Volume 330, number 3, 297–301 FEBS 12988 September 1993

Turnover rates of the canine cardiac Na,K-ATPases

J.M. Maixent[a], I. Berrebi-Bertrand[b,*]

[a]*Université Aix Marseille II, Faculté de Médecine Secteur Nord, Boulevard P. Dramard, 13916 Marseille Cedex 20, France*
[b]*Laboratoire de Pharmacologie des Transports Ioniques Membranaires, Hall de Biotechnologies, Tour 54, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France*

Received 16 June 1993

Two functional isoforms α (α_1) and α^+ (α_3) of the Na,K-ATPase catalytic subunit coexist in canine cardiac myocytes [J. Biol. Chem. (1987) 262, 8941–8943] The in vitro turnover rates of ATP hydrolysis have been determined in sarcolemma preparations by comparing [^{3}H]ouabain-binding and Na,K-ATPase activity at various doses of ouabain (0.3–300 nM). The correlation between the occupancy of the ouabain-binding sites and the degree of Na,K-ATPase inhibition was not linear. The results showed that the form of low-affinity for ouabain (K_d = 300–700 nM) exhibited a lower turnover rate (88 ± 10 vs. 147 ± 15 molecules of ATP hydrolyzed per second per ouabain-binding site) than the high affinity form (K_d = 1–8 nM). Thus our results indicate this specific isoform kinetic difference could contribute to differences in the cardiac cellular function.

Digitalis; Molecular activity, Heart; Isoform

1. INTRODUCTION

The Na,-K-ATPase (EC 3.6.1.3) is the in vitro manifestation of the Na^+ pump responsible in vivo for the ATP-dependent transport of both sodium and potassium ions [1]. This system specifically inhibited by digitalis, consists of two subunits: a catalytic α-subunit bearing the digitalis binding site, and a glycosylated β-subunit. Three distinct isoforms of the α-subunit (α_1, α_2 and α_3) have been identified by immunological techniques and molecular genetics [2–4].

These isoforms are clearly distinguished by differences in electrophoretic mobility (reviewed in [2]), affinity for ouabain, N-terminal amino acid sequence and antigenic determinants. They also differ in their sensitivity to proteases, *N*-ethylmaleimide, pyrithiamine, hormonal regulations with insulin and thyroid hormone and affinity for Na^+ ions [2,5]. In adult cardiac myocytes from different species; rat, dog and ferret [6–10], two functional isoforms of the α-subunit were also detected. α_1 with faster electrophoretic mobility had a low affinity for ouabain and α_3 with slower mobility exhibited a high affinity for ouabain.

In dog heart, we have shown that the high-affinity molecular form (α^+) was the pharmacological receptor exclusively related to positive inotropy of digitalis whereas occupation by digitalis of the low-affinity form (α_1) led to toxicity [8,11]. The α^+ form corresponds to α_3 [12].

Inasmuch as ouabain specifically inhibits the Na,K-ATPase activity, a constant ratio representing the turnover number should exist between the ouabain-sensitive ATP hydrolysis and the ouabain-binding capacity. This is exemplified by the linear relationship found with isolated enzyme preparations [13–19]. According to these different authors, the turnover rates of the Na,K-ATPases varied from 60 to 200 s^{-1}. However, this wide range appeared to be dependent upon the species sensitivity to ouabain. As shown in Table I, the purified Na,K-ATPase of high affinity for digitalis (K_d) values of the 10 nM) isolated from pig brain, beef heart and human erythrocytes, had a higher turnover rate (150–200 s^{-1}) than the enzyme of low affinity for ouabain (μM range) (60–108 s^{-1}) as found in the rectal gland of the dogfish, dog kidney and duck nasal gland.

Our objective was to document whether the functional ATPase isoforms α_1 and α_3 (of low and high affinity for digitalis) present in canine cardiac ventricular membrane preparations exhibited the same turnover rates. Our results show that two isoforms exhibit different turnover numbers: 88 ± 10 s^{-1} and 147 ± 15 s^{-1} for α_1 and α_3, respectively.

2. MATERIALS AND METHODS

2.1. *Sarcolemmal preparations*

Na,K-ATPase enriched sarcolemma preparations from canine ventricular muscle were isolated as previously described [8,11]. Left ventricular tissue was obtained from pentobarbital anaesthetized mongrel dogs. The microsomal fraction (a 32,000 × *g*, 30-min pellet) resuspended in 100 mM NaCl, 250 mM sucrose and 30 mM imidazole, pH 7.4, was frozen in liquid nitrogen and stored at −80°C.

2.2. *Measurements of sensitivity to ouabain*

One day after the preparation, the vesicles were subjected to SDS

*Corresponding author Fax: (33) 9109 0506.

treatments (0.2 mg/mg of protein, 30 min at 20°C) to render them permeable to substrates and ligands prior to [^{3}H]ouabain binding studies and ATPase assays.

2.2.1. [^{3}H]ouabain binding assays

Equilibrium binding of [^{3}H]ouabain was measured as previously reported [8]. After a 60 min incubation at 37°C in 1 ml of a medium containing 0.04 mg of protein with increasing ouabain concentrations from 0.3 nM to 0.1 μM and specific radioactivity varying from 19 Ci/mmol to 0.6 Ci/mol, either in buffer 1: 4 mM $MgCl_2$, 4 mM ATP, 100 mM $NaCl_2$ and 40 mM imidazole-HCl, pH 7.4, or in buffer 2: 1 mM $MgCl_2$, 1 mM inorganic phosphate and 50 mM Tris-HCl, pH 7.4. The receptor concentration was 170 pM except for [^{3}H]ouabain concentrations less than 1 nM. Under these conditions the final volume was 10 ml instead of 1 ml and the receptor concentration was 17 pM.

2.2.2. Sensitivity of Na,K-ATPase to ouabain

Two different protocols have been devised. In protocol 1, ouabain was added after initiation of the enzymatic reaction and the enzyme inhibition was continuously monitored for 60 min. In protocol 2, membranes were first preincubated for 60 min at 37°C with ouabain in the presence of buffer 1 (ATP, Mg^{2+}, Na^+) and then assayed for enzyme activity. The Na,K-ATPase activity in the absence or presence of various concentrations of ouabain from 0.3 nM to 30 μM was determined using a coupling assay method as previously described [20]. The final concentration of active Na,K-ATPase molecules was 15 pM.

2.3. *Analysis methods*

The experimental data were fitted using Enzfitter (Biosoft, Elsevier). The following model was used.

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

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 least-squares regression. The experimental data were also fitted with one or a sum of two functions assuming the presence of one or two sites [21]. The K_d and B_{max} values were computed assuming equilibrium conditions, using the same program.

3. RESULTS

In the conditions used, equilibrium binding or steady state level of enzyme inhibition was reached within 60 min of incubation at 37°C. [^{3}H]ouabain binding measured with concentrations between 1 and 100 nM after 120- or 180-min incubations reached similar levels to those found after 60 min (data not shown).

Enzyme activity and [^{3}H]ouabain binding capacities were determined under conditions as similar as possible, i.e. at equilibrium, after the same period of time, at the closest receptor concentration (i.e. 10–20 pM for enzymatic assays and 17–170 pM for binding assays) and at the lowest receptor concentration compatible with detection of both inhibition and binding signals.

Since ouabain-binding occurs on the phosphorylated enzyme, ouabain-binding was carried out in the presence of either ATP + Mg^{2+} + Na^+ (complex 1, forward phosphorylation). Note that to induce the formation of complex 1, a pretreatment of the vesicles by SDS was necessary to facilate free access of ATP to its site. In contrast, this pretreatment was not required for the backward phosphorylation (complex 2) in the presence of Mg^{2+} and P_i.

For the same enzyme preparation, the maximal levels of ouabain bound at equilibrium to complex 1 or to complex 2 were the same (47 + 2.8 pmol/mg of protein).

As illustrated in Fig. 1, under both conditions, they exhibited the same respective affinities and the same proportional contributions (Table II).

High (α_3) and low affinity (α_1) sites exhibited K_d values of 3 ± 2 and 500 ± 200 nM, respectively. The contribution of the high-affinity sites represented 28 pmol/mg of protein, i.e. 60% of the total binding. Regardless of the protocols used, no enzyme/ouabain preincubation in the absence of K^+, the dose–response curves of Na,K-ATPase to ouabain were very similar. The IC_{50} values were 2 ± 1 nM and 300 ± 100 nM, for the high- and low-affinity forms, respectively. The high-affinity form representing 66% of the total inhibitory process. Note that the IC_{50} values in the presence of ATP + Mg^{2+} + Na^+ + K^+ were similar to the K_d values found by [^{3}H]ouabain-binding measurements in the presence of either Mg^{2+} + P_i or ATP + Mg^{2+} + Na^+ (Fig. 1).

When Na,K-ATPase inhibition was plotted as a function of the occupancy of the specific [^{3}H]ouabain- binding sites, the correlation was not linear (Fig. 2). This indicates that the rates of ATP hydrolysis associated with each site type were not the same. For the α_3-subunit form involved at low ouabain concentrations, the turnover rate was 147 ± 12 s^{-1} (i.e. 147 mol of ATP hydrolyzed per second per mol of ouabain binding site).

At higher ouabain concentrations (100 nM), the high-affinity form was completely inhibited and the remaining activity corresponded to the low-affinity form (α_1). The turnover data for this site type was 88 ± 10 s^{-1}. The rate of ATP hydrolysis per ouabain-binding site was 1.6-fold faster for the α_3 form than the α_1 form.

4. DISCUSSION

The data presented in this paper show that the two canine cardiac, Na,K-ATPase isoenzymes have differ-

Table I

Comparison of turnover rates, sensitivities of Na,K-ATPase preparations from different sources

Species	Sensitivity to digitalis	Turnover number (s^{-1})	References
Pig brain	High	203	[13]
Beef heart	High	149	[14]
Beef heart	High	142	[15]
Human red cells	High	200	[15]
Dog fish rectal gland	Low	98	[16]
Dog kidney	Low	108	[17]
Dog kidney	Low	60	[18]
Duck nasal salt gland	Low	91	[19]

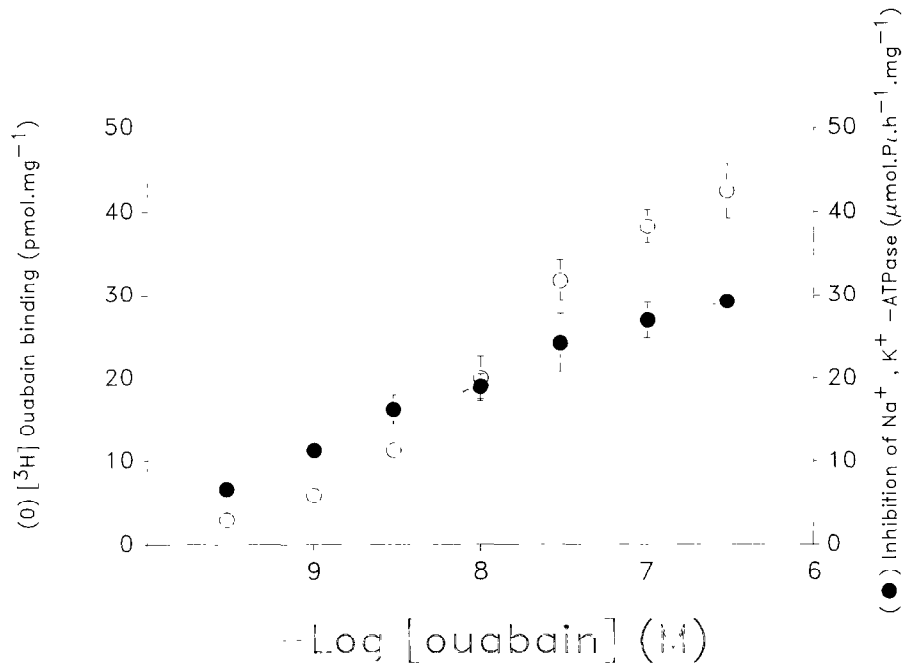

Fig. 1. Concentration dependence of ouabain binding and inhibition of canine heart membrane-bound Na,K-ATPase. Inhibition of Na,K-ATPase activity (●) was assessed during ATP hydrolysis either in the presence of 100 mM NaCl, 10 mM KCl, 4 mM ATP, 4 mM $MgCl_2$ and the other constituents used to couple the hydrolysis of ATP to the oxidation of NADH or after a 60 min preincubation period without KCl in the presence or absence of various ouabain concentrations. [^{3}H]Ouabain-binding equilibrium was activated in the same buffer without KCl (○) or in 1 mM $MgCl_2$, 1 mM P_i and 50 mM Tris-HCl, pH 7.4. The average ± S.E. of at least 4 independent determinations are shown. The curves represent the best fit of the data to a two-component inhibition model.

ent turnover rates. The turnover rate was higher for the α_3 form with high affinity for ouabain (147 ± 15 s^{-1}) as compared to that found for the α_1 form with low affinity for ouabain (88 ± 10 s^{-1}).

The first evidence for different turnover rates as measured in vitro is supported by the comparison of drug binding and enzyme assays carried out under very similar conditions. However, several limitations associated with the method(s) used to determine the turnover numbers may have induced erroneous differences between the isoforms.

One possibility is that ouabain-binding experiments had not been carried out at equilibrium, and subsequently one population of binding sites was underestimated. This is unlikely for the high-affinity (fast association and slow dissociation) and the low-affinity forms (slow association and fast dissociation) as similar results were obtained after incubations of 60, 120 and 180 min. The binding equilibrium has already been reached in 60 min. Note that the sensitivity of Na,K-ATPase activity to ouabain was also measured during 60 min.

Table II

Apparent affinities for ouabain and respective contributions by the two digitalis receptor forms and turnover rates

	IC_{50} (nM)	Contribution (% of activity)	K_d (nM)	Contribution (% of binding)	Turnover number (s^{-1})
$\alpha_{2/3}$ form	1–8	58–72	1–5	50–70	147 ± 15
α_1 form	100–500	23–33	300–700	30–50	88 ± 10

Inasmuch as K^+ ions, known to slow the ouabain-association process [22] were present in the enzyme assay medium but not during the binding, it is possible that enzyme inhibition by ouabain was not complete during the 60 min assay period. This possibility is also unlikely. Indeed, conditions in protocol 2, (i.e. a 60 min preincubation without K^+ ions) also led to the same dose-response curves as in protocol 1 (data not shown). Thus K^+ did not interfere in the level of equilibrium of ouabain-binding.

Another objection would be that the K_d values and the number of low-affinity sites could not be accurately measured by tritiated ouabain-binding methods due to the very high level of non-specific binding (at least 50% at 3 μM for [^{3}H]ouabain). This objection is valid with this type of site in rat, but not in the dog since in the latter species the low-affinity site has a K_d value of 0.3 μM for ouabain. However, inasmuch as the total number of low-affinity sites can only be estimated, the true turnover rate of this type of site might be slightly different from that found here.

A reproductible and selective inactivation of the

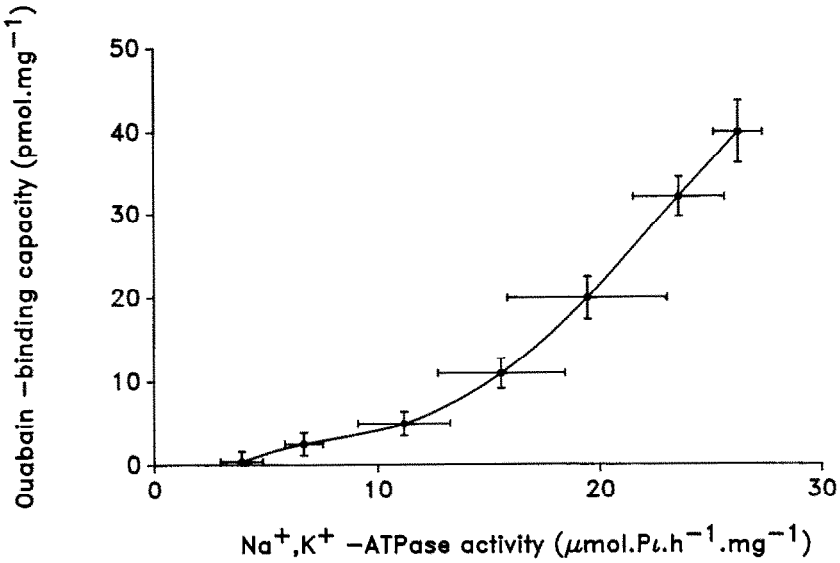

Fig. 2. Ouabain binding and inhibition of Na,K-ATPase enzymatic activity in dog myocardial cell membranes. The two measurements were determined with the same microsomal membrane preparations under equilibrium conditions. Specific [^{3}H]ouabain-binding is not linearly correlated to inhibition of Na,K-ATPase At low concentrations (less than 10 nM), a linear relation has been computed which corresponds to 147 ± 15 molecules of ATP hydrolyzed per second per ouabain-binding site. The same linear regression applied between 20 and 30 units of enzyme activity corresponding to 20 to 40 pmol ouabain per mg of protein, gave a turnover rate of 88 ± 12 s^{-1}. Results are means ± S.E.

Na,K-ATPase with low affinity for ouabain would lead to similar results. The specific enzymatic activity associated with Na,K-ATPase is more labile than the ouabain-binding capacity. We can preserve the ouabain-binding capacity for several weeks while no Na,K-ATPase activity could be detected. Such an inactivation of this enzyme isoform could occur during the sarcolemma isolation procedure, before storage and assay. These possibilities have been minimized, but not excluded since we have used the same membrane fractions at the same time (one day after the membrane isolation) for the determination of both parameters, sensitivity and binding. Conversely, note that the turnover rate found for α_3 in dog heart is close to that reported in the literature: 150 s^{-1} per site compared to 203 in pig brain, 149 in beef heart and 200 in human red cells (Tables I and II). All these Na,K-ATPases of high turnover rates are of high affinity for digitalis (K_d) lower than 10 nM).

The optimal conditions for evaluating one of the isoforms could artifactually decrease its contribution in the total process. This possibility cannot be excluded here and is exemplified with guinea-pig cardiac Na,K-ATPase [23]. The two types of Na,K-ATPases with high and low sensitivity to ouabain have half-maximal phosphorylation stimulated by 69 and 4.5 mM Na$^+$, respectively. If this is also true in dog heart, at 100 mM Na$^+$, as used here, the turnover rate of the α_3 isoform would in turn be underestimated. This in turn will further increase the differences observed between α_1 and α_3. A pattern for differential Na$^+$ affinity between α_1 and α_3 has been demonstrated in rat brain [24–26].

If one takes into account the fact that the ouabain-binding reactions and enzymatic measurements are dependent upon the formation of the phosphorylated enzyme intermediate, the difference in turnover rates could mean that α_1 and α_3 catalytic subunit in dog heart differ in their phosphorylation and dephosphorylation rates. The difference in the turnover rates seems to be an intrinsic property of the protein. Consistent with our data, it has been reported that in terms of phosphorylation, the α_3 form in shrimp has a better apparent affinity for ATP than the α_1 form [27].

Our results strongly suggest that the Na,K-ATPase of high affinity for ouabain (α_3) have a higher turnover rate than the α_1 isoforms. The low turnover rate of 88 s^{-1} is consistent with the turnover rate found for α_1 in canine kidney (Table I). These enzymes forms with relatively similar low turnover rates, apparently exhibit the same range of low affinities for ouabain.

The involvement of these isoforms of different turnover rates in the global Na pump activity in vivo as a function of the ionic environment remains an open question.

Acknowledgements We are grateful to Dr Richard M. Kawamoto (Procter and Gamble Pharmaceuticals Inc., Norwich, NY, 13915 USA), Pr Lionel G. Lehèvre (université Paris 7) for helpful discussions and for revising the English. We also thanks Michel Ninot and Renaud Mougenot for their technical assistance and their in help in preparing the figures.

REFERENCES

[1] Skou, J.C., Norby, J.G., Maunsbach, A.B. and Esmann, M. (1988) The Na^+,K^+-Pump, Progress in Clinical and Biological Research, Vol 268A-B, Alan R. Liss, Inc., New York.
[2] Sweadner, K.J. (1989) Biochim. Biophys. Acta 988, 185–220.
[3] Shyjan, A.W. and Levenson, R. (1989) Biochemistry 28, 4531–4535.
[4] Lingrel, J.B., Orlowski, J., Shull, M.M. and Price, E.M. (1990) Prog. Nucleic Acid Res. Mol. Biol 38, 37–89.
[5] Horisberger, J.D., Lemas, V., Krachenbühl, J.P. and Rossier, B. (1991) Annu. Rev. Physiol. 53, 565–584.
[6] Charlemagne, D., Mayoux, E., Poyard, M., Oliviero, P. and Geering, K. (1987) J. Biol. Chem. 262, 8941–8943.
[7] Sweadner, K J. and Farshi, S.K. (1987) Proc. Natl. Acad. Sci. USA 84, 8408–8407.
[8] Maixent, J.M , Charlemagne, D., De la Chapelle, B. and Lelièvre, L.G. (1987) J. Biol. Chem. 262, 6842–6848.
[9] Matsuda, T., Iwata, H and Cooper, J.R (1984) J Biol. Chem. 259, 3858–3863.
[10] Ng, Y C. and Akera, T. (1987) Am. J Physiol. 252, 1016–1022.
[11] Maixent, J.M. and Lelièvre, L.G. (1987) J. Biol. Chem. 262, 12458–12462
[12] Maixent, J.M. and Berrebi-Bertrand, I. (1992) J. Mol Cell. Cardiol. 24, S26.
[13] Nakao, T., Nakao, M , Nagai, F., Kawai, K., Fijihara, Y., Hara, Y. and Fujita, M. (1973) J. Biochem. 73, 781–791.
[14] Pitts, B.J R., Lane, L.K. and Schwartz, A. (1973) Biochem. Biophys. Res Commun. 53, 1060–1067.
[15] Erdmann, E., Werdan, K. and Brown, L. (1984) Eur. Heart J. 5, 297–302.
[16] Hokin, L E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973) J. Biol. Chem. 248, 2593–2603.
[17] Lane, L.K., Copenhaver Jr., J.H., Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 7197–7200.
[18] Kyte, J. (1972) J. Biol. Chem. 247, 7634–7641.
[19] Hopkins, B.E., Wagner Jr., H. and Smith, T.W. (1976) J. Biol. Chem. 251, 4361–4371.
[20] Lelièvre, L.G., Maixent, J.M., Lorente, P., Mouas, C. Charlemagne, D. and Swynghedauw, B. (1986) Am. J. Physiol. 250, 923–931.
[21] Maixent, J.M., Fénard, S. and Kawamoto, R.M. (1991) J. Receptor Res. 11, 687–698.
[22] Akera, T. and Brody, T.M. (1978) Pharmacol. Rev. 29, 187–200.
[23] Fricke, U. and Klaus, W. (1977) Br. J. Pharmacol. 61, 423–428
[24] Feige, G., Lentert, T. and De Pover, A. (1988) in: The Na^+/K^+ pump, Progress in Clinical and Biological Research, Vol. 268A-B, Alan R Liss, Inc., pp. 377–384, New York.
[25] Shyjan, A.W., Cena, V., Klein, D C. and Levenson, R. (1990) Proc. Natl Acad. Sci. USA 87, 1178–1182.
[26] Brodsky, J.L. and Guidotti, G. (1990) Am. J. Physiol. 258, 803–811.
[27] Churchill, L. (1982) J. Exp. Zool. 231, 335–341