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Immunodetection and enzymatic characterization of the α3-isoform of Na,K-ATPase in dog heart

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Abstract

The expression of the canine α 2 and 3 subunit isoenzymes of NA,K-ATPase has been investigated in plasma membranes isolated from dog heart, brain and kidney by immunoblotting, employing polyclonal anti rat fusion protein, and enzymological techniques. Western blot analysis revealed with purified dog membrane Na,K-ATPase preparations, one immunoreactive signal with rat specific α_3 antisera in cardiac tissues, and two immunoreactive signals with rat α_2 and α_3 antisera in cerebral tissues. These findings suggested the specific expression of α_3 polypeptide in dog heart (99 kDa), whereas dog brain expressed the α 2 and 3 polypeptides. The stained bands were superimposed. The antibody to rat brain α_1 fusion protein did not cross-react with dog antigens whatever the three tissues tested. Expression of the α_3-subunit isoform in dog heart membranes was consistent with a high affinity digitoxigenin-sensitive class of Na,K-ATPase (IC_{50} = 7 ± 2 nM). A single component with low affinity to digitoxigenin (IC_{50} = 110 ± 10 nM) characterized the α_1 kidney form. The mixture of α_2 and α_3 isoforms in dog brain exhibited an apparent affinity for digitoxigenin (IC_{50} = 17 ± 5 nM) lower than the heart. The sodium dependences of the high affinity digitoxigenin sites were for the cardiac $\alpha_3$form ($K_{0.5}$ = 10 ± 1.9 mM) and for the cerebral α_2 and α_3 mixture ($K_{0.5}$ 19.6 ± 4.9 mM). The sensitivities for Na^+ of the low affinity sites (α_1) were: 6.7 ± 1.4 mM, 6.3 ± 1.2 mM and 11.6 ± 2.9 mM in heart, brain and kidney respectively. This is the first report of the catalytic characteristics of the α3 subunit isoenzyme in canine cardiac plasma membranes.

Key words: Na,K-ATPase; Isoform; Isoenzyme; Heart; Brain; Kidney; Sodium sensitivity; Ouabain affinity

1. Introduction

Na,K-ATPase is a membrane-bound enzyme that couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane. The enzyme exists as a heterodimer consisting of a large catalytic α subunit (100 kDa) and a smaller glycosylated α subunit (55 kDa). Three alpha (α_1, α_2, α_3) and three beta (β_1, β_2, β_3) subunits have been identified from cDNA cloning [1]. The α_1 isoform is only expressed in the kidney, whatever the species considered whereas in the brain, two other isoforms (α_2 and α_3) are concomitantly expressed [2]. However, each of them can be expressed separately in other tissues such as α_2 in the cardiac and skeletal muscle [2], α_3 in the pineal gland [3], and macrophages [4]. In the heart throughout development, the neonatal α_3 isoform declines rapidly and is replaced by α_2 in the adult [5–7]. In an opposite way, the adaptative response of the heart to pressure overload by hypertrophy consists principally of an expression of the neonatal α_3 isoform [8,9]. Recently, it has been shown that neonatal and adult ferret heart express simultaneously the α_3 isoforms and not the α_2 isoforms [10]. The relationship between the expression of isoforms of Na,K-ATPase and species or tissues is still poorly understood. This issue seems to be important since we and other recently demonstrated expression of the three functional α isoforms in human heart in approximately equal amounts [11,12].

The most striking difference observed among the three isoforms is in their apparent affinity for Na^+. The ouabain-sensitive isoform with a very low sensitivity to Na^+ found by different investigators in rat brain [13–15] and in rat pineal gland [3] is either the α_3 isoform or the α_2 isoforms depending on the system of enzyme expression. Two biochemically distinct isoforms of the α subunit (α and α^+) have been described in dog heart through their inhibition profile with digitalis drugs and their mobilities on SDS-PAGE [17]. Since it was immunologically demonstrated that the α^+ form could contain a mixture of α_2 and α_3 isoforms [2], it is our objective to further investigate in dog heart and brain the nature and function of these two isoforms. We have used the panel of antibodies specific for the three rat α subunit isoforms [18] in Western blot analysis of canine microsomal fractions from kidney, brain and heart. In these 3 tissues, functional differences were investigated in terms of sensitivity to digitalis and Na^+.

2. Materials and methods

2.1. Microsomal preparations from canine tissues

They were obtained from sodium pentobarbital (30 mg/kg) anesthetized adult mongrel dogs of either sex (3 males and 3 females; 10–15 kg). The same rapid isolation procedure [19] (less than 2 h) was applied to

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the different tissues to eliminate a possible selective loss of one isoform during purification, except for the first step. The first step used 10 g of cardiac ventricles, 5 g of whole brain, 5 g of outer medulla kidney. The tissues were minced and homogenized in 10 (cardiac)–20 volumes (brain and kidney) of ice-cold buffer containing 20 mM sodium pyrophosphate, 0.1 mM phenylmethane sulfonyl fluoride, 250 mM sucrose, 80 mM KCl and 20 mM imidazole-HCl (pH 7.4 at 25°C) with a polytron PT.20 (10 s; setting 5) for muscle (5 s; setting 5) for brain and kidney. For the three tissues, the homogenates were subfractionated by two sequential differential centrifugations at 7,000 × *g* for 15 min and 46,000 × *g* for 30 min. The pellet was resuspended in 100 mM NaCl, 250 mM sucrose and 30 mM imidazole-HCl pH 7.4 and stored frozen in liquid N_2. The yields in mg of proteins from homogenates to microsomes (approximately 1–2%) were similar for the different tissues. Protein concentration was determined by the method of Lowry [20].

2.2. Microsomal preparations from rat tissues

The methods for the rat brain membrane preparations were those previously described [21]. The specific activities of the Na,K-ATPase in those preparations were 90 ± 15 μmol P_i/mg protein × h.

2.3. SDS-PAGE and Western blots

Microsomal preparations from kidney, brain, and cardiac muscles were electrophoresed and immunoblotted with polyclonal antisera. The samples were reduced with 20 mM dithiotreitol and solubilized by SDS (2%).

The samples (from 10 to 60 μg) were resolved in a 6–12% linear polyacrylamide gradient. After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose sheets at 100 V for 90 min in 25 mM Tris, 192 mM glycine, 20% methanol according to the method described previously [22]. Saturation of the blot was performed by incubation overnight at 4°C in phosphate-buffered saline (PBS): 0.1 M Na-Phosphate, 0.5 M NaCl pH 7.4, containing 3% fat-free milk. The nitrocellulose sheets were incubated for 2 h at 37°C with α1, 2 and 3 anti-Na,K-ATPase antisera. Blots were incubated with biotinylated-donkey anti-rabbit IgG (dil. 1/400) (Amersham) washed three fold with the PBS fat-free milk 3% then followed by horseradish peroxydase conjugated to streptavidin (dil. 1/1000) (Amersham) followed by 2 washings with TBS (0.5 M NaCl 20 mM, Tris-HCl pH 7.5). Specific interactions were detected with treatment with 15 mg of 4-chloro-1-naphtol (Bio-Rad) dissolved in 5 ml of ice-cold methanol and added to 25 mg of TBS containing 0.002% hydrogen peroxide. Peroxidase activity was stopped by acetic acid 1% (v/v). Specific antisera were purchased from Upstate Biotechnology Inc., (New-York), anti-rat Na,K-ATPase α_1, lot no. 10452; anti-α_2, lot no. 10453; anti-α_3, lot no. 10454. A monoclonal antibody specific for α_2 isoform was kindly provided by Kathleen Sweadner (Harvard University, Cambridge, MA).

2.4. Enzyme assays

The enzymatic assays were performed with vesicles treated with SDS (0.2–0.3 mg/mg of protein for 30 min at 20°C).

The Na,K-ATPase activity was measured at 37°C in an ATP regenerating medium by continuously recording NADH oxidation with a coupled assay method using a Beckman DU70 spectrophotometer [17,19]. Each cell contained 100 mM NaCl, 2 mM phosphoenolpyruvate, 10 mM KCl, 4 mM ATP, 4 mM $MgCl_2$, 30 mM imidazole-HCl (pH 7.4), 0.4 mM NADH, 3.5 units pyruvate kinase and 5 units of lactate dehydrogenase. The enzymatic reaction was initiated by the addition from 0.5 to 5 μg of protein. The Na,K-ATPase activity was defined as the activity inhibitable by 0.5 mM ouabain or digitoxigenin. When the decrease in absorbance was linear versus time, this indicated that a new steady-state level between drugs and Na,K-ATPase had been reached. The specific activities of the Na,K-ATPase were respectively 28 ± 9, 70 ± 13 and 65 ± 12 μmol P_i/mg protein × h for dog heart, brain and kidney microsomes. Inhibition percentages were calculated by comparing the activities in the presence or absence of digitoxigenin after correcting for the ouabain-insensitive ATPase activity. The experimental data were fitted using ENZFITTER (Biosoft, Elsevier). The following model was used:

$$V = V_{max}C/(IC_{50} + C) \quad (1)$$

where V is the observed velocity at a given inhibitor concentration (C) and V_{max} is the maximal velocity of the Na,K-ATPase. The best-fit curve was calculated using non-linear regression. The experimental data were also fitted with one or a sum of two functions assuming the presence of one or two sites [19]. Na^+-dependent ATPase activities were assayed with the same microsomal fractions. Modifications in the assay medium were introduced to decrease the Na^+ to 0.6 mM. K^+ salts of the phosphoenolpyruvate and NADH were used instead of Na^+ salts. The pH was adjusted to pH 7.4 with KOH.

Na^+-dependent ATPase activity was taken as the difference in activity in the absence and presence of 0.5 mM ouabain. Assays were performed in the absence and presence of 100 nM and 0.05 mM digitoxigenin to rapidly inactivate the high and low affinity isozymes, respectively. The curves were calculated using an equation derived from Michaelis–Menten kinetics with a model of highly cooperative sites (Equation 2).

$$A = A_{max}[Na^+]^3/(K + [Na^+]^3) \quad (2)$$

where A was the activity at various concentrations of Na^+, A_{max} was the maximal value of A. The $K_{0.5}$, i.e. the substrate concentration at which the Na,K-ATPase activity was half maximal, could easily be calculated from the value of K in Equation 2 as $K_{0.5} = \sqrt[3]{K}$.

3. Results

3.1. Localization of the Na,K-ATPase α_2 and 3 isoforms in dog tissues

Fusion protein antisera to α_1, α_2 and α_3 were used to analyze by immunoblotting the corresponding polypeptide expression in microsomes prepared from adult dog heart. The α_3 fusion protein antiserum stained a single polypeptide band of 99 kDa (apparent molecular mass) in dog heart and brain whereas the antiserum α_2 reacts with a polypeptide of 99 kDa only in dog brain (Fig. 1). With the antisera used, no α_1 subunit was detectable in the different dog membrane preparations whatever the amount of protein used (from 10 to 60 μg) (data not shown). The two polyclonal anti-rat brain α_2 and α_3 fusion proteins were specific only for the α_2 and α_3 bands with apparent molecular weights around 100 kDa, since we did not observe any staining of other protein with anti α_2 and 3 on the blots. We verified the specicifity of the α_3 antisera by demonstrating that this antibody does not recognize the canine α_2 isoform present in skeletal muscle (data not shown). Another α_2-specific monoclonal antibody, McB2*, showing specificity for the α_2 canine cerebral isoforms did not react with the α^+ isoform of dog heart (data not shown). These results indicate that α_3 subunit isoform is expressed in dog heart muscles, and α_2 and α_3 subunits coexist in brain. These isoforms are expressed in a tissue-specific fashion.

3.2. Enzymatic properties of the α_2 and α_3 subunits isoenzymes (comparison to α_1 kidney form)

We took advantage of the predominance of α_1 in the kidney and α_3 in the heart to compare the functional properties of Na,K-ATPase isoenzymes.

*McB2 is a monoclonal antibody specific for α2 with broad species cross-reactivity.

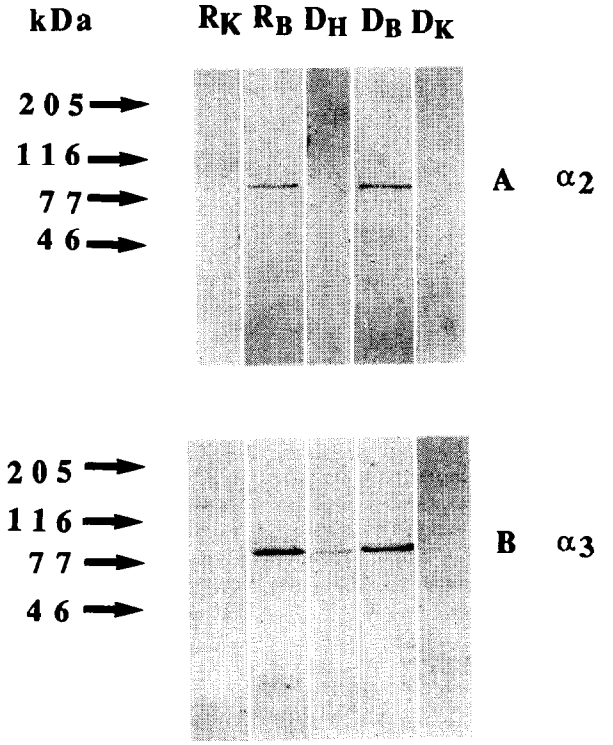

Fig. 1. Expression of Na,K-ATPase α subunits in dog cardiac microsomes. Solubilized microsomal proteins were fractionated by electrophoresis through an SDS-containing 6–12% polyacrylamide gel, then transferred to a nitrocellulose filter and probed with the rat α subunit specific antisera indicated on the right. The positions of the molecular mass markers are indicated on the left. Rat brain and kidney microsomes were run in control lanes. The identification of the lines are: R_K, rat kidney; R_B, rat brain; D_H, dog heart; D_B, dog brain and D_K, dog kidney.

3.3. Sensitivity to digitoxigenin

We compared digitoxigenin-sensitive Na,K-ATPase activity in microsomal membrane fractions from dog heart, brain and kidney.

The results normalized in percent of Na,K-ATPase activity for each membrane fraction and fitted by least squares analysis (Equation 1) are presented in Fig. 2.

Microsomes from dog kidney exhibited, whatever the one to three-site model, a single component of digitoxigenin-sensitive Na,K-ATPase activity with a contribution of 90% of the total activity. IC_{50} was 0.10 ± 0.01 μM.

Data obtained from dog cerebral and cardiac microsomes were best-fitted by a two-site model (IC_{50} I = 17 ± 5 nM and IC_{50}II = 1.3 ± 0.5 μM), and (IC_{50}I = 7 ± 2 nM and IC_{50}II = 0.63 ± 0.17 μM), respectively, suggesting at least two components of ouabain sensitive Na,K-ATPase activity. This profile was found similar for heart and aorta. A three-site model described and used for the rat brain membrane data analyses [23–25] was unsuccessful in discriminating the cerebral α 2 or 3 isoforms (data not shown).

3.4. Na ± affinity

To ascertain whether there are other changes in the pump characteristics, a kinetic study was performed to estimate the apparent affinity of the Na,K-ATPase for Na^+. The requirement of isoenzyme was determined by measuring ATPase activity at a constant K^+ (20 mM) and varying Na^+ (0.6–100 mM) concentrations. To distinguish between the two α forms of the Na,K-ATPase, two different concentrations of digitoxigenin have been chosen [24,25] and (see Fig. 2); a low concentration (100 nM) which inhibits almost all the high affinity sites (93%), and a few percent of the low affinity sites; a high digitalis concentrations (50 μM) inhibits the whole activity. By difference between the two digitoxigenin concentrations, this activity principally yielded the α_1 subunit. The discrimination by digitoxigenin reveals a different pattern of Na^+ affinity for heart and brain (Fig. 3 and Table 1). In heart, two Na^+ affinities of 6.7 and 10 mM were attributed to the α_1 and α_3 subunits. For brain enzymes which contain in addition to the α_1 and α_3 cardiac subunits the α_2 isoform, the high affinity inhibitory sites to digitoxigenin (the α_2 and α_3 subunits) exhibits a $K_{0.5}$ for Na^+ comprised between 15–25 mM, and a $K_{0.5}$ around 6 mM for the low affinity sites to digitoxigenin.

4. Discussion

We took advantage of antisera specific for the α 2 and α3 subunits of the Na,K-ATPase to identify for the first

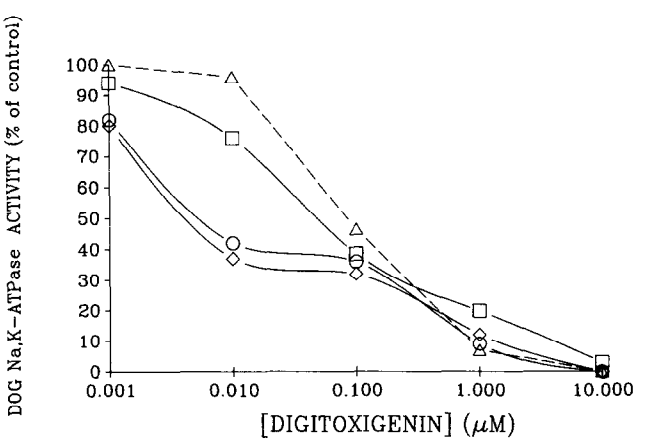

Fig. 2. Na,K-ATPase inhibition by digitoxigenin in microsomal membranes from dog brain, heart, aorta and kidney. Data are expressed as percent of total Na,K-ATPase activity relative to V_{max}. The V_{max} are respectively 28 ± 9, 70 ± 13 and 65 ± 12 μmol $P_i \times mg^{-1}$ protein $\times h^{-1}$ for dog heart, brain and kidney preparations. All, Na,K-ATPases were normalized to V_{max} of the 0.5 mM ouabain-sensitive activity. Parameters were estimated by using Equation 1 (section 2). These plots represent one experiment, each repeated at least three times with comparable results with three separate membrane preparations. For heart and brain, the curves represent the best fit of the data obtained from a two-site model whereas a one-site model was used for kidney. The IC_{50} values are listed in Table 1. Brain (B) is denoted by -□-; heart (H) by ◇; kidney (K) by △; Aorta (A) by ○.

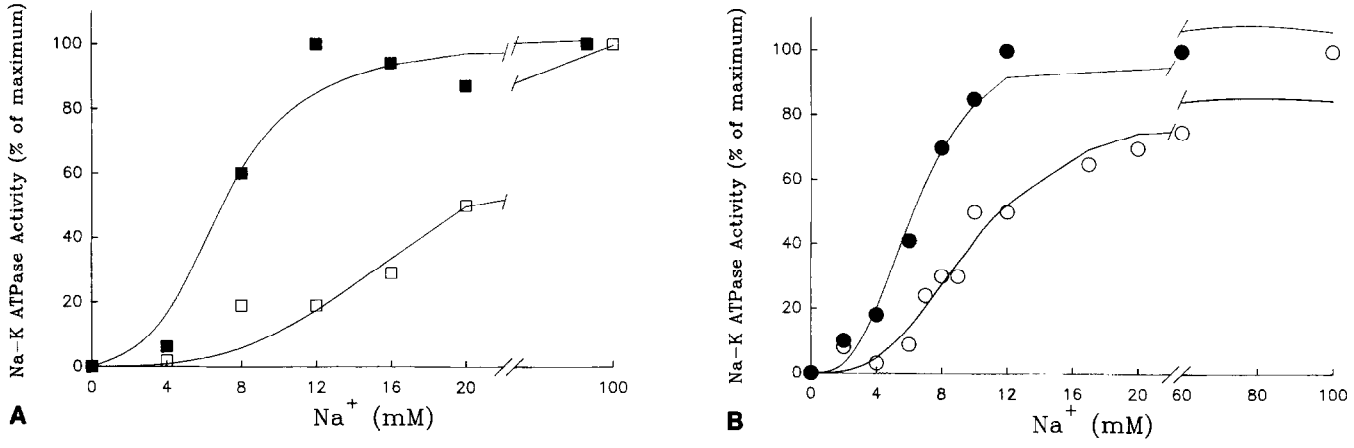

Fig. 3. Sodium dependencies of Na,K-ATPase activity in microsomal membranes from dog heart and brain. The rate of ouabain inhibitable was measured at 37°C using the enzyme-coupled spectrophotometric assay. (A) Brain: (-□-), ATPase activity is expressed as percent of maximal activities measured in the presence of 100 nM digitoxigenin; and (-■-), ATPase activity between 100 nM and 50 μM digitoxigenin. (B) Heart: (-○-), measured in the presence of 100 nM digitoxigenin; and (-●-) measured between 100 nM and 50 μM digitoxigenin. Symbols are normalized values from three separate experiments. Two separate membrane preparations were assayed. Parameters (presented in Table 1) were estimated by using Equation 2 (section 2). The curves represent the best fit of the data to a model of highly cooperative sites.

time in dog heart membranes the presence of a single immunoreactivity with the antisera specific for α3. Furthermore there is evidence for a tissue heart (vs. brain) specific pattern of expression of the isoforms.

It is possible that anti α_3 and anti α_2 antibodies raised against one rat form (α 2 or 3) crossreact, to some extent, with the two dog antigens. We can rule out this hypothesis, since we demonstrated that these antibodies always react against one polypeptide (Fig. 1). Furthermore in dog, the α_2 presence in smooth muscles has been identified with the rat antibodies [26].

Another possibility would be that the antibodies specific for rat α_3 subunit lose their specificity for this subunit isotype in the dog. This leads to a characterization of the dog α_2 subunit both in the presence of α_3 and α_2 subunit specific antisera. In this study, it cannot be excluded on the basis of a different molecular weight like in rat brain, since a very similar apparent molecular weight of 98–99 kDa was found. However, in dog brain this seems unlikely since monoclonal α_3 antibody and anti-α_3 specific antibodies derived from rat cloned fusion proteins has been found to only cross react with α_3 subunits in bovines [27] and humans [28]. Besides the proved existence of α_3 isoform with the Northern blot criteria is not confined to rat brain or nerves since α_3 mRNA has been clearly demonstrated in the human heart [29,30]. More recently in the ferret heart, the peptide mapping of the α_3 immunological signals has been shown to correspond to the α_3 and not to the α_2 subunit [10]. An unambiguous and direct proof in dog will be provided by peptide mapping analysis of α_2 and α_3 polypeptides after electrophoresis, and by Northern blot analysis with the α_3 subunit cDNA fragments used as probes.

The presence of α_3 subunit polypeptides might result from contaminations of our membranes by nerve membranes or conducting tissues containing significant amounts of the α_3 subunit [2,31]. This possibility should be excluded because predominant source of proteins in

Table 1
Sodium and digitoxigenin affinities of α_1, α_2 and α3 in canine brain, heart and kidney membranes

Tissue	α subunit isoform	Digitoxigenin			Sodium	
		V_{max} (%) H.A.	IC_{50} (nM) H.A.	IC_{50} (μM) L.A.	$K_{0.5}$ (mM) H.A.	$K_{0.5}$ (mM) L.A.
Heart	α_1, –, α_3	67	7 ± 2	0.63 ± 0.17	10.0 ± 1.9	6.7 ± 1.4
Brain	α_1, α_2, α_3	68	17 ± 5	1.3 ± 0.5	19.6 ± 4.9*	6.3 ± 1.2
Kidney	α_1, –, –	100	110 ± 10	–	11.6 ± 2.9	–

All data were analyzed by the non-linear least squares procedures. The IC_{50} and V_{max} result from the analysis with a two-site model for heart and brain and a one-site model for kidney. $K_{0.5}$ values are the best-fit of the curves (presented in Fig. 3) according to a model with 3 non-interacting Na^+ ions required for activity. H.A. (high affinity) and L.A. (low affinity) were defined by the Na,K-ATPase activity inhibited at 100 nM digitoxigenin and 50 μM ouabain respectively.

*Digitoxigenin inhibitable activity of mixture of α_2 and α_3 subunits.

our membranes are provided by cardiac cells far more abundant by their volume than the other cell type. In addition, we have already demonstrated that cardiomyocytes isolation without contamination by nerve cells prior to membrane purification does not show a difference in the electrophoretic pattern at the level of the α subunit doublet of the Na,K-ATPase to the purified membranes from the whole heart [17].

On the other hand, we show that there is no cross reactivity between rat specific α_1 antibodies and dog in three tissues tested: heart, brain and kidney. This would mean that the epitope constituted by the rat α_1 subunit might present a species specificity. This has already been evidenced for other antibodies specific for rat α_1 subunit [2,10].

Recent studies have demonstrated substantial differences in the functions of α_2 and α_3 isoforms. Immunochemical demonstration of α_3 subunit of Na,K-ATPase in dog heart is consistent with previous studies that described the expression of high affinity ouabain-sensitive class of Na,K-ATPase. If isoforms in each tissue possess the same substrate and inhibitor dependence properties, it is tempting to speculate about the relationship of isoforms to the enzymatic properties. The comparison of the IC_{50} and contributions between brain, heart and kidney, reveals differences that suggest that the dog brain α_2 isoenzyme, although not discriminated by a three site model, present a lower digitoxigenin sensitivity than the α_3 high affinity component of ouabain-sensitive Na,K-ATPase activity present in dog heart. This is in agreement with ouabain-binding studies done in rat brain and heart membranes [32].

Concerning the Na^+ affinity, it is often reported in the literature that the Na,K-ATPase from mammalian kidney (α_1) had a lower affinity than the enzyme from nervous tissues (α_2 and α_3 isoforms) [13,33,34]. Because the cerebral enzymes are constituted of two α_2 and α_3 entities in rat, the discrimination of the two subtypes α_2 and α_3 and their comparison with the α_1 kidney form show similar and opposite results to previous reports. A lower [15] or a higher [16] affinity to Na^+ for α_3 than α_1 has been observed. Our results partially confirm that the α_3 isoform confers a lower Na^+ affinity than α_1 as described in the first studies.

Our comparison of the low-affinity inhibitory sites for digitalis between organs shows an heterogeneity between the tissues tested, kidney, brain and heart (Table 1). This indicates that parameters may have a large influence on the apparent affinity for sodium. Some of them (α–α composition) have been extensively discussed [15]. Parameters other than the α subunit such as $\alpha\beta$ complexes, environment of the pump [25,35] may have a large influence on the apparent affinity for sodium. One of these is the presence of two possible complexes $\alpha_1\beta_1$ or $\alpha_1\beta_2$ in heart since the β_2 protein was detected in our canine preparations (J.M.M., unpublished observation). So to relate this observed difference of Na^+ affinities with α subunits, we must practise some caution. One important factor is the variation of the apparent affinity with the ionic sidedness of distribution of Na^+ and K^+ found in cells. Our experiments with membranes were done without a membrane potential, under constant ATP, Na^+ and K^+ concentrations. Identical concentrations of each ion were present on both sides of the membranes.

Various combinations of Na^+ and K^+ concentrations that maintain a total concentration of 150 mM shifted the apparent affinity to higher values for the three isoforms (kidney α_1, 46 mM; brain $\alpha_2 + \alpha_3$, 25 mM; and heart $\alpha_1 + \alpha_3$, 40 mM) [13]. Indeed, in vitro we observed that differences could be due to the conditions necessary for the enzymatic assay. For example, the replacement of imidazole by HEPES desensitized the enzyme to Na^+ ions (data not shown). On the other hand, the addition of choline chloride to maintain the iso-osmolarity with dog brain membranes modified the shape and shifted to the left the Na^+ sensitivity curves. This can be explained by a sodium-like effects of imidazole and choline chloride as described by Robinson [36].

The physiological relevance of the substrate difference and distinct distribution of α subunits in dog tissues reinforce those reviewed in [37]. The α_1 form of high affinity for sodium (6 mM) will pump under physiological condition near its $K_{0.5}$ value where maximum changes in activity can be achieved by a minimal intracellular change. In heart, therapeutic concentrations of digitalis produce their inotropic effects through an enhanced sodium transient and a limited myocardial Na^+ accumulation (0.3 mM), whereas toxic concentrations of digitalis lead to at least a ten-fold higher Na^+ accumulation than inotropic doses. Activity of the Na pump is first determined by $(Na^+)_i$, the Na pump isozymes may be differentially and early activated during each cycle of myocardial function [38]. The α_3 form of the enzyme would be maximally active only when beneath the membranes the sodium reaches its maximum following the depolarisation phase. It will be of interest to characterize directly, in the whole cardiomyocyte with intact ionic gradients, the functional relevance of the Na,K-ATPase α-isoforms. Some other physiological relevance of α2 and α3 protein expression in cardiac function can be inferred by the analogy with the fact that the switch between 7 and 15 days after birth is synchronous with the shortening of heart- action potential duration in rat [1] and age-related changes in electromechanical properties such as the higher digitalis tolerance in neonatal dogs as compared with adult dogs [39]. Thus, the α_3 expression in dog might be synchronous with a slow action potential. In heart, the α_1 form has a $K_{0.5}$ for sodium of 6 mM, which means that it participated in sodium extrusion under physiological conditions (Na_{in}: 7 mM). The α3 on the other hand will be maximally active when $(Na^+)_{in}$ reaches its maximum following the depolarisation phase. This could ex-

plain the biphasic increase in $(Na^+)_{in}$ after addition of 1 nM to 100 μM ouabain to isolated chicken myocytes [40] and an enhanced sodium transient and a limited myocardial Na^+ accumulation resulting from $\alpha 3$ inhibition during the positive inotropic effects of digitalis [18].

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