Labeling of a cysteine in the cardiotonic glycoside binding site by the steroid derivative HDMA

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Abstract The digoxigenin derivative *N*-hydroxysuccinimidyl digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproate (HDMA) has been shown to covalently label the ouabain binding site of the Na,K-ATPase ε subunit [Antolovic et al. (1995) Eur. J. Biochem. 227, 61–67]. In the present study we observed both, labeling and inactivation of the activity, of wild type Na,K-ATPase overexpressed in *Xenopus* oocyte. In contrast, no significant inhibition and no labeling could be detected when a Cys-113 of the first transmembrane segment was mutated to serine, although the affinity of this mutant for digoxigenin or HDMA measured in acute inhibition experiments was similar to the wild type. This indicates that after docking of its genin moiety, HDMA can form a thioester bond with Cys-113.

Key words: Na,K-ATPase; Cardiac steroid; *Xenopus laevis* oocyte; Site-directed mutagenesis; Affinity labeling

1. Introduction

The Na,K-ATPase is an integral membrane protein responsible for the transport of Na^+ and K^+ across the cell membrane against their electrochemical gradient. It is also the specific target of widely used drugs, the cardiac steroids. A natural endogenous cardiac steroid may also be involved in the physiological control of the Na,K-ATPase activity [1].

The structure of the binding site of cardiotonic steroid on the α subunit has been extensively studied [2,3] and it is now clear that several of the short extracellular loops connecting the transmembrane segments of the α subunit are involved. Mutations affecting several amino acids of the H1-H2 loop [3], a tyrosine in the H3-H4 loop [4], a threonine in the H5-H6 [5,6] and an arginine of the H7-H8 loop [7] significantly modify the cardiotonic steroid affinity.

At least two amino acids of the outer third of the first transmembrane segment (H1) appear to be important determinants of ouabain binding. Canessa et al. [8] have identified a cysteine which was mutated into a phenylalanine or a tyrosine in an ouabain-resistant MDCK cell line. Similarly Askew and Lingrel [9] observed the Cys to Tyr mutation at the same position in an ouabain-resistant HeLa cell line. Site directed mutations at the corresponding sites in *Xenopus* [8] or sheep [10] α1 subunits yielded an ouabain-resistant phenotype. Similarly, the tyrosine at position 108 in the sheep α1 subunit was also shown to be a determinant of ouabain binding [10].

Naturally occurring cardiotonic steroids are glycosylated with 1–3 sugar residues in position 3 of the steroid nucleus and the presence of these sugar groups results in a roughly 20–50-fold increase in affinity compared to that of the corresponding aglycone [11]. Modification of the protein domain that binds the sugar moiety of glycoside compounds would be expected to alter the affinity of glycosides without changing the affinity for the corresponding aglycone. Up to now investigators have failed to identify mutants or isoforms with a significantly altered glycoside/aglycone affinity ratio [6,8,10] although there was a very large range of affinity for ouabain in the studied mutants. Therefore the nature of the sugar binding site remains to be determined.

Antolovic et al. [12] have recently shown that it is possible to label the α subunit of purified pig kidney Na,K-ATPase by exposure to the cardiac steroid derivative *N*-hydroxysuccinimidyl digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproate (HDMA), a compound in which a reactive hydroxysuccinimidyl group is located at the end of a 6-carbon chain which is attached to position 3 of the steroid ring of digoxigenin. They proposed that HDMA could form a thioester with a sulfhydryl group located in one of the first two transmembrane domains of the α subunit, e.g. with Cys-104 in H1 or Cys-138 in H2. Since it was not possible to determine which one of the two cysteines was modified by sequence analysis of HDMA-labeled peptides, we investigate in this study the binding of HDMA to wild type *Xenopus* α1 subunit and mutants in which Cys-113 was replaced by either a serine or a tyrosine residue (C113S, C113Y). The *Xenopus* oocyte expression system allowed us to study both the labeling of the α subunit protein and the functional effects of HDMA binding, and to demonstrate that HDMA does indeed form a bond with Cys-113 in H1 but not with Cys-147 in H2 and thereby inhibits the Na,K-pump function.

2. Material and methods

2.1. Expression of wild-type and mutant Na^+/K^+-ATPase

Stage V–VI *Xenopus* oocytes were obtained, prepared and maintained as described earlier [13], and injected with 7 ng of cRNA of the α subunit (wild-type or mutant) and 1 ng of cRNA of the β1 subunit (wild-type) of the *Xenopus laevis* Na^+/K^+-ATPase [14]. The oocytes were kept in amphibian Ringer solution for 3 days to allow the expression of the exogenous Na^+/K^+-ATPase and then loaded with sodium by exposure to a K-free solution (in mM: Na-gluconate 80, $NaHCO_3$ 9.6, $MgCl_2$ 8.2, Na-MOPS 20, pH 7.0). Na^+-loaded oocytes were exposed to 100 nM HDMA in the same solution for 12 h at 19°C and used either for electrophysiological measurements or for measurement of labeling.

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Abbreviations: HDMA, *N*-hydroxysuccinimidyl digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproate. *Enzyme:* Na^+/K^+-ATPase, Na^+/K^+-transporting ATPase (EC 3.6.1.37).

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2.2. Electrophysiological measurements

The Na^+/K^+-pump current was measured as described earlier [13] using the 2-electrode voltage clamp technique as the outward current induced by addition of 10 mM K^+ to K^+-free solution (in mM): Na^+ 80, Ba^{2+} 5, Mg^{2+} 0.82, tetraethylammonium (TEA) 10, Ca^{2+} 0.41, [*N*-morpholino]propanesulfonic acid (MOPS) 5, Cl^- 22, gluconate 80, pH 7.0. Potassium was added as K-gluconate. We have shown earlier that the Na,K-pump current measured in these conditions at a membrane potential of −50 mV is a reliable measure of the Na,K-pump activity present in the oocyte membrane [13,15].

2.3. Analysis of membrane labeling

Forty to 50 oocytes of each experimental group were homogenized at 4°C with a Pasteur pipette in 25 μl/oocyte of HEPES buffer, pH 7.0, containing (in mM): NaCl 50, $MgCl_2$ 10, PMSF 0.5 and 5 μg/ml of each leupeptin, antipain and pepstatin. The homogenate was centrifuged twice at 1000 × *g* for 10 min and the supernatant at 10,000 × *g* for 20 min at 4°C as described [16]. For SDS-PAGE the pellet was dissolved in 0.5 μl/oocyte of 3.7% SDS. Electrophoresis was done according to the procedure of Laemmli [17] in 8–13% and 10% SDS-polyacrylamide gels. Western blotting was carried out according to the prescription of Boehringer Mannheim using the Dig-detection kit for chemiluminescence. Briefly after, the SDS-PAGE protein was transferred on the nitrocellulose membrane. The membrane was blocked with the Boehringer blocking solution and additionaly incubated with specific polyclonal anti-digoxigenin-POD antibodies [12]. The HDMA labeled α subunit was detected with polyclonal antibodies against the α subunit of Na^+/K^+-ATPase from *Bufo marinus* [18] with the chemiluminescence method described above.

Results are given as mean ± S.E.M. Statistical calculation for significant differences between group means were made using the Student's *t*-test using the InStat version 2 of GraphPad Software.

3. Results

3.1. Affinity labeling of wild-type and mutant Xenopus α1 subunits

When oocytes were treated with 0.1 μM HDMA covalent labeling could only be detected in oocytes overexpressing the Na,K-pump after wild-type α subunit cRNA injection (Fig. 1B). No HDMA labeling could be detected in oocytes expressing the C113S mutant. HDMA labeling was also absent in non-injected control oocytes probably because of the relatively low level of expression of the endogenous Na,K-pump. An excess of ouabain present during HDMA labeling protected against the incorporation of the digoxigenin derivative. Therefore, it was possible to detect HDMA affinity labeling of the cardiac steroid binding site of the *Xenopus* α1 subunit expressed at a high level in oocyte as well as in isolated pig kidney Na^+/K^+-ATPase [12] and in embryonic chick heart cells [19]. Differences in the labeling of α subunits in oocytes expressing the wild-type and the C113S mutant α subunit are not due to variations in protein expression as shown by immunoblotting with anti-α antibodies (Fig. 1A) nor to failure of expression of active Na,K-pump mutants at the oocyte surface (see legend of Fig. 1).

3.2. Functional measurements

Electrophyiological measurements of the K^+-stimulated outward current showed first that wild-type and mutant Na,K-ATPases were expressed at a high level, clearly larger than the level of the endogenous Na,K-pump (Fig. 2). Overnight exposure to 100 nM HDMA treatment induced partial inactivation of the Na,K-pump activity both in the non-injected oocyte (inactivation of the endogenous Na,K-ATPase) and in oocytes expressing the wild-type α1 *Xenopus*. A much smaller inhibition

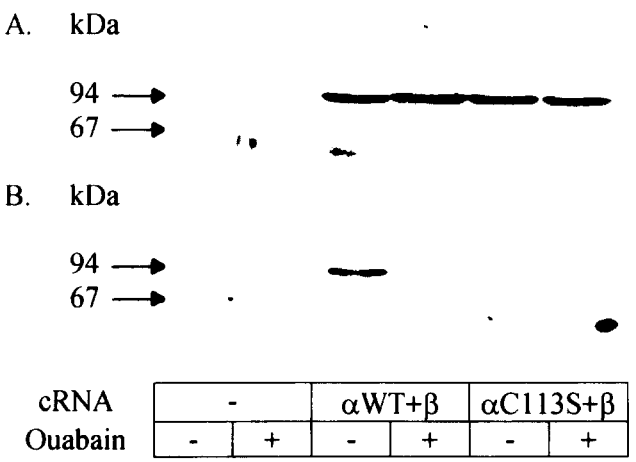

Fig. 1. Western blots of HDMA-labeled wild-type and mutant *Xenopus laevis* α subunits. *Xenopus* oocytes were injected with *Xenopus* wild-type (WT) or mutant (C113S) α subunit, and wild-type β subunit cRNA as described in Material and Methods. After 12 h incubation in 100 nM HDMA, microsomes were prepared, subjected to SDS-PAGE and transferred to nitrocellulose. (A) Immunodetection of the α subunit with specific anti-α antibodies after stripping of the membrane shown in B. (B) Labeling of the α subunit with HDMA. Labeling was revealed with anti-digoxigenin antibodies. Measurements of the Na,K-pump currents in oocytes from the same batch and treated in the same manner yielded the following values: 85 ± 12 nA in non injected oocytes, 423 ± 50 nA in oocytes injected with wild-type α1 cRNA and 303 ± 61 nA in oocytes injected with the C113S mutant α1 cRNA. The presence of 1 mM ouabain in the solution containing HDMA is indicated in the bottom line. This is the result of 1 out of 4 similar experiments.

of the Na,K-pump function was observed in oocytes expressing the C113S or C113Y mutants (Fig. 2). The small decrease of the Na,K-pump current observed in these groups can entirely be attributed to inactivation of the endogenous (wild-type) Na,K-ATPase of the oocyte.

HDMA affinity labeling of the Na,K-ATPase can be prevented by ouabain and it is therefore probable that the genin moiety of HDMA must first dock into the cardiac steroid binding site before the thioester bond can form between the hydroxysuccinimidyl reactive group and a nearby -SH group [12]. Failure of HDMA labeling in the mutants might then be explained either by low affinity of the steroid for the mutant or by the absence or inaccessibility of the -SH group. The former possibility is, however, unlikely for the C113S mutant since we had shown earlier that the affinity of the C113S for ouabain or strophanthidin was very close to that of the wild-type [8]. To confirm that this applied also to the genin of HDMA we determined the inhibition kinetics of wild-type and mutants α subunit for digoxigenin. The Na,K-pump current was measured in the absence and presence of 0.1, 1.0 and 10 μM of digoxigenin. These experiments showed no difference in the half maximal inhibition of the wild-type (1.16 ± 0.61 μM, $n = 7$) and the C113S mutant (0.97 ± 0.24 μM, $n = 7$). The C113Y mutant was, however, more resistant to the digoxigenin (21.7 ± 10.4 μM, $n = 3$). Finally we measured the apparent affinity of HDMA for the wild-type and C113S mutant in acute inhibition experiments. Oocytes expressing high level of artificially expressed Na,K-pump (i.e., with an Na,K-pump current more than twice larger than the current in oocyte injected with the β subunit alone) were exposed to concentrations of 0.1, 1.0

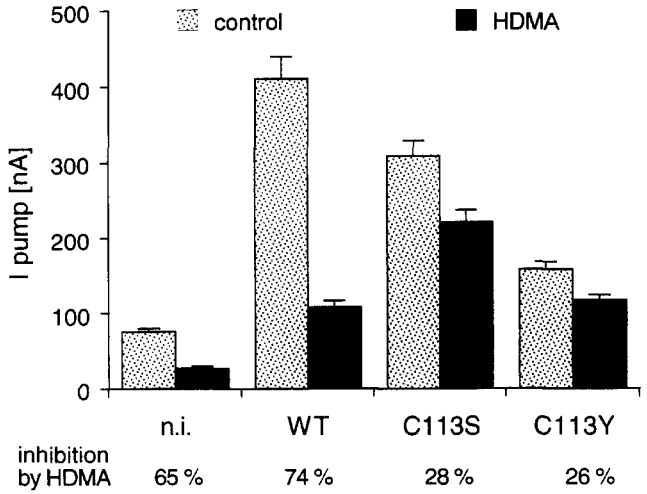

Fig. 2. Inactivation of the Na,K-pump current by HDMA in *Xenopus laevis* oocytes expressing endogenous, exogenous wild-type and C113S and C113Y mutant α subunits of *Xenopus laevis*. The Na,K-ATPase activity was measured as the Na,K-pump current (nA) after overnight exposure to 100 nM HDMA (dark columns) or in non-exposed control oocytes (light columns). The effect of HDMA is shown in non-injected oocytes (n.i., control $n = 15$, HDMA $n = 15$), in oocytes injected with wild-type α and β cRNA (WT, control $n = 13$, HDMA $n = 15$), or in oocytes injected with mutant α cRNA and wild-type β cRNA (C113S: control $n = 14$, HDMA $n = 13$; C113Y: control $n = 13$, HDMA $n = 10$). The percentage of inhibition induced by HDMA is indicated below the columns.

and 10 μM HDMA for 3–5 min at each concentration. The half inhibition constant of HDMA was 3.9 ± 0.6 ($n = 7$) in wild-type and 2.5 ± 0.25 in the C113S mutant ($P = 0.035$), indicating a slightly higher affinity for the C113S mutant. Both these values were slightly higher than those obtained for digoxigenin which may be explained by a slower rate constant due to the steric hindrance caused by the additional side chain in HDMA.

We also attempted to label the α1 subunit of *Bufo marinus*, an isoform which is moderately ouabain-resistant (K_i around 50 μM) [20]. The conserved cysteine of H1 is present in the *Bufo* α1 subunit sequence (Cys-111). No specific labeling could be detected after overnight exposure to 100 nM HDMA (data not shown) and functional measurements could not detect any inhibition of the *Bufo* Na,K-ATPase expressed in oocytes (Na,K-pump current 263 + 22 nA ($n = 27$) after HDMA vs. 244 + 25 nA ($n = 25$) control, $P > 0.1$).

4. Discussion

The present study shows first that it is possible to use the protein reactive HDMA to specifically label the cardiac steroid binding site of an Na,K-ATPase artificially expressed in *Xenopus laevis* oocytes. Affinity labeling was detectable only in a ouabain-sensitive (*Xenopus* α1) but not in an ouabain-resistant (*Bufo marinus* α1) isoform, indicating that sufficient binding of the cardiac steroid is necessary before the covalent labeling can occur. This also suggests that Cys-113 is not easily accessible as predicted by its location inside the membrane in the presently best accepted topology models. In fact, other -SH reagents, such as the fluorescent coumarinyl maleimido derivative do not label this cysteine [21]. It is also possible that formation of the thioester can occur only after a cardiotonic steroid-induced conformational change. HDMA not only labeled the wild-type α subunit of *Xenopus* Na,K-ATPase but also partially inhibited the transport function of this enzyme.

Both results of affinity labeling and of functional studies confirm the hypothesis put forward by Antolovic et al. [12] that HDMA forms a thioester bond with the cysteine located in the outer half of first transmembrane segment, Cys-113 in the *Xenopus* α1 sequence. Mutation of Cys-113 to serine prevents labeling. However, it does not affect the acute inhibition of digoxigenin or of HDMA (as shown above) by non-covalent binding either to ouabain or to strophanthidin [8]. Together with the biochemical data of Antolovic et al. [12], this result indicates that the hydroxysuccinimidyl group of HDMA can form a thioester bond with Cys-113 in the first transmembrane segment after the genin moiety of HDMA has been bound to the cardiac steroid site on the α subunit. Another explanation for the present observation could be that the C113S mutant exists in an altered conformation in which the labeling site is not exposed to the reactive group of HDMA. This seems highly unlikely considering that the Na,K-pump function of this mutant is very similar to the wild-type [4], the binding of aglycone is not modified [4] and the labeled cysteine is located in the first pair of the transmembrane segment [12].

Studies by Canessa et al. [8] and O'Brien et al. [22] on the effects of mutations of the cysteine in H1 of *Xenopus* or sheep α1 subunit and the amino acids in the extracellular H1-H2 loop show that the affinity of an aglycone (strophanthidin or ouabagenin) is lowered to the same extent as that of a glycoside (ouabain). If any of these amino acids were directly involved in the recognition of the sugar moiety of the glycoside, a stronger effect on the glycoside would be expected. The authors therefore conclude that the sugar moiety does not bind to these residues. Moreover, in a recent report on the effect of the mutation of Cys-104 of the human α1 subunit in H1C1 cells on the discriminatory power between digoxin and digitoxin, Askew and Lingrel [9] proposed that Cys-104 may interact with the C-14-OH group between the C and the D rings. These conclusions are somewhat in conflict with the affinity labeling data of McParland et al. [23] which showed that the protein-reactive 24-azido 3β-digitoxoside labeled the sequence W310LEA313 in the extracellular H3-H4 loop. The latter sequence is adjacent to the sequence Y308TW310 which has a sequence similarity with the steroid dehydrogenase, the h-progesterone and the h-glucocorticoid receptor and has been proposed to interact with the unsaturated lactone ring of cardiac steroids through a triple hydrogen bond [24].

Arystarkhova et al. [25] conclude from their studies with the VG4 antibody directed towards the extracellular H1-H2 loop and enhancing cardiac glycoside binding that this loop may play a modulary role rather than directly participating in the binding site structure for cardiac glycosides. Such data point to the possibility that changes in the free energy of interaction with the cardiac steroids digoxin and digitoxin observed after single site mutation may reflect the sum of dipole interactions and Van der Waals forces rather than hydrogen bonds.

We could previously show by molecular modeling that the length and the proper spacial orientation of the sulfhydryl-reactive substituents at the C-3-OH group of the A ring of digoxigenin is of prime importance for affinity labeling [12]. Comparison of the 3-dimensional models of the various protein-reactive digoxigenin derivatives with digoxigenin-3-*O*-di-

gitoxoside led to the suggestion that the reactive SH group was also involved in the recognition of the sugar moiety of the cardiac glycoside [12]. Although the results of the present study cannot resolve the question of the binding site for the sugar part of the cardiac glycoside, the data in this study and in the preceding paper [12] show that the genin moiety is bound to the α subunit in such a position, that the Cys-113 of H1 is located within reach of a 4- to 7-carbon chain from the C3 position of the steroid ring.

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