Change of Na^+ pump current reversal potential in sheep cardiac Purkinje cells with varying free energy of ATP hydrolysis

H. G. Glitsch and A. Tappe

Department of Cell Physiology, Ruhr-University, D-44780 Bochum, Germany

1. The Na^+–K^+ pump current, I_p, of cardioballs from isolated sheep cardiac Purkinje cells was measured at 30–34 °C by means of whole-cell recording.

2. Under physiological conditions I_p is an outward current. Experimental conditions which cause a less negative free energy of intracellular ATP hydrolysis (ΔG_{ATP}) and steeper sarcolemmal gradients for the pumped Na^+ and Cs^+ ions evoked an I_p in the inward direction over a wide range of membrane potentials. The reversal of the I_p direction was reversible.

3. The inwardly directed I_p increased with increasingly negative membrane potentials and amounted to $-0{\cdot}13 \pm 0{\cdot}03$ μA cm^{-2} (mean ± S.E.M.; $n = 6$) at −95 mV.

4. The reversal potential (E_{rev}) of I_p was studied as a function of ΔG_{ATP} at constant sarcolemmal gradients of the pumped cations.

5. In order to vary ΔG_{ATP} the cell interior was dialysed with patch pipette solutions containing 10 mM ATP and different concentrations of ADP and inorganic phosphate. The media were composed to produce ΔG_{ATP} levels of about −58, −49 and −39 kJ mol^{-1}.

6. A less negative ΔG_{ATP} shifted E_{rev} to more positive membrane potentials. From measurements of I_p as a function of membrane potential E_{rev} was estimated to be −195, −115 and −60 mV at ΔG_{ATP} levels of approximately −58, −49 and −39 kJ mol^{-1}, respectively. The calculated E_{rev} amounted to −224 mV at $\Delta G_{ATP} \approx -58$ kJ mol^{-1}, −126 mV at $\Delta G_{ATP} \approx -49$ kJ mol^{-1} and −24 mV at $\Delta G_{ATP} \approx -39$ kJ mol^{-1}.

7. Possible reasons for the discrepancy between estimated and calculated E_{rev} values are discussed.

8. Shifting ΔG_{ATP} to less negative values not only altered E_{rev} but also diminished I_p at each membrane potential tested. The maximal I_p ($I_{p,max}$), which can be activated by external Cs^+ (Cs^+_o), decreased under these conditions, whereas $[Cs^+]_o$ causing half-maximal I_p activation remained unchanged. Similarly, the voltage dependence of I_p activation by Cs^+_o was unaffected.

9. It is concluded that E_{rev} of I_p varies with ΔG_{ATP} at constant sarcolemmal gradients of the pumped cations. This agrees with thermodynamic considerations.

The energy required for active Na^+ and K^+ transport across cell membranes is provided by the free energy of intracellular ATP hydrolysis (ΔG_{ATP}). According to thermodynamic considerations it should be possible to reverse the direction of the active cation transport and to produce ATP from ADP and inorganic phosphate (P_i) at the expense of a downhill movement of Na^+ and K^+ through the Na^+–K^+ pump. In fact, Garrahan & Glynn (1966, 1967) demonstrated the synthesis of ATP by the pump in erythrocytes which were pretreated to have low intracellular Na^+ and ATP but high K^+, ADP and P_i concentrations. In K^+-free media with a high Na^+ concentration these cells incorporated labelled P_i into ATP by a mechanism blocked by cardiac glycosides which are known to be specific inhibitors of the Na^+–K^+ pump (Schatzmann, 1953). During the following years experiments on red cells (Lant & Whittam, 1968; Lew, Glynn & Ellory, 1970) and non-myelinated nerve fibres (Chmouliovsky & Straub, 1974) confirmed that lowering the free energy of ATP hydrolysis combined with steep

transmembranal gradients for the transported ions forces the Na^+–K^+ pump to run backwards and synthesize ATP.

A backwards-running Na^+–K^+ pump should produce an inwardly directed I_p. The reversal potential (E_{rev}) of the Na^+ pump current is the membrane potential at which ΔG_{ATP} equals the energy required for the active transport of Na^+ and K^+, and I_p vanishes (cf. De Weer, Gadsby & Rakowski, 1988*b*):

$$\Delta G_{ATP} + m\left(-FE_{rev} + RT \ln \frac{[Na^+]_o}{[Na^+]_i}\right) + n\left(FE_{rev} + RT \ln \frac{[K^+]_i}{[K^+]_o}\right) = 0. \quad (1)$$

$[Na^+]_o$ and $[K^+]_o$ represent the extracellular and $[Na^+]_i$ and $[K^+]_i$ the intracellular ion concentrations, m and n stand for the number of Na^+ and K^+, respectively, which are transported per pump cycle and F, R and T have their usual meanings. Rearranging and introducing the $3Na^+$: $2K^+$ stoichiometry of the active Na^+–K^+ exchange yields:

$$E_{rev} = \frac{\Delta G_{ATP}}{F} + 3E_{Na} - 2E_K. \quad (2)$$

E_{Na} and E_K denote the Nernst potential of the respective ions. E_{rev} is calculated to be −200 to −240 mV under physiological conditions (De Weer *et al.* 1988*b*). I_p is inward negative to E_{rev} but outward at more positive potentials. Thus, under physiological conditions, I_p is an outward current in the range of membrane potentials which are experimentally accessible. In order to measure an inward I_p, E_{rev} has to be shifted to more positive potentials. According to eqn (2) this can be achieved by producing a less negative ΔG_{ATP} and a *simultaneous* increase in the Na^+ and K^+ gradients across the cell membrane. A few authors have chosen to use this procedure and reported on an inwardly directed I_p over the whole potential range which can be studied (De Weer & Rakowski, 1984; Bahinski, Nakao & Gadsby, 1988; Efthymiadis & Schwarz, 1991). The aim of the present study was to show by measurements of I_p the reversibility of this procedure and to demonstrate predictable variations of E_{rev} following changes in ΔG_{ATP} at *constant* sarcolemmal ionic gradients in cardiac cells. Some of the results have already been published in abstract form (Glitsch & Tappe, 1993*b*, *c*, 1994*a*, *b*).

METHODS

Preparation of single cells

Single Purkinje cells were isolated from sheep cardiac Purkinje fibres dissected from either ventricle at the local slaughterhouse. The enzymatic treatment has been described earlier in detail (Glitsch, Krahn & Pusch, 1989). In short, cell isolation was performed in a laminar flow cabinet at 37 °C. Ca^{2+}-free Tyrode solution containing various concentrations of protease, collagenase and elastase were applied to the fibres for about 100 min. Afterwards the extracellular Ca^{2+} concentration was increased in six steps by superfusing the fibres for 20 min with each of six different mixtures of Ca^{2+}-free Tyrode solution and culture medium (Medium 199; Gibco, Paisley, UK), supplemented with 5% fetal calf serum (Product No. 210463; Boehringer, Mannheim, FRG) and 1% Nutridoma-SR (Product No. 1271091; Boehringer). The final medium was composed of Ca^{2+}-free Tyrode solution and culture medium in equal amounts. The Purkinje cells were then squeezed out of the fibres into culture dishes (diameter 35 mm; Falcon, Becton Dickinson, Plymouth, UK) and cultured in culture medium for up to 7 days in an incubator (Heraeus B 5060 EK/CO_2; Hanau, FRG) at 95% humidity, 2% CO_2 and 37 °C. All media contained gentamycin (Product No. 16-760-45; Flow Laboratories, Irvine, UK) and kanamycin (K-0129; Sigma, St Louis, MO, USA), 10 μg ml^{-1} each. After 24–40 h in culture isolated Purkinje cells display a spherical shape with a diameter of 40–70 μm ('cardioballs', cf. Bechem, Pott & Rennebaum, 1983). For geometrical reasons they are better suited for whole-cell recording (see below) than freshly isolated Purkinje cells. We have previously shown that the control of the subsarcolemmal ionic concentration by the patch pipette solution is better in the spherical cardioballs than in cylindrical cells of considerable length and rather small diameter (Bielen, Glitsch & Verdonck, 1991*a*; Glitsch & Tappe, 1991). The experiments were carried out on cells after 1–5 days in culture.

Solutions

Reversal of I_p. For the experiments aimed at the reversal of the Na^+–K^+ pump current, media were used which were designed to steepen the sarcolemmal gradients of the transported Na^+ and Cs^+ and to lower simultaneously the free energy of ATP hydrolysis. The external (Cs^+-free) solution contained (mM): 140 NaCl, 10 NaOH, 6 TEACl, 1·5 $MgCl_2$, 0·9 $CaCl_2$, 2 $BaCl_2$, 5 $NiCl_2$ and 20 Hepes. $BaCl_2$ and $NiCl_2$ were added in order to suppress sarcolemmal K^+ conductances and the Na^+–Ca^{2+} exchange, respectively. The composition of the (Na^+-free) patch pipette medium was (mM): 110 CsCl, 30 CsOH, 15 TEACl, 5 TrisADP, 2·5 Cs_2HPO_4, 2·5 CsH_2PO_4, 3 $MgCl_2$ (free Mg^{2+} ~0·6 mM), 6 EGTA, 21 Hepes, 5 2-deoxy-D-glucose (added directly) and 0·002 carbonylcyanide-*m*-chlorophenyl-hydrazone (from a 2 mM stock solution in dimethylsulphoxide). The latter two substances block the ATP synthesis via oxidative phosphorylation and glycolysis. Dimethylsulphoxide (1 ‰) had no effect on I_p. In order to abolish the reversed I_p and to evoke I_p in the (physiological) outward direction, solutions which induce low sarcolemmal ionic gradients and increase the free energy of ATP hydrolysis were applied to the cardioballs. The external medium contained 6 mM CsCl instead of TEACl; otherwise the composition was the same as given above. The patch pipette solution now contained (mM): 130 CsCl, 10 CsOH, 15 NaOH, 10 MgATP, 10 glucose, 3 $MgCl_2$ (free Mg^{2+} ~2·1 mM), 6 EGTA and 26 Hepes.

E_{rev} as a function of ΔG_{ATP}. The external medium containing 6 mM CsCl was used throughout. The patch pipette solutions for ΔG_{ATP} levels of ~−58, ~−49 and ~−39 kJ mol^{-1} are given in Table 1. In addition, I_p was studied at two ΔG_{ATP} levels as a function of $[Cs^+]_o$ in superfusion media containing either 40 or 60 mM CsCl plus TEACl: variation of [CsCl] in the two solutions was compensated by changing [TEACl] in order to keep [CsCl] + [TEACl] constant at either 40 or 60 mM. The pH of all solutions was adjusted to pH 7·4, either with HCl or with the hydroxide of the main cation.

Table 1. Patch pipette solutions for varying ΔG_{ATP}

ΔG_{ATP} (kJ mol^{-1})	CsCl (mM)	CsOH (mM)	NaOH (mM)	MgATP (mM)	TrisADP (mM)	Cs_2HPO_4 (mM)	CsH_2PO_4 (mM)	$MgCl_2$ (mM)	EGTA (mM)	Hepes (mM)
~−58	110	30	10	10	0·3	0·15	0·15	3	6	56
~−49	127	10	10	10	2·0	1·00	1·00	3	6	10
~−39	60	50	10	10	20·0	10·00	10·00	3	6	36

Calculation of ΔG_{ATP}

ΔG_{ATP} can be calculated from the composition of the various patch pipette solutions by:

$$\Delta G_{ATP} = \Delta G^{\circ}_{ATP} + RT \ln \frac{[ADP][P_i]}{[ATP]}, \quad (3)$$

where ΔG°_{ATP} denotes the standard free energy of ATP hydrolysis. [ATP], [ADP] and [P_i] represent the respective concentrations of adenosine triphosphate, adenosine diphosphate and inorganic phosphate in the patch pipette media. R and T have their usual meaning. ΔG°_{ATP} was derived according to the procedure of Guynn & Veech (1973) who related the equilibrium dissociation constant, K_{ATP}, of the ATP hydrolysis at any [H^+] and [Mg^{2+}] of the media to K_{ATP} measured under standard conditions defined by the authors. Knowing K_{ATP} at given [H^+] and [Mg^{2+}] permits the calculation of the actual ΔG°_{ATP} by:

$$\Delta G^{\circ}_{ATP} = -RT \ln K_{ATP}. \quad (4)$$

In order to calculate K_{ATP}, various stability constants of ATP, ADP, P_i and EGTA compounds with ionic constituents of the solutions have to be known. These constants were computed by means of a program published by Fabiato (1988). Table 2 presents values of ΔG°_{ATP} and ΔG_{ATP} calculated for three patch pipette solutions and the corresponding E_{rev} which was derived by means of eqn (1). The free [Mg^{2+}] of the different solutions varies. Previous unpublished measurements (H. G. Glitsch & T. Krahn) revealed no effect on I_p following variation of [Mg^{2+}] in the pipette solution between 0 and 11 mM Mg^{2+}.

Experimental procedure and whole-cell recording

A culture dish containing a few hundred cardioballs was installed on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, FRG). The external medium (prewarmed to 30–34 °C) was pumped into the dish at 0·5–0·7 ml min^{-1} and removed by suction opposite to the inflow. The cardioball under study was additionally superfused by the external medium with or without dihydroouabain (DHO; Product No. 7197, Roth, Karlsruhe, FRG; 2×10^{-4} M) via a multibarrelled pipette (internal tip diameter ~200 μm) nearby. The release of the solution from the pipette was carried out at 0·1–0·2 ml min^{-1} by gravitational force and controlled by a command valve unit. Solution change was complete within 400 ms as judged from the variation in membrane current upon switching between media containing different [K^+].

Current (I)–voltage (V) relationships were obtained by whole-cell recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) from the cardioballs. The initial resistance of the patch pipettes filled with one of the patch pipette media was measured to be 1–3 MΩ. The pipettes were fixed in a holder designed for intracellular perfusion with various solutions. The holder was originally described by Soejima & Noma (1984) and was modified as reported earlier (Glitsch *et al.* 1989). Starting from a holding potential of −20 mV voltage pulses were applied to the cell studied. The resulting membrane current was measured by means of an L/M EPC-7 patch clamp amplifier (List-Medical, Darmstadt, FRG). The clamp potential and the corresponding membrane current were displayed on an oscilloscope (model 5111A; Tektronix, Beaverton, OR, USA) and recorded on a pen recorder (R-50; Rikadenki Kogyo Co., Tokyo, Japan) or stored by means of an A/D converter (DT 2801A; Data Translation, Marlborough, MA, USA) in a personal computer. In some experiments, the membrane current was only measured at the holding potential under various conditions. The Na^+–K^+ pump current I_p was identified as the current activated by external Cs^+ and inhibited by the cardiac glycoside DHO. The surface of the cells was estimated from the diameter measured through the microscope assuming a regular geometrical shape for the cardioballs (cf. Glitsch *et al.* 1989).

Statistics

Whenever possible data are presented as means ± S.E.M. The S.E.M. is shown in the figures only if it is larger than the size of the symbols. Differences between means and zero were checked for significance by Student's one-sample, two-tailed t test. They were deemed significant if $P \leqslant 0{\cdot}05$. n denotes the number of cardioballs studied.

RESULTS

Measurement of I_p

As mentioned above, I_p was measured as current activated by Cs^+_o and blocked by the cardiac glycoside DHO. The lower trace of Fig. 1*A* displays the membrane current of a cardioball at the holding potential of −20 mV 6 min after establishing the whole-cell configuration. The upper trace

Table 2. Calculated free Mg^{2+} concentration, ΔG°_{ATP}, ΔG_{ATP} and E_{rev} for the pipette solutions used to study E_{rev} as a function of ΔG_{ATP}

Approximate ΔG_{ATP} (kJ mol^{-1})	Pipette solution ATP (mM)	ADP (mM)	P_i (mM)	Free Mg^{2+} (mM)	ΔG°_{ATP} (kJ mol^{-1})	ΔG_{ATP} (kJ mol^{-1})	E_{rev} (mV)
~−58	10	0·3	0·3	1·99	−28·7	−58·18	−224
~−49	10	2·0	2·0	1·46	−28·9	−48·75	−126
~−39	10	20·0	20·0	0·35	−30·7	−38·87	−24

indicates $[Cs^+]_o$. Initially the cell was superfused with a Cs^+-free solution, which contained no activator cation of the pump. Switching to a medium containing 40 mM Cs^+ caused an outward shift of the holding current which coincided with the activation of the Na^+–K^+ pump and thus probably represents I_p. Due to variations of the subsarcolemmal Na^+ concentration I_p reached its steady state only after many seconds (cf. Bielen *et al.* 1991*a*; Glitsch & Tappe, 1991). Addition of 2×10^{-4} M DHO (horizontal bar between the traces) blocked I_p as expected and therefore shifted the membrane current in the inward direction to the level observed in Cs^+-free solution where I_p is absent. This level remained nearly constant throughout the experiment. The block of I_p by DHO is partially reversible. The outward current component reappears in drug-free medium containing Cs^+. However, the amplitude of the outward current evoked by Cs^+_o is smaller at the end than at the beginning of the record. This 'run-down' of I_p has already been described for cardioballs (Glitsch & Tappe, 1993*a*). Three times during the experiment, voltage steps of 200 ms duration were applied from the holding potential (indicated by the vertical lines labelled *a*–*c*). In order to establish the voltage dependence of the membrane current, the current amplitude at the end of each clamp pulse was plotted *versus* the clamp potential during the pulse. Figure 1*B* shows the resulting *I*–*V* curves. The first *I*–*V* relationship was measured in the external medium containing 40 mM Cs^+ (○; *a* in Fig. 1*A*). The second *I*–*V* curve was obtained during the inhibition of the Na^+–K^+ pump by DHO (●; *b* in Fig. 1*A*). Due to the blockade of I_p which is an outward current under the conditions chosen, the membrane current was shifted to the inward direction over the whole range of membrane potentials tested. The final *I*–*V* relationship was recorded after partial recovery of I_p (◇; *c* in Fig. 1*A*). The membrane current was more outward again but, in general, did not reach the initial amplitudes.

In order to derive the I_p–*V* curve the difference between the current amplitudes *a* and *b* (Fig. 1*A*) in media with or without DHO was plotted *versus* the membrane potential in Fig. 1*C*. The resulting curve shows a nearly constant I_p at positive potentials and a decrease of I_p at negative clamp potentials.

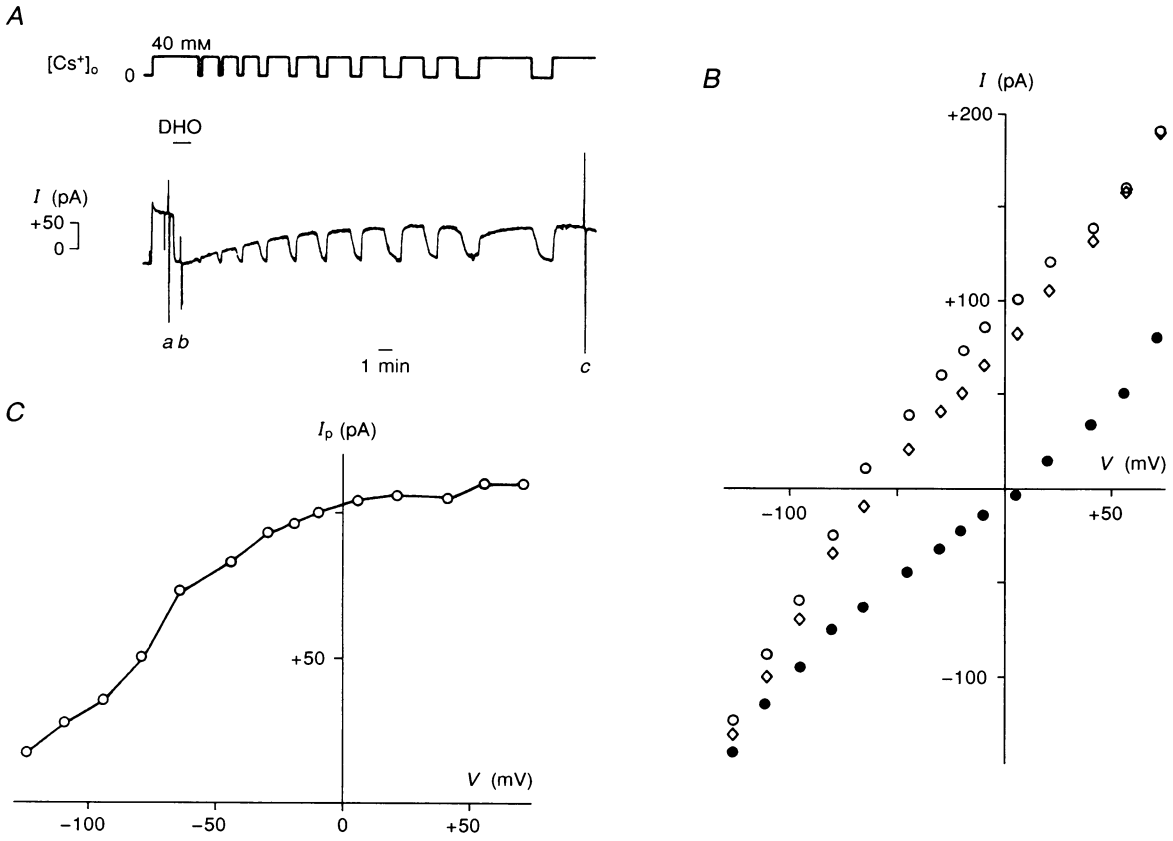

Figure 1. Measurement of I_p in a cardioball

A, membrane current at various $[Cs^+]_o$. Upper trace, $[Cs^+]_o$; lower trace, membrane current at −20 mV. Current–voltage relationships are measured at *a*, *b* and *c* and indicated by vertical lines. Bar above the current trace states application of DHO-containing solution (2×10^{-4} M). $\Delta G_{ATP} \approx -58$ kJ mol^{-1}. *B*, current–voltage curves recorded before (*a* in *A*; ○), during (*b* in *A*; ●) and after (*c* in *A*; ◇) application of DHO. *C*, I_p–*V* relationship. I_p derived from the difference between membrane current before (○ in *B*) and during (● in *B*) application of DHO.

It is a prerequisite for a study of I_p–V curves to ensure that the shape of the I_p–V relationship under a chosen condition remains unchanged during the experiment. Figure 2*A* displays the membrane current of a cardioball superfused with a solution containing 6 mM Cs^+. The bars above the current traces indicate the application of 2×10^{-4} M DHO. The record starts at holding potential (−20 mV) 10 min after establishing the whole-cell configuration. First, the I–V relationship was measured in drug-free solution (*a*). Thereafter the cardioball was superfused with a medium containing DHO and a second I–V curve was recorded (*b*). After washing off the drug for more than 10 min the procedure was repeated (*c* and *d*). The I_p–V curves derived from the differences $a - b$ (○) and $c - d$ (●) are presented in Fig. 2*B*. The I_p amplitudes of each curve are normalized to the corresponding amplitude at +5 mV, which were measured to be 31 (○) and 24 pA (●). Both curves exhibit a positive slope at negative membrane potentials and little change at positive voltages. The shape of the curve is nearly the same, regardless of whether measurements were made 10 or 22 min after establishing the whole-cell configuration. Thus, 10 min was long enough for complete equilibrium to be reached between cytosol and patch pipette solution. Furthermore the shape of a cell's I_p–V relationship does not change with time.

Reversal of I_p

Figure 3*A* shows five sets of membrane current (*a*–*e*) measured in a cardioball which was clamped to the membrane potentials indicated on the extreme left of the figure. The sample records start 11 min after access to the cytosol was obtained. The cell interior was perfused with an ATP-free patch pipette solution containing blockers of the physiological ATP synthesis, high concentrations of ADP and P_i, Cs^+ as the main cation and no Na^+. The Cs^+-free superfusion medium contained 150 mM Na^+ as the principal cation. Any external activator cation of the Na^+–K^+ pump was absent. Thus, the sarcolemmal gradients of the transported cations were very steep and ΔG_{ATP} was less negative than under physiological conditions. Under the conditions specified above, the left set of membrane currents (*a*) was obtained as a control in the drug-free superfusion solution. A few seconds later a superfusion medium containing 2×10^{-4} M DHO (bar above the current traces) was applied. Compared with the control the membrane current (*b*) was little affected at the holding potential (−20 mV), suggesting that I_p is small. This is also true for positive and other moderately negative clamp potentials. However, at very negative potentials the membrane current under DHO is more *outward* than the corresponding controls. Therefore I_p must be an inward current in this potential range. The third set of currents (*c*) represents a second control obtained 11 min after switching to the DHO-free superfusion medium. As in the first control the membrane current at very negative voltages was more *inward* than in DHO-containing solution, whereas the current showed little difference at other clamp potentials. Immediately after the second control the patch pipette medium (for *intracellular* perfusion) was changed to a solution containing 15 mM Na^+, 10 mM ATP and 10 mM glucose, but no ADP, P_i or metabolic inhibitors. The main cation was still Cs^+ (140 mM). The (external) superfusion solution now contained, in addition, 6 mM Cs^+. The alterations in the composition of the media *decreased* the sarcolemmal

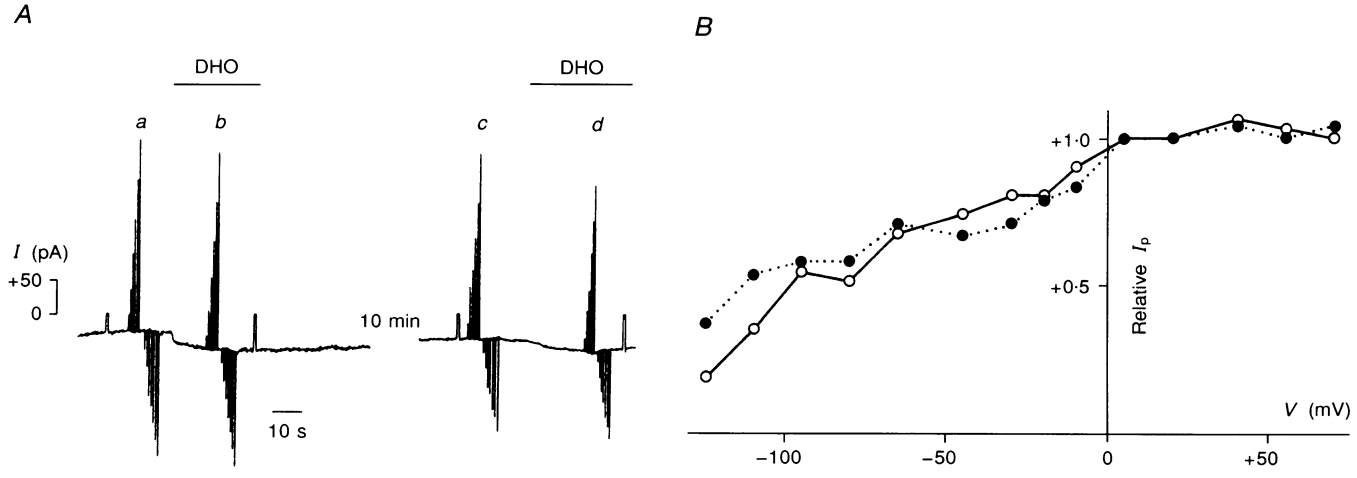

Figure 2. The shape of the I_p–V curve does not change with time

A, two sample records of membrane current from a cardioball superfused with DHO-free or DHO-containing medium at −20 mV. $\Delta G_{ATP} \approx -58$ kJ mol^{-1}. Interval between the records was 10 min. Bars above the current traces indicate application of 2×10^{-4} M DHO. Small rectangular signals indicate zero current level. Current–voltage relationships are measured at *a*, *b*, *c* and *d*. *B*, normalized I_p–V curves. I_p normalized to the respective I_p amplitude at +5 mV, arbitrarily set to 100%. I_p derived at different times during the experiment (○, *a*–*b*; ●, *c*–*d*).

gradients of the pumped cations and *increased* the free energy of ATP hydrolysis. Following 10 min application of the new media a fourth I–V curve was measured in DHO-free solution (*d*). Clearly, not only the holding current at −20 mV, but also the membrane current at positive and other moderately negative clamp potentials are shifted in the *outward* direction. A final application of DHO-containing superfusion medium now generally induced an inward shift of the membrane current at the various membrane potentials (*e*).

Two I_p–V curves derived from the sample record in Fig. 3*A* are depicted in Fig. 3*B*. They were obtained by subtracting, at each potential, the current amplitude measured at the end of the clamp pulse in DHO-containing medium from the corresponding membrane current recorded before in DHO-free solution. The filled circles represent the I_p–V relationship of the cardioballs under conditions which lower the free energy of ATP hydrolysis and steepen the sarcolemmal gradients of the transported cations (*a* − *b* in Fig. 3*A*). I_p reverses its direction near zero potential. The current is outward at positive voltages and inward at negative membrane potentials. The I_p–V curve measured later on at lower ionic gradients and more negative ΔG_{ATP} (*d* − *e* in Fig. 3*A*) is represented by open circles. The curve displays a positive slope at negative potentials up to −30 mV and little change of I_p at more positive voltages. This shape of the I_p–V relationship is characteristic for cardioballs from cardiac Purkinje cells under 'physiological' conditions (Glitsch *et al.* 1989).

Figure 3*C* shows the mean I_p–V curve from six cells studied at increased sarcolemmal Na^+ and Cs^+ gradients

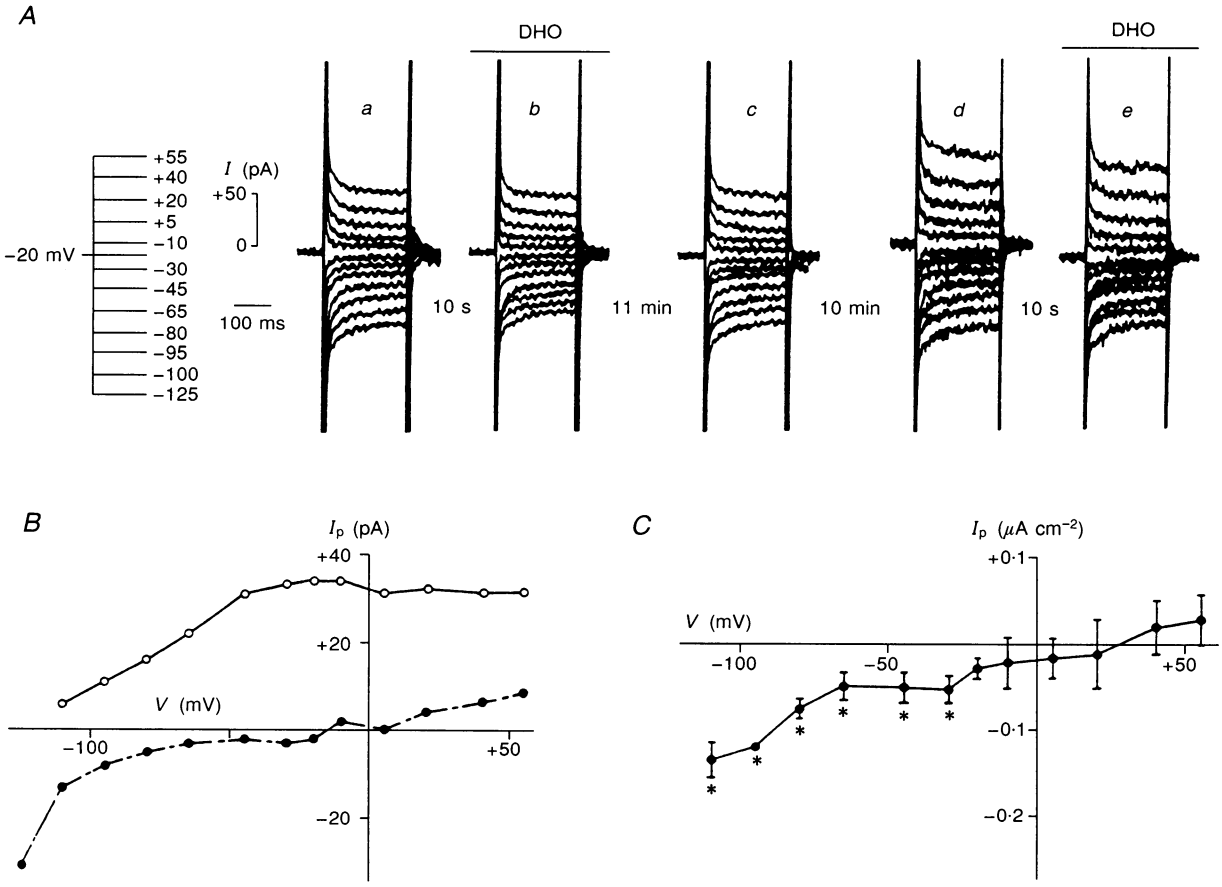

Figure 3. Reversal of I_p

A, five sets of membrane currents (*a*–*e*) measured at the clamp potentials indicated on the left. Bars above the current traces denote application of 2×10^{-4} M DHO. Gaps in the trace of holding current represent omission of record for the time given below the trace. The first three sets of membrane current (*a*–*c*) are taken at low free energy of ATP hydrolysis and steep ionic gradients; *d* and *e* are measured at more negative ΔG_{ATP} and flat ionic gradients. *B*, I_p–V curves derived either from the I–V relationships *a* and *b* in *A* (●) or from the I–V curves *d* and *e* in *A* (○). *C*, normalized mean I_p–V curve of six cardioballs at low free energy of ATP hydrolysis and steep ionic gradients. The I_p density of each cell is normalized to the corresponding I_p density at −95 mV, arbitrarily set to 100%, and scaled with the mean I_p density at this potential (−0·13 μA cm^{-2}). Asterisks indicate I_p densities different from 0 μA cm^{-2}.

and decreased free energy of ATP splitting. In order to allow for the necessary exchange between cytosol and patch pipette solution the measurements were started 10–15 min after establishing the whole-cell configuration. The I_p–V relationship displays a negative I_p at voltages negative to +30 mV. The negative I_p density increases with increasingly more negative clamp potentials and amounts to $-0{\cdot}13 \pm 0{\cdot}03$ μA cm^{-2} ($n = 6$) at −95 mV.

I_p–V relationship at various ΔG_{ATP} levels

According to eqn (2) the reversal potential (E_{rev}) of I_p depends on the sarcolemmal gradients of the transported cations and on ΔG_{ATP}. Consequently, keeping the ionic gradients constant, E_{rev} should vary with ΔG_{ATP}. In order to test this prediction we studied E_{rev} at three different ΔG_{ATP} levels (~−39, ~−49 and ~−58 kJ mol^{-1}) and constant ionic gradients. Thus, under all conditions, the patch-pipette media contained 140 mM Cs^+ and 10 mM Na^+, whereas the external solutions always included 150 mM Na^+ and 6 mM Cs^+. As an example, Fig. 4 illustrates an estimation of E_{rev} at a ΔG_{ATP} of ~−39 kJ mol^{-1}. Fig. 4*A* displays two sets of membrane current (right) measured in media with (bar) or without 2×10^{-4} M DHO. The various clamp potentials at which the currents were recorded are indicated on the left. Generally speaking, the difference between the currents obtained at the same potential in solution with or without DHO is small and thus I_p is small. The voltage dependence of I_p is shown in Fig. 4*B*. I_p reverses its direction near the holding potential. The pump current is outward at more positive potentials and is inwardly directed at more negative voltages. Similar experiments were performed at $\Delta G_{ATP} \approx -49$ and −58 kJ mol^{-1}. Figure 5 summarizes the mean I_p–V curves derived from measurements at the three different ΔG_{ATP} levels. Each curve is based on the results from five cells. The I_p amplitudes observed at the various clamp potentials under each ΔG_{ATP} are first normalized to the corresponding amplitude of I_p at +5 mV and then scaled with the mean I_p density at this potential under the condition chosen. The uppermost curve (●) represents the I_p–V relationship of the cardioballs at $\Delta G_{ATP} \approx -58$ kJ mol^{-1}. The I_p density displays a maximum around zero potential ($0{\cdot}35 \pm 0{\cdot}07$ μA cm^{-2} at +5 mV), a small decrease at positive voltages and a more pronounced decrease at negative potentials. Over the whole range of membrane potentials tested I_p is an outward current. A linear extrapolation of the I_p–V curve suggests an E_{rev} of about −195 mV. The voltage dependence of I_p at $\Delta G_{ATP} \approx -49$ kJ mol^{-1} is illustrated by the middle curve (◆). I_p is smaller over the whole potential range tested ($0{\cdot}19 \pm 0{\cdot}07$ μA cm^{-2} at +5 mV) but the general shape of the I_p–V curve is essentially unchanged. I_p seems to reverse its direction at about −115 mV. However, the I_p density is not significantly different from 0 μA cm^{-2} between −80 and −125 mV (asterisks). The bottom curve (▲) represents the I_p–V relationship at $\Delta G_{ATP} \approx -39$ kJ mol^{-1}. The amplitude of I_p is further diminished at all potentials ($0{\cdot}11 \pm 0{\cdot}02$ μA cm^{-2} at +5 mV). I_p is nearly constant at potentials positive to −10 mV and is more and more reduced at increasingly more negative voltages. E_{rev} seems to be at about −60 mV. It is pertinent, however, to note that I_p does not differ significantly from 0 μA cm^{-2} between −20 mV and −95 mV (asterisks). The current is significantly inward at more negative potentials and significantly outward at more positive voltages. Again the general shape of the I_p–V curve is unaffected. Thus decreasing the free energy of ATP hydrolysis at constant

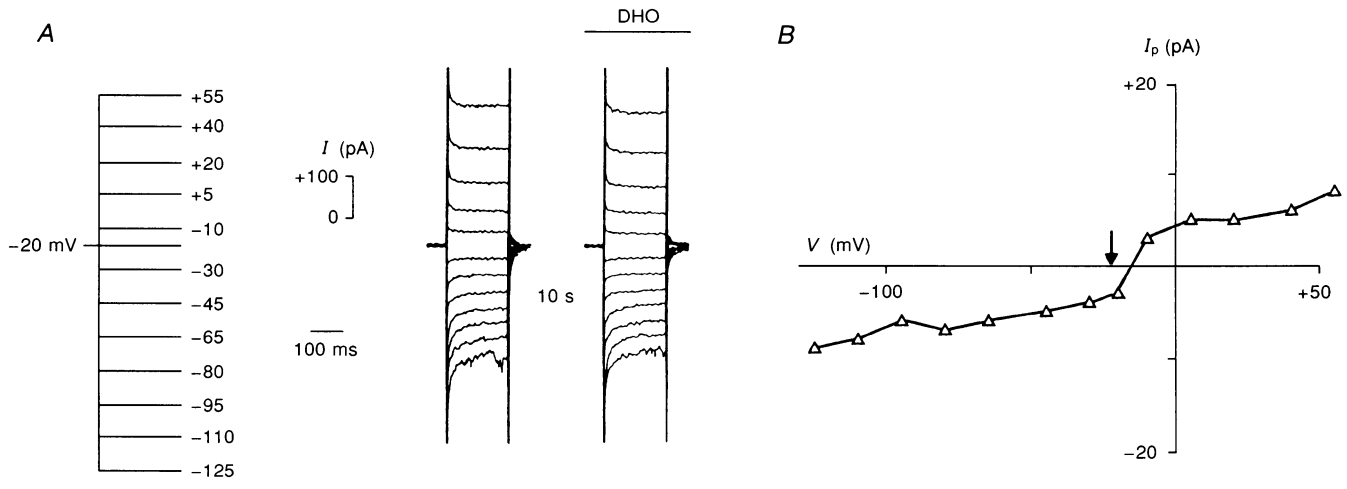

Figure 4. I_p–V curve of a cardioball at $\Delta G_{ATP} \approx -39$ kJ mol^{-1}

A, sample records of membrane currents measured at the clamp potentials indicated on the left. Bar above the current traces denotes application of 2×10^{-4} M DHO. Interval between the sets of current traces 10 s. *B*, I_p–V curve derived from the difference between corresponding currents measured in DHO-free or DHO-containing medium and displayed in *A*. The arrow indicates E_{rev} calculated according to eqn (2) (−24 mV).

gradients of the transported cations shifts the I_p–V relationship of the cardioballs in the inward direction.

Activation of I_p by Cs_o^+ at different ΔG_{ATP} levels

As can be seen from Fig. 5, variation of ΔG_{ATP} does not only shift E_{rev} but also diminishes the amplitude of I_p over the whole voltage range tested. In order to exclude that the kinetics of I_p activation by Cs_o^+ varies with ΔG_{ATP} we studied the kinetics at $\Delta G_{ATP} \approx -58$ and -49 kJ mol^{-1}. A sample record from an experiment under the former condition is shown in Fig. 6*A*. The clamp potential was manually changed to preset values, as indicated by the upper trace. The lower trace represents the membrane current. At each potential the cardioball was successively superfused with Cs^+-free solution and media containing 5, 10, 20 or 40 mM Cs^+. Each Cs^+ concentration applied evoked an outward current which was considered to be I_p. The amplitude of I_p depends on $[Cs^+]_o$ and membrane potential. Figure 6*B* presents the I_p density as a function of $[Cs^+]_o$ at zero potential. Mean values from four cells each at $\Delta G_{ATP} \approx -58$ (●) and -49 kJ mol^{-1} (◆) are shown. The curves fitted to the data obey the Hill equation:

$$I_p = \frac{I_{p,max}}{1 + \left(\frac{K_{0.5}}{[Cs^+]_o}\right)^h}, \quad (5)$$

where $I_{p,max}$ denotes the maximum I_p density, $K_{0.5}$ is $[Cs^+]_o$ for half-maximum I_p activation and h represents the Hill coefficient. $K_{0.5}$ is much the same under both conditions (19·5 mM Cs_o^+ for $\Delta G_{ATP} \approx -58$ kJ mol^{-1}; 19·4 mM Cs_o^+ for $\Delta G_{ATP} \approx -49$ kJ mol^{-1}), whereas $I_{p,max}$ decreases (from 0·50 to 0·37 μA cm^{-2}) at the lower ΔG_{ATP}.

The voltage dependence of $K_{0.5}$ under both conditions is plotted in Fig. 6*C* ($\Delta G_{ATP} \approx 58$ kJ mol^{-1}, ●; $\Delta G_{ATP} \approx -49$ kJ mol^{-1}, ◆). A single curve can be fitted to both sets of data. The curve represents the equation:

$$K_{0.5} = K_{0.5(V=0\ mV)} \exp(\alpha FV/RT). \quad (6)$$

α denotes (for monovalent ions) a fraction of an elementary charge and amounts to 0·32 for the curve shown. R, T and F have their usual meaning. Thus the results presented in Fig. 6 demonstrate that variation of ΔG_{ATP} between ~ -58 and ~ -49 kJ mol^{-1} has no effect on the $K_{0.5}$ of I_p activation by Cs^+ within the potential range studied.

DISCUSSION

Measurement of I_p

Figure 1 shows that the current activated by Cs_o^+ is blocked by the cardiac glycoside DHO and thus probably represents I_p. This finding is in line with previous measurements of I_p on isolated guinea-pig ventricular myocytes (Gadsby, Kimura & Noma, 1985), cardiac Purkinje cells (Bielen, Glitsch & Verdonck, 1991*b*), squid giant axons (Rakowski, Gadsby & De Weer, 1989) and oocytes of *Xenopus laevis* (Lafaire & Schwarz, 1986). A constant coupling ratio between active Na^+ efflux and active K^+ influx under a variety of experimental conditions is a prerequisite for considering I_p a reliable indicator of the Na^+–K^+ pump activity and for predicting E_{rev} according to eqns (1) and (2). Earlier reports have demonstrated that the coupling ratio is independent of

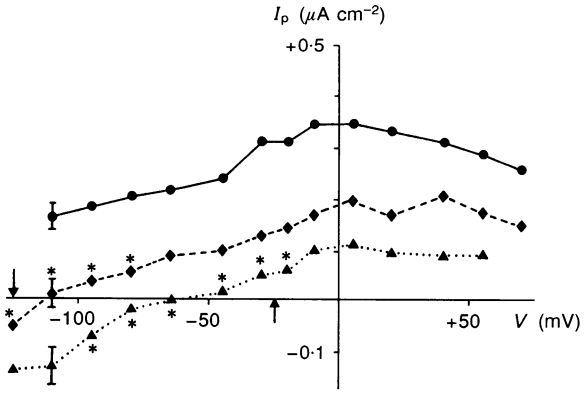

Figure 5. Normalized I_p–V relationships of cardioballs at different ΔG_{ATP} levels

I_p density observed at the various clamp potentials is normalized to the respective I_p density at +5 mV, arbitrarily set to 100%, and then scaled with the mean I_p density at +5 mV under the condition chosen. ●, $\Delta G_{ATP} \approx -59$ kJ mol^{-1}; ◆, $\Delta G_{ATP} \approx -49$ kJ mol^{-1}; ▲, $\Delta G_{ATP} \approx -39$ kJ mol^{-1}. Asterisks mark means not significantly different from 0 μA cm^{-2}. Sarcolemmal gradients of Na^+ and Cs^+ are constant throughout. The arrows indicate the theoretical E_{rev} (calculated according to eqn (2)) at $\Delta G_{ATP} \approx -49$ kJ mol^{-1} (−126 mV) and at $\Delta G_{ATP} \approx -39$ kJ mol^{-1} (−24 mV). E_{rev} at $\Delta G_{ATP} \approx -59$ kJ mol^{-1} is calculated as −224 mV and is not marked in the figure. For clarity error bars are shown only for one mean value in each curve. $n = 5$ for each ΔG_{ATP} level.

changes in the intracellular ATP concentration (Eisner & Richards, 1981; Goldshleger, Shahak & Karlish, 1990), in extra- and intracellular ionic concentrations and in membrane potential (see De Weer, Gadsby & Rakowski, 1988*a* for a review). Accordingly, a constant coupling ratio is assumed in discussing the results described above.

Reversal of I_p

Equation (2) predicts a shift of E_{rev} for I_p to more positive membrane potentials by lowering the free energy of ATP hydrolysis and/or steepening the sarcolemmal gradients of the pumped cations. Steep ionic gradients were created by application of a Na^+-free pipette solution and a Cs^+-free external medium. In order to diminish ΔG_{ATP} an ATP-free pipette solution containing inhibitors of glycolysis and oxidative phosphorylation was used. Previous experiments revealed that this treatment effectively decreases the outward directed I_p of cardioballs (Glitsch & Tappe, 1993*a*). Moreover, the addition of ADP and P_i to the patch pipette solution further reduced the free energy of ATP hydrolysis and provided at the same time the substrates for the ATP synthesis by the backward-running Na^+–K^+ pump. As can be seen from Fig. 3*C* these experimental conditions caused a dramatic shift of E_{rev} to more positive membrane potentials. Over

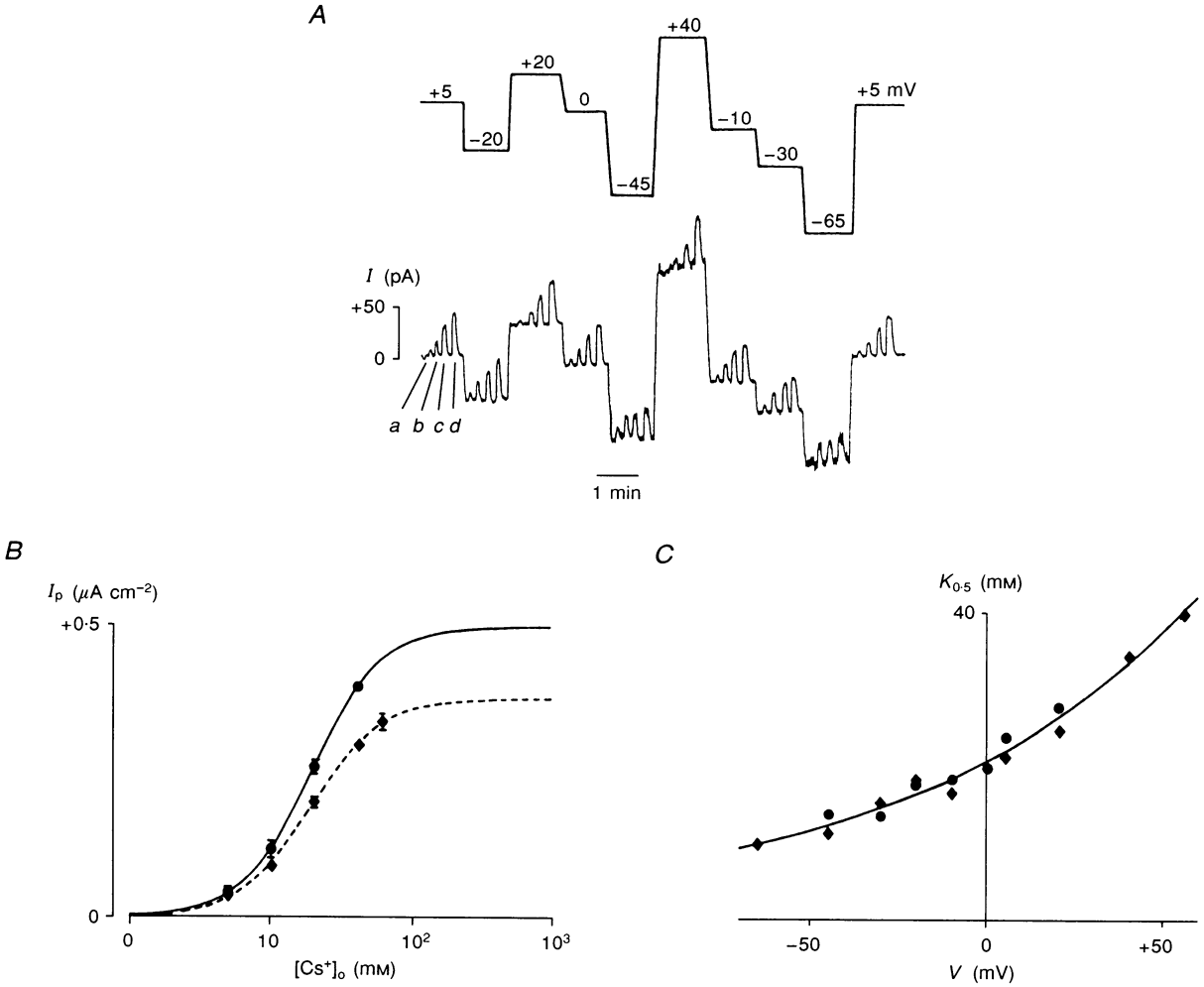

Figure 6. I_p activation depends on $[Cs^+]_o$ and membrane potential

A, I_p activation in a cardioball at various $[Cs^+]_o$ and clamp potentials. Upper trace, clamp potential; lower trace, membrane current. At each potential I_p is activated by 5 (*a*), 10 (*b*), 20 (*c*) and 40 mM (*d*) Cs^+_o. I_p is extremely small at +40 mV and 5 mM Cs^+_o. *B*, semilogarithmic plot of mean I_p densities *versus* $[Cs^+]_o$ at $\Delta G_{ATP} \approx -49$ (◆) and -58 kJ mol^{-1} (●). Clamp potential 0 mV. I_p amplitudes normalized to the corresponding amplitude at 40 mM Cs^+_o and scaled with the respective I_p density at this $[Cs^+]_o$ (●, $0{\cdot}39 \pm 0{\cdot}08$ μA cm^{-2}; ◆, $0{\cdot}29 \pm 0{\cdot}11$ μA cm^{-2}). $n = 4$ for both curves. The curves represent Hill equations (eqn (5)) with $K_{0·5} = 19{\cdot}5$ mM Cs^+_o, $I_{p,max} = 0{\cdot}50$ μA cm^{-2}, $h = 1{\cdot}84$ and $r^2 = 1{\cdot}0$ for ● and $K_{0·5} = 19{\cdot}4$ mM Cs^+_o, $I_{p,max} = 0{\cdot}37$ μA cm^{-2}, $h = 1{\cdot}82$ and $r^2 = 1{\cdot}0$ for ◆. *C*, $K_{0·5}$ varies with membrane potential. $K_{0·5}$ values for I_p activation by Cs^+_o plotted *versus* membrane potential. Symbols and n as in *B*. The curve obeys eqn (6) with $\alpha = 0{\cdot}32$, $r^2 = 0{\cdot}98$.

the whole range of membrane potentials studied I_p is significantly inward (asterisks) or insignificantly different from 0 μA cm^{-2}. The inward direction of I_p can be reversed to the 'physiological' outward direction during continuous whole-cell recording after switching to intra- and extracellular media which increase the free energy of ATP splitting and lower the sarcolemmal cationic gradients (Fig. 3*B*). The first reported measurement of a reversed I_p was performed on squid giant axon (De Weer & Rakowski, 1984). Superfusion with a K^+-free solution and internal dialysis with an ATP-free medium containing ADP, P_i and inhibitors of the ATP synthesis caused an inwardly directed I_p. Further experiments (Rakowski, De Weer & Gadsby, 1988) revealed I_p to be zero at about +30 mV and to increase at negative membrane potentials towards a maximum of $-0{\cdot}24$ μA cm^{-2} between −80 and −100 mV. Bahinski *et al.* (1988) were the first to describe a reversed I_p in cardiac cells under similar experimental conditions. The reversed I_p displayed a maximum of about $-0{\cdot}32$ μA cm^{-2} at −100 mV and a monotonic decrease at more positive potentials. According to Efthymiadis & Schwarz (1991) a reversed I_p is also observed in *Xenopus* oocytes containing low Na^+ and ATP but high ADP concentrations if they are superfused with K^+-free solution. The reversed I_p increased with increasingly more negative clamp potentials and amounted to $\sim -0{\cdot}1$ μA cm^{-2} at −100 mV. However, the current exhibited no maximum at negative voltages up to −140 mV.

A characteristic common to all reversed I_p–V curves published so far is the increase of the inward I_p with increasingly more negative membrane potentials. A backward-running Na^+–K^+ pump transports external Na^+ into the cell. The I_p amplitude depends on the Na^+ concentration at the extracellular Na^+ binding sites of the pump. These sites seem to be connected via an 'access channel' to the external medium (e.g. Gadsby, Rakowski & De Weer, 1993). Both hyperpolarization of the cell membrane and an increase of the external Na^+ concentration tend to augment the Na^+ concentration at the Na^+ binding sites of the pump and thereby the amplitude of the reversed I_p. The slightly different shapes of the reversed I_p–V curve in various cells may reflect – apart from species differences – the different extracellular Na^+ concentrations present during the measurements of I_p.

E_{rev} as a function of ΔG_{ATP}

In order to demonstrate the dependence of E_{rev} on ΔG_{ATP}, I_p was measured at three ΔG_{ATP} levels and constant sarcolemmal gradients of the pumped cations. The control of the subsarcolemmal ion concentrations was facilitated by applying a relatively low concentration of external Cs^+ in order to activate I_p (cf. Glitsch & Tappe, 1991). The different ΔG_{ATP} levels were established by using pipette solutions containing a physiological ATP concentration (~10 mM; e.g. Kammermeier, Schmidt & Jüngling, 1982) and various concentrations of ADP and P_i. Previous experiments (Glitsch & Tappe, 1993*a*) suggested that it is difficult to lower effectively the ATP concentration at the ATP-binding site of the Na^+–K^+ pump by means of an ATP-free patch pipette solution, most probably due to functional compartmentation.

ΔG_{ATP} is estimated to be between −55 and −60 kJ mol^{-1} in cardiac cells under physiological conditions (e.g. Kammermeier *et al.* 1982). Cardioballs internally perfused with a pipette solution composed to establish a $\Delta G_{ATP} \approx -58$ kJ mol^{-1} display an I_p–V relationship which is very similar to that found in cardioballs (Glitsch *et al.* 1989) and freshly isolated cardiac Purkinje cells (Bielen *et al.* 1991*b*) when a systematic control of ΔG_{ATP} is not attempted. I_p is outwardly directed over the whole potential range studied and displays a maximum near zero potential. It decreases slightly at more positive voltages and strongly at more negative voltages (Fig. 5). The linearly extrapolated E_{rev} of −195 mV is not too far from the calculated value of −224 mV. Comparable I_p–V curves have been reported for guinea-pig cardiac ventricular cells (Gadsby *et al.* 1985), oocytes of *Xenopus laevis* (Lafaire & Schwarz, 1986) and for the squid giant axon (Rakowski *et al.* 1989). Small differences in the shape of the I_p–V relationships may be due to the different concentrations of the external pump activator cation used (cf. Bielen, Glitsch & Verdonck, 1993).

A ΔG_{ATP} of ~ -49 kJ mol^{-1} shifts the measured E_{rev} to about −115 mV (Fig. 5), in good agreement with the calculated reversal potential which amounts to −126 mV. From Fig. 5, E_{rev} at $\Delta G_{ATP} \approx -39$ kJ mol^{-1} is estimated to be about −60 mV, whereas the calculated value turned out to be −24 mV. However, it is helpful to realize that the mean values of the measured I_p are not significantly different from zero between −20 and −95 mV (Fig. 5). The discrepancy between the calculated and the measured E_{rev} at the various ΔG_{ATP} levels is probably – at least partly – due to experimental difficulties in determining E_{rev} exactly because of the small I_p amplitudes in an appreciable range of membrane potentials in the vicinity of E_{rev}. In addition, we cannot exclude the possibility that the subsarcolemmal ΔG_{ATP} differs slightly from that predicted from the composition of the pipette solution. Finally it is obvious that the linear extrapolation of the I_p–V curve observed at $\Delta G_{ATP} \approx -58$ kJ mol^{-1} can only result in a rough guess of E_{rev}. In fact a computer model of the Na^+–K^+ pump studied by Chapman, Johnson & Kootsey (1983) predicts a linear I_p–V relationship near E_{rev} only at low ΔG_{ATP} (see Fig. 5, ▲) but a non-linear I_p–V curve in this potential range at higher (more physiological) ΔG_{ATP}. Considering these shortcomings the observed similarity between the calculated and the measured (or extrapolated) values of E_{rev} seems quite satisfying.

I_p decreases at less negative ΔG_{ATP}

Lowering the free energy of ATP hydrolysis not only shifts E_{rev} towards more positive membrane potentials but also reduces the amplitude of I_p (Fig. 5). Interestingly this behaviour of the I_p–V relationship has already been postulated by Chapman *et al.* (1983) from their aforementioned computations of electrogenic Na^+ pumping (see their Fig. 6). Figure 6 demonstrates that neither $[Cs^+]_o$ for half-maximal I_p activation ($K_{0.5}$) nor its voltage dependence change upon variation of ΔG_{ATP} whereas the maximal I_p amplitude decreases at $\Delta G_{ATP} \approx -49$ kJ mol^{-1}. It is known from previous work of other authors (e.g. Apell, Nelson, Marcus & Läuger, 1986; Kennedy, Lunn & Hoffman, 1986) that the Na^+–K^+ pump activity is changed under these conditions and the active cation fluxes through the pump are diminished. However, the underlying mechanism (inhibition of K^+ deocclusion and/or decreased phosphorylation of the Na^+–K^+ pump) is under debate. Our experiments were not designed to differentiate between these possibilities and thus the mechanism remains to be elucidated. The Cs^+_o concentration which causes half-maximal I_p activation is voltage dependent. The dependence can be described according to eqn (6) with $\alpha = 0{\cdot}32$. This finding is in line with the hypothesis that extracellular cations have to pass through an 'access channel' in order to reach their binding sites at the Na^+–K^+ pump. The concentration of the external cations at the binding sites varies with membrane potential and therefore the apparent concentration for half-maximal I_p activation by activator cations like Cs^+_o also depends on voltage (cf. Läuger & Apell, 1986). Similar observations and comparable α values have been reported from studies on *Xenopus* oocytes (Rakowski, Vasilets, La Tona & Schwarz, 1991; Omay & Schwarz, 1992) and freshly isolated rabbit cardiac Purkinje cells (Bielen *et al.* 1993). The main conclusion from our results is, however, that E_{rev} of I_p varies with ΔG_{ATP} if the sarcolemmal gradients of the pumped cations are kept constant. This is what one expects from thermodynamic considerations.

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