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Progesterone Binding to the α 1-Subunit of the Na/K-ATPase on the Cell Surface: Insights from Computational Modeling

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Abstract

Progesterone triggers the resumption of meiosis in the amphibian oocyte through a signaling system at the plasma membrane. Analysis of [³H]ouabain and [³H]progesterone binding to the plasma membrane of the *Rana pipiens* oocyte indicates that progesterone competes with ouabain for a low affinity ouabain binding site on a 112 kDa α 1-subunit of the membrane Na/K-ATPase. Published amino acid sequences from both low and high affinity ouabain binding α 1-subunits are compared, together with published site-directed mutagenesis studies of ouabain binding. We propose that the progesterone binding site is located in the external loop (23 amino acids) between the M1-M2 transmembrane helices. Analysis of loop topology and the countercurrent hydrophobicity/polarity gradients within the M1-M2 loop further suggest that the polar β and hydrophobic α surfaces of the planar progesterone molecule interact with opposite sides of the amino acid loop. The 19-angular methyl group of progesterone is essential for activity; it could bind to the C-terminal region of the M1-M2 loop. Maximum biological activity requires formation of hydrogen-bond networks between the 3-keto group of progesterone and Arg¹¹⁸, Asp¹²⁹ and possibly Glu¹²²⁻¹²⁴ in the C-terminal region of the loop. The 20-keto group hydrogen may in turn hydrogen bond to Cys¹¹¹ near the M1 helix. Peptide flexibility undergoes a maximal transition near the midway point in the M1-M2 loop, suggesting that folding occurs within the loop, which further stabilizes progesterone binding.

Keywords

Progesterone; oocyte; ouabain; plasma membrane; receptor; topology; α 1-subunit Na/K-ATPase

1. Introduction

Steroids affect cell metabolism through two different pathways. Cytosol steroid receptors which bind to DNA and alter gene expression are characterized by a latent period between steroid uptake and cell response (reviewed in [1]). The second pathway acts via receptors at the plasma membrane, is associated with a much more rapid response, and does not directly affect transcription (reviewed in [2-4]). Progesterone-induced meiosis in the *Rana pipiens* oocyte was one of the first demonstrations of a steroid response system originating in the plasma membrane [5,6]. The meiotic divisions in amphibian oocytes are initiated by progesterone, secreted by ovarian follicle cells in response to pituitary gonadotropins. As demonstrated with enucleated oocytes, progesterone induces changes in the oocyte membrane and cytosol which,

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in intact oocytes, lead to completion of the first meiotic division and arrest at metaphase II [7].

The present study discusses evidence for the interaction of progesterone with a specific site on an integral plasma membrane protein, the ouabain-binding $\alpha 1$ -subunit of the Na/KATPase. We find that progesterone competes with ouabain for a low affinity, but not a high affinity, binding site on the oocyte plasma membrane [8]. This suggests that the low affinity ouabain binding site may correspond to the progesterone receptor on the oocyte surface. Evidence from other laboratories indicates that the low affinity and a high affinity sites are on different $\alpha 1$ -subunits [9]. Since the low and high affinity ouabain binding $\alpha 1$ -subunits differ by only a few amino acids, largely concentrated in the first external loop between the first and second transmembrane helices, it should be possible to identify the amino acids associated with progesterone binding. A comparison of topology and site-directed mutagenesis data of ouabain binding in the low and high affinity forms are used to propose a model for the interaction of progesterone with a specific site on the oocyte plasma membrane.

Two other steroid binding sites have been proposed for the vertebrate oocyte plasma membrane. A G-protein-linked 40 kDa peptide with seven transmembrane domains has been isolated from fish ovarian segments and shown to bind steroids (reviewed in [10]). Alternatively, it has been proposed that the classical progesterone (cytosol) receptor mediates the non-genomic action that triggers meiosis in *X. laevis* oocytes [11]. Thus, including the steroid binding Na/K-ATPase $\alpha 1$ -subunit described here, there are at least three candidate membrane receptor peptides associated with non-genomic steroid events in the amphibian/fish oocyte.

2. Experimental

The present study uses: 1) our published data on progesterone-ouabain competition for a binding site on the external surface of isolated plasma membranes from *Rana pipiens* oocytes [8,12], 2) site-directed mutagenesis studies of ouabain binding to sheep and human Na/K-ATPase $\alpha 1$ -subunits by other investigators (e.g. [13]), and 3) the known amino acid sequence of $\alpha 1$ -subunit isoforms from protein data banks. Previously unpublished data describes the meiotic inducing activity and electron spin resonance (ESR) data for 3-DOXYL-17 β -OH-5 α -androstane (Table 1).

2.1 Use of *Rana pipiens* to study the meiotic divisions

Rana pipiens (Leopard frog) has been widely used in developmental biology. Its oocytes are particularly advantageous for the study of steroid action at the plasma membrane. Mature females contain up to several thousand fully grown, yolk-filled oocytes, arrested in prophase of the first meiotic division. In contrast to *Xenopus laevis* (African clawed frog), *Rana* ovulates only once a year (late spring for females from the Northeastern United States), following winter hibernation. However, oocytes can be removed from the ovary of the hibernating *R. Pipiens* female from October thru May, and, immersed in Ringer's solution containing progesterone, will complete the first meiotic division and arrest at metaphase II *in vitro* (reviewed in [14]). The chromosomes are tightly condensed and remain condensed throughout the meiotic divisions. Since all fully grown oocytes are in exactly the same stage, all synchronously complete the first meiotic division and advance to metaphase of the second meiotic division. The disappearance or "breakdown" of the nuclear membrane is generally used as the criterion for initiation of the first meiotic division. This can be monitored by dissection of heat-fixed oocytes. (Completion of the second meiotic division is triggered by sperm entry). *R. pipiens* nuclear membrane breakdown occurs 8-10 h after exposure to progesterone at 20° C, compared to about 3 h in fully grown Stage VI *X. laevis* oocytes. (The oocyte nucleus has been referred to as the germinal vesicle and germinal vesicle breakdown as GVBD.)

2.2 Isolation of Intact Plasma Membrane from Amphibian Oocytes

The large size of the *R. pipiens* oocyte (1.8-2.2 mm diameter) and nucleus (0.4 mm diameter) are particularly useful for biochemical, biophysical and microinjection studies. Intact or enucleated oocytes can be pulse-labeled with radiolabeled compounds or studied noninvasively using nuclear magnetic resonance techniques (reviewed in [14]). Prophase oocytes free of adherent follicle cells (referred to as “denuded”) can be obtained after brief exposure to EDTA-containing Ca^{2+} -free Ringer's solution (Masui et al. (cit. [15])). (This avoids modification of plasma membrane proteins by prolonged treatment with collagenase, often employed to free *X. laevis* oocytes from follicle and thecal cells.)

The ligand-binding experiments described here were carried out with plasma-vitelline membranes from fully grown, denuded *R. pipiens* oocytes. Membranes were isolated, one at a time, from denuded oocytes immersed in isotonic sucrose containing 1.0 mM CaCl_2 . Each oocyte was manually penetrated with a fine-tipped pipette, and the yolk contents carefully rinsed out with the sucrose solution, leaving a ghostlike translucent sphere [15,16]. The presence of the vitelline membrane, an acellular fibrous mesh-like glycoprotein structure overlying the plasma membrane, supports the plasma membrane and prevents fragmentation. Care was taken to avoid contact of the membrane preparation with the air-water interphase (if this occurs there is a rapid dispersal of membrane fragments as an oily layer on the surface of the aqueous phase).

2.3 Peptide Topology Analysis

The UniprotKB/Swiss-Prot/EMBL data base (www.expasy.org/uniprot) was the source for sequence data for α -subunit isoforms of the Na/K-ATPase from a variety of species. The sequence data for rat, sheep, frog and human Na/K-ATPase α 1-subunit (see Accession numbers indicated in the appropriate text) were used to plot the conformational differences between the low vs. high affinity ouabain binding forms that are shown in the figures below. SIB BLAST searches were performed using the BLAST network service: NCBI BLAST program reference [PMID:9254694]. No X-ray crystallographic or nuclear magnetic resonance protein structure studies have been published for the α 1-subunits of the Na/K-ATPase from any species.

The Residue-based Diagram editor (RbDe) Web site maintained by the Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY was employed to illustrate the topology of the Na/K-ATPase α -subunits of various species [17]. Chem 3D Ultra v. 10 (Cambridgesoft, Cambridge Scientific Computing, Cambridge, MA) was used to visualize the structure of external loops and helices of the α 1-subunit of Na/K-ATPase shown in Figures 3 and 8. Hydrophobic and polarity gradients within the loops were measured as described by Kyte and Doolittle [18] and of Grantham [19], respectively. Hydrophobic cluster analysis [20] on the amino acid sequence of the first external loop (M1-M2) was carried out using the internet site bioserv.rpbs.jussieu.fr. The modeling software used in this study are either programmed for single letter or three letter amino acid codes. Therefore, the amino acid codes used in each computer generated figure are also used in the corresponding discussion.

3.0 Comparison of Meiotic Inducing Activity of Progesterone and Cardiotonic Steroids on the Amphibian Oocyte

Figure 1 compares the structure of Δ^4 -pregnane 3,20-dione (progesterone) with that of the steroid, ouabain (G-strophanthin). Ouabain is a digitalis derivative, frequently used in the laboratory as an inhibitor of the Na/K-ATPase (Na^+ -pump). It differs from progesterone in that it contains rhamnose in the 3 position, an unsaturated lactone ring in the 20 position, and hydroxyl groups on carbons 1, 5, 11, 14 and 21 of the steroid nucleus. Ouabain-like immunoreactivity has been found in vertebrate tissues and in plasma; the highest levels being

found in the adrenal cortex, hypophysis and hypothalamus (reviewed in [21]). Its concentration in plasma varies from pmolar to nmolar responding to physiological stress, causing it to be considered for hormone status. Endogenous mammalian ouabain appears to be identical to plant-derived ouabain [21]. Pregnenolone and progesterone have both been shown to be precursors of endogenous ouabain in vertebrates [22-24]. Smaller amounts of related compounds (bufadienolides, marinobufogenin, 19-norbufalin) have also been identified in vertebrates (cit [21]).

The meiotic activity of two cardiotonic steroids (ouabain and digitoxigenin) is compared with that of progesterone in Table 1. Vitto and Wallace found that while ouabain alone will not induce meiosis in *X. laevis* oocytes, it will induce nuclear membrane breakdown in *Xenopus* oocytes after a brief exposure to progesterone [25]. Cartaud et al. [26] subsequently confirmed the ouabain facilitation effect and found that digitoxigenin, a digitalis steroid that differs from ouabain in that it lacks hydroxyl groups in the 1, 5 and 11 positions, also induced nuclear membrane breakdown in *X. laevis* oocytes with a half-maximal response of 3×10^{-5} M. These findings suggest that initial progesterone binding permits the plasma membrane receptor to subsequently bind ouabain.

4.0 Comparison of Progesterone and Ouabain binding to the *Rana pipiens* Oocyte Plasma Membrane

4.1 Progesterone Binding to the Isolated Plasma-Vitelline Membrane

The surface of the prophase *R. pipiens* oocyte is covered with numerous microvilli that increase the effective surface area of the prophase oocyte 10-12 fold [27]. The isolated plasma-vitelline membrane complex does not metabolize progesterone [15], which facilitates accurate determination of [³H]progesterone binding. Table 2 summarizes the progesterone binding characteristics of the oocyte plasma-vitelline membrane. We find that progesterone binds to a single site ($K_d = 5 \times 10^{-7}$ M) on the plasma membrane of the *R. pipiens* oocyte which is specific, saturable and which has a slow off-rate [15]. Photoaffinity labeling with the progestin R5020 demonstrates a major 110-114 kDa component in these membranes [16]. Similarly, Western blots of plasma-vitelline membranes, using SDS/PAGE, contain a single 110-114 kDa band which binds an antibody to the steroid-binding C-terminal domain of the human/rat progesterone cytosol receptor [8]. In contrast, a polyclonal antibody to an N-terminal region (sequence 375-564) of the human progesterone receptor immunoreacted with oocyte cytosol but not with oocyte plasma membrane proteins [8]. Thus, the oocyte plasma membrane receptor seems to be homologous to carboxyterminal steroid binding region of the human/rat intracellular progesterone receptor but differs in the N-terminal region of the oocyte and rat/human cytosol progesterone receptor.

The progestin R5020 also binds to a single class of sites on both 80 and 110 kDa components in 105,000g cytosol prepared from *R. pipiens* oocytes denuded of follicle cells [28]. These binding sites have a K_d (3×10^{-8} M) that is an order of magnitude higher than that of the plasma membrane receptor [15]. Therefore, based on K_d and Western blot analysis, the 110-114 kDa progesterone-binding component present in the oocyte cytosol appears to be distinct from that found in the plasma membrane. A progesterone receptor recently cloned from *X. laevis* oocytes [29] may represent the oocyte cytosol receptor [28], rather than a plasma membrane receptor.

4.2 Ouabain Binding to the *R. pipiens* Plasma-Vitelline Membrane

The outer surface of the amphibian oocyte plasma membrane is known to have binding sites for both progesterone [15] and ouabain [12]. Table 2 summarizes the dissociation constants, binding capacity and estimated molecular weights of the ouabain binding component in plasma-vitelline membranes from prophase-arrested (unstimulated) *R. pipiens* oocytes.

Ouabain binding to the oocyte plasma membrane was measured after incubation of prophase-arrested oocytes (denuded of follicle cells) in [³H]ouabain, followed by the rapid manual isolation of plasma membranes [12]. Scatchard analysis of [³H]ouabain binding to the isolated plasma membrane indicates the presence of at least two components: a K⁺-sensitive, high affinity site ($K_d = 3 \times 10^{-8}$ M) linked to inhibition of the pump, and a low affinity site ($K_d = 2 \times 10^{-6}$ M) which does not appear to be associated with transport inhibition [12]. The binding capacity for the low-affinity ouabain site and the progesterone site are comparable. However, progesterone has a K_d one order of magnitude greater than that for low affinity ouabain binding site (Table 2).

Pretreatment of prophase *Rana* oocytes (denuded of follicle cells) with 10 μ M exogenous progesterone for 60 min eliminates the low affinity, but not the high affinity ouabain binding site (Table 1). This finding, plus the fact that the estimated molecular weight of the oocyte plasma membrane progesterone binding component and that of the steroid-binding α 1 subunit of the Na/K-ATPase are comparable (112 kDa, reviewed in [30]), and that ouabain and its analogues either facilitate or induce meiosis (Table 1), suggests that progesterone binds to the steroid-binding α -subunit of the Na/K-ATPase.

The total surface area of the prophase-arrested *Rana* oocyte can be estimated to be about 0.6 cm² per oocyte, based on capacitance measurements [27]. Scatchard analysis of low affinity ouabain binding and progesterone binding to the prophase-arrested oocyte indicates that 4,280 \pm 720 and 3,960 \pm 910 (mean \pm SD, N = 3) molecules of ouabain and progesterone, respectively, are bound per μ m². This indicates that progesterone and ouabain bind in a 1:1 ratio, consistent with a common binding site.

5.0 The Plasma Membrane Na/K-ATPase: Cardiotonic Steroid Binding to the α -Subunits of the Na/K-ATPase

5.1 Properties of the α -subunits of the Na/K-ATPase

Four genes encoding the Na/K-ATPase α -subunit have been cloned in vertebrates (reviewed in [31-34]). Evidence to date indicates that α -subunit isoforms with low affinity (certain α 1 isoforms) and high affinity (α 1, α 2, α 3 and α 4 isoforms) for the steroid, ouabain, are present in a variety of animal species (reviewed in [32-34]). Isoform-1 of the α -subunit is ubiquitous and has been considered the “housekeeping” form of the enzyme and it has been proposed that the high affinity ouabain binding α 1-subunit of Na/K-ATPase may serve to maintain low “bulk” cytosolic Na⁺ concentrations [9]. The α 4-subunit has been reported only in testis [34]. The neonate and the adult rat hearts exhibit both low and high affinity ouabain binding sites (e.g. [35]), although the low affinity isoform constitutes about 80-90% of the total; thus the rat heart is relatively insensitive to ouabain. The rat kidney and several other rat tissues contain only the low affinity isoform [32]. Thus, the adult rat heart and kidney are relatively insensitive to ouabain and may be used as a sources for the low affinity ouabain binding α 1-isoform.

Pralong-Zamofing et al. [33] have identified the α 1 isoform in a plasma membrane-rich sucrose gradient fraction prepared from Stage VI (fully grown) *X. laevis* oocytes using anti- α 1-serum. Ouabain sensitivity of endogenous *X. laevis* ovarian α 1 isoform was not measured., although ouabain binding to intact oocytes following expression of cRNA's encoding kidney α 1-isoform has been reported [36]. No published information was found relating to the α 2, α 3 and/or α 4 isoforms in vertebrate oocytes or ovaries.

5.2 The Structure of the α 1-Subunit of the Na/K-ATPase

Figure 2 illustrates the primary amino acid sequence of the α 1 subunit of *X. laevis* Na/K-ATPase (primary accession # **Q92123**) in Scalable Vector graphics (SVG) format [17]. Projection A

(left) shows the complete $\alpha 1$ -subunit, and projection B (right) represents a truncated version with emphasis on the external loops and transmembrane helices. The N and C termini of the α -subunit of the Na/K-ATPase are intracellular. The peptide has the 10 transmembrane domains and the 2 large intracellular loops found in all α -isoforms (reviewed in [30-32]). Transmembrane domains are α -helices composed primarily of non-polar amino acids. The transmembrane domain is stable across the fluid lipid bilayer because the hydrophobic effect operating on the side chains provides ~ 40 kcal mol⁻¹ of favorable free energy [36].

Conventional α -helices also occur within the loops between the transmembrane domains and will be considered separately as progesterone binding sites in section 6.1, below.

The primary amino acid sequence (1023 residues) of the $\alpha 1$ -subunit of *X. laevis* has a 93% sequence homology with that from both sheep (primary accession # **P04074**) and human (primary accession # **P05023**). For comparison, *Bufo marinus* (Giant toad) (primary accession # **P30714**) has a 94% sequence homology with the Na/K-ATPase $\alpha 1$ -subunit of *X. laevis*. An amino acid sequence for the Na/K-ATPase $\alpha 1$ -subunit from *R. pipiens* has not been published. Since *R. pipiens* (Leopard frog) is closely related to *X. laevis* (African clawed frog), the amino acid sequence for the $\alpha 1$ -subunit of *X. laevis* is taken as representative of the frog.

5.3 The Ouabain Binding Site on the $\alpha 1$ -Subunit

Three general approaches have been used to describe the high affinity binding site on the ouabain-sensitive $\alpha 1$ -subunit of the Na/K-ATPase. Mutagenesis of an $\alpha 1$ -subunit Na/K-ATPase gene of the H1C1 cell line [37] and random mutagenesis of the high affinity ouabain binding sheep $\alpha 1$ isoform [13], coupled with expression, has shown that the amino acids which alter ouabain binding and/or sensitivity cluster in two major regions. The first ouabain binding region comprises the extracellular loop joining the first two membrane spanning domains in the N-terminal region. A second ouabain-binding region is formed by the extracellular portions of transmembrane domains four, five, six and seven. In a second approach, the 2.6 Å resolution X-ray crystallographic structure of the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum (10 transmembrane domains) was used to infer the ouabain binding site in the Na/K-ATPase [38]. The luminal and cytoplasmic loop topology of the two peptides is remarkably similar (compare primary accession numbers **O14983** (Ca-pump) and **P05023** (Na-pump)). However, the Ca²⁺-ATPase does not bind ouabain, and except for a seven residue phosphorylation site on a cytoplasmic loop, there is a relatively low (20-25%) sequence homology between the Ca²⁺- and the Na/K-ATPase with less than 10% sequence homology in the first and second luminal loops identified as putative ouabain binding sites. The crystallographic data may, however, predict an arrangement of the ten $\alpha 1$ -subunit helices within the plane of the lipid bilayer that is common to both enzymes. In a third approach, chimeras of individual Na/K-ATPase loop regions were inserted into either the skeletal muscle sarcoplasmic reticulum/endoplasmic reticulum Ca²⁺-ATPase (SERCA1) [39,40] or the gastric H/K-ATPase [41,42]. The results suggest that M1-M2, M3-M4 and M5-M6 extracellular loops (see Figure 2) are important for ouabain sensitivity.

Few studies have been designed to elucidate the ouabain binding site on the low affinity ouabain binding isoform of the $\alpha 1$ -subunit. Lingrel et al. [13] suggested that the M1-M2 region of the $\alpha 1$ -subunit of sheep Na/K-ATPase, when dephosphorylated, is the low affinity ouabain-binding site, whereas in the high affinity (phosphorylated) conformation a single ouabain molecule spans both the M1-M2 and M4-M5-M6 regions. However, as outlined above, the concept of two unique $\alpha 1$ proteins with different sensitivities to ouabain best fits the data from a number of laboratories (reviewed in [9]). This implies that the low affinity ouabain binding region is restricted to the extracellular loop joining the first two membrane spanning domains in the N-terminal region.

6. Characterization of Progesterone Binding to the α 1-Subunit of Na/K-ATPase

6.1 Progesterone Binding Sites(s) on the *In Situ* α 1-Isoform

If progesterone selectively binds to the low affinity ouabain site on the α -subunit, a comparison of the amino acid sequence of the α 1 isoform with low ouabain affinity (rat; primary accession #P06685) with that for an α 1 isoform with high ouabain affinity (sheep; primary accession #P04074) could indicate the loci at which progesterone binding occurs. The amino acid sequence for the successive external loops of the α 1 isoforms from rat and sheep is illustrated in Table 3 (the sequence for the α 1 isoform from *X. laevis* kidney epithelial cells [43] and neural plate cells [44] (primary accession #Q92123) is shown for comparison). The amino acid sequence of the α 1-subunit in *R. pipiens* and *X. laevis* should be highly conserved. The amino acids highlighted in red indicate those residues identified as determinants of ouabain binding in the sheep enzyme [13,45], whereas those highlighted in green indicate residues that differ in rat (low affinity) compared to sheep (high affinity). Underlined residues in the C-terminal region of the M5-M6 loop indicate residues that are part of the transmembrane helix.

Based on RbDe and UniprotKB/Swiss-Prot/EMBL projections, the five external loops illustrated in Figure 2 contain 23, 12, 10, 51 and 15 residues going respectively from N to C terminal ends. The amino acid sequences of the 5 external loops of the α 1 isoforms of rat, sheep and *X. laevis* differ primarily in the 23 amino acid external loop between M1 and M2. The residues identified as determinants of cardiac glycoside binding in the sheep and human enzymes [45,46]; are C¹⁰⁹, Y¹¹³, Q¹¹⁶, A¹¹⁴, P¹²³, D¹²⁶, N¹²⁷, Y³¹³, W³¹⁵, T⁸⁰², and R⁸⁸⁵ (sheep numbering system; highlighted in red, see Table 3). In the first external loop, 5 of the 23 amino acids in rat differ from sheep, 3 of which have been identified as determinants of ouabain binding. In loops 2 and 3, all 3 amino acids identified by site-directed mutagenesis are the same in rat and sheep. In the largest external loop (M7-M8), only one residue has been identified by site-directed mutagenesis. It is the same in rat, sheep and *X. laevis*. Finally, in loop 5, no amino acids have been identified by site-directed mutagenesis. Loop 3 is unique in that the one residue identified by mutagenesis (T) is within the outer part of the M6 transmembrane helix. A comparison of the amino acids required for ouabain binding in Table 2 suggest that: 1) if ouabain binds to a site on the external loops of the α 1 isoform, and 2) if rat and sheep α 1 isoforms represent low and high affinity ouabain binding forms, respectively, then the primary low affinity ouabain binding site may lie in the external loop between M1 and M2.

6.2 Topology of the First External Loop of the α 1-Subunit

Figure 3 compares the 3D projection of the first external loop of the human α 1 isoform (upper) with that of the rat α 1 isoform (lower) of the Na/K-ATPase using Chem3D Ultra v. 10.0. As can be seen, the first external loop of the isoform that binds ouabain with low affinity (rat, $K_d = 10^{-6}$ M) is more tightly organized, with a large α -helical content. In addition, there is a hook in the c-terminal region due to the Pro¹²⁷-Pro¹²⁸ sequence in the low affinity ouabain binding form. In contrast, the first external loop of the isoform that binds ouabain with high affinity (sheep, human, $K_d = 10^{-9}$ M) contains only two small helical elements.

As noted above, an analysis of differences in the amino acid sequences between low and high affinity ouabain binding isoforms should indicate the peptide sequence important in progesterone binding. To this end, an expanded amino acid SVG plot of the M1 and M2 helices from the high ouabain affinity α 1 subunit, together with the extracellular loop that joins the two transmembrane domains is illustrated in Figure 4. The diamonds indicate the positions of the random amino acid substitutions that confer the greatest ouabain resistance: which include Cys¹¹³, Tyr¹¹⁷, Gln¹²⁰, Pro¹²⁷, Asp¹³⁰, and Asn¹³¹ [45] (positions indicated in Figure 4 are

based on *X. laevis* α 1-subunit numbering). In the low affinity ouabain form (rat) Gln¹²⁰ becomes Arg, Ala¹²¹ becomes Ser, and Asn¹³¹ becomes Asp. Based on the M1-M2 rat sequence shown in Table 3, a consensus sequence for the progesterone binding site in the α 1-subunit may therefore include the following CFLAYGIRSATEEEPPNDD (residues 113 to 131).

6.3 Hydrophobicity and Polarity Gradients within the First External Loop

Structure-function studies indicate that the spatial arrangement of the polar substituents on the upper or β face of the steroid molecule is of critical importance for progesterone binding [47]. The 3, 20-dione, 21-ol configuration is the most active. An unsubstituted α or lower surface is also required for activity; introduction of a polar group diminishes or abolishes activity. The upper or β surface may interact with a polar region, whereas the lower or α surface may interact with a hydrophobic region. Figure 5 compares the hydrophobicity (upper panel) polarity (lower panel) gradients as a function of position from residue 110 to residue 134 (abscissa). The hydrophobicity and polarity scores were calculated using the ProtScale Tool (ExPASy) based on Kyte and Doolittle [18] and Grantham [19], respectively. The plots indicate that a steep gradient exists within the loop. The N-terminal region of the loop is hydrophobic whereas the C-terminal region is highly polar.

The 23 amino acid sequence in the first external loop in rat can also be written as a classical α -helix (3.6 amino acids per turn) on the surface of a cylinder. After 5 turns, residues I¹⁰⁹ and P¹²⁶ have similar positions parallel to the axis of the cylinder. Gaboriaud et al. [20] have shown that if the cylinder is cut parallel to its axis and unrolled, sets of adjacent hydrophobic residues can be encircled and termed hydrophobic clusters (see Figure 6). As some adjacent amino acids (e.g. G¹¹⁶ and I¹¹⁷) are widely separated by the unfolding of the cylinder, the representation is duplicated in the lower projection, making the sequence easier to follow and giving a better impression of the microenvironment of individual amino acids.

Sets of adjacent hydrophobic residues in the pattern are encircled and termed hydrophobic clusters (Figure 6). I, L, F, and Y were considered to be hydrophobic amino acids, whereas P (star) is primarily considered as a breaker of these clusters, and A and C as mimetic, i.e. hydrophobic only in a hydrophobic environment. Consistent with the method of Gaboriaud et al. [20], different symbols are used for proline (star), glycines (square), which are often present in loops, and cysteines (C) which may be involved in disulfide bond formation. As shown in the lower portion of Figure 6, a large hydrophobic cluster exists in the N-terminal region of the first external loop. This predicts that the lower or α surface of the steroid molecule interacts with the hydrophobic region (residues 110-120). The upper or β face of progesterone would be expected to interact with the more polar portion of the loop (residues 121-134).

6.4 Changes in Flexibility within the First External Loop

Figure 7 illustrates the average flexibility score [48] as a function of position along the first external loop in the *Xenopus laevis* α 1-subunit of Na/K-ATPase. The average flexibility index for the sequence (residues 110-132, abscissa) was calculated using the algorithm of Bhaskaran and Ponnuswamy [48] with ProtScale (expasy.org). The positions of the amino acids (Arg¹¹⁸, Asp¹²⁹, Cys¹¹¹) at which site-directed mutagenesis studies indicate that substitutions confer the greatest ouabain resistance, are indicated by arrows. Regions of low flexibility occur in the hydrophobic region whereas the highest flexibility occurs near the mid-point of the loop, suggesting that folding occurs within the loop, further stabilizing the progesterone binding site.

7.0 Progesterone Binding to the $\alpha 1$ -Subunit of the Na/K-ATPase

7.1 Molecular Modeling of the Progesterone Binding Site

Figure 8 illustrates the proposed progesterone binding site within the first external loop of the low affinity ouabain binding isoform of the $\alpha 1$ -subunit. As mentioned above, X-ray crystallographic data for the $\alpha 1$ -subunit of the Na/K-ATPase are not available. It is not possible, therefore, to develop a molecular model similar to that published for the cytosolic progesterone receptor [49]. The graphic in Figure 8 represents a view looking down on the cell surface, with an end view of the first (M1) and second (M2) transmembrane helices and the interconnecting loop of 23 amino acids. The peptide bonds between the helices and the external loop amino acids are discontinuous to allow for 90 degree rotation of the loop sequence. The amino acids thought to be critical for ouabain binding are indicated in single letter codes (C¹¹¹, Y¹¹⁵, R¹¹⁸, S¹²², P¹²⁸, D¹²⁹, D¹³¹). The planar progesterone is shown on its side with the α hydrophobic face upward facing left and the polar β surface facing down and to the right. The arrows indicate the direction of insertion of the planar progesterone molecule.

The ouabain binding domain of Na/K-ATPase and the classical cytosolic steroid receptors appear to share amino acid sequence homologies, as reported by LaBella and Templeton [50] and Seccombe et al. [51]. In the ouabain radioligand assay used by Seccombe et al. [51], the potencies of a series of steroid moieties suggest that the steroid-binding domain of the Na/K-ATPase can accommodate compounds with planar configurations. Progesterone, dehydroepiandrosterone, 11-hydroxycortisol and 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) were found to have digoxin-like immunoreactivity at low concentrations in mammals. (Digoxin differs from ouabain in that it has 2 additional sugar moieties in the 3-position and lacks three hydroxyl groups). Furthermore, progesterone and 18-OH-DOC were most efficient in displacing radiolabeled-ouabain from canine kidney Na/K-ATPase. These observations are consistent with our previous ouabain competition studies [8], as well as with our earlier findings of the order of efficiency of different steroids in inducing amphibian meiosis [47].

Previous studies in this laboratory have shown that three structural features of the steroid molecule are essential for induction of meiosis [47]: 1) a planar ring system, 2) a hydrophobic lower or α surface of the steroid, and 3) an angular methyl group at C-10. Thus, testosterone or androstanediol are effective inducers, 19-nor-testosterone or 19-nor-androstanediol are inactive (Table 2). Although the 3,20-dione, 21-ol pregnane configuration is the most active in meiotic induction [47], steroids with a lactone in the 17-position will also induce nuclear membrane breakdown (digitoxigenin, Table 2).

Steroids with a nitroxide ring (DOXYL) in the 3-position will also induce meiosis (3-DOXYL-17 β -OH-5 α -androstane, Table 2). 3-DOXYL-17 β -OH-5 α -androstane (Cat. #25,354-5, Aldrich Chemical Co., Milwaukee, WI) is readily taken up by isolated *R. pipiens* plasma-vitelline membranes. The order parameter, S, can be measured using electron spin resonance techniques [52] [the order parameter is a general concept, and ranges from a value of 1.0 (no motion) to 0 (complete averaging)]. In contrast to an S value of 0.681 \pm 0.010 (N = 5) for the 5-DOXYLstearic acid probe taken up by the isolated plasma membrane, the EPR signal for 3-DOXYL-17 β -OH-5 α -androstane indicated no motion. In other words, the 3-DOXYL moiety of the steroid was inserted into a totally restricted environment in the oocyte plasma membrane. The 3-DOXYL-17 β -OH-5 α -androstane spin probe could be recovered from the isolated plasma membranes by extraction with CHCl₃:CH₃OH (2:1)

If a strict geometric fit is assumed between the shape of the steroid molecule and the receptor, then the structure-function restrictions outlined here and elsewhere [47] suggest that the steroid is inserted into the first external loop with the upper or β face conforming to a polar region (residues 123-132, Figure 5) and the lower or α face closely associated with a hydrophobic

region (residues 113-122, Figure 5). Replacement of Gln¹²⁶ with Pro¹²⁶ in the low affinity ouabain binding isoform introduces a sharp bend in the peptide chain at the N-terminal end of the first external loop (lower cartoon, Figure 3). We propose (Figure 8) that the hydrophobic α surface interacts with the hydrophobic clusters in the N-terminal region (Figure 6), and that the angular methyl group at C-10 interacts with the Leu¹³²-Gly¹³³-Val¹³⁴ sequence at the beginning of the M2 helix.

The sharp bend produced by the Pro¹²⁵-Pro¹²⁶ sequence would also place the 3-keto group in juxtaposition to a Glu¹²²-Glu¹²³-Glu¹²⁴ sequence on one side of the bend, and an Asp¹²⁸-Asp¹²⁹ sequence on the other. The optimal inducing activity of the 3,20-keto steroids such as progesterone could be due to hydrogen bonding between the carbonyl dipole and adjacent polar amino acids (Glu¹²²-Glu¹²³-Glu¹²⁴ and Asn¹²⁷-Asp¹²⁸) within the bend. Such bonds are highly directional and would project above the β -plane of the steroid. Cys¹¹¹ at the interface with the external loop, M1, is known to be essential for ouabain binding/inhibition [45] and could participate in hydrogen bonding to the 20-keto group of progesterone, as has been proposed for progesterone binding to the cytosol receptor [49]. As noted above, maximum flexibility occurs near the midpoint of the external loop (Figure 8), suggesting that if M1 and M2 moved towards each other, folding would occur within the loop and further stabilize progesterone binding.

7.2 Role of Plasma Membrane Lipids in Progesterone Binding

The molecular model illustrated in Figure 8 represents an integral membrane protein with multiple transmembrane domains within the lipid bilayer. In non-dividing cells, phosphatidylcholine (PC), sphingomyelin (SM) and glycosphingolipids are primarily localized in the outer (exoplasmic) lipid leaflet, whereas phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol are generally enriched in the cytoplasmic leaflet [53]. The inner and outer regions of the $\alpha 1$ -subunit are therefore exposed to different lipid environments.

The model in Figure 8 does not consider possible contributions by other hydrophobic membrane constituents (e.g. PE, SM, cholesterol) on progesterone binding to an exoplasmic peptide loop associated with the outer bilayer. A number of reports indicate that the topological organization, and thus the function of many membrane proteins, are not only determined by the amino acid sequence of the protein but also by the lipid composition of the membrane (reviewed in [54]). Major changes in the functional properties and topological organization of transport proteins such as GABA permease and the phenylalanine transporter of *E. Coli* have been reported to occur when membrane PE is altered [54].

We have found that progesterone binding to the plasma membrane increases several fold during the first 4-5 h, during which time PE is converted to PC and there is rise in SM (reviewed in [3]). These changes could alter the microenvironment of the external peptide loops of the $\alpha 1$ -subunit of Na/K-ATPase and contribute to increased progesterone binding. Changes in the lipid microenvironment could also modulate $\alpha 1$ -subunit interaction with other integral membrane proteins. Phospholipid changes could contribute to the observed progesterone-induced increases in integral membrane enzymes such as PE-N-methyl transferase (4 transmembrane domains) and sphingomyelin synthase (1 transmembrane domain) (reviewed in [3]).

8.0 Comparison of the $\alpha 1$ -Subunit Progesterone Binding Site with Other Putative Progesterone Receptors

As noted in the introduction, at least two other plasma membrane receptors have been proposed to explain steroid induction of the meiotic divisions in fish and *Xenopus*. A 40 kDa G-protein

linked membrane steroid receptor has been described in fish ovary [55]. More recently, it has been proposed that the classical *Xenopus* progesterone receptor (named xPR-1) can interact with the plasma membrane through its ligand-binding domain [11]. Overexpression of xPR-1 accelerates progesterone-induced oocyte maturation (as does ouabain, Table 2). Injection of antisense oligonucleotides almost completely blocks progesterone-induced nuclear membrane breakdown.

Steroid induction of meiosis and/or binding in fish and *Xenopus* has been generally described as if the rapid response to progesterone (or a progesterone metabolite) was sufficient to complete the first meiotic division. However, it was first shown by Dettlaff [56] that gonadotropin (responsible for progesterone synthesis and release from the follicle cells) must be present throughout the period leading to nuclear membrane breakdown in *Rana temporaria* follicles. Dettlaff termed this the “hormone-dependent period”. We confirmed this finding with *Rana pipiens* follicles [57] and found that at physiological (nmolar) concentrations, extracellular progesterone must be present throughout the first 4-5 h for nuclear membrane breakdown to occur 7 - 8 h after the initial exposure [8,57]. Similar experiments with Stage VI *X. laevis* follicles (unpublished) indicate that a continuous exposure to low levels of progesterone for at least 90 min is necessary for nuclear membrane breakdown to occur at about 3 h.

We have found that, in the gonadotropin-stimulated *Rana pipiens* ovarian follicle, progesterone levels increase several-fold during the hormone-dependent period both *in vivo* and *in vitro* [8]. The progesterone continuously synthesized by the follicle cells becomes largely bound to the oocyte plasma membrane over the first 4-5 h. At 5-6 h, about 95% the membrane-bound progesterone is internalized along with about 60% of the plasma membrane. The internalized progesterone is rapidly metabolized to highly polar (polyhydroxylated) derivatives [58]. Since plasma membrane bound-progesterone is internalized prior to nuclear membrane breakdown in *Rana* oocytes, the appearance of xPR-1-bound steroid could reflect a transfer between ligands following steroid internalization.

A broad range of steroids, including progesterone, induce nuclear membrane breakdown in human chorionic gonadotropin (hCG)-primed fish oocytes [55]. The finding that radiolabeled progesterone is converted to 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S) (as well as to at least one other unidentified polar steroid) by ovarian segments (taken from the Atlantic Croaker) has been cited as evidence that 20 β -S is the maturation-inducing steroid in fish. However, 20 β -S will not induce nuclear membrane breakdown unless the fish ovarian segments have been pretreated with hCG for 9-12 h. As noted by Patino and Thomas [55], “maximal response to 20 β -S was seen in ovaries with oocytes showing the first signs of morphological (meiotic) maturation”. Furthermore, radiolabeled 20 β -S was extracted and identified 1-2 h after completion of nuclear membrane breakdown in the fish ovarian segments. Thus, the steroid-dependent events monitored in the Atlantic croaker may occur after the hormone-dependent period and the appearance of 20 β -S may coincide with conversion of internalized progesterone to polar steroids in *Rana pipiens* oocytes [58]. The finding that a polyhydroxylated steroid (ouabain) will potentiate progesterone induction of meiosis (Table 2) suggests that the polar (ouabain-like) steroids produced following internalization of membrane-bound progesterone may be necessary but are not sufficient by themselves to induce meiosis.

9.0 Summary and Conclusions

Progesterone induces the meiotic divisions in the amphibian oocyte by activating a signaling system in the plasma membrane. We present evidence that the progesterone receptor on the oocyte surface is located in an isoform of the α 1-subunit of Na/K-ATPase, which is characterized by a low affinity for ouabain ($K_d = 2 \times 10^{-6}$ M, Table 1). Progesterone competes

in vitro with ouabain for this low affinity site on the $\alpha 1$ -isoform; however under physiological conditions the nmolar concentrations of circulating ouabain would not affect binding by μ molar levels of progesterone ($K_d = 5 \times 10^{-7}$ M for progesterone, Table 1). In comparing the low and high ouabain binding α -isoforms, we find a sequence difference of six amino acids localized in the M1-M2 N-terminal external loop. Seven amino acids in this loop of the high ouabain affinity isoform have been identified as determinants of ouabain binding in the high affinity isoform (Table 3 and Figure 4).

Based on these differences, we conclude that at least seven of the 23 amino acids in the first external M1-M2 loop of the low ouabain affinity form may be involved in progesterone binding. The findings predict that the polar β and the hydrophobic α surfaces of the planar progesterone molecule interact with opposite sides of the folded M1-M2 loop (Figure 8). This would be consistent with the countercurrent hydrophobicity and polarity gradients within the loop (Figure 5) as well as with the hydrophobic clusters within the N-terminal region of the loop (Figure 6). The molecular model (Figure 8) further suggests that the angular methyl group between the A and B rings forms hydrophobic bonds with the Leu¹³²-Gly¹³³-Val¹³⁴ sequence at the N-terminal region of the M2 helix. The model also predicts that the 3-keto group of progesterone hydrogen-bonds to residues Arg¹¹⁸, Asp¹²⁹ and possibly Glu¹²²⁻¹²⁴. Similarly, Cys¹¹¹ could hydrogen bond to the 20-keto group.

We propose that the low affinity ouabain $\alpha 1$ -isoform is a progesterone receptor, whereas the high affinity ouabain $\alpha 1$ -isoform regulates Na⁺-pump activity. As noted above, even at maximal physiological ouabain concentrations in the blood (nmolar [21]), there would be no significant binding of the cardiotonic steroids to the low affinity ouabain $\alpha 1$ -isoform. Our structure-function studies with the oocyte indicate that classical steroids other than progesterone may also bind to the first external loop of the $\alpha 1$ -subunit of the Na/K-ATPase [47]. Since C-21 (pregnanes) and C-19 (androstanes) steroids often have overlapping biological activities, the low affinity ouabain $\alpha 1$ -subunit of the Na/K-ATPase in the plasma membrane may serve a broader function in nongenomic (plasma membrane) responses to steroids by interacting with tissue-specific integral membrane proteins.

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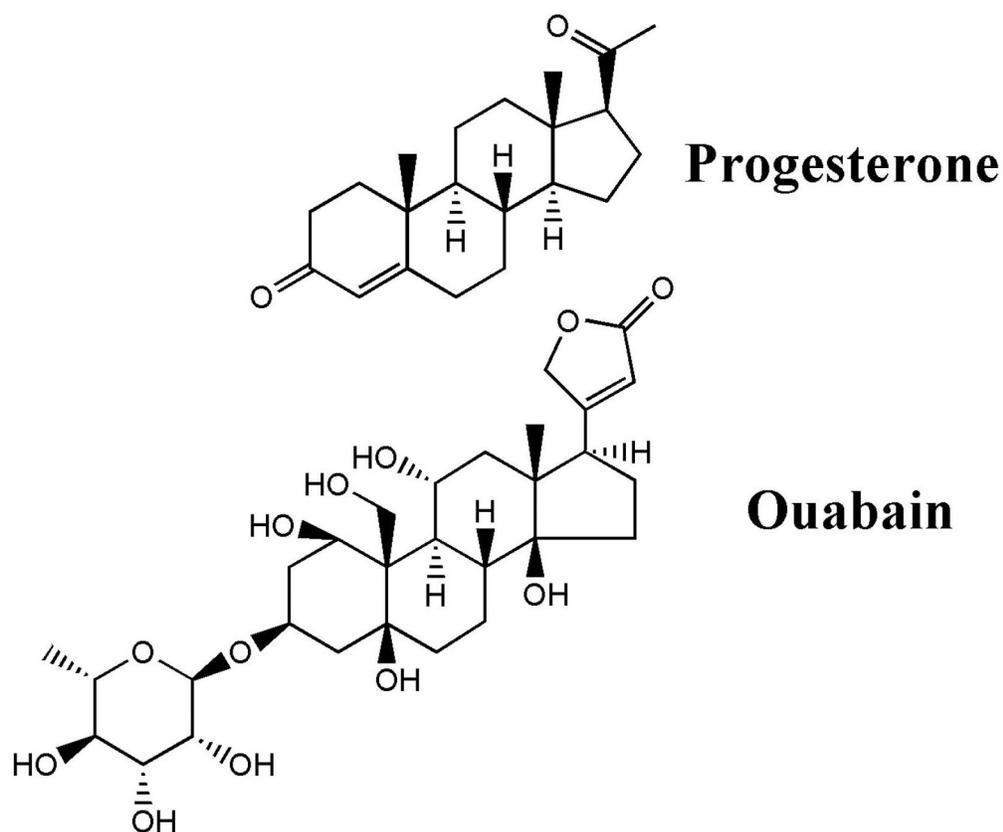


Figure 1. Structures of Δ^4 -pregnane-3,20-dione (progesterone) and the cardiotonic steroid ouabain. Compounds with an unsaturated five-membered lactone ring are cardenolides and those with an unsaturated six-membered lactone ring are bufadienolides. In the formulae, bonds or atoms above the plane of the ring system are depicted as solid wedges; those below the plane are depicted as open, dashed wedges.

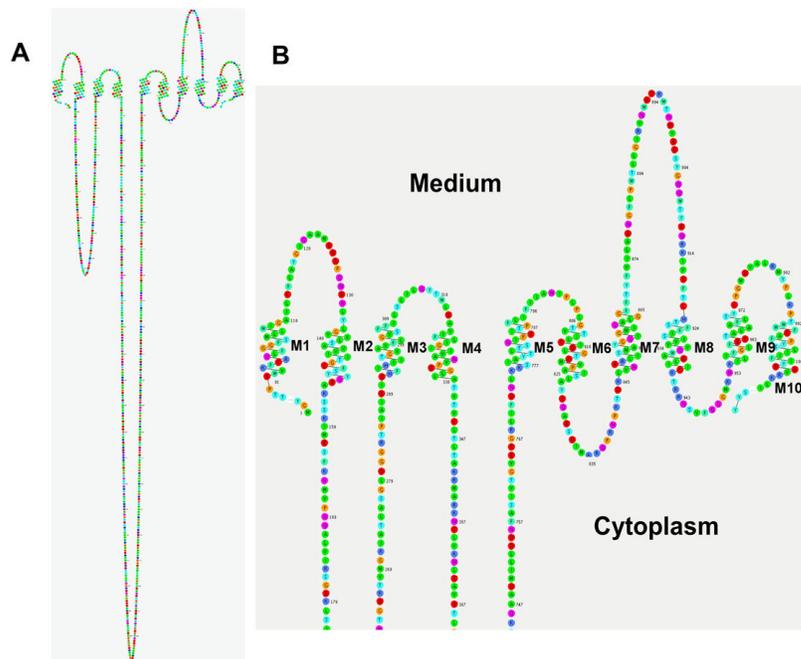


Figure 2.

The primary amino acid sequence of the complete (A) and truncated (B) graphics of the α -1 subunit of *Xenopus laevis* Na/K-ATPase in Scalable Vector graphics (SVG) format [17]. The sequence (primary accession # **Q92127**) was obtained from the nucleotide sequence of *Xenopus laevis* kidney epithelium [43] and neural plate cells [44]. The N and C termini of the α -subunit of the Na/K-ATPase are located intracellularly and the protein has 10 transmembrane domains and 2 large intracellular loops (see text). The intracellular N-terminal and C-terminal ends contain a white spacer to shorten the terminal sequence due to space limitations.

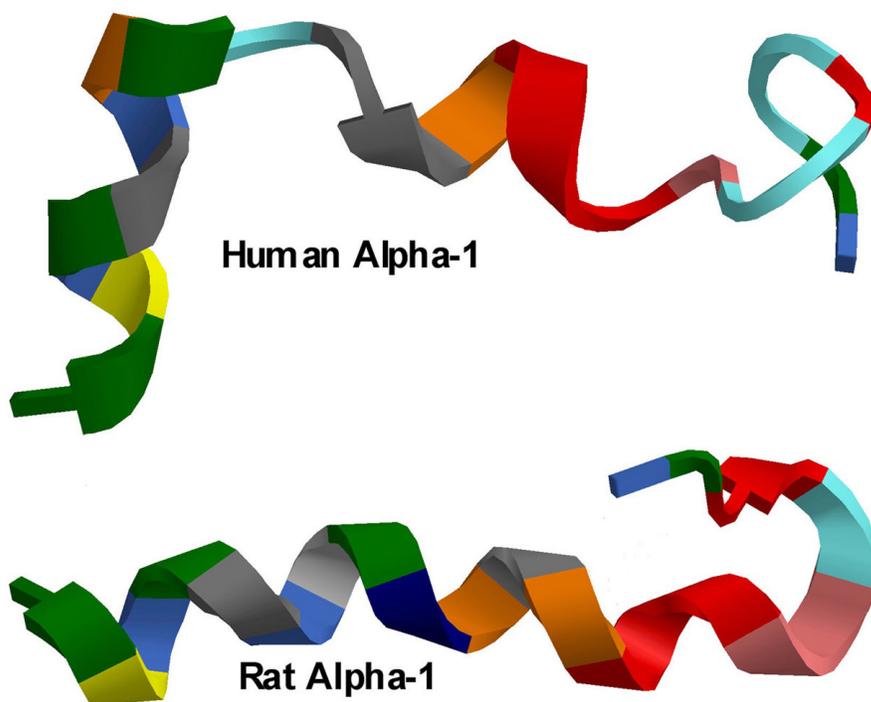


Figure 3. A comparison of 3D projection of the 23 amino acid sequence in the first external loop of the $\alpha 1$ subunit of the Na/K-ATPase of the high affinity ouabain binding isoform (human, upper cartoon) and low affinity ouabain binding isoform (Rat, lower cartoon). Plots were generated using Chem 3D Ultra v. 10.0 (Cambridgesoft.com). Colors indicate individual amino acids.

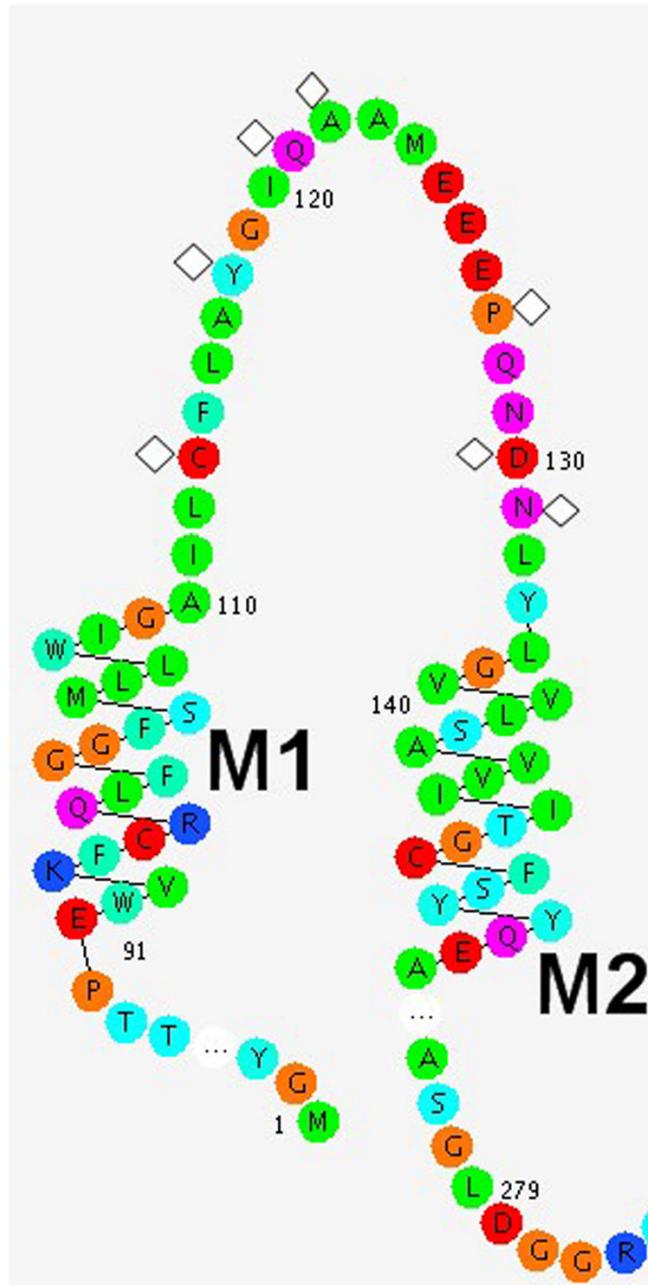


Figure 4.

An expanded amino acid Scalable Vector Graphics (SVG) plot of the M1-M2 domain with the extracellular loop that joins the two transmembrane domains for the $\alpha 1$ -isoform from *X. laevis* (Primary accession #**Q92127**). Arrows indicate residues identified as determinants of ouabain sensitivity for the sheep enzyme [13]; Cys¹¹³, Tyr¹¹⁷, Gln¹²¹, Pro¹²⁷, Asp¹³⁰, and Asn¹³¹. Residue numbers are based on the sequence for *X. laevis* [43,44]. The N-terminal intracellular domain (80 residues, primary accession # **P04074**) of the sheep $\alpha 1$ subunit is about 9 residues shorter than that of the *Xenopus* $\alpha 1$ subunit (89 residues).

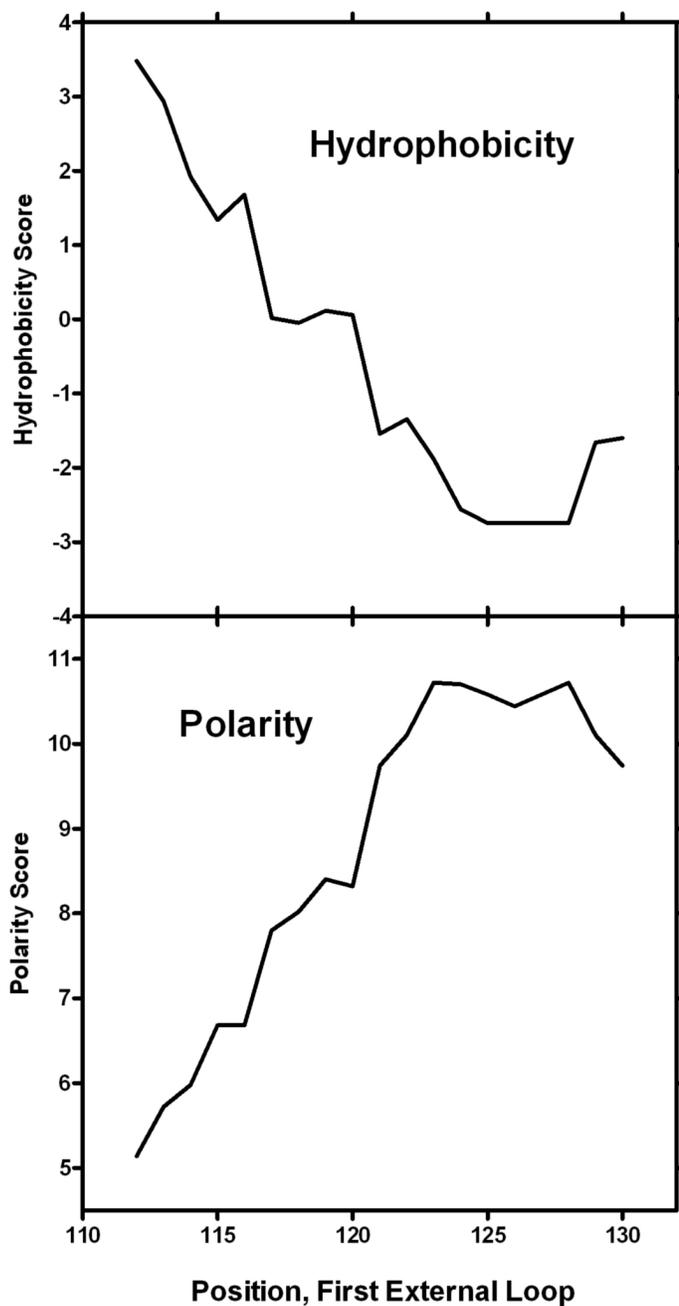


Figure 5.

The hydrophobicity (upper panel) and polarity (lower panel) gradients within the first external loop of the gradient for the $\alpha 1$ isomer illustrated in Figure 5. The sequence (residues 110-132, abscissa) was analyzed using ProtScale (expasy.org) and is shown as a function of the hydrophobicity or polarity score on the ordinate. The window was set at 5 and the algorithm of Kyte and Doolittle [18] was used to estimate hydrophobicity whereas the algorithm of Grantham [19], was used to measure polarity.

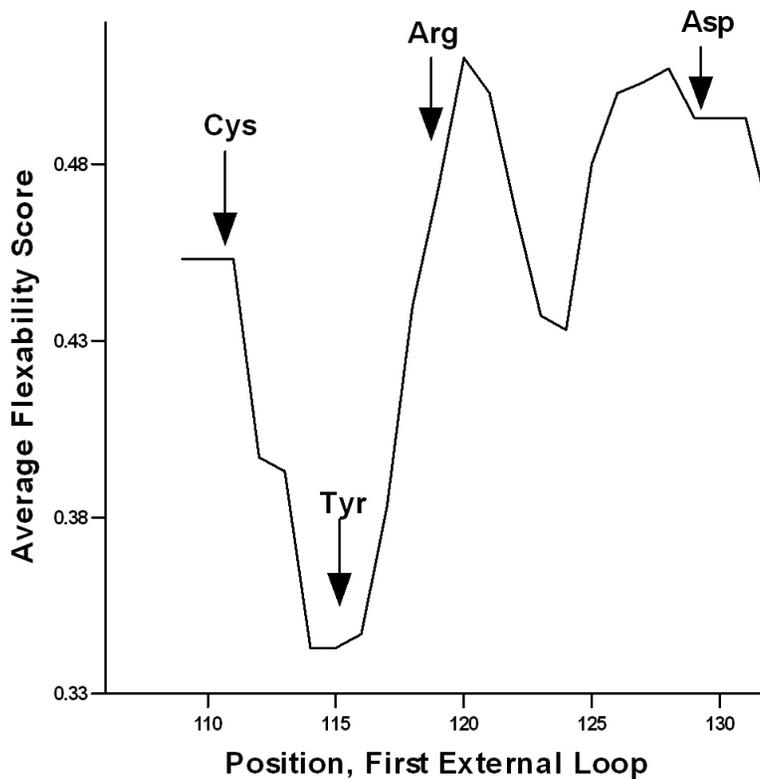


Figure 7. The average flexibility index along the first external loop is illustrated as a function of amino acid position in *Xenopus laevis* α 1-subunit of Na/K-ATPase. The average flexibility index for the sequence (residues 110-132, abscissa) was calculated by the algorithm of Bhaskaran and Ponnuswamy [48] using ProtScale (expasy.org).

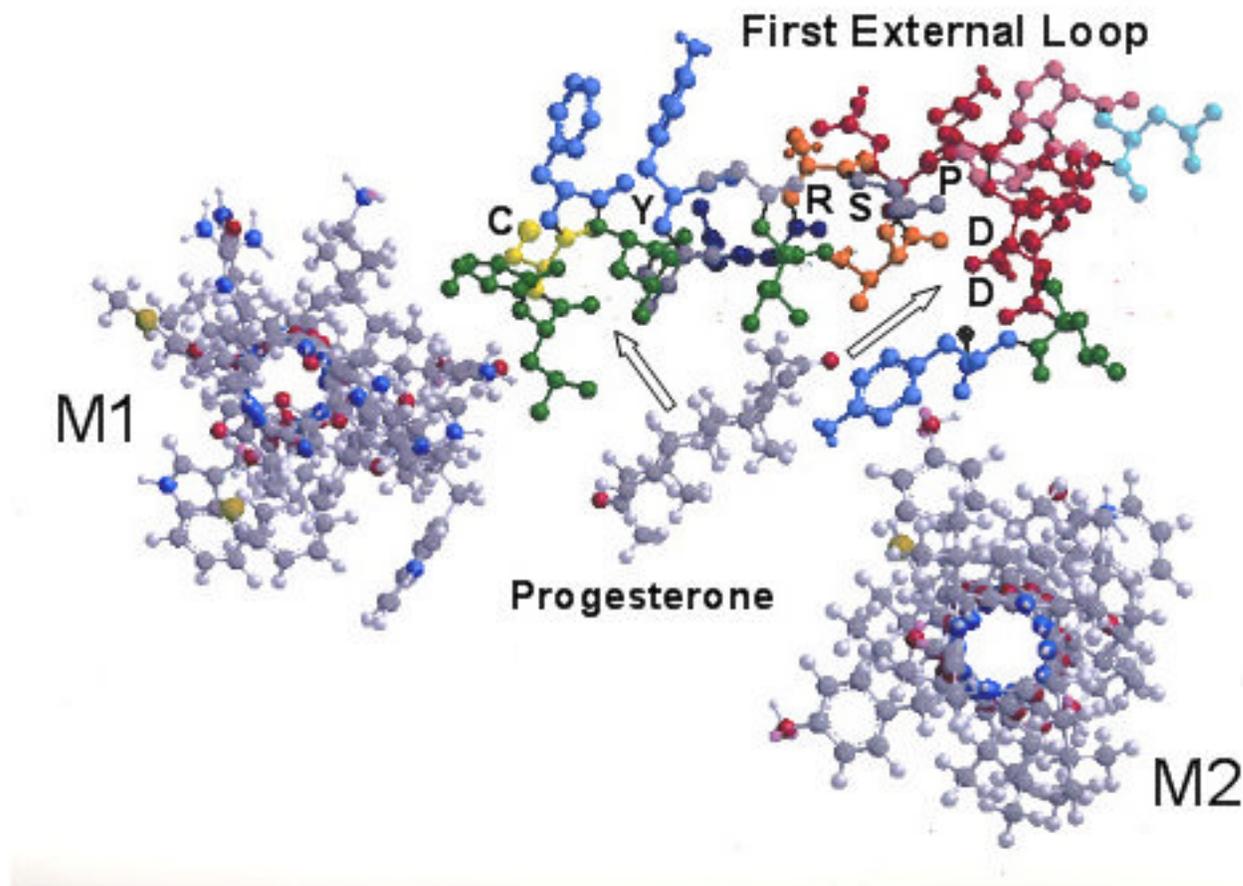


Figure 8.

An illustration of the possible progesterone binding site within the first external loop of the low affinity ouabain binding isoform of the $\alpha 1$ -subunit of the Na/K-ATPase. The graphic represents a view looking down on the cell surface, with an end view of the first (M1) and second (M2) transmembrane helices and the interconnecting loop of 23 amino acids. The peptide bonds between the helices and the external loop amino acids are discontinuous to allow for 90 degree rotation of the loop sequence. The arrows indicate the direction of insertion of the planar steroid into the loop. The amino acids in the external loop are color coded and the amino acids thought to be critical for ouabain binding are indicated in single letter codes (C¹¹¹, R¹¹⁸, S¹²², P¹²⁸, D¹²⁹, D¹³¹). The planar progesterone (shown on its side with the α hydrophobic face upward and to the left) interacts with hydrophobic clusters in the N-terminal region. The 19-methyl group (facing down and to the right) interacts with residues at the beginning of the transmembrane helix (M2). As proposed, a hydrogen-bonding network forms between the 3-keto group and the conserved residues Gln¹²⁰, Asn¹³¹ and Tyr¹¹⁸ in the first external loop of the α -1 subunit of the rat Na/KATPase.

Comparison of [³H]Progesterone and [³H]Ouabain Binding to Isolated Plasma-viteline Membranes from *Rana pipiens* Oocytes

Table 1

Steroid	[Progesterone] ₀ , μM	Oocyte Plasma-viteline Membrane		M.W.	Ref
		Kd	fmols/oocyte		
Progesterone	$5.1 \pm 1.1 \times 10^{-7}$ M	66 ± 19	110-114 kDa	15
Ouabain	0	$3.2 \pm 0.5 \times 10^{-8}$ M	15 ± 4		8,12
	0	$2.0 \pm 0.4 \times 10^{-6}$ M	80 ± 9	112 kDa ¹	8,12
	1.6 ²	$1.8 \pm 0.26 \times 10^{-8}$ M	28 ± 5		8

¹ M.W. of α-subunit of Na/K-ATPase [27].

² Oocytes were pretreated with 1.6 μM progesterone for 1 h.

Table 2

Steroid	C-3	Δ^4 DB	C-1	C-10	C-5	C-13	C-11	C-14	C-17	Response ¹	Ref
Pregnane	keto	Yes	H	CH ₃	H	CH ₃	H	H	20-keto	Induces	44
Progesterone	Sugar	No	OH	CHOH	OH	CH ₃	OH	OH	Lactone	Potentiated	21
Ouabain	OH	No	H	CH ₃	H	CH ₃	H	OH	Lactone	Induces	22
Digitoxigenin	DOXYL	Yes	H	CH ₃	H	CH ₃	H	H	OH	Induces	...
3-DOXYL-17 β ol-5 α -androsterane	OH	Yes	H	CH ₃	H	CH ₃	H	H	OH	Induces	44
5 α -androsterane-3,17-diol	OH	Yes	H	CH ₃	H	CH ₃	H	H	OH	Induces	44
19-Nor-androstane-3,17-diol ²	OH	Yes	H	H	H	CH ₃	H	H	OH	Inactive	44

¹ Nuclear membrane breakdown in amphibian oocytes *in vitro* (24h at 20-22° C).

² 19-Nor-testosterone is also inactive (ref. 44).

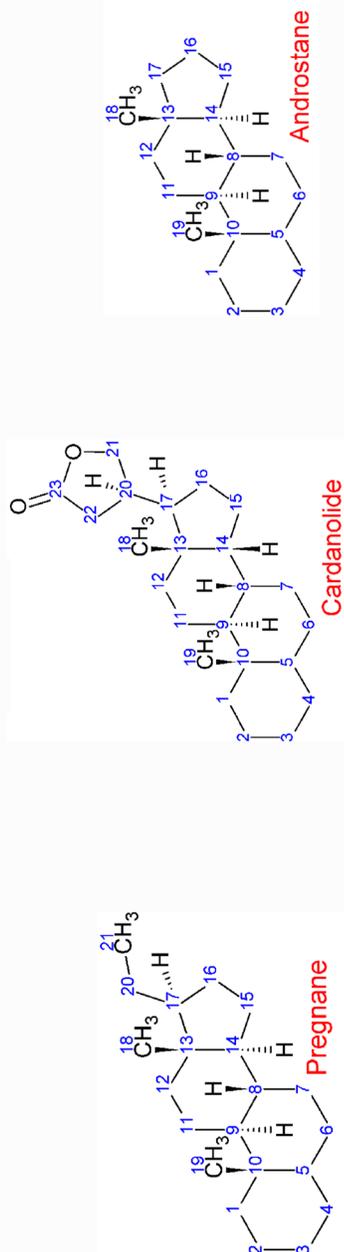


Table 3Variations¹ in the External Loops of the α 1-Subunit of the Na/K-ATPase of Sheep, Xenopus and Rat

M1-M2 External Loop	
Sheep	VLCFLAYYGIQAATEEEPQNDNLY
Xenopus	ILCFLAYYGIQAAMEEEPQNDNLY
Rat	ILCFLAYGIRSAATEEEPPNDDLY
M3-M4 External Loop	
Sheep	LSLILEYTWLEA
Xenopus	LSLILQYTWLEA
Rat	LSLILEYTWLEA
M5-M6 External Loop	
Sheep	FILIANIPLPLGTV
Xenopus	FILIANIPLPLGTV
Rat	FILIANIPLPLGTV
M7-M8 External Loop	
Sheep	FTYFVIMAENGFLPNHLLGIRVTWDDRWINDVEDSYGQQ
	WTYEQRKIVEFTC
Xenopus	FTYFVILAENCFLPWTLLGIRVNWDDRWTNDVEDSYGQQ
	WTYEQRKIVEFTC
Rat	FTYFVILAENGFLPFHLLGIRETWDDRWINDVEDSYGQQ
	WTYEQRKIVEFTC
M9-M10 External Loop	
Sheep	CPGMGAALRMYP L K P
Xenopus	CPGM D VALRMYP L K P
Rat	CPGMGAALRMYP L K P

¹ Amino acids highlighted in red indicate residues identified as determinants of ouabain binding in the sheep enzyme (e.g. [13]), whereas those highlighted in green indicate residues that differ in Rat compared to sheep. Underlined sequences in C-terminal region of the M5-M6 loop indicate residues that are part of the transmembrane helix.