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Ouabain- and Ca^{2+}-sensitive ATPase activity of chimeric Na- and Ca-pump molecules

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Chimeric ion-pumps, consisting of the N-terminal 2/3 of the α_1-subunit of the ouabain-sensitive chicken Na^+,K^+-ATPase and the C-terminal 1/3 of the sarcoplasmic reticulum Ca^{2+}-ATPase, were expressed in ouabain-insensitive mouse L cells. These chimeric molecules exhibited ouabain-sensitive ATPase activity very similar to that of the wild-type chicken Na^+,K^+-ATPase. This ATPase activity could be stimulated by adding Ca^{2+} to the assay system. These results suggest that the sites for ouabain-inhibition are restricted to the N-terminal 2/3 of the Na-pump, and the C-terminal 1/3 of the Ca-pump interacts with Ca^{2+}.

Na^+,K^+-ATPase; Ca^{2+}-ATPase; Ouabain; Chimeric molecule; Chicken

1. INTRODUCTION

Recent cDNA cloning and sequencing studies have shown that a variety of E1-E2 type ATPases (ion pumps) may form very similar higher order structures, even though the primary amino acid sequences of these ATPases are rather different [1]. On the other hand, these ATPases show distinct specificities for particular ions, inhibitors and subunits. One approach to identifying the functional domains that determine selectivity of ion transport is to construct chimeric molecules between distinct functional proteins that have very similar structures. This type of approach has been successfully applied to structure/function analysis of several membrane proteins [2–6].

Proposed models of the higher order structures of E1-E2 ATPases predict multiple (7–10) transmembrane segments, and conserved amino acid sequences for the ATP-binding- and phosphorylation-domains between the fourth and the fifth potential transmembrane domains (M_4 and M_5) (Fig. 1). The region just before M_5 is also very well conserved among these distinct ion-pumps [1]. We have taken advantage of this, and constructed a set of chicken sodium pump/calcium pump chimeric cDNAs that would preserve the overall topological structure of the encoded chimeric proteins as ion pumps. Mouse L cells that express the chicken β-subunit [7] were transfected with these chimeric cDNAs. Because the Na^+,K^+-ATPase of mouse L cells is relatively ouabain-resistant, this avian/mouse hybrid system allows us to monitor enzymatic functions of the relatively ouabain-sensitive chimeric molecules.

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Abbreviations: ATPase, adenosine-tri-phosphatase; FITC, fluorescein-isothiocyanate; FSBA, 5′-(*p*-fluorosulfonyl)benzoyl-adenosine; PM, plasma membrane; SR, sarcoplasmic reticulum; TRITC, tetramethylrhodamine

2. MATERIALS AND METHODS

2.1. *Construction of mutant cDNAs encoding chicken Na-pump/Ca-pump chimeric molecules*

Wild-type cDNAs encoding the chicken sodium pump α_1-subunit [8] and the chicken SR calcium-pump [9] were used for construction of chimeric cDNAs. A unique restriction site, *Eco*NI, which is endogenous to both the SR calcium pump cDNA and the PM sodium pump α_1-subunit cDNA, was used as an exchange point for recombination. This exchange occurred at nucleotide 2134 on the α_1-subunit cDNA and at nucleotide 2170 on the calcium pump cDNA, both of which encode lysine* residues within the evolutionarily conserved FSBA-binding domain [1,10] (-Pro-Ala-Leu-Lys*-Lys-Ala-) just before M_5 of the two proteins. These chimeric cDNAs as well as wild type cDNAs were cloned into an expression vector [7] derived from a plasmid pSV_2CAT [11].

2.2. *Transfection and cell culture*

The detailed methods for cell culture were described previously [7,8]. Mouse L cells expressing the β_1-subunit of the chicken Na^+, K^+-ATPase [7] were co-transfected by the calcium phosphate precipitation method, as described by Small and Scangos [12], with 0.1 μg of pSVneo DNA and about 1 μg of chimeric cDNA/100 mm diameter tissue culture plate. The transfected cells were selected in a medium containing G418 sulfate (Gibco) (~500 μg/ml), and then screened for expression of the chicken chimeric molecules using chicken specific monoclonal antibodies (see below).

2.3. *Immunofluorescence microscopy*

Monoclonal antibodies 7C (specific for the chicken α_1-subunit [8]) and 5D2 (specific for the chicken SR-calcium pump [13]), and TRITC-labeled goat-anti-mouse IgG were used to identify the chicken proteins in mouse cells. Fluorescence images were obtained with a laser scanning confocal microscope (BioRad MRC 600). Further details of immuno-fluorescent staining were described in previous reports [7,8].

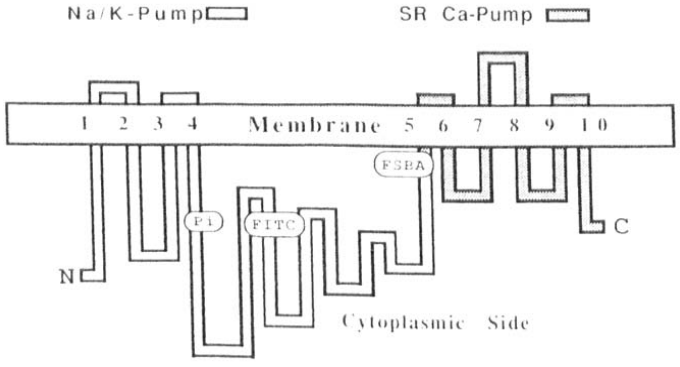

Fig. 1. Construction of chicken chimeric ion-pumps. Schematic representation of a possible structure of chimera I. Chimera II (not shown) consists of the N-terminal 2/3 of the SR calcium-pump and the C-terminal 1/3 of the sodium pump. Phosphorylation site (Pi) and FITC- and FSBA-binding domains are shown, and potential transmembrane segments are numbered.

2.4. *ATPase activity assays*

The rate of ATP hydrolysis was determined with an enzyme coupled system, for each ADP produced in the assay, a molecule of NADH was oxidized to NAD^+ and this was monitored spectroscopically by a decrease in absorbance at 340 nm. [14–16]. The ATPase assay medium (1 ml) contained 5 μl of cell homogenate (300–500 μg total protein), 120 mM NaCl, 15 mM KCl, 30 mM triethanolamine, pH 7.4, 4 mM $MgCl_2$, 3 mM Na_2ATP, 0,5 mM EGTA, 2 mM NaN_3, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH, 10 U/ml pyruvate kinase, and 30 U/ml lactate dehydrogenase. Na^+,K^+-ATPase activity was obtained by calculating the difference in activity in the presence and absence of 1 mM ouabain. Protein concentration was determined by the method of Bradford [17].

3. RESULTS AND DISCUSSION

Utilizing an avian/murine hybrid expression system [7,8,18], relatively ouabain-sensitive chicken sodium-pump functions can be studied in relatively ouabain-insensitive mouse L cells. When a chicken β_1-subunit cDNA was stably transfected into mouse L cells, the chicken β_1-subunit protein was expressed on mouse cell surfaces associated with the mouse α-subunit. These cells are designated as Ltk$^-\beta$ cells [7]. When an additional chicken α_1-subunit cDNA was introduced into these mouse Ltk$^-\beta$3 cells, the chicken α1-subunit was expressed both on the mouse cell surface and inside the cells. These cells are designated as Ltk$^-\beta$neo$^+\alpha$ cells [18]. Normally, mouse cells transfected with the chicken α_1-subunit cDNA express 10^5–10^6 ouabain-sensitive sites per cell during 10 mM butyrate treatment [8]. When mouse cells are transfected with a cDNA encoding chicken SR calcium pump, the avian calcium pump protein was always expressed inside the cells [9]. These cell lines were used as control cells in this study.

Fig. 2 shows confocal fluorescence micrographs of the cloned mouse cells expressing chicken chimeric ion pumps (Ltk$^-\beta$neo$^+$ChiI and Ltk$^-\beta$neo$^+$ChiII). Chimera I (containing the N-terminal 2/3 of the sodium pump α_1-sununit) was found only internally, while chimera II (containing the N-terminal 2/3 of the calcium pump) was found both on the plasma membrane and inside the cell. Extensive studies of the expression of sodium-pump subunits in a variety of systems have demonstrated that the α-subunit contains exclusively the domains conferring ouabain-sensitivity and catalytic activity to the sodium pump [5,8,19–21], while the β-subunit is critically involved in the stabilization and transport of the α-subunit to the PM [18,19,22,23]. A recent study of expression of sodium/calcium pump chimeric molecules in *Xenopus* oocytes demonstrated that the C-terminal 1/3 of the α-subunit contains the domain(s) that assemble with the β-subunit [24]. Subcellular distributions of chimeras I and II (Fig. 2) are consistent with these results. Our in-

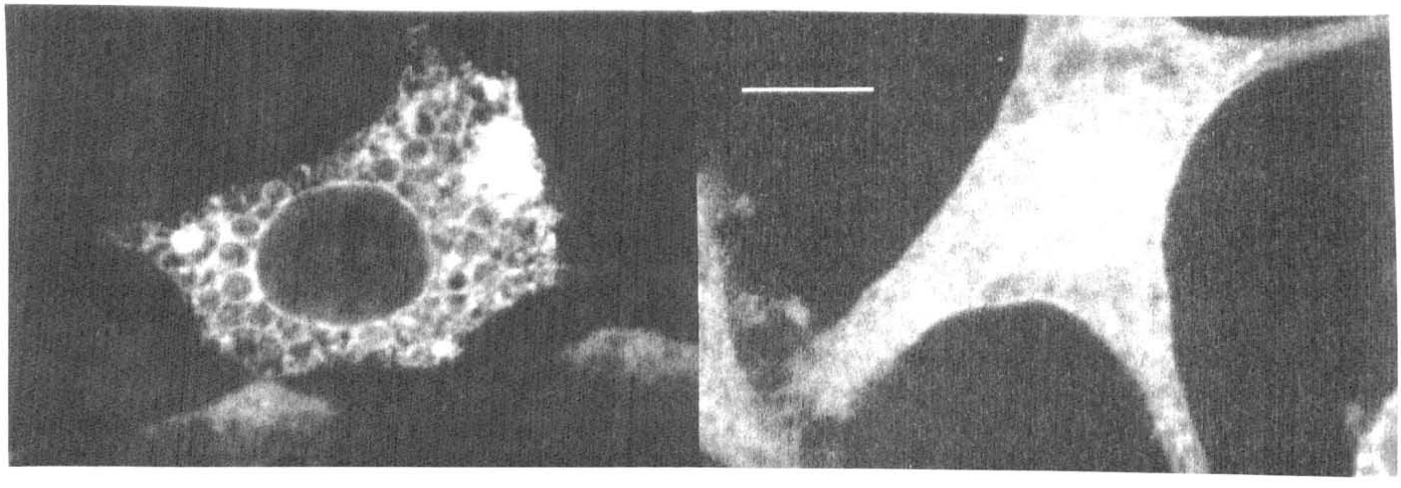

Fig. 2. Laser confocal micrographs of cloned mouse L cells that express the chicken Na-pump/Ca-pump chimeric molecules. (Left) Cells transfected with chimera I cDNAs were fixed, permeabilized, and stained with monoclonal IgG 7C specific to the chicken α_1-subunit [8]. (Right) Cells transfected with chimera II cDNAs were fixed, permeabilized, and stained with monoclonal IgG 5D2 specific to the chicken SR calcium pump [13]. Scale bar = 10 μm.

terpretation is that chimera I lacks the domains that assemble with the β-subunit, and is, therefore, not transported to the plasma membrane, while chimera II acquires the assembling domains and is transported to the cell surface.

Fig. 3 illustrates the effects of varying concentrations of ouabain on the ATPase activity (measured in the presence of Na^+ and K^+ and absence of Ca^{2+}) of cloned mouse L cells transfected with various chicken cDNAs. The inhibition of the ATPase activity of both $Ltk^-\beta neo^+\alpha$ and $Ltk^-\beta neo^+$ ChiI cells by ouabain is biphasic; ~60% shows IC_{50} of <10 μM and the rest IC_{50} of ~300 μM. On the other hand, ouabain has a monophasic inhibitory effect (IC_{50}: ~300 μM) in untransfected mouse Ltk^- and $Ltk^-\beta neo^+$ ChiII cells. At present, functions of chimera II distinct from those of the endogenous mouse ATPase cannot be detected in our system (see also below). However, it has been known that the calcium-pump does not bind ouabain, and that the endogenous mouse sodium pump does not have high affinity sites for ouabain. Thus, this new high affinity site for ouabain in transfected cells must come from the chicken wild-type α_1-subunit and chimera I. Recently, using chimeras and site-directed mutagenesis, Price and Lingrel [5] have demonstrated that the extracellular domain between M1 and M2 consisting of 11 amino acids (especially Arg-116 and Asp-127) is the major ouabain binding domain. However, whether ouabain binds only to this site or to additional site(s) in order to exert its action remains to be determined. The results from the inhibitory effect of ouabain on chimera I suggest that ouabain does not have to bind to the ectodomains after M_5.

Some of the potential transmembrane segments, including M_4, M_5, M_6, of both sodium- and calcium-pumps contain glutamic and aspartic acid residues. Mutagenesis experiments have demonstrated that these charged amino acids are critical for both sodium pump [25] and calcium pump activities [26]. In our chimeric molecules, these amino acids are preserved. Therefore, it is interesting to identify the ion specificity of these chimeric molecules. Fig. 4 shows the effect of Ca^{2+} ions on the chimeric molecules, illustrating that Ca^{2+} ions added to the assay system stimulated the ATPase activity of chimera I. Under the same conditions, neither the chicken wild-type nor the mouse endogenous ATPase showed this level of stimulation by Ca^{2+}. Thus, when combined with the N-terminal 2/3 of the sodium pump α_1-subunit, the C-terminal 1/3 of the calcium pump appears to undergo the proper protein folding required for Ca^{2+}-stimulation of the ATPase activity.

We have established many mouse L cell lines stably transfected with the chicken wild-type Ca^{2+}-ATPase cDNA as well as chimera II cDNA. However, we have not been able to detect chicken calcium-pump specific

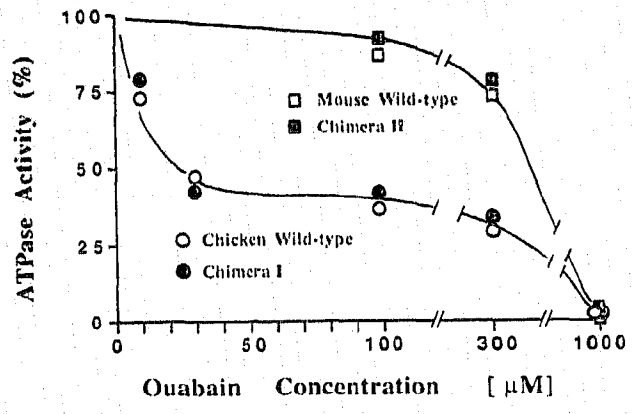

Fig. 3. Dose/response relationships for ouabain-inhibition of the ATPase activity of homogenate from untransfected (Ltk^-) and transfected ($Ltk^-\beta neo^+$ ChiI, $Ltk^-\beta neo^+$ ChiII, and $Ltk^-\beta neo^+\alpha$) mouse cells. ATPase activities of cell homogenate were measured in the presence of 0.2% Triton X-100 which partially solubilizes and clears turbid sample and allows chemicals access to both sides of the membrane. At appropriate concentrations, detergents have been found to activate the Na,K-ATPase [27]. In our system, 0.2% Triton X-100 increases ATPase activity on the average 5-fold in the presence of Ca^{2+}- and mitochondrial ATPase inhibitors (0.5 mM EGTA, 2 mM NaN_3). The ATPase activity (60–80 nmol ADP cleaved/mg protein/min) that can be inhibited by 1 mM ouabain was defined as 100%. Ouabain-sensitive ATPase activity was ~64% of overall ATPase activity measured in the presence of 0.5 mM EGTA and 2 mM NaN_3.

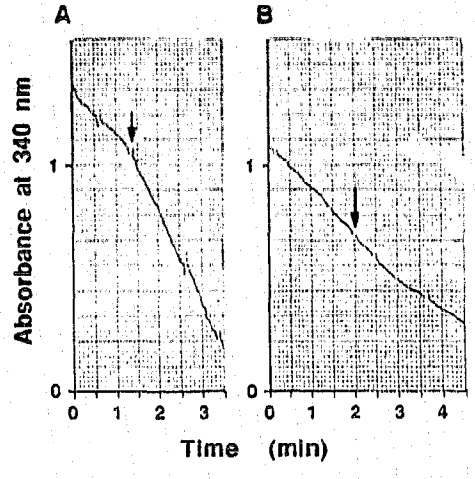

Fig. 4. Effect of calcium ions on the ATPase activity of homogenate from transfected cells. The same ATPase assay system as described in Fig. 3 (in the presence of Na and K ions) was activated at time 0, and then 1 μl of 0.5 M $CaCl_2$ was added to the 1 ml assay system after a few minutes (shown by arrows in the figure). This created a final free Ca^{2+} level of ~4 μM after EGTA chelation. The addition of the same level of free Ca ions to the assay system leads to maximal stimulation of the Ca^{2+}-ATPase activity of biochemically purified chicken skeletal SR Ca^{2+}-ATPase. (A) $Ltk^-\beta neo^+$ ChiI cells. Sample contained 450 μg protein and calibration (adding 50 nmol of ADP to sample after the assay's completion) determined 0.222 AU to equal 50 nmol ADP. ATPase activity before and after Ca^{2+}-addition was 92.6 and 211 nmol ADP cleaved/mg protein/min, respectively. (B) $Ltk^-\beta$ cells. Sample contained 296 μg protein and calibration determined 0.355 AU to equal 50 nmol ADP in this assay. ATPase activity before and after Ca^{2+} addition was 86.7 and 94.1 nmol ADP cleaved/mg protein/min, respectively.

ion-stimulated ATPase activity for unknown reasons. The only method currently available for detecting the chicken Ca^{2+}-ATPase activity (suggested to us by Dr. G. Inesi, Univ. Maryland) is to express them in COS-1 cells [26]. Probably expression of the chimeric and the wild type molecules in COS-1 cells will be useful for further characterization of ion specificity.

The Na^+,K^+-ATPase is supposed to exist as an α-/β-subunit complex in order to exert its full activity [1], while the SR Ca^{2+}-ATPase does not require any additional subunits for its full functioning. In this sense, it is intriguing that chimera I (N-terminal sodium-/C-terminal calcium-pump) exhibits ouabain-sensitive and Ca^{2+}-stimulated ATPase activity. Further detailed analysis of chimera I, as well as careful characterization of chimera II as a 'Ca^{2+}-ATPase' and a 'Na^+, K^+-ATPase', should provide critical information about the structure, conformational changes, and functional relationships of the E1-E2 ATPases.

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