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# The amino-terminal 200 amino acids of the plasma membrane $Na^+,K^+$-ATPase $\alpha$ subunit confer ouabain sensitivity on the sarcoplasmic reticulum $Ca^{2+}$-ATPase

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ABSTRACT **Cardiac glycosides such as G-strophanthin (ouabain) bind to and inhibit the plasma membrane $Na^+,K^+$-ATPase but not the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase, whereas thapsigargin specifically blocks the SR $Ca^{2+}$-ATPase. The chimera [n/c]CC, in which the amino-terminal amino acids $Met^1$ to $Asp^{162}$ of the SR $Ca^{2+}$-ATPase (SERCA1) were replaced with the corresponding portion of the $Na^+,K^+$-ATPase $\alpha$1 subunit ($Met^1$ to $Asp^{200}$), retained thapsigargin- and $Ca^{2+}$-sensitive ATPase activity, although the activity was lower than that of the wild-type SR $Ca^{2+}$-ATPase. Moreover, this $Ca^{2+}$-sensitive ATPase activity was inhibited by ouabain. The chimera NCC, in which $Met^1$–$Gly^{354}$ of the SR $Ca^{2+}$-ATPase were replaced with the corresponding portion of the $Na^+,K^+$-ATPase, lost the thapsigargin-sensitive $Ca^{2+}$-ATPase activity seen in CCC and [n/c]CC. [$^3$H]Ouabain binding to [n/c]CC and NCC demonstrated that the affinity for this inhibitor seen in the wild-type chicken $Na^+,K^+$-ATPase was restored in these chimeric molecules. Thus, the ouabain-binding domains are distinct from the thapsigargin sites; ouabain binds to the amino-terminal portion ($Met^1$ to $Asp^{200}$) of the $Na^+,K^+$-ATPase $\alpha$1 subunit, whereas thapsigargin interacts with the regions after $Asp^{162}$ of the $Ca^{2+}$-ATPase. Moreover, the amino-terminal 200 amino acids of the $Na^+,K^+$-ATPase $\alpha$1 subunit are sufficient to exert ouabain-dependent inhibition even after incorporation into the corresponding portion of the $Ca^{2+}$-ATPase, and the segment $Ile^{163}$ to $Gly^{354}$ of the SR $Ca^{2+}$-ATPase is critical for thapsigargin- and $Ca^{2+}$-sensitive ATPase activity.**

The plasma membrane $Na^+,K^+$-ATPase and the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase are distinct membrane-bound enzymes with ≈30% identity in their amino acid sequences. Both enzymes belong to a family of ion-transporting ATPases (P- or $E_1E_2$-type ATPases) that have an obligatory step to form a phosphorylated intermediate during the course of cation transport (1). Distinct inhibitors have been found and are used widely in basic and clinical sciences; thapsigargin and ouabain are specific for the SR $Ca^{2+}$-ATPase (2–4) and the $Na^+,K^+$-ATPase (5), respectively. Recent successful attempts to construct and express chimeric ion pumps (6) between these enzymes have led us to elucidation of subunit-assembly domains of the $Na^+,K^+$-ATPase $\alpha$ subunit (7) and $Ca^{2+}$-binding domains of the $Ca^{2+}$-ATPase (8). In this report, we employ this type of approach and identify the regions responsible for inhibition by cardiac glycosides.

In order to identify the domains responsible for ouabain inhibition, recombinant cDNAs encoding chicken chimeric ion pumps were constructed (Fig. 1*A*) according to the strategy described previously (8), in which the amino-terminal amino acids of the SR $Ca^{2+}$-ATPase (SERCA1) were replaced with the corresponding portions of the $Na^+,K^+$-ATPase $\alpha$1 subunit. This strategy depends upon the existence of four highly homologous regions in a variety of P-type ATPases, many of which are thought to contribute to the ATP hydrolysis site: i.e., the region between the second and the third membrane-spanning domains, the phosphorylation domain (11), the fluorescein isothiocyanate-binding domain (12), and the 5′-(*p*-fluorosulfonyl)benzoyladenosine-binding region (13). On the basis of a comparison of the hydrophobicity plots of the amino termini between the chimera and the wild-type $Ca^{2+}$-ATPase (Fig. 1*B*), it is expected that these conserved regions can be used as exchange junctions to produce chimeric molecules without perturbing the general membrane topology of the enzymes.

## MATERIALS AND METHODS

**Oligonucleotide-Directed Mutagenesis.** Site-directed mutagenesis using the polymerase chain reaction (PCR) was employed to create unique endonuclease recognition sites, *Eco*RV and *Kpn* I, at the positions encoding $Asp^{200}$ and $Gly^{377}$ of the $Na^+,K^+$-ATPase $\alpha$1-subunit, respectively. Since the SR $Ca^{2+}$-ATPase cDNA contains a unique *Eco*RV site at the position for $Asp^{162}$, only the *Kpn* I site was created for $Gly^{354}$. In [n/c]CC, the nucleotide sequence encoding $Met^1$ to $Asp^{200}$ of the $Na^+,K^+$-ATPase $\alpha$1 subunit was amplified by PCR using 50 ng each of deoxyoligonucleotide primers 5′-CCAAGCTTGGCTCGAGGTCGACGG-3′ (vector primer) and 5′-CCGGATATCAGCTGGAAT-3′ (*Eco*RV site is underlined) and 50 ng of $Na^+,K^+$-ATPase $\alpha$1-subunit cDNA, and placed into the corresponding region of the SR $Ca^{2+}$-ATPase cDNA by using the *Eco*RV site as a junction. For the construction of NCC, the nucleotide sequences encoding $Met^1$ to $Gly^{377}$ of the $Na^+,K^+$-ATPase $\alpha$1 subunit and $Thr^{355}$ to $Ala^{994}$ of the SR $Ca^{2+}$-ATPase were synthesized by PCR using the appropriate primers, including 5′-AGGGTACCT-GTTTTGTCAGAACA-3′ for the $Na^+,K^+$-ATPase $\alpha$1 subunit and 5′-ACCGGTACCCTCACCACCAACC-3′ for the SR $Ca^{2+}$-ATPase (*Kpn* I site is underlined). Nucleotide sequences of PCR fragments were confirmed by the dideoxy technique (14). The final chimeric cDNAs were ligated into one of the polylinker sites in a mammalian expression vector, pRc/CMV (Invitrogen, no. V750-20).

**Establishment of Permanent Cell Lines.** By the methods previously described (15), the chimeric chicken cDNA constructs were introduced into mouse L cells (Ltk$^+\beta$3; termed Ltk cells hereafter) that had been transfected with a cDNA encoding the chicken $Na^+,K^+$-ATPase $\beta$1 subunit (16). The cells were selected for high-level expression of the chimeric ATPase by immunofluorescence microscopy and immunoprecipitation using a monoclonal antibody, 5D2, specific to the chicken SR $Ca^{2+}$-ATPase (SERCA1) (17).

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Abbreviations: SR, sarcoplasmic reticulum; SER, SR/endoplasmic reticulum.

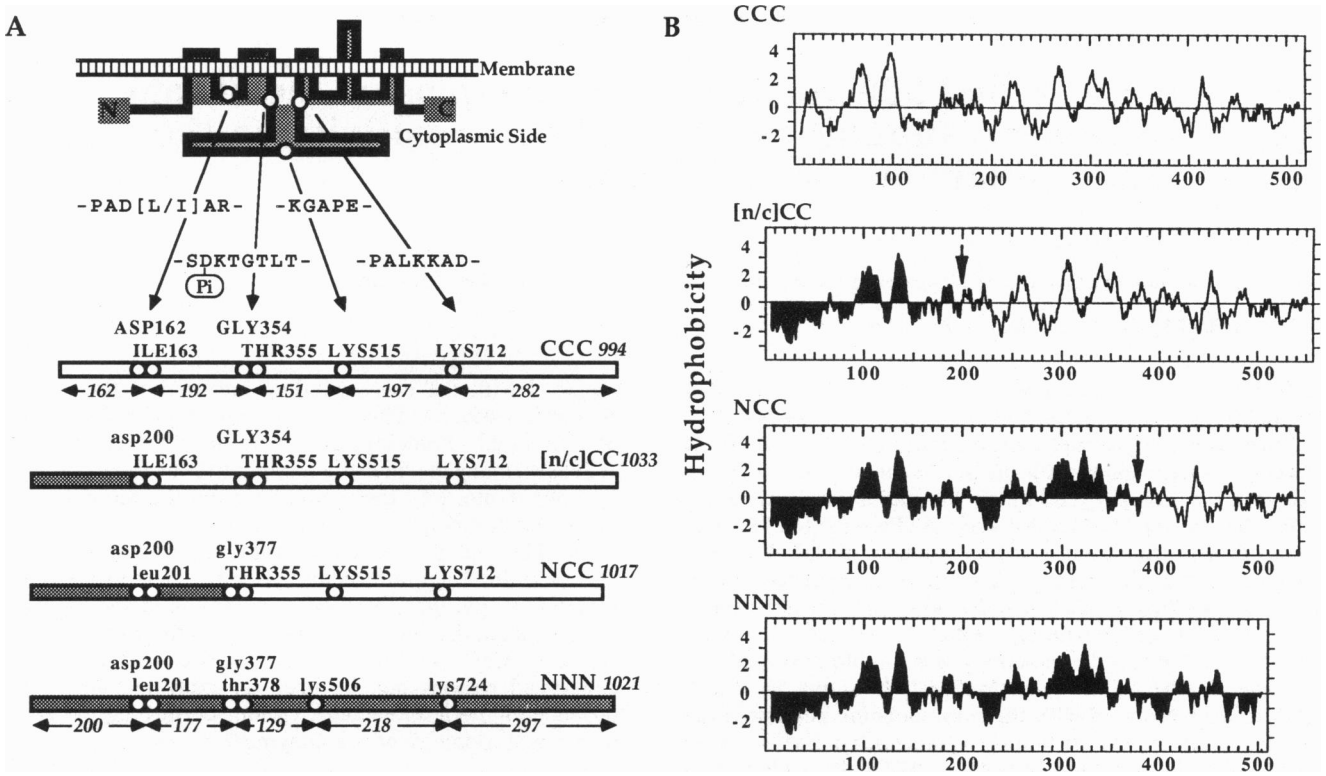


FIG. 1. (*A*) The topology model proposed for the wild-type SR $Ca^{2+}$-ATPase (9) is adapted to all the constructs on the basis of reported results (7), and the amino acid sequences of four evolutionarily conserved domains are indicated in one-letter designation. By using the first two conserved domains, two chimeric proteins were constructed, [n/c]CC and NCC, with total numbers of amino acids of 1033 and 1017, respectively. For these constructs three uppercase letters represent amino-terminal, central, and carboxyl-terminal domains of the $Na^+,K^+$-ATPase (N) and $Ca^{2+}$-ATPase (C). Bracketed lowercase letters replace an uppercase letter when junctions between the two ATPases occur within the amino-terminal domain. The chimeric junctions are shown by three-letter notation: asp200/ILE163 for [n/c]CC and gly377/THR355 for NCC, where asp200 and gly377 come from the $Na^+,K^+$-ATPase (NNN), and ILE163 and THR355 originate in the $Ca^{2+}$-ATPase (CCC). The numbers of amino acids between four conserved domains are shown in NNN and CCC. (*B*) Hydrophobicity plots of NNN, NCC, [n/c]CC, and CCC amino termini were compared by the Kyte and Doolittle program (10) with a window set for 11 amino acids. Amino acid numbers are shown on the *x* axis, ending at the fluorescein isothiocyanate-binding LYS515 or lys506; positive values on the *y* axis indicate hydrophobic regions. Arrows indicate chimeric junctions.

**Crude Membrane Preparation.** Monolayers of cells were treated in 5 mM Tris·HCl (pH 7.5) for 20 min on ice, scraped from the bottom of the culture dish, and centrifuged at 15,000 rpm (Sorvall SA-600 rotor) for 15 min. The pellet was suspended to give ≈1 mg of protein per ml in TME buffer (75 mM Tris·HCl, pH 7.5/12.5 mM $MgCl_2$/1.5 mM EDTA) and used for ATPase assays. For [$^3$H]ouabain-binding, this preparation was further homogenized [Tekmar (Cincinnati) Tissuemizer]. Protein concentration was determined by the method of Lowry *et al.* (18).

**Binding of [$^3$H]Ouabain.** This was measured by using crude membrane preparation and various concentrations of [$^3$H]ouabain in 0.5 ml of binding medium containing 50 mM Tris maleate (pH 7.2), 5 mM $MgCl_2$, and 0.1 mM sodium vanadate as described (19). The specific activity was adjusted to 15,000–18,000 dpm/pmol. The reaction was started by addition of [$^3$H]ouabain (1 nM to 1 μM). After incubation for 90 min at 37°C, binding was terminated by dilution with 4 ml of ice-cold wash buffer (5 mM $MgCl_2$/5 mM Tris phosphate/50 mM Tris maleate, pH 7.4), and samples were filtered on Whatman G/F filters presoaked in 0.1% bovine serum albumin. The filters were washed three times with 4 ml of wash buffer, and the radioactivity remaining on the filters was detected in a liquid scintillation counter (Packard 2500 TR). Specific binding was calculated by subtracting the nonspecific binding measured in the presence of excess unlabeled ouabain (2 mM) from the total binding obtained at a given concentration of [$^3$H]ouabain.

**$Na^+,K^+$-ATPase Activity.** $Na^+,K^+$-dependent ATPase activity was measured by monitoring the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP (Amersham, PB218) in 0.5 ml of assay medium containing crude membrane preparation (≈0.1 μg of protein), 100 mM NaCl, 5 mM KCl, 50 mM Tris·HCl (pH 7.4), 2 mM ATP (containing 100 μM NaOH and [$\gamma$-$^{32}$P]ATP), 3 mM $MgCl_2$, 5 mM $NaN_3$, and 1 mM EGTA (pH adjusted to 7.4 with ≈260 μM NaOH) (20). Thus, this assay medium contained 105.4 mM $Na^+$, 5 mM $K^+$, and no free $Ca^{2+}$. Assays were conducted at 37°C for 30 min. The reaction was terminated by addition of 0.5 ml of 8% perchloric acid. The cleaved $^{32}P_i$ was converted to phosphomolybdate and extracted into 2-methylpropanol, and the radioactivity was determined in a liquid scintillation counter. Ouabain-sensitive $Na^+,K^+$-ATPase activity was defined as the difference in the $Na^+,K^+$-ATPase activities measured in the presence and absence of 5 mM ouabain.

**$Ca^{2+}$-ATPase Activity.** $Ca^{2+}$-ATPase activity was measured in a crude membrane preparation by monitoring the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP at 37°C (21). The assay system (0.5 ml) consisted of membranes (≈0.1 μg of protein), 100 mM KCl, 50 mM Tris·HCl (pH 7.4), 2 mM ATP (containing 100 μM KOH and [$\gamma$-$^{32}$P]ATP), 3 mM $MgCl_2$, 2 μM A23187, 1 mM $NaN_3$, and appropriate amounts of $CaCl_2$ and EGTA (pH adjusted to 7.4 with ≈29 μM KOH) to produce the required free $Ca^{2+}$ concentration. Thus this assay medium contained 100.13 mM $K^+$, 1 mM $Na^+$, and the necessary concentration of free $Ca^{2+}$. The reaction was terminated at intervals by the addition of 0.5 ml of 8% perchloric acid. The

cleaved $^{32}P_i$ was recovered in the same way as for $Na^+,K^+$-ATPase activity.

The $Ca^{2+}$- and thapsigargin-sensitive SR ATPase [SR/endoplasmic reticulum (SER) $Ca^{2+}$-ATPase] activity was defined as a difference in the $Ca^{2+}$-ATPase activities measured in the presence and absence of 500 nM thapsigargin. The $Ca^{2+}$-ATPase activity in the absence of thapsigargin was determined as a difference in the activities in the presence of a given concentration of free $Ca^{2+}$ or 5 mM EGTA without $Ca^{2+}$. The $Ca^{2+}$-ATPase activity in the presence of thapsigargin was determined as a difference in the activities in the presence of a given concentration of free $Ca^{2+}$ and 500 nM thapsigargin or 5 mM EGTA (no free $Ca^{2+}$) and 500 nM thapsigargin. The ouabain-sensitive SER $Ca^{2+}$-ATPase activity was defined as a difference in the SER $Ca^{2+}$-ATPase activities measured in the presence and absence of 4 mM ouabain. All assays were started after preincubation with or without thapsigargin for 5 min at 37°C.

## RESULTS

Fig. 2 illustrates the binding of [$^3$H]ouabain to five selected cell lines which express recombinant proteins at a similar level; Ltk (control for endogenous mouse enzymes), NNN (expressing wild-type $Na^+,K^+$-ATPase $\alpha$1 subunits), NCC, [n/c]CC, and CCC (expressing wild-type $Ca^{2+}$-ATPase, SERCA1). All these constructs, except CCC, exhibited high-affinity [$^3$H]ouabain binding. Scatchard plot analysis of the specific binding yielded a dissociation constant ($K_d$) of ≈350 nM common for NNN, NCC, and [n/c]CC. The maximum number of binding sites was 170–200 fmol/mg of protein after 48 hr of incubation in Dulbecco's modified Eagle's medium plus 10 mM butyrate, consistent with the level of expression in mouse L cells obtained elsewhere (15). These results indicate that the short amino-terminal fragment ($Met^1$ to $Asp^{200}$) of the $Na^+,K^+$-ATPase $\alpha$1 subunit is sufficient for accepting ouabain with high affinity.

Functionality of these chimeric molecules was assessed by two types of ATPase activities: the $Na^+,K^+$-ATPase activity, defined as the ouabain-inhibitable ATP cleavage, and the SER $Ca^{2+}$-ATPase activity, defined as the thapsigargin-inhibitable $Ca^{2+}$-dependent ATP cleavage (see *Materials and Methods*). Only NNN, and not [n/c]CC, NCC, or CCC, showed detectable $Na^+,K^+$-ATPase activity over the background of endogenous mouse $Na^+,K^+$-ATPase activity (Fig. 3*B*), whereas CCC and [n/c]CC, but not NNN or NCC, in which amino acids $Ile^{163}$ to $Gly^{354}$ of the $Ca^{2+}$-ATPase were further substituted with the corresponding portion of the $Na^+,K^+$-ATPase, exhibited significantly higher SER $Ca^{2+}$-ATPase activity than the mouse endogenous enzyme (Fig. 3*A*). NCC can bind [$^3$H]ouabain with high affinity, like NNN and [n/c]CC (Fig. 2), suggesting that the overall topological structure of the amino-terminal region is preserved. It might be that the sites for thapsigargin reside between $Ile^{163}$ and $Gly^{354}$ of SERCA1. Therefore, NCC, which lacks this region, cannot exhibit thapsigargin sensitivity. Alternatively, thapsigargin sites could be localized at further C-terminal domains, and the segment $Ile^{163}$ to $Gly^{354}$ of SERCA1 might be critical for the $Ca^{2+}$-dependent ATPase activity (also see *Discussion*). A recent study on chimeric molecules between $Ca^{2+}$-ATPase isoforms (SERCA1, -2, and -3) has suggested that the middle segment of the $Ca^{2+}$-ATPase is responsible for efficient linkage between $Ca^{2+}$-binding and ATP-hydrolysis activities (22).

Fig. 4 illustrates the effect of ouabain on the thapsigargin-sensitive $Ca^{2+}$-ATPase activity in transfected mouse L cells. The $Ca^{2+}$-ATPase activity observed in [n/c]CC was drastically reduced to the level of the endogenous activity by the addition of 4 mM ouabain in the assay medium, while the SER $Ca^{2+}$-ATPase activities in Ltk and CCC-transfected cells

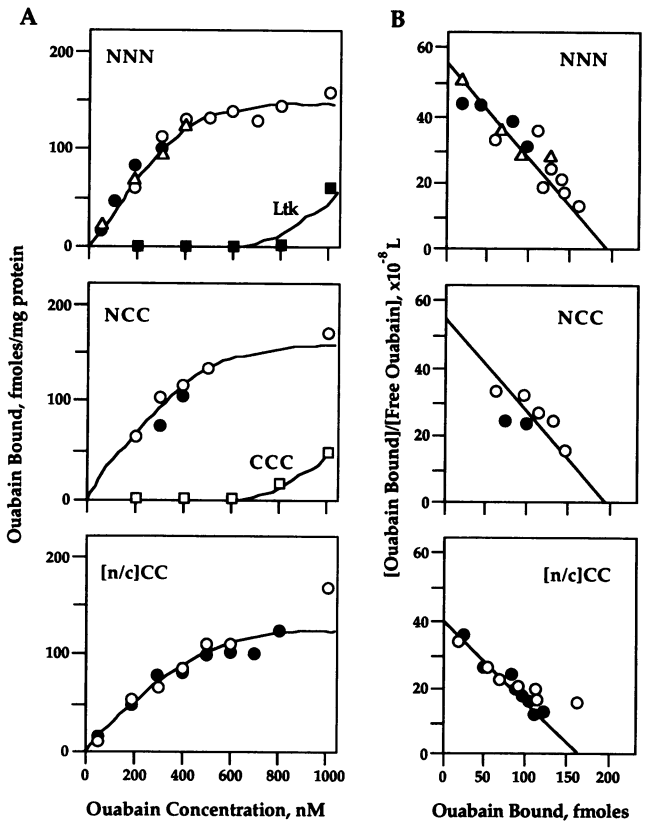


FIG. 2. [n/c]CC and NCC possess high-affinity ouabain-binding sites. The levels of expression of the various chimeric constructs were found to be very similar on the basis of immunofluorescence and immunoprecipitation assays using monoclonal antibodies IgG 5D2 and IgG 5. (*A*) [$^3$H]Ouabain binding to the homogenate of mouse L cells transfected with cDNAs for CCC, [n/c]CC, NCC, or NNN was measured at various ouabain concentrations. The ouabain-binding property of mouse L cells expressing CCC is essentially the same as that seen in untransfected mouse L cells and $Ltk^+\beta$3 cells, while the ouabain binding detected in cells expressing [n/c]CC and NCC exhibits characteristics of the chicken wild-type $Na^+,K^+$-ATPase, NNN. Different symbols indicate the values obtained from separate experiments. (*B*) Scatchard plots give a $K_d$ of ≈350 nM and a maximum binding of 170–200 fmol/mg of protein for NNN, [n/c]CC, and NCC, verifying the similar levels of expression. At higher concentration (>800 $\mu$M), [$^3$H]ouabain starts to bind to the endogenous mouse $Na^+,K^+$-ATPase. Therefore, the obtained $K_d$ values are the possible largest values affected by the endogenous mouse component at high [$^3$H]ouabain concentrations.

were not affected at all. The $IC_{50}$ value for ouabain in this assay (in medium with 100 mM $K^+$ and 5 $\mu$M free $Ca^{2+}$) is ≈800 $\mu$M (Fig. 5). This value is considerably larger than the $K_d$ value obtained in the absence of $K^+$ and $Ca^{2+}$ (Fig. 2), implying that these ions may play a role in setting the affinity for ouabain in [n/c]CC. It has been reported that $K^+$ and $Ca^{2+}$ ions markedly influence the binding of ouabain to the $Na^+,K^+$-ATPase (23–25), although the sites of ion effects have not been identified.

## DISCUSSION

Mouse L cells transfected with chicken cDNAs encoding ion-translocating ATPases can be used for studies on structure and function of these exogenous molecules. Introduction of chimeric cDNAs encoding portions of the $Na^+,K^+$- and the $Ca^{2+}$-ATPase in this heterologous expression system revealed that the binding site(s) for ouabain should be restricted to within the amino-terminal 200 amino acids of the $Na^+,K^+$-ATPase $\alpha$1 subunit and that this stretch of amino acids can

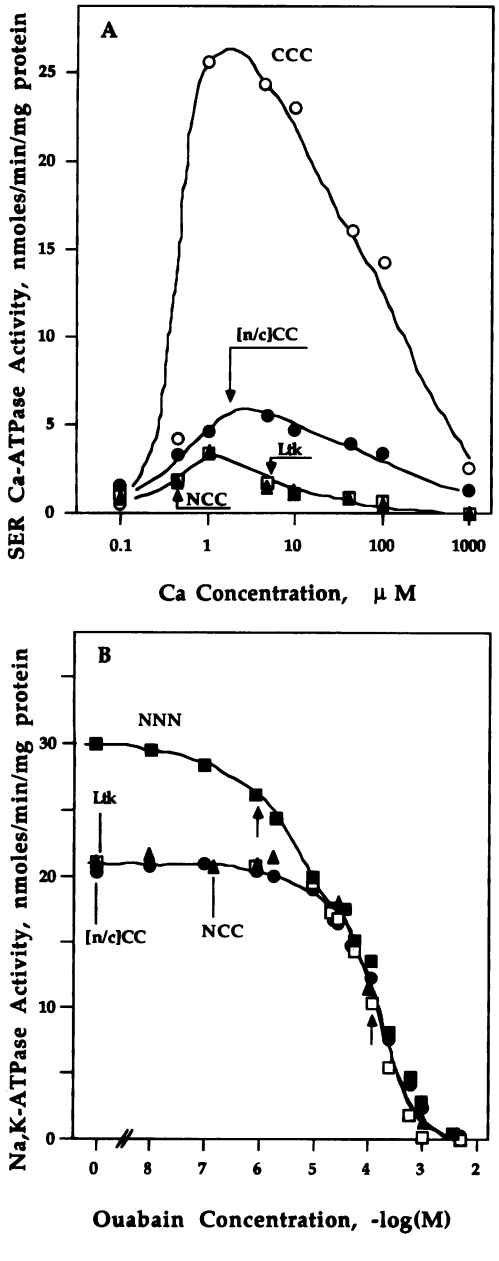


FIG. 3. (*A*) [n/c]CC is a thapsigargin-sensitive and $Ca^{2+}$-dependent ATPase. Thapsigargin was used as a specific inhibitor for the SR $Ca^{2+}$-ATPase (2), and the thapsigargin-inhibitable $Ca^{2+}$-sensitive ATPase activity was defined as the SER $Ca^{2+}$-ATPase activity. The $Ca^{2+}$-ATPase activities of NCC- and NNN-transfected cells at various $Ca^{2+}$ concentrations were essentially identical to those of mouse Ltk cells. Therefore, the data points obtained from NNN-transfected cells are not shown. (*B*) Ouabain-sensitive ATPase activity detected in transfected mouse cells. The $Na^+,K^+$-ATPase activities of [n/c]CC- and NCC-transfected cells were essentially identical to those of mouse Ltk cells, with a single ouabain-sensitive component ($IC_{50}$ value of ≈100 μM), whereas the $Na^+,K^+$-ATPase activity of NNN-transfected cells was ≈50% higher than that of Ltk cells and contained two ouabain-sensitive components with $IC_{50}$ values of ≈1 μM and ≈100 μM (indicated by arrows). The $Na^+,K^+$-ATPase activity with an $IC_{50}$ value of ≈1 μM was a result of expression of chicken wild-type $Na^+,K^+$-ATPase α1 subunit, being consistent with a previous report (15).

exert inhibitory function upon ouabain binding even after substitution for the corresponding region of the SR $Ca^{2+}$-ATPase. Further application of this type of approach will provide critical information on the structure–function relationship of P-type ATPases in general.

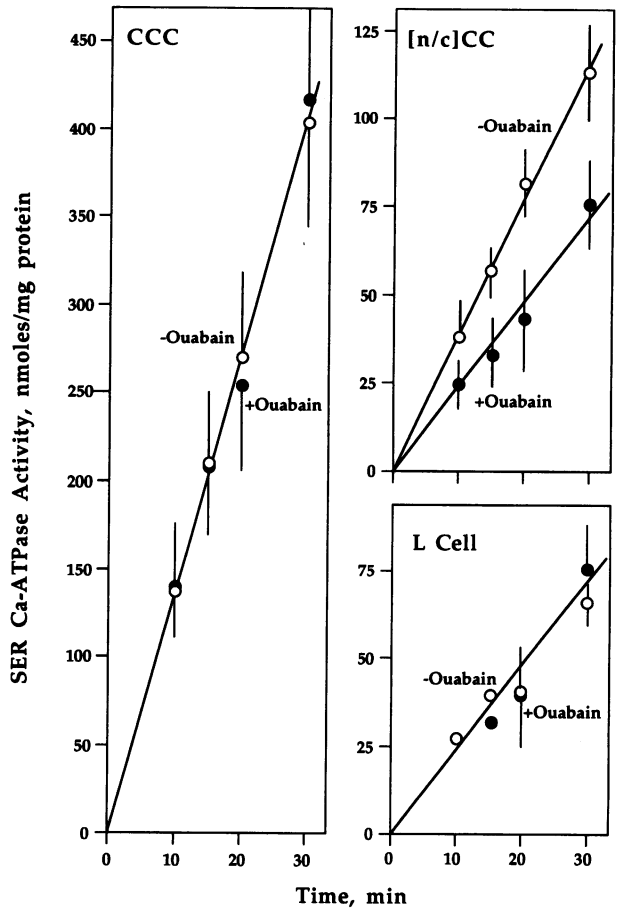


FIG. 4. [n/c]CC is a ouabain-sensitive $Ca^{2+}$-ATPase. Effects of ouabain (4 mM) on the rates of thapsigargin-sensitive $Ca^{2+}$-ATPase in transfected mouse L cells were monitored in the presence of 5 μM free $Ca^{2+}$. The SER $Ca^{2+}$-ATPase activity of [n/c]CC-transfected cells was drastically reduced to the level of endogenous mouse enzymes. The rates in the absence (and presence) of ouabain for CCC, [n/c]CC, and Ltk are respectively 13.6 ± 0.9 (13.5 ± 1.0), 3.8 ± 0.6 (2.3 ± 0.5), and 2.0 ± 0.4 (2.2 ± 0.6) nmol/min per mg of protein (mean ± SD, $n = 3$).

**Ouabain Binding Does Not Require β Subunits of the $Na^+,K^+$-ATPase.** NNN is expected to assemble with the β subunit, whereas CCC, NCC, and [n/c]CC lack the domain required for assembly with the β subunit (7). High-affinity binding of [$^3$H]ouabain to NCC and [n/c]CC indicates that the amino-terminal fragment comprising $Met^1$–$Asp^{200}$ of the $Na^+,K^+$-ATPase α1 subunit is sufficient for accepting ouabain with high affinity without the β subunit. This is intriguing with respect to previous reports that the β subunit was required for the α subunit to acquire the ouabain-sensitive and $Na^+,K^+$-dependent ATPase activity (26–28). It might be that the carboxyl-terminal region of the wild-type α subunit can induce a conformational change in remote regions of the α subunit upon assembling with the β subunit and, thus, influence the ouabain-binding ability of the amino-terminal domain. The sensitivity of ouabain binding to the concentration of $K^+$ can be modulated by different combination of β subunits—e.g., either $Na^+,K^+$-ATPase β1 subunit or $H^+,K^+$-ATPase β subunit (29). Therefore, the ouabain-binding region of NNN, but not of NCC or [n/c]CC, might require the β subunit for its correct conformation. In any case, the β subunit is not required for ouabain binding *per se*, although it may modulate the binding.

**Where Within the 200-Amino Acid Segment Might Ouabain Binding Occur?** Studies employing site-directed mutagenesis demonstrated the importance of the charged amino acids located at the two borders of the extracellular domain that lies

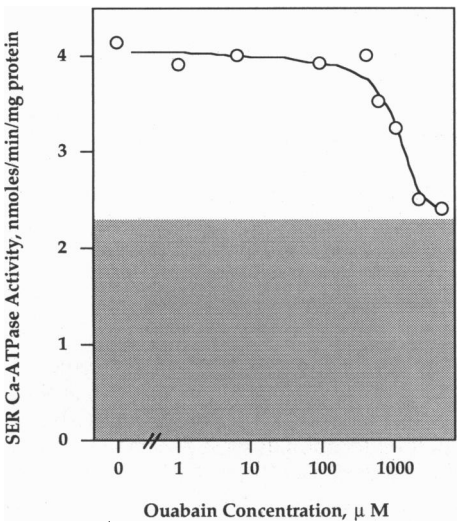


FIG. 5. Relationship between ouabain concentration and SER $Ca^{2+}$-ATPase activity of [n/c]CC. The SER $Ca^{2+}$-ATPase activities in the presence of various concentrations of ouabain were determined over a period of 30 min. [n/c]CC-transfected cells exhibit ouabain-sensitive ($IC_{50}$ value of ~800 μM) and ouabain-insensitive SER $Ca^{2+}$-ATPase activities, and the mouse endogenous enzyme is responsible for the ouabain-insensitive component (shaded; the value of 2.3 nmol/min per mg of protein is taken from Fig. 4).

between the first (M1) and second (M2) transmembrane domains in setting the affinity for ouabain (30). This idea was further supported by amino acid sequence comparisons of various $Na^+,K^+$-ATPase α subunits, indicating that the M1–M2 segment of the α subunit is the most variable ectodomain between different species and among different isoforms with different ouabain sensitivities (31, 32). On the other hand, site-directed monoclonal antibodies against the M1–M2 region enhance ouabain binding, instead of inhibiting it (33), suggesting that ouabain binds to regions other than this extracellular domain, although the M1–M2 region affects the affinity for ouabain. A set of mutations within the M1 and M2 transmembrane segments have been found to confer ouabain resistance on ouabain-sensitive cultured cells (34–36). These reports imply that ouabain might interact with hydrophobic transmembrane domains via interaction with membrane lipids. Our present results complement these findings and clearly indicate that the amino-terminal 200 amino acids of the $Na^+,K^+$-ATPase α1 subunit are sufficient for ouabain binding even in an isolated environment—i.e., NCC and [n/c]CC. A study employing expression of several truncation mutants in *Xenopus* oocytes has demonstrated that ouabain binding can be retained after removal of the amino-terminal 37 amino acids (37). This leaves a stretch of 166 amino acids, $Lys^{35}$ to $Asp^{200}$, as the possible domain responsible for ouabain binding.

**Thapsigargin-Binding Sites Are Distinct from Ouabain-Binding Sites.** The amino-terminal 200 amino acids of the $Na^+,K^+$-ATPase α1 subunit incorporated into the corresponding portion of SERCA1 can actually confer ouabain-dependent inhibitory function upon SER $Ca^{2+}$-ATPase activity that is thapsigargin-sensitive (Figs. 4 and 5). This indicates that [n/c]CC possesses two distinct binding sites, one for ouabain and the other for thapsigargin. Taking into account the recently reported result that CNC, in which the middle $Ca^{2+}$-ATPase segment ($Gly^{354}$-$Lys^{712}$) is replaced with the corresponding domain of the $Na^+,K^+$-ATPase, can cleave ATP in a $Ca^{2+}$- and thapsigargin-dependent fashion (38), thapsigargin should interact with the $Ca^{2+}$-ATPase domains—either $Ile^{163}$ to $Gly^{354}$ (including M3 and M4) or $Lys^{712}$ to the carboxyl end (including M5–M10). This conclusion complements the available information about critical point mutations of the SR $Ca^{2+}$-ATPase (39), since the charged amino acids within the transmembrane segments M4, M5, M6, and M8 have been postulated to play a key role for $Ca^{2+}$ binding in the SR $Ca^{2+}$-ATPase, and it has been shown that $Ca^{2+}$ competes with thapsigargin in ATP catalysis (40).

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