Br. J. Pharmacol. (1988), **93**, 803–810

High sensitivity of the Na^+, K^+-pump of human red blood cells to genins of cardiac glycosides

Nathalie Senn, *Lionel G. Lelièvre, **Pierre Braquet & [1]Ricardo Garay

INSERM U7, Hôpital Necker, 161, rue de Sèvres, 75015 Paris, *INSERM U127, Hôpital Lariboisière, Université Paris VII, 41, boul. de la Chapelle, 75010 Paris and **Institut Henri Beaufour, 17, av. Descartes, 92350 Le Plessis-Robinson, France

1 Four different cardiac glycosides (ouabain, digitoxin, digoxin and gitoxin) and their corresponding genins were tested on Na^+, K^+-pump fluxes measured under steady-state and initial rate conditions (non equilibrium conditions) in human and rat erythrocytes and in mouse macrophages.

2 In human red cells, Na^+, K^+-pump fluxes exhibited up to 8 fold higher sensitivity to genins than to glycosides. In addition genins, but not the corresponding glycosides, exhibited double reactivity with regard to the erythrocyte Na^+, K^+-pump (with the exception of gitoxigenin). A weak reactivity component was similar to the one of the corresponding glycosides (IC_{50} of about 10^{-6} M) and a high reactivity component exhibited IC_{50} values varying from 0.1 to 0.5×10^{-6} M for digitoxigenin and ouabagenin respectively.

3 In contrast with human red cells, the initial rate of Na^+, K^+-pump fluxes in rat erythrocytes and mouse macrophages was less sensitive to genins than to the corresponding cardiac glycosides.

4 Dihydroouabain was 3, 10 and 75 times less active than ouabain in inhibiting the initial rate of Na^+, K^+-pump fluxes in human and rat erythrocytes and in mouse macrophages respectively.

5 In conclusion, Na^+, K^+-pump fluxes measured under initial rate conditions in human erythrocytes exhibit an unusually high sensitivity to genins of cardiac glycosides. This property probably results from the fast binding rate constants of genins and the slow association rates of glycosides to human red cells.

Introduction

Several authors have reported that plasma and/or urine extracts from human and animals submitted to a variety of pathophysiological conditions are able to inhibit Na^+, K^+-ATPase activity (for review see DeWardener & Clarkson, 1985). However, in spite of extensive chemical purification and analysis, the molecular structure of these 'endogenous digitalis-like' (EDL) factors remains unknown.

Most of the above studies were based on the assay of partially purified biological samples on Na^+, K^+-ATPase activity. We have recently introduced a different approach based upon the 'screening' of structurally defined natural products obtained by synthesis (lignans and others) on the initial rate of fluxes catalyzed by the Na^+, K^+-pump in human red cells (Braquet *et al.*, 1986). One guiding criterion was the structural relationship of these compounds to cardiac glycosides and to other well known pump-inhibitors in heart membranes (for data on cardiac glycosides in human red cells see Kahn & Acheson, 1955; Glynn, 1957; Belz, 1981; Brown & Erdmann, 1984). This focussed our attention on the fact that, in contrast to lignans and other synthetic aglycones, cardiac glycosides contain a sugar residue in $C_3\beta$ position which strongly contributes to the drug-receptor interaction in heart membranes (Tamm, 1963; Repke, 1963; Yoda & Yoda, 1974; Wallick *et al.*, 1974; Thomas *et al.*, 1979).

The important role of $C_3\beta$-glycosylation was apparently confirmed in human red cells by previous investigators working on equilibrated cardenolide-binding (Belz, 1981; Brown & Erdmann, 1984). However, it is important to note that these results were obtained after prolonged preincubation (up to 3–4 h) with different concentrations of drugs (from 0.1 nM to 100 μM) and therefore the internal ion contents were probably not the same in all the cell samples.

[1] Author for correspondence at: INSERM U7, Hôpital Necker, 161, rue de Sèvres, Paris 75015, France.

We therefore decided to reinvestigate the role of $C_3\beta$-glycosylation in human red cells by measuring the effect of four different cardiac glycosides and their corresponding genins on Na^+ efflux and Rb^+ influx catalyzed by the Na^+, K^+-pump under steady-state and initial rate conditions (non equilibrium conditions). The results were compared with those obtained under the same kinetic conditions in two other cell preparations: rat erythrocytes and mouse macrophages. The data show that the initial rate of fluxes catalyzed by the Na^+, K^+-pump exhibit an unusual high sensitivity to genins in human red cells, but not in rat erythrocytes or mouse macrophages.

Methods

Preparation of red cells

Venous blood from healthy donors was collected in heparinized tubes. Arterial blood from Wistar rats was sampled under pentobarbitone anaesthesia (intraperitoneal dose of 60 mg kg^{-1}) from a catheter implanted in the aorta. Blood was centrifuged at 1750 *g* for 10 min at 4°C. The plasma and buffy coat were aspirated and the red cell pellet was used immediately.

Preparation of mouse macrophages

Mouse peritoneal macrophages were obtained according to a previously published method (Diez *et al.*, 1985). Briefly, 2–5 ml of sterile thioglycollate medium (Institut Pasteur, Paris) was injected into the peritoneal cavity of female mice 5–8 weeks old, of the 57 BL/5(H-2^b) and DBA/2(H-2^d) inbred strains. Elicited cells were collected 3 to 5 days later by washing the peritoneal cavity with Hank's balanced salt solution (HBSS). HBSS media were freshly prepared by adding $NaHCO_3$ (final concentration: 4.2 mM) to a basal medium of the following composition (mM): NaCl 137, KCl 5.3, Na_2HPO_4 0.34, $MgSO_4$ 0.32, KH_2PO_4 0.32, glucose 5.5, $CaCl_2$ 1.3 and $MgCl_2$ 0.5. A pool of 10–20 mice was used in each experiment. The collected suspension was immediately centrifuged at 1000 *g* for 1 min at 4°C and the supernatant was removed. Red cells were lysed by suspending the cell pellet in distilled water for 20 s. The cells were then washed twice with NaCl (150 mM). The number of cells collected per mouse was 10–20 $\times$ 10^6 and of these more than 80% had the morphological aspect of macrophages.

Measurement of Na^+, K^+-pump activity

Na^+, K^+-pump activity was equated to: (i) initial rate of ouabain-sensitive Na^+ efflux in human red cells (see for details Garay *et al.*, 1985) and (ii) initial rate of ouabain-sensitive Rb^+ influx in rat erythrocytes and mouse macrophages.

Measurement of Na^+, K^+-pump activity in human red cells

Fresh human erythrocytes were washed five times with cold $MgCl_2$ (110 mM) and resuspended in Mg-sucrose medium at a hematocrit of 20–25%. The Mg-sucrose medium contained (mM): $MgCl_2$ 75, sucrose 85, MOPS-Tris buffer (pH 7.4 at 37°C) 10 and glucose 10. The osmolality was adjusted to 295 $\pm$ 5 mOsm. A portion of the cell suspension was set aside to measure haematocrit, intracellular Na^+ and K^+ by flame photometry and haemoglobin absorbance at 541 nm by spectrophotometry.

An aliquot (0.5 ml) of the cell suspension in Mg-sucrose medium was added to two tubes containing 2 ml of Mg-sucrose with KCl 2 mM and to two tubes containing 2 ml of Mg-sucrose media with KCl 2 mM and 100 μM ouabain. The tubes were incubated for 30 min at 37°C (in control experiments we observed that fluxes were linear for more than 30–40 min and that no evidence of red cell lysis during the incubation in the efflux media could be detected). At the end of the incubation period the tubes were chilled at 4°C for 1 min and then centrifuged at 1750 *g* for 4 min at 4°C. The supernatants were transferred into tubes for Na^+ analysis in an Eppendorf flame photometer. Na^+ standards (checked with commercial standards, Merck, Darmstadt) were prepared in water and compared with those prepared in the different efflux media.

Ouabain-sensitive Na^+ efflux was calculated from the difference in external Na^+ concentration between tubes in the presence and absence of ouabain (see for details Garay *et al.*, 1985).

Measurement of Na^+, K^+-pump activity in rat red cells

Fresh rat erythrocytes were washed four times with cold NaCl (150 mM) medium and resuspended in the same NaCl solution at a haematocrit of 20–25%; 0.5 ml of the cell suspension was added to two tubes containing 2 ml of sodium-rubidium medium and two other tubes containing 2 ml of sodium-rubidium medium with 2 mM ouabain. The sodium-rubidium medium contained (mM): NaCl 145, RbCl 4, $MgCl_2$ 1, MOPS-Tris buffer (pH 7.4 at 37°C) 10 and glucose 10. The osmolality was adjusted to 300 $\pm$ 5 mOsm. The cells were incubated for 15 min at 37°C (in

control experiments we observed that fluxes were linear for more than 15–20 min and that no evidence of red cell lysis during the incubation in the efflux media could be detected). At the end of the incubation period the tubes were chilled at 4°C for 1 min and the cells were then washed three times with cold NaCl (150 mM). The red cell pellets were haemolyzed with 3 ml of Acationox 0.02% and centrifuged at 2250 *g* for 10 min at 4°C. The supernatants were transferred to tubes containing CsCl (final concentration of CsCl was 1 mM) and Rb^+ contents were measured in an IL 457 atomic absorption spectrophotometer. Rb^+ standards (checked with commercial standards, Merck, Darmstadt) were prepared in water and compared with those prepared in the different efflux media. An aliquot of supernatant was diluted 1 : 10 with distilled water and used to measure haemoglobin absorbance at 541 nm by spectrophotometry. A dilution factor was obtained by comparing these haemoglobin absorbances with the one obtained from the original suspension (of known haematocrit) and used to calculate the erythrocyte Rb^+ content.

Ouabain-sensitive Rb^+ influx was calculated from the difference in internal Rb^+ contents between tubes with and without ouabain.

Measurement of Na^+, K^+-pump activity in mouse macrophages

Washed fresh mouse macrophages were preincubated at 37°C for 30 min in a sodium-potassium medium containing (mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1, MOPS-Tris buffer (pH 7.4 at 37°C) 10 and glucose 10. The cells were then washed twice with cold NaCl (150 mM) medium and resuspended in the same NaCl solution at a phagocrit of 2–5%; 100 µl of the cell suspension were added to two tubes containing 1 ml of sodium-rubidium medium and to two other tubes containing 1 ml of sodium-rubidium medium with 2 mM ouabain. The sodium-rubidium medium contained (mM): NaCl 145, RbCl 4, $MgCl_2$ 1, MOPS-Tris buffer (pH 7.4 at 37°C) 10 and glucose 10. The osmolality was adjusted to 300 ± 5 mOsm. The cells were incubated for 10 min at 37°C (in control experiments we observed that fluxes were linear for more than 15–20 min and that internal Na^+ contents remained almost constant during the incubation period). At the end of the incubation period the tubes were chilled at 4°C for 1 min and the cells were then washed three times with cold NaCl 150 mM. The cell pellets were lysed with 1.1 ml of Acationox 0.02%, frozen and thawed three times, sonicated and then centrifuged at 2250 *g* for 10 min at 4°C. The supernatants were transferred into tubes for Rb^+ analysis in an IL 457 atomic absorption spectrophotometer. Intracellular Rb^+ contents were calculated by dividing the Rb^+ readings by the final phagocrit.

Ouabain-sensitive Rb^+ influx was calculated from the difference in internal Rb^+ contents between tubes with and without ouabain.

Drugs

All chemicals were either from Merck or Sigma (distributed through Coger, Paris, France). Concentrated solutions of drugs in water or dimethylsulphoxide (DMSO) were freshly prepared every day. Different amounts of drugs were added directly to the flux media provided that the final concentration of DMSO had no effect *per se* on Na^+ and K^+ fluxes. Fluxes in the presence of different concentrations of drugs were measured using the above protocols.

Data analysis

The dose-response curves reflect the ratios between ion translocation rates measured in the presence (v) or absence of drug (v_0), respectively. Pump-fluxes are non competitively inhibited by digitalis drugs (Joiner & Lauf, 1978). As a result, the initial rate v_0 is decreased by the factor $(IC_{50}/(IC_{50} + [drug]))$; where IC_{50} is the drug concentration ([drug]) inhibiting 50% of transport activity. Dose-response curves were analyzed assuming that the relation between the drug concentration and the inhibitory effect within the flux period could be considered either as being due to one, or to the sum of two independent inhibitory processes.

In the case of one process:

$$v/v_0 = IC_{50}/(IC_{50} + [drug]) \quad (1)$$

In the case of two processes:

$$v/v_0 = (A(IC_{50})_a/((IC_{50})_a + [drug])) + (B(IC_{50})_b/((IC_{50})_b + [drug])) \quad (2)$$

where A and B represent the percentage of each inhibitory process in the total inhibition (A + B being equal to 1).

Results

Inhibition of Na^+, K^+-pump activity by cardenolides in human red cells

Figure 1a shows the effect of cardiac glycosides on the initial rate of ouabain-sensitive Na^+ efflux in human erythrocytes. Note that the drugs were added at zero time without any preincubation with the cells. Analysis of the data depicted in Figure 1a by

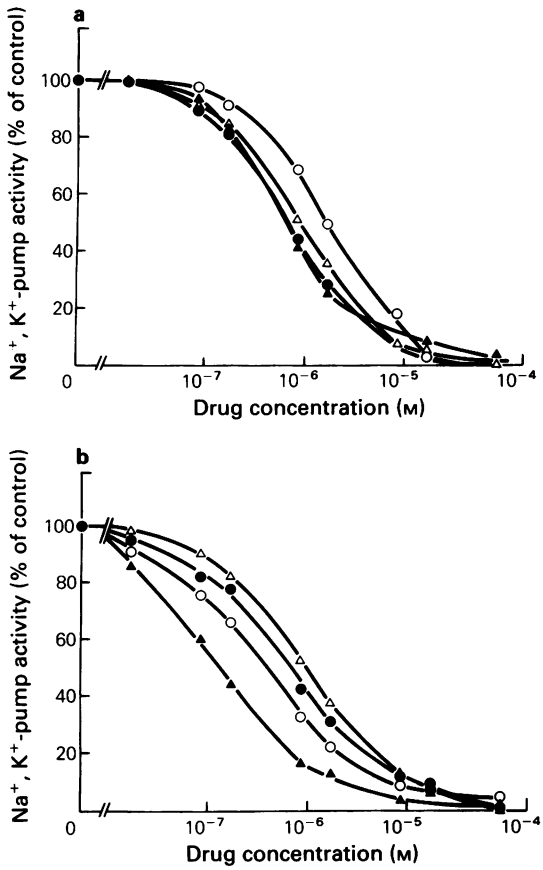

Figure 1 (a) Inhibition of Na^+, K^+-pump activity by cardiac glycosides in human red cells. Na^+, K^+-pump activity was equated to the initial rate of ouabain-sensitive Na^+ efflux. Values are given as means of 3 to 6 experiments. For most drugs the ranges were similar in size to the experimental points (ranges omitted for the sake of simplicity): (●) ouabain; (○) digoxin; (▲) digitoxin; (△) gitoxin. (b) Inhibition of Na^+, K^+-pump activity by genins of cardiac glycosides in human red cells. Values are given as means of 3 to 6 experiments. Comparison with (a) shows that genins were equal or higher in potency than the corresponding cardiac glycosides. (●) ouabagenin; (○) digoxigenin; (▲) digitoxigenin; (△) gitoxigenin.

using equation 1 showed a one site affinity to cardiac glycosides. Ouabain, digitoxin and gitoxin inhibited Na^+, K^+-pump activity with IC_{50} of about 0.8–1.1 $\times 10^{-6}$ M. Digoxin was slightly less effective (IC_{50} of about 2.2 $\times 10^{-6}$ M).

Figure 1b shows the effect of the corresponding genins. Analysis of the data depicted in this figure by using equation 1 showed a single inhibitory process only for gitoxigenin (IC_{50} of about 1.1 $\times 10^{-6}$ M). Conversely multiple (i.e. 2) inhibitory processes were observed with the three other genins. As shown in Table 1, the weak affinity component was similar to that of the corresponding glycosides (IC_{50} of about 10^{-6} M). In addition all these three genins exhibited a high affinity component with IC_{50} values varying from 0.1 to 0.5 $\times 10^{-6}$ M for digitoxigenin and ouabagenin respectively.

Comparison of Figures 1a and b show that genins were equal or higher in potency than the corresponding cardiac glycosides. In particular digitoxigenin and digoxigenin were one order of magnitude more active than digitoxin and digoxin.

Inhibition of Na^+, K^+-pump activity by cardenolides in rat red cells

Figures 2a and 2b show the effect of cardiac glycosides and corresponding genins on ouabain-sensitive Rb^+ influx in rat erythrocytes. As a general rule, all these cardenolides (particularly gitoxin and gitoxigenin) were much less active than in human red cells. IC_{50} values found in rat erythrocytes were between 10^{-4} and 10^{-3} M and therefore at least 100 times higher than in human red cells.

Table 2 shows that, generally speaking, all cardenolides exhibited a single affinity towards rat erythrocytes (with the exception of gitoxin and gitoxigenin for which no inhibition could be found).

Comparison of Figures 2a and b show that genins have slightly (digoxigenin and digitoxigenin) or markedly (ouabagenin) lower potency than the corresponding cardiac glycosides.

Inhibition of Na^+, K^+-pump activity by cardenolides in mouse macrophages

In previous screening of glycosilated lignans we have observed that some of them were active in mouse macrophages but not in human (or rat) erythrocytes (data not shown). It is for this reason that we investigated the effect of genins and corresponding cardiac glycosides in this cell type.

Table 3 shows the effect of cardenolides on ouabain-sensitive Rb^+ influx in mouse macrophages. It can be seen that all these compounds exerted an inhibitory activity intermediate between the one in human red cells and that in rat erythrocytes. The IC_{50} values varying between 8×10^{-6} and 5×10^{-5} M for digoxin, digitoxin and ouabain; gitoxin had an IC_{50} of about 10^{-3} M. As a general rule, genins were less active than the corresponding cardiac glycosides (Table 3). In particular, ouabage-

Table 1 Reactivity of genins and cardiac glycosides in relation to the initial rate of Na^+, K^+-pump fluxes in human red blood cells

	IC_{50} (M)	
Compound	*High reactivity*	*Low reactivity*
Ouabain	—	0.85×10^{-6}
Ouabagenin	5×10^{-7} (40%)	10^{-6} (60%)
Digoxin	—	2.2×10^{-6}
Digoxigenin	2×10^{-7} (50%)	10^{-6} (50%)
Digitoxin	—	0.85×10^{-6}
Digitoxigenin	10^{-7} (20–25%)	0.85×10^{-6} (75 – 80%)
Gitoxin	—	1.1×10^{-6}
Gitoxigenin	—	1.1×10^{-6}

Values in this table are given as mean of 3 to 6 experiments. The percentage of each population of sites is indicated in parentheses.

nin was 100 times less potent than ouabain (Table 3). Digoxigenin and digitoxigen were 3–10 fold less potent than the corresponding glycosilated derivatives.

Inhibition of Na^+, K^+-pump activity by dihydro-ouabain

Since all our originally tested lignans were butanolide (saturated lactone ring) and not butenolide derivatives (Braquet *et al.*, 1986), we tested the effect of dihydro-ouabain (saturated lactone ring of ouabain) in all the three cell preparations. Table 4 shows that dihydro-ouabain was 3, 10 and 75 times less active than ouabain in inhibiting Na^+, K^+-pump activity in human and rat erythrocytes and in mouse macrophages respectively.

Table 2 Reactivity of genins and cardiac glycosides with regard to the initial rate of Na^+, K^+-pump fluxes in rat red blood cells

	IC_{50} (M)	
Compound	*High reactivity*	*Low reactivity*
Ouabain	10^{-5} (20%)	1.6×10^{-4} (80%)
Ouabagenin	—	$>10^{-3}$
Digoxin	—	1.2×10^{-4}
Digoxigenin	—	2.4×10^{-4}
Digitoxin	—	2.1×10^{-4}
Digitoxigenin	10^{-4} (20%)	4×10^{-4} (80%)
Gitoxin	—	—
Gitoxigenin	—	—

Values in this table are given as mean of 3 to 5 experiments. The percentage of each population of sites is indicated in parentheses.

Discussion

The main result of our study was that in human red blood cells genins are more efficient than the corresponding cardiac glycosides in inhibiting the initial rate of Na^+, K^+-pump fluxes. The three genins of the most widely used glycosides: ouabain, digoxin and digitoxin, exert significant pump-inhibition at doses up to 8 fold lower than those required for glycosides. In addition, these genins, but not the glycosides, exhibit two site binding kinetics with regard to the human erythrocyte Na^+, K^+-pump. Two IC_{50} values could be therefore determined from the dose-response curves of genins (Figure 1b and Table 1).

None of the above two properties of genins: increased inhibitory potency and two-site binding against the initial rate of Na^+, K^+-pump fluxes, were observed in rat red blood cells or in mouse macrophages.

The unusual pump sensitivity to genins in human erythrocytes can be explained by two hypotheses: (i)

Table 3 Reactivity of genins and cardiac glycosides with regard to the initial rate of Na^+, K^+-pump fluxes in mouse macrophages

Compound	IC_{50} (M)
Ouabain	$8.0 \pm 3.5 \times 10^{-6}$
Ouabagenin	$8.0 \pm 2.1 \times 10^{-4}$
Digoxin	$4.3 \pm 1.5 \times 10^{-5}$
Digoxigenin	$1.1 \pm 0.5 \times 10^{-4}$
Digitoxin	$1.8 \pm 0.4 \times 10^{-5}$
Digitoxigenin	$2.0 \pm 0.5 \times 10^{-4}$
Gitoxin	$>10^{-3}$
Gitoxigenin	$>10^{-3}$

Values in this table are given as mean ± range of 3 to 5 experiments.

Table 4 Reactivity of dihydro-ouabain with regard to the initial rate of Na^+, K^+-pump fluxes in human and rat erythrocytes and in mouse macrophages

Compound	IC_{50} (M) Human red cells	Rat red cells	Mouse macrophages
Dihydro-ouabain	2.1×10^{-6}	10^{-3}	6×10^{-4}
Ouabain	6.5×10^{-7}	10^{-4}	8×10^{-6}

Values in this table are given as means of 3 to 5 experiments.

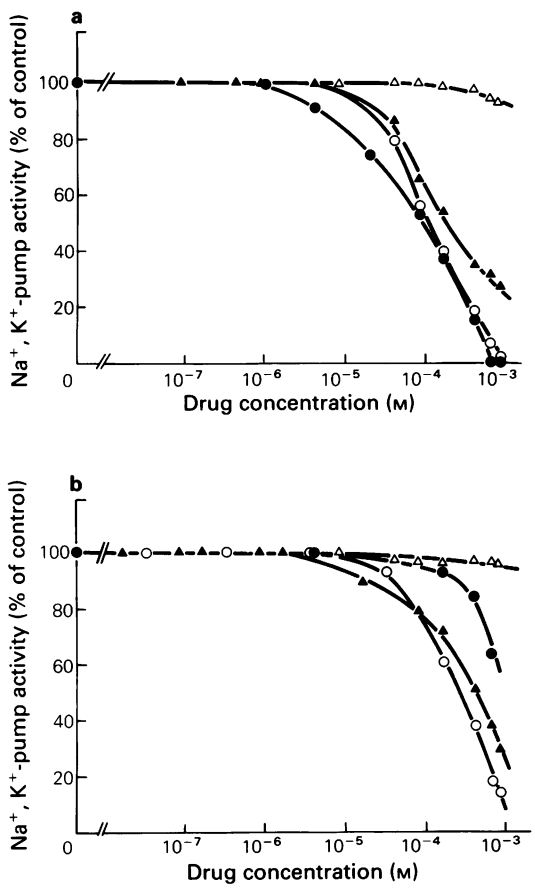

Figure 2 (a) Inhibition of Na^+, K^+-pump activity by cardiac glycosides in rat red cells. Na^+, K^+-pump activity was equated to the initial rate of ouabain-sensitive Rb^+ influx. Values are given as means of 3 to 5 experiments: (●) ouabain; (○) digoxin; (▲) digitoxin; (△) gitoxin. (b) Inhibition of Na^+, K^+-pump activity by genins of cardiac glycosides in rat red cells. Comparison with (a) shows that genins have slightly (digoxigenin and digitoxigenin) or markedly (ouabagenin) lower potency than the corresponding cardiac glycosides: (●) ouabagenin; (○) digoxigenin; (▲) digitoxigenin; (△) gitoxigenin.

the experimental conditions of flux measurement reveal differences in (association-dissociation) rate constants between genins and glycosides and/or; (ii) two types of digitalis-receptor sites may exist.

In human erythrocytes, as in other cells of digitalis-sensitive species (for review see Schwartz *et al.*, 1975), pump inhibition by glycosides increases with time. This is not the case with genins that exhibit higher association (dissociation) rate constant(s) than cardiac glycosides (Wallick *et al.*, 1974; Yoda & Yoda, 1974; Thomas *et al.*, 1979).

Binding equilibrium is reached after 3–5 h of incubation of human red cells with cardiac glycosides (Brown & Erdmann, 1984). The apparent dissociation constants estimated here, in the micromolar range (Table 1), are therefore much higher than those determined at binding equilibrium, which are in the nanomolar range (Belz, 1981; Brown & Erdmann, 1984).

The 30 min period that we used for measuring the initial rate of pump fluxes in human red cells may therefore reveal the high association rate constants of genins.

In cells from rodents and other digitalis-resistant species, cardiac glycosides reach equilibrium in about 5 min (Lelièvre *et al.*, 1979; Mansier & Lelièvre, 1982). A flux experiment of 10–15 min (see Methods) cannot reveal the high association rate constants of genins in rat erythrocytes or mouse macrophages.

Another experimental condition that can influence the measured IC_{50} values is the potassium (or rubidium) concentration in the assay medium. However, this ion decreases the apparent affinities for genins more than the affinities for glycosides (Akera *et al.*, 1978), and we observed the opposite situation (Table 1). The unusual pump sensitivity to genins in human red cells cannot therefore be explained by the influence of potassium.

The above difference in binding rate constants cannot itself explain the two-site binding seen with genins with regard to the initial rate of Na^+, K^+-pump fluxes in human red cells. Indeed, a difference in binding rate constants should induce parallel shifts of the dose-response curves. Comparison of the

curves depicted in Figures 1a and b (digoxin vs. digoxigenin and digitoxin vs. digitoxigenin) clearly shows that this is not the case.

The multiple binding of genins argues in favour of our second hypothesis, i.e. that two types of digitalis-receptor sites may exist, one of low reactivity (IC_{50} values very similar to those found with glycosides) and another of high reactivity (IC_{50} values 2 to 8 fold lower than glycosides). This is illustrated in Table 1 by digitoxigenin (IC_{50} values of 0.1 and 0.85 μM) and digitoxin (IC_{50} values of 0.85 μM).

Flux experiments performed at binding equilibrium were unable to reveal two types of digitalis-receptor sites in human red cell membranes. So far the simplest interpretation is that genins can initially interact (in a first association step) with a pump-site not accessible to glycosides.

Two kinds of digitalis receptor sites have been detected previously, in brain (Sweadner, 1979) and in heart (Brown & Erdmann, 1984; Erdmann *et al.*, 1984; 1985; Maixent *et al.*, 1987). However, these experiments were performed under equilibrium conditions. Interestingly, dihydro-ouabain, a specific ligand for high affinity cardiac digitalis receptor sites (Finet *et al.*, 1983) exhibited a single binding site in human red cells (Table 4). This suggests that: (i) if a second site does exist, it is recognized by a free-OH group in $C_3\beta$ and (ii) this high reactivity site of genins may correspond to the low affinity receptor site of heart. Further investigation is therefore required before it is possible to conclude that there are two kinds of digitalis receptor sites in human red blood cells.

An important question is whether an assay based upon the measurement of the initial rates of pump-fluxes in human erythrocytes is a good pharmacological model (of intact cells) for the screening of inotropic (and other pump-inhibiting) drugs. Indeed, the human erythrocyte is the most rapid and precise model for the screening of loop diuretics on the Na^+, K^+ cotransport system and other assays (see for instance Ellory & Stewart, 1982; Garay *et al.*, 1986). In addition it has been shown that the order of potency of different digitalis molecules is the same in human erythrocyte and human cardiac preparations (Brown & Erdmann, 1984).

Screening of putative pump-inhibitors in human red cells cannot be carried out by performing fluxes under equilibrium conditions because: (i) this requires previous knowledge of the time for reaching binding equilibrium and (ii) long-term preincubation induces changes in erythrocyte Na^+ content which can themselves cause important changes in pump fluxes (Garay & Garrahan, 1973).

Chemical synthesis of glycosides (or glucuronoconjugates) introduces a further, sometimes very complicated step. The fact that in human erythrocytes, aglycones appear to be efficient pump-inhibitors may allow a rapid decision for selecting molecules that can be glycosilated (or glucuronoconjugated) in a second step. These compounds can subsequently be tested for inotropic and/or natriuretic activities.

In conclusion, Na^+, K^+-pump fluxes measured under initial rate conditions in human erythrocytes exhibit an unusual high sensitivity to genins of cardiac glycosides. This property probably results from the fast binding rate constants of genins and the slow association of glycosides to human red cells. An assay based upon the measurement of initial rates of pump-fluxes in human erythrocytes represents a good pharmacological intact cell model for the rapid screening of aglycones of putative inotropic drugs or endogenous ouabain-like factors.

References

AKERA, T., TEMMA, K., WIEST, S.A. & BRODY, T.K. (1978). Reduction of the equilibrium binding of cardiac glycosides and related compounds to Na^+, K^+-ATPase as a possible mechanism for the potassium-induced reversal of their toxicity. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **304**, 157–165.

BELZ, G.G. (1981). Rubidium uptake in erythrocytes. In *Cardiac Glycosides*, Part 1. Experimental Pharmacology. pp. 95–113. ed. Greeff, K. Berlin: Springer-Verlag.

BRAQUET, P., SENN, N., ROBIN, J.P., ESANU, A. & GARAY, R. (1986). Endogenous lignans—a potential endogenous digitalis. *J. Hypertension*, **4** (suppl. 5), S161–S164.

BROWN, L. & ERDMANN, E. (1984). Comparison of the affinity of human, beef and cat heart ($Na^+ + K^+$)-ATPase for different digitalis derivatives. *Drug Res.*, **34**, 1314–1318.

DEWARDENER, H.E. & CLARKSON, E.M. (1985). Concept of Natriuretic Hormone. *Physiol. Rev.*, **65**, 658–759.

DIEZ, J., BRAQUET, P., VERNA, R., NAZARET, C. & GARAY, R. (1985). The effect of cyclic AMP on Na^+ and K^+ transport systems in mouse macrophages. *Experientia*, **41**, 666–667.

ELLORY, J.C. & STEWART, G.W. (1982). The human erythrocyte Cl-dependent Na–K cotransport system as a possible model for studying the action of loop diuretics. *Br. J. Pharmacol.*, **75**, 183–188.

ERDMANN, E., WERDAN, K. & BROWN, L. (1985). Multiplicity of cardiac glycoside receptors in the heart. *Trends Pharmacol. Sci.*, **71**, 293–295.

ERDMANN, E., WERDAN, K. & KRAWIETZ, W. (1984). Influence of digitalis and diuretics on ouabain binding sites on human erythrocytes. *Klin. Wochenschr.*, **62**, 87–92.

FINET, M., GODFRAIND, T. & NOEL, F. (1983). The inotropic effect of ouabain and its antagonism by dihydroouabain in rat isolated atria and ventricules in relation to specific binding sites. *Br. J. Pharmacol.*, **80**, 751–759.

GARAY, R., DIEZ, J., NAZARET, C., DAGHER, G. & ABITBOL, J.P. (1985). The interaction of canrenone with the Na^+, K^+-pump in human red blood cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **329,** 311–315.

GARAY, R. & GARRAHAN, P. (1973). The interaction of sodium and potassium with the sodium-pump in red cells. *J. Physiol.*, **231,** 297–325.

GARAY, R., HANNAERT, P.A., NAZARET, C. & CRAGOE, E.J. Jr. (1986). The significance of the relative effects of loop diuretics and anti-brain edema agents on the Na^+, K^+, Cl^--cotransport system and the $Cl^-/NaCO_3^-$ anion exchanger. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **334,** 202–209.

GLYNN, I.M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol.*, **136,** 148–173.

JOINER, C.H. & LAUF, P.K. (1978). The correlation between ouabain binding and potassium pump inhibition in human and sheep erythrocytes. *J. Physiol.*, **283,** 155–175.

KAHN, J.B. & ACHESON, G.H. (1955). Effects of cardiac glycosides and other lactones, and of certain other compounds on cation transfer in human erythrocytes. *J. Pharmacol.*, **115,** 123–130.

LELIEVRE, L.G., ZACHOWSKI, A., CHARLEMAGNE, D., LAGET, P. & PARAF, A. (1979). Inhibition of Na^+, K^+-ATPase by ouabain: involvement of calcium and membrane proteins. *Biochem. Biophys. Acta*, **557,** 399–408.

MAIXENT, J.M., CHARLEMAGNE, D., DE LA CHAPELLE, B. & LELIEVRE, L.G. (1987). Two ($Na^+ + K^+$)-ATPase isoenzymes in canine cardiac miocytes. Molecular basis of inotropic and toxic effects of digitalis. *J. Biol. Chem.*, **262,** 6842–6848.

MANSIER, P. & LELIEVRE, L.G. (1982). Ca^{2+}-free perfusion of rat heat reveals a ($Na^+ + K^+$)-ATPase form highly sensitive to ouabain. *Nature*, **300,** 535–537.

REPKE, K. (1963). Metabolism of cardiac glycosides. In *New Aspects of Cardiac Glycosides.* ed. Wilbrandt, W. & Lindgren, P. pp. 47–73. Oxford: Pergamon Press.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975). The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.*, **27,** 3–134.

SWEADNER, K.J. (1979). Two molecular forms of ($Na^+ + K^+$)-stimulated ATPase in brain. *J. Biol. Chem.*, **254,** 6060–6070.

TAMM, C.H. (1963). The stereochemistry of the glycosides in relation to biological activity. In *New Aspects of Cardiac Glycosides.* ed. Wilbrandt, W. & Lindgren, P. pp. 11–26. Oxford: Pergamon Press.

THOMAS, R., ALLEN, J.C., PITTS, B.J.R. & SCHWARTZ, A. (1979). Cardenolide analogs. An explanation for the unusual properties of AY 22241. *Eur. J. Pharmacol.*, **53,** 227–237.

WALLICK, E.T., DOWD, F., ALLEN, J.C. & SCHWARTZ, A. (1974). The nature of the transport adenosine triphosphatase-digitalis complex. VII. Characteristics of ouabagenin-Na^+,K^+-adenosine triphosphatase interaction. *J. Pharmacol. Exp. Therap.*, **189,** 434–442.

YODA, A. & YODA, S. (1974). Structure-activity relationships of cardiotonic steroids for the inhibition of Na^+ and K^+-dependent adenosine triphosphatase. *Mol. Pharmacol.*, **10,** 494–501.

(Received August 21, 1987
Accepted November 16, 1987)