The C-terminal 165 amino acids of the plasma membrane Ca^{2+} -ATPase confer Ca^{2+} /calmodulin sensitivity on the Na^+, K^+ -ATPase α -subunit

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The C-terminal 165 amino acids of the rat brain plasma membrane (PM) Ca^{2+} -ATPase II containing the calmodulin binding auto-inhibitory domain was connected to the C-terminus of the ouabain sensitive chicken Na^+, K^+ -ATPase $\alpha 1$ subunit. Expression of this chimeric molecule in ouabain resistant mouse L cells was assured by the high-affinity binding of [^3H]ouabain. In the presence of Ca^{2+} /calmodulin, this chimeric molecule exhibited ouabain inhibitable Na^+, K^+ -ATPase activity; the putative chimeric ATPase activity was absent in the absence of Ca^{2+} /calmodulin and activated by Ca^{2+} /calmodulin in a dose-dependent manner. Furthermore, this chimeric molecule could bind monoclonal IgG 5 specific to the chicken Na^+, K^+ -ATPase $\alpha 1$ subunit only in the presence of Ca^{2+} /calmodulin, suggesting that the epitope for IgG 5 in this chimera is masked in the absence of Ca^{2+} /calmodulin and uncovered in their presence. These results propose a direct interaction between the calmodulin binding auto-inhibitory domain of the PM Ca^{2+} -ATPase and the specific regions of the Na^+, K^+ -ATPase $\alpha 1$ subunit that are structurally homologous to the PM Ca^{2+} -ATPase. A comparison of the deduced amino acid sequences revealed several possible regions within the Na^+, K^+ -ATPase that might interact with the auto-inhibitory domain of the PM Ca^{2+} -ATPase.
Key words: Ca^{2+} /calmodulin/cardiac glycoside/ Na^+, K^+ -ATPase/PM Ca^{2+} -ATPase

Introduction

The plasma membrane (PM) calcium pump is a member of the P-type ion-transporting ATPases which undergo a series of conformational changes, including formation of a phosphorylated intermediate, during the course of ATP catalysis and cation transport (Pedersen and Carafoli, 1987). It differs from the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase in its subcellular localization, and consists of a single catalytic subunit in contrast to the multi-subunit H^+, K^+ - and Na^+, K^+ -ATPases. An additional distinct feature of the PM Ca^{2+} -ATPase is its stimulation by Ca^{2+} /calmodulin (Carafoli, 1992).

Cloning and sequencing of cDNAs encoding the PM Ca^{2+} -ATPase revealed several isoforms with ~90% amino acid sequence identity (Greeb and Shull, 1989). This is a

similar degree of sequence homology to that observed among isoforms of the SR Ca^{2+} -ATPase (Brandl *et al.*, 1986) and among isoforms of the Na^+, K^+ -ATPase α subunit (Shull *et al.*, 1986). The deduced amino acid sequences of PM Ca^{2+} -ATPase isoforms share only ~25% identity with those of other cation-transporting ATPases, while overall higher order structures are suggested to be rather similar (Shull and Greeb, 1988). A striking difference between the primary structures of PM Ca^{2+} -ATPases and other cation-transporting ATPases is at the C-terminus, where the PM Ca^{2+} -ATPase carries an extra ~150 amino acids, including two possible binding domains for calmodulin (Vorherr *et al.*, 1990). Proteolytic cleavage of this region of the PM Ca^{2+} -ATPase results in a loss of calmodulin sensitivity, making the molecule fully active in the absence of calmodulin. Synthetic peptides that correspond to the calmodulin binding domains of the PM Ca^{2+} -ATPase inhibit the activity of this truncated ATPase (Enyedi *et al.*, 1989; James *et al.*, 1989). These results have led to the proposal of the existence within the PM Ca^{2+} -ATPase molecule of auto-inhibitory domains which interact with the calmodulin binding domains within the ATPase and suppress the activity in the absence of calmodulin. The binding of calmodulin to these domains would remove this auto-inhibition and stimulate enzyme activity.

Several attempts have been made to elucidate possible regions within the PM Ca^{2+} -ATPase that might interact with the 'calmodulin binding domains (CBD)', and some possible candidate fragments have been postulated (Carafoli, 1992; Falchetto *et al.*, 1991). One such region between the second (M2) and the third transmembrane segment (M3) is conserved among different P-type ATPases. These results raise the question of whether different P-type ATPases possess a potential ability to interact with the CBD. If so, studies on the mechanism by which CBD inhibits various P-type ATPases will provide useful information on the general regulatory mechanisms of Ca^{2+} /calmodulin. This question has recently been partly answered by the demonstration that synthetic peptides that copy the CBD can effectively inhibit both PM and SR Ca^{2+} -ATPases (Enyedi and Penniston, 1993).

We have been able to incorporate the specific domains of Na^+, K^+ -ATPase into the structurally related regions of the SR Ca^{2+} -ATPase and study the activity of the resulting enzyme. This strategy (Luckie *et al.*, 1992) has enabled us to identify some of the critical domains of the Na^+, K^+ -ATPase, such as those responsible for ouabain binding and inhibition (Ishii and Takeyasu, 1993), Na^+ detection (Ishii *et al.*, 1994) and assembly with the β subunit (Lemas *et al.*, 1992, 1994). We constructed and expressed a chimeric ATPase in which the C-terminal region (Arg1043–Leu1198 including CBD) of the rat brain PM

Fig. 1. Schematic representation of the chimeric protein NNN-CBD. The calmodulin binding domain (CBD, Arg1043–Leu1198) of the rat PM Ca^{2+} -ATPase II is attached to the C-terminus (Pro1011) of the chicken $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit (NNN), and the CBD is lightly shaded in the model. A 10 transmembrane model (Lemas *et al.*, 1992; Takeyasu *et al.*, 1990) is adapted to the structure of the $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit, and the potential transmembrane segments are numbered. The phosphorylation site (P) as well as fluorescein isothiocyanate (FITC) (Farley *et al.*, 1984) and 5'-*p*-fluorosulfonyl-benzoyl adenosine (FSBA) binding regions (Ohta *et al.*, 1986) is also indicated. Three interchangeable segments (Ishii *et al.*, 1994) between different P-type ATPases include (i) the N-terminal region from Met1 to the phosphorylation site (N--), (ii) the middle cytoplasmic domain from the phosphorylation site to the FSBA binding site (N-), and (iii) the C-terminal region after the FSBA binding site (--N), and form an entire ATPase molecule (NNN). Homologous amino acid residues identified in chicken $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit (Takeyasu *et al.*, 1988), rabbit SR Ca^{2+} -ATPase (MacLennan *et al.*, 1985) and rat PM Ca^{2+} -ATPase II (Greeb and Shull, 1989) molecules are shaded in the model and shown below by one letter notation.

Ca^{2+} -ATPase II was connected to the ouabain sensitive chicken $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit at the C-terminus (Pro1011; Figure 1). The chimeric molecule retained the ouabain binding ability, but its ouabain inhibitable $\text{Na}^{+},\text{K}^{+}$ -ATPase activity was detectable only in the presence of Ca^{2+} /calmodulin. These results suggest that the $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit contains all the necessary molecular machinery except CBD for stimulation by Ca^{2+} /calmodulin. This modification at the C-terminus of the $\text{Na}^{+},\text{K}^{+}$ -ATPase α subunit affected ouabain binding and subunit assembly at remote regions. The possible domains within the $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit that might interact with the CBD are discussed. This success in functional expression of the $\text{Na}^{+},\text{K}^{+}$ - and PM Ca^{2+} -ATPase chimera provides a new strategy in which the Ca^{2+} -ATPase properties can be studied in terms of modified $\text{Na}^{+},\text{K}^{+}$ -ATPase activity.

Results

P-type ATPase activities in mouse L cells

Functional properties of a chimeric molecule consisting of portions of different types chicken P-type ATPases (calmodulin sensitive PM Ca^{2+} -ATPase in the ouabain sensitive $\text{Na}^{+},\text{K}^{+}$ -ATPase) were studied in murine Ltk

cells. The assay conditions were, therefore, rather complicated, and it was essential to characterize the endogenous murine $\text{Na}^{+},\text{K}^{+}$ -ATPase and Ca^{2+} -ATPase activities before making detailed measurements on the transfected cells.

$\text{Na}^{+},\text{K}^{+}$ -ATPase activity was defined as ouabain inhibitable and responsible for $\text{Na}^{+},\text{K}^{+}$ -dependent ATP-hydrolysis; SERCA-ATPase activity was thapsigargin inhibitable and responsible for Ca^{2+} -dependent ATP hydrolysis; PM Ca^{2+} -ATPase activity was thapsigargin insensitive and showed calmodulin and Ca^{2+} -dependent ATP hydrolysis. Figure 2 summarizes the three different endogenous P-type ATPase activities in mouse Ltk cells. The calmodulin sensitive PM Ca^{2+} -ATPase activity showed the lowest activity (maximum 0.9 ± 0.1 nmol/min/mg protein). The pattern of dependence on Ca^{2+} concentration was very similar to that reported for PM Ca^{2+} -ATPase from red blood cells (Niggli *et al.*, 1981). Activity of SERCA-ATPase was ~2.5 times higher than that of PM Ca^{2+} -ATPase (maximum 2.3 ± 0.3 nmol/min/mg protein), consistent with previous reports (Ishii and Takeyasu, 1993; Ishii *et al.*, 1994). Ouabain inhibitable $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity was ~20 times higher than PM Ca^{2+} -ATPase activity in mouse Ltk cells (maximum 20.5 ± 1.8 nmol/min/mg protein). The affinity for ouabain of the endogenous murine $\text{Na}^{+},\text{K}^{+}$ -ATPase was much lower than that of chicken enzyme, and the activities of the endogenous murine SERCA- and $\text{Na}^{+},\text{K}^{+}$ -ATPases are known to be unchanged in mouse Ltk cell lines transfected with unrelated cDNAs (Ishii and Takeyasu, 1993; Ishii *et al.*, 1994). It should also be noted that endogenous mouse PM Ca^{2+} -ATPase activity is almost undetectable at free Ca^{2+} concentrations <100 nM, concentrations used for the activation of the NNN-CBD chimera in later experiments (Figures 4–6). At the same concentrations of free Ca^{2+} the level of the endogenous SERCA-ATPase activity was ~5% of the maximum $\text{Na}^{+},\text{K}^{+}$ -ATPase activity. Since PM Ca^{2+} -ATPase and the SERCA-ATPase are ouabain insensitive, the endogenous mouse PM Ca^{2+} - and SERCA-ATPase activities are unlikely to contaminate the measurements of ouabain inhibitable $\text{Na}^{+},\text{K}^{+}$ -ATPase activity described below.

As reported for cardiac SERCA-ATPase (Katz and Remtulla, 1978; Xu *et al.*, 1993), the thapsigargin inhibitable and ouabain insensitive SERCA-ATPase activity of mouse Ltk cells was slightly enhanced by the presence of calmodulin in the assay medium (Figure 2). The molecular mechanisms underlying this effect are not well understood: explanations include phosphorylation via phospholamban (or similar molecules), direct phosphorylation of SERCA-ATPase via Ca^{2+} /calmodulin-kinase, and others (Katz and Remtulla, 1978; Xu *et al.*, 1993). Addition of Ca^{2+} /calmodulin to the assay medium, however, had little effect, and if anything slightly inhibited endogenous murine $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity.

Ouabain binding to chimeric and wild-type molecules

Mouse Ltk cells (Takeyasu *et al.*, 1987) were transfected with cDNAs encoding NNN (chicken wild-type $\text{Na}^{+},\text{K}^{+}$ -ATPase $\alpha 1$ subunit) and NNN-CBD (chimeric molecules), and Ltk/NNN and Ltk/NNN-CBD cell lines were isolated. NNN exhibited a high-affinity [^3H]ouabain

Fig. 2. Comparison of various P-type ATPase activities in mouse Ltk cells. Bars represent the standard deviations obtained from four to six separate experiments. (A) Na⁺,K⁺-ATPase activity defined as ouabain inhibitable and Na⁺,K⁺-dependent ATP hydrolysis. The presence of 1 μM calmodulin and 20 nM free Ca²⁺ in the assay system has little effect on the ouabain inhibitable Na⁺,K⁺-ATPase activity, although the activity tends to be slightly depressed in the presence of Ca²⁺/calmodulin. The addition of thapsigargin (500 nM) to the assay systems did not affect the data points at all (Table I). The Na⁺,K⁺-ATPase assay buffer contained 100 mM NaCl, 5 mM KCl, 50 mM Tris-HCl (pH 7.4), 2 mM ATP (containing 100 μM NaOH and [³²P]ATP), 3 mM MgCl₂, 5 mM NaN₃ and 1 mM EGTA. In the presence of 1 μM calmodulin and 20 nM free Ca²⁺, 1 mM EGTA was substituted for 1 μM calmodulin, 900 μM EGTA and 100 μM CaCl₂. (B) SERCA-ATPase activity defined as thapsigargin inhibitable and Ca²⁺-dependent ATP hydrolysis. Ca²⁺-dependent ATPase activity was measured in a buffer containing 100 mM KCl, 50 mM Tris-HCl (pH 7.4), 2 mM ATP (containing 100 μM KOH and [³²P]ATP), 3 mM MgCl₂, 2 μM A23187, 1 mM NaN₃, and the appropriate amount of CaCl₂ and EGTA to produce the required free Ca²⁺ concentration (Horiuchi, 1986; Tada *et al.*, 1988). The SERCA-ATPase activity is the difference in the Ca²⁺-dependent ATPase activities measured in the presence and absence of 500 nM thapsigargin. Calmodulin (1 μM) tends to enhance the SERCA-ATPase activity, particularly at higher Ca²⁺ concentrations, consistent with previous reports (Katz and Remtulla, 1978; Xu *et al.*, 1993). The addition of ouabain (4 mM) to the assay systems had no effect on data points (not shown). (C) PM Ca²⁺-ATPase activity defined as thapsigargin insensitive and calmodulin/Ca²⁺-dependent ATP hydrolysis. All the points were measured as the differences in the thapsigargin insensitive Ca²⁺-dependent ATPase activities in the presence and absence of 1 μM calmodulin.

binding with a K_d of ~300 nM in a buffer containing 50 mM Tris-maleate (pH 7.2), 5 mM MgCl₂ and 0.1 mM sodium vanadate (Figure 3A), consistent with previous reports (Takeyasu *et al.*, 1988; Ishii and Takeyasu, 1993; Ishii *et al.*, 1994). The affinity of NNN-CBD for ouabain was lower than that of NNN, although still higher than that of endogenous mouse enzymes. The K_d of the mouse endogenous Na⁺,K⁺-ATPase for ouabain is probably >10 μM and impossible to estimate from [³H]ouabain binding assays; [³H]ouabain starts to bind to murine Na⁺,K⁺-ATPase only at concentrations >1 μM (Ishii and Takeyasu, 1993).

To further characterize NNN-CBD, the effects of Ca²⁺/calmodulin on the binding of [³H]ouabain (800 nM) to NNN and NNN-CBD were compared (Figure 3B). [³H]ouabain binding in transfected mouse L cells at this concentration represents only that due to high-affinity exogenous chicken Na⁺, K⁺-ATPase (Figure 3A), since assays of the ouabain sensitive Na⁺,K⁺-ATPase activity under these conditions indicated that the number of Na⁺,K⁺-ATPase molecules does not change (see Figures 4–6) and since 800 nM is not a saturating concentration. Binding of [³H]ouabain to both NNN and NNN-CBD was drastically reduced in the presence of Ca²⁺/calmodulin (Figure 3B). This can be attributed to the component of the buffer; e.g. 100 μM CaCl₂, 900 μM EGTA and 200 μM KOH, which gives 20 nM free Ca²⁺ under the present conditions. This decrease in [³H]ouabain binding may be due to the combined effects of some of these components, particularly Ca²⁺ and K⁺. These ions are thought to induce an unstable conformational state (E₂P) required for ouabain binding (Tobin *et al.*, 1973; Askari *et al.*, 1983; Forbush, 1983; Hansen, 1984). Calmodulin itself is unlikely to

affect the binding of [³H]ouabain to NNN and NNN-CBD since, in the presence of 1 μM calmodulin alone, the binding was decreased to the same degree (~20%) as in the presence of 60 nM CaCl₂ [1 μM calmodulin (Calbiochem #208694) contains ~60 nM CaCl₂]. In summary, the characteristics of [³H]ouabain binding to NNN and NNN-CBD were similar, although the affinity of the latter for [³H]ouabain was slightly lower. This can be attributed to modification at the cytoplasmic C-terminal region of the Na⁺/K⁺-ATPase α subunit, which affects its assembly with the β subunit (see Figure 7).

Ouabain inhibitable Na⁺,K⁺-ATPase activity expressed in NNN and NNN-CBD

SERCA-ATPase and PM Ca²⁺-ATPase activities of Ltk/NNN (expressing chicken wild-type Na⁺,K⁺-ATPase α1 subunit) and Ltk/NNN-CBD (expressing chimeric molecules) cell lines were essentially identical to those of Ltk cells (data not shown), indicating that the activities of endogenous mouse P-type ATPases were not affected. In the absence of Ca²⁺/calmodulin (Figure 4), the Na⁺,K⁺-ATPase activity of Ltk/NNN-CBD cells was essentially identical to that of mouse Ltk cells (Figure 2A) with a single ouabain sensitive component (IC₅₀ ~100 μM), while the Na⁺,K⁺-ATPase activity of Ltk/NNN cells was ~40% higher and contained two ouabain sensitive components (IC₅₀ <1 μM and ~100 μM). The Na⁺,K⁺-ATPase activity with an IC₅₀ <1 μM was a result of expression of chicken wild-type Na⁺,K⁺-ATPase α1 subunit, consistent with previous reports (Takeyasu *et al.*, 1988; Emerick and Fambrough, 1993; Ishii and Takeyasu, 1993). This can be easily seen after subtraction of mouse background activity (IC₅₀ ~100 μM; Figure 4 inset). As shown in Figure 3,

Fig. 3. (A) The affinities of ouabain binding to NNN-CBD and NNN are different. [³H]Ouabain binding to homogenates of mouse L cells transfected with cDNAs for NNN (○) and NNN-CBD (▲) was measured as described in Materials and methods. The affinity for ouabain is too low in untransfected mouse L cells (■) to detect under these conditions (Takeyasu *et al.*, 1988; Ishii and Takeyasu, 1993; Ishii *et al.*, 1994), while affinities in NNN and NNN-CBD are high enough for specific [³H]ouabain binding to be detected. The affinity for ouabain of the chicken wild-type Na⁺,K⁺-ATPase α 1-subunit, NNN, is consistent with previous reports (Takeyasu *et al.*, 1988; Ishii and Takeyasu, 1993; Ishii *et al.*, 1994), whereas the affinity of NNN-CBD is slightly lower than that of NNN and cannot be subjected to a Scatchard analysis within the practical concentration range of [³H]ouabain used in the present experiments. (B) Specific [³H]ouabain binding at 800 nM to the homogenates of Ltk/NNN (mouse L cells transfected with cDNAs for NNN) and Ltk/NNN-CBD under different conditions. Control [³H]ouabain binding was measured as in A. [³H]ouabain binding was also measured in a buffer containing 50 mM Tris-maleate (pH 7.2), 5 mM MgCl₂ and 0.1 mM sodium vanadate with the specific ions and ligands present in the assay medium as shown in the figure. Calmodulin (1 μ M) and CaCl₂ (60 nM) were added directly to the assay system, while 20 nM free Ca²⁺ was obtained by addition of 100 μ M CaCl₂, 900 μ M EGTA and 0.1 mM sodium vanadate with the specific ions and ligands present in the assay medium as shown in the figure. Calmodulin (1 μ M) and CaCl₂ (60 nM) were added directly to the assay system, while 20 nM free Ca²⁺ was obtained by addition of 100 μ M CaCl₂, 900 μ M EGTA and 200 μ M KOH to the assay medium. Bars represent the standard deviations obtained from 3–6 separate experiments. **P* < 0.05–0.01 compared with the control binding.

Ltk (control for endogenous mouse enzymes) possesses no high-affinity binding site for ouabain, while Ltk/NNN and Ltk/NNN-CBD possess a significant number of high-affinity sites (~200 and ~145 fmol/mg protein, respectively, at 800 nM). These results indicate that the chicken wild-type molecule is an active ouabain sensitive Na⁺,K⁺-ATPase, whereas NNN-CBD is inactive in the absence of Ca²⁺/calmodulin, even though it exhibits high-affinity [³H]ouabain binding.

In the presence of calmodulin (1 μ M) and free Ca²⁺

Fig. 4. The ouabain sensitive Na⁺,K⁺-ATPase activity of NNN-CBD can be regulated by Ca²⁺/calmodulin. Ouabain-sensitive Na⁺,K⁺-ATPase activity was measured in the same buffer system as in Figure 2 in the presence and absence of free Ca²⁺ (20 nM) and calmodulin (1 μ M). Membrane preparations from NNN (A) and NNN-CBD (B) were used, and 20 nM free Ca²⁺ was obtained by addition of 100 μ M CaCl₂, 900 μ M EGTA and 200 μ M KOH to the assay medium. Inset figures show the Na⁺,K⁺-ATPase activities after subtraction of the mouse endogenous activity presented in Figure 2. It should be noted that, compared with the Ca²⁺/calmodulin stimulated level of NNN-CBD, the level of the mouse endogenous SERCA- and PM Ca²⁺-ATPase activities are too low (Figure 2), and that the addition of thapsigargin (500 nM) to the assay systems did not affect the data points (Table I). Bars represent the standard deviations obtained from four separate experiments. **P* < 0.05–0.01 compared with the activity in the absence of Ca²⁺/calmodulin.

(20 nM), the chimeric NNN-CBD exhibited detectable ouabain sensitive Na⁺,K⁺-ATPase activity over the mouse background (Figure 4B, compare with Figure 2A). This ouabain sensitive enzyme was slightly stimulated by the addition of 20 nM free Ca²⁺ (Figure 4B), although to a lesser degree than the stimulation seen with Ca²⁺/calmodulin. The activation of NNN-CBD by 20 nM free Ca²⁺ or Ca²⁺/calmodulin was inhibited by ouabain (Figure 4B) but not by thapsigargin (500 nM; Table I), consistent with the fact that the Na⁺,K⁺-ATPase is thapsigargin insensitive (Lyttton *et al.*, 1991). NNN-CBD therefore potentially possesses Na⁺,K⁺-ATPase but not SERCA-ATPase activity. Because the endogenous murine Na⁺,K⁺-ATPase was not stimulated by 20 nM free Ca²⁺ or Ca²⁺/calmodulin (Figure 2A), the increase in Na⁺,K⁺-ATPase activity in Ltk/NNN-CBD is due to the increased activity of the NNN-CBD enzyme.

Fig. 5. Calmodulin dependency of the Na^+, K^+ -ATPase activity of NNN-CBD. Ouabain sensitive Na^+, K^+ -ATPase activity was measured in the same buffer system as in Figure 2 in the presence of free Ca^{2+} (20 nM) and various concentrations of calmodulin. Inset figures show the Na^+, K^+ -ATPase activities after subtraction of the mouse endogenous activity presented in Figure 2. Bars represent the standard deviations obtained from three to four separate experiments. * $P < 0.05$ – 0.01 compared with the activity in the absence of calmodulin.

The IC_{50} for ouabain inhibition of the Na^+, K^+ -ATPase activity in the presence of Ca^{2+} /calmodulin was considerably lower than that of the chicken wild-type enzyme (see Discussion). Nevertheless, it should be emphasized that the NNN-CBD can become active in the presence of Ca^{2+} /calmodulin, and that the level of endogenous PM Ca^{2+} -ATPase activity is much lower (Figure 2C).

Neither wild-type NNN (Figure 4) nor murine Na^+, K^+ -ATPase (Figure 2) was stimulated by 20 nM free Ca^{2+} or Ca^{2+} /calmodulin; addition of free Ca^{2+} or Ca^{2+} /calmodulin often slightly decreased enzyme activity, and significantly inhibited the ouabain sensitive NNN, in strong contrast to its stimulation of NNN-CBD. Figure 4 (inset) shows Na^+, K^+ -ATPase activity after subtraction of endogenous enzyme activity (Figure 2A). IC_{50} values for ouabain inhibition were $\sim 10 \mu\text{M}$ and $< 1 \mu\text{M}$ in the presence and absence of Ca^{2+} /calmodulin. The molecular mechanisms underlying the effects of Ca^{2+} /calmodulin on wild-type Na^+, K^+ -ATPase are not well understood (see Discussion). The effects of 20 nM free Ca^{2+} in the presence and absence of 1 μM calmodulin are apparently the same in Ltk and Ltk/NNN cells, implying that further addition of calmodulin has no effect. However, it is also possible that the existence of endogenous calmodulin in our crude membrane preparations may contribute to the effect of Ca^{2+} . This could also explain the slight activation of the ouabain inhibitable and thapsigargin insensitive Na^+, K^+ -ATPase activity seen in NNN-CBD.

Calmodulin and Ca^{2+} dependency of the Na^+, K^+ -ATPase activity of NNN-CBD

The effects of various concentrations of calmodulin on Na^+, K^+ -ATPase of Ltk, NNN and NNN-CBD cells were

examined in the presence of 20 nM free Ca^{2+} (Figure 5). Calmodulin up to 800 nM had no detectable effect in NNN and Ltk cells, but significantly stimulated the enzyme in NNN-CBD cells in a dose-dependent manner ($\text{EC}_{50} \sim 400$ nM). The concentration of calmodulin required for full activation of the enzyme in these cells was very similar to that previously reported for PM Ca^{2+} -ATPase (Filoteo *et al.*, 1992). In addition, the effect of various concentrations of free Ca^{2+} on Na^+, K^+ -ATPase activity of the above cell lines in the presence of a fixed concentration of calmodulin (1 μM) was studied. Figure 6A demonstrates a biphasic effect of Ca^{2+} on calmodulin-stimulated Na^+, K^+ -ATPase in NNN-CBD cells, with stimulation at lower free Ca^{2+} concentrations and inhibition at higher concentrations. No stimulation was seen in NNN and Ltk cells, although inhibition occurred in the same concentration range of free Ca^{2+} . Ca^{2+} has been reported to inhibit Na^+, K^+ -ATPase activity by reducing the rate of phosphorylation via an interaction with Na^+ and Mg^{2+} ions (Tobin *et al.*, 1973). The Hill coefficients (0.5–0.6) for the inhibitory effects of Ca^{2+} ions were similar for both wild-type and chimeric ATPases (Figure 6B). The Na^+, K^+ -ATPase activity of NNN-CBD cells was ~ 10 times more sensitive to Ca^{2+} ions (IC_{50} value of $\sim 100 \mu\text{M}$) than that in NNN and Ltk cells ($\text{IC}_{50} \sim 1$ mM).

Antibody binding to the chimeric and wild-type molecules

Antibody binding properties of NNN and NNN-CBD expressed in mouse L cells were examined by immunoprecipitation using chicken-specific monoclonal antibodies, IgG 24 and IgG 5 (Figure 7). In transfected cells, the wild-type chicken Na^+, K^+ -ATPase $\alpha 1$ subunit (NNN) as well as the mouse endogenous α subunit is co-immunoprecipitated with the chicken β subunit by the anti- β subunit monoclonal antibody IgG24 (Lemas *et al.*, 1992; Takeyasu *et al.*, 1987). This procedure has been used as an operational definition of subunit assembly (Fambrough and Bayne, 1983; Tamkun and Fambrough, 1986; Takeyasu *et al.*, 1987; Lemas *et al.*, 1992). Monoclonal antibody IgG5 is known to recognize the region between Thr338 and Lys724 of the chicken Na^+, K^+ -ATPase $\alpha 1$ subunit, NNN (Ishii *et al.*, 1994; Lemas *et al.*, 1992). Indeed, in the present study (Figure 7A), NNN with an expected molecular weight of ~ 100 kDa was detected in the immunoprecipitated materials using our routine procedure with IgG24 and IgG5. On the other hand, the same procedures were not successful (Figure 7A) either in co-immunoprecipitation of NNN-CBD (an expected molecular weight of ~ 116 kDa for total 1167 amino acids) with the anti- β antibody IgG24, or in immunoprecipitation with the anti- α antibody IgG5, although the assembly domain of the $\alpha 1$ subunit (Asn894–Ala919) with the β subunit (Lemas *et al.*, 1994) and the epitope for IgG5 exists in the NNN-CBD chimera. The inability of IgG5 in immunoprecipitation parallels the failure in immunostaining of NNN-CBD (see Materials and methods).

Initially (Figure 7A), 1% Triton X-100 was used to solubilize the cells expressing NNN and NNN-CBD. After considering the possibility that the concentration of detergents used in the solubilization/purification steps may affect the efficiency of immunoprecipitation, the

Fig. 6. Calcium ion dependency of the Na⁺,K⁺-ATPase activity of NNN-CBD. (A) Ouabain sensitive Na⁺,K⁺-ATPase activity was measured in the presence of calmodulin (1 μM) and various concentrations of free Ca²⁺. Inset figures show the Na⁺,K⁺-ATPase activities after subtraction of the mouse endogenous activity presented in Figure 2. Bars represent the standard deviations obtained from four separate experiments. **P* < 0.01 compared with the activity in the absence of Ca²⁺. (B) Hill plot of the data in (A) according to the equation: $\log\{[B]/([B_0]-[B])\} = n_H \cdot \log[\text{free Ca}^{2+}] - n_H \cdot \log[IC_{50}]$, where [B] and [B₀] represent the Na⁺,K⁺-ATPase activity in the presence and absence of free Ca²⁺, and *n_H* represents the Hill coefficient. The maximum Na⁺,K⁺-ATPase activity obtained at 20 nM free Ca²⁺ was taken as the [B₀] value for NNN-CBD.

Fig. 7. Expression of NNN and NNN-CBD detected by metabolic labeling followed by immunoprecipitation. Monoclonal antibodies used for isolation are shown at the bottom of the figure. The isolated materials were labeled with ■ (for NNN) and * (for NNN-CBD) on the left side of the bands. (A) Mouse Ltk (lanes 1 and 2), NNN-CBD-transfected (lanes 3 and 4) and NNN-transfected cells (lanes 5 and 6) were labeled with [³⁵S]Translabel, solubilized in 1% Triton X-100, and the labeled materials were immunoprecipitated using anti-α (lanes 2, 4 and 6) and anti-β subunit monoclonal antibodies (lanes 1, 3 and 5). It should be noted that only NNN could be immunoprecipitated by using IgG5 under these conditions, and that IgG24 could co-immunoprecipitate NNN and endogenous mouse α subunit (Lemas *et al.*, 1992; Takeyasu *et al.*, 1987, 1988) but not NNN-CBD. (B) Mouse Ltk (lanes 1 and 4), NNN-CBD-transfected (lanes 2 and 5) and NNN-transfected cells (lanes 3 and 6) were labeled with [³⁵S]Translabel, solubilized in 0.3% Triton X-100, and the labeled materials were immunoprecipitated using anti-α subunit monoclonal antibody IgG5 in the absence (lanes 1-3) and presence of Ca²⁺/calmodulin (lanes 4-6). In this set of experiments, 10 times more cells than in (A) were used based on the consideration of the solubilization efficiency in 0.3% Triton X-100 and the loss of materials by proteolysis in the presence of Ca²⁺/calmodulin. NNN-CBD was successfully affinity purified only in the presence of Ca²⁺/calmodulin. Although significant protein degradation was observed, the amount of expressed ATPase could be estimated to be within ~2-fold differences between cell lines expressing NNN and NNN-CBD.

concentration of Triton X-100 was reduced to 0.3% (Figure 7B). The NNN was immunoprecipitated by IgG5 with a slightly increased background as expected, whereas IgG5 still failed to bind to the NNN-CBD chimera. However, by the addition of Ca²⁺/calmodulin throughout the purification steps, the NNN-CBD was successfully isolated. The concentrations of Ca²⁺/calmodulin were very similar to those used for maximal stimulation of Na⁺,K⁺-ATPase activity of NNN-CBD (see Figure 4). Under the same conditions (i.e. in the presence of 0.3% Triton X-100 and Ca²⁺/calmodulin), IgG24 still did not co-immunoprecipitate the NNN-CBD (data not shown). These results suggest that the modification of the C-terminus of the Na⁺,K⁺-ATPase α subunit (NNN) with the calmodulin binding domain of the PM Ca²⁺-ATPase

results in a specific configuration of NNN and affects the antibody binding, and that NNN-CBD may not assemble with the β subunit or assembles with low affinity, resulting in a failure of co-immunoprecipitation.

Discussion

The four major characteristics of chimeric NNN-CBD are (i) lower affinities for the β subunit and [³H]ouabain than the wild-type chicken Na⁺,K⁺-ATPase (NNN); (ii) inability to bind a monoclonal antibody specific to the chicken Na⁺,K⁺-ATPase α subunit in the absence of Ca²⁺/calmodulin; (iii) very little or no Na⁺,K⁺-ATPase activity in the absence of Ca²⁺/calmodulin; (iv) stimulation of Na⁺,K⁺-ATPase activity by Ca²⁺/calmodulin.

Table I. Ouabain inhibitable Na^+/K^+ -ATPase activity (nmol/min/mg protein)

Cell line	Control (+ thapsigargin)	+ Ca^{2+} /Calmodulin (+ thapsigargin)
Ltk	19.6 ± 0.9 (19.7 ± 1.0) ^a	18.3 ± 0.9 (18.2 ± 0.8) ^a
NNN-CBD	17.7 ± 0.8 (18.0 ± 0.9) ^a	25.7 ± 1.2^b (25.8 ± 0.8) ^{a,b}
NNN	29.5 ± 1.0 (29.6 ± 0.9) ^a	25.3 ± 1.4^b (25.5 ± 1.5) ^{a,b}

Ouabain inhibitable Na^+/K^+ -ATPase activity was measured as the difference between the Na^+/K^+ -dependent ATPase hydrolysis activities in the presence and absence of 5 mM ouabain (see Materials and methods). Free Ca^{2+} (20 nM), calmodulin (1 μM) and thapsigargin (500 nM) were used as in Figures 2–4. The values represent mean $\pm$ standard deviation of four independent measurements.

^aAddition of thapsigargin did not exert any significant effects.

^b $P < 0.01$ compared with the values obtained in the absence of Ca^{2+} /calmodulin.

C-terminal modification of the Na^+/K^+ -ATPase α -subunit affects the affinities for β subunit and ouabain

The search for ouabain binding domains has led to a proposal that they are localized within the N-terminal region rather than the C-terminal region (Price and Lingrel, 1988). As an extension of this line of evidence, we have previously demonstrated that the N-terminal 200 amino acids of the Na^+/K^+ -ATPase are sufficient to bind ouabain and exert inhibitory function even after incorporation into the corresponding region of the SR Ca^{2+} -ATPase (Ishii and Takeyasu, 1993). Another line of evidence has indicated that the β -subunit of the Na^+/K^+ -ATPase can modulate the characteristics of ATPase functions including affinities for K^+ and ouabain (Eakle *et al.*, 1992; Jaisser *et al.*, 1992). We have previously demonstrated that the C-terminal 161 amino acids of the α subunit (Lemas *et al.*, 1992), especially the extracellular 26 amino acids (Asn894–Ala919) between M7 and M8 (Lemas *et al.*, 1994), are responsible for assembly with the β subunit. Point mutations within the extracellular domain between M7 and M8 change the affinity for ouabain (Schultheis *et al.*, 1993). Thus, the ouabain binding property of the N-terminal region may be affected through long range conformational changes upon assembly with the β subunit at the extracellular C-terminal region.

The present results suggest that the affinity for ouabain can be modified by a modification of the cytoplasmic C-terminal region (Figure 3), probably through a long range interference. Immunoprecipitation using a monoclonal IgG specific to the β subunit has demonstrated that the chimeric NNN–CBD does not assemble with the β subunit (or assembles weakly; Figure 7). Therefore, the apparent lower affinity for ouabain of NNN–CBD may partly be due to a defect in assembly with the β subunit. In this sense, it is intriguing that the addition of 165 extra amino acids at the cytoplasmic C-terminus of the Na^+/K^+ -ATPase α subunit affects the ability of its extracellular critical domain (Asn894–Ala919) to assemble with the β subunit.

The calmodulin binding domain of the PM Ca^{2+} -ATPase interacts with the Na^+/K^+ -ATPase α -subunit and regulates the Na^+/K^+ -ATPase activity in a Ca^{2+} /calmodulin-dependent manner

Monoclonal antibody IgG5 recognizes the chicken Na^+/K^+ -ATPase α subunit, but not mammalian isoforms (Takeyasu *et al.*, 1988). The epitope for this antibody resides between Thr338 and Lys724 of the chicken Na^+/K^+ -ATPase α 1 subunit (Luckie *et al.*, 1992; Sumbilla

et al., 1993; Ishii *et al.*, 1994). Further detailed analysis on the location of the IgG5 binding site has indicated that the epitope is localized within 100 amino acids before Lys724 (D.Fambrough, personal communication). This epitope is naturally included in the middle portion of the chimeric NNN–CBD (the epitope-containing fragment is underlined). However, our routine immunoprecipitation assay using IgG5 failed to detect NNN–CBD in the absence of Ca^{2+} /calmodulin (Figure 7), indicating that the epitope in this chimera is not accessible to IgG5 under these conditions. NNN–CBD was, however, immunoprecipitated by IgG5 in the presence of Ca^{2+} /calmodulin (Figure 7B); i.e. the epitope is now identified by this antibody. These data together suggest that the epitope for IgG5 in NNN–CBD is masked by CBD in the absence of Ca^{2+} /calmodulin and uncovered in the presence of Ca^{2+} /calmodulin.

Ca^{2+} /calmodulin inhibited wild-type Na^+/K^+ -ATPase (Figure 6), but had a dual effect (activation and inhibition) on the chimeric ATPase. The stimulatory effect of Ca^{2+} /calmodulin was seen only in the chimeric ATPase, NNN–CBD. This indicates that Ca^{2+} /calmodulin interacts directly with the NNN–CBD via the CBD incorporated in the C-terminus of the α 1 subunit. The regulation of NNN–CBD by Ca^{2+} /calmodulin may involve a molecular mechanism similar to that of the wild-type PM Ca^{2+} -ATPase: the calmodulin binding domain of NNN–CBD functions as a repressor, and the binding of calmodulin to this domain removes the auto-inhibition. Effects of Ca^{2+} ions on the Na^+/K^+ -ATPase activity of NNN–CBD were biphasic, as seen for the PM Ca^{2+} -ATPase, with activation at low concentrations and inhibition at high concentrations.

In crude systems such as the cell homogenate used in the present study, the activity of Na^+/K^+ -ATPase can be regulated by protein kinases including PKA (Chibalin *et al.*, 1991; Satoh *et al.*, 1992; Horiuchi *et al.*, 1993) and PKC (Chibalin *et al.*, 1991, 1992) via phosphorylation at positions other than Asp395. This might explain the inhibitory effect of Ca^{2+} /calmodulin on both the wild-type and chimeric Na^+/K^+ -ATPase. Alternatively, Ca^{2+} at high concentrations may simply reduce the rate of phosphorylation (Tobin *et al.*, 1973).

Possible domains of the Na^+/K^+ -ATPase α -subunit that might interact with the calmodulin binding domain of the PM Ca^{2+} -ATPase

A recent report has demonstrated that a synthetic peptide containing the sequence of the CBD of the PM Ca^{2+} -ATPase inhibits SR Ca^{2+} -ATPase activity (Enyedi and Penniston, 1993). Our present study indicates that the

Na⁺,K⁺-ATPase α 1 subunit can be regulated by Ca²⁺/calmodulin if the α subunit is connected properly to the C-terminal region of the PM Ca²⁺-ATPase that is responsible for the binding of Ca²⁺/calmodulin. This, in turn, suggests the existence of a direct interaction between the calmodulin binding regions of the PM Ca²⁺-ATPase and the Na⁺,K⁺-ATPase α 1 subunit, similar to that proposed for the calmodulin binding regions and their interacting sites within the PM Ca²⁺-ATPase (Carafoli, 1992). These results together suggest that these P-type ATPases (Na⁺,K⁺-ATPase and PM and SR Ca²⁺-ATPases) may possess a common acceptor site(s) for the CBD. A comparison of the primary structures among these ATPases (rat brain PM Ca²⁺-ATPase II, rabbit SR Ca²⁺-ATPase and chicken Na⁺,K⁺-ATPase α 1 subunit) identifies five homologous domains that may be candidates for 'acceptor' sites of the CBD. One is located between M2 and M3, and others are located in the catalytic domain between M4 and M5.

Results of previous studies using a photo-activatable derivative of synthetic calmodulin binding regions (Falchetto *et al.*, 1991; Carafoli, 1992; Carafoli *et al.*, 1992) suggest that the CBD might interact with two regions, one at Ile206–Val271 and the other at Cys537–Thr544 (the amino acid number is based on the human PM Ca²⁺-ATPase IV sequence). These sequences have been proposed to be the first and the second receptor site for the CBD, respectively (Carafoli *et al.*, 1992). It is also possible, however, that acceptor sites and effective sites for the CBD might be different (Carafoli, 1992; Falchetto *et al.*, 1991). It is very interesting that the amino acid residues Ile206–Val271 between M2 and M3 are within one of the highly homologous domains in PM Ca²⁺-ATPase, SR Ca²⁺-ATPase and Na⁺,K⁺-ATPase (Figure 1), whereas the amino acid residues Cys537–Thr544 between the phosphorylation and FITC binding sites are more variable among the P-type ATPases. It is also noteworthy that the CBD seriously affected the binding site for monoclonal antibody IgG5, which resides near Lys724. Taking all the evidence together, it is probable that the calmodulin binding domain folds over extensive protruding regions, including a large catalytic domain between M4 and M5 and a short intracellular loop between M2 and M3.

Materials and methods

Construction of chimeric cDNA

For the construction of chimeric NNN–CBD (Figure 1), unique endonuclease recognition sites, *Xho*I and *Sal*I, were created at position Pro1011 in the chicken Na⁺,K⁺-ATPase α 1 subunit (Takeyasu *et al.*, 1988) and Arg1043 in the rat brain PM Ca²⁺-ATPase II (Greeb and Shull, 1989), respectively, by oligonucleotide derived mutagenesis. The nucleotide sequence encoding Met1 to Pro1011 of the Na⁺,K⁺-ATPase α 1 subunit was synthesized by polymerase chain reaction (PCR) using two oligonucleotide primers; 5'-CCAAGCTTGGCTCGAGGTGCG-ACGG-3' (vector primer) and 5'-CCAACCTCGAGGTTGCG-3' (*Xho*I site is underlined). The nucleotide sequence for Arg1043–Leu1198 of PM Ca²⁺-ATPase II was synthesized by PCR using two primers; 5'-TACCAGTCTGACTCAAGTTTCTGAA-3' (*Sal*I site is underlined) and 5'-ACAGAGATCTCAGCTAAAGCGACG-3' (including stop codon). These two PCR fragments were ligated using compatible *Xho*I/*Sal*I sites as a junction. The nucleotide sequence of PCR fragments was determined by a standard DNA sequencing technique (Sanger *et al.*, 1977). When mutated sequences were found, they were replaced with the wild-type sequences using appropriate restriction enzyme sites available near the

mutated regions. The final construction of chimeric cDNA was ligated into one of the polylinker sites in a mammalian expression vector, pRC/CMV (Invitrogen Corp. cat#V750–20), and was introduced into mouse L cells (Ltk) that had been transfected with a cDNA encoding the chicken Na⁺,K⁺-ATPase β 1 subunit (Takeyasu *et al.*, 1987).

Transfection and cloning of cell lines

Chimeric cDNA was transfected into mouse Ltk cells (5 × 10⁵ cells/100 mm culture dish) using the calcium/phosphate precipitation method as described previously (Takeyasu *et al.*, 1987, 1988), and 50 individual G418 resistant cell lines were isolated. In our previous studies (Takeyasu *et al.*, 1988; Ishii and Takeyasu, 1993; Ishii *et al.*, 1994), we were able to screen transfected cells by immunostaining with a monoclonal antibody, IgG5, which is specific to the chicken Na⁺,K⁺-ATPase α subunit (Takeyasu *et al.*, 1988). However, in the present study, IgG5 could not be used to detect the expressed chicken chimeric protein (NNN–CBD) by immunofluorescence microscopy, even though this chimeric molecule contained the epitope for this antibody. This can be attributed to the fact that IgG5 binds to NNN–CBD only under highly restricted conditions (for details, see Figure 7). Therefore, in order to ensure the expression of NNN–CBD we had to rely on the ability of NNN–CBD molecule to bind [³H]ouabain with high affinity. By further screening the 50 G418 resistant cell lines for high levels of [³H]ouabain binding at 800 nM, 10 cell lines were identified as good expressors of NNN–CBD. At this concentration range the chicken α 1 subunit, but not mouse endogenous α subunit, binds [³H]ouabain due to species-specific differences (Takeyasu *et al.*, 1987, 1988; Ishii and Takeyasu, 1993; Ishii *et al.*, 1994). Since all the cell lines expressing NNN–CBD exhibited essentially the same qualitative characteristics in terms of ouabain binding and ATPase activity, the cell line (Ltk/NNN–CBD) expressing the chimeric molecule at the highest level was used for further analysis in this study. The cell line (Ltk/NNN) that expresses the wild-type chicken Na⁺,K⁺-ATPase α 1 subunit (NNN) has been described previously (Ishii and Takeyasu, 1993).

Maintenance of cell lines and crude membrane preparation

The cloned cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) and G418 (100 µg/ml) at a density of 5 × 10⁵–10⁶ cells/100 mm culture dish. Keeping cells in a healthy condition is extremely important for obtaining steady expression levels of transfected and endogenous proteins. Usually for one set of experiments, we performed three to five independent measurements using separate culture dishes containing 5 × 10⁵–10⁶ cells (i.e. measurements were repeated three to five times using freshly and independently prepared cultures). For this, the monolayers were treated with 5 mM Tris–HCl (pH 7.5) for 20 min on ice, scraped off from the bottom of the culture dish, and centrifuged at 15 000 r.p.m. (Du Pont, Sorvall SA-600) for 15 min. The pellet was resuspended to give ~1 mg protein/ml in TME buffer (75 mM Tris–HCl, pH 7.5, 12.5 mM MgCl₂, 1.5 mM EDTA) and used as crude membrane preparation for a single ATPase assay. Protein concentration was determined by the method of Lowry *et al.* (1951).

Na⁺,K⁺-ATPase assay using crude membrane preparation

We define the Na⁺,K⁺-ATPase activity as the ouabain inhibitable and Na⁺,K⁺-dependent ATP hydrolysis. Na⁺,K⁺-dependent ATPase activity was measured at 37°C for 30 min by monitoring the release of inorganic ³²P from [γ -³²P]ATP (Amersham, PB218) in 0.5 ml of assay medium containing crude membrane preparation (~0.1 µg protein), 100 mM NaCl, 5 mM KCl, 50 mM Tris–HCl (pH 7.4), 2 mM ATP (containing 100 µM NaOH and [γ -³²P]ATP), 3 mM MgCl₂, 5 mM Na₂SO₄ and 1 mM ethyleneglycol-bis-*N,N,N',N'*-tetra-acetic acid (EGTA). Assays were conducted at 37°C for 30 min and reaction was terminated by addition of 0.5 ml of 8% perchloric acid. The cleaved ³²P was converted to phosphomolybdate, extracted into 2-methyl-propanol, and the radioactivity was determined in a liquid scintillation counter. The rates of ATP hydrolysis were linear over this time course of experiments. In some experiments, appropriate amounts of CaCl₂ and EGTA (pH adjusted to 7.4 with ~200 µM KOH) were added to produce the required free Ca²⁺ concentration. The necessary concentrations of CaCl₂ and EGTA were calculated using a computer program developed by Horiuchi (1986) which has been widely used in the studies of muscle contraction under a variety of free Ca²⁺ concentrations (e.g. see Kitazawa *et al.*, 1989). The background for the Na⁺,K⁺-ATPase was determined in the presence of 5 mM ouabain. Thus, the ouabain sensitive Na⁺,K⁺-ATPase activity was defined as the difference in the Na⁺,K⁺-dependent ATP hydrolysis measured in the presence and absence of 5 mM ouabain.

Ca²⁺-ATPase activity

We defined SERCA-ATPase activity as the thapsigargin inhibitable and Ca²⁺-dependent ATP hydrolysis, and the PM Ca²⁺-ATPase activity as thapsigargin insensitive/calmodulin/Ca²⁺-dependent ATP hydrolysis. Ca²⁺-dependent ATPase activity was measured in crude membrane preparations by monitoring the release of inorganic ³²P from [γ -³²P]ATP (Amersham, PB218) over a period of time at 37°C (Tada *et al.*, 1988). The assay system (0.5 ml) consisted of membranes (~0.1 μ g), 100 mM KCl, 50 mM Tris-HCl (pH 7.4), 2 mM ATP (containing 100 μ M KOH and [γ -³²P]ATP), 3 mM MgCl₂, 2 μ M A23187, 1 mM NaN₃, and an appropriate amount of CaCl₂ and EGTA to produce required concentrations of free Ca²⁺ (Horiuchi, 1986; Tada *et al.*, 1988). The background Ca²⁺-dependent ATPase activity was determined in the presence of 5 mM EGTA. Thus, the Ca²⁺-ATPase activity was defined as the difference in the Ca²⁺-dependent ATP hydrolysis measured in the presence and absence of 5 mM EGTA. The SERCA-ATPase activity was defined as the difference in the Ca²⁺-dependent ATPase activities measured in the presence and absence of 500 nM thapsigargin. A fraction of the Ca²⁺-dependent ATPase activities that cannot be inhibited by thapsigargin contains PM Ca²⁺-ATPase activity. The PM Ca²⁺-ATPase activity was therefore defined as the difference in the thapsigargin-insensitive Ca²⁺-dependent ATPase activities measured in the presence and absence of 1 μ M calmodulin. All Ca²⁺-ATPase activity assays were conducted after 5 min preincubation of the crude membrane preparation at 37°C in the presence or absence of 500 nM thapsigargin.

Ouabain binding

For [³H]ouabain binding, the crude membrane preparation was further homogenized using a Tekmar Tissuemizer. Binding of [³H]ouabain (Du Pont-New England Nuclear) to the crude membrane preparations was measured in 0.5 ml of binding media containing 50 mM Tris-maleate (pH 7.2), 5 mM MgCl₂, 0.1 mM sodium vanadate and various concentrations of [³H]ouabain (Lucchesi and Sweadner, 1991). After incubation for 90 min at 37°C, the binding was terminated by dilution with 4 ml of ice-cold washing buffer (5 mM MgCl₂, 5 mM Tris-phosphate, and 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 washing buffer, and the radioactivity remaining on the filters was measured. Specific binding was calculated by subtracting the non-specific binding measured in the presence of excess unlabeled ouabain (2 mM) from the total binding obtained at a given concentration of [³H]ouabain.

Metabolic labeling and immunoprecipitation

Immuno-beads coupled with monoclonal antibodies were prepared by reductive amination as described (Stults *et al.*, 1989). Briefly, 1 mg of purified antibody and 1 g of activated beads (oxidized cross-linked Sepharose CL-6B) were incubated for 24 h in the presence of 30 mM pyridine borane. After a thorough wash in 0.2 M sodium phosphate, the monoclonal antibody-coupled beads were pre-incubated with 5% horse serum and then used for the following immunoprecipitation. Cells (1×10⁵ cells/60 mm culture dish and 1×10⁶ cells/100 mm dish for solubilization in 1 and 0.3% Triton X-100, respectively) were labeled in methionine-free DMEM (GIBCO) containing 20–40 μ Ci/ml [³⁵S]Translabel (ICN Pharmaceuticals) for 24 h, and solubilized in 5 vol. of buffer solution containing Triton X-100 (1 or 0.3%), 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EGTA and 2 mM phenyl-methyl-sulfonyl-fluoride at 4°C. In some experiments, EGTA (5 mM) was replaced by CaCl₂ (100 μ M) and calmodulin (2 μ M). The solubilized materials were incubated for 24 h at 4°C with activated beads coupled with either monoclonal antibody IgG5 [specific for the chicken Na⁺,K⁺-ATPase α subunit (Takeyasu *et al.*, 1988)] or IgG24 [specific for the chicken Na⁺,K⁺-ATPase β subunit (Fambrough and Bayne, 1983; Tamkun and Fambrough, 1986; Takeyasu *et al.*, 1987)]. The beads were then washed 4 times with solubilization buffer and once with unbuffered 1 or 0.3% Triton X-100. After elution in 0.5% deoxycholate/0.1 M triethylamine (pH 11.5) and neutralization with Tris-HCl (pH 7.4) as described (Fambrough and Bayne, 1983), the purified materials were analyzed by SDS-polyacrylamide gel electrophoresis (5.5% polyacrylamide) and fluorography.

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