Discrepancy between the short and long term effects of ouabain on the sodium pumps of human cells grown in culture

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1 Human cells (HeLa) were cultured for periods up to 48 h in growth medium in the absence or presence of a range of concentrations of cardiac glycosides. In some experiments the potassium concentration of the medium was varied between 0.3 mM and the usual 5 mM.

2 For periods up to 2 h in ouabain the association and dissociation rate constants were measured and the equilibrium binding constant (K_D) calculated; the apparent equilibrium binding constant (K'_D) was measured after 1–2 days growth in ouabain.

3 Ouabain had a K'_D after 2 days of 2–6 nM in 5 mM K^+ growth medium, a 4 fold greater blocking effect on sodium pumps after 2 days than expected from the association and dissociation rate constants measured in untreated or previously ouabain-treated cells.

4 This effect was: (a) approximately the same over a range of external potassium concentrations from 0.3 to 5 mM, although the absolute effect of ouabain over this range of potassium was much different; (b) probably not due to different isoforms of pumps in cells grown in ouabain compared to untreated cells; (c) apparently not a consequence of internalisation of pump-glycoside complexes.

5 We conclude that ouabain has only a limited access to sodium pumps in whole cells; this could be because sodium pumps cycle continuously through an inaccessible region of the plasma membrane. This effect needs to be considered both in the assessment of the magnitude of the long term effects of cardiac glycosides on cells, and in the measurement of the glycoside affinities of various isoforms of the pump.

Keywords: Sodium pumps; ouabain; Na^+ , K^+ -ATPase; cardiac glycosides; internalisation

Introduction

In cultured human cells, as in most other animal cells, active sodium and potassium ion transport is mediated by a Na^+ , K^+ -ATPase (the sodium pump) situated in the plasma membrane, which is specifically inhibited by the cardiac glycosides. The sodium pump has two types of protein subunit, alpha and beta; the alpha subunit (112 kDa) contains the binding sites for adenosine 5'-triphosphate (ATP) and cardiac glycosides, and is often loosely known as the 'catalytic subunit'. The beta subunit (35 kDa) is a glycoprotein that seems to be required for enzymatic activity, but its exact function is unknown. It was initially thought that the sodium pumps from all tissues and species were the same, only varying in their sensitivity to cardiac glycosides, but in the last few years it has become apparent that there are at least three different molecular forms of the alpha subunit, now called $\alpha 1$, $\alpha 2$ and $\alpha 3$ (Sweadner, 1989).

Considerable efforts are now being made to identify the distribution of these isozymes in various species and tissues by use of cDNA probes to identify their mRNA, and antibodies to demonstrate and measure their quantities in the plasma membranes. Concomitant with these studies the kinetic and functional differences of these isozymes are being re-measured to relate the molecular form of the isoform to its pharmacological properties.

HeLa cells, an epithelial cell line derived from human cervix, have only one alpha isoform of around 3.7 kb (Cutler *et al.* 1988), probably equivalent to $\alpha 1$ in rat (Emanuel *et al.*, 1986), so that the pharmacological properties of the glycoside- Na^+ , K^+ -ATPase interaction of these cells can be unambiguously related to this isoform. It is of particular interest to study this isoform for we had long noticed that the effect of cardiac glycosides on these and other cultured human cells under equilibrium conditions (see e.g. Lamb & McCall, 1972) seemed to be much larger than either that reported in the literature or that expected from the usual short term measurements. We describe here a detailed study of this effect.

Methods

Cell culture

Outline: HeLa (human epitheloid carcinoma cervix) cells were cultured routinely in tissue culture flasks (Sterilin) in Minimum Essential Medium (MEM) with Earle's salts containing 10% newborn calf serum, 1% non-essential amino acids and kanamycin 50 μ g/ml. For experiments, cells were subcultured into 12 or 24 well multiwell plates (some earlier experiments used 3 cm diameter petri dishes) at typical seeding densities of $3\text{--}4 \times 10^4$ cells cm^{-2} . The plates were incubated at 37°C in an atmosphere of 95% air/5% CO_2 . Cells were grown for 2–4 days until near confluence; the medium was then aspirated and replaced with fresh normal or experimental medium for the next 0.5 to 2 days. A stock of HeLa cells was also kept in liquid N_2 to ensure continuity of the cell line in case of infection.

Measurement of numbers of sodium pumps

Sodium pump numbers were estimated by measuring the specific [^3H]-ouabain binding. Cells were incubated for 20 min at 37°C in 200 nM [^3H]-ouabain in a modified Krebs-Hensleit solution containing either no potassium (0 K^+), or 15 mM potassium (15 K^+) or 1 mM unlabelled ouabain. The specific binding was taken as the difference between the total (0 mM K^+) and non-specific (15 mM K^+ or 1 mM ouabain) binding. Usually the 'non-specific' binding in excess ouabain was about half that in 15 mM potassium, but as the non-specific binding was much less than 10% of the total binding, this difference did not affect the conclusions drawn. The [^3H]-ouabain bound in 15 mM K^+ solution was directly related to the total binding, whereas that in excess ouabain was not, possibly indicating that 15 mM K^+ did not block all the [^3H]-ouabain binding to the pump. After incubation with [^3H]-ouabain, the plates were rinsed with ice-cold Krebs solution to remove extracellular isotope and the cells were then detached with trypsin/EDTA (0.25% trypsin, 2 mM EDTA in basal salt solution without Ca^{2+} or Mg^{2+}) to obtain a single cell suspension. Cell numbers and volume were then measured

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Figure 1 Direct and indirect measures of sodium pumps with [^3H]-ouabain. The ordinate scale shows the number of sodium pumps occupied by [^3H]-ouabain directly (■), or the disappearance of the unoccupied pumps (●) with time; the indirect measure. The first order rate equation fitted to the direct measure had a rate of association of $0.209 \pm 0.033 \text{ min}^{-1}$ and a maximum value of $704 \pm 55 \times 10^3$ pumps per cell; the indirect measure gave values of 0.276 ± 0.014 and 597 ± 19 respectively. As neither the rate nor maximum are significantly different the mean values are 0.243 min^{-1} (i.e. a $t_{1/2}$ of 2.9 min) and maximum pump numbers of 650×10^3 per cell.

with a Coulter Counter (Model ZM) and channelyser (Model 256). The amount of isotope was determined with a liquid scintillation Spectrometer (Packard Tricarb).

In some conditions it was not possible to measure the total number of sodium pumps directly, so an indirect method was used. In this we first bound non-radioactive ouabain or other glycosides to the cells and then estimated the unoccupied pumps by binding [^3H]-ouabain to them; the original number of pumps occupied by the non-radioactive ouabain (or other glycoside) was then calculated by measuring the total pumps in control cells. In cells treated chronically with glycosides this method is only applicable if the total number of pumps does not change much; this has been found to be so by Pollack *et al.* (1981). Figure 1 shows that in untreated cells direct and indirect procedures both give the same total number of pumps and the same initial rate of binding. The mean density of sodium pumps in untreated cells was $726,000 \pm 36,000$ (mean $\pm$ s.e.mean, $n = 25$) per cell.

Measurement of pump activity and cell sodium and potassium

Pump activity was assessed by measuring the ouabain-sensitive potassium influx (with ^{86}Rb as the tracer) for 5 min. This influx time gave sufficient counts and was short in comparison to the equilibrium time ($> 1 \text{ h}$). We took account of our earlier evidence that ouabain sometimes stimulates co-transport, and so included bumetanide in the influx solutions to block this effect (Bakker-Grunwald *et al.*, 1982).

To measure cell sodium and potassium, plates were rinsed with an ice-cold isotonic sorbitol solution (275 mM sorbitol, 15 mM Tris-HCl; pH 7.4 at 4°C) and then lysed with ion-free distilled water for 1 h or more. The sodium and potassium concentrations were then measured by flame photometry. The cell water was obtained by multiplying the total cell volume by 0.8. Much previous work by us on these cells has shown that the Coulter system used, as described, gives a good measure of the cell volume; a direct measure of the cell water with labelled 3-O-methyl [^{14}C]-glucose sugars confirmed this method (see Aiton & Simmons, 1983).

Equilibrium constant for ouabain binding

In the absence of internalisation the dissociation constant, K_D , for the ouabain-ATPase complex formation is given by k_{-1}/k_1 where k_1 and k_{-1} are the association and dissociation rate constants respectively. The sodium pumps of HeLa cells, however, are being continuously turned over by the process of internalisation of membrane pumps and synthesis of new ones (Cook *et al.*, 1982), so this needs to be considered when measuring the binding of ouabain and other ligands. The apparent dissociation constant, K'_D , for this process is given by $(k_{-1} + k_{\text{int}})/k_1$ where k_{int} is the rate constant of internalisation. Substituting for k_1 yields the expression $K'_D = K_D (1 + k_{\text{int}}/k_{-1})$. Thus it follows that internalisation always increases the apparent equilibrium constant (i.e. decreases the apparent affinity; Klausner *et al.*, 1985). In almost all the experiments described in this paper we measure $(k_{-1} + k_{\text{int}})$ and not k_{-1} alone.

This equation assumes that all the pumps which are internalised are returned to the membrane, either by new synthesis or by recycling, so that the total density of the pumps on the membrane is constant over time. We tested this directly by measuring the rate of recovery of unoccupied pumps after a digoxin block and comparing it with the loss of digoxin from the cells (Algharably *et al.*, 1986). The rate of recovery of unoccupied pumps was $7.7 \pm 0.6\% \text{ h}^{-1}$, not different from the mean rate of loss of digoxin (which measures both internalisation and dissociation, see later) from the cells.

Plasma membrane isolation

Cells were grown on microcarrier beads, and the membranes prepared as follows. A 10 ml sample from the culture was collected in a sterile universal container. This sample was washed 4 times with cold 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) buffered saline (0.9% NaCl, 5 mM Tris) at pH 8. Between each wash the microcarrier beads were allowed to settle out before the supernatant was removed (around 3 min). The sample was incubated with ice-cold hypotonic solution (1 mM Tris-HCl, pH 8.0) for 30 min and then washed twice in ice-cold 10 mM Tris-HCl solution, pH 8.0. Then 10 mM Tris was added, the cells vortexed for 10 s to complete cell lysis. The beads with the attached membranes were then washed twice more, resuspended in 10 mM Tris-HCl pH 8.0 to give a 50% suspension. This was then sonicated for 10 s at 20 W and washed twice in ice-cold buffer. Analysis of isolated membranes showed that there was about a 25% recovery of membrane markers, with a purity similar to other methods (see Algharably *et al.*, 1986 for other details).

Experimental design and analysis of results

Experiments were designed to reduce the inter-cell differences as far as possible. With multi-well plates it was possible to examine several conditions on the same plate; if this was not possible then we handled different plates in as similar a way as possible. All comparisons between different conditions were made with the same batch of cells in the same experiment. The commercial package 'Enzfitter' was used on a desktop computer to fit curves and compute errors. This package fits equations to the raw data without any transformation, using a 'non-linear regression' method. It allows the error structure to be varied to check the 'robustness' of the conclusion for different assumptions about variability. Little difference was found; the curves shown were those fitted with constant variance (no weighting). The rectangular hyperbolae shown have a linear X-axis rather than the more common log axis. For simple t tests the commercial package 'Minitab' was used.

Drugs and materials

Tissue culture plastic ware, i.e. flasks, Petri dishes and 12 or 24 multiwell plates, were supplied either by Sterilin or Flow

Laboratories (Linbro). Culture media were obtained from Life Technologies. Cardiac glycosides and the Cytodex Microcarrier beads were bought from Sigma Chemical Co. [21, 22- ^3H]-ouabain and $^{86}\text{RbCl}$ were bought from Amersham International. All other chemicals used were of Analar grade from BDH.

Solutions

The modified Krebs solution contained (mM): NaCl 137, KCl 5.4, CaCl_2 2.8, MgSO_4 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, Tris 15, HCl 12 and glucose 10 (pH 7.4). For 0 mM K^+ Krebs, K^+ salts were omitted and replaced by Na^+ salts.

Results

Steady state effects of cardiac glycosides

Figure 2 shows an experiment in which the effects of exposure of cells to various cardiac glycosides were studied over 48 h in the same batch of cells. The order of potency was ouabain, digitoxin, digoxin, acetylstrophanthidine and dihydro-ouabain. For a detailed comparison of short and long term exposure we used ouabain. Figure 3 shows that exposure of cells to a ouabain concentration of a few nM for 48 h reduces the unoccupied pump numbers to half; the best-fit equilibrium binding constant (K_D) being 2.6 ± 0.7 nM. In two of the experiments in which there were sufficient data points the K_D values were 2.5 and 2.9 nM, so that amalgamating the data did not distort the result. At higher ouabain concentrations than shown (>27 nM) the cells died, and even at 27 nM they did not divide; this may explain the poorish fit of the curves at these higher concentrations. When experiments were done with a single concentration of ouabain, we used 10 nM as a compromise between seeing an effect and not causing too much cell deterioration.

In some experiments cells were only grown for 24 h in ouabain, and in these the best-fit K_D was around 10 nM so that the steady state had not been reached in this time. In Appendix 1 we show that this time discrepancy would be expected to have only a minor effect.

Effect of serum and the potassium concentration

The serum concentration of the growth medium affects the density of sodium pumps (Aiton & Lamb, 1984), so we examined the effects of growth in ouabain at different serum

Figure 2 Effect of growth in the presence of various cardiac glycosides on unoccupied sodium pumps. HeLa cells were incubated in normal growth medium containing a range of concentrations of ouabain (●); dihydro-ouabain (○); digoxin (▲); digitoxin (△); or acetylstrophanthidine (■) for 48 h. The unoccupied pumps were estimated by incubation with 200 nM [^3H]-ouabain. The results are expressed as a % of the control value. Each point is the mean of 3 measurements. The digitoxin concentration is corrected for protein binding. All measurements were made on the same batch of cells. Lines fitted by eye; the s.e.mean was less than 5% of the mean.

Figure 3 The effect of growing HeLa cells for 48 h in a range of ouabain concentrations on unoccupied pump numbers. After 4 days of growth in normal medium the cells were transferred to growth medium containing ouabain; 48 h later the unoccupied pumps were measured with 200 nM [^3H]-ouabain. The hyperbolic curve shown has a K_m of 2.5 ± 0.7 nM.

concentrations. Over 48 h, raising serum from 0.1% to 20% increased pump density by 30% but 10 nM ouabain produced the same block (66%) at both serum concentrations, with no interaction (by ANOVA). Similar observations were made in 2 experiments in which cells were grown for 48 h in digoxin.

A low concentration of potassium in the growth medium upregulates the density of sodium pumps (Boardman *et al.*, 1974) in HeLa, and most cultured cells. Figure 4 shows that over 24 h a low potassium concentration greatly increased the absolute ouabain binding as well as upregulating pump density, so that ouabain had a much larger absolute and proportional effect at low potassium concentrations than at high ones.

Short term experiments with ouabain

In order to check if the expression for K_D (see Methods) is a good description of the steady state binding of ouabain to the

Figure 4 Effect of the potassium concentration of the medium on the density of unoccupied sodium pumps on HeLa cells in the presence and absence of various ouabain concentrations. Cells were grown for 4 days in normal medium and then transferred for 24 h to media containing various combinations of potassium shown; the ouabain concentrations in the growth medium were 0 (●); 2 (■); 5 (△) and 10 (□) nM. The unoccupied sodium pumps were then measured by exposure to [^3H]-ouabain. Each point is the mean of 3 replicate observations; typical s.e.means are shown; others were less than 5% of the mean.

Figure 5 [^3H]-ouabain binding to untreated HeLa cells from a potassium-free medium. The first order rate equation fitted gives a $V_{\max}$ of $746 \pm 13 \times 10^3$ sites per cell and an association rate of $0.159 \pm 0.009 \text{ min}^{-1}$; a $t_{1/2}$ of 4.4 min. The inset shows a log transformation of the data.

sodium pumps of HeLa cells we needed to measure the association rate constant ($k_1 = \ln 2/t_{1/2}$), the sum of internalisation and dissociation rates constants ($k_{-1} + k_{\text{in}}$), and the apparent equilibrium binding constant (K'_D), as has been done for the sodium pumps of red blood cells (Furukawa *et al.*, 1980). However, the apparent dissociation of ouabain from the pumps of human cultured cells is very slow, so that it takes many hours to reach equilibrium. At equilibrium the cell sodium is high and the potassium low, so that the cells are no longer in a normal state. The best that can be done is to measure the association and dissociation rates as usual, and the apparent binding constant under non-equilibrium conditions.

The rate of association of ouabain with the sodium pump is first order with respect to the ouabain concentration (Baker & Willis, 1970; Furukawa *et al.*, 1980; a point we also checked) and, in HeLa cells at least, is very sensitive to the concentration of potassium in the bathing medium, a condition which is used to estimate the non-specific binding. We made measurements of the rate of association in zero and 5 mM potassium both in untreated cells and in cells that had been grown in ouabain for 24–48 h.

Figure 5 shows the time course of ouabain binding to untreated cells in a nominally potassium free medium with 200 nM [^3H]-ouabain; the inset shows that the binding follows a log curve. The $t_{1/2}$ of this was 4.4 min, giving an association rate constant of $4.7 \times 10^7 \text{ h}^{-1} \text{ M}^{-1}$. Figure 6 shows the rates of binding in 0 and 5 mM potassium of 200 nM [^3H]-ouabain in cells which had been grown for 48 h in 10 nM ouabain. The $t_{1/2}$ values are around 4 and 86 min respectively (association rate constants of $4.8 \times 10^7 \text{ h}^{-1} \text{ M}^{-1}$ and $2.4 \times 10^6 \text{ h}^{-1} \text{ M}^{-1}$; a potassium effect of 20 fold). We concluded from these experiments that there was no difference in the association rate constants of the ouabain-ATPase complex of untreated cells and those grown in ouabain for 48 h. The overall mean rates of association in all experiments, averaged over untreated and ouabain-treated cells was $6.2 \pm 1.0 \times 10^7 \text{ h}^{-1} \text{ M}^{-1}$ ($n = 6$) in 0 mM potassium and $4.5 \pm 0.7 \times 10^6 \text{ h}^{-1} \text{ M}^{-1}$ ($n = 7$) in 5 mM potassium, a mean potassium effect of 14 times from 0 to 5 mM.

Dissociation rates

Ouabain bound to the pumps of HeLa cells is mainly internalised by the cells and then processed through a lysosomal com-

Figure 6 [^3H]-ouabain binding to HeLa cells grown in ouabain. The cells were grown for 3 days in normal medium and then for 2 days in medium containing 10 nM ouabain. [^3H]-ouabain binding was then measured as in the legend to Figure 5 both in potassium-free and in 5 mM potassium containing Krebs. In potassium-free Krebs ($\bullet$) $V_{\max}$ is $353 \pm 5 \times 10^3$ sites per cell and the rate constant is $0.159 \pm 0.01 \text{ min}^{-1}$, and in 5 mM potassium ($\blacksquare$) $V_{\max}$ is $385 \pm 118 \times 10^3$ and the rate constant is $0.008 \pm 0.003 \text{ min}^{-1}$. The lines drawn are best-fit lines to the rate equations (Enzfitter).

partment (Cook *et al.*, 1982). The main determinant of ouabain dissociation from the cells is thus by post plasma membrane events, so that ouabain cannot be used to measure a dissociation rate from the sodium pumps on the plasma membrane. Digoxin is handled by the cell via a similar route but at a much faster rate, for the lysosomal step does not impede the loss of digoxin from the cell (Griffiths *et al.*, 1983). In previous work we showed that the plasma membrane of HeLa cells loses ouabain and digoxin at the same rate (about $9\% \text{ h}^{-1}$; Algharably *et al.*, 1986; a result also confirmed in the present experiments), and that the rate of loss of digoxin from whole cells is the same as from the membrane; whereas ouabain is lost much more slowly from whole cells. The loss of digoxin from whole cells is therefore a measure of ($k_{-1} + k_{\text{in}}$).

In Figure 7 the loss of digoxin from untreated cells is compared with that from cells treated for 48 h with 10 nM ouabain; the rates of loss were 9.6 ± 0.9 and $12.2 \pm 0.9\% \text{ h}^{-1}$ respectively. We also checked if the degree of the digoxin block, used to measure the efflux, altered the rate of efflux, and so did a number of experiments varying the proportion of pumps blocked by digoxin. The results were: untreated cells 9.3 ± 0.8 ($n = 7$), chronic cells 11.8 ± 0.6 ($n = 4$), both with all pumps blocked by digoxin, and untreated cells with trace digoxin block (5–10%) $9.6 \pm 2.2\% \text{ h}^{-1}$ ($n = 5$). Although there is a suggestion that the cells adapt to chronic block, the difference is not statistically significant.

Apparent K'_D in short term experiments

The ratio of the (mean) dissociation and (individual) association rate constants measured in these short term experiments gives an apparent binding constant, K'_D , for ouabain in normal growth medium ($[\text{K}]_0$ 5 mM) of $24 \pm 4 \text{ nM}$ (s.e.mean; $n = 7$), a value some 9 times higher than the 2.6 nM found in equilibrium conditions: the value in 0 K^+ medium is $1.92 \pm 0.23 \text{ nM}$ ($n = 6$).

To try to obtain a direct measure of the binding constant we measured binding curves for [^3H]-ouabain with incubation periods of 0.25 to 2 h. Figure 8 shows a typical experiment in which cells were exposed to a range of ouabain concentrations for 0.5 h in K^+ -free medium. Half saturation of the pumps occurs at 34 nM. Increasing the time to 4 h only reduced the half block concentration to 18 nM, but the cells

Figure 7 Rate of loss of digoxin from untreated and ouabain-treated cells. Cells were grown for 3 days in normal growth medium and then either changed into a similar or 10 nM ouabain medium for a further 2 days. After rinsing the cells were then put into 200 nM [^3H]-digoxin in potassium-free Krebs for 15 min, then washed at 37°C for 15 min to remove the non-specifically bound digoxin and then returned to the efflux medium. At the times indicated, aliquot plates were removed for counting of cell numbers and activity. Control cells (■) had an initial digoxin binding of $465 \pm 13 \times 10^3$ sites per cell, and a rate of loss of digoxin of $9.6 \pm 0.9\% \text{ h}^{-1}$, and ouabain-treated cells (●) values of $265 \pm 6 \times 10^3$ sites per cell and a rate of loss of $12.2 \pm 0.9\% \text{ h}^{-1}$. Each point is the mean of 3 observations, and the lines were fitted to an exponential dissociation equation by Enzfitter.

were then not in a good condition. In a similar experiment in 5 mM potassium, half block occurred at 100 nM.

The results so far show that there is a large discrepancy between the K_D calculated from short term measurements and that found under equilibrium conditions in 5 mM potassium growth medium. We then extended these measurements over a range of $[K]_o$ values down to the lowest the cells would survive in, around 0.3 mM and so made a direct comparison in

Figure 8 [^3H]-ouabain binding to HeLa cells after 0.5 h incubation. The main figure shows the number of molecules bound for each ouabain concentration on a linear scale, and the inset the usual log dose-response curve. The V_{max} is $858 \pm 17 \times 10^3$ sites per cell and the K_m 34 ± 3 nM. Each point is a single value, and the Michaelis-Menten lines fitted by Enzfitter. The s.e.mean was less than 5% of the mean.

Figure 9 Effect of potassium on K_D determined after chronic or acute exposure to ouabain in HeLa cells. The ordinate scale shows the apparent K_D and the abscissa scale the potassium concentration in the growth or experimental medium. The data for chronic exposure to ouabain are taken from Figure 4, the acute results were obtained in the same experiment by measuring the initial rate of ouabain binding at potassium concentrations of 0.5, 1, 2.5 and 5 mM; the K_D was then calculated using the mean value of dissociation rate of $9\% \text{ h}^{-1}$. The lines are drawn by eye; (●) chronic exposure to ouabain; (■) acute exposure to ouabain. Also shown on the figure are single values from other chronic experiments (Δ), and other acute experiments ($\square$).

the same experiment of the effect of potassium on chronic and acute binding. Figure 9 shows this comparison between the apparent K_D found in 24 h chronic growth and that calculated from the initial rates of binding from 200 nM ouabain in the same potassium concentrations. It is clear that chronic growth binding decreases the apparent K_D by a similar factor (around 4) at all potassium concentrations measured. As this experiment was only carried on for 24 h a rather larger discrepancy would be expected after 48 h.

Effect of pump occupancy on potassium influx

A possible explanation for the present results of a discrepancy in sensitivity between long and short term growths in ouabain is that HeLa cells have two isoforms for the sodium pump of differing sensitivities. This seems unlikely because cDNA probes only show one major band ($\alpha 1$) on Northern blots (see Discussion). Other evidence on this point can be obtained by examining the effect of an increasing block of sodium pumps on the active potassium influx, for two different sensitivities should show up as a discontinuous curve. Figure 10 shows the effect of the % occupancy of the sodium pumps by these cardiac glycosides on the ouabain-sensitive potassium influx, both in the presence and absence of bumetanide. There is no obvious discontinuity in the potassium influx with increasing block of the pump, a result consistent with the presence of only one isoform. If co-transport is present low concentrations of glycosides appear to 'stimulate' the ouabain-sensitive potassium influx (really an effect on co-transport; see Brown & Lamb, 1978; Bakker-Grunwald *et al.*, 1982). This perhaps explains the periodic claim that low concentrations of cardiac glycosides have their therapeutic action by pump stimulation.

In small cells such as HeLa, with a high surface to volume ratio, the most sensitive indicator of pump activity are changes in cell sodium and potassium. Figure 11 shows results from two experiments on different batches of cells done at different times. Both show a similar pattern; the potassium concentration falls by almost a fifth at the lowest ouabain concentration used (0.3 nM) and then continues to fall as the ouabain concentration increases; the sodium concentration shows an initial variable change and then a progressive rise. In other, less complete experiments, we found that 10 nM ouabain significantly increased the sodium concentration whereas 2 nM ouabain had a variable effect.

Figure 10 Effect of pump occupancy by various glycosides on the ouabain-sensitive potassium influx. HeLa cells were grown normally for 3 days and then for a further 2 days in various concentrations of ouabain (●); dihydro-ouabain (○); digoxin (▲); digitoxin (△); or acetylthiocholine (■). The plates were rinsed three times; half were then used to measure unblocked pumps and the other half to measure the potassium influx. Potassium influx (estimated with trace amounts of ^{86}Rb in 5 mM potassium salt solution) was measured in cells with no further drugs added or with 1 mM ouabain or with 1 mM ouabain and 10 μM bumetanide; calculation then gave ouabain-sensitive + co-transport influx (a) or ouabain-sensitive influx (b). Each point is the mean of 3 measurements made separately but on the same batch of cells. The s.e.mean of the values around 100% was 10%, composed of an error due to the influx and binding measurements. As the variance was constant this was also the error of the lowest values shown.

Association and dissociation experiments using membranes absorbed onto beads

Membranes prepared as described under Methods were loaded for various times up to equilibrium in 200 nM [^3H]-

Figure 11 The effect of chronic ouabain treatment on cell sodium and potassium. Cells were grown normally for 3 days and then changed to medium containing the ouabain concentrations shown. Two days later the sodium and potassium contents were measured. In parallel plates cell numbers and water were measured. Two separate experiments on different batches of cells are shown. Each point was the mean of 4 replicate measurements; the variance was constant at 7.8 for sodium, and 22 for potassium; s.e.means are shown. The cell chloride in medium was 52 mM and in Krebs 74 mM; (●, ○), potassium; (■, □), sodium.

ouabain. At equilibrium the total binding was in the range of 22 to 32 pmol mg^{-1} protein, which corresponds to around 500,000 sodium pumps per cell; a normal value for intact cells. The ouabain dissociated from the membranes with a $t_{1/2}$ of around 3 h (a rate constant of $0.22 \pm 0.01 \text{ h}^{-1}$), a rate 2 to 3 times faster than from intact cells. In a single experiment in potassium-free conditions ouabain bound to the membranes with a $t_{1/2}$ of around 2 min (a rate constant of $0.37 \pm 0.10 \text{ min}^{-1}$). The calculated equilibrium binding constant from these values is 2.02 nM, a value similar to the 1.92 nM found in whole cells also in potassium-free conditions. These experiments suggest that the exchanges of ouabain between the medium and membranes on beads are somewhat faster than those with whole cells, but the K_D is of the same order. They do not explain the discrepancy between the short and long term effects of ouabain.

Discussion

The main point shown here is that equilibrium binding constants found by growth of cells in ouabain are much lower (by a factor of at least 4) than those expected from the ratios of the association and dissociation rate constants measured independently in short term experiments, either in intact cells or isolated membranes. This is different from the extensive study of the interaction of ouabain with Na^+ , K^+ -ATPase in turkey red cells (Furukawa *et al.*, 1980), which showed that the equilibrium dissociation constant (K_D) for the interaction predicted from the ratio of the dissociation and association rate constant agreed closely with independently measured values of K_D measured directly under equilibrium binding conditions. There are a number of differences between HeLa and turkey red cells, some of which might account for the observed difference; we have investigated a number of these differences.

(1) Long term growth in cardiac glycosides could have led to an alteration in the state of the cells, for as previously noted such growth greatly reduces cell division (Lamb & McCall, 1972) probably due to a reduction in protein synthesis. We find, however, that there is only a substantial effect on cell growth (probably via changes in cellular ions affecting cell division) at high levels of glycosides, so this cannot explain the effects at low glycoside concentrations. We think that this does affect the goodness of fit of the rectangular hyperbola at such high blocks; this may correspond to the toxic effects seen clinically.

Another effect of a rise in cell sodium following partial block of the pumps might be to increase the rate or magnitude of glycoside binding by increasing the fraction of time the pump was in the E2 state, as well as perhaps recruiting 'spare pumps'. This is unlikely, firstly because the rate of binding in fresh and ouabain-treated cells in these experiments was the same, and secondly we find that loading HeLa cells with sodium, by use of potassium-free medium, does not alter the sensitivity to ouabain (unpublished experiments).

(2) In short-term experiments, glycoside binding to the sodium pump in human, and some other cells is very sensitive to the potassium concentration in the medium, a finding confirmed in this study. However, it is not known if there is a quantitatively similar effect in longer term experiments such as those done here. If the potassium effect were much smaller, perhaps because ouabain binding could occur during the occasional backwards working of the pump which is known to occur normally, then this might explain our results. Our experiments show that although the potassium effect is perhaps somewhat smaller in long term experiments, the difference is less than a factor of two and so is unlikely to be the major explanation. This conclusion agrees with the clinical finding that low serum potassium potentiates glycoside effects.

(3) Internalisation of glycoside-pump complexes occurs in HeLa cells but not in red cells, and this could affect the process: (a) If each internalised pump is replaced by a new one then internalisation should decrease the affinity to cardiac gly-

cosides, rather than increase it, for it provides another way of replacing blocked pumps (Klausner *et al.*, 1985), although the effect should be quite small in HeLa cells with their slow rate of internalisation. (b) If some of the internalised pumps are not replaced by new ones then the total density of pumps (blocked + free) on the cell surface would be reduced, and this could provide an explanation for our observation. Pollack *et al.* (1981), however, showed that chronic growth marginally increases the density (by less than 20%), rather than decreases it, and we could find no change in the total number of membrane pumps on chronic growth (Owler, 1986). Our present results also show that the disappearance of blocked pumps equals the reappearance of fresh ones, implying a steady state of pump density. (c) Another possibility is that as internalisation probably involves various compartments, glycoside growth might alter rates between these so as to produce a false equilibrium constant. Mathematically we can find no way by which this can be made to alter the equilibrium values of blocked pumps, and so had to abandon this explanation.

For these various reasons we do not think that internalisation is a factor in the apparent increase in sensitivity to cardiac glycosides observed in prolonged growth.

(4) Another possible explanation for the present results would be for HeLa cells to have two isoforms for the sodium pump of differing sensitivities, and for the proportion of these isoforms expressed in the plasma membrane to differ between the acute and chronic treatment regimes. We investigated the following points in considering this explanation: (a) Northern blots of HeLa cell mRNA with a general α -cDNA probe (pHank) only show one major band (Cutler *et al.* 1988), indicating only one α -subunit, probably equivalent to $\alpha 1$ (Sweadner, 1989); (b) the association and dissociation rate constants of fresh cells and those treated chronically with ouabain are the same; (c) an increasing block of the sodium pumps with cardiac glycosides causes a continuous decrease in the active potassium influx, with no sign of the kind of discontinuity expected from two or more isoforms.

(5) The rectangular hyperbola is the appropriate representation of a reaction between one ligand and one receptor when equilibrium is reached. Before equilibrium is reached the curve is not of this form and values of rate constants obtained by fitting such a curve would be inaccurate. The magnitude of such errors, and the internal evidence for them, are explored in Appendix 1. They cannot explain our findings.

The temperature sensitivity of ouabain binding to HeLa cells measured at 2, 13 and 37°C gave an activation energy of 27.6 kcal mol⁻¹, a figure very similar to that obtained by Furukawa *et al.* (1980) for the binding of ouabain to turkey red blood cells, so that this aspect of our measurements agreed.

We think that the most likely explanation for the apparent difference in sensitivity of short and long term sensitivity to cardiac glycosides involves the following combination of events in cells grown chronically in cardiac glycosides: a slightly reduced sensitivity of cardiac glycoside binding to potassium, slower removal of glycosides from the membranes due

to raised cell sodium (Pollack *et al.*, 1981) and better access of cardiac glycosides to the sodium pump. This latter factor probably is largely responsible for underestimating the association rate constant in short term experiments and so underestimating the sensitivity.

A possible drawback to this latter explanation has been pointed out by Klausner *et al.* (1985) in a slightly different context. This is, that such diffusional slowing should also affect the rate of dissociation and so not have an effect on the equilibrium dissociation constant. We think that this does not apply to these cells, for we are measuring the sum of dissociation and internalisation and think that internalisation dominates the reaction. Indeed it may be that the diffusional delay impedes dissociation so much that the membrane is largely cleared by internalisation.

However, the cause of this delay has not been established in this work. The possibilities are either that all the pumps are partially hidden from the bathing medium, perhaps by the normal coating of molecules found on cells, or that a proportion of the pumps are not accessible to the bathing medium and so can only be reached rather slowly. We examine such models mathematically in Appendix 2 and show that if pumps cycle into and out of such an inaccessible region this would explain our results. Possible regions are the coated pits which exist on the cell surface of most cells.

Although the explanation for these results is not clear, it is obvious that the isoform present in these human cultured cells is very sensitive to cardiac glycosides when measured in normal growth medium containing 5 mM potassium under equilibrium conditions. As the apparent sensitivity of the HeLa Na⁺, K⁺-ATPase in short term experiments is similar to that of other types of human cells (heart cell membranes; Erdmann, 1981; leucocytes; Boon *et al.*, 1984); it may well be that the true sensitivity of these other cells is also some 4 to 10 times the apparent value. This sensitivity is such that if this isoform is present in human heart the normal plasma concentrations of cardiac glycosides of a few nM should give a substantial block of the sodium pumps. This finding also does have the consequence that it is not a straightforward matter to obtain a correct measure of the pharmacological properties of the various isoforms of the sodium pump in short term experiments for comparison with the other measures of these isoforms.

These results were obtained on cells in culture, so the question arises as to whether similar results would be obtained in animals *in vivo*. In dogs treated chronically with digoxin to test if the sodium pumps adapt to the continued presence of therapeutic doses, an incidental finding of Ku *et al.* (1977), not discussed in the paper, was that the sensitivity to chronic digoxin was some 2 to 15 times greater than to acutely applied digoxin. This suggests that cultured cells are a good model for the *in vivo* situation.

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Appendix 1

Chronic observations made before equilibrium is reached

The time dependent solution of the simple reaction equation, $S + R \overset{k_1}{\rightleftharpoons} \overset{k_{-1}}{SR}$, gives the proportion of pumps that are blocked after time *t* in concentration *s* of ouabain as

$$b(t) = \frac{s}{(k_{-1}/k_1 + s)} [1 - e^{-(k_1s + k_{-1})t}]$$

$$= B_{\text{equil}} [1 - e^{-(k_1s + k_{-1})t}]$$

As a function of *s*, this differs from the equilibrium equation because of the distorting factor

$$[1 - \exp^{-(k_1s + k_{-1})t}].$$

If the experimenter believes the reaction to be in equilibrium after time *t* and fits a rectangular hyperbola *Vs/(K_D + s)* to data obtained at that time from cells exposed to a range of concentrations *s*, the estimates *V* and *K_D* will be biased as estimates of the true *V* = *N* and *K_D* = *k₋₁/k₁*. Clearly the extent of the bias depends primarily on the value of *k₁t*, the dominant term in the exponent when *k₋₁* is small, as in these experiments.

The results of generating data by this equation and fitting a rectangular hyperbola are shown below, for a typical acute experiment, with five levels of *s*: 0, 1, 3, 9, 27 nM, the true *K_D* = 2.7 nM and the true *V* = 1000.

The shorter the duration of the experiment, the more will the *K_D* from a fitted rectangular hyperbola overestimate the true *K_D*. For the fitted value to be double the true value, *k₁t* has to be as low as 0.25 nm⁻¹ (e.g. *k₁* = 0.001 nm⁻¹ min⁻¹ after 4 h). The fact that the rectangular hyperbola is the wrong form of curve for the data will be difficult to infer from the residuals in the presence of typical experimental variation.

If equilibrium has not been reached within 24 h then the true *K_D* will be less than the estimate obtained from fitting a rectangular hyperbola to the 24 h data. However, with the true *k₁* = 0.001 nm⁻¹ min⁻¹, at 24 h *k₁t* = 1.44 nm⁻¹ and the

effect of stopping early on *K_D* or *V* can be seen from Table 1 to be trivial.

Appendix 2

Models with inaccessibility

The observation that the equilibrium *K_D* in chronic experiments is less than the ratio *k₋₁/k₁* of rate constants in acute experiments can be generated by the following mechanism.

Suppose that there is a continual movement of all pumps from an accessible state *A* to an inaccessible state *I* at rate *θ₁*, the reverse movement occurring at rate *θ₂*. Ouabain can be associated with, or dissociated from, a pump only while it is accessible, an inaccessible pump being locked in the state, free *F* or blocked *B*, it was in when it became inaccessible. One, but not the only, possibility is that a pump becomes inaccessible when it enters a coated pit. Although such pits are usually considered as the location of internalisation, no internalisation need be invoked to generate the required property. We describe three models differing in the behaviour of blocked pumps. They have particularly simple solutions if it is assumed that equilibrium has been reached between *A_F* and *I_F* before the cell is placed in the glycoside medium. First assume that transition rates between *A* and *I* are the same for free or blocked pumps.

The proportion of sites in the accessible state remains constant at *θ₂/(θ₁ + θ₂)*. Solution of the resulting differential equations gives the proportion of blocked pumps at time *t* as

$$\frac{k_1s}{(k_1s + k_{-1})} [1 - ce^{-\alpha_1t} - (1 - c)e^{-\alpha_2t}]$$

where *α₁*, *α₂* are the real, positive, unequal roots of the quadratic *x*² - (*θ₁* + *θ₂* + *k₁s* + *k₋₁*)*x* + *θ₂* (*k₁s* + *k₋₁*) = 0 and *c* = [*θ₂* (*k₁s* + *k₋₁*) - *α₂* (*θ₁* + *θ₂*)]/[(*α₁* - *α₂*)(*θ₁* + *θ₂*)].

Table 1

<i>k₁t</i>	<i>K_D</i>	<i>V</i>	Residual <i>s.s.</i>	Residuals about fitted hyperbola				
				0	1	3	9	27 nM
1.9	2.70	1000.2	0.03	0	-0.1	0.1	0	0
1.5	2.71	1000.3	0.56	0	-0.6	0.4	0.2	-0.2
1.1	2.74	1002.4	9.1	0	-2.3	1.5	0.8	-0.9
0.7	2.92	1011.8	122	0	-8.1	3.9	5.0	-4.1
0.3	4.38	1071.7	1160	0	-18.0	-4.8	25.0	-13.4
0.1	13.34	1292.5	220	0	-6.5	-8.6	9.7	-2.8

This has the same equilibrium relation (t large) as the simple exponential model

$$\frac{k_1 s}{(k_1 s + k_{-1})} [1 - e^{-(k_1 s + k_{-1})t}]$$

Moreover an acute experiment will be virtually unable to distinguish between the two models if c is close to zero or to 1. For example, with $(k_1 s + k_{-1}) = 0.2 \text{ min}^{-1}$, $\theta_1 = 0.1875$ and $\theta_2 = 0.1125$ so that 5/8 of pumps are inaccessible at any instant then $c = 0.9375$, $\alpha_1 = 0.05$, $\alpha_2 = 0.45$ and the mixture of exponentials is almost indistinguishable, on the basis of an acute experiment lasting up to 1 h, from a single exponential with rate constant of 0.058, a value that is roughly one-quarter of that (0.2) in the model with inaccessible states. The values in the example were selected to correspond to values in Klausner *et al.* (1985).

The second model postulates that blocked pumps cannot become inaccessible. The proportion of blocked pumps at time t is again a mixture of two exponentials

$$\frac{k_1 s}{[k_1 s + k_{-1}(1 + \theta_1/\theta_2)]} [1 - ce^{-\alpha_1 t} - (1 - c)e^{-\alpha_2 t}]$$

where α_1 , α_2 are the real, positive, unequal roots of the quadratic

$$x^2 - (\theta_1 + \theta_2 + k_1 s + k_{-1})x + [\theta_2(k_1 s + k_{-1}) + \theta_1 k_{-1}]$$

and

$$c = [(k_1 s - \alpha_2) + k_{-1}(1 + \theta_1/\theta_2)]/(\alpha_1 - \alpha_2).$$

Although, when the dissociation rate k_{-1} is small, the asymptote and the exponents differ little from the earlier model, the mixing constant c is different. With the values used above $c = 0.42$. Such an equal mixture of the two exponentials is more readily distinguishable from a single exponential.

Moreover, the best-fitting exponential has an estimated rate constant 0.16, not greatly different from the 0.2 used to generate the data. Thus this model does not generate patterns which match the experimental results described in the main paper.

A third model of this type postulates that inaccessible blocked pumps revert to accessibility only by losing their bound ligand:

One possible mechanism for this is that the inaccessible sites are internalised pumps, models for which are discussed in detail by Klausner *et al.* (1985). In this case radioligand binding will measure A_B only, not $(A_B + I_B)$ as assumed in the other models. In general the rates of internalisation may be different for unblocked and for blocked pumps, denoted θ_1 and α_1 respectively, and the transitions from I_F and I_B to A_F are likely to be replacement by new pumps, occurring at a constant rate β . However, in such a model the number of accessible pumps will vary with the degree of blocking: at equilibrium

$$A_F + A_B = \frac{\beta}{\theta_1} \left[1 - \frac{k_1 s(\alpha_1 - \theta_1)}{(\alpha_1 k_1 s + \alpha_1 \theta_1 + \theta_1 k_{-1})} \right]$$

The time-dependent behaviour of the proportion of blocked pumps is again a mixture of two exponentials which can have properties of either of the previous models according to the numerical values of the parameters.

The first and third mechanisms thus provide possible explanations of the difference between acute and chronic experiments.