

# Characterisation of Na/K-ATPase, its isoforms, and the inotropic response to ouabain in isolated failing human hearts

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**Objective:** The aim was to determine whether failing human hearts have increased sensitivity to the inotropic and toxic effects of ouabain, and to examine alterations in Na/K-ATPase that might explain the observed higher ouabain sensitivity. **Methods:** For contractility studies, a total of 57 trabeculae were isolated from two non-failing (death from head injury) and 10 terminally failing, explanted human hearts. After the experiment, each trabecula was inspected under the light microscope for morphological alterations consistent with heart failure. Samples for biochemical and molecular studies were obtained from five non-failing and 13 failing hearts. Total Na/K-ATPase was measured in desoxycholate treated homogenates and expressed per unit of tissue wet or dry weight, DNA, protein, or myosin. Interference from residual bound digoxin due to previous therapy was excluded. The expression of the three  $\alpha$  isoforms was studied at both the mRNA level using northern blots and the protein level by analysis of dissociation kinetics of the [ $^3$ H]ouabain-enzyme complex. **Results:** Trabeculae showing morphological alterations and decreased contractility were sensitive to lower concentrations of ouabain (3-100 nM) than control trabeculae (100-1000 nM); the inotropic  $EC_{50}$  and the minimum toxic concentration were both reduced. [ $^3$ H]Ouabain binding was significantly lower ( $p < 0.001$ ) in failing than in non-failing hearts, at  $293(SD 74) v 507(48) \text{ pmol}\cdot\text{g}^{-1}$  wet weight. No significant change was observed in maximum ATPase turnover rate, or in sensitivities to  $\text{Na}^+$ ,  $\text{K}^+$ , vanadate, and dihydro-ouabain. All three  $\alpha$  isoforms were expressed at the mRNA level in both normal and failing hearts. **Conclusions:** This study shows conclusively, for the first time, that failing human hearts are more sensitive to ouabain. This may be at least partly due to a mean reduction of 42% (95% confidence interval, 26 to 56%) in the concentration of Na/K-ATPase (decrease in Na,K pump reserve), but not to an alteration in its catalytic properties or in its isoform composition.

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Cardiac glycosides have been widely used in the treatment of congestive heart failure. Their basic mechanism of action consists in inhibiting Na/K-ATPase, which leads to a rise in intracellular free  $\text{Na}^+$  concentration and, through alteration of Na/Ca exchange, to the enhancement of  $\text{Ca}^{2+}$  transients and of cardiac contraction.<sup>1</sup> However, excessive inhibition induces  $\text{Ca}^{2+}$  overload, causing arrhythmia, a decrease in developed tension, and contracture.<sup>2</sup> Although it is known that patients with advanced heart disease are more susceptible to glycoside induced arrhythmias,<sup>3</sup> the direct effect of the drugs on the diseased myocardium is still poorly documented. While one study has shown that papillary muscle strips isolated from terminally failing hearts responded to the inotropic action of ouabain at lower concentrations than strips from non-failing hearts, the interpretation of this difference was complicated by the fact that the inotropic action showed an all or nothing effect in non-failing hearts instead of a typical concentration dependent response, and also by the absence of arrhythmia data.<sup>4</sup> In biochemical studies, it is still uncertain for methodological and statistical reasons whether the density of the glycoside receptor, the Na/K-ATPase, on the cardiac cell membrane decreases, and if so to what extent.<sup>5</sup> (This point

will be discussed in detail below.) Furthermore, the catalytic properties of Na/K-ATPase have not yet been studied in diseased human hearts, and although the expression of three  $\alpha$  isoforms at the mRNA level has been reported,<sup>6,7</sup> no information is available at the protein level.

In the present study on Na/K-ATPase, contractility experiments revealed a population of failing heart trabeculae with increased sensitivity to the inotropic and toxic effects of ouabain. In these experiments, concentration dependent inotropic effects were obtained with both failing and non-failing hearts, allowing quantitative comparison. The recent availability of relatively large samples of human heart from patients undergoing cardiac transplantation has made it possible to undertake a comprehensive study of Na/K-ATPase properties. Thus we decided to test different hypotheses that could account for the increased ouabain sensitivity: (1) a switch in isoform gene expression, (2) a decreased density of ouabain binding sites, (3) reduced Na/K-ATPase turnover rate, and (4) reduced  $\text{Na}^+$  sensitivity. For this study to be valid, it was critical to demonstrate the complete recovery of Na/K-ATPase from tissue samples, the independence of the results from the reference used to express the data (for example, tissue wet weight), and the

absence of interference from residual bound digoxin resulting from drug therapy prior to surgery. Some of the discrepancies reported in previous studies of Na/K-ATPase in human heart may be explained by the fact that these variables were not considered, as well as by the differences in the methods used. The present results support the hypothesis that a decrease in Na/K-ATPase density underlies the greater sensitivity of severely failing hearts to cardiac glycosides. These investigations were approved by the local ethics review committees. Preliminary results have been reported.<sup>8,9</sup>

### Methods

#### Contractility study

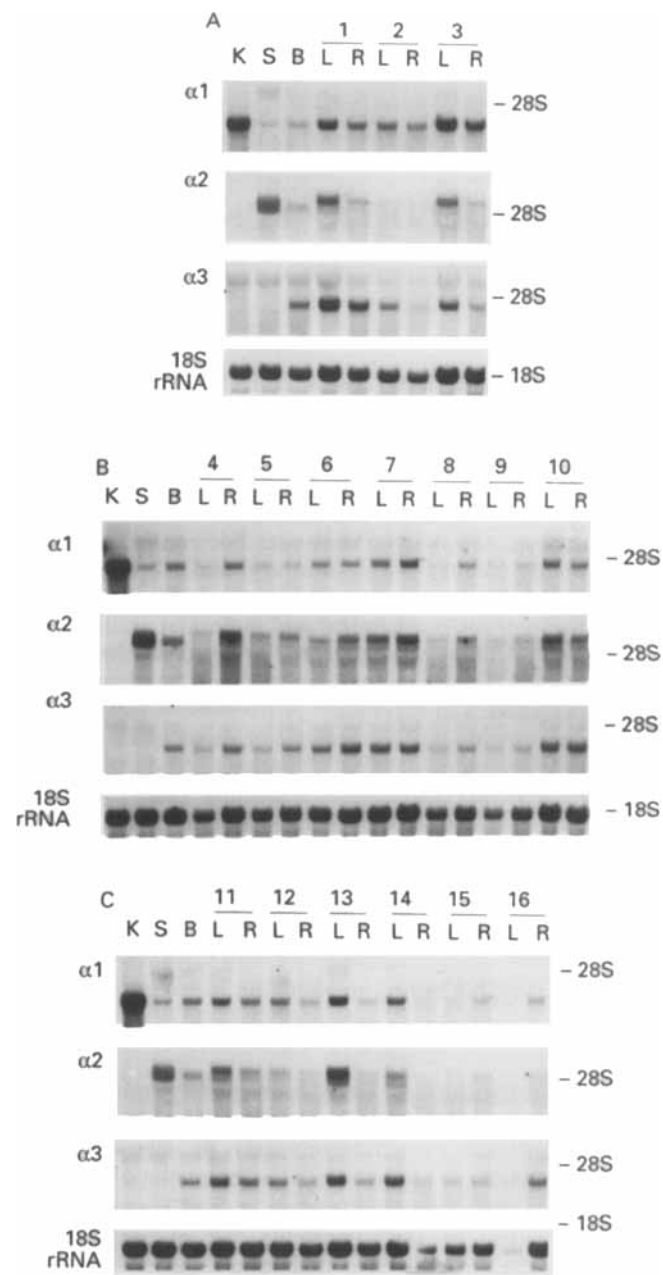
Trabeculae were taken from explanted hearts obtained in collaboration with the University of Cincinnati cardiac transplantation programme. Failing hearts carried the general pathological diagnoses of ischaemic, hypertrophic, or dilated cardiomyopathy. Non-failing hearts (death from head injury) could not be implanted for technical reasons. The hearts were placed in ice cold Krebs-Henseleit solution in the operating theatre immediately after removal. The mural trabeculae were excised from the walls of both right and left atria and ventricles and stored for a short time on ice before being mounted and suspended in a 70 ml assay bath at 35°C. We chose trabeculae 1 mm wide and 5 mm long. No Purkinje fibres or visibly necrotic fibres were used. Contractility measurements were performed as previously described.<sup>10</sup> Briefly, the trabeculae were placed in a muscle holder in contact with two pointed platinum electrodes used for stimulation, connected to a force transducer (Grass FT 03C), and studied under isometric conditions: initially they were stretched to a length at which force was maximal ( $L_{max}$ ). The stimulation rate was 1.0 Hz, 2 to 5 ms duration, with a voltage 20% above threshold. Contractile force and its first derivative  $dF/dt$  were recorded on a Grass P7 polygraph. After equilibration for 60 min, cumulative ouabain dose-response curves were obtained by adding ouabain in increasing concentrations from 1 to 1000 nM every 40-60 min. The duration of exposure was long enough to bring the trabeculae close to their maximum response at each concentration. When clear signs of toxicity became apparent (increase in resting tension, decrease in active tension, irregularities in contractility and/or contracture) the experiment was terminated.

#### Tissue acquisition for northern analysis and biochemical studies

Besides the heart samples used above for contractility studies, samples from additional hearts were procured over a later time period for RNA isolation and biochemical studies. Samples from three failing and 13 non-failing hearts were collected in conjunction with the University of Cincinnati cardiac transplantation programme. Samples from two normal hearts that were used for biochemical studies only were supplied by the International Institute for the Advancement of Medicine (Essington, PA, USA). The material included end stage failing hearts from patients with diagnoses of ischaemic, idiopathic, idiopathic dilated, dilated, hypertensive, or hypertrophic cardiomyopathy, and non-failing hearts (death from head injury) that could not be implanted for technical reasons. All patients with heart failure had undergone left and right heart catheterisation, echocardiography, and multiple gated nuclear angiographic scanning. Mean right atrial pressure and mean pulmonary arterial pressure were recorded from fluid filled pressure transducers zeroed at mid-axillary level in the supine position. Cardiac output was determined by thermodilution, using right atrial injection and a pulmonary arterial catheter tip thermistor. Left ventricular ejection fraction was determined from the ratio of radioisotopic activity at end systole to that in end diastole in the left ventricular profile at nuclear angiography. Clinical data are summarised in table II. The principal therapy prior to cardiac transplantation had been diuretics and converting enzyme inhibitors; five patients had also been receiving digoxin. On explantation, samples from each heart were immediately frozen in liquid nitrogen and stored at -70°C.

#### Northern analysis

Total cellular RNA was isolated from tissue samples and northern blot analyses carried out as described.<sup>6,11</sup> Data obtained from three non-failing hearts in the present study have already been reported<sup>9</sup> and are shown for comparison only (fig 1A). Briefly, 10 µg of total RNA was isolated and fractionated on agarose gels and transferred to nylon membranes (Magna NT, MSI). Total RNA from both the left and right ventricles of 13 diseased hearts was used to make two sets of three identical blots. Each set was hybridised with  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  specific 60mer oligonucleotide probes (described in detail in<sup>6</sup>) and end labelled to a uniform specific activity with ( $\gamma^{32}P$ )-ATP (3000 Ci-mmol<sup>-1</sup>, New England Nuclear). Northern blots were exposed to x ray film for autoradiography and to a Phosphor screen for quantification with a



**Figure 1** Northern blot analysis of Na/K-ATPase  $\alpha$  isoform mRNA expression in human heart. Each lane contains 10 µg of total cellular RNA isolated from human control tissues (kidney (K), skeletal muscle (S), and brain (B)) and from left and right ventricular free wall (L and R, respectively) from three non-failing human hearts (panel A, hearts 1, 2, and 3)<sup>9</sup> and from 13 diseased hearts (panels B and C, hearts 4-16). The classification of the diseased hearts according the type of cardiomyopathy was as follows: five ischaemic (hearts 5, 7, 9, 10, and 15), three idiopathic (hearts 8, 13, 16), one dilated (heart 4), two idiopathic dilated (hearts 11, 12), one hypertensive (heart 14), and one hypertrophic (heart 6). Blots were probed with  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  isoform-specific <sup>32</sup>P labelled probes and exposed to x ray film for five days, then stripped and reprobed with a <sup>32</sup>P labelled 18S rRNA probe, which showed that some samples were more degraded than others.

PhosphorImager (Molecular Dynamics), then stripped and reprobed with a <sup>32</sup>P labelled 18S rRNA 20mer. As controls, 10 µg of total RNA from human kidney (expresses  $\alpha 1$ ), skeletal muscle (expresses  $\alpha 1$  and  $\alpha 2$ ), and brain (expresses  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) were included on each blot.

#### Tissue homogenisation and detergent treatment

Pieces of frozen tissue were rapidly thawed in saline. Fat, vessels, and connective tissue were removed as well as possible with scissors. Unless otherwise stated, the following procedure was performed at

0–6°C. A sample weighing 0.5–2 g was minced and homogenised in 10 ml of chilled buffer per g wet weight using a Polytron homogeniser, 2 s at maximum setting, twice (buffer composition: 2 mM EDTA and 10 mM Tris, pH adjusted to 7.4 with HCl). The homogenate was diluted to 30 ml with buffer and centrifuged at 100 000 g for 30 min. The pellet was homogenised in 6.5 ml buffer per g sample, with a Teflon/glass Potter Elvehjem homogeniser. Aliquots of these two homogenates were pooled and used for determinations of DNA, total protein, myosin, and creatine phosphokinase (CPK). The second homogenate was mixed with 3.5 ml (per g sample) of buffer supplemented with 2.86 mg·ml<sup>-1</sup> sodium desoxycholate (DOC). After 30 min incubation at room temperature, this mixture was frozen and kept overnight at –30°C. This treatment, used to permeabilise vesicles, revealed maximum latent Na/K-ATPase activity (data not shown). After thawing, the mixture was centrifuged at 100 000 g for 2 h. The pellet was resuspended in 10 ml of buffer per g sample (buffer composition: 10 mM Tris, pH adjusted to 7.4 with HCl) and kept at –70°C until use. Na/K-ATPase activity was stable for more than a year under these conditions. Four samples were processed per experimental run. No significant activity was found in the supernatants, indicating virtually complete recovery of the Na/K-ATPase in the final suspension. In the following procedures, homogenates were stirred with vortex immediately before pipetting. Occasionally, minute fragments of non-homogenised tissue blocked the pipette tip and had to be removed. To avoid loss of material, no further attempt was made to remove unhomogenised tissue (for example, by low speed centrifugation or filtration), since we had observed that these procedures affected Na/K-ATPase recovery differently in normal and diseased hearts. For all samples, Na/K-ATPase activity and [<sup>3</sup>H]ouabain binding were assayed in two to three separate experiments.

#### Na/K-ATPase assay

Na/K-ATPase activity was determined by measuring inorganic phosphate (Pi) release from the ouabain sensitive hydrolysis of ATP by DOC treated homogenates as described.<sup>12</sup> Protein (10–12 µg) was incubated for 60 min at 37°C in 0.1 ml medium containing (in mmol·litre<sup>-1</sup>) ATP 5, MgCl<sub>2</sub> 5, NaN<sub>3</sub> 5, NaCl 145, KCl 20, EGTA 1, and maleic acid 10 (pH adjusted to 7.4 with Tris). ATP hydrolysis never exceeded 20%. Basal activity was determined in the presence of 1 mM ouabain and represented about one third of total activity. Na/K-ATPase activity was linear with respect to time and protein concentration for all samples. Enzyme activation by Na<sup>+</sup> and K<sup>+</sup> was studied, according to the method of Skou,<sup>13</sup> at a constant ionic strength by maintaining the total [Na<sup>+</sup> + K<sup>+</sup>] = 150 mM while the Na<sup>+</sup> and K<sup>+</sup> concentrations were varied. Na<sup>+</sup> was varied by increasing NaCl from 0 to 145 mM, while NaN<sub>3</sub> remained constant at 5 mM. Maximum turnover rate was calculated from the ratio of maximum activity (V<sub>max</sub>) to maximum [<sup>3</sup>H]ouabain binding (B<sub>max</sub>), assuming one ouabain binding site per catalytic subunit. Dihydro-ouabain inhibition curves were plotted at 5 mM KCl. This glycoside was used instead of ouabain or digoxin because it binds quickly to Na/K-ATPase.

#### [<sup>3</sup>H]Ouabain binding assay

[<sup>3</sup>H]Ouabain binding to DOC treated homogenates was assayed by a filtration technique as described.<sup>14</sup> Briefly, 2.5–3 mg protein was incubated at 37°C in 5 ml medium containing (in mmol·litre<sup>-1</sup>): MgCl<sub>2</sub> 3, phosphoric acid 3, EGTA 1, and maleic acid 10 (pH adjusted to 7.4 with Tris), plus 1 µM [<sup>3</sup>H]ouabain (3–6 Ci·mmol<sup>-1</sup>, Amersham batch diluted with ouabain). Under these conditions, complete saturation of ouabain receptors was obtained (K<sub>D</sub> < 0.01 µM) and the specific binding represents B<sub>max</sub>. After 10 min incubation, 0.2 ml aliquots were filtered through Whatmann GF/F glass fibre filters previously soaked in 0.3% polyethylenimine. Non-specific binding was determined in the presence of 0.1 mM ouabain and did not exceed 10% of total binding. For [<sup>3</sup>H]ouabain dissociation kinetics, 0.1 mM ouabain was added after the 10 min incubation, and 0.2 ml aliquots were rapidly filtered at various times.

#### Other biochemical assays

For DNA and creatine phosphokinase (CPK) determinations, sample aliquots were diluted in appropriate buffers and centrifuged for 2 min in an Eppendorf centrifuge. DNA was determined as described<sup>15</sup> and CPK assayed using Boehringer Mannheim kits at 22–23°C. For total protein and myosin determinations, proteins were extracted with 2% SDS. Total protein was estimated by the bicinchoninic acid method<sup>16</sup> (reagents from Pierce, Rockford, Illinois, USA) and myosin levels were determined by densitometry after SDS electrophoresis according to Laemmli.<sup>17</sup> Tissue dry weight was measured after freeze drying 0.2 ml of homogenate.

#### Statistical analysis

Clinical data were disclosed at the end of the study. The significance of observed differences between failing and non-failing hearts (unpaired) and between left and right ventricles (paired) was verified using appropriate *t* tests. *F* tests were used to check for differences linked with the aetiology of the disease (multiple groups). Differences

were accepted as significant at the *p* < 0.05 level. Unless otherwise indicated, data represent means(SD). In figs 3 and 4, SEM is shown instead of SD to make the graphs easier to read.

#### Curve fitting analysis

Lindenmayer and Schwartz have shown that Na/K-ATPase activation by Na<sup>+</sup> and K<sup>+</sup> followed the relationship  $y = V_{max}/(a \cdot b \cdot c)$ , where the term *a* described the activation for three equivalent Na<sup>+</sup> sites and the terms *b* and *c* the activation for two non-equivalent K<sup>+</sup> sites.<sup>18</sup> Since the resolution of this equation requires a large number of experiments, to evaluate the two cations parsimoniously we replaced *a* and *b*·*c* by the logistic relationship, which is equivalent to the equation of Hill,<sup>19</sup> leading to

$$y = A + \frac{D}{\left[1 + \left(\frac{C_{Na}}{x}\right)^{B_{Na}}\right] \left[1 + \left(\frac{C_K}{150-x}\right)^{B_K}\right]}$$

where *y* is the predicted effect, *A* the basal ATPase activity, B<sub>Na</sub> and B<sub>K</sub> the Hill coefficients, C<sub>Na</sub> and C<sub>K</sub> the concentrations for half maximum activity, *D* the maximum activity (V<sub>max</sub>), *x* the variable Na<sup>+</sup> concentration, and 150–*x* the variable K<sup>+</sup> concentration. V<sub>max</sub> was 20% higher than the optimal activity measured at 130 mM Na<sup>+</sup> and 20 mM K<sup>+</sup>. The data fitted to this empirical function over a wide range of cation concentrations (fig 3). Note that although the above equation applies to the case where Na<sup>+</sup> and K<sup>+</sup> bind simultaneously, it does not rule out a sequential mechanism.<sup>18</sup>

Na/K-ATPase inhibition curves were fitted to the logistic function<sup>19</sup>

$$y = A + \frac{D}{1 + \left(\frac{C}{x}\right)^{-B}}$$

where *y* is the predicted effect, *A* the basal ATPase activity, *B* the Hill coefficient, *C* the concentration for half maximum inhibition, *D* the control specific activity, and *x* the variable concentration.

[<sup>3</sup>H]Ouabain dissociation kinetics were fitted to the one site function<sup>20</sup>  $y = A + D_1 e^{-k_1 x}$  or to the function for two independent sites<sup>20</sup>  $y = A + D_1 e^{-k_1 x} + D_2 e^{-k_2 x}$  where *y* is the predicted effect, *A* the non-specific binding, *k*<sub>1</sub> and *k*<sub>2</sub> the first order dissociation rate constants, *D* the binding at 0 time, and *x* the variable time.

For statistical analysis, curves were fitted by using individual sample or individual heart data whenever possible, and mean values and SD were calculated for groups of hearts. For [<sup>3</sup>H]ouabain kinetics, two site fit could not be performed with some samples because the binding was too low. In these cases, curves were fitted by using mean values of groups of hearts.

## Results

### Contractility

In table I the inotropic responses to ouabain in trabeculae from non-failing and failing hearts are listed. The data from atrial and ventricular trabeculae were pooled, since there was no statistical difference between the chambers. The distinction between moderately and severely altered trabeculae was made after the experiment, using a light microscope to evaluate the state of the myofibrils in the contraction bands, endocardial thickening, vacuolisation, scar formation, interstitial oedema, and mitochondrial and nuclear damage. Seventeen trabeculae from seven explanted hearts were rated as moderately altered, and nine trabeculae from four hearts as severely altered. It is clear that minimal inotropic effects of ouabain occurred at 3 and 30 nM in the severely and moderately altered fibres compared to 100 nM in the normal ones. The EC<sub>50</sub> and the minimal ouabain toxicity showed a similar relationship. In addition, the severely altered fibres responded with a significantly reduced maximum effect to both calcium (not shown) and ouabain. Trabeculae from two non-failing hearts served for comparison.

### Na/K-ATPase $\alpha$ isoform mRNA expression

We have previously shown that all three  $\alpha$  isoforms of Na/K-ATPase mRNAs are expressed in normal human left and right ventricle (LV and RV, respectively) at high levels.<sup>6</sup> Northern blots of this result (fig 1A) are shown in this

**Table 1** Ouabain inotropism in human heart trabeculae. Values in parentheses are SD of 9-18 trabeculae.

|   | Non-failing hearts (n = 2) | Terminally failing hearts (n = 10) |                               |                             |
|---|----------------------------|------------------------------------|-------------------------------|-----------------------------|
|   |                            | Apparently normal trabeculae       | Moderately altered trabeculae | Severely altered trabeculae |
| Number of trabeculae                      |                            |                                    |                               |                             |
| atrial                                    | 6                          | 2                                  | 8                             | 3                           |
| ventricular                               | 12                         | 11                                 | 9                             | 6                           |
| total                                     | 18 (2 hearts)              | 13 (5 hearts)                      | 17 (7 hearts)                 | 9 (4 hearts)                |
| Developed tension (mg)                    |                            |                                    |                               |                             |
| at control                                | 665(140)                   | 691(133)                           | 568(120)                      | 554(129)                    |
| at ouabain maximum inotropy               | 1800(513)                  | 1736(497)                          | 1425(416)                     | 732(273)                    |
| Ouabain inotropic response (% of maximum) |                            |                                    |                               |                             |
| Concentration (nM):                       |                            |                                    |                               |                             |
| 1   | 0                          | 0                                  | 0                             | 2                           |
| 3   | 0                          | 0                                  | 1                             | 12                          |
| 10  | 1                          | 1                                  | 2                             | 25                          |
| 30  | 4                          | 5                                  | 32                            | 60                          |
| 100                                       | 29                         | 24                                 | 92                            | 100                         |
| 300                                       | 96                         | 100                                | 100                           | —                           |
| 1000                                      | 100                        | 98                                 | —                             | —                           |
| EC <sub>50</sub> (nM)                     | 122(26)                    | 137(22)                            | 60(22)                        | 34(14)                      |
| Minimum toxic concentration (nM)          | 1077(86)                   | 1008(96)                           | 376(148)                      | 144(50)                     |

Data from atrial and ventricular trabeculae were pooled. Trabeculae from failing hearts were classified in three groups according to the degree of morphological alteration determined by microscopic inspection (see text).

manuscript to allow comparison of these data to the diseased heart expression pattern. Northern blots of left and right ventricle samples from 13 transplant recipient hearts show that mRNAs for all three  $\alpha$  isoforms are expressed in diseased human heart (fig 1B and C, hearts 4-16). The quality of the RNA varied considerably from sample to sample, as could be seen on ethidium bromide stained check gels (data not shown) and from the variability in the 18S rRNA signals (that is, heart 9 (LV), heart 14 (RV), and heart 16 (LV)).

For each heart, the contribution of each individual  $\alpha$  isoform to the total  $\alpha$  isoform mRNA pool was calculated by dividing the normalised (to 18S rRNA) signal for each isoform by the sum of the normalised signals for all three. The mean and standard deviation for left and right ventricle normal heart samples (n=6) and diseased heart samples (n=22, four samples were too degraded for quantification) were calculated for comparison purposes. For the normal hearts, the results for  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  isoform mRNAs were 48(SD 21)%, 26(13)%, and 27(10)% of the total  $\alpha$  isoform pool, respectively. For diseased hearts, the results for  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  mRNAs were 18(6)%, 30(12)%, and 52(12)%, respectively. From these results, it appears that in normal heart the  $\alpha 1$  mRNA represents about half the pool, whereas in diseased heart  $\alpha 3$  represents half the pool. However, these data must be interpreted with caution due to the small sample size for the normal hearts as well as the large variability between samples. The large variability may reflect heart to heart variation, regional variation within one heart, preferential degradation of one or more of the isoforms, or a combination of these factors. Due to the variable levels of degradation, absolute levels of expression between the different hearts were not compared.

#### Na/K-ATPase quantification

Samples were obtained from various parts of the left and right ventricle free wall and from the septum (total numbers = 27, 20, and 10, respectively; 1-6 samples per heart). Their numbers and locations were not predetermined, raising the question whether the data were truly representative of the hearts. However, differences between left and right ventricles and septum were not statistically significant. (This is in agreement with animal studies.<sup>21</sup>)

Furthermore, the within heart variation did not affect the conclusions regarding the effect of disease on Na/K-ATPase. Therefore for the sake of simplicity mean values were calculated from the different samples, reducing the data to one observation per heart. Expressed per g wet weight, [<sup>3</sup>H]ouabain binding decreased from 507(48) pmol in non-failing to 293(74) in failing hearts (-42%, p<<0.001). The disparity was not related to age, which ranged from 1.5 to 60 years (table II). Although a steep decrease in ouabain binding during the first six months of life has been reported, we did not test infant hearts.<sup>22</sup> When hearts were grouped according to aetiology, an analysis of variance showed that the variability was dependent on the type of cardiomyopathy (p=0.006). Table III shows that the effect of disease was also highly significant when binding data were expressed per tissue dry weight, DNA, myosin, or total protein, instead of tissue wet weight. Thus we could reasonably exclude the involvement of tissue swelling or necrosis in this effect. Na/K-ATPase activity correlated closely with [<sup>3</sup>H]ouabain

**Table II** Clinical data for the hearts used in biochemical study.

| No <sup>a</sup> | Diag | Age | Sex | CO  | LVEF | RAP | PAP | OBG |
|-----------------|------|-----|-----|-----|------|-----|-----|-----|
| —               | NF   | 2   | M   | NA  | NA   | NA  | NA  | 526 |
| 3               | NF   | 15  | F   | NA  | NA   | NA  | NA  | 511 |
| 2               | NF   | 27  | M   | NA  | NA   | NA  | NA  | 446 |
| 1               | NF   | 39  | F   | NA  | NA   | NA  | NA  | 572 |
| —               | NF   | 60  | F   | NA  | NA   | NA  | NA  | 478 |
| 15              | IS   | 41  | M   | 5.7 | 14   | 2   | 25  | 301 |
| 7*              | IS   | 48  | M   | 6.0 | 13   | 12  | 42  | 260 |
| 9               | IS   | 52  | M   | 3.4 | 16   | 7   | 35  | 184 |
| 10              | IS   | 54  | M   | 4.1 | 17   | 8   | 27  | 308 |
| 5               | IS   | 58  | M   | 3.5 | 9    | 18  | 36  | 290 |
| 16*             | ID   | 34  | M   | 6.8 | 16   | 3   | 26  | 320 |
| 13              | ID   | 38  | M   | 2.9 | 23   | NA  | 42  | 330 |
| 8               | ID   | 47  | M   | 3.7 | 20   | 10  | 35  | 358 |
| 4*              | D    | 44  | M   | NA  | 20   | 12  | 45  | 289 |
| 11              | DID  | 20  | M   | 4.4 | 15   | 6   | 31  | 217 |
| 12*             | DID  | 54  | F   | 2.8 | 17   | 10  | 36  | 222 |
| 6               | H    | 32  | M   | 3.9 | 21   | 8   | 31  | 477 |
| 14*             | HT   | 39  | M   | 2.7 | 7    | 20  | 50  | 246 |

<sup>a</sup> refers to heart numbers used in fig 1.

\* Indicates previous digitalis therapy.

CO = cardiac output (litres·min<sup>-1</sup>); LVEF = left ventricular ejection fraction (%); RAP = right atrial pressure (mm Hg); PAP = pulmonary artery pressure (mm Hg); OBG = [<sup>3</sup>H]ouabain binding (pmol·g<sup>-1</sup> wet weight); NA = no available data; DIAG = cardiomyopathy type; NF = non-failing; IS = ischaemic; ID = idiopathic; D = dilated; DID = dilated idiopathic; H = hypertrophic; HT = hypertensive.

**Table III** Na/K-ATPase activity and other biochemical variables in human heart samples. Values are mean(SD), except those indicated by an asterisk, which are median (IQR).

|  | Non-failing hearts<br>(n = 5) | Failing hearts<br>(n = 13) | p       | Decrease<br>(%) |
|--|-------------------------------|----------------------------|---------|-----------------|
| Tissue dry weight (mg·g <sup>-1</sup> w wt)              | 251(37)                       | 255(33)                    | 0.83    |                 |
| DNA (mg·g <sup>-1</sup> w wt)                            | 2.32(0.64)                    | 2.03(0.17)                 | 0.13    | 12              |
| Protein (mg·g <sup>-1</sup> w wt)                        | 119(5)                        | 106(13)                    | 0.05    | 11              |
| Myosin (mg·g <sup>-1</sup> w wt)                         | 19.7(4.8)                     | 17.9(3.8)                  | 0.41    | 9               |
| Creatine phosphokinase (IU·g <sup>-1</sup> )             |                               |                            |         |                 |
| per g dwt  | 2.93(0.85)                    | 2.05(0.33)                 | 0.009   | 30              |
| per g w wt   | 722(177)                      | 550(122)                   | 0.03    | 24              |
| per mg DNA   | 338(130)                      | 274(65)                    | 0.18    | 19              |
| per mg protein   | 6.1(1.7)                      | 5.3(1.4)                   | 0.30    | 13              |
| per mg myosin  | 37(6)                         | 33(12)                     | 0.44    | 11              |
| Na/K-ATPase activity (μmol Pi·min <sup>-1</sup> )        |                               |                            |         |                 |
| per g dwt  | 12.4(2.0)                     | 6.6(1.9)                   | <<0.001 | 47              |
| per g w wt*  | 3.06(0.33)                    | 1.64(0.37)                 | <<0.001 | 46              |
| per mg DNA   | 1.41(0.40)                    | 0.84(0.26)                 | 0.003   | 40              |
| per mg protein*  | 0.0252(0.0021)                | 0.0149(0.0037)             | 0.002   | 41              |
| per mg myosin*   | 0.156(0.025)                  | 0.088(0.033)               | 0.006   | 44              |
| [ <sup>3</sup> H]Ouabain binding B <sub>max</sub> (pmol) |                               |                            |         |                 |
| per g dwt  | 2050(320)                     | 1140(270)                  | <<0.001 | 44              |
| per g w wt   | 507(48)                       | 293(74)                    | <<0.001 | 42              |
| per mg DNA   | 232(61)                       | 146(37)                    | 0.002   | 37              |
| per mg protein   | 4.37(0.66)                    | 2.79(0.80)                 | 0.001   | 36              |
| per mg myosin  | 26.5(4.5)                     | 17.2(6.5)                  | 0.01    | 35              |
| Turnover rate (s <sup>-1</sup> )                         | 121(7)                        | 117(19)                    | 0.54    | 3               |

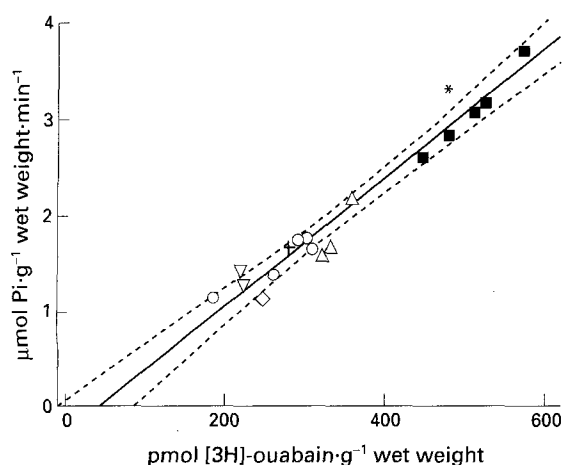
Na/K-ATPase activity was determined at 150 mM Na<sup>+</sup> and 20 mM K<sup>+</sup>.

Turnover rate = Na/K-ATPase activity/[<sup>3</sup>H]ouabain B<sub>max</sub>; w wt = tissue sample wet weight; dwt = dry weight.

binding (fig 2), indicating that the apparent concentration of Na/K-ATPase was altered by disease, but not its molecular turnover rate. For comparison, creatine kinase activities are also reported in table III. A statistically significant decrease of this enzyme was observed in failing hearts only when tissue wet or dry weight was used as reference.

#### Potential interference from digoxin therapy

In hearts from patients previously treated with digoxin, the drug may have remained bound to Na/K-ATPase and interfered with the determinations. To check for such interference, we measured 1 μM [<sup>3</sup>H]ouabain binding at two incubation times: 5 min (sufficient for complete saturation of Na/K-ATPase with [<sup>3</sup>H]ouabain) and 4 h (sufficient for virtually complete dissociation of digoxin binding). Thus if



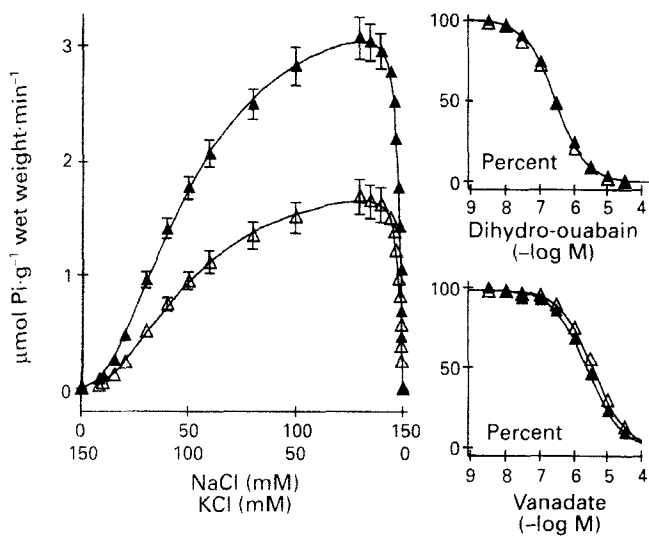
**Figure 2** Relation between Na/K-ATPase activity (vertical axis) and [<sup>3</sup>H]ouabain binding in non-failing (filled squares), ischaemic (circles), idiopathic (triangles), idiopathic-dilated (inverted triangles), hypertensive (diamond), hypertrophic (\*), and dilated (+) human hearts. Na/K-ATPase activity was determined at 150 mM Na<sup>+</sup> and 20 mM K<sup>+</sup>. The symbols represent individual hearts. The dotted lines represent 95% confidence limits.

digoxin was bound to Na/K-ATPase, [<sup>3</sup>H]ouabain binding should have increased after the longer incubation. We observed no difference between the binding data after 5 min and 4 h in any of the 18 hearts used in this study, irrespective of whether digoxin had been prescribed before surgery (data not shown).

We further checked whether digoxin could have been released from Na/K-ATPase during the membrane preparation as a result of homogenisation in hypotonic buffer, ultracentrifugation, and/or treatment of the homogenate with detergent. To estimate this effect, control homogenates were incubated with 1 μM digoxin for 5 min in the presence of MgCl<sub>2</sub> and inorganic phosphate (Pi) (this is sufficient for complete saturation of Na/K-ATPase with digoxin), submitted to ultracentrifugation, and treated with DOC exactly as described in Methods. These homogenates were then challenged with 1 μM [<sup>3</sup>H]ouabain for 5 min. Digoxin pretreatment inhibited 60% of the specific [<sup>3</sup>H]ouabain binding, indicating that DOC treatment results in the release of 40% of the bound digoxin. After a 4 h incubation, digoxin pretreatment had no effect on [<sup>3</sup>H]ouabain binding, indicating complete release of the drug. Thus in samples from digoxin treated patients part of the specifically bound digoxin gets washed out during sample preparation.

#### Kinetics of Na/K-ATPase activation and [<sup>3</sup>H]ouabain binding

No significant difference was observed in enzyme and binding kinetics in left ventricle, right ventricle, and septum; therefore, mean values were calculated for each heart. Figure 3 illustrates the activation of Na/K-ATPase by Na<sup>+</sup> and K<sup>+</sup> and its inhibition by dihydro-ouabain and vanadate. V<sub>max</sub> decreased from 3.6(0.5) μmol Pi·g<sup>-1</sup> wet weight·min<sup>-1</sup> in non-failing hearts to 2.0(0.4) in failing hearts (p<<0.001), while activation and inhibition constants and Hill coefficients did not change. The Na<sup>+</sup> and K<sup>+</sup> sensitivities and the molecular turnover rate of Na/K-ATPase agree well with data from purified preparations,<sup>12-14, 23</sup> showing that DOC treated homogenates are suitable for Na/K-ATPase



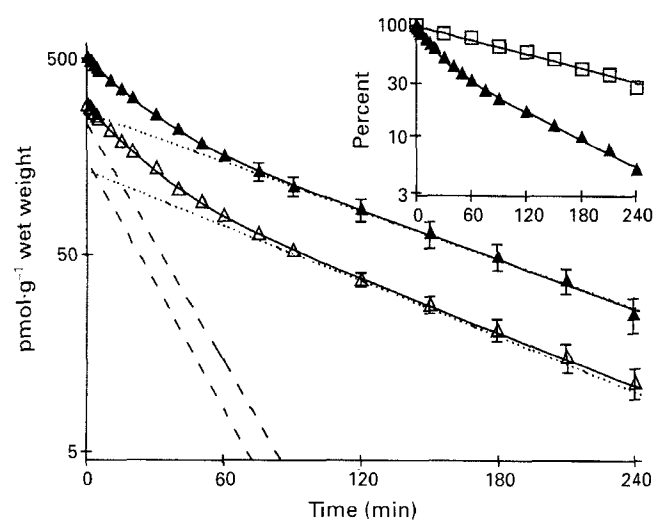
**Figure 3** Na/K-ATPase activation by Na<sup>+</sup> and K<sup>+</sup> (left panel) and inhibition by dihydro-ouabain (right upper panel) and vanadate (right lower panel) in non-failing ( $n = 5$ , filled triangles) and failing ( $n = 13$ , empty triangles) human hearts. Error bars = SEM.

characterisation. It is interesting to note that  $\alpha$  isoforms have different Na<sup>+</sup> sensitivities.<sup>24</sup>

Figure 4 shows dissociation kinetics of the Na/K-ATPase-[<sup>3</sup>H]ouabain complex. As previously reported, dissociation kinetics can be fitted to a two site model in human heart membranes<sup>14</sup> and to a one site model in kidney.<sup>25</sup> This tissue difference can be accounted for by different isoenzyme compositions. The slow complex represented 49% and 52% of the specific sites, respectively, in non-failing ( $k_1 = 0.047\cdot\text{min}^{-1}$ ;  $k_2 = 0.009\cdot\text{min}^{-1}$ ) and failing hearts ( $k_1 = 0.052\cdot\text{min}^{-1}$ ;  $k_2 = 0.011\cdot\text{min}^{-1}$ ). It is clear that a third high affinity site indistinguishable from the other two may be present, as suggested by the mRNA analysis, and that makes the identification of the binding sites uncertain.

### Discussion

Our initial studies showed that failing human hearts contain trabeculae that are more sensitive to the inotropic and toxic effects of ouabain, and we examined various hypotheses that could account for the difference. An original feature of this study was that the fibres were inspected under the light microscope after the contractility experiments. Thus, although necrotic fibres were discarded, the experiments were not designed to look at the "best case" response to inotropic stimulation. We found two types of fibres in diseased hearts; apparently normal fibres and fibres showing morphological alterations characteristic of diseased heart. Only visibly damaged fibres showed functional changes: (1) reduced maximum effect of ouabain, and (2) inotropic and toxic responses at markedly lower concentrations of ouabain. The fact that the inotropic responses to calcium and ouabain were similarly reduced suggests that this reduction may be related to loss of contractile material and/or to reduced myofibrillar ATPase activity.<sup>26</sup> It is crucial to note that the inotropic effect of ouabain was concentration dependent in non-failing fibres, in agreement with animal studies.<sup>27</sup> The unexplained all or nothing inotropic response of non-failing human papillary muscle in a previous study<sup>4</sup> did not allow a valid comparison with failing hearts. Thus the present study shows conclusively, and for the first time, an increased sensitivity of failing human fibres to digitalis.



**Figure 4** Dissociation kinetics of the Na/K-ATPase-[<sup>3</sup>H]ouabain complex in non-failing ( $n = 5$ , empty triangles) and failing ( $n = 13$ , filled triangles) human hearts. Error bars = SEM. Dotted lines represent the computed functions for two sites. Inset: Comparison between human heart (filled triangles) and human kidney (squares).

To explain this observation, we first hypothesised that failing hearts might express a higher affinity isoform of the Na/K-ATPase. Indeed, three  $\alpha$  isoforms with varying affinities for cardiac glycosides have been observed in mammalian tissues.<sup>28</sup> For example, the affinity of  $\alpha 1$  expressed in the kidney varies greatly among species,<sup>25</sup> ranging from the human  $\alpha 1$ , which is one of the most sensitive to ouabain (1 nM), to the rat  $\alpha 1$ , which is highly resistant (10  $\mu\text{M}$ ). In the rat,<sup>28</sup> the affinities for  $\alpha 2$  and  $\alpha 3$  are respectively 100 and 1000 times more sensitive than the affinity for  $\alpha 1$ . In other species, the affinities for  $\alpha 2$  and  $\alpha 3$  are less precisely known, but analysis of inhibition and binding kinetics<sup>25</sup> in tissues predominantly expressing these isoforms suggests high affinities similar to those for rat  $\alpha 2$  and  $\alpha 3$ . In human heart membranes, the dissociation kinetics of the [<sup>3</sup>H]ouabain enzyme complex show a complex pattern that is compatible with the presence of different isoforms, in agreement with mRNA analysis. The slower phase (higher affinity) is close to the monophasic dissociation observed in human kidney, suggesting that it may represent  $\alpha 1$ . Thus, although this deduction still has to be verified experimentally,<sup>5</sup> it appears that human  $\alpha 1$  could be 5 to 10 times more sensitive to ouabain than human  $\alpha 2$  and  $\alpha 3$ ; therefore an increase in  $\alpha 1$  expression could augment the ouabain sensitivity of failing human hearts.

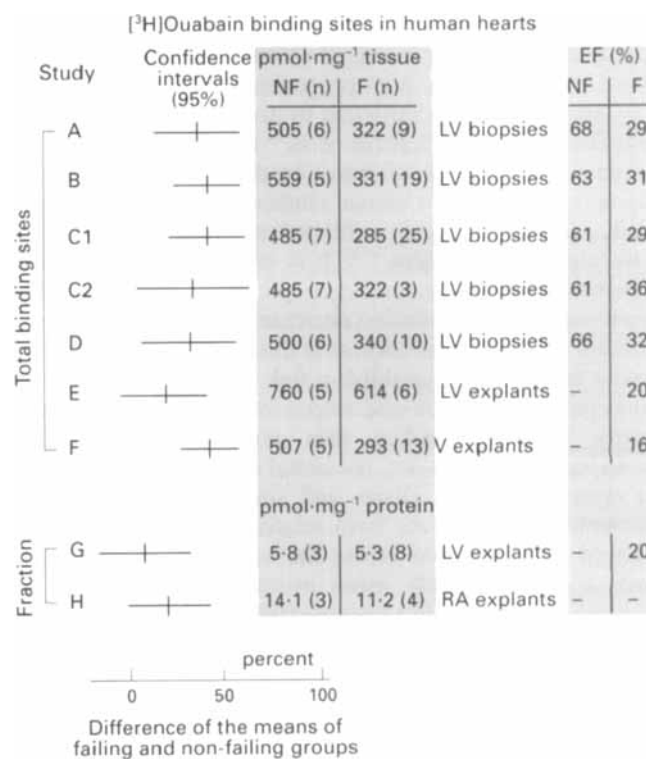
In this study, we have confirmed previous reports that the three  $\alpha$  isoform mRNAs are expressed in both failing<sup>7</sup> and non-failing hearts.<sup>6,7</sup> When we quantified the mRNA levels for each  $\alpha$  isoform the errors were quite large. The  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  mRNAs comprised 48(SD 21)%, 26(13)%, and 27(10)%, respectively, of the total  $\alpha$  isoform pool in normal hearts, and 18(6)%, 30(12)%, and 52(12)%, respectively, in diseased hearts. Due to the large variability in the isoform levels, we are not certain that the apparent shift towards less  $\alpha 1$  and more  $\alpha 3$  in disease is significant. Our results, including the large variability, are similar to those published by Allen *et al.*,<sup>7</sup> who report a 1:0.3:0.5 ratio for  $\alpha 1$ : $\alpha 2$ : $\alpha 3$  mRNA expression in both normal and diseased heart. Their variability, when converted from SEM to SD, is similar to ours. Although the large variability in mRNA levels of each isoform may reflect heart to heart variation or regional



variation within one heart, it may also be caused by selective degradation of one or more isoforms. The degradation level from sample to sample varied considerably, as evidenced by a 20-fold range in 18S rRNA levels per 10  $\mu\text{g}$  of total RNA. In fact, we were surprised at the variability that Allen *et al*<sup>7</sup> observed in the  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  mRNA levels, because their evaluation was done from slot blots instead of northern blots, and their samples were stored in liquid nitrogen instead of at  $-70^\circ\text{C}$ . Since we used northern blots, we considered our results to be semiquantitative, especially for the  $\alpha 2$  mRNAs which run at 5.7 and 6.1 kb<sup>6</sup>; the signals tend to be slightly smeared by the 28S rRNA band and may not transfer as efficiently as mRNAs that do not run with the rRNAs. Moreover, in our hands, the RNA from heart samples stored at  $-70^\circ\text{C}$  tended to be more degraded than that from samples stored in liquid nitrogen (data not shown). We were also concerned about the specificity of the probes used, since they came from rat cDNAs, which are not identical to human sequences; the regions the probes are derived from were not specified and the primary data used to determine probe specificity were not shown or referenced. For our study, we used 60mer oligonucleotides derived from published human sequences, which we had previously shown to be isoform specific using northern analysis.<sup>6</sup>

As it is not known whether the correlation between mRNA and the expressed protein is maintained in diseased hearts, we have attempted to study the Na/K-ATPase isoforms at the protein level by analysing the dissociation kinetics of the [<sup>3</sup>H]ouabain enzyme complex (a technique much more sensitive than Scatchard analysis to distinguish between forms with similar binding site affinities<sup>7-14</sup>). The complex pattern of dissociation kinetics is not changed in failing hearts. Therefore there is no evidence for any change in isoform expression at the protein level that could account for the increased digitalis sensitivity.

We then hypothesised that failing hearts may have a lower Na/K pump reserve capacity resulting from a decrease in Na/K-ATPase density such that less inhibition is required to produce pharmacological responses. Previous studies on [<sup>3</sup>H]ouabain binding site concentration in failing hearts have led to contradictory conclusions, for example that both significant and non-significant decreases can occur.<sup>4 30-34 36</sup> Figure 5 shows that these studies are not contradictory from a statistical standpoint,<sup>29</sup> since decreases between 26% and 32% fall within the confidence intervals. However, there are important methodological differences. In one group of studies (A to E, inclusive), small samples of tissue were frozen, thawed, and incubated for hours in the presence of [<sup>3</sup>H]ouabain, and binding data were expressed per g wet weight. In studies A,<sup>30</sup> B,<sup>31</sup> C,<sup>32</sup> and D<sup>33</sup> it was concluded there were significant decreases in Na/K pumps in failing hearts, while study E<sup>34</sup> showed no significant decrease. It is uncertain whether this discrepancy is due to the use of explants in study E instead of biopsies or of  $F_{ab}$  fragments to eliminate bound digoxin resulting from previous therapy, or to insufficient statistical power (large variability and small number of hearts). This uncertainty is compounded by the failure of other reports, independent of the water content of the samples, to express binding data. The potential drawback of using small (a few mg) samples has been briefly discussed by this group in an earlier study on necropsy samples,<sup>35</sup> but verification of the water content was not reported in A to E. In another group of studies indicating that there are non-significant decreases in binding sites (G<sup>36</sup> and H<sup>4</sup>), large pieces of tissue were used (several grams), but [<sup>3</sup>H]ouabain binding was measured in a membrane fraction instead of in



**Figure 5** Comparison between [<sup>3</sup>H]ouabain binding data from this and from previous studies (for references see text). Confidence intervals of the differences between non-failing and failing groups were calculated according to Gardner and Altman<sup>29</sup> by using the means, SEM, and numbers of hearts reported. Note that this comparison is valid in so far as methods are comparable and random sampling applied. Data have been normalised and expressed as percent of the means of the non-failing groups. Ejection fraction was not determined for non-failing hearts in studies E to H and is assumed to be normal (about 60%). Diagnoses were dilated (A, C1, D, G, and H), idiopathic dilated (B), and ischaemic (C2, E) cardiomyopathies. Methodological differences between studies are indicated in the figure. Studies A to D: biopsies were obtained from cardiac patients divided among groups according to their ejection fractions: many patients were treated with digitalis in failing and non-failing groups; the total concentration of specific sites was determined in intact endocardium and corrected for methodological errors. Study E: tiny pieces of myocardium were obtained from explanted hearts ("explants"); methodology as in preceding studies except that tissue was washed with digoxin  $F_{ab}$  prior to binding assay. Study F: present study. Studies G and H: ouabain binding was measured to a membrane fraction isolated from left ventricular (LV) or right auricular (RA) myocardium of explanted hearts; failing patients were treated with digitalis in G, but not in H.

whole tissue extracts and expressed per mg of membrane protein. The possibility of Na/K-ATPase redistribution between membrane fractions was not examined. In the present study, we took care to check or minimise the influence of such pitfalls by using large samples, ensuring complete recovery of Na/K-ATPase, using different references to express data, and checking that interference of bound digoxin was negligible. We further examined the maximum turnover rate or the sodium sensitivity of Na/K-ATPase, which might be affected by changes in the lipid composition of the sarcolemma, or by the action of proteases. We did not find any change in these important variables and, although we did not examine the ATP binding site directly, the vanadate or phosphate binding site was not altered. Our results suggest a mean 42% decrease of the

enzyme concentration in failing hearts (ranging from 26% to 56%), as opposed to a change in isozyme composition, or some other alteration in its properties.

Is such a decrease sufficient to account for the increased digitalis sensitivity? Schmidt *et al*<sup>34</sup> have estimated that the occupation of digitalis receptors by digoxin is about 24% in digitalised patients. In animal studies, 50-65% inhibition of Na/K-ATPase has been estimated to produce arrhythmias with digitalis glycosides.<sup>37,38</sup> It is clear that less glycoside may be required to produce inotropic and toxic effects if the Na/K-ATPase concentration decreases by 26-56%. However, because of limitations inherent in this study, additional proof would be needed to establish a link between Na/K-ATPase concentration and inotropic responsiveness in failing human hearts. Ouabain binding data represent apparent Na/K-ATPase concentrations, not actual densities, since changes in myocyte number, shape, and size were not taken into account. Our data were from relatively large samples and showed little variation across the ventricles, in agreement with a previous study using necropsy samples,<sup>35</sup> but in contrast with the results of contractility experiments in which trabeculae from a failing heart differed markedly in their inotropic responses. Furthermore, a single (hypertrophic) failing heart showed normal Na/K-ATPase activity. Therefore it is unlikely that a decrease in Na/K-ATPase alone explains the increased digitalis sensitivity. Changes distal to Na/K-ATPase affecting, for example, sodium permeability, Na/Ca exchange, or ATP content may also be involved.

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Key terms: cardiomyopathy; heart failure; ouabain; cardiac glycosides; Na/K-ATPase; Na/K-pump; ouabain binding; heart trabeculae; human heart.

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