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Isoform specificity of cardiac glycosides binding to human Na^+,K^+-ATPase $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$

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Abstract

Cardiac glycosides inhibit the Na^+,K^+-ATPase and are used for the treatment of symptomatic heart failure and atrial fibrillation. In human heart three isoforms of Na^+,K^+-ATPase are expressed: $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$. It is unknown, if clinically used cardiac glycosides differ in isoform specific affinities, and if the isoforms have specific subcellular localization in human cardiac myocytes. Human Na^+,K^+-ATPase isoforms $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ were expressed in yeast which has no endogenous Na^+,K^+-ATPase. Isoform specific affinities of digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain were assessed in [^{3}H]-ouabain binding assays in the absence or presence of K^+ (each n=5). The subcellular localizations of the Na^+,K^+-ATPase isoforms were investigated in isolated human atrial cardiomyocytes by immunohistochemistry. In the absence of K^+, methyldigoxin ($\alpha_1>\alpha_3>\alpha_2$) and ouabain ($\alpha_1=\alpha_3>\alpha_2$) showed distinct isoform specific affinities, while for digoxin, digitoxin and β-acetyldigoxin no differences were found. In the presence of K^+, also digoxin ($\alpha_2=\alpha_3>\alpha_1$) and β-acetyldigoxin ($\alpha_1>\alpha_3$) had isoform specificities. A comparison between the cardiac glycosides demonstrated highly different affinity profiles for the isoforms. Immunohistochemistry showed that all three isoforms are located in the plasma membrane and in intracellular membranes, but only $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are located in the T-tubuli. Cardiac glycosides show distinct isoform specific affinities and different affinity profiles to

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Na^+,K^+-ATPase isoforms which have different subcellular localizations in human cardiomyocytes. Thus, in contrast to current notion, different cardiac glycoside agents may significantly differ in their pharmacological profile which could be of hitherto unknown clinical relevance.

Keywords

Heart failure; Digoxin; Digitoxin; β-Acetyldigoxin; Methyldigoxin

1. Introduction

Cardiac glycosides have been used for pharmacological treatment of heart failure for more than 200 years (Sterkowicz, 1985). Today they are still indicated for heart rate control in atrial fibrillation especially when concomitant heart failure is present. The current guidelines for the treatment of heart failure recommend the use of cardiac glycosides (at low concentrations) if serious heart failure symptoms remain even after optimal adjustment of all other therapeutic options (Hunt et al., 2005; Swedberg et al., 2005). There was only one large randomized clinical trial, the DIG-trial, in which treatment with cardiac glycosides (digoxin) was compared to placebo (Digitalis Investigation Group, 1997). The results showed overall an unaltered mortality of patients treated with digoxin, but significantly reduced hospitalizations.

Thus, digitalis is a class I or IIa recommendation for the treatment of symptoms of heart failure in patients with sinus rhythm according to the American Heart Association (AHA) and European Society of Cardiology (ESC) guidelines, respectively (Hunt et al., 2005; Swedberg et al., 2005), and it is considered class I for the treatment of heart failure in the presence of atrial fibrillation. However, no distinction between different types of cardiac glycosides is made. In the treatment of atrial fibrillation digoxin is recommended for heart rate control (Fuster et al., 2006), but in clinical practice all cardiac glycosides are used alike. This is based on pure assumption, since so far no comparative studies on different glycoside agents were performed. For the molecular structure of the cardiac glycoside see Fig. 1.

One putative difference of the agents may be a different preference for the isoforms of the Na^+,K^+-ATPase. In human heart, mainly three different alpha-isoforms (α_1, α_2 and α_3) are expressed as a heteromer together with the β_1-isoform (Wang et al., 1996).

It is not clear if only one isoform mediates the positive inotropic action of the cardiac glycosides or if it is an effect of several isoforms. It seems that in mice both, α_1 and α_2, are involved in the regulation of the cardiac contractility (James et al., 1999; Dostanic et al., 2004).

In the present study, the binding affinities of different, clinically used cardiac glycosides (i.e. digoxin, digitoxin, methyldigoxin and β-acetyldigoxin) to each individual human cardiac Na^+,K^+-ATPase isoform were assessed for the first time. This was done by using a yeast expression system of the isoforms. Due to the fact that yeast has no endogenous Na^+,K^+-ATPase, the experiments were specific for the tested human isoform. Furthermore, the specific subcellular distribution of Na^+,K^+-ATPase isoforms α_1, α_2 and α_3 were investigated

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in human cardiac myocytes as a putative underlying cause for specific function in humans. The results contribute to a better understanding of the underlying mechanism of cardiac glycosides action in heart failure and might lead to more differentiated therapeutical strategies in the use of these agents.

2. Materials and methods

The use of human tissue was approved by the local ethics committee. Written informed consent of the patients was obtained prior to the procedures.

2.1. Materials

Bacto-Peptone and yeast extract, were purchased from Difco (Heidelberg, Germany). [^{3}H]-ouabain (specific activity 17 Ci/mmol, concentration 1 mCi/ml) and enhanced Chemiluminescence kit (ECL) were purchased from Amersham Life Science, Buckinghamshire, United Kingdom. Methyldigoxin and lactate dehydrogenase were from Roche, Mannheim, Germany. Coomassie Brilliant Blue R, sodium dodecyl sulfate (SDS), and N,N,N′,N′-tetramethylendiamine (TEMED) were from Serva Feinbiochemika, Heidelberg, Germany. Polyvinyldienfluoride (PVDF)-membrane and Kaleidoscope prestained standards (≊7–205 kDa) were from BioRad, Munich, Germany. Custodiol® was purchased from Köhler Chemie, Alsbach-Haehnlein, Germany. The monoclonal IgG1 α_1-antibody (McK1) and the monoclonal IgG1 α_2-antibody (McB2) were kindly provided by Kathleen Sweadner, Boston, USA. The polyclonal α_3-antibody (Anti-TED) was kindly provided by Thomas Pressley, Lubbock, USA. All other materials were either purchased from Sigma (Taufkirchen, Germany), Merck-Eurolab (Munich, Germany) or Roth (Karlsruhe, Germany).

2.2. Human myocardium and kidney tissue

Human right myocardial tissue was obtained from the right auricles of patients undergoing coronary artery bypass graft surgery. The patients gave a written informed consent prior to surgery. The patients received beta-blockers, angiotensin-converting enzyme-inhibitors (ACE-inibitors), aldosterone-antagonists, statins and diuretics. None of the patients was treated with cardiac glycosides. Anaesthesia was achieved by injection of flunitrazepam, fentanyl and pancuronium bromide with enflurane or isoflurane. Cardiac arrest was achieved by performing intraoperative perfusion of the heart of cardioplegic solution (Custodiol®) while patients were connected to a cardiopulmonary bypass.

2.3. Expression of human Na^+,K^+-ATPase in yeast and membrane preparation

$\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ heteromers of the human Na^+,K^+-ATPase were expressed in yeast (*Saccharomyces cerevisiae* 30-4) as previously described (Müller-Ehmsen et al., 2001a). A microsomal membrane fraction of yeast cells was prepared (Eakle et al., 1992) and expression of Na^+,K^+-ATPase was confirmed by ouabain binding experiments. Control western blots were performed in order to verify specific expression of isoforms of Na^+,K^+-ATPase (Fig. 2). For further experiments, the 4 clones with the highest expression level were pooled in order to get sufficient amounts of protein.

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2.4. Ouabain binding experiments in yeast membranes

Ouabain binding experiments were performed as previously described (Erdmann and Schoner, 1973) (Schwinger et al., 1991; Müller-Ehmsen et al., 2001a,b). For each binding reaction 200 μg of membrane protein was incubated with 20 nM [^{3}H]-ouabain (specific activity 17 Ci/mmol, concentration 1 mCi/ml). The incubation buffer consisted of 4 mM H_3PO_4, 4 mM $MgCl_2$, and 50 mM Tris–HCl (pH 7.4, final concentrations, total volume 1ml). Digoxin, digitoxin, methyldigoxin and β-acetyldigoxin were added at increasing concentrations (10 different concentrations, 0–10 μM) to inhibit ouabain binding. Unspecific binding was assessed in the presence of 1 mM unlabeled ouabain.

The affinities of the ligands for the specific Na^+,K^+-ATPase heteromers were assessed with the computerized assistance of Graph Pad Prism® (one-site competition) using the previously established K_D for ouabain to the individual heteromers (13 nM for $\alpha_1\beta_1$, 33 nM for $\alpha_2\beta_1$ and 17 nM for $\alpha_3\beta_1$) (Müller-Ehmsen et al., 2001a). In addition, the binding affinities were assessed in the presence of K^+ in order to obtain results under more physiological conditions. However, a concentration of 4mM K^+ suppressed the [^{3}H]-ouabain binding in a way that made a proper statistical analysis impossible. Due to these experimental setup problems 1mM K^+ were chosen for further experiments.

2.5. Western blot

After protein determination by the Lowry method (Lowry et al., 1951) equal amounts of protein (75 μg) were mixed with loading buffer containing Tris–HCl, glycerol, mercaptoethanol and bromophenol blue. Samples were vortexed and boiled at 95 °C before being subjected to SDS-PAGE. Samples were loaded on a 4% SDS stacking electrophoresis gel (1 h at 80 mA) and subsequently separated on a 12% SDS gel for 3–5 h at 110–130 mA (electrophoresis chamber Hoefer, Munich, Germany). The proteins were then transferred to a PVDF (polyvinyldienfluoride) membrane by tank blotting (16 h at 65 mA). Efficiency of the blotting was confirmed by Coomassie blue staining of the gel and Ponceau® staining of the membrane. Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder solution in Tris buffered saline containing Tween® (TBS-T) for 15–24 h at 4 °C. Subsequently, the membrane was incubated for 12–18 h at 4 °C with the respective first antibody at indicated concentrations. After washing in fresh, cold TBS-T the blots were blocked again with 5% fat dry milk powder solution in TBS-T for 20 min at room temperature. Following a second washing step, the membranes were subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Hyperfilm ECL) using an enhanced chemiluminescence system (both, Amersham Life Science, Buckinghamshire, United Kingdom).

2.6. Single cell preparation of cardiomyocytes

Human myocardium was cut in approximately 1 mm^3 thin pieces while being incubated in single cell media (containing NaH_2PO_4 0.33 mM, NaCl 135 mM, KCl 4 mM, HEPES 10 mM, $MgCl_2$ 1 mM, glucose 10 mM at pH 7.3). The media was changed every 3 min. After addition of isolation media 1 (12 mg collagenase type II+2 mg protease type 24 in 10 ml single cell media) cells were incubated for 20 min under O_2-gasing at 37 °C. Subsequently,

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cells were washed twice with single cell media. Cells were incubated at 37 °C with isolation media 2 (10 mg collagenase type II in 10 ml single cell media) for 10 min. The supernatant was tested for dead cells using the microscope Axiovert 25 from Zeiss (Jena, Germany). After centrifugation of the supernatant (5 min at 800×*g*) the resulting supernatant was resuspended in 1 ml acetone for max. 1 min. After a second centrifugation step cells were resuspended in PBS. This suspension was again centrifuged at 800 ×g for 5 min and the prepared cardiomyocytes were resolved in 500 μl PBS for another microscopic control.

2.7. Immunohistochemical preparation

Cardiomyocytes were washed 3 × 10 min with TBS and incubated for 20 min with 20 ml methanol+5 ml 3% H_2O_2 in order to block endogenous peroxidase. After 2 × 10 min of washing with TBS, washing with 0.5 M ammonium chloride+0.25% triton X in TBS for 10 min was performed. This step promotes the release of antigen and increases the cell membrane permeability. Two further washing steps (10 min in TBS) were followed by 60 min charge neutralization in 5% BSA solution. Cells were incubated overnight at 4 °C with the respective first antibody (McK1 1:200, McB2 1:200 or anti-TED 1:100+0.8% BSA in TBS). For negative control experiments only 0.8% BSA in TBS was added. The next day after several washing steps the cells were incubated for 60 min with the secondary antibody (biotinylated anti-mouse IgG-antibody, 1:400 in TBS). After 4 × 10 min washing with TBS cells were incubated for 60 min with horseradish peroxidase-complex (1:150 in TBS) and washed again. Peroxidase activity was measured using the DAB (Diaminobenzidine) method. After successful staining, which was microscopically controlled, cells were put in xylol and embedded using Entellan®. Cardiomyocytes were visualized using the Axiovert 25 (magnification 40×).

2.8. Statistical analysis

Data are expressed as means±standard error of means (S.E.M.). Group comparisons were analyzed by Student's *t*-test (Graph Pad Prism, version 4). Values with $P<0.05$ were considered statistically significant. In order to calculate the IC_{50} values the dissociation constant (K_D) of digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain for each isoform in the absence or presence of K^+ were calculated according to the equation of Johnson et al. using Graph Pad Prism version 4 (each n=5) (Johnson et al., 1995).

3. Results

3.1. Specific expression of isoforms of Na^+,K^+-ATPase in yeast

Human brain contains all three isoforms of Na^+,K^+-ATPase, while human kidney tissue contains only the $\alpha_1\beta_1$ isoform. Therefore, in the Western blot experiments human brain and human kidney preparations were used as positive controls and non-transfected yeast extract as negative control. We used the monoclonal IgG1 α_1-antibody (McK1) and the monoclonal IgG1 α_2-antibody (McB2) as well as the polyclonal α_3-antibody (Anti-TED) as first antibodies and appropriate horseradish peroxidase conjugated secondary antibodies. As expected we found all three isoforms in human brain tissue but only α_1 in human kidney (Fig. 2). No signal was found in the non-transfected yeast cells. These results show that our detecting system was valid and specific for detection of Na^+,K^+-ATPase isoforms. Fig. 2

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also shows that transfection had been performed successfully and the isoforms were specifically expressed in yeast cell clones. After treatment with the respective isoform specific antibody only the expected bands were detected. In accordance with previous findings of our group similar expression levels were found for α_1 and α_3 whereas the expression of α_2 was somewhat lower (Müller-Ehmsen et al., 2001a). In summary, the Western Blots showed that the prepared clones allowed isoform specific observations for human Na^+,K^+-ATPase.

3.2. Ouabain binding experiments in yeast membranes

Binding experiments of radioactive [^{3}H]-ouabain (20 nM) for each cardiac glycoside (digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain) in increasing concentrations (0–40 nM) for each of the three isoforms were performed. The results could be fitted into one-site competition curves. Fig. 3 shows the binding curves of digoxin to the three Na^+,K^+-ATPase isoforms as a representative example of the original data. In this case, the curve for α_1 binding was shifted towards higher concentrations and therefore increased K_D values compared to α_2 and α_3. The comparison of all K_D values is shown in Fig. 4 A–E. The results demonstrate that several of the cardiac glycosides possess different affinities to the three isoforms of Na^+,K^+-ATPase (Fig. 4 A–E). In the absence of K^+ digoxin, digitoxin and β-acetyldigoxin showed no isoform specific affinities, in contrast to methyldigoxin and ouabain. Methyldigoxin had a higher affinity to α_1 ($P<0.01$ vs. α_2 and α_3) and ouabain to α_2 ($P<0.01$ vs. α_1 and α_3). It is of note that the maximal binding capacities were higher in yeast membranes that contained $\alpha_1\beta_1$ or $\alpha_3\beta_1$ heterodimers than in those that contained the $\alpha_2\beta_1$ heteromers (Bmax 4.95±2.8 vs. 1.32±0.5 vs. 4.51±2.6 pmol/mg protein, $P<0.001$).

The presence of K^+ decreased the affinity of all 5 cardiac glycosides to the human Na^+,K^+-ATPase as expected (Fig. 4 A–E). A significant isoform specific difference of affinity could now be found for digoxin and β-acetyldigoxin. Digoxin had a lower affinity to α+K^+ 1 ($P<0.05$ vs. α_2+K^+ and α_3+K^+, Fig. 4) and β-acetyldigoxin to α_3+K^+ ($P<0.01$ vs. α_1+K^+, Fig. 4). Digitoxin showed no isoform specific affinity in the absence or in the presence of K^+ (Fig. 4 B).

3.3. Comparison of K_D of the different cardiac glycosides

The comparison of the K_D values of digoxin, digitoxin, β-acetyldigoxin, methyldigoxin, and ouabain for each isoform in the presence or absence of K^+ revealed highly significant differences between the glycosides (Fig. 5 A–F and Table 1). In the absence of K^+ β-acetyldigoxin showed a significant lower affinity to $\alpha_1$compared to digitoxin and ouabain (Fig. 5 A and Table 1). Concerning α_2 in the absence of K^+, methyldigoxin possesses the lowest affinity and the differences to all other cardiac glycosides were highly significant (Fig. 5 B and Table 1). The affinity to α_3 in the absence of K^+ was significantly different among digoxin and ouabain and also among β-acetyldigoxin and ouabain (Fig. 5 C and Table 1).

Under more physiological conditions in the presence of K^+, digoxin showed the lowest affinity for α_1 of all cardiac glycosides studied ($P<0.001$, Fig. 5 D and Table 1). Ouabain had the highest affinity reaching the level of significance compared with β-acetyldigoxin and

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methyldigoxin (and of course digoxin), but not digitoxin (Fig. 5 D and Table 1). For the affinity to α_2 in the presence of K^+, our experiments revealed the highest affinity for ouabain and the lowest for methyldigoxin with significant or highly significant differences to the other cardiac glycosides (Fig. 5 E and Table 1). The experiments in the presence of K^+ showed for the α_3 isoform that β-acetyldigoxin had the lowest affinity (significant vs. digoxin, digitoxin and ouabain, but not methyldigoxin), and again ouabain had the highest affinity. Methyldigoxin had a lower affinity than digoxin and ouabain, and digitoxin had a lower affinity than ouabain. Thus, the order of affinities among cardiac glycosides differs from isoform to isoform.

3.4. Immunohistochemical staining and subcellular localization of Na^+,K^+-ATPase isoforms

The immunofluorescence staining of single cell cardiomyocytes showed an accumulation of antibody in the plasma membrane, T-tubuli, and intracellular membranes (Fig. 6 A–C). All three isoforms of the human Na^+,K^+-ATPase were located at the plasma membrane and intracellular membranes of the cardiomyocyte (Fig. 6 A–C). However, only $\alpha_1\beta_1$ and $\alpha_2\beta_1$, but not $\alpha_3\beta_1$, were also located in the T-tubuli (Fig. 6 A+B).

4. Discussion

The aim of the study was to investigate if clinically used cardiac glycosides differ in Na^+,K^+-ATPase isoform specific affinities, and to analyze if isoforms of the Na^+,K^+-ATPase have a specific subcellular localization in human cardiac myocytes. The main findings of the present study are that in the absence of K^+ the two cardiac glycosides methyldigoxin and ouabain in fact show isoform specificity, and that in the presence of K^+ two other cardiac glycosides (digoxin and β-acetyldigoxin) have isoform specific affinities. Furthermore, the isoforms showed different profiles regarding their affinities towards the different cardiac glycosides, and we found evidence that the isoforms of the human Na^+,K^+-ATPase have a different subcellular localization within the human cardiac myocyte. Thus, in contrast to what has been thought for a long time, it seems that cardiac glycosides have different pharmacological profiles and are not simply exchangeable. In addition, the different subcellular distribution of Na^+,K^+-ATPase isoforms may indicate that the different isoforms of human Na^+,K^+-ATPase may indeed have a different physiological function, as previously postulated for mice (James et al., 1999).

The results of the radioligand binding experiments in the absence of K^+ showed that besides ouabain, which was already known to have some isoform specificity (Müller-Ehmsen et al., 2001a), only methyldigoxin among the other cardiac glycosides has an isoform specific affinity profile. Interestingly, the isoform specific affinity profiles changed in the presence of K^+ with digoxin and β-acetyldigoxin showing isoform specific properties under these more physiological conditions. Such influence of K^+ could at least partly be explained with the different affinities of K^+ to the Na^+,K^+-ATPase isoforms which were previously found in heterologous expressed Na^+,K^+-ATPase isoforms (yeast, *Xenopus* oocytes) (Müller-Ehmsen et al. 2001a; Crambert et al., 2000). In yeast K^+ had a higher affinity to α_2 than α_3 and α_1 (K_D: 0.5 mM vs. 2.5 mM and 3 mM). Thus, in the presence of a constant concentration of

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K^+ (as in our experiments, but also as in the human body) the affinities of the cardiac glycosides towards α_2 should decrease the most, and this should be the case for all cardiac glycosides. However, this was not the case, e.g. for digoxin the affinity shift in the presence vs. the absence of K^+ was most pronounced for the α_2 isoform, while for digitoxin it was most pronounced for α_3. Therefore, substance specific interactions with the isoforms in the presence of K^+ seem to play a role. Lingrel et al. hypothesized that in mutant α_1 Na^+,K^+-ATPase different affinities between digoxin and digitoxin could be explained by hydrogen bond forming between the hydroxyl group at C-12 of digoxin and Cys-108 of the enzyme but they also could not rule out other molecular interactions such as dipole interaction or van der Waals forces (Askew and Lingrel, 1994). The differences we found could possibly as well be explained by dipole interactions or van der Waals forces between the isozymes and the cardiac glycosides. However, the exact molecular mechanism remains unknown.

The K_D-ranges we observed for the cardiac glycosides binding to the isoforms are all within one order of magnitude. Mostly, they are within a factor of 2–4, and the greatest difference is for $\alpha_1\beta_1$ in the presence of K^+ which is bound at 6-fold lower concentrations by ouabain (K_D 19 nM) as compared to digoxin (K_D 110 nM). However, also these rather small differences might be of clinical relevance given the steep concentration-dependent binding of the glycosides.

The clinical relevance of the observed differences between the cardiac glycosides depends on the distinct isoform specific function. To date the function of the isoforms is still unclear, and in case of functionally equivalent isoforms, our finding of different isoform binding profiles of the glycosides might be without clinical consequence. However, it seems extremely unlikely that the presence of different isoforms and their distribution occurs in a random fashion, since different Na^+,K^+-ATPase isoforms are found in completely unrelated species such as mammals, birds, crustaceans, platyhelminths, etc. (Blanco and Mercer, 1998). It is believed that the ubiquitous $\alpha_1\beta_1$-isozyme could have the role of the housekeeping Na^+,K^+-ATPase whereas $\alpha_2\beta_1$ and $\alpha_3\beta_1$ could mediate more tissue specific tasks. In neurons where all three isoforms are expressed $\alpha_3\beta_1$ with its relatively lower affinity to cations seems to function as a spare pump which will only be activated during depolarisation (Blanco and Mercer, 1998). Another indicator for isoform specific function can be found during development where a change in the relative amount of Na^+,K^+-ATPase isoforms takes place. For example, in rat hearts, a switch from α_3 to α_2 as the dominant isoform was found between weeks two and three of life (Blanco and Mercer, 1998). In humans, Na^+,K^+-ATPase isoforms are specifically regulated in response to diseases such as heart failure, indicating that they may definitely play specific functional roles in humans (Schwinger et al., 1999a,b; Müller-Ehmsen et al., 2001b; Müller-Ehmsen et al., 2002).

In human heart three different isoforms of the Na^+,K^+-ATPase: $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are expressed (Wang et al., 1996). Previous studies showed that a specific regulation of these enzymes occurs during heart failure (Müller-Ehmsen et al., 2001b; Schwinger et al., 1999a,b). Regarding subcellular localization as a possible indicator for an isoform specific function, McDonough et al. showed a distribution of α_1 and α_2 in the T-tubuli and the plasma membrane of cardiomyocytes of rats and guinea pigs. In rat cardiomyocytes the staining of α_1 in T-tubuli seemed to be slightly increased suggesting an accumulation of this

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isoform in this region (McDonough et al., 1996). We could now show for the first time the different subcellular localization of the Na^+,K^+-ATPase isoforms in human cardiomyocytes. Our experiments revealed for the α_3-isoform a differing distribution compared to the two other isoforms: α_1 and α_2 were located at the T-tubuli, whereas α_3 was not. All isoforms were equally found in the plasma membrane. Interestingly, α_1 and α_2 showed the same subcellular localization like in rat ventricular cardiomyocytes (McDonough et al., 1996).

The group of Lingrel et al. hypothesized previously, that in mice the action of cardiac glycosides is only mediated by the isoform $\alpha_2\beta_1$ (James et al., 1999). In more recent work with knock-in-mice with genetically engineered ouabain sensitive α_1- and ouabain resistant α_2-isoforms, they seem to question their own theory of a α_2-mediated cardiac glycoside effect in mice (Dostanic et al., 2004). However, in wild type rodents it is not feasible to demonstrate an isoform specific functional role by using cardiac glycosides due to the insensitivity of the $\alpha_1\beta_1$-isoform to them (Dostanic et al., 2004). Since mice do not express α_3, also no conclusions on the role of this isoform can be drawn from their study.

Taking our results of the different subcellular localization and the findings of other groups together this might lead to the assumption of a, yet unclear, specific physiological function of the isoforms and eventually also to different roles of the isoforms in cardiac glycoside action. Under this presumption the observed differences in isoform specificity among clinically used cardiac glycosides could in fact gain relevance for the treatment of patients. If the drug action is not mediated via all isoforms equally, a substance that inhibits the relevant isoform(s) more potently than the others would probably be the best choice for treatment.

In summary, clinically used cardiac glycosides show distinct isoform specific affinities to Na^+,K^+-ATPase isoforms. The binding affinities reflect the inhibitory potencies of the agents, and we conclude that, if the isoforms have different functions in the human heart, the different cardiac glycosides cannot be regarded as equivalent agents. In support of isoform specific function we found that the Na^+,K^+-ATPase isoforms have different subcellular localizations in human cardiac myocytes. The present study demonstrates first evidence that, in contrast of current clinical practice, the different cardiac glycosides might differ under pharmacological and clinical aspects.

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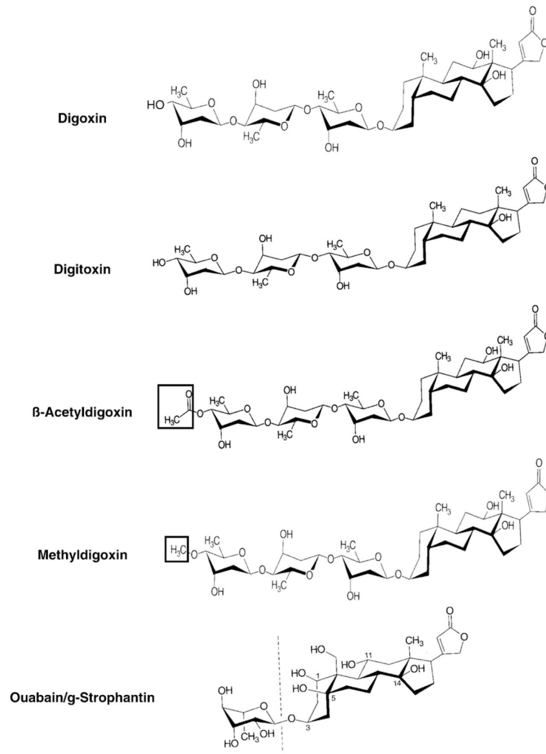

Fig. 1.

Molecular structures of the used cardiac glycosides. (Modified from Roth, Arzneistoffe, pages 361–362, 1994).

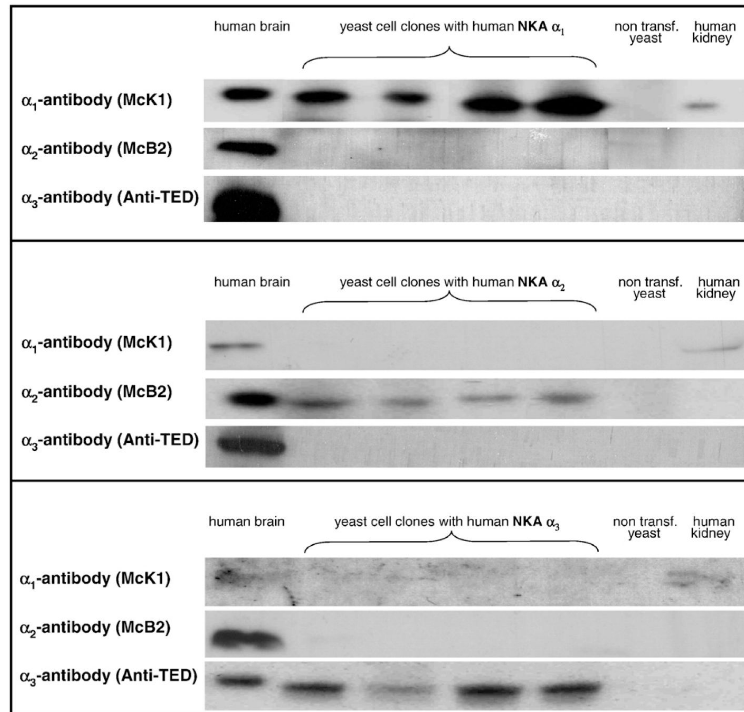

Fig. 2.

Western blot analysis of isoform specific expression of Na^+,K^+-ATPase in yeast cells (4 clones for each isoform, for further experiments the four clones were pooled). Human brain expressing α_1, α_2 and α_3 was used as a positive control for all isoforms. Human kidney expressing α_1 only was used as a positive control for that isoform. Non-transfected yeast was used as negative control.

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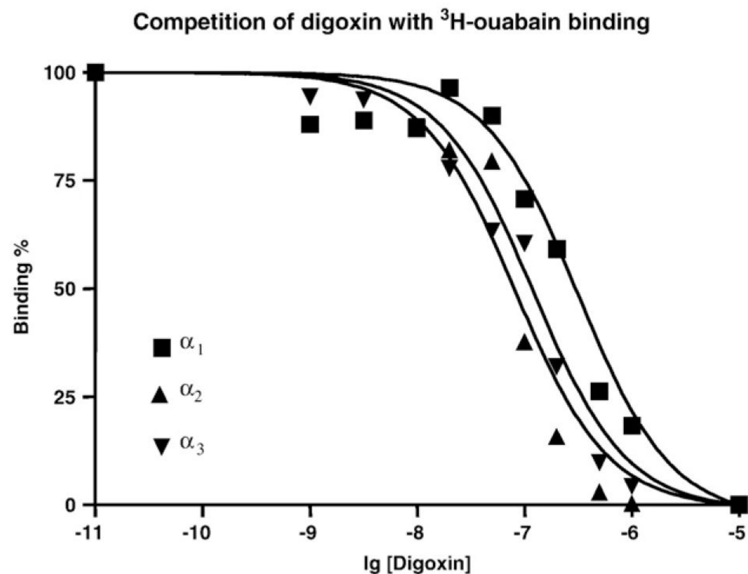

Fig. 3.

Example of a competition curve of a cardiac glycoside with [^{3}H]-ouabain (20 nM) binding in yeast expressed Na^+,K^+-ATPase isoforms. One representative competition curve of digoxin for each isoform $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ is shown. The experiments were performed in the presence and absence of K^+ and besides digoxin for all other clinically used cardiac glycosides digitoxin, β-acetyldigoxin, methyldigoxin and ouabain. Using these curves the respective dissociation constants (K_D) were calculated.

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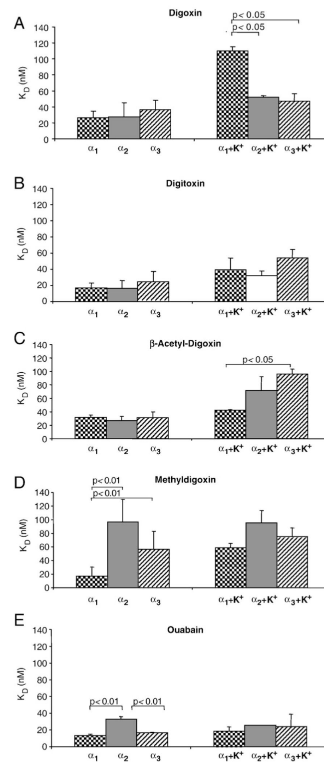

Fig. 4.

K_D in nM±S.E.M. of digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain in the absence and presence of K^+ (each n=5). K_D was calculated based on the equation of Johnson et al. All results are summarized in Table 1.

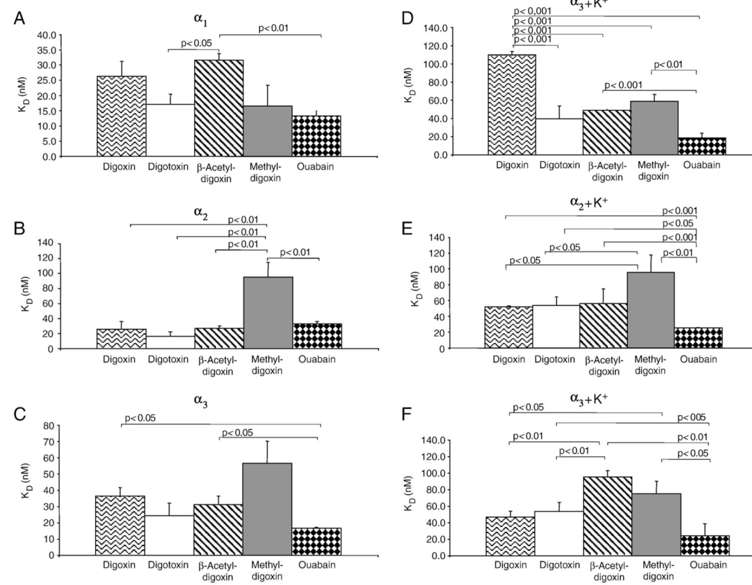

Fig. 5.

Comparison of the five cardiac glycosides digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain for all three isoforms (α_1, α_2 and α_3) as K_D in nM±S.E.M. in the absence or presence of K^+ (each n=5).

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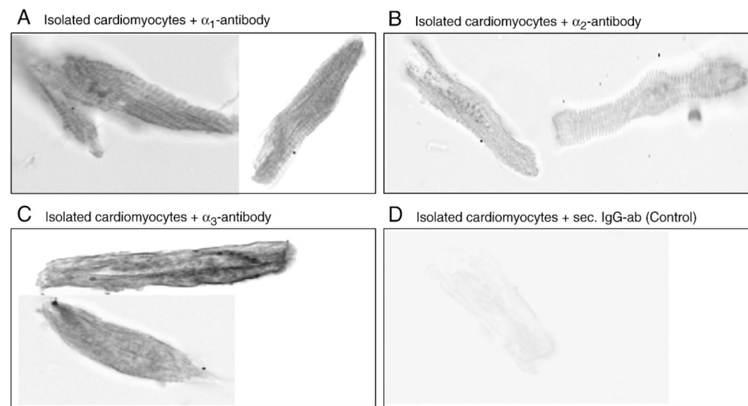

Fig. 6.

Immunhistochemical preparations (magnification: 40×) of isolated cardiomyocytes after treatment with isoform specific antibodies (McK1 1:200, McB2 1:200 or anti-TED 1:100) and biotinylated anti-mouse IgG-antibody. Fig. 5D shows the negative control. One representative of 3 experiments is shown for each isoform.

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Table 1

K_D in nM±S.E.M. of digoxin, digitoxin, β-acetyldigoxin, methyldigoxin and ouabain in the absence and presence of K^+ (each *n*=5).

	Human NKA	Digoxin	Digitoxin	β-Acetyldigoxin	Methyldigoxin	Ouabain
No K^+	$\alpha_1\beta_1$	26.4±4.8	17.1±3.4[e]	31.6±2.2	16.5±6.7[a]	13.3±1.7[f]
	$\alpha_2\beta_1$	25.7±10.3	16.4±5.5	26.8±3.8	95.5±19.3[g]	32.8±3.4[b]
	$\alpha_3\beta_1$	36.5±6.8	24.5±7.6	31.3±5.1	56.6±13.6	16.7±0.6[h]
K^+ (1 mM)	$\alpha_1\beta_1$	110.0±3.9[c,i]	47.4±10.	42.4±1.1	58.7±8.0	18.9±4.8[j,k]
	$\alpha_2\beta_1$	52.2±1.4	32.4±3.9	71.8±20.4	95.4±22.3[l,m]	25.5±0.1[n,o]
	$\alpha_3\beta_1$	50.0±4.2[q]	53.1±11.0	95.6±7.8[d,p]	75.4±14.8	24.5±14.6[r,s]

K_D was calculated based on the equation of Johnson et al.

[a] $P<0.01$ vs. α1 and α3,

[b] $P<0.01$ vs.α1 and α3,

[c] $P<0.05$ vs.α2 and α3,

[d] $P<0.001$ vs. α1,

[e] $P<0.05$ vs. β-acetyldigoxin,

[f] $P<0.01$ vs. β-acetyldigoxin,

[g] $P<0.01$ vs. digoxin, digitoxin, β-acetyldigoxin and ouabain,

[h] $P<0.05$ vs. digoxin and β-acetyldigoxin,

[i] $P<0.001$ vs. digitoxin, β-acetyldigoxin, methyldigoxin and ouabain,

[j] $P<0.001$ vs. β-acetyldigoxin,

[k] $P<0.01$ vs. methyldigoxin,

[l] $P<0.05$ vs. digoxin and digitoxin,

[m] $P<0.01$ vs. ouabain,

[n] $P<0.001$ vs. digoxin and β-acetyldigoxin,

[o] $P<0.05$ vs. digitoxin,

[p] $P<0.01$ vs. digoxin, digitoxin and oubain,

[q] $P<0.05$ vs. methyldigoxin,

[r] $P<0.05$ vs. digitoxin and methyldigoxin,

[s] $P<0.01$ vs. β-acetyldigoxin.