The effect of cardiac glycosides on the Na^+ pump current–voltage relationship of isolated rat and guinea-pig heart cells

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1. Whole-cell recording from isolated rat and guinea-pig ventricular myocytes revealed a change of the cardiac Na^+ pump current (I_p)–voltage (V) relationship by cardiac glycosides, specific inhibitors of the Na^+–K^+ pump.

2. Dihydro-ouabain (DHO) diminished I_p in rat ventricular cells at 0 mV in a concentration-dependent manner.

3. The concentration–response curve of I_p inhibition caused by DHO was shifted to higher [DHO] at higher extracellular K^+ concentrations ($[K^+]_o$) or at more negative membrane potentials.

4. In rat myocytes, DHO immediately flattened the normalized cardiac I_p–V curve and evoked or enhanced a region of negative slope.

5. Ouabain, at concentrations which caused a comparable inhibition of I_p, exerted DHO-like effects on the I_p–V relationship of rat ventricular myocytes. However, the effects developed more slowly.

6. A slowly developing alteration of the I_p–V curve was also observed upon application of DHO to guinea-pig ventricular cells. The range of [DHO] used was about 100-fold lower than that applied to rat ventricular cells, but was equally effective for I_p inhibition.

7. Increasing the K^+ concentration of DHO-containing media affected the existing equilibrium of DHO binding to the cardiac Na^+–K^+ pump. A new equilibrium was reached within about 3 s in rat ventricular myocytes, but only within about 50 s in guinea-pig ventricular cells under the experimental conditions chosen.

8. It is concluded that the changes of the cardiac I_p–V curve induced by cardiac glycosides are mediated by voltage-dependent variations of the local $[K^+]_o$ at the K^+ binding sites of the Na^+–K^+ pump in an 'access channel'. The variations were estimated by means of the Boltzmann equation. The estimations agreed with those derived from the measured DHO binding to the Na^+–K^+ pump at various $[K^+]_o$. A new equilibrium of glycoside binding to the pump is established at the altered $[K^+]_o$. The time necessary to reach the new binding equilibrium varies with the cardioactive steroid, its concentration and the glycoside sensitivity of the cardiac cells.

The Na^+–K^+ pump of animal cells generates a current, the Na^+ pump current (I_p). The amplitude of I_p is voltage dependent. Under physiological conditions, I_p decreases strongly with increasingly more negative membrane potentials but remains unchanged at positive voltages in cardiac cells. Monovalent cations are known to change the Na^+ pump current–voltage (V) curve of various cells. Their effects were studied in some detail in cardiac ventricular myocytes (Nakao & Gadsby, 1989), cardiac Purkinje cells (Bielen, Glitsch & Verdonck, 1991*a*), squid axons (Rakowski, Gadsby & De Weer, 1989), *Xenopus* oocytes (Omay & Schwarz, 1992) and in Na^+–K^+ pump molecules of *Torpedo electroplax* expressed in *Xenopus* oocytes (Vasilets, Ohta, Noguchi, Kawamura & Schwarz,

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1993). For example, a region of negative slope appears in the I_p–V relationship at low extracellular K^+ concentration ($[K^+]_o$). This phenomenon is caused by voltage-dependent K_o^+ binding to the Na^+–K^+ pump (Rakowski, Vasilets, La Tona & Schwarz, 1991). K_o^+ binding is diminished at positive membrane potentials. The potential-dependent K_o^+ binding is probably mediated by voltage-induced changes of the local $[K^+]_o$ at the K_o^+ binding sites of the pump located in a narrow 'access channel' (Omay & Schwarz, 1992). Apart from the cations, only few substances have been reported so far to change the voltage dependence of I_p. According to Vasilets & Schwarz (1992), stimulation of protein kinases varies the I_p–V curve of endogenous *Xenopus* pumps and of Na^+–K^+ pumps of *Torpedo* expressed in *Xenopus* oocytes. The results to be described below demonstrate that cardiac glycosides, specific inhibitors of the Na^+–K^+ pump, alter the I_p–V relationship of cardiac cells and neurones.

METHODS

Isolation of single cells

Rats were killed by cervical dislocation in deep ether anaesthesia; guinea-pigs were killed by a blow on the head. Ventricular myocytes were isolated from the hearts by means of a procedure described in detail previously (Bielen *et al.* 1991*a*). After an enzymatic treatment of the hearts during a Langendorff perfusion with Ca^{2+}-poor media at 35 °C, the ventricles were cut into pieces. The pieces were gently stirred and the dissociated myocytes were transferred to culture dishes (3·6 cm diameter) where the Ca^{2+} concentration of the bathing solution was increased stepwise at room temperature to 1 mM for rat cells and to 1·8 mM for guinea-pig myocytes. A dish containing isolated, single cells was mounted on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, Germany). A plastic ring was pressed to the bottom of the dish. Thus, the volume of the dish was reduced to about 0·3 ml. The cells were superfused at 2 ml min^{-1} with media prewarmed to 32–34 °C.

Solutions

In order to make sure that no constituent alters qualitatively the cell response studied, two solutions of different composition were used for intracellular perfusion during whole-cell recording. Patch pipette solution A contained (mM): 110 caesium aspartate, 40 NaOH, 10 EGTA, 40 Hepes, 5 $MgCl_2$, 5 glucose, 5 Mg-ATP, 5 sodium creatine phosphate (adjusted to pH 7·35 with HCl; free Mg^{2+} concentration about 2 mM). The composition of solution B was (mM): 20 CsCl, 100 NaCl, 0·15 $CaCl_2$, 5 EGTA, 40 Hepes, 6 $MgCl_2$, 5 glucose, 5 Mg-ATP (adjusted to pH 7·35 with CsOH; free Ca^{2+} concentration about 10 nM, free Mg^{2+} concentration about 4 mM). Pipette solutions containing high Na^+ concentrations were used in order to diminish the effect of changes of the subsarcolemmal Na^+ concentration during variations of the Na^+–K^+ pump activity (cf. Bielen, Glitsch & Verdonck, 1991*b*). Intracellular perfusion with either medium resulted in very similar effects of cardiac glycosides on the I_p–V relationship of the cells. The standard (external) superfusion medium contained (mM): 144 NaCl, 0·5 $MgCl_2$, 1·8 $CaCl_2$, 10 Hepes, 10 glucose (adjusted to pH 7·35 at 32–34 °C with NaOH). Choline chloride (plus 5 μM atropine sulphate; pH adjusted with LiOH) replaced NaCl in Na^+-free solutions. The superfusion media applied via one or two multibarrelled pipettes to the cell under study contained in addition 0–5·4 mM KCl and, in order to diminish K^+ and Ca^{2+} conductances and the Na^+–Ca^{2+} exchange of the cell membrane, 2 mM $BaCl_2$ and 5 mM $NiCl_2$, respectively. Control measurements by atomic absorption spectrometry revealed K^+ concentrations of up to 0·015 mM in nominally K^+-free solutions. Dihydro-ouabain (DHO) or ouabain was added to the external media from aqueous stock solutions.

Whole-cell recording

Current–voltage curves of the cells were measured by means of whole-cell recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) as described previously (Bielen *et al.* 1991*a*; Bielen, Glitsch & Verdonck, 1993). The initial resistance of the patch pipettes filled with either pipette solution varied between 2 and 4 MΩ. A holding potential of −20 mV was maintained before and after measurements of the I_p–V relationships. Starting from the holding potential, the cell membrane was clamped to preset potentials by manual variations of the command potential. The clamped membrane potential and the resulting membrane current were measured by means of an Axoclamp 2A voltage clamp amplifier (Axon Instruments, Foster City, CA, USA) and recorded on a pen recorder (Watanabe Multicorder, Tokyo, Japan). The Na^+ pump current was measured as current activated by external K^+, which can be blocked by cardiac glycosides. There was no noticeable difference in the I_p–V curves regardless of whether I_p was estimated by pulses of K^+-free solution during superfusion of myocytes with K^+-containing media or by pulses of the latter solutions during superfusion with K^+-free medium.

The cell surface area was calculated from the capacitive charge flowing during small hyperpolarizing voltage pulses. The capacitance was assumed to be 1 μF cm^{-2}. The surface area of rat ventricular cells was estimated to be $(88 \pm 2) \times 10^{-6}$ cm^2 ($n = 50$) and the surface area of guinea-pig ventricular myocytes estimated to be $(91 \pm 4) \times 10^{-6}$ cm^2 ($n = 24$).

Statistics

Data are presented as means ± S.E.M.; n indicates the number of cells studied.

RESULTS

DHO changes the rat cardiac I_p–V curve; the DHO effect depends on $[K^+]_o$

The upper trace of Fig. 1*A* displays the membrane potentials to which the cell membrane of a rat ventricular myocyte was clamped starting from the holding potential of −20 mV. At each clamp potential, short pulses of media containing 1 mM K^+ with or without 1 mM DHO were applied to the cell via a multibarrelled pipette (indicated below the lower trace). As can be seen from the lower trace, the pulses of K_o^+-containing solutions evoke step-like shifts of the membrane current in the outward direction. The K^+-induced current, which can be inhibited

by cardioactive steroids (right part of the figure), represents I_p (e.g. Gadsby & Nakao, 1989; Bielen *et al.* 1991*a*).

Mean I_p–V curves of rat ventricular myocytes derived from measurements under the conditions illustrated in Fig. 1*A* are presented in Fig. 1*B*. The ordinate indicates the I_p density (μA cm^{-2}) and the abscissa shows the clamped membrane potential (V_c, mV). Since the I_p amplitude may vary during a long-lasting pulse of K^+-containing solution (cf. Bielen *et al.* 1991*b*) the initial I_p amplitude was used to calculate the I_p density. The I_p–V relationship observed at 5·4 mM K_o^+ (●) exhibited the typical positive slope at voltages negative to the holding potential (−20 mV) and small changes of I_p at more positive membrane potentials. Addition of 1 mM DHO to the medium (○) decreased the I_p density at potentials positive to −60 mV by about 20%.

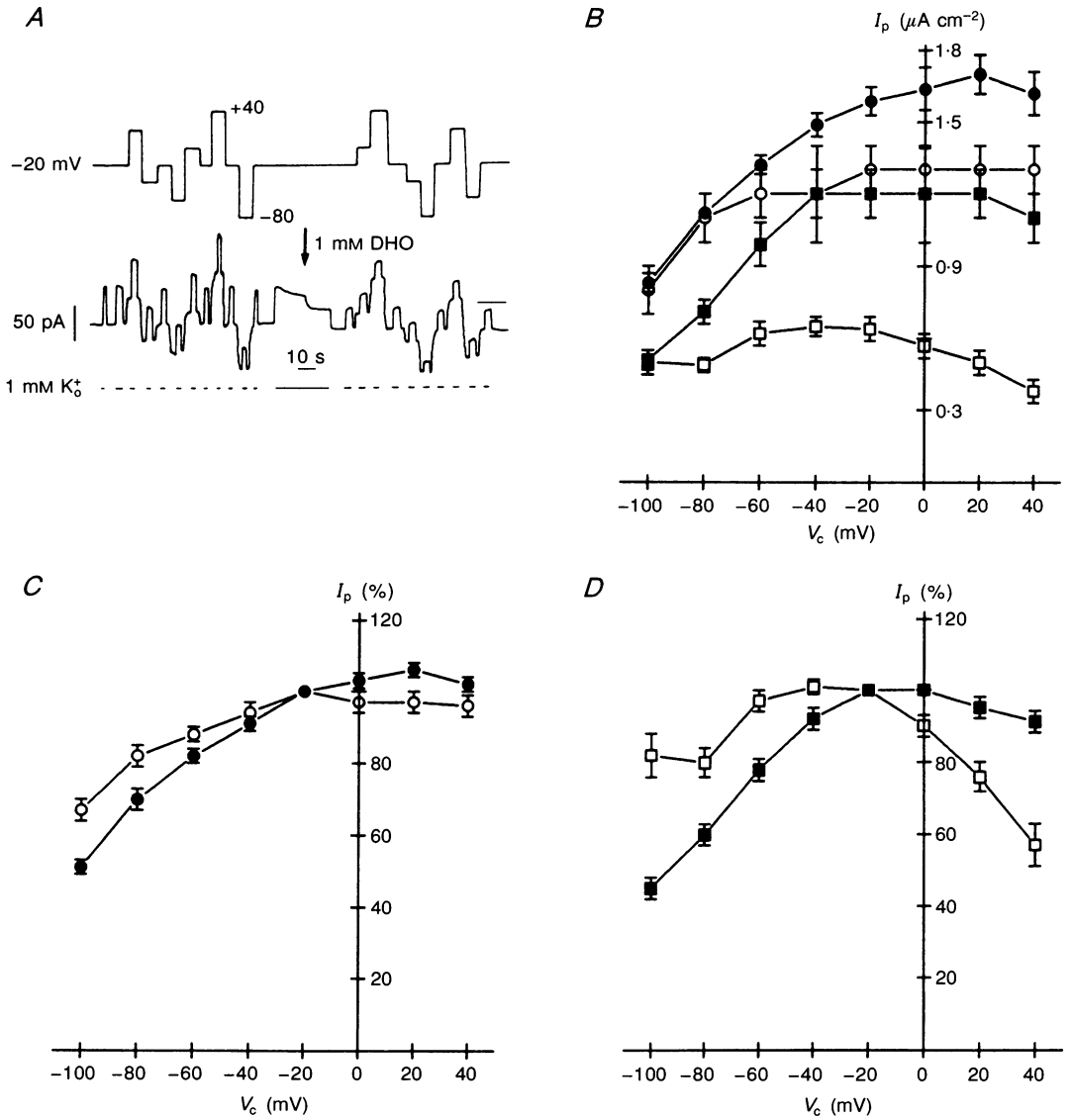

Figure 1. I_p inhibition by DHO depends on membrane potential and $[K^+]_o$
A, sample record. Clamped membrane potential (V_c, upper trace) and membrane current (lower trace) of a rat ventricular myocyte. Arrow marks addition of 1 mM DHO. Cell superfused with K^+-free solution in the presence (right part of the figure) or absence (left part) of DHO. I_p identified by short applications of a medium containing 1 mM K^+, as indicated below the current trace. Horizontal bar above the trace shows zero current level. *B*, mean I_p–V relationships of rat ventricular myocytes at two $[K^+]_o$ in the presence or absence of 1 mM DHO. ●, 5·4 mM K_o^+ ($n = 5$); ○, 5·4 mM K_o^+ plus DHO ($n = 5$); ■, 1 mM K_o^+ ($n = 6$); □, 1 mM K_o^+ plus DHO ($n = 6$). In this and the following figures S.E.M. is only shown where it exceeds the size of the symbols. *C* and *D*, normalized mean I_p–V curves of rat ventricular myocytes. I_p amplitudes normalized to the corresponding I_p values at −20 mV (see *B*), arbitrarily set to 100%. Same data and symbols as in *B*. Patch pipette solution B in *A*–*D*.

However, I_p was essentially unchanged at more negative voltages. Lowering $[K^+]_o$ to 1 mM (■) diminished the I_p density at all clamp potentials tested, whereas the general shape of the I_p–V curve was little affected. The shape was drastically altered in the solution containing 1 mM K^+ plus 1 mM DHO (□). At zero potential, DHO decreased I_p to about 50% of the amplitude measured in the DHO-free medium. The inhibition of I_p increased with increasingly more positive potentials and decreased with more negative voltages. At −100 mV, I_p was not at all affected by 1 mM DHO. A region of negative slope appeared in the I_p–V curve at potentials positive to −60 mV. Obviously, the inhibition of I_p by DHO depends on both clamp potential and $[K^+]_o$. In order to demonstrate more clearly the effect of DHO on the shape of the I_p–V relationship, Fig. 1*C* and *D* display normalized I_p–V curves from the data already shown in Fig. 1*B*. The I_p amplitude measured at each clamp potential was normalized to the mean value of the I_p amplitudes recorded at the holding potential immediately before and after the clamp step. Due to this procedure, the shape of the normalized I_p–V relationships differs slightly from that expected on the basis of Fig. 1*B*. In solution containing 5·4 mM K^+ (Fig. 1*C*), DHO flattened the I_p–V relationship (○) and thus shifted the curve upwards at clamp potentials negative to the holding potential, if compared with the drug-free medium (●). However, the glycoside caused a downward shift and induced a minimal negative slope of the I_p–V relationship at positive voltages. Figure 1*D* reveals a small negative slope of the I_p–V curve at 1 mM K_o^+ (■) at clamp potentials positive to the holding potential (−20 mV). The same DHO concentration (1 mM) exerted a stronger effect on the I_p–V curve at the lower $[K^+]_o$ (□). Both the flattening of the I_p–V relationship at negative potentials and the negative slope of the curve at positive voltages were more distinct. Thus, a low $[K^+]_o$ strengthened the DHO effect on the I_p–V curve. The modulation by $[K^+]_o$ of the DHO-induced changes of the I_p–V relationship was not limited to the range of $[K^+]_o$ described above. In Na^+-free solution where the pump's affinity for K_o^+ is increased by a factor of 10 (Nakao & Gadsby, 1989), the modulatory action of K_o^+ was present below 0·5 mM (not illustrated).

The effect of DHO on the I_p–V relationship of rat ventricular myocytes is concentration dependent

Mean I_p–V curves of rat myocytes ($n = 3$) superfused with media containing 2 mM K^+ are shown in Fig. 2*A* and *B*. The alterations of the I_p–V relationship caused by three DHO concentrations are depicted. At the holding potential of −20 mV, the I_p density amounted to 1·2 ± 0·1 (2 mM K_o^+), 0·91 ± 0·09 (2 mM K_o^+ plus 0·5 mM DHO), 0·82 ± 0·08 (2 mM K_o^+ plus 1 mM DHO) and 0·32 ± 0·06 μA cm^{-2} (2 mM K_o^+ plus 5 mM DHO). As can be seen from Fig. 2*A*, the I_p density decreased with increasing DHO concentration at membrane potentials positive to −60 mV. In the drug-free solution the I_p–V curve displayed a conventional shape (●). The I_p amplitude decreased markedly at clamp potentials negative to the holding potential but changed very little

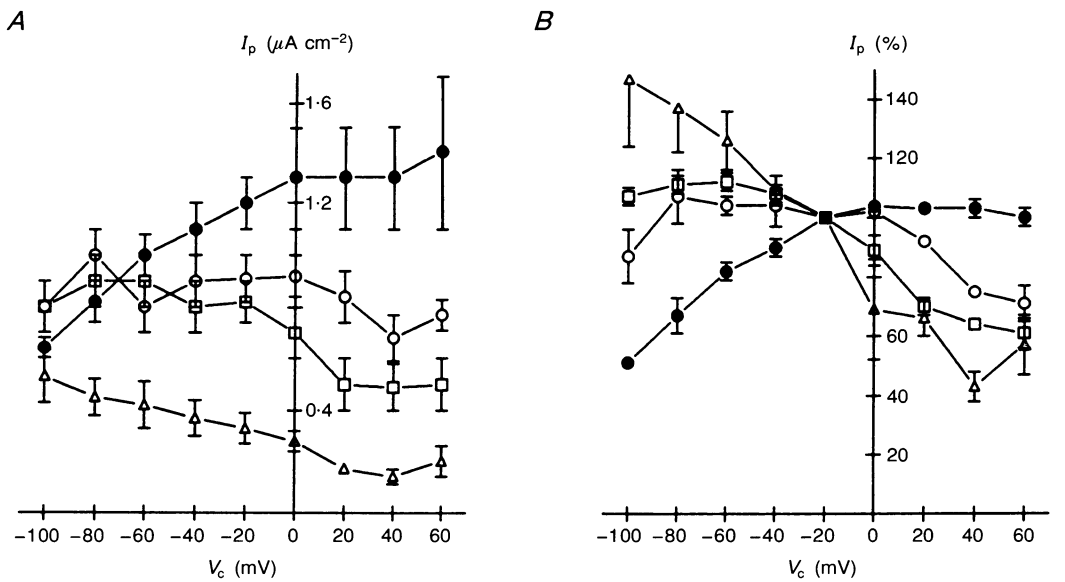

Figure 2. The effect of DHO on the I_p–V curve is concentration dependent
Mean I_p–V relationships of rat ventricular myocytes at 2 mM K_o^+ and various [DHO]. *A*, mean I_p densities as a function of membrane potential. *B*, normalized mean I_p–V curves. I_p amplitudes normalized to the respective I_p values at −20 mV (see *A*), arbitrarily set to 100%. ●, control in DHO-free medium; ○, 0·5 mM DHO; □, 1 mM DHO; △, 5 mM DHO ($n = 3$); patch pipette solution A in *A* and *B*.

at more positive voltages. A region of negative slope appeared in the I_p–V relationship of the myocytes if the medium contained 0·5 mM DHO (○). Both the negative slope of the I_p–V curve and the potential range where this slope is observed increased at higher DHO concentrations. This is most clearly seen in Fig. 2*B*, where normalized mean I_p–V relationships are depicted. Thus, the DHO effect on the cardiac I_p–V relationship depends on the concentration of the drug. Furthermore, it is obvious that the DHO effect also depends on voltage. At a given DHO concentration the inhibition of I_p is strong at positive membrane potentials and is barely detectable at potentials negative to −80 mV. Similar changes of the I_p–V curve were found in two series of experiments on rat myocytes superfused with Na^+-free media containing 0·07 mM K^+ ($n = 4$) or 0·1 mM K^+ ($n = 5$) with or without DHO concentrations ranging from 0·5 to 4 mM (pipette solution B in both series). It is worth mentioning, however, that the concentration ranges of DHO and K_o^+ where the effect of DHO on the cardiac I_p–V relationship can be observed may vary from one cell preparation to another. For example, a concentration-dependent effect of DHO on the mean I_p–V curve of three rat myocytes superfused with a medium containing 150 mM Na^+ and 1·1 mM K^+ became apparent only at concentrations > 2 mM DHO. Thus, this group of cells was less DHO sensitive than that from which the results shown in Fig. 2 were obtained.

The effect of DHO on the I_p–V relationship is not limited to rat cardiac cells. We noticed corresponding changes of the I_p–V curve of rat dorsal root ganglion neurones. The cells were superfused with media containing 2 mM K^+ in the absence or presence of DHO (1 mM DHO, $n = 6$; or 5 mM DHO, $n = 2$).

Variation of $[K^+]_o$ or membrane potential exerts an equivalent effect on the I_p inhibition by DHO

Figures 1 and 2 show that the inhibition of I_p by DHO (and the corresponding alterations of the I_p–V relationship) in rat ventricular myocytes not only depends on [DHO] but also on $[K^+]_o$ and membrane potential. At a given [DHO], the I_p inhibition increases with decreasing $[K^+]_o$ or increasing depolarization. Lowering $[K^+]_o$ or depolarizing the sarcolemma seems to exert a similar effect on the inhibition of I_p by the cardiac glycoside. In order to test this equivalence of changes in $[K^+]_o$ and membrane potential, we measured the inhibition of I_p by three [DHO] either at four $[K^+]_o$ and fixed membrane potential (0 mV) or at four clamp potentials and fixed $[K^+]_o$ (0·5 mM). Some of the results are presented in Fig. 3. The sample record in Fig. 3*A* shows the effect of two [DHO] on the I_p amplitude of a rat myocyte in a medium containing 0·5 mM K^+ at three clamp potentials: +40, 0 and −40 mV. First, a drug-free medium containing 0·5 mM K^+ was used as superfusion fluid. DHO (2 or 1 mM) was then added to the solution as indicated by the horizontal bars above the traces. The drug partially inhibited the Na^+–K^+ pump in a concentration-dependent manner and thereby caused an inward shift of the membrane current. The residual I_p was estimated by short pulses of K^+-free media containing the respective [DHO]; this is indicated by the horizontal bars beneath each current trace. I_p was completely blocked during the pulses. The results of similar experiments at two $[K^+]_o$ but constant membrane potential are shown in Fig. 3*B*. The figure displays the steady-state inhibition of I_p by DHO at 1 (□; $n = 5$) and 5·4 mM K_o^+ (▲; $n = 4$ or 5) and a clamp potential of 0 mV. The ordinate gives the I_p inhibition as a percentage of the I_p amplitude measured in the corresponding drug-free, K^+-containing solution. The sigmoid curves fitted to the data are computed assuming simple one-to-one binding of DHO to the Na^+–K^+ pump molecules (cf. Bielen, Glitsch & Verdonck, 1992). They are drawn in order to visualize the shift of the concentration–response curve. The apparent K_D values ([DHO] for half maximal I_p inhibition) increased with increasing $[K^+]_o$, and amounted to 1·1 mM at 1 mM K_o^+ and 2·8 mM at 5·4 mM K_o^+. Measurements at 0·5 ($n = 6$) and 2 mM K_o^+ ($n = 7$) yielded K_D values of 1·1 and 1·9 mM DHO, respectively (for clarity, not illustrated). The voltage dependence of the K_D value at 0·5 mM K_o^+ is demonstrated in Fig. 3*C* by results obtained at two clamp potentials. The K_D value for I_p inhibition at +40 mV (□) was estimated to be 0·88 mM DHO ($n = 4$). At −80 mV (▲) half-maximal I_p inhibition was caused by 2·2 mM DHO ($n = 2$ or 3). For clarity, apparent K_D values measured at two additional clamp potentials are not shown. These K_D values amounted to 1·1 (0 mV, $n = 6$) and 1·7 mM DHO (−40 mV, $n = 3$). Clearly, the data suggest that a hyperpolarization of the sarcolemma or an increase of $[K^+]_o$ similarly affect the inhibition of I_p by the cardiac glycoside.

The probable cause of this similarity is that the local $[K^+]_o$ at the extracellular K^+ binding sites of the cardiac Na^+–K^+ pump varies with membrane potential. The binding sites seem to be connected by an 'access channel' to the bulk extracellular space (cf. Läuger, 1991). Ions travelling through the channel sense about 0·26 of the potential gradient across the cell membrane (Bielen *et al.* 1991*a*, 1993). Hyperpolarization increases and depolarization decreases the local $[K^+]_o$ at the K^+ binding site of the cardiac Na^+–K^+ pump. Therefore, hyperpolarization and an increase in $[K^+]_o$ have equivalent actions on the I_p inhibition by DHO. According to recent experiments (Hermans, Glitsch & Verdonck, 1994), the K_D values for the I_p inhibition by DHO as a function of $[K^+]_o$ exhibit saturation kinetics in rat ventricular myocytes. The K_D value observed at 5·4 mM K_o^+ amounts to 70% of the value found at 10·8 mM K_o^+. To a first approximation the function is nearly linear between 0·5 and 2 mM K_o^+. If the local variation of $[K^+]_o$ with clamp potential were the cause of the varying K_D values, $[K^+]_o$ and K_D should

display the same dependence on membrane potential in this range of $[K^+]_o$. This seems to be the case, as shown in Table 1. The table presents apparent K_D values (left column) and local K_o^+ concentrations (columns a and b) for various membrane potentials normalized to the respective numbers at 0 mV. The K_D values were taken from the measurements illustrated in Fig. 3*C*. The relative local K_o^+ concentrations at the external K^+ binding sites of the

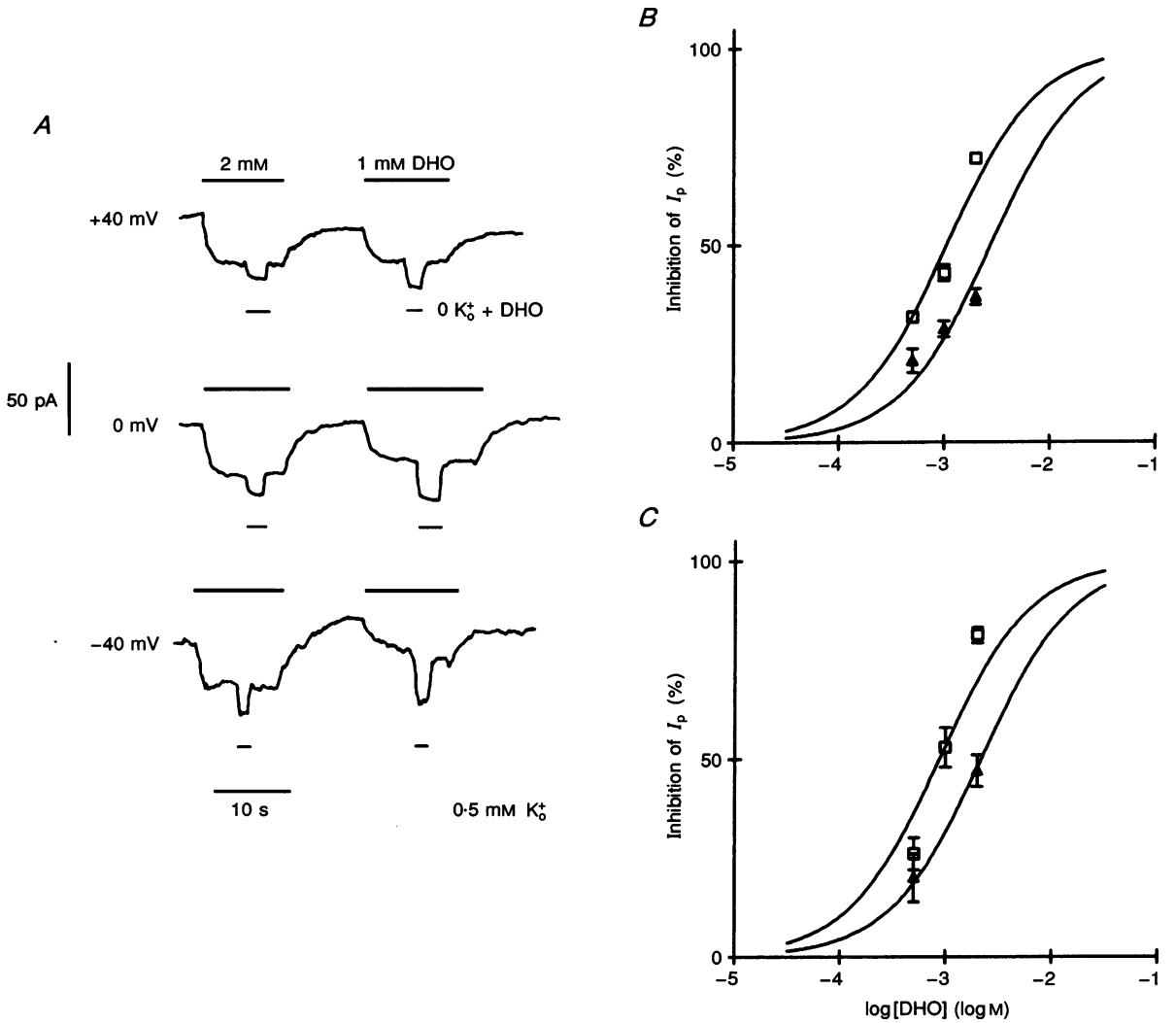

Figure 3. The concentration–response curve of I_p inhibition by DHO in rat ventricle cells varies with $[K^+]_o$ and membrane potential

A, sample record. Membrane current of a rat ventricular myocyte superfused with a medium containing 0·5 mM K^+ at various clamp potentials. Horizontal bars above the current traces mark application of 2 mM DHO (left part of the figure) or 1 mM DHO (right part). I_p estimated by means of short pulses of K^+-free solution containing the respective [DHO]. The pulses are indicated beneath the current traces. I_p is blocked during the pulses. *B*, mean concentration–response curves of I_p inhibition by DHO at two $[K^+]_o$. Semilogarithmic plot. Complete inhibition of I_p in K^+-free solution is arbitrarily set to 100%. In drug-free solution, the I_p density was measured as $0{\cdot}53 \pm 0{\cdot}05$ μA cm^{-2} at 1 mM K_o^+ ($n = 4$) and $1{\cdot}3 \pm 0{\cdot}2$ μA cm^{-2} at 5·4 mM K_o^+ ($n = 5$). Sigmoid curves fitted to the data are computed assuming one-to-one binding of DHO to the Na^+–K^+ pump molecules. K_D values amount to 1·1 mM DHO at 1 mM K_o^+ (□; $n = 5$) and to 2·8 mM DHO at 5·4 mM K_o^+ (▲; $n = 4$ or 5). Data obtained at 0 mV. *C*, mean concentration–response curve of I_p inhibition by DHO at 0·5 mM K_o^+ and two clamp potentials. Semilogarithmic plot. □, at +40 mV; ▲, at −80 mV. Complete I_p inhibition in K^+-free medium is arbitrarily set to 100%. In drug-free solution, the I_p density was measured as $0{\cdot}42 \pm 0{\cdot}04$ μA cm^{-2} at +40 mV ($n = 4$) and $0{\cdot}17 \pm 0{\cdot}02$ μA cm^{-2} at −80 mV ($n = 3$). K_D values amount to 0·88 mM DHO at +40 mV ($n = 4$) and to 2·2 mM DHO at −80 mV ($n = 2$ or 3). Curves computed as in *B*. Patch pipette solution A in *A–C*.

Table 1. Normalized K_D values of I_p inhibition by DHO and normalized local $[K^+]_o$ at various clamp potentials

V_c (mV)	$K_{D(V_c)}/K_{D(V_c = 0\ mV)}$*	$[K^+]_{o(V_c)}/[K^+]_{o(V_c = 0\ mV)}$†	
		a‡	b§
+40	0·77	0·68	0·67
0	1·00	1·00	1·00
−40	1·54	1·45	1·62
−80	1·93	2·20	2·30

* From measurements illustrated in Fig. 3*C*. † $[K^+]_{o(V_c = 0\ mV)}$ assumed to be identical to $[K^+]_o$ of the extracellular solution (0·5 mM). ‡ Numbers calculated by means of eqn (1). § Numbers derived from $K_{D(V_c)}/K_{D(V_c = 0\ mV)}$ and K_D values as a function of $[K^+]_o$ at 0 mV (Hermans *et al.* 1994).

Na^+–K^+ pump were calculated (column a) from the same experiments by means of the Boltzmann equation:

$$[K^+]_{oV_c} = [K^+]_{o(V_c = 0\ mV)} \exp(\delta V_c F/RT), \quad (1)$$

where $[K^+]_{oV_c}$ is the local K_o^+ concentration within the 'access channel' at the clamp potential V_c, $[K^+]_{o(V_c = 0\ mV)}$ denotes the local $[K^+]_o$ at 0 mV, δ indicates the fraction (0·26) of the electric field across the sarcolemma sensed by K_o^+, and F, R and T have their usual meanings. The relative local K_o^+ concentrations listed in column b were obtained from the measured K_D values at the various clamp potentials (left column) and the above-mentioned relationship between K_D values and $[K^+]_o$ (Hermans *et al.* 1994.). In all calculations it is assumed that $[K^+]_{o(V_c = 0\ mV)} = 0{\cdot}5$ mM, i.e. the K^+ concentration of the external solution. The agreement between the numbers at each test potential is satisfactory and supports the view that potential-dependent changes of the local $[K^+]_o$ at the extracellular K^+ binding sites of the Na^+–K^+ pump probably cause the voltage dependence of the interaction between DHO and the pump molecules. Thus, the increase of I_p inhibition with depolarization at a given [DHO] (Figs 1 and 2) is probably due to a decrease of the local $[K^+]_o$ at the K^+ binding sites of the pump with increasingly more positive clamp potentials. The diminished $[K^+]_o$ strengthens the inhibition of I_p (Fig. 3*B*) and reduces the I_p amplitude. Furthermore, a reduced $[K^+]_o$ diminishes *per se* the amplitude of I_p. As a consequence, a region of negative slope appears in the I_p–V relationship of rat ventricular myocytes superfused with DHO-containing solution (Figs 1 and 2).

Equipotent DHO and ouabain concentrations evoke different effects on the I_p–V curve of rat ventricular cells

The upward shift of the I_p–V relationship at potentials negative to the holding potential, and the downward shift at more positive voltages in DHO-containing solution, were essentially time independent. Regardless of whether the I_p amplitude of a myocyte in drug-containing medium was recorded at the beginning or at the end of a clamp pulse, the amplitude remained constant. This is shown on the sample record of Fig. 4*A*. A rat ventricular myocyte was superfused with a solution containing 1 mM K^+ plus 1 mM DHO. I_p is estimated at various clamp potentials (upper trace) by short pulses of a K^+-free, DHO-containing medium, as indicated below the lower trace which represents the membrane current. Although the I_p amplitude differed at the different membrane potentials, it showed no consistent variation during the clamp pulses. Accordingly, the I_p–V relationship of the cell superfused with DHO-containing medium was unaltered whether I_p was recorded at the beginning or at the end of the clamp pulse to the various membrane potentials. This can be seen in Fig. 4*B*, where mean normalized I_p–V curves are depicted. The curves were obtained at 1 mM K_o^+, either in drug-free solution (●) or at two different times (◇, 2 s after the start; □, 20–25 s after the start) during clamps in DHO-containing medium. The I_p amplitude at each clamp potential is normalized to the corresponding I_p value at the holding potential. The two I_p–V relationships recorded in DHO-containing solutions superimpose and differ clearly from the control I_p–V curve. There was no time-dependent shift of the I_p–V relationship under DHO. Similarly the shape of the control I_p–V curve did not change with time in the experiments.

To our initial surprise, the effect of ouabain on the I_p–V relationship of rat ventricular myocytes was quite different. The sample record of Fig. 4*C* shows the membrane current (lower trace) at various clamp potentials (upper trace) of a myocyte superfused with a solution containing 1 mM K^+ plus 0·05 mM ouabain. I_p was estimated by short pulses of a K^+-free, drug-containing medium that blocked I_p. The application is indicated by the horizontal bars below the current trace. Hyperpolarization reduced I_p compared with the control at the holding potential of −20 mV. However, in contrast to the results obtained in DHO-containing medium, I_p increased clearly during hyperpolarization. Moreover, if the cell

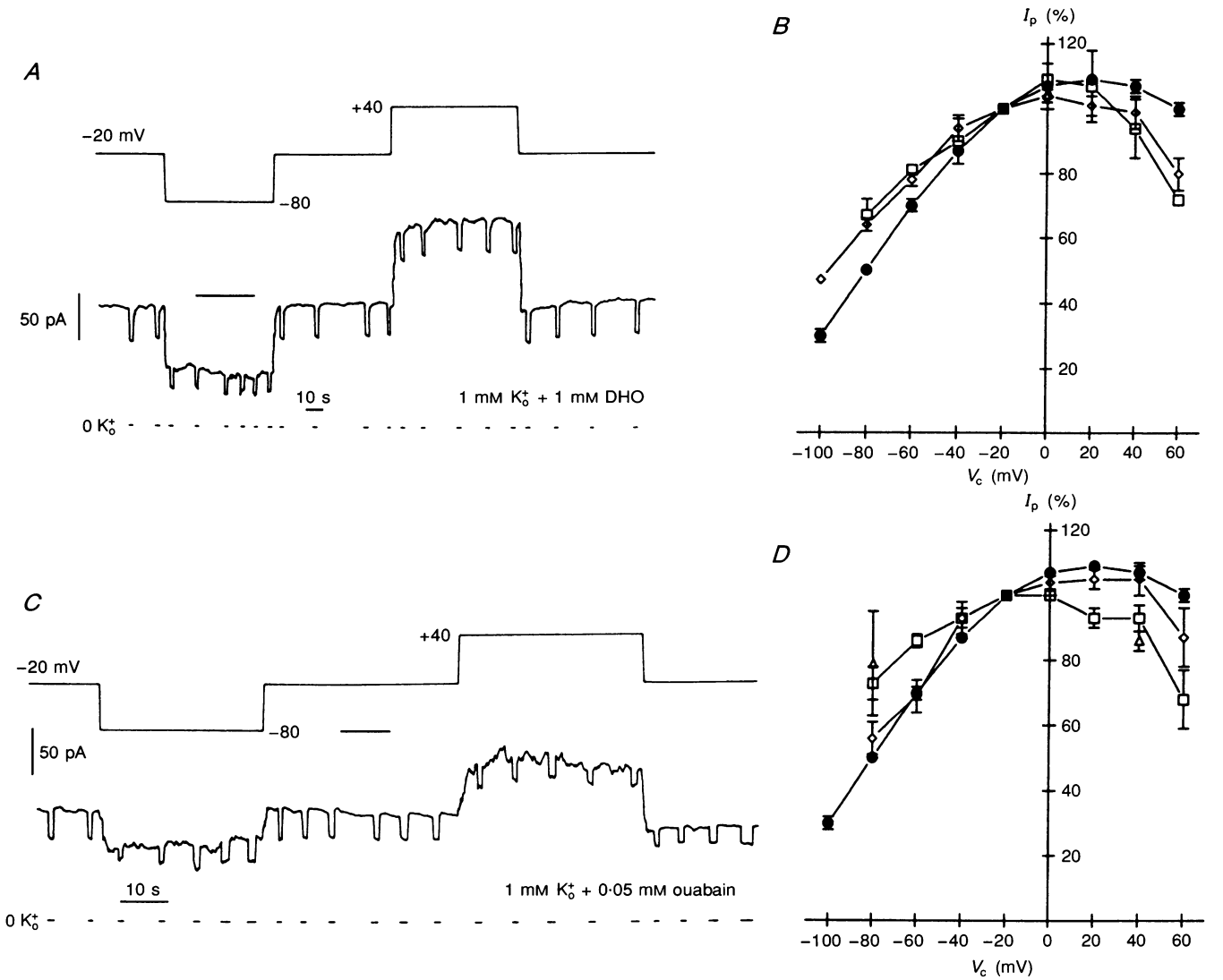

Figure 4. Different effects of DHO and ouabain on the I_p–V relationship of rat ventricular cells
A, *B*, the effect of DHO on the rat cardiac I_p–V curve is independent of time. *A*, sample record. Lower trace, membrane current of a rat ventricular myocyte superfused with a medium containing 1 mM K^+ plus 1 mM DHO. Horizontal bar above the trace marks zero current level. I_p estimated by short pulses of K^+-free solution containing 1 mM DHO as indicated below the trace. Upper trace, clamp potential. *B*, normalized mean I_p–V relationships of rat ventricular myocytes at 1 mM K_o^+ in the presence or absence of 1 mM DHO. I_p amplitudes normalized to the corresponding I_p amplitudes at −20 mV, which amounted to $0{\cdot}69 \pm 0{\cdot}03$ μA cm^{-2} ($n = 29$) in drug-free solution and to $0{\cdot}38 \pm 0{\cdot}03$ μA cm^{-2} ($n = 10$) in the medium containing DHO. ●, control at 1 mM K_o^+ without drug ($n = 23$–26). ◇, 1 mM DHO; I_p measured within 2 s after the start of a clamp step ($n = 7$–8). □, 1 mM DHO; I_p measured 20–25 s after the start of a clamp step ($n = 1$–3). *C*, *D*, the effect of ouabain on the rat cardiac I_p–V relationship depends on time. *C*, sample record. Lower trace, membrane current of a rat ventricle cell superfused with a medium containing 1 mM K^+ plus 0·05 mM ouabain. Horizontal bar above the trace marks zero current level. I_p estimated by short applications of K^+-free solution containing 0·05 mM ouabain, as shown beneath the trace. Upper trace, clamp potential. *D*, normalized mean I_p–V curves of rat ventricular myocytes at 1 mM K_o^+ in the presence or absence of 0·05 mM ouabain. I_p amplitudes normalized to the respective values at −20 mV which are arbitrarily set to 100%. The I_p density at −20 mV was measured as $0{\cdot}27 \pm 0{\cdot}02$ μA cm^{-2} ($n = 9$) in the medium containing 1 mM K^+ plus 0·05 mM ouabain ($0{\cdot}69 \pm 0{\cdot}03$ μA cm^{-2} ($n = 29$) in drug-free solution). ●, control at 1 mM K_o^+ without drug ($n = 23$–26). ◇, 0·05 mM ouabain; I_p measured within 2 s after the start of a clamp step ($n = 4$–8). □, 0·05 mM ouabain; I_p measured 30–40 s after the start of a clamp pulse ($n = 2$–7). △, 0·05 mM ouabain; I_p measured 2 min after the start of the clamp step ($n = 2$). Patch pipette solution A in *A*–*D*.

membrane was clamped back to the holding potential, I_p was initially larger than later on. This too is in contrast with the I_p changes observed in DHO-containing solution where I_p immediately reached its control value after clamping back to the holding potential (Fig. 4*A*). The slow I_p variation during the clamp steps to various potentials resulted in a time-dependent ouabain effect on the I_p–V relationship of the rat ventricular myocyte. Figure 4*D* displays this effect. Mean normalized I_p–V curves are presented. The I_p amplitude at each clamp potential is plotted relative to the corresponding I_p value at the holding potential. With I_p amplitudes recorded 2 s after the beginning of the clamp steps, the resulting I_p–V relationship (◇) was very much the same as the I_p–V curve in drug-free solution (●). If, however, I_p values obtained later during the clamp steps (□, 30–40 s after the start; △, 2 min) were used for the construction, the I_p–V relationship showed an upward shift at potentials negative to the holding potential and a downward shift at more positive voltages (□, △). In drug-free medium, the I_p amplitude at each potential remained constant during the clamp pulse.

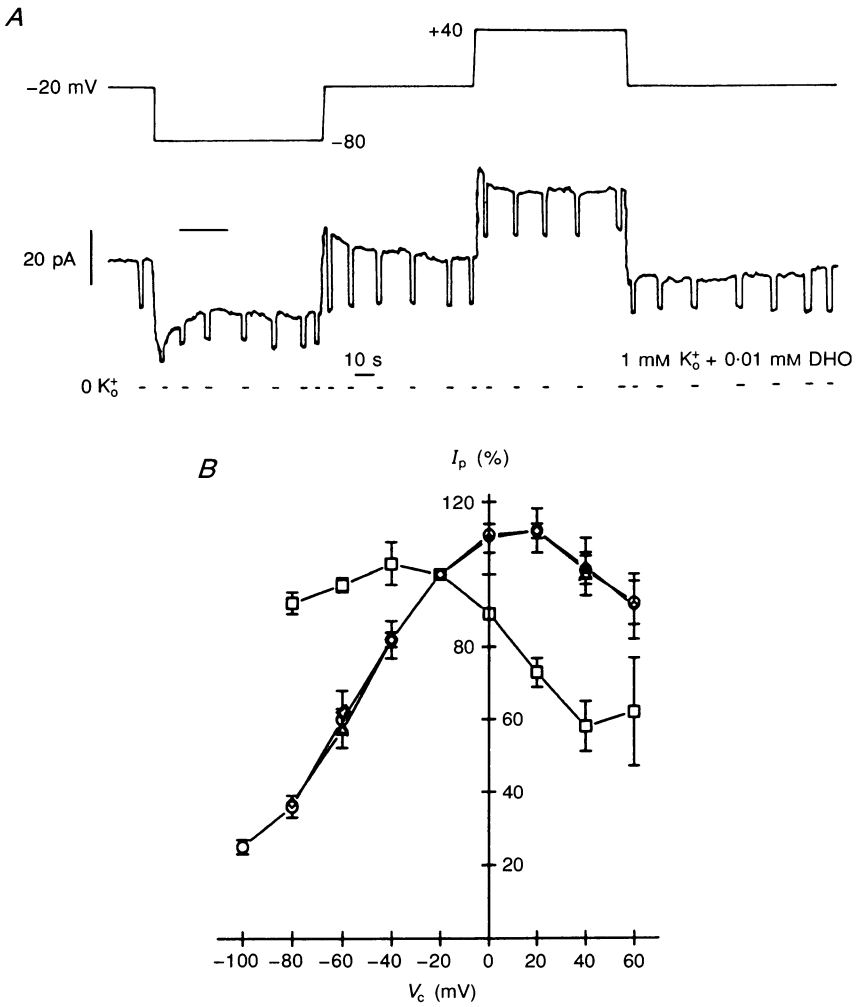

Figure 5. The effect of DHO on the I_p–V relationship of guinea-pig ventricular cells is time dependent

A, sample record. Upper trace, clamp potential; lower trace, membrane current. Horizontal bar above the lower trace marks zero current level. I_p estimated by short pulses of K^+-free solution containing 0·01 mM DHO as indicated below the trace. *B*, normalized mean I_p–V curves of ventricular myocytes at 1 mM K_o^+ in the presence or absence of 0·01 mM DHO. The I_p amplitude at each clamp potential is normalized to the corresponding I_p amplitude at −20 mV which is arbitrarily set to 100%. The I_p density at −20 mV amounted to 0·47 ± 0·03 μA cm^{-2} ($n = 15$) in drug-free solution and to 0·17 ± 0·01 μA cm^{-2} ($n = 6$) in the medium containing DHO. ○, control without DHO ($n = 13$–15). ◇, 0·01 mM DHO; I_p measured within 2 s after the start of a clamp step ($n = 4$–6). □, 0·01 mM DHO; I_p measured 60 s after the start of the clamp step. ($n = 2$–3). △, I_p supplementary measured 60 s after changing V_c from −20 mV to −60 or +40 mV under control conditions ($n = 2$). Patch pipette solution A in *A*–*B*.

Different kinetics of glycoside binding to the cardiac Na^+–K^+ pump cause different effects on the I_p–V curve of glycoside-sensitive and -insensitive species

The different actions of DHO (K_D value for I_p inhibition at 1 mM K_o^+ and −20 mV: 1·3 mM) and of ouabain (K_D value: 0·037 mM) on the I_p–V relationship of rat ventricular myocytes prompted the question of whether the different kinetics of drug binding to the Na^+–K^+ pump could be responsible for these effects. We had previously observed that DHO binding to the Na^+–K^+ pump of a cardiac glycoside-insensitive species (rat) is characterized by a smaller association rate constant and a larger dissociation rate constant if compared with the corresponding constants of a cardiac steroid-sensitive species (guinea-pig, K_D value: 8·6 μM; Hermans *et al.* 1994). Therefore, we

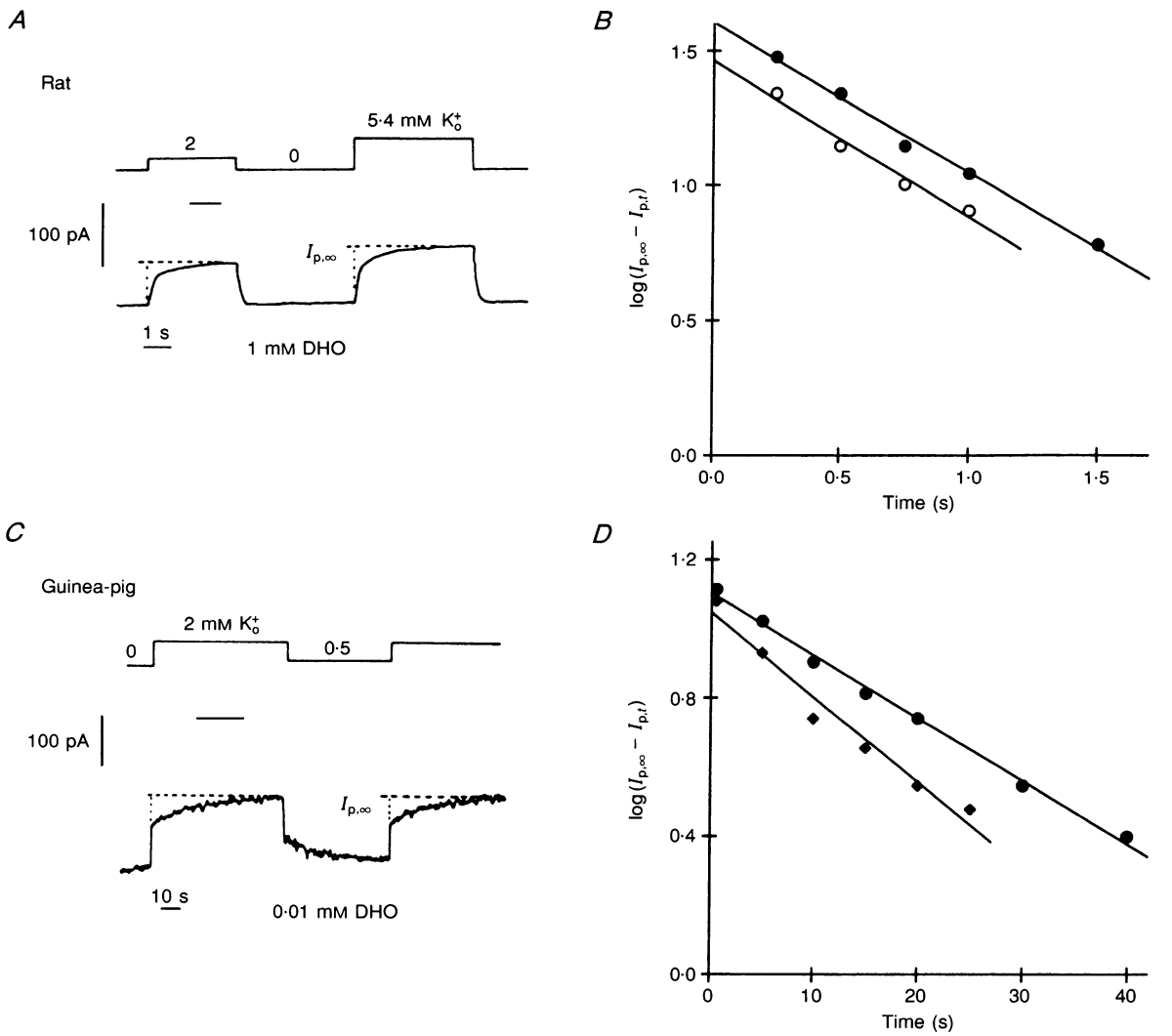

Figure 6. Change of I_p in ventricular myocytes following variation of $[K^+]_o$

A, *B*, time course of I_p change upon variation of $[K^+]_o$ in a rat ventricular myocyte at 1 mM DHO. *A*, I_p activated by 2 or 5·4 mM K_o^+. Upper trace, $[K^+]_o$. Lower trace, membrane current at −20 mV. The steady-state amplitude of the slow component of I_p increase ($I_{p,\infty}$) estimated as shown by the broken lines. Horizontal bar above the lower trace marks zero current level. *B*, semilogarithmic plot of the slow component of the I_p increase. ●, I_p activated by 5·4 mM K_o^+ ($t = 0{\cdot}8$ s). ○, I_p activated by 2 mM K_o^+ ($t = 0{\cdot}7$ s). Data fitted by regression lines ($r^2 = 0{\cdot}98$). *C*, *D*, time course of I_p change in a guinea-pig ventricular cell at 0·01 mM DHO. *C*, I_p activated by 2 mM K_o^+ from 0 mM (left) or 0·5 mM K_o^+, respectively. Upper trace, $[K^+]_o$. Lower trace, membrane current at −20 mV. Horizontal bar above the lower trace indicates zero current level. $I_{p,\infty}$ estimated as shown by the broken lines. Note the different time scale in *A* and *C*. *D*, semilogarithmic plot of the slow change of I_p increase induced by 2 mM K_o^+. ●, following an increase of $[K^+]_o$ from 0 to 2 mM ($t = 24$ s). ◆, following an increase of $[K^+]_o$ from 0·5 to 2 mM ($t = 18$ s). Data fitted by regression lines ($r^2 = 0{\cdot}97$–$0{\cdot}99$). Patch pipette solution B in *A*–*D*.

studied the I_p–V curve of guinea-pig ventricular cells as affected by DHO. Figure 5*A* displays a sample record. The upper trace indicates the various clamp potentials whereas the lower trace shows the corresponding membrane current. The ventricular cell was superfused with a solution containing 1 mM K^+ plus 0·01 mM DHO. As indicated beneath the lower trace, I_p was estimated by short pulses of a K^+-free medium (containing 0·01 mM DHO) which completely blocks I_p. Clearly, hyperpolarization caused a slow increase in the initially reduced I_p and depolarization induced a decrease in the pump current during the clamp pulse. Figure 5*B* presents three normalized mean I_p–V relationships, one measured in drug-free solution containing 1 mM K^+ (○), the others in a medium additionally containing 0·01 mM DHO. In the DHO-containing solution, the I_p amplitude was recorded either at the beginning (◇) or at the end (□) of a 60 s clamp pulse. The I_p amplitude observed at each potential was normalized to the corresponding I_p value at the holding potential (−20 mV). The action of DHO on the I_p–V curve of the glycoside-sensitive guinea-pig ventricular cells resembled the effect of ouabain on the I_p–V relationship of the insensitive rat myocytes. The I_p–V curve measured at the beginning of the clamp pulses (◇) in DHO-containing medium was nearly identical to the I_p–V relationship recorded in the drug-free solution (○). However, the curve observed at the end of the clamp pulses (□) displayed an upward shift at potentials negative to the holding potential and a downward shift at more positive voltages, the typical effects of DHO on the cardiac I_p–V relationship. Note that the I_p–V curve measured in drug-free solution remained unchanged regardless whether I_p was recorded at the beginning (○) or at the end (△) of a 60 s clamp step. Figure 6 further illustrates the different kinetics of the DHO interaction with the Na^+–K^+ pump of rat and guinea-pig ventricular myocytes in media containing equieffective DHO concentrations. The lower trace of Fig. 6*A* depicts the membrane current of a rat cell at holding potential (−20 mV). The upper trace indicates the K^+ concentration of the extracellular medium containing 1 mM DHO. First, the myocyte was superfused with a K^+-free solution where I_p was absent. Application of a medium containing 2 mM K^+ evoked an outward current which represents I_p. The increase of I_p occurred in two phases. There was an initial step-like I_p activation which was followed by a slower increase of I_p. The steady-state I_p amplitude was reached within 3 s and depended on $[K^+]_o$. As can be seen from Fig. 6*B*, the slow component of the I_p increase obeyed an exponential function. The ordinate gives the logarithm of the difference between the steady-state I_p amplitude ($I_{p,\infty}$) and the momentary I_p amplitude ($I_{p,t}$) of the slow component. The time constant of the process was independent of $[K^+]_o$ between 1 and 5·4 mM and amounted to 0·6 ± 0·05 s ($n = 8$) at 1 mM K_o^+, 0·5 ± 0·1 s ($n = 4$) at 2 mM K_o^+ and to 0·6 ± 0·1 s ($n = 4$) at 5·4 mM K_o^+. The lower trace of Fig. 6*C* displays the membrane current of a guinea-pig ventricular cell superfused with solutions containing 0·01 mM DHO plus different K^+ concentrations as indicated by the upper trace. First, the cell was superfused with a K^+-free medium where I_p was not activated. Application of a solution containing 2 mM K^+ reactivated I_p which reached its steady-state value only after about 55 s. Thus, the time required to obtain the steady-state I_p amplitude was approximately 20 times longer than in the corresponding experiment with a rat ventricular myocyte (Fig. 6*A*). Lowering $[K^+]_o$ to 0·5 mM strongly reduced I_p. The final I_p amplitude was observed after about 52 s in the low K^+ medium. Reapplication of the solution containing 2 mM K^+ and 0·01 mM DHO observed I_p again and the steady-state I_p amplitude was observed once more only after many seconds. Due to the poor time resolution of the sample record, the initial step-like variation of I_p upon alterations of $[K^+]_o$ is not properly depicted. Figure 6*D* shows the exponential time course of the slow component. The time constants derived (23 ± 3 s; $n = 13$) are definitively larger than those calculated for the changes of I_p in rat myocytes (Fig. 6*B*). It therefore turns out that, at equieffective concentrations, the kinetics of the interaction between DHO and the Na^+–K^+ pump are clearly slower in guinea-pig than in rat ventricular cells. Correspondingly, a new steady state of DHO binding, following a change of $[K^+]_o$, is reached more slowly in guinea-pig myocytes.

DISCUSSION

The inhibition of I_p depends on [DHO]

DHO exerts a concentration-dependent inhibition of I_p. At a given $[K^+]_o$ and membrane potential the inhibition increases with [DHO] (e.g. Fig. 2*A* at $V_c = 0$ mV). Of course, the inhibitory effect of various [DHO] can be predicted, in principle, by means of a concentration–response curve of the type depicted in Fig. 3*B*. However, the action of DHO on the cardiac I_p–V relationship depends not only on [DHO] but is additionally modulated by $[K^+]_o$ and membrane potential. The I_p inhibition by DHO is less evident at very negative clamp potentials and in a certain range of [DHO] (Figs 1*B* and 2*A*).

Modulation of the DHO effect by $[K^+]_o$ and membrane potential

Figure 1 shows that membrane potential and $[K^+]_o$ modulate the inhibition of I_p by a given [DHO]. This modulation causes typical alterations of the cardiac I_p–V curve by the cardiac glycoside. Hyperpolarization of the sarcolemma diminishes the I_p inhibition by a given [DHO] whereas depolarization strengthens the inhibitory effect. Similarly, an increased $[K^+]_o$ reduces the inhibition caused by DHO and a low $[K^+]_o$ intensifies the inhibitory action of the drug on I_p. The equivalence of changes in $[K^+]_o$ or membrane potential with respect to the inhibition of the

cardiac Na^+–K^+ pump by the cardioactive steroid suggests that the voltage-dependent effects are mediated by alterations of $[K^+]_o$. Voltage-dependent binding of extracellular K^+ to the Na^+–K^+ pump was first discussed by Rakowski *et al.* (1991) in order to explain the negative slope of the I_p–V relationship of *Xenopus* oocytes. It was likewise considered by Bielen *et al.* (1991*a*, 1993) in the discussion of corresponding observations on cardiac Purkinje cells at low $[K^+]_o$. As pointed out by Omay & Schwarz (1992), voltage-dependent K_o^+ binding may be mediated by potential-dependent variations of the local $[K^+]_o$ at the extracellular K^+ binding sites of the Na^+–K^+ pump in an 'access channel'. The present findings are easily explained within the framework of this hypothesis, which is supported by the numbers listed in Table 1. Hyperpolarization augments the local $[K^+]_o$. As a consequence, the inhibition of the pump by a cardiac glycoside decreases (Fig. 3*B*). In addition, the increased local $[K^+]_o$ activates *per se* Na^+–K^+ pumping. Depolarization reduces the $[K^+]_o$ in the 'access channel' and strengthens thereby the I_p inhibition induced by the drug. Furthermore, a low local $[K^+]_o$ *per se* slows down the activity of the pump (Fig. 1). These mechanisms produce the negative slope of the cardiac I_p–V relationship observed in media containing cardiac glycosides (e.g. Figs 1 and 2). The effect of the drugs on the cardiac I_p–V curve is less prominent at high $[K^+]_o$ ($\geqslant$ 5·4 mM) than at low $[K^+]_o$ (Fig. 1) for several reasons. First, as mentioned above, the inhibition of I_p by a given concentration of a cardioactive steroid is smaller at higher $[K^+]_o$ (Fig. 1). Second, variation of an augmented $[K^+]_o$ causes *per se* less alteration of the I_p amplitude than a corresponding change of a low $[K^+]_o$ because I_p activation as a function of $[K^+]_o$ exhibits saturation kinetics. Thus, it is expected that at high $[K^+]_o$, voltage-dependent glycoside effects on the I_p–V curve too will be less pronounced (Fig. 1*C* and *D*) since they are mediated by variations of the local $[K^+]_o$. If the hypothesis of voltage-dependent local alterations of $[K^+]_o$ is correct, it follows that the glycoside binding site of the Na^+–K^+ pump directly or indirectly senses these alterations in the 'access channel'. The apparent K_D value for I_p inhibition by DHO and the activation of I_p display a similar dependence on $[K^+]_o$. The similarity suggests that the occupancy of the pump's K_o^+ binding sites determines the DHO interaction with the pump. Once the K_o^+ binding sites are saturated, a further increase of $[K^+]_o$ has no effect on I_p or K_D and, consequently, the DHO effect on the I_p–V relationship remains unchanged.

The effects of cardiac glycosides on the cardiac I_p–V curve are time dependent

In seeming contrast to the action of DHO, the effect of ouabain on the I_p–V relationship of rat ventricular cells is time dependent (compare Fig. 4*B* and *D*). This is also true for the alteration of the I_p–V curve caused by DHO in guinea-pig ventricular myocytes (Fig. 5*B*). The time course of the I_p change upon an increase of $[K^+]$ in media containing DHO reveals a marked difference between rat and guinea-pig cardiac cells. The time to reach the new steady-state amplitude of I_p is longer by more than one order of magnitude in the latter cells (Fig. 6). According to Bielen *et al.* (1992), the interaction between DHO and the receptor on the cardiac Na^+–K^+ pump can be described as a reversible one-to-one binding reaction. The exponential time course of DHO binding towards the equilibrium of the reaction is determined by the rate constant:

$$k = k_1[\mathrm{DHO}] + k_2, \quad (2)$$

where k_1 represents the association rate constant and k_2 stands for the dissociation rate constant. The kinetics of the interaction between DHO and the cardiac Na^+–K^+ pump of the glycoside-sensitive guinea-pig are characterized by a 6 times larger k_1 and a 14-fold smaller k_2 if compared with the DHO binding to the Na^+–K^+ pump of the glycoside-insensitive rat (Hermans *et al.* 1994). It is clear from eqn (2) that the DHO concentration applied is important for the value of k. In the experiments shown in Fig. 6, the solutions contained DHO concentrations near the respective K_D values for the inhibition of I_p by DHO at 2–5·4 mM K^+ (rat: 1 mM; guinea-pig: 0·01 mM). The new equilibrium of the DHO binding reaction following changes of $[K^+]_o$ appears because k_1 varies with $[K^+]_o$. k_2 is essentially K_o^+ independent, at least in the concentration range under consideration (Bielen *et al.* 1992). The reason why the effect of DHO on the I_p–V curve of rat ventricular cells is apparently independent of time is that the new equilibrium of the DHO binding reaction is reached very quickly after a variation of the clamp potential (equivalent to an alteration of the local $[K^+]_o$). The binding of DHO to guinea-pig ventricular myocytes proceeds more slowly and the same is true for the interaction between ouabain and rat ventricular cells. It may be argued that slow changes of $[K^+]_o$ within the 'access channel' rather than the kinetics of cardiac glycoside binding cause the variation of the I_p amplitude with time following a clamp step. However, as mentioned above, I_p remained constant during clamp pulses in drug-free media.

To summarize, cardiac glycosides diminish the I_p amplitude of ventricular myocytes. The effect depends on $[K^+]_o$ and membrane potential. The dependence on voltage is probably mediated by local changes of $[K^+]_o$ at the K_o^+ binding sites of the cardiac Na^+–K^+ pump in an 'access channel'. The variation of the local $[K^+]_o$ disturbs the existing equilibrium of glycoside binding to the pump and a new equilibrium is established. The time required to reach the new binding equilibrium varies with the cardioactive steroid, its concentration and the glycoside sensitivity of the cardiac cells.

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