Regional Expression of Sodium Pump Subunit Isoforms and Na^+-Ca^{++} Exchanger in the Human Heart

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Abstract

Cardiac glycosides exert a positive inotropic effect by inhibiting sodium pump (Na,K-ATPase) activity, decreasing the driving force for Na^+-Ca^{++} exchange, and increasing cellular content and release of Ca^{++} during depolarization. Since the inotropic response will be a function of the level of expression of sodium pumps, which are $\alpha\beta$ heterodimers, and of Na^+-Ca^{++} exchangers, this study aimed to determine the regional pattern of expression of these transporters in the heart. Immunoblot assays of homogenate from atria, ventricles, and septa of 14 nonfailing human hearts established expression of Na,K-ATPase α1, α2, α3, β1, and Na^+-Ca^{++} exchangers in all regions. Na,K-ATPase β2 expression is negligible, indicating that the human cardiac glycoside receptors are $\alpha1\beta1$,, $\alpha2\beta1$, and $\alpha3\beta1$. α3, β1, sodium pump activity, and Na^+-Ca^{++} exchanger levels were 30–50% lower in atria compared to ventricles and/or septum; differences between ventricles and septum were insignificant. Functionally, the EC_{50} of the sodium channel activator BDF 9148 to increase force of contraction was lower in atria than ventricle muscle strips (0.36 vs. 1.54 μM). These results define the distribution of the cardiac glycoside receptor isoforms in the human heart and they demonstrate that atria have fewer sodium pumps, fewer Na^+-Ca^{++} exchangers, and enhanced sensitivity to inotropic stimulation compared to ventricles. (*J. Clin. Invest.* 1996. 98:1650–1658.) Key words: Na,K-ATPase • cardiac contractility • digitalis receptor • BDF 9148

Introduction

Cardiac glycosides have been recognized to have a positive inotropic effect on the failing heart for the past 70 yr (1–3), and digoxin is one of the most commonly prescribed drugs in the United States. Despite the emergence of other therapies for congestive heart failure, clinical studies have demonstrated that digoxin withdrawal from patients receiving other therapies (such as angiotensin-converting enzyme inhibitors) is associated with significant worsening of the heart failure (4), indicating that this therapy will continue to be widely prescribed.

The only known cellular receptor for cardiac glycosides is the plasma membrane sodium pump, also known as Na,K-ATPase, which transports Na^+ out of the cell and K^+ into the cell against their concentration gradients coupled to hydrolysis of ATP. The cardiac sodium pumps, located in both the T tubules and the peripheral sarcolemma (5), are heterodimers composed of an α catalytic subunit and a β glycoprotein subunit. Multiple isoforms of each subunit are expressed in developmental and tissue-specific patterns (6). Cardiac glycosides bind to the extracellular face of the α subunit of Na pumps and inhibit their transport and enzymatic activity. In isolated cardiac tissue, this inhibition reduces the driving force for Na entry and coupled Ca^{++} extrusion via the Na^+-Ca^{++} exchanger, and leads to increased cellular stores of Ca^{++} that produce the positive inotropic effect that is the hallmark of this therapy (2, 3, 7). The relationship between the change in intracellular sodium and developed tension can be remarkably steep: force can be doubled with as little as a 1-mM increase in the activity of intracellular sodium (1).

Despite the long history of cardiac glycoside therapy and the numerous investigations examining its cellular mechanisms of action, important questions persist regarding its precise therapeutic target. The therapeutic range is low and very narrow (1–2 nM), and many factors (hypokalemia, hypothyroidism, amiodarone therapy) predispose the patient to digoxin toxicity in this therapeutic range (8). Sensitivity and range are likely to be inversely related to the levels of expression of both sodium pump isoforms and Na^+-Ca^{++} exchanger in the target tissue, as well as differences between sodium pump isoforms in affinity for the drug. The cardiac glycoside sensitivity of the individual human isoforms have not been determined, although it is generally agreed that all three are high affinity (7, 9). This contrasts with the rat heart, where there is a 1,000-fold range in affinities for ouabain among the three isoforms (reviewed in references 6–8). It is not known whether there is regional specificity to the therapeutic effects of digoxin in the human heart, but studies in the guinea pig heart demonstrate that atrial muscle, compared with ventricular muscle, has less than half the Na,K-ATPase activity and 3 mM higher intracellular Na^+ activity associated with three times higher sensitivity to the cardioactive steroid dihydroouabain (10). Besides issues of cardiac regional sensitivity, it has also been suggested that digoxin improves hemodynamics by inhibition of extracardiac Na,K-ATPase, which restores the baroreceptor reactivity that is depressed in congestive heart failure (11).

In the human heart, α1, α2, and α3 catalytic isoforms of the sodium pump have been detected in atrial and ventricle samples at both the mRNA and protein levels (9, 12, 13). To ad-

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This paper contains data from the doctoral thesis of K. Frank (Universität zu Köln, Köln, Germany).

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Received for publication 13 May 1996 and accepted in revised form 25 July 1996.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/96/10/1650/09 $2.00
Volume 98, Number 7, October 1996, 1650–1658

Table I. Clinical Characteristics of Organ Donors

Donor	Sex	Age	Cause of Death	Reason rejected for transplant
1	F	59	Subarachnoid bleeding	Virus infection, alcohol abuse
2	F	58	Subarachnoid bleeding	Virus infection, alcohol abuse
3	F	50	Not available	Coronary sclerosis
4	F	33	Not available	Excessive doses of dopamine
5	M	44	Not available	Pathologic ECG, high doses of dopamine
6	M	55	Intercerebral bleeding	Mesenteric infection
7	F	52	Coronary sclerosis	Not available
8	M	47	Subarachnoid bleeding	Fever shortly before explantation
9	F	42	Multisystem injuries	Alcohol abuse
10	F	37	Intercerebral bleeding	Operation not performed for technical reason
11	M	57	Multisystem injuries	Pericardial effusion, probably due to traumatic injury
12	M	51	Subarachnoid bleeding	Fever shortly before explantation
13	F	53	Intracerebral bleeding	Suspected coronary sclerosis
14	M	44	Multisystem injuries	Operation not performed because of logistic problems
Mean±SD		49±8		

dress the specific molecular mechanisms that are responsible for digoxin action in the human heart, the current study aimed to establish the pattern of expression of the sodium pump α and β isoform subunits, ouabain binding, Na,K-ATPase activity, and Na^+-Ca^{++} exchanger expression in the left and right atria, ventricles, and septum (LA, RA, LV, RV, and S)[1] from a significant number of nonfailing human hearts. In addition, we examined the potency of the inotropic response to the sodium channel activator BDF 9148 in atria vs. ventricles.

Methods

Tissues. Nonfailing human hearts were obtained from 14 organ donors with brain death caused by traumatic injury. Table I summarizes the characteristics of the individual donors and the reasons why the hearts were not used for transplantation. The cardioplegic solution was a modified Bretschneider solution containing (in mM): NaCl, 15; KCl, 10; $MgCl_2$, 4; histidine-HCl, 180; tryptophan, 2; mannitol, 30; and potassium dihydrogen oxoglutarate, 1. Samples from the LA, RA, LV, RV, and S were dissected and frozen at −80°C pending analysis. Samples of human brain and kidney were obtained from tissues that were discarded at surgery. These investigations were approved by the local ethics review committees of both the University of Köln and University of Southern California.

Immunoblot analysis. Immunoblot analysis was conducted on samples of unfractionated homogenate to avoid the issue of differential recoveries of membranes from various regions of the heart. Samples of heart tissue (0.1–0.2 g) were rapidly thawed, dissected free of all visible fat and vessels, weighed, and homogenized on ice for 2 min with either a Polytron (Brinkmann Instruments, Inc., Westbury, NY) at a setting of 5 or a Tissuemiser (Janke and Kunkel, Germany) for 3 min at a thyristor setting of 55, both at 1:20 (wt:vol) in 5% sorbitol, with 25 mM imidazole/histidine (pH 7.4), 0.5 mM Na_2EDTA, and proteolytic enzyme inhibitors (1 μg/ml leupeptin, 0.5 mM PMSF, and 1 mM 4-aminobenzamidine dihydrochloride). Protein concentrations of homogenates were determined by the method of Lowry et al (14).

Membrane fractions from human brain and kidney and from rat brain were prepared as previously described (15) and used as positive controls for the detection of sodium pump isoforms. Sarcolemmal membranes from dog heart (for antibody positive control) were prepared according to the method of Frank et al. (16), and those from human heart were prepared as described recently (17).

Homogenate protein was resolved by SDS-PAGE, and the gels were electrophoretically blotted onto an Immobilon P membrane. 25 and 50 μg of each homogenate sample were resolved for detection of sodium pump subunits, and 50 and 100 μg were resolved for Na^+-Ca^{++} exchanger detection. Blots were probed with one of the following antibodies: 464.6, a mouse monoclonal against α1 (1:100) from M. Kashgarian (Yale University, New Haven, CT) (also called 6H; 18, 19); McB2, a mouse monoclonal against α2 (1:100) from K. Sweadner (Harvard University, Boston, MA) (20); anti-TED, a rabbit polyclonal against α3 (1:200) (21); SpETβ1 and SpETβ2, rabbit polyclonals against β1 and β2, respectively (1:2,000) (22); and two different anti–Na^+-Ca^{++} exchanger antibodies: a rabbit polyclonal called π (1:1,000) and a mouse monoclonal called C2C12, (1:5,000), both obtained from K. Philipson (University of California, Los Angeles, CA) (23, 24). Each blot was probed with antibody only once. For initial characterization of β1 and β2 subunit expression in the human heart, crude membrane fractions were prepared and deglycosylated with *N*-glycanase, as described previously (25). All blots were prepared and processed as described previously (26) using enhanced chemiluminescence (Amersham, Arlington Heights, IL) for initial antibody characterization and ^{125}I-protein A and autoradiography for quantitation of antibody–antigen complexes.

For quantitation of immunoblots, the linearity of the signal as a function of the amount of antigen loaded was assured by both establishing the relationship between antigen concentrations and signal, and by generating multiple exposures of autoradiograms to demonstrate that signals were within the linear range of the film. Immunoblots were quantified with Imaging Densitometer GS670 and Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Ouabain-binding assay. Myocardial tissue was placed in 30 ml of ice-cold homogenization buffer (50 mM Tris-HCl, 10 mM EDTA, 1 mM DTT, pH 8.0). Connective tissue was trimmed away, minced with scissors, and homogenized with a motor-driven glass-Teflon homogenizer for 1 min, then homogenized by hand for 1 min with a glass-glass homogenizer. The homogenate was spun at 480 *g* for 15 min, the supernatant was filtered through two layers of gauze, and the pellet was discarded. The supernatant was diluted with an equal volume of ice-cold 1 M KCl, stored on ice for 15 min, then centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in homogenization buffer and centrifuged again at 100,000 *g* for 30 min. Finally, the pel-

1. *Abbreviations used in this paper:* LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; S, septa.

let was resuspended in 3–5-ml storage buffer containing (in mM): Tris-HCl, 50; $MgCl_2$, 10 (pH 7.4). Membranes were stored at −80°C pending analysis. Protein concentration (14) ranged between 3 and 13 mg/ml.

Maximal ouabain-binding capacity (B_{max}) was measured under conditions that allowed complete equilibration of the receptor with the radioligand. 300 μg of membrane protein was diluted into 2 ml incubation buffer (3 mM $MgCl_2$, 3 mM imidazole-H_3PO_4 [pH 7.4], 50 mM imidazole-HCl [pH 7.4]), 1.5 nM [^{3}H]ouabain (43 Ci/mmol), and various concentrations of cold ouabain (0, 2, 4, 7.5, 15, and 30 nM) for 3 h at 37°C. Nonspecific ouabain binding, estimated by adding 1 mM cold ouabain, never exceeded 1% of the specific binding. Reaction was stopped by incubation on ice for 15 min and rapid vacuum filtration through GF/C filters (Whatman Chemical Co., Clifton, NJ). The filters were washed immediately three times with 6 ml ice-cold incubation buffer, dried at 90°C, put into 5 ml Quickszint 501 (Zinsser Analytics, Frankfurt, Germany), and ^{3}H was determined by liquid scintillation counting. [^{3}H]ouabain-binding sites were determined according to the method of Erdmann and Schoner (27).

Na,K-ATPase activity assay. Sodium pump ATPase activity was measured with an NADH-coupled optical assay, as detailed previously (27–29). Myocardial membranes, prepared as described under *Ouabain-binding assay*, were preincubated on ice with or without 5 mM ouabain for 1 h. The assay was started by adding 50 or 100 μg of preincubated membrane protein to 750 μl of reaction solution containing (in mM): imidazole-HCl, 100 (pH 7.25); NaCl, 150; KCl, 10; NH_4Cl, 100; $MgCl_2$, 5; ATP, 5; NADH, 0.5; phospho(enol)pyruvate, 2.5; 10 μl pyruvate kinase-lactate dehydrogenase (Sigma P0294). The decrease in absorbance was simultaneously registered with a photometer (344 nm; Beckman Instruments, Inc., Fullerton, CA), and the kinetic values between 2 and 8 min were obtained for further evaluation. The reaction was linear between 1 and 15 min, and linear with protein concentration between 10 and 250 μg. The ouabain-inhibitable component of total ATPase activity was always > 30%. The specific Na,K-ATPase activity was calculated by subtracting the ouabain-insensitive activity from total activity.

Electrically driven human atrial and ventricular muscle strips. Experiments were performed as described in detail previously (30). Immediately after excision, the atrial muscle strips and ventricular papillary muscle strips were placed in ice-cold, preaerated Tyrode's solution and delivered to the laboratory within 10 min. Under microscopic control, each muscle was split into thin strips (0.6–0.8 mm wide and 8–10 mm long), with muscle fibers running approximately parallel to the length of the strips. The muscles were suspended in an organ bath (10 ml) maintained at 37°C containing a modified Tyrode's solution (in mM): NaCl, 119.8; KCl, 5.4; $MgCl_2$, 1.05; $CaCl_2$, 1.8; $NaHCO_3$, 22.6; NaH_2PO_4, 0.42; glucose, 5.05; ascorbic acid, 0.28; Na_2EDTA, 0.05. The bathing solution was continuously aerated with 95% O_2 and 5% CO_2. The muscles were stimulated by two platinum electrodes using field stimulation from a Grass (Quincy, MA) S 88 stimulator (frequency, 1 Hz; duration, 5 ms; intensity, 10–20% above threshold). The developed force was measured isometrically with an inductive force transducer (W. Fleck, Mainz, Germany) attached to either a Hellige or Gould recorder or by the IOA 5300 organ bath system (FMI, Egelsbach, Germany). The resting tension applied to the muscles was 8.0±0.5 mN. Preparations were allowed to equilibrate for at least 90 min, and bathing solution was changed once after 45 min. Concentration–response curves were determined by adding BDF 9148 cumulatively to the organ bath after an apparent equilibration of the previous effect. Each muscle was used for one concentration–response curve only.

Statistical analysis. Because immunoblot signals yield data in arbitrary units, establishing the region-specific pattern of expression required normalizing signals within each heart. All regions from a heart (LV, RV, S, LA, and RA) were analyzed on the same blot, and a different blot was conducted for each sodium pump subunit or Na^+-Ca^{++} exchanger. In other words, five blots of each heart were analyzed for α1, α2, α3, β1, and Na^+-Ca^{++} exchanger, respectively. Within each heart blot, signals were normalized to the mean signal in LV, defined as 1.0. The normalized values of these measurements were used for all analyses, while ouabain-binding B_{max} and ATPase activity data were kept as original. To assess intraheart variability in the LV immunoblot signals, all 14 of the LV samples were reanalyzed together on five blots and were probed individually for the sodium pump subunits and Na^+-Ca^{++} exchanger.

For each measurement, one-way repeated ANOVA was used to detect the regional differences among RV, S, LA, and RA (not including LV). Further pairwise comparison with multiple comparison adjustments (with Tukey Studentized range test) was used when the overall F test was significant. The comparisons between LV and RV, S, LA, and RA were done by testing whether the normalized mean values from RV, S, LA, and RA were equal to 1 (the normalized value for LV) with type I error adjusted for four multiple comparisons (significant if $P < 0.0125$).

Materials. Chemicals were reagent grade, spectroquality, or electrophoresis purity reagents. SDS-PAGE reagents were from Bio Rad. The Immobilon-P transfer membrane was from Millipore Corp. (Bedford, MA). ^{125}I-Protein A was from ICN (Irvine, CA). Rabbit anti–mouse secondary antibody was from Calbiochem-Novabiochem Corp. (La Jolla, CA). *N*-glycanase was from Genzyme Corp. (Cambridge, MA), and proteolytic enzymes, phospho(enol)pyruvate, and pyruvate kinase-lactate dehydrogenase were from Sigma Immunochemicals (St. Louis, MO). [21,22-^{3}H]ouabain was obtained from Amersham International (Amersham, UK). Ouabain was obtained from Herbert Pharma (Wiesbaden, Germany). BDF 9148 (4-(3-(1-diphenylmethyl-azetidin-3-oxy)-2-hydroxy-propoxy)-1 H-indol-2-carbonitril) was kindly provided by Prof. Mest (Beiersdorf AG, Hamburg, Germany).

Results

Detection of sodium pump subunits in the human heart. The isoform specificity of the anti-α antibodies for human α subunits is summarized in Fig. 1. The samples that were probed included the positive controls of rat kidney, where only α1 is expressed, and rat brain, in which all three isoforms are detected. As expected, α1 was detected in all samples probed, α2 and α3 were detected in rat and human brain, but not in the rat or human kidney, and α2 and α3 were detected in the human heart. The faint signals in the (α3 blots of human kidney at a slightly lower apparent molecular weight can be attributed to binding of the secondary antibody used to detect this polyclonal antiserum by ECL (the monoclonal anti-α1 and -α2 antibodies were

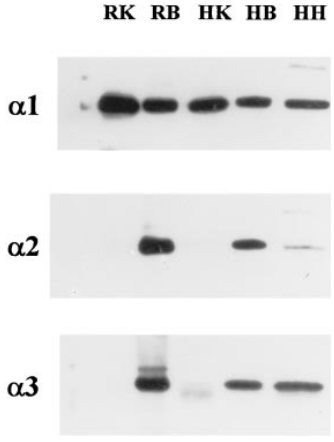

Figure 1. Immunodetection of Na,K-ATPase α isoforms in human tissues. The following crude tissue homogenates were resolved by SDS-PAGE and blotted onto nitrocellulose membranes: *RK*, 5 μg rat kidney; *RB*, 5 μg rat brain; *HB*, 5 μg human brain; *HK*, 5 μg human kidney; *HH*, 25 μg human heart. Three blots were prepared and incubated with anti–α1 mAb (464.6, 1:100), anti–α2 antiserum (McB2, 1:100), or anti–α3 antiserum (anti-TED, 1:200). Antibody–antigen complexes were detected by enhanced chemiluminescence using a kit from Amersham.

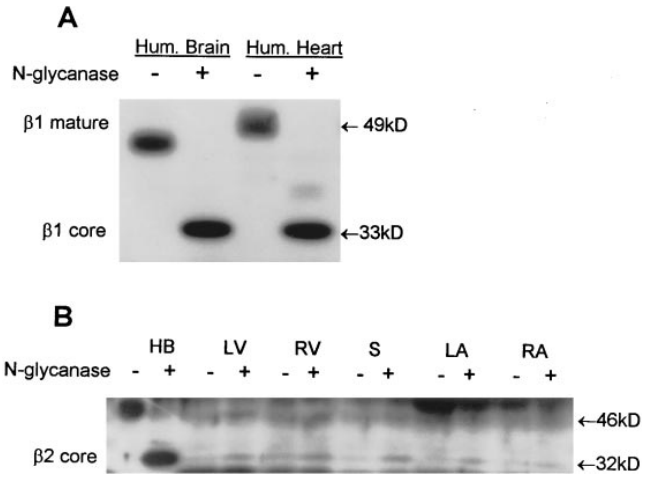

Figure 2. Identification of Na,K-ATPase β isoforms in nonfailing human hearts. (*A*) Crude membrane samples from the human brain (5 μg) and nonfailing human heart RV (50 μg) were treated with (+) or without (−) *N*-glycanase to remove sugars from the β subunit, as described in Methods, and analyzed by immunoblotting with SpETβ1 antibody (1:2,000). (*B*) Crude membranes from human brain (5 μg) and from five different regions of a nonfailing human heart (50 μg each): LV, RV, S, LA, and RA were treated as described in *A* and probed with SpET β2 antibody (1:2,000).

incubated with a different secondary antibody). These results demonstrate that α1, α2, and α3 proteins are expressed in the human heart, confirming the finding of Sweadner et al. (12), and they illustrate that the isoforms can be readily detected in crude homogenates of human heart. While the antibody-binding signals cannot be used to compare levels of expression of one isoform to another (because of antibody-specific affinity for epitopes), they can be used to compare levels of expression within an isoform between the heart and brain. In Fig. 1, the autoradiographic signals from 25 μg of human heart are equivalent to that from 5 μg human brain for both α1 and α3, and the signal is much lower for α2 in human heart relative to the brain. These results and other similar comparisons indicate to us that the heart/brain ratio of expression is similar for α1 and α3, and lower for α2.

It has not been previously addressed whether β1 and/or β2 proteins are expressed in human heart. β subunits are not easily characterized because, since they are glycoproteins, there are tissue-specific variations in mobility assessed by SDS-PAGE, and also because the sugar residues can interfere with antibody binding. Thus, heart samples were deglycosylated with *N*-glycanase to improve detection and to demonstrate glycosylation (by shift to lower apparent molecular weight). Fig. 2 *A* shows that the mature glycosylated β1 subunit is detected in both human brain membranes (5 μg) and human heart homogenate (50 μg) between 46 and 49 kD, and that both bands are reduced to the same apparent molecular mass of 33 kD after deglycosylation. Based on these results, we concluded that the 49-kD band is β1 and need not be treated with *N*-glycanase for detection. Fig. 2 *B* illustrates that mature and deglycosylated β2 can be readily detected in the human brain at 46 and 32 kD, respectively, but that only a faint β2 signal was evident in any region of the heart, and only after deglycosylation. Detection of β2 was equivalent in all five regions of the heart that were assayed, and similar results were obtained in samples from additional hearts. Based on these results, we conclude that expression of β2 in the human heart is very low. The corollaries to this conclusion are that α1, α2, and α3 most likely form heterodimers with β1, and that β1 protein levels can be used to estimate the total sodium pump pool size in the human heart.

Detection of sodium calcium exchanger in human heart. Purified Na^+-Ca^{++} exchanger from dog sarcolemmal exhibits major bands on SDS gels at 120 and 70 kD in both dog and rat heart (23, 26). The 70-kD band is a proteolytic fragment of the 120-kD exchanger and also possesses exchanger activity (23). The purified exchanger has been used to generate both polyclonal and monoclonal antibodies (23, 24). By immunoblot, the polyclonal antibody named π detects both major bands in rat and dog heart (23, 26). Two related studies (31, 32), however, reported that this same polyclonal antibody detected the 120-kD band in human heart, did not detect the 70-kD band, but did detect a 40-kD band that was postulated to be a fragment of the exchanger analogous to the 70-kD band. In contrast, using the same π antibody, we detect the 120-kD band in all human heart samples, and variable detection of the 70- and 40-kD bands (Fig. 3). This issue was further explored by assuming that any fragments of the Na^+-Ca^{++} exchanger would be localized to the sarcolemma, and determining whether the 70- and/or the 40-kD bands are successively enriched between homogenate, crude membrane fractions, and purified sarcolemma. Blots of these fractions were probed in parallel with either the π antiserum or C2C12, an mAb also raised against the canine exchanger. In the blot probed with π, there is significant and progressive enrichment of both the 120- and 70-kD bands with sarcolemmal purification, but there is also depletion of the 40-kD band, which is absent from the purified sar-

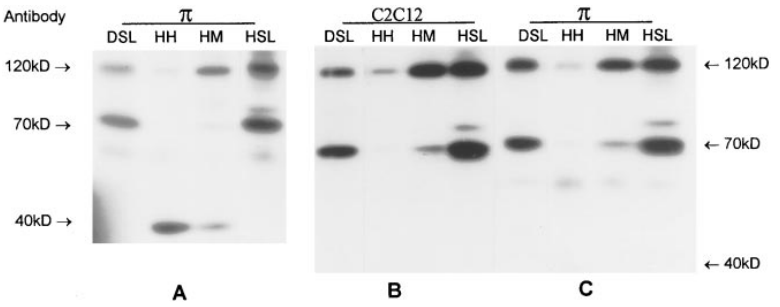

Figure 3. Identification of Na/Ca exchanger in a nonfailing human heart. Purified dog heart sarcolemmal membrane as positive control (*DSL*, 40 μg) and human heart homogenate (*HH*, 50 μg), crude membranes (*HM*, 50 μg), and purified sarcolemmal membrane (*HSL*, 50 μg) were resolved by SDS-PAGE, blotted onto Immobilon-P, and incubated with either the polyclonal antiserum π (1:1,000) or monoclonal C2C12 (1:5,000); all were detected with ^{125}I-protein A. Apparent molecular masses (indicated in kilodaltons) were determined against a panel of standards that were analyzed on the same blots (left margin for blot *A* and right margin for blots *B* and *C*). *B* and *C* are the same heart samples probed with the two different antibodies. *A* is from a different heart. In *A* the 120- and 40-, but not 70-kD bands are evident in HH; in *C* the 120- and 70-, but not 40-kD bands are evident.

colemma. The 40-kD band was also not evident in purified sarcoplasmic reticulum (not shown). The monoclonal C2C12 antibody detected both the 120- and 70-kD bands, but did not recognize the 40-kD band, indicating that the antigenic epitope that is present on both the 120- and 70-kD bands is not present on the 40-kD band. The fold enrichment of the 120- and 70-kD bands was the same with either antibody. Based on the following observations, we conclude that both the 120- and 70-kD bands of the Na^+-Ca^{++} exchanger are present in samples prepared from human heart, and that the 40-kD band is not a fragment of the exchanger: (*a*) purification of sarcolemmal membranes resulted in the enrichment of the 120- and 70-kD bands and the loss of the 40-kD band; (*b*) an mAb to the exchanger detected 120-, 70-, and not 40-kD bands; and (*c*) the intensity of the 40-kD band in homogenate samples was too great to be a fragment of the exchanger—it was equivalent to the signal obtained from the 120- and 70-kD bands of the purified dog sarcolemma (the positive control). It is possible that the 40-kD protein may have a similar antibody-binding epitope to a region of the Na^+-Ca^{++} exchanger. When care is taken to prevent proteolysis during sample preparation, the intact 120-kD Na^+-Ca^{++} exchanger is the predominant species of the exchanger that is detected in human heart homogenates. In subsequent assays, the abundance of the Na^+-Ca^{++} exchanger is based on the sum of the densities of the major 120- and minor 70-kD band in each sample.

Immunoblot calibration. The linearity of the autoradiographic signals for α1, α2, α3, β1, and Na^+-Ca^{++} exchanger as a function of amount of ventricle homogenate protein loaded is illustrated in Fig. 4 (*upper panel autoradiogram*, *lower panel calibration*). Choosing a region where the slope of the signal vs. amount loaded was close to unity, 25- and 50-μg samples were assayed for quantitation of sodium pump subunits, and double these amounts (50- and 100-μg samples) were chosen for quantitation of Na^+-Ca^{++} exchanger to reduce exposure time because the autoradiographic signals were lighter than that of the sodium pump subunits.

Regional expression of sodium pump subunits and Na^+-Ca^{++} exchanger. The regional pattern of expression of sodium pump subunits was determined in samples of homogenate protein from each of the five regions (LV, RV, S, LA, and RA) of nonfailing hearts (n = 14), as described in Methods. Fig. 5 contains typical immunoblots of each protein and summarizes the normalized results. The statistical analysis of the patterns was carried out by ANOVA followed by pairwise comparison with multiple comparison adjustment when the results of the ANOVA were significant, all as described in Methods. The significant differences are summarized in Table II. Abundance of α1 in LA is significantly lower than in RA or RV, and α2 is lower in RA when compared to LV, but all other comparisons for α1 and α2 are nonsignificant. Relative abundance of α3 is significantly lower in both RA and LA compared to LV, RV, or S. Likewise, β1 pool size, postulated to be a marker of total sodium pump expression, is lower in the atria than in the ventricles or septum. In summary, there is a region-specific pattern of expression of the sodium pump isoforms: while no differences were noted between LV, RV, and S for any sodium pump subunit, α3 and β1 are lower in the atria than in the ventricles or septa, α1 is lower only in the LA, and α2 is lower only in the RA. In comparison, Na^+-Ca^{++} exchanger expression was significantly lower in the LA and RA only when compared to S.

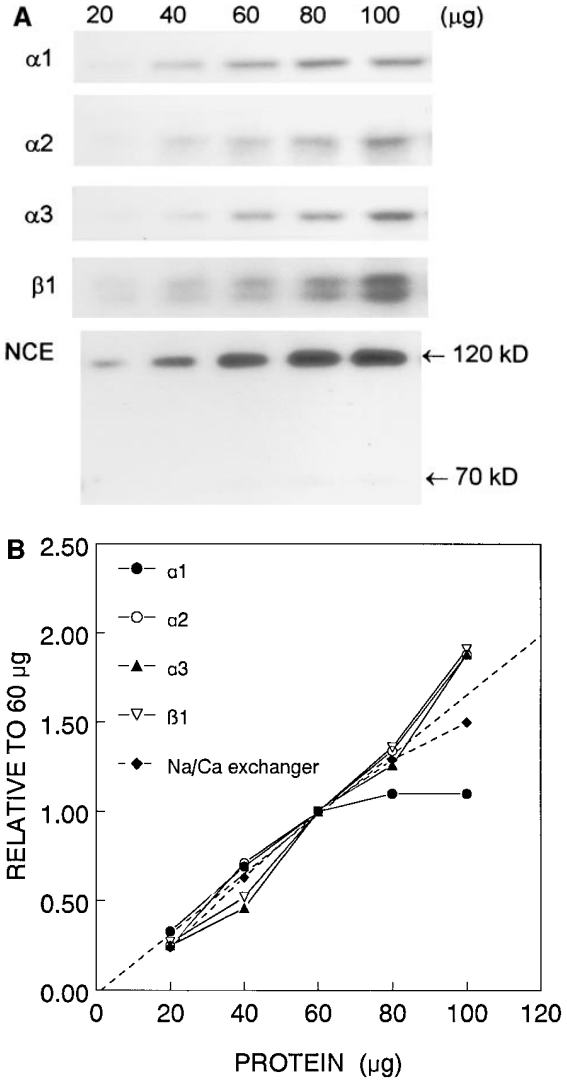

Figure 4. Calibration of detection system. (*A*) Autoradiographic signals as a function of amount of protein loaded. Sets of 20–100 μg of human heart left ventricular homogenate were resolved by SDS-PAGE, blotted, probed with subunit specific antibodies (π used to detect Na/Ca exchanger), and visualized with ^{125}I-protein A. (*B*) Densitometric data were expressed relative to the autoradiographic signal from 60 μg homogenate, arbitrarily defined as 1.0. A dotted line with a slope of 1.0 is included for reference.

Regional expression of sodium pump activity and ouabain binding. Sodium pump activity and maximal ouabain binding (B_{max}) were assayed in nine of the same hearts to provide functional measures of the sodium pump in the five regions. These data were summarized without normalizing, since they are expressed in absolute measures. Na,K-ATPase activity was significantly lower in both LA and RA compared to S (Fig. 6*A* and Table II), in agreement with the lower atrial Na,K-ATPase activity measured in guinea pig hearts (10). ^{3}H-ouabain B_{max} was significantly lower only in RA compared to S, even though the mean values in LA and RA were very close (Fig. 6 *B* and Table II). Schmidt et al. (33) reported that in porcine and canine hearts, ouabain binding site concentration was 1.4–2.5 times larger in the ventricles than in the atria, similar to the difference we measure in human hearts. The *K*d for ouabain was not

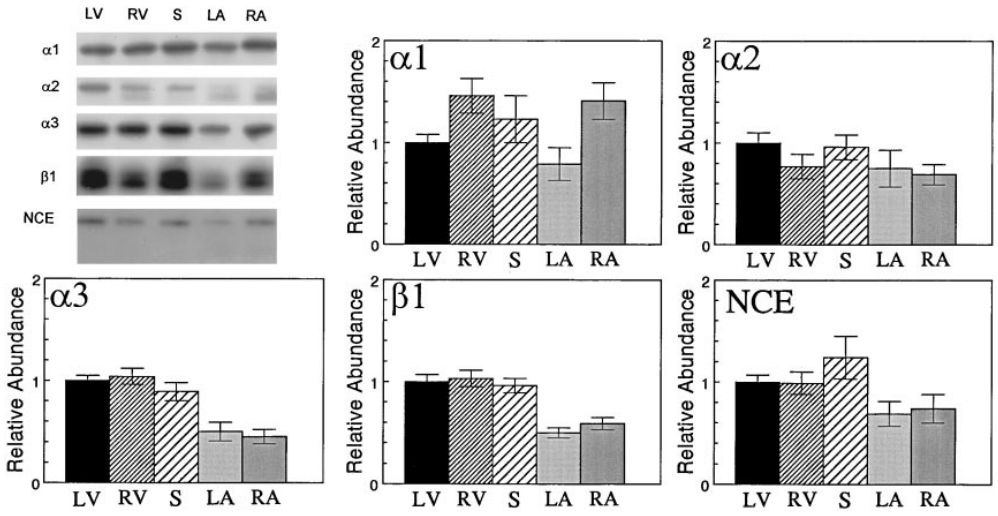

Figure 5. Regional expression of sodium pump α and β subunits and Na/Ca exchanger (*NCE*) in a nonfailing human heart. In the first panel, representative autoradiograms of regional expression of sodium pump subunits and NCE in nonfailing human heart. Samples shown are 25 μg/lane for Na,K-ATPase α and β subunit analysis, and 50 μg/lane for NCE analysis from the following regions: LV, RV, S, LA, and RA. Samples were resolved by SDS-PAGE, blotted, probed with the specific antibodies described in the legends to Figs. 1–3 (Na^+-Ca^{++} exchanger probed with C2C12), and visualized with ^{125}I-protein A. Each panel represents five regions from a single heart. Autoradiograms from each heart were quantitated by scanning densitometery. The data, expressed as mean±SEM, were collected in arbitrary units, processed as described in Methods, and normalized to LV protein abundance defined as 1 within each heart, $n = 14$. Statistical significance was assessed by analysis of variance, and results are summarized in Table II.

region specific, ranging between 10.65±2.24 nM in RV and 11.09±2.01 nM in RA. These functional results correlate with the lower levels of α3, α2, and β1 measured in the atria, and contrast to the higher levels of α1 in the RA.

The significant correlation between sodium pump activity and ouabain binding B_{max} ($r = 0.26$, $P < 0.0005$) is illustrated in Fig. 7 *A*. While no significant correlation emerged between individual α subunits and Na,K-ATPase activity or ouabain binding, there was a significant correlation between Na,K-ATPase activity and β1 expression ($r = 0.47$, $P < 0.0006$) (Fig. 7 *B*). This relationship supports the notion that β1 pool size is a good indicator of the sum total of α1β1, α2β1, and α3β1 heterodimers.

Regional sensitivity to the inotropic actions of BDF 9148. Inotropic sensitivity of the myocardium to a Na^+ channel activator will be a function of the difference between the sodium entry rate governed by the number of sodium channels, as well as active sodium extrusion rate governed by the number of sodium pumps. This difference will ultimately determine the transmembrane Na^+ gradient driving the Na^+-Ca^{++} exchanger (34, 35). We tested the prediction that the lower levels of Na,K-ATPase activity, α3, and β1 and Na^+-Ca^{++} exchanger in the atria would be associated with an increased sensitivity to the Na^+ channel activator BDF 9148, compared to ventricles. The clinical and hemodynamic data of the nonfailing hearts

Table II. Summary of Significant Differences in Regional Expression of Sodium Pump Subunit and Na^+-Ca^{++} Exchanger Abundance, Na,K-ATPase Activity, and Ouabain Binding in the Human Heart

Measurement	Comparison	*P*
α1	LA vs. RV	0.008
	LA vs. RA	0.02
α2	RA vs. LV	0.007
α3	LA or RA vs. LV, RV, S	0.0001
β1	LA or RA vs. LV, RV, S	0.0001
Na,K-ATPase activity	LA, RA vs. S	0.01
Ouabain binding	RA vs. S	0.01
Na^+-Ca^{++} exchanger	LA or RA vs. S	0.0004

The comparisons refer to the data summarized in Figs. 5 and 6, conducted as described in Methods.

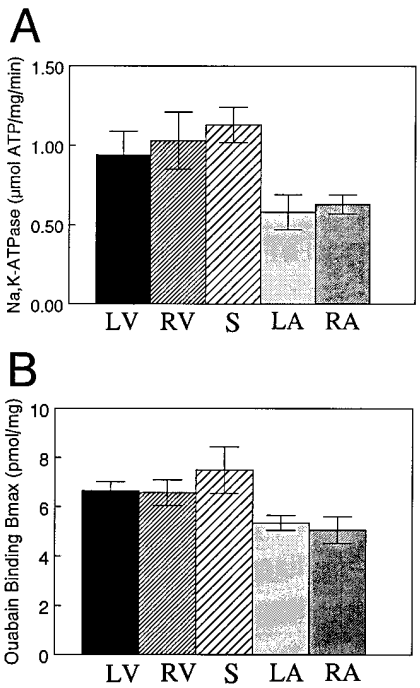

Figure 6. Regional distribution of Na,K-ATPase activity and maximal ouabain-binding capacity in the nonfailing human heart. (*A*) Na,K-ATPase activity measured with a NADH-coupled assay, as described in Methods. (*B*) Maximal ouabain-binding capacity (B_{max}) was measured with ^{3}H-ouabain under the conditions described in Methods. Legend is the same as in Fig. 5. Data are expressed as mean±SEM, $n = 9$. Statistical significance was assessed by ANOVA, and the results are summarized in Table II.

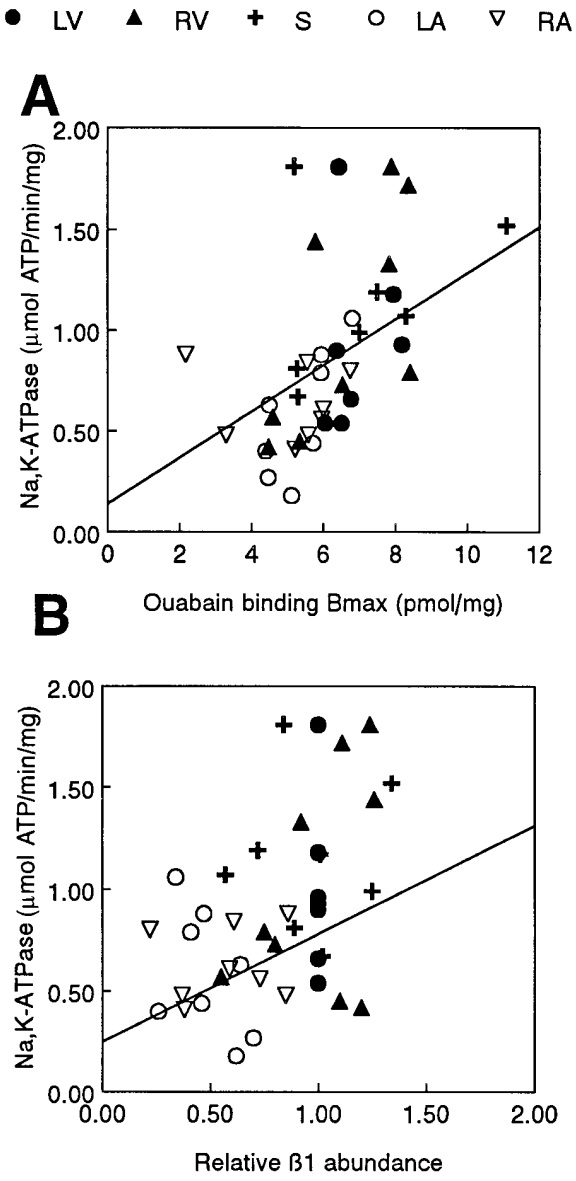

Figure 7. Relationships among Na,K-ATPase activity, maximal ouabain-binding capacity (B_{max}) and Na,K-ATPase β1 protein abundance in nonfailing human hearts. (*A*) Na,K-ATPase activity vs. B_{max}, $r = 0.53$, $P = 0.0005$. (*B*) Na,K-ATPase activity vs. the relative β1 protein abundance, $r = 0.47$, $P = 0.0006$.

studied are summarized in Table III. The ejection fraction and basal force of contraction of the hearts used for atrial vs. ventricular studies were not significantly different. However, BDF 9148 was more potent at increasing the force of contraction in atrial muscle strips (Fig. 8). The EC_{50} values were 0.36 μM (95% confidence limits, 0.21–0.61) in atrial strips and 1.54 μM (95% confidence limits, 0.96–2.47) in ventricular strips. There was no significant difference in the maximal positive inotropic effect of BDF 9148 in the atrial vs. ventricular muscle (Table III). These results support the prediction that the lower levels of sodium pump and Na^+-Ca^{++} exchanger expression in the atria contribute to increased sensitivity to inotropic stimulation; however, the possibility that the increased sensitivity is also a function of a difference in channel abundance between the regions cannot be excluded.

Table III. Clinical and Hemodynamic Data of Patients Studied

	RA	LV
Number/patients	7/3	5/4
Female/male	2/1	2/2
Age (yr)±SEM	56.3±3.5	38.5±2.8
EF (%)±SEM	73.3±6.3	75.8±1.5
Basal FOC (mN)±SEM	1.9±0.5	1.3±0.4
Max PIE (mN)±SEM	4.3±0.3	5.3±1.2

EF, ejection fraction; basal FOC (mN) basal force of contraction in millinewtons; *max PIE*, maximal positive inotropic effect in mN.

Discussion

Sodium pump isoforms expressed in the human heart. Cardiac glycosides are widely prescribed to increase contractility of the failing human heart, and it is known that sodium pumps are specific receptors for these drugs. While cardiac glycosides bind to the α-catalytic subunit (6), the β isoform can influence the kinetics of ouabain binding (36). The existence of three α-catalytic subunits and two β-glycoprotein subunits indicated the possibility for six αβ heterodimer combinations with six different affinities for cardiac glycosides. While cardiac β1 has been detected in every species examined, including the human hearts examined in this study, β2 protein was detected at only negligible levels (Fig. 2). Thus, this study establishes that there are apparently only three significant heterodimers in the human heart: α1β1, α2β1, and α3β1. A corollary to this conclusion is that β1 can be used as an indicator of total sodium pump pool size.

Previous investigation of sodium pump subunits at the protein level in the human heart is limited to a single sample of LV in which there was strong labeling of α1 and faint detection of α2 and α3 (β was not studied; 20). Our results also indicate low levels of α2 expression, but suggest that both α3 and α1

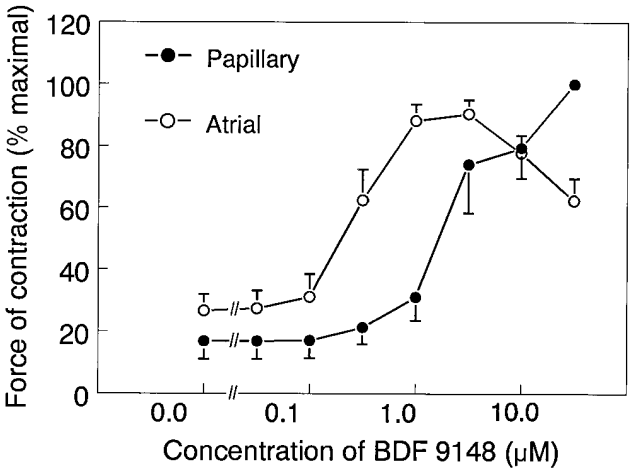

Figure 8. Concentration–response curves for the effects of the inotropic sodium channel activator BDF 9148 on the force of contraction in isolated, electrically driven atrial and papillary muscle strips from nonfailing human hearts isolated and assayed as described in Methods. Basal force of contraction and maximal positive inotropic effect are provided in Table III.

are expressed at significant levels in the human heart, based on the relative levels of expression in human heart vs. human brain (Fig. 1). Expression of all three α isoforms is also observed in the primate heart, where α2 expression is likely to be even lower than in the human heart (12).

The regional expression of sodium pump isoforms. Using ouabain binding to assay the number of regional sodium pumps in intact myocardial samples of porcine and canine hearts, Schmidt et al. (33) determined that there were 50% fewer sodium pumps in the atria than in the ventricles or septa, and Wang et al. (10) measured lower Na,K-ATPase activity in guinea pig atria compared to ventricles. The current study shows that Na,K-ATPase activity was nearly 50% lower in atria than ventricles in the human heart as well (Fig. 6 *A*), even though the magnitude of difference in ouabain binding was much smaller. Only β1 and α3 levels were 40–50% lower in the atria than in the ventricles or septum, leading us to postulate that the lower levels of Na,K-ATPase and ouabain binding measured in the atria are caused by lower levels of α3β1 (Fig. 5, Table II). Lower expression of α3, not α1, in atria was also observed in a dissected macaque heart (12).

A comment is warranted regarding the respective advantages and disadvantages of the various assays of myocardial sodium pumps. Ouabain binding and Na,K-ATPase activity measure only functioning sodium pumps that are capable of phosphorylation–dephosphorylation cycling; pumps that are acutely or chronically inhibited are not measured. In addition, isoform specificity of these assays has not been established in human samples. Ouabain binding to intact cells or bits of myocardium (33) detect only functioning pumps in the sarcolemma. Acute redistribution of sodium pumps between plasma membrane and intracellular pools has been demonstrated in skeletal muscle (37) and remains a possibility in the heart. In contrast to ouabain binding, immunoblot analysis of homogenates measures the total pool of a specific sodium pump isoform subunit, whether enzymatically active or assembled into functional heterodimers or not, and whether inside the cell or on the surface. Using these assays in concert provides complimentary information. For example, since Na,K-ATPase activity, ouabain binding, and α3 and β1 expression are all lower in the RA than in the S while α1 and α2 are not, one can postulate that α1 and α2 are minor isoforms in the atria and that α3 predominates, or alternatively, that there are nonfunctional pools of excess α1 and α2 subunits in the atria.

Na^+-Ca^{++} exchanger in human heart. The regional pattern of expression of Na^+-Ca^{++} exchangers in the human heart is similar to that of α3, β1, Na,K-ATPase activity, and ouabain binding: equivalent in ventricles and septum and lower in the atria compared to the septum. The ratio of expression of sodium pumps to exchangers is likely to influence cell calcium stores and contractility (12, 26, 38). The lower levels of both sodium pumps and Na^+-Ca^{++} exchangers in the atria suggest that the rates of uphill sodium and calcium efflux would be less in the atria than in the ventricles. We predict that while homeostasis could be maintained under normal circumstances, the atria would be more sensitive to inotropic stimulation than the ventricles because of reduced capacity to restore intracellular sodium and calcium levels. In fact, the lower Na,K-ATPase activity measured in guinea pig atria is associated with increased intracellular Na^+ activity, even in quiescent muscles (10).

Regional sensitivity to inotropic drugs. The force of myocyte contraction is a function of cell calcium stores. The inotropic drug BDF 9148 increases the force of contraction by prolonging the open state of the Na^+ channel, increasing sodium influx relative to efflux via the sodium pumps thus elevating intracellular Na^+, which increases the driving force for Ca^{++} uptake and decreases the driving force for Ca^{++} extrusion via the Na^+-Ca^{++} exchanger. Calcium content of the sarcoplasmic reticulum is increased, leading to increased Ca^{++} release during depolarization and increased force of contraction (39). The lower sodium pump expression observed in the atria suggested that this region would be more sensitive to this inotropic drug than ventricles, and the somewhat lower Na^+-Ca^{++} exchanger expression in the atria would amplify this effect. Indeed, the potency of BDF 9148 to increase the force of contraction was four times higher in atrial than ventricular muscle (Fig. 8). The critical determinant may not be lower levels of pumps and exchangers in the atria per se, but rather, a higher ratio of sodium channels to sodium pumps. Information on the relative abundance of sodium channels in atria vs. ventricles in the human heart is not available at this time. Enhanced sensitivity may also result from a lower driving force for Na^+-H^+ exchangers, which would lead to intracellular acidification and increased calcium sensitivity of the myofilaments (40). Finally, sensitivity may be influenced by cell volume considerations, but while the atria have a smaller diameter than ventricular cells, they also have less T tubules, resulting in a similar ratio of surface area to cell volume in atrial and ventricular cardiac myocytes (41).

The lower levels of sodium pumps in the atria predict that the sensitivity to cardiac glycosides will be higher than in the ventricles because the inhibitors would further lower the capacity of the atria to pump sodium and transport calcium out of the cells. In fact, the potency of dihydroouabain is threefold higher in guinea pig atria, which have significantly lower Na,K-ATPase activity compared to ventricles (10), and the time needed to achieve maximal force in the presence of 0.1 μM ouabain is significantly shorter in atrial than ventricular muscle from human heart (42, 43).

The existence of isoforms suggests the potential for differential function, location, and regulation; however, the significance of specific α isoforms in the heart remains to be established. While there are no significant differences in α or β isoform subunits or Na^+-Ca^{++} exchanger pool sizes between the left and right ventricle or septum, there may be differences at the cellular level, e.g., conduction tissue vs. nonconducting tissue (13, 44) or subcellular level in peripheral sarcolemma vs. T tubule membranes, as in the rat (5, 45). α subunit expression in the heart is variable among species: α1 is expressed ubiquitously, and neither α2 nor α3 are expressed in all hearts: adult rat and mouse express α1 and α2 (α3 expression is primarily restricted to the neonatal heart), dog and ferret express α1 and α3, and sheep and guinea pig may express just α1 (reviewed in reference 12). The relationship among cardiac glycoside therapy, inhibition of the three isoforms expressed in human heart, and increased cardiac contractility remains to be defined.

Acknowledgments

Our special thanks go to Prof. Dr. B. Reichart (Department of Cardiac Surgery, University of Munich; Munich, Germany); Prof. E.R. De Vivie (Department of Cardiac Surgery, Universität zu Köln, Köln, Germnay), and their colleagues for providing the myocardial tissue, as well as to Dr. M. Levy (Department of Neurosurgery, University of Southern California Medical School), for providing the brain tissue.

This work was supported by a fellowship award to J.N. Wang from the American Heart Association, Greater Los Angeles Affiliate, the National Institutes of Health (HL 44404 and DK 34316 to A. McDonough), the Deutsche Forschungsgemeinschaft (DFG Schw 607/1-1), and the Zentrum für Molekulare Medizen der Universität zu Köln (ZMMK to R.H.G. Schwinger).

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