *Xenopus* oocytes can be described by the exponential function:

$$K_{0.5} = K_{0.5(V_c=0mV)} \times \exp(\alpha F V_c / RT). \quad (2)$$

$K_{0.5(V_c=0mV)}$ gives the cation concentration causing half-maximal I_p activation at 0 mV clamp potential (V_c); α denotes (for monovalent cations) a fraction of an elementary charge and determines the steepness of the function; F, R and T have their usual meanings. The $K_{0.5}$ values for K^+ and the other activator cations were calculated by means of eqn (1). By fitting a curve according to eqn (2) to the $K_{0.5}$ values derived for K^+ at the different membrane potentials α was calculated to be 0·24. Thus 0·24 of an elementary charge apparently crosses the cell membrane during K^+ binding to external binding sites of the Na^+–K^+ pump. Possible interpretations of α values are considered below. Figure 5*A* shows the $K_{0.5}$ values for K^+ and Tl^+ as a function of membrane potential. The voltage dependence of the $K_{0.5}$ values for the

other K^+ congeners is illustrated in Fig. 5*B*. Obviously, all $K_{0.5}$ values increase with increasingly positive membrane potentials. The concentrations for half-maximal I_p activation at zero membrane potential ($K_{0.5(V_c=0\,mV)}$) differ by a factor of ~ 30; Tl^+ being the most potent, Cs^+ the least potent pump activator (Table 1). Compared to the data obtained in Na^+-containing media at -20 mV (Bielen *et al.* 1991*a*) the present $K_{0.5}$ values are smaller by a factor of 10–20. Little difference exists between the α values listed in Table 1 for the various cations. The mean value amounts to 0.26 ± 0.03 ($n = 4$).

DISCUSSION

I_p amplitude reflects Na^+–K^+ pump activity

The fundamental assumption underlying the following discussion of the results is, that the amplitude of I_p is a reliable measure of the Na^+–K^+ pump activity and alterations of the electrogenic fraction, i.e. the portion of pumped Na^+ that constitutes I_p, do not occur under the experimental conditions chosen. This important issue has been intensively considered in previous reports on I_p measurements carried out in Na^+–containing solution (e.g. Schwarz & Gu, 1988; Rakowski, Gadsby & DeWeer, 1989; Bielen *et al.* 1991*a*; review: Gadsby, 1984) or in Na^+-free media (Rakowski *et al.* 1989; Omay & Schwarz, 1992; Vasilets & Schwarz, 1992). The conclusion of the authors is that the electrogenic fraction is most probably independent of the external concentration of monovalent cations and the membrane potential. Therefore, the basic assumption seems acceptable.

I_p–V_c relationships of cells in Na^+-free solution

I_p–V_c curves of cardiac Purkinje cells were measured in Na^+-free solutions containing 2 mM Ba^{2+} and 5 mM Ni^{2+} in order to suppress the K^+ conductance and the Na^+–Ca^{2+} exchange, respectively. Control experiment with two cells in a medium containing 20 mM Cs^+ revealed no effect of 2 mM Ba^{2+} on the shape of the I_p–V_c relation. Furthermore, it was previously shown that 5 mM Ni^{2+} does not affect I_p of Purkinje cells in Na^+-free solutions (Bielen *et al.* 1991*a*). Application of Na^+-free media generally reduces the voltage dependence of I_p found in Na^+-containing solution at negative membrane potentials in *Xenopus* oocytes (Schweigert, Lafaire & Schwarz, 1988), squid giant axons (Rakowski *et al.* 1989), cardiac ventricular myocytes (Gadsby & Nakao, 1987, 1989) and Purkinje cells (Bielen *et al.* 1991*a*). However, the effect seems to be somewhat smaller in Purkinje cells than in ventricular cells.

Components of the I_p–V_c curve

Cardiac Purkinje cells in *Na^+-rich* solutions containing activator cations $> K_{0.5(V_c=-20\,mV)}$ display a characteristic normalized I_p–V_c relation. I_p increases with depolarization at negative membrane potentials up to ~ -20 mV and remains essentially unchanged upon further depolarization. At external concentrations of K^+ or its congeners $< K_{0.5(V_c=-20\,mV)}$ the I_p–V_c curve of the cells exhibits a negative slope at positive membrane potentials (Bielen *et al.* 1991*a*). Basically, the same components of the I_p–V_c relation were observed here in Purkinje cells superfused with *Na^+-free* media. In solutions containing K^+ or K^+ congeners at concentrations $> K_{0.5(V_c=0\,mV)}$

the I_p–V_c curve of the cells exhibited a positive slope at negative voltages (though less steep than in Na^+-containing bathing fluid) and little consistent variation at more positive potentials (Figs 1*C* and 3). At low concentrations of external activator cations ($< K_{0.5(V_c=0mV)}$) the I_p–V_c relationships often showed a negative slope over the whole range of clamp potentials tested (Figs 1*C* and 3*B* and *C*) in contrast to the observations for cells in Na^+-rich solution. Interestingly, even under these conditions the I_p–V_c curve occasionally displayed a positive slope at membrane potentials negative to −20 mV (Figs 2 and 3*A* and *B*). This finding is at variance with the reports by Rakowski *et al.* (1991) and Omay & Schwarz (1992) on I_p–V_c relationships of *Xenopus* oocytes in Na^+-free media. According to the authors the I_p–V_c curves exhibit a negative slope over the whole potential range accessible ($\sim +50$ mV to −150 mV) at activator cation concentrations which barely or moderately activate I_p.

The binding of external K^+ and its congeners to the cardiac Na^+–K^+ pump determines one component of the I_p–V_c curve in Na^+-free media

The variation of the normalized I_p–V_c curves with the external concentration of activator cations (Figs 1–3) indicates that, at a given membrane potential, a process which depends on these cations determines the amplitude of I_p. Vice versa, at a given external concentration of K^+ or its congeners, the effect of this process on the shape of the I_p–V_c depends on membrane potential. Since the translocation of K^+ and its congeners into cells is supposed to be voltage independent (Goldshlegger, Karlish, Rephaeli & Stein, 1987; Bahinski, Nakao & Gadsby, 1988), it is generally assumed that binding of the activator cations to the Na^+–K^+ pump (or their subsequent occlusion within the pump molecules) probably is the potential-dependent process involved (Rakowski *et al.* 1991; Stürmer, Bühler, Apell & Läuger, 1991). It seems possible to study the binding in some quantitative details by analysing the effects of external K^+ and K^+ congeners on that component of the I_p–V_c curve which reflects the binding. A prerequisite for a proper analysis is to avoid the range of membrane potentials in which a different process, presumably Na^+ release from the pump, dominates the shape of the I_p–V_c relationship and causes a positive slope of the I_p–V_c curve. Therefore, the $K_{0.5}$ values of the different activator cations for I_p activation were calculated only at membrane potentials positive to −40 mV.

$K_{0.5}$ values at zero membrane potential

The early report by Post, Merrit, Kinsolving & Albright (1960) demonstrated competition between external Na^+ and K^+ for a common binding site at the Na^+–K^+ pump of erythrocytes and an appreciably lower $K_{0.5}$ value for the activation of the pump by extracellular K^+ in Na^+-free medium than in Na^+-containing solution. In accordance with these observations the present experiments yielded $K_{0.5}$ values for the I_p activation by K^+ and its congeners in Na^+-free medium which are lower by a factor of 10–20 than the corresponding values derived from experiments in Na^+-containing solution (cf. Bielen *et al.* 1991*a*). A similar decrease of the $K_{0.5}$ value for the activation of I_p by external K^+ in Na^+-free or extremely Na^+-poor media was found in squid giant axons (Rakowski *et al.* 1989) and cardiac ventricular cells (Nakao & Gadsby, 1989). The $K_{0.5(V_c=0mV)}$ value reported here (0·08 mM K^+) agrees reasonably well with the values published by Nakao & Gadsby (1989; 0·22 mM K^+)

and Bielen *et al.* (1991*a*; 0·13 mM K^+). The $K_{0.5}$ values for K^+ and K^+ congeners presented above are clearly lower than those reported by Omay & Schwarz (1992) for the activation of I_p in *Xenopus* oocytes in Na^+-free media. However, the sequence of potency for I_p activation of $Tl^+ > K^+ > NH_4^+ > Cs^+$ (Eisner & Lederer, 1979, 1980) is the same for both cell species and may point to a common mechanism involved in cation binding to the Na^+–K^+ pump (see below).

Calculation of $K_{0.5}$ values at various clamp potentials

The results shown in Figs 1–3 reveal that the potency of monovalent cations for I_p activation decreases with depolarization. In order to derive the $K_{0.5}$ values for the different activator cations at membrane potentials positive to −40 mV, the Hill coefficients (h) obtained by applying eqn (1) to the data measured at zero membrane potential were used. The procedure seems adequate since calculations did not disclose any systematic variation of h values with membrane potential. The mean h value for the activator cations at zero membrane potential amounted to $1{\cdot}16 \pm 0{\cdot}13$ ($n = 4$). Omay & Schwarz (1992) assumed a constant h value of 1·3 for all activator cations and membrane potentials in *Xenopus* oocytes. In agreement with the reports by Schwarz and his colleagues (Rakowski *et al.* 1991; Omay & Schwarz, 1992) our calculations yielded increasing $K_{0.5}$ values with depolarization. The voltage dependence of the $K_{0.5}$ values can be described by an exponential function according to eqn (2). The steepness of the function is determined by the α value. The α values derived from the data presented varied between 0·18 and 0·32 of an elementary charge (mean, $0{\cdot}26 \pm 0{\cdot}03$; $n = 4$). An obvious correlation does not exist between the values obtained for α and $K_{0.5(V_c=0\,mV)}$ (cf. Table 1). In experiments on *Xenopus* oocytes Omay & Schwarz (1992) found α values between 0·20 and 0·47 (mean, 0·39) of an elementary charge for various external activator cations.

Possible meaning of α values

As mentioned in the previous section, in cardiac Purkinje cells binding of external K^+ or K^+ congeners to the Na^+–K^+ pump is accompanied by the apparent transfer of about 0·26 elementary charge across the sarcolemma. An interpretation of the finding would be that cation binding occurs in an 'ion well' (Läuger & Apell, 1986) located within the electric field in the sarcolemma. The activator cations have to travel through a narrow 'high-field access channel' to reach the binding site and sense thereby a part of the electrical field across the cell membrane. A positive membrane potential hampers the travel. Stürmer *et al.* (1991) substantiated this hypothesis by impressing experimental evidence. Other experimental results are also in keeping with the idea of an 'ion well' within the membrane. They include the observations on the binding of K^+ and its congeners to the Na^+–K^+ pump in *Xenopus* oocytes by Rakowski *et al.* (1991) and Omay & Schwarz (1992). The latter authors pointed out that the apparent affinities of the binding site for the pump activator cations display the same sequence as does the permeability of the delayed rectifying K^+ channels of neurons towards cations. Clearly, the identical sequence supports the hypothesis of a binding site in an 'ion well' accessible via a channel-like structure. However, certain data are difficult to reconcile with a simple 'ion well' concept. For example, in a recent paper Vasilets & Schwarz (1992) reported that one mechanism

of K^+ binding to *Torpedo* pumps expressed in *Xenopus* oocytes is probably accompanied by the apparent transfer of 2·46 elementary charges across the cell membrane. The observation is unexpected because it is generally accepted that *two* K^+ are pumped into the cell per pump cycle and therefore more than two elementary charges should not move during K^+ binding and/or occlusion. Thus mechanisms different from simple cation binding in an 'ion well', such as additional voltage-dependent changes in the conformation of an external binding site, which cause potential-dependent alterations of the affinity towards cations, cannot be ruled out at present.

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