Carboxy-Terminal Regions of the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}- and the Na^+/K^+-ATPases Control Their K^+ Sensitivity†

Toshiaki Ishii,‡,§ Fumiaki Hata,§ M. Victor Lemas,|| D. M. Fambrough,|| and Kunio Takeyasu*,‡,⊥

Department of Medical Biochemistry and Biotechnology Center, The Ohio State University, Columbus, Ohio 43210, Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218, Research Institute for Advanced Science and Technology and Department of Veterinary Pharmacology, University of Osaka Prefecture, Sakai, Osaka 591, Japan, and Department of Natural Environment Sciences, Faculty of Integrated Human Studies, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

Received March 15, 1996; Revised Manuscript Received November 15, 1996⊗

ABSTRACT: The Na^+,K^+-ATPase and the sarcoplasmic/endoplasmic reticulum Ca^{2+}- (SERCA-) ATPase belong to a family of P-type ATPases that undergo a cycle of conformational changes between the phosphorylated and dephosphorylated stages in an ion-specific manner. The ouabain-inhibitable Na^+,K^+-ATPase activity requires Na^+ and K^+. On the other hand, the Ca^{2+}-dependent and thapsigargin-inhibitable activity of the SERCA-ATPase does not depend upon Na^+ and K^+ for its basal activity. However, the SERCA-ATPase and Ca^{2+}-transport activities can be further activated either by K^+ in a two-step fashion with high ($ED_{50} \sim 20$ mM) and low affinity ($ED_{50} \sim 70$ mM) or by Na^+ in a one-step fashion with an ED_{50} value of ~50 mM. A chimera, in which the carboxy-terminal region (Leu861-COOH) of the Na^+,K^+-ATPase α1 subunit replaced the corresponding region (Ser830-COOH) of the SERCA1-ATPase, lacked the low-affinity K^+ activation of the SERCA-ATPase but displayed a higher-affinity ($ED_{50} < 10$ mM) activation by K^+, similar to that of the Na^+,K^+-ATPase, whereas activation by Na^+ was not affected. The replacement of the large cytosolic loop (Gly354−Lys712) and the amino-terminal regions (Met1−Asp162) of the SERCA1-ATPase with the corresponding portions of the Na^+,K^+-ATPase α1 subunit did not affect the sensitivity of the SERCA-ATPase activity to K^+. Thus, the carboxy-terminal regions of both the SERCA1 and the Na^+,K^+-ATPase α1 subunit are critical for K^+ sensitivity. Analysis of additional (Ca^{2+}/Na^+,K^+)-ATPase chimeras demonstrated that the carboxy-terminal 102 amino acids (Phe920−Tyr1021) of the Na^+/K^+-ATPase α1 subunit are sufficient to shift the K^+ affinity for activation of the SERCA-ATPase without the *β* subunit. No change in the two-step activation of SERCA-ATPase by K^+ was seen when residues Thr871−Thr898 of the SERCA1-ATPase were replaced with residues Asn894−Ala919 of the Na^+,K^+-ATPase α1 subunit, a region known to bind the Na^+,K^+-ATPase *β* subunit [Lemas, M. V., et al. (1994) *J. Biol. Chem. 269*, 8255−8259]. Thus, the Na^+,K^+-ATPase subunit-assembly domain and the K^+-sensitive region are distinct within the carboxy-terminal 161 amino acids of the Na^+,K^+-ATPase.

Both the Na^+- and K^+-dependent ATPase (Dean, 1941; Skou, 1957) and the Ca^{2+}-dependent ATPase (SERCA-ATPase) (Ebashi & Lipman, 1962; Hasselbach & Makinose, 1961) undergo a series of conformational changes during their ion-transport cycles. Their reaction mechanisms are thought to be similar (Albers, 1967; De Meis & Vianna, 1979; Inesi & De Meis, 1989; Post et al., 1972). A central question in the physiology and biochemistry of these ion-transporting ATPases has been the following. How do different ions and inhibitors exert their specific effects on a particular ion pump (Bodemann & Hoffman, 1976; Hansen, 1984; Jørgensen & Andersen, 1988; Kijima et al., 1991; Lytton et al., 1991; Post & Jolly, 1957; Sagara et al., 1992)? Recombinant DNA technology has made it possible to address this question in more direct ways by functional expression of manipulated encoding DNAs.

Our approach to the identification of distinct functional domains of these ion pumps has been to express and monitor the specific functions of chimeric ATPases constructed between the Na^+,K^+-ATPase catalytic α1 subunit and the SERCA1 ATPase (Ishii & Takeyasu, 1993; Ishii et al., 1994; Lemas et al., 1992, 1994). In designing chimeric molecules, we have relied on the following characteristics of the P-type ATPases (Jørgensen & Andersen, 1988). (i) All of the ion pumps share a common pattern of hydrophobicity. (ii) Evolutionarily conserved sequences (~30% of total amino acids) form the ATP-catalytic domain in the middle portion of the enzymes. (iii) The Na^+,K^+-ATPase activity is ouabain-inhibitable (Bodemann & Hoffman, 1976; Hansen, 1984; Post & Jolly, 1957), whereas the SERCA-ATPase activity is thapsigargin-sensitive (Lytton et al., 1991; Kijima et al., 1991; Sagara et al., 1992). (iv) Different ion pumps have regions of very distinct amino acid sequences that could be responsible for ion and inhibitor specificities. Using the fast form of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase

† This work was supported by the National Institutes of Health (GM44373 to K.T. and NS23241 to D.M.F.) and by the Japanese Ministry of Education, Science and Culture (to K.T.).

* Correspondence: Dr. Kunio Takeyasu, Biotechnology Center, 125 Rightmire Hall, The Ohio State University, 1060 Carmack Road, Columbus, OH 43210. Phone: 614-292-8542. Fax: 614-292-5379.

‡ The Ohio State University.

§ University of Osaka Prefecture.

|| The Johns Hopkins University.

⊥ Kyoto University.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1997.

S0006-2960(96)00644-7 CCC: $14.00

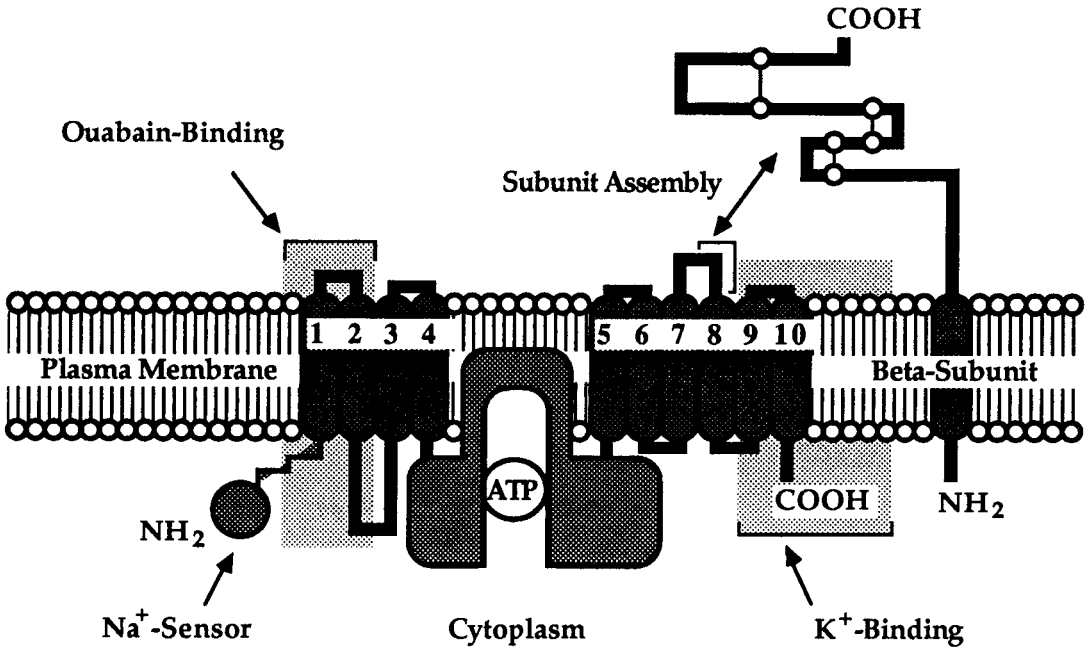

FIGURE 1: Functional domain model (working model) for the structure and function of the Na/K-ATPase that accommodates all the results obtained with recombinant chimeric molecules between the Na/K- and the SR Ca-ATPase (Ishii & Takeyasu, 1993; Ishii et al., 1994; Lemas et al., 1992, 1994; this study). The topology model proposed for the SR Ca-ATPase (Mathews et al., 1990) is adapted to the Na/K-ATPase α subunit on the basis of the previously reported results (Fambrough et al., 1994). Accordingly, the transmembrane segments are numbered (M1−M10). The model includes Na^+ sensor, ouabain-binding, and subunit-assembly domains and a K^+-binding region. Two additional ion-binding sites for K^+ and Na^+ are possibly localized between M3 and M6 (not shown in the figure) on the basis of the analogy to the minimum structural requirement for SERCA-ATPase activity. The channel-like structure identified by atomic force microscopy (Paul et al., 1994) was schematically incorporated into the large cytoplasmic loop between M4 and M5. Three S−S bonds (○−○) are shown in the smaller β subunit.

(SERCA1) as a parental molecule and replacing the middle phosphorylation domain (Gly354−Lys712) and the amino- (Met1−Asp162) and carboxy-terminal regions (Ser830-COOH) with the corresponding portions of the Na^+,K^+-ATPase α1 subunit, we have identified the domains within these two ATPases responsible for (i) activation by Ca^{2+} and inhibition by thapsigargin (the segments Ile163−Gly354 and Lys712−Ser830) (Ishii et al., 1994), (ii) Na^+ sensitivity (Met1−Leu69) (Ishii et al., 1994), (iii) ouabain binding (Ala70−Asp200) (Ishii & Takeyasu, 1993; Ishii et al., 1994), and (iv) subunit assembly (Asn894−Ala919) (Lemas et al., 1992, 1994) (Figure 1). These results together suggest that the catalytic subunit of P-type ATPases consists of several distinct functional domains that retain their structural−functional integrity when transferred into other P-type ATPase molecules.

Here we report extensions of this type of approach that identify K^+-sensitive domains within the carboxy-terminal regions of both Na^+,K^+- and SERCA-ATPase molecules. We also report that the carboxy-terminal K^+-binding regions are distinct from the assembly domain of the Na^+,K^+-ATPase α subunit. The K^+ sensitivity of the SERCA-ATPase has not been explored previously. It was intriguing to identify it obviously, and only because of the use of chimeric ATPases did the observation come about. The biological significance of the K^+ sensitivity of the SERCA-ATPase is not yet known. However, the existence of the effect, demonstrated in the present study, may have implications for the general mechanism of ion transport in P-type ATPases. On the basis of these results and previous findings, we propose a functional-domain model for the Na^+,K^+- and SERCA-ATPase that may be useful in elucidation of the structure−function relationships of the P-type ATPase in general.

EXPERIMENTAL PROCEDURES

Preparation of SR Membranes. Adult chicken SR membranes were obtained by the method described earlier (Kaprielian & Fambrough, 1987; Meisnner, 1975). Briefly, breast muscles were isolated, rinsed in 0.1 M EDTA (pH 7.0) on ice, and trimmed of fat and connective tissues. The trimmed muscles (100 g) were homogenized in 300 mL of buffer containing 10 mM histidine, 10% sucrose, and 0.1 M EDTA (pH 7.0) and centrifuged at 15000*g* for 20 min. The supernatant was filtered through six layers of gauze and centrifuged at 40000*g* for 90 min. The resulting pellet was washed in 40 mL of 0.6 M KCl/10 mM histidine buffer (pH 7.0), resuspended in 0.88 M sucrose and 10 mM MOPS (pH 7.0) to give a protein concentration of 10−30 mg/mL, and stored at −70 °C.

Expression of Chicken Chimeric cDNAs in Mouse L Cells. By using the SERCA1-ATPase as a parental molecule and replacing the phosphorylation domain (Gly354−Lys712) and the amino- (Met1−Asp162) and carboxy-terminal regions (Ser830-COOH) with the corresponding portions of the Na^+,K^+-ATPase α1 subunit, we constructed several functional chicken chimeric ATPases. The chimeric chicken cDNA constructs encoding CCC (Karin et al., 1989), [n/c]CC (Ishii & Takeyasu, 1993), CNC (Ishii et al., 1994), CC[c/n/n] (Lemas et al., 1992), and CC[c/n/c] (Lemas et al., 1994) (C and c stand for portions of the SERCA1, N and n for portions of the Na^+,K^+-ATPase) were cloned into a mammalian expression vector, pRc/CMV (Invitrogen, V750-20), introduced into mouse L cells that had been transfected with

a cDNA encoding the chicken Na^+,K^+-ATPase β1 subunit (Takeyasu et al., 1987), and the cell lines that showed a high level of expression of the encoded chimeric ATPase were selected. The fundamental characterization of these cell lines has been reported (Ishii & Takeyasu, 1993; Ishii et al., 1994; Lemas et al., 1992, 1994), and these cell lines were used in the present study.

Affinity Purification of Chicken ATPases Expressed in Mouse Cells. Immunobeads coupled with monoclonal antibodies, IgG 5D2, IgG α5, or IgG 24 (monoclonal antibody specific to the chicken Na^+,K^+-ATPase β subunit), were prepared by reductive amination as described (Stults et al., 1989). Transfected cells from two culture plates (5×10^6 cells per 150 mm dish) were treated with 5 volumes of solubilization solution containing C12E8 (75 μg/mL), 10 mM Tris-HCl (pH 7.5), and 150 mM NaCl at 4 °C for 1 h. The solubilized materials were incubated with immunobeads for 24 h at 4 °C. The beads were then washed five times with the solubilization solution, resuspended in TME buffer [75 mM Tris-HCl (pH 7.5), 12.5 mM MgCl2, and 1.5 mM EDTA] to give ~200 ng of protein per 100 μL, and used for the determination of protein content and the measurement of SERCA-ATPase activity (see below).

Determination of the Amount of Expressed Chicken Molecules. The purified chicken ATPase molecules were eluted in 0.5% deoxycholate/0.1 M triethylamine (pH 11.5) (Fambrough & Bayne, 1983) from one-half of the immunobeads suspension (see above). The eluted materials were neutralized with Tris-HCl (pH 7.4) and subjected to SDS−PAGE followed by silver staining (for IgG 5D2- and IgG α5-purified materials) or Western blot analysis (IgG 24-purified materials). For silver staining, Silver Stain Plus (Bio-Rad) was used and the visualized protein bands were quantified by scanning densitometery with rabbit phosphorylase *b* as a calibration standard. The Ca^{2+}-ATPase (SERCA1) purified from SR can be also used as a calibration standard to give essentially the same results. For Western blot analysis, the IgG 24-purified materials were transferred to nitrocellulose membranes at 4 °C in transfer solution containing 25 mM Tris-HCl (pH 8.4), 192 mM glycine, 20% methanol, and 0.025% SDS and then probed with IgG 5D2 in PBS containing 0.05% Tween 20, followed by goat anti-mouse IgG conjugated to horseradish peroxidase. The final protein/IgG complexes were detected by the reaction to 3,3′-diaminobenzidine tetrahydrochloride (BRL-Gibco), and the visualized bands on the membranes were analyzed by Image Scanner (EPSON, GT6500) with purified chicken SERCA1 as a calibration standard. The band densities were quantified with image analysis software (NIH, Image).

Crude Membrane Preparation. Monolayers of cells were treated in 5 mM Tris-HCl (pH 7.5) for 20 min on ice, scraped from the culture dish, and centrifuged at 15 000 rpm (Du Pont, Sorvall SA-600 instrument) for 15 min. The pellet was resuspended to give ~1 mg of protein/mL in TME buffer and used for ATPase assays.

Thapsigargin-Sensitive Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity in crude membrane preparations or affinity-purified fractions (see above) was measured by monitoring the kinetics of release of ^{32}Pi from [γ-^{32}P]ATP (Amersham, PB218) at 37 °C. The basic assay system (0.5 mL) consisted of membranes (~0.1 mg of protein) [or immunobeads (~200 ng of protein)], 100 mM KCl, 50 mM Tris-HCl (pH 7.4), 2 mM ATP (containing 100 μM KOH and [γ-^{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 calculated with the software developed by Horiuchi (1986). When a higher ionic strength or a different ionic component was used, the amount of KCl was adjusted to the requirement (see the legends for the figures). The Ca^{2+}- and thapsigargin-sensitive ATPase (SERCA-ATPase) activity was defined as a difference in the Ca^{2+}-ATPase activities measured in the presence and absence of 500 nM thapsigargin as previously described (Ishii & Takeyasu, 1993). Addition of ouabain (100 nM to 4 mM) to the assay medium did not affect the above-defined SERCA-ATPase activity of all tested constructs except [n/c]CC. [n/c]CC is a Ca^{2+}-, thapsigargin-, Na^+-, and ouabain-sensitive ATPase (Ishii & Takeyasu, 1994). All assays were started with 5 min of preincubation at 37 °C in the presence or absence of thapsigargin.

Thapsigargin-Sensitive Ca^{2+}-Transport Activity. Uptake of ^{45}Ca was measured at 25 °C by microfiltration methods as described by Zhang et al. (1995). The basic reaction mixture contained 20 mM MOPS (pH 7.0), 180 mM KCl (or NaCl plus choline chloride), 5 mM $MgCl_2$, 5 mM potassium oxalate, 3 mM ATP, membrane vesicles (~10 μg/mL), and appropriate amounts of $CaCl_2$ and EGTA to produce the required free Ca^{2+} concentration (Horiuchi, 1986). The thapsigargin-sensitive Ca^{2+}-transport activity was defined as a difference in the Ca^{2+}-transport activities measured in the presence and absence of 500 nM thapsigargin. The reaction was started by addition of oxalate and ATP after 5 min of preincubation at 37 °C in the presence or absence of thapsigargin and was terminated at intervals by filtration using Millipore filters (0.45 μm). The filters were washed twice with 2 mL of 2 mM $LaCl_3$ and 10 mM MOPS (pH 7.0), and the remaining radioactivity was quantified in a scintillation counter.

RESULTS

Effects of K^+ and Na^+ on the SERCA-ATPase Activity. The SERCA-ATPase activity of chicken SR membranes was relatively low in the absence of K^+ and Na^+ but markedly increased in the presence of K^+ or Na^+. At the optimum Ca^{2+} concentration, Na^+ and K^+ independently enhanced the SERCA-ATPase activity in a one- and two-step manner, respectively (Figure 2A). The stimulation by Na^+ showed a single ED_{50} value of ~50 mM. The initial activation by K^+ had an ED_{50} value of ~20 mM and subsequent full activation an ED_{50} of >50 mM. These effects of K^+ and Na^+ were also detected on ^{45}Ca-transport activity of SR vesicles (panels C and D of Figure 2). It should be noted that the activity level induced by 100 mM Na^+ in the absence of K^+ is very similar to that induced by the high-affinity effect of K^+. When high concentrations of K^+ (e.g., 100 mM) were present in the assay systems (for both SERCA-ATPase and ^{45}Ca-transport activities), the stimulatory effect of Na^+ was abolished (panels B and D of Figure 2). On the other hand, when saturating concentrations of Na^+ were present, only the high-affinity effect of K^+ was abolished and the low-affinity stimulation by K^+ was still observed (panels A and C of Figure 2).

These effects of K^+ and Na^+ were also observed in mouse L cells transfected with cDNA encoding the chicken SERCA1 (Figure 3). Similarly, the SERCA-ATPase activity

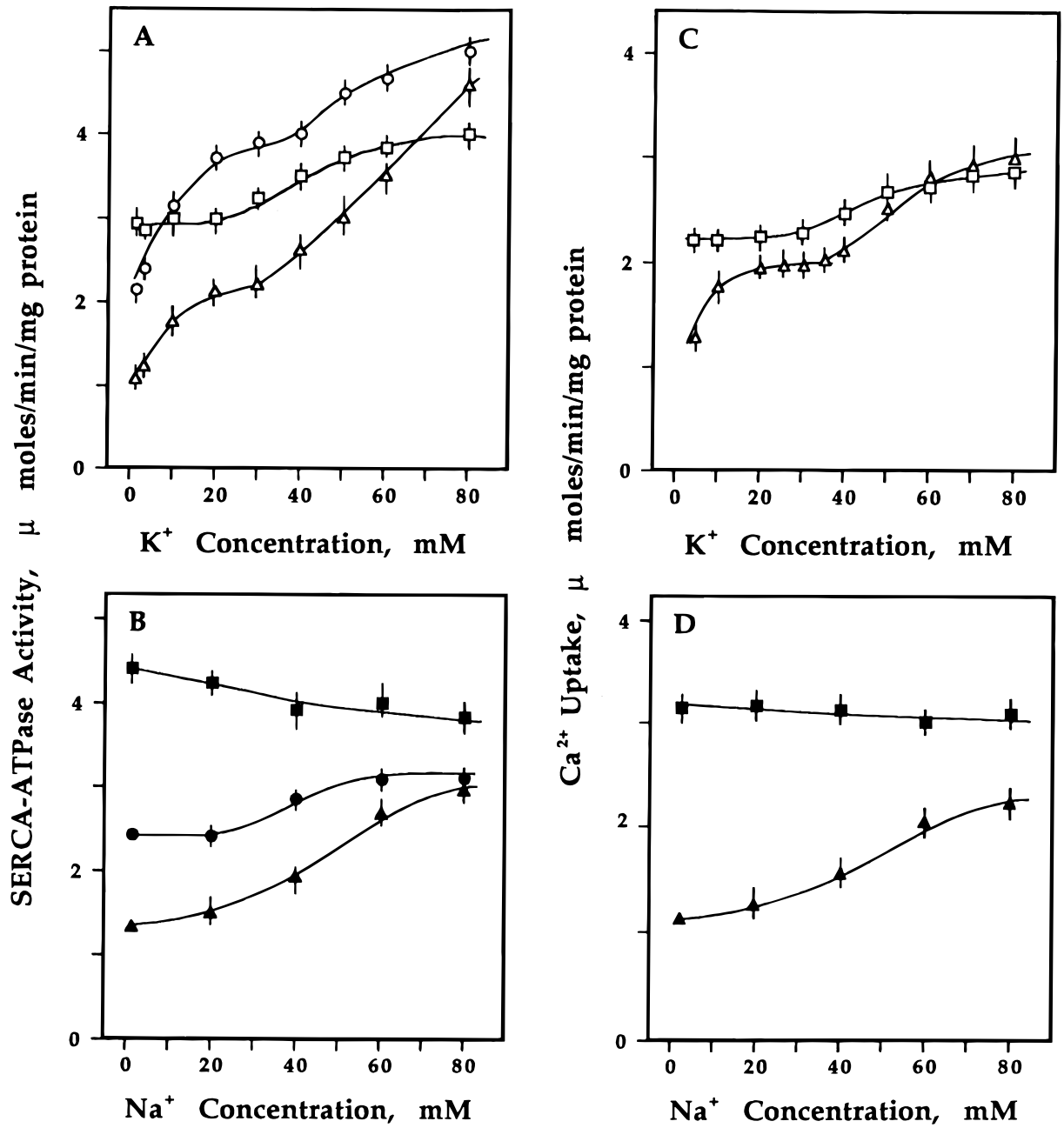

FIGURE 2: Stimulatory effects of Na^+ and K^+ on the thapsigargin-inhibitable and Ca^{2+}-sensitive Ca^{2+}-ATPase (SERCA-ATPase) and Ca^{2+}-transport activities. The total ionic strength was kept constant at 180 mM with 100 mM KCl (or NaCl) and 80 mM (NaCl (or KCl) plus choline chloride (Ch-Cl) (Jewell & Lingrel, 1991). (A) K^+- and (B) Na^+-dependent activity of SERCA-ATPase were measured in the chicken SR membrane vesicles in the presence of 2 μM A23187. SERCA-ATPase activity was measured at 5 μM free Ca^{2+} and different concentrations of Na^+ and/or K^+. (C) K^+- and (D) Na^+-dependent activity of Ca^{2+}-transport were measured in the chicken SR membrane vesicles at 5 μM free Ca^{2+} and different concentrations of Na^+ and/or K^+. All curves were fitted by eye using the data (mean ± standard deviation) obtained from six to eight independent measurements. Addition of 4 mM ouabain (Na^+,K^+-ATPase inhibitor) to the assay systems did not influence the effects of Na^+ and K^+ (data not shown): (○) [KCl] + [Ch-Cl] = 100 mM, (△) [KCl] + [Ch-Cl] = 180 mM, (□) [KCl] + [Ch-Cl] + 100 mM NaCl = 180 mM, (●) [NaCl] + [Ch-Cl] = 100 mM, (▲) [NaCl] + [Ch-Cl] = 180 mM, and (■) [NaCl] + [Ch-Cl] + 100 mM KCl = 180 mM.

of control mouse L cells was regulated by K^+ and Na^+, although the level of SERCA-ATPase activity is much lower than that in the transfected cells. As reported previously, the activity level of the mouse endogenous SERCA-ATPase was not changed by transfection with unrelated cDNAs, such as those encoding the wild-type and mutated Na^+,K^+-ATPase subunits (Ishii & Takeyasu, 1993; Ishii et al., 1994)

The simplest interpretation of these results is that the SR Ca^{2+}-ATPase possesses two different population of K^+-binding sites, one of which (the higher-affinity K^+ site) can also bind Na^+. This population of K^+/Na^+-binding sites possesses a higher affinity for K^+ than for Na^+. To search for the possible sites, we have chosen to utilize a set of chicken SERCA/Na^+,K^+-ATPase chimeras (Ishii & Takeyasu, 1993; Ishii et al., 1994). In these chimeras, parts of the wild-type SERCA1-ATPase (CCC) were replaced with the corresponding portions of the wild-type Na^+,K^+-ATPase α1 subunit (NNN) as follows. In chimera CNC, the large cytoplasmic phosphorylation domain (Gly354−Lys712) was replaced; in chimera [n/c]CC, the amino-terminal domain (Met1−Asp162) was replaced, and in chimera CC[c/n], the carboxy-terminal domain (Ser830-COOH) was replaced.

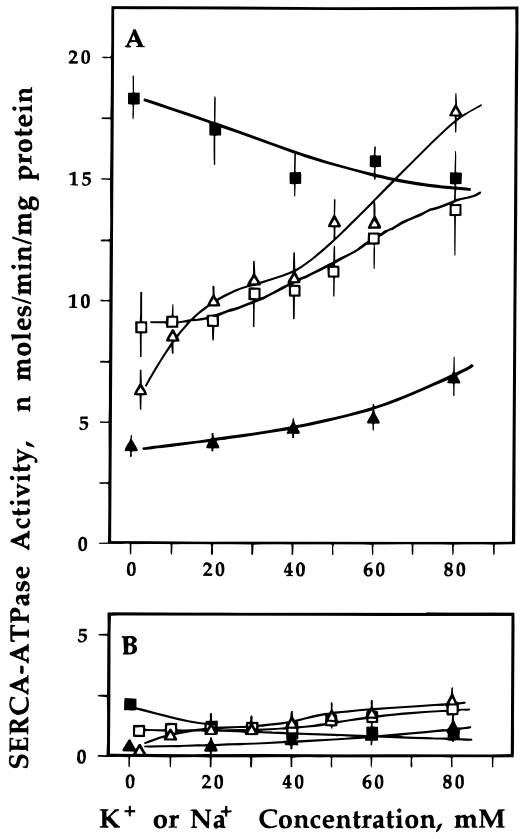

FIGURE 3: Stimulatory effects of Na^+ and K^+ on the thapsigargin-inhibitable and Ca^{2+}-sensitive Ca^{2+}-ATPase (SERCA-ATPase) activity in crude membranes of mouse L cells before (B) and after (A) transfection with cDNAs encoding the chicken SERCA1, CCC. SERCA-ATPase activity was measured at 5 μM free Ca^{2+} and different concentrations of Na^+ and/or K^+ ions. The total ionic strength was kept constant at 180 mM as it was in Figure 2. All curves were fitted by eye using the data (mean ± standard deviation) obtained from four to six independent measurements. Addition of 4 mM ouabain (Na^+,K^+-ATPase inhibitor) to the assay systems did not influence the effects of Na^+ and K^+ (data not shown): (△) [KCl] + [Ch-Cl] = 180 mM, (□) [KCl] + [Ch-Cl] + 100 mM NaCl = 180 mM (▲) [NaCl] + [Ch-Cl] = 180 mM, and (■) [NaCl] + [Ch-Cl] + 100 mM KCl = 180 mM. It should be noted that the SERCA-ATPase activity at a few millimolar K (in the presence of 100 mM Na) of overexpressed proteins was higher than that in the absence of Na (A). On the other hand, the SERCA-ATPase activity at 80 mM K (in the presence of 100 mM Na) was lower than that in the absence of Na (A). These were true for the SERCA-ATPase activity of the SR membrane (Figure 2A). However, the crossover points of these two curves between the SR ATPase and the overexpressed SERCA-ATPase were somewhat different, and the overexpressed SERCA-ATPase activities at 10−50 mM K (in the presence of 100 mM Na) were slightly lower than those in the absence of Na (cf. Figure 2 and this figure). The possible reasons for this include the following. (i) SR-associated proteins (which may be different from those associated with ER of tissue cultured cells) might influence the SERCA-ATPase activity. (ii) The difference in lipid compositions of SR and ER might affect the ATPase activity.

Each chimera was stably expressed in mouse L cells, and all were found to show thapsigargin-sensitive Ca^{2+}-ATPase activity (i.e., SERCA-ATPase, but not ouabain-sensitive Na^+/K^+-ATPase) with very similar ED_{50} values of 1−5 μM for Ca^{2+} activation and IC_{50} values of ∼1 nM for thapsigargin inhibition (Ishii & Takeyasu, 1993; Ishii et al., 1994). Using these chimeric molecules, the effects of K^+ and Na^+ on their SERCA-ATPase activities were further examined.

Search for the Low-Affinity K^+ Site. In strong contrast to the transfected chicken SERCA1 (CCC) (Figure 3A) and the native chicken SERCA-ATPase in SR membranes (Figure 2), the SERCA-ATPase activity of CC[c/n] was regulated in a single-step fashion by K^+ with an ED_{50} value of ∼20 mM and in a single-step fashion by Na^+ (panels A and B of Figure 4). These results suggest that the carboxy-terminal 180 amino acids of the SERCA1 include the K^+-sensitive region with lower affinity, and that a substitution of this region with 161 amino acids of the Na^+/K^+-ATPase α1 subunit apparently removes this low-affinity activation by K^+. If this is the case, chimera CC[c/n] would possess only the high-affinity K^+ site that can be blocked by Na^+. Indeed, the SERCA-ATPase activity of CC[c/n] in the presence of 100 mM Na^+ was stimulated by neither low nor high concentrations of K^+ (panels C and D of Figure 4).

To identify more precisely the carboxy-terminal regions of the Na^+,K^+-ATPase and/or the SERCA-ATPase that are responsible for the low-affinity activation by K^+, we analyzed an additional set of chimeras (CC[c/n/c] and CC[c/c/n]) together with CCC and CC[c/n/n] (originally named CC[c/n]). The schematic structures of CC[c/n/c] (26 amino acids of the α subunit), CC[c/c/n] (103 amino acids of the Na^+,K^+-ATPase α subunit), and CC[c/n/n] (161 amino acids of the α subunit) are depicted in Figure 5. As shown in Figure 6, CC[c/n/c] did undergo a second activation by K^+ at higher concentrations just like the wild-type SERCA1 (CCC), whereas CC[c/c/n] did not, indicating that the carboxy-terminal 97 amino acids (Thr898−Ala994) of the SERCA1 are responsible for the low-affinity activation by K^+.

What roles does the corresponding region (Phe920−Tyr1021) of the Na^+,K^+-ATPase α1 subunit play? When incorporated in the SERCA1, this region apparently removes the low-affinity activation by K^+ (Figure 6). However, the interpretation of this result needs careful consideration. At present, at least two different regions within the Na^+,K^+-ATPase α subunit have been shown to be important for K^+ sensitivity; one region includes Glu327 in M4 and Ser775 in M5 (Kuntzweiler et al., 1995; Arguello & Lingrel, 1995), and the other is the carboxy-terminal region including M7−M9 (Goldshleger et al., 1992; Capasso et al., 1992; Lutsenko & Kaplan, 1993). Therefore, it could be possible that the incorporation of this carboxy-terminal region (Phe920−Tyr1021) confers high-affinity K^+ activation on the SERCA1. Indeed, the ED_{50} value for K^+ activation was much lower (<10 mM) in CC[c/c/n] than in the wild-type CCC (∼20 mM) (Figure 6).

Functional Properties of Affinity-Purified Chimeras. Overall SERCA-ATPase activities in transfected mouse Ltk cells assessed in crude membrane fractions (Figures 3, 4, and 6) vary depending upon the expression levels of transgenes and the specific activities of the gene products. The levels of expression were assessed as described (Ishii et al., 1994) by immunoprecipitation of metabolically labeled materials using chicken SERCA1-specific monoclonal IgG 5D2 (Kaprielian & Fambrough, 1987) and were found to be within the range of 2−3-fold differences among the wild-type (CCC) and chimeric molecules (e.g., CC[c/n/n], CC[c/n/c], and CC[c/c/n]). The intrinsic SERCA-ATPase activities associated with chimeras were assessed by SERCA-ATPase assays after affinity purification (Figure 7). In this assay system, the expressed chimeric molecules were first solubilized with C12E8 as described (Lemas et al., 1992, 1994) and then bound to immunobeads coupled with IgG 5D2. The resultant affinity-purified materials on immunobeads were divided into

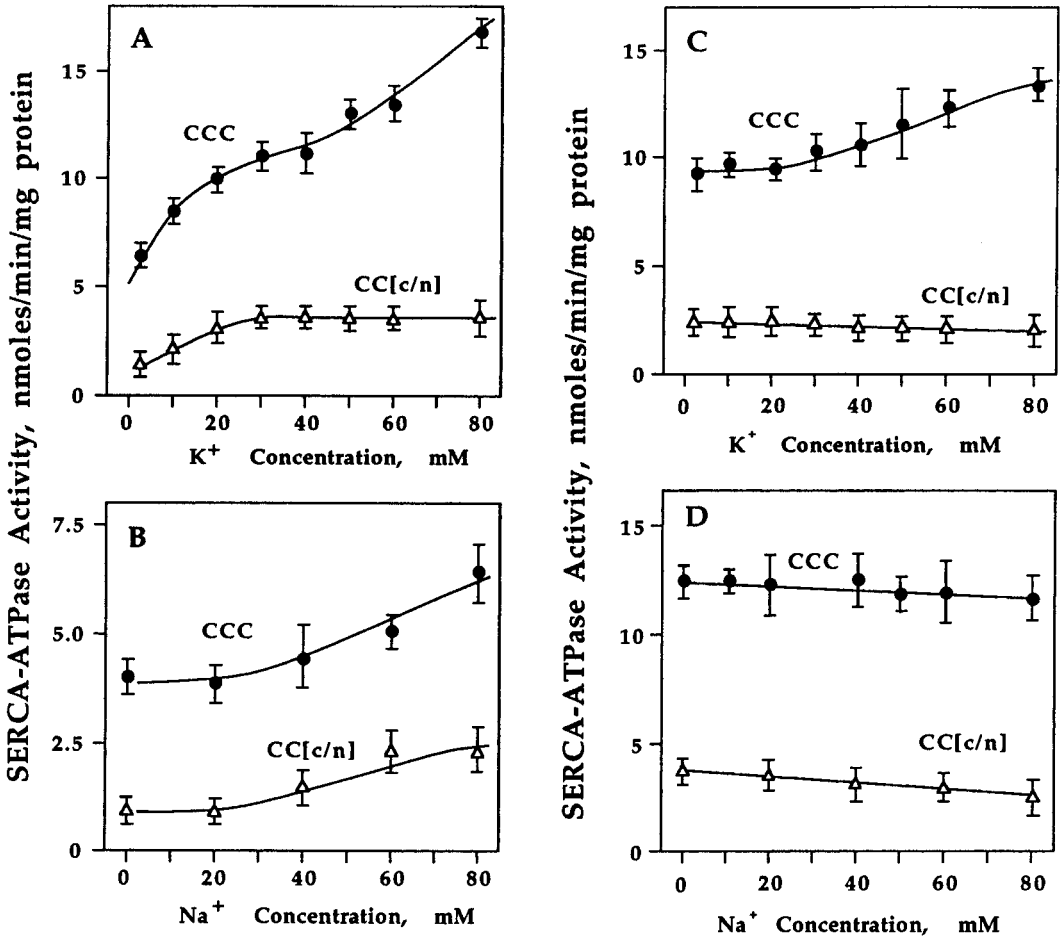

FIGURE 4: Carboxy-terminal regions of the SERCA-ATPase contain low-affinity sites for K^+. Thapsigargin- and Ca^{2+}-sensitive (SERCA-) ATPase activities of CCC and CC[c/n] were measured at 5 μM free Ca^{2+} and different concentrations of Na^+ and/or K^+ ions. All data points were obtained by subtracting the mouse endogenous SERCA-ATPase activity (Figure 3B) from the total SERCA-ATPase activity of transfected cells: (A) effect of K^+ in the absence of Na^+, and (B) effect of Na^+ in the absence of K^+, (C) effect of K^+ in the presence of 100 mM Na^+, and (D) effect of Na^+ in the presence of 100 mM K^+. In A and B, the total ionic strength was kept at 180 mM with KCl (or NaCl) plus choline chloride. In C and D, the total ionic strength was kept constant at 180 mM with 100 mM KCl (or NaCl) and 80 mM of NaCl (or KCl) plus choline chloride (Jewell & Lingrel, 1991). The SERCA-ATPase activity of CC[c/n] was not stimulated by K^+ in the presence of 100 mM Na^+ (C) but was stimulated in a single-step fashion in the absence of Na^+ (A). All curves were fitted by eye using the data (mean $\pm$ standard deviation) obtained from six to eight independent measurements. Addition of 4 mM ouabain (Na^+,K^+-ATPase inhibitor) to the assay systems did not influence the effects of Na^+ and K^+ (data not shown).

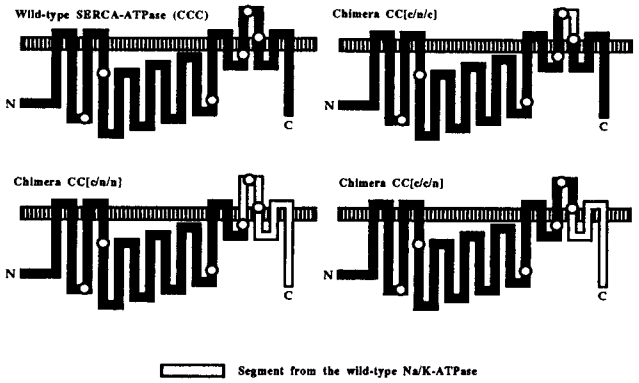

FIGURE 5: Schematic representation of a new set of chimeras: CCC, CC[c/n/n], CC[c/n/c], and CC[c/c/n]. (○) Possible chimeric junction.

two parts; the one part was used for estimation of the protein concentration on SDS−PAGE (Figure 7A), and the other part was used for kinetic analysis of Ca^{2+}- and thapsigargin-sensitive ATP hydrolysis in the presence of 4 mM ouabain (Figure 7B). This procedure allowed us to monitor the exogenous SERCA-ATPase activities of transfected mouse Ltk cells without contamination of endogenous mouse enzymes (e.g., SERCA-ATPase, Na^+,K^+-ATPase, and others) and enabled us to calculate chicken SERCA-ATPase activity per unit mass of purified protein.

The chicken wild-type SERCA1-ATPase (CCC) and chimera CC[c/c/n] exhibited the highest activities of 2.2 and 1.9 μmol min^{-1} (mg of protein)$^{-1}$, respectively, after being purified with IgG 5D2 (Figure 7B). These specific activities are comparable to the reported values for SERCA-ATPase activity in rabbit skeletal muscles (Sasaki et al., 1992). Chimera CC[c/n/n] showed an activity [0.7 μmol min^{-1} (mg of protein)$^{-1}$] $^1/_3$ of that of CCC, and chimera CC[c/n/c] had no detectable activity after IgG 5D2 purification (Figure 7B). This result with CC[c/n/c] is unexpected because all chimeras were active as SERCA-ATPase in crude membrane preparations (Figures 4 and 6). We have previously shown that CCC and CC[c/c/n] do not assemble with the Na^+,K^+-ATPase β subunit, but CC[c/n/n] and CC[c/n/c] do (Lemas et al., 1994). However, the affinity for this chimeric subunit assembly was found to be lower than that for wild-type assembly (Lemas et al., 1992, 1994). Thus, it could be possible that affinity purification with chicken SERCA1-specific IgG 5D2 preferentially enriches unassembled chimeras (i.e., free CC[c/n/n] and free CC[c/n/c] without the

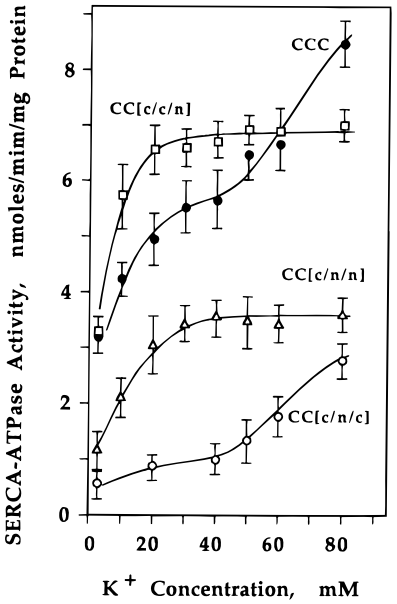

FIGURE 6: Existence of a distinct K^+-binding site at the carboxy-terminal region (~100 amino acids) of the Na/K- and the SERCA-ATPase. Stable cell lines that express CCC, CC[c/n/n], CC[c/n/c], and CC[c/c/n] (Figure 4) were used for the measurement of their thapsigargin- and Ca^{2+}-sensitive (SERCA-) ATPase activities as described (Ishii & Takeyasu, 1993). The SERCA-ATPase activities were measured at 5 μM free Ca^{2+} and different concentrations of K^+ in the absence of Na^+. The total ionic strength was kept at 180 mM with KCl plus choline chloride. Since the activity of the mouse endogenous SERCA-ATPase (Figure 3 in the original application) is very low in mouse L cells and remains unchanged after transfection (Ishii et al., 1994), the SERCA-ATPase activities intrinsic to the chicken chimeric molecules were calculated by subtracting the SERCA-ATPase activity of the mouse Ltkβ3 cells (that had been transfected with the chicken Na^+,K^+-ATPase β subunit) from the total SERCA-ATPase activity. The SERCA-ATPase activity of CCC is expressed as 50% of its real activity to fit in a comparable scale. All curves were fitted by eye to the data points (mean ± standard deviation) obtained from six separate measurements.

Na^+,K^+-ATPase β subunit) over assembled ones. Therefore, we examined the SERCA-ATPase activities of only chimeras that assembled with the Na^+,K^+-ATPase β subunit after affinity purification using chicken-specific anti-β subunit IgG 24. Since this procedure purified any materials (i.e., chicken chimeras as well as mouse endogenous Na^+,K^+-ATPase α subunits in this study) that can assemble with the Na^+,K^+-ATPase β subunit (Fambrough et al., 1994), the amount of purified chimeras was determined by Western blot analysis using chicken-specific monoclonal antibody, IgG 5D2, following SDS−PAGE (Figure 8A), and the intrinsic ATPase activity of these assembled chimeras was assessed by SERCA-ATPase assays in the presence of 4 mM ouabain that do not measure the activity associated with endogenous mouse Na^+,K^+-ATPase α subunits (Figure 8B). As a result, CC[c/n/c] and CC[c/n/n] clearly exhibited SERCA-ATPase activities nearly as high as those of the wild-type SERCA1 in the presence of the Na^+,K^+-ATPase β subunit.

Possible Location of the High Affinity K^+ Site. Chimera CC[c/n/n] (═CC[c/n]) lost its low-affinity K^+ site but still retained the high-affinity K^+ site (Figure 4A). These results suggest that the high-affinity site for K^+ might be localized in the regions of the SERCA-ATPase excluding Ser830-COOH. Therefore, effects of K^+ as well as Na^+ on the SERCA-ATPase activities of [n/c]CC and CNC were further

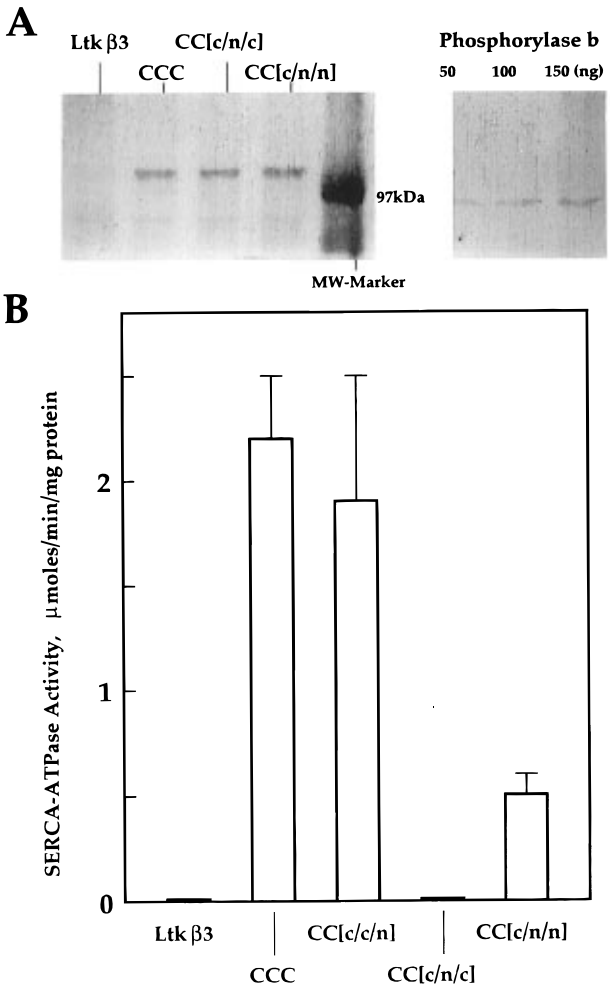

FIGURE 7: Characterization of affinity-purified chicken SERCA-ATPase and its chimeric derivatives. The wild-type SERCA-ATPase (CCC) and its carboxy-terminal chimeras (CC[c/c/n], CC[c/n/c], and CC[c/n/n]) were purified by using immunobeads coupled with IgG 5D2 that recognizes the large cytosolic phosphorylation domain (-C-) (Kaprielian & Fambrough, 1987). The SERCA-ATPase activities of the purified materials attached to the immunobeads were measured (B), and the amount of the purified materials was determined (A) as described in Experimental Procedures. (A) An example for detection of chicken proteins expressed in mouse Ltkβ3 cells that express the chicken Na^+,K^+-ATPase β subunit (Takeyasu et al., 1987). The silver-stained bands (~100 kDa) were subjected to quantification of the materials that were used for SERCA-ATPase assays. (B) Comparison of SERCA-ATPase activities associated with the affinity-purified materials, showing that chimera CC[c/c/n] was as active as the wild type, CCC. However, the other chimera, CC[c/n/n], was less active, and chimera CC[c/n/c] had no activity. Note that the column for Ltkβ3 shows, for a comparison, that the absolute null activity associated with the immunobeads used for the Ltkβ3 extract. Bars represent standard deviations of at least four independent assays.

examined. As seen Figure 9, K^+ and Na^+ were found to exert one- and two-step stimulation, respectively, as observed for the endogenous mouse Ca^{2+}-ATPase, and also for the transfected chicken SERCA1 (CCC) and the native chicken SERCA-ATPase in SR membranes (Figure 2). When the SERCA-ATPase activities of [n/c]CC and CNC were measured in the presence of a high concentration of Na^+ (100 mM), the high-affinity effect of K^+, but not the low-affinity activation by K^+, was abolished (Figure 9C). These results suggest that the domains responsible for high-affinity stimulation by K^+ should reside within regions common to all the chimeric SERCA-ATPases, i.e., segments between

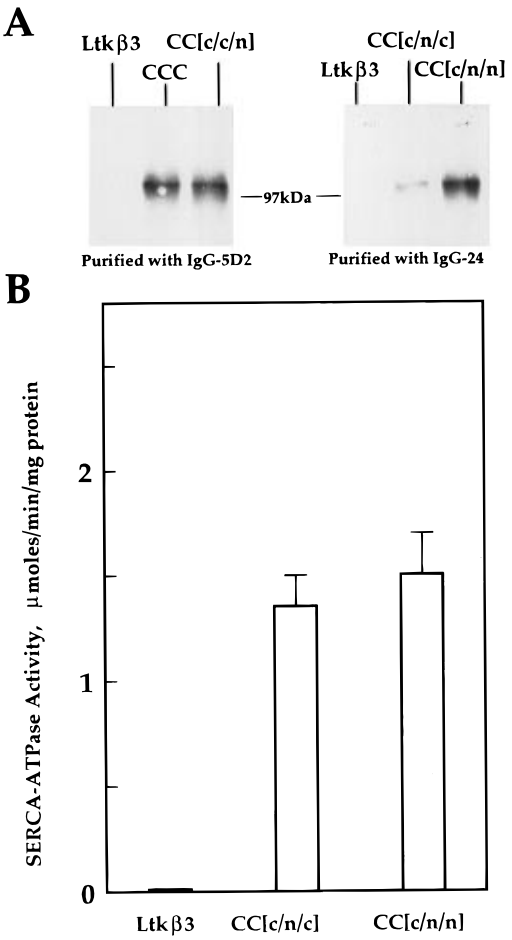

FIGURE 8: SERCA-ATPase activities of the chimeras, CC[c/n/c] and CC[c/n/n], assembled with the Na^+,K^+-ATPase β subunit. The chimeric molecules, CC[c/n/c] and CC[c/n/n], were purified by using immunobeads coupled with IgG 24 that recognizes the Asn894−Ala919 sequence [-/n/-] of the Na^+,K^+-ATPase α subunit (Lemas et al., 1994). The SERCA-ATPase activities of the purified materials attached to the immunobeads were measured in the presence of 4 mM ouabain (B), and the amount of the purified materials was determined (A) as described in Experimental Procedures. (A) Detection of chicken proteins expressed in mouse Ltkβ3 cells that express the chicken Na^+,K^+-ATPase β subunit (Takeyasu et al., 1987). An example of Western blot analysis detects the bands (∼100 kDa), which were then subjected to quantification of the materials that were used for SERCA-ATPase assays. (B) Comparison of SERCA-ATPase activities associated with the affinity-purified materials, showing that chimeras CC[c/n/n] and CC[c/n/c] were highly active SERCA-ATPases. Note that the column for Ltkβ3 shows, for a comparison, that the absolute null activity associated with the immunobeads used for the Ltkβ3 extract. Bars represent standard deviations of three independent assays.

Ile163 and Gly354 and/or between Lys712 and Ser830 of the SERCA1. Thus, the occupation of these regions by Na^+ is expected to prohibit additional K^+ binding to these regions, thus abolishing the high-affinity effect of K^+.

On the other hand, preoccupation of the high-affinity site by K^+ would be expected to block the Na^+ binding to this site. Indeed, the stimulatory effect of Na^+ was abolished in CNC when the SERCA-ATPase activity was measured in the presence of a high concentration of K^+ (100 mM) (Figure 9D). In [n/c]CC, due to the existence of a Na^+ sensor within the amino-terminal 69 amino acids of the Na^+,K^+-ATPase α subunit (16), the SERCA-ATPase activity of [n/c]CC was markedly enhanced in the presence of Na^+ (panels C and D of Figure 9).

DISCUSSION

Using chimeric ATPase molecules, we have previously demonstrated that the segments between Ile163 and Gly354 and between Lys712 and Ser830 of the SERCA1-ATPase are responsible for Ca^{2+}- and thapsigargin-sensitive ATPase activity, because all the chimeric molecules ([n/c]CC, CNC, and CC[c/n/n]) exhibited thapsigargin- and Ca^{2+}-sensitive ATPase activities similar to that of wild-type SERCA1, CCC (Ishii et al., 1994). The present study suggests that the same regions of the SERCA1-ATPase also include the sites for Na^+ and K^+. The carboxy-terminal regions of the SERCA1-ATPase contain an additional K^+-sensitive domain. The carboxy-terminal regions of the Na^+,K^+-ATPase α subunit are also suggested to contain a K^+-sensitive domain which is distinct from the assembly domain with the β subunit. These results together with other findings suggest a functional-domain model for the P-type ATPase (Figure 1). The biological significance of the K^+ sensitivity of the SERCA-ATPase is not yet known. However, the existence of the effect, demonstrated in the present study, may have implications for the mechanism of ion transport in P-type ATPases in general, and this will be explored further in the future by looking at the K^+ sensitivity of the phosphorylated intermediate and whether K^+ can become occluded in SERCA pumps.

The SERCA-ATPase Activity Can Be Regulated by Two Distinct K^+ Bindings. The two-step activation of SERCA-ATPase by K^+ (Figure 2) has not been described before in the literature, although some kinetic analyses have been conducted; i.e., the K_m values for K^+ activation of several partial reactions of the SERCA-ATPase have been reported to be 15−50 mM (Shigekawa et al., 1978). Only a very few studies have explored the mechanisms of SERCA-ATPase activation by monovalent cations (such as K^+) and reported that the stimulatory effect of K^+ on the rabbit SERCA-ATPase activity was monophasic when K^+ was substituted with Li^+ (Shigekawa et al., 1978) instead of choline$^+$ (this study). There are alternative mechanisms that might explain the effects of these monovalent cations. (i) There is a species-specific difference, and (ii) choline$^+$ and Li^+ behave differently. We have tested these two possibilities using SR membranes form rabbit and found that the activities of the rabbit SERCA-ATPase are regulated by different ions basically in the same way as in the chicken SERCA1 shown in Figure 2. Namely, choline$^+$ does not stimulate the SERCA-ATPase activity; however, Li^+ behaves like K^+ (unpublished observations). Therefore, we conclude that the SERCA-ATPase possesses two different populations of K^+-binding sites in general.

Since all the chimeric molecules, [n/c]CC, CNC, and CC[c/n] (=CC[c/n/n]), exhibited high sensitivity for K^+, and since the high-affinity stimulatory effect of K^+ was blocked by Na^+, Na^+ and K^+ should compete for their effect within a region common to all the chimeric SERCA-ATPases, i.e., the region between Ile163 and Gly354 and/or between Lys712 and Ser830 (containing the transmembrane segments M3 and M4 and M5 and M6, respectively). Since CC[c/n] (=CC[c/n/n]) shows a one-step, high-affinity stimulation by K^+ while lacking the low-affinity activation by K^+, the carboxy-terminal 180 amino acids of the SERCA1 apparently include the K^+-sensitive region with a lower affinity. Substitution of this region with 161 amino acids of the

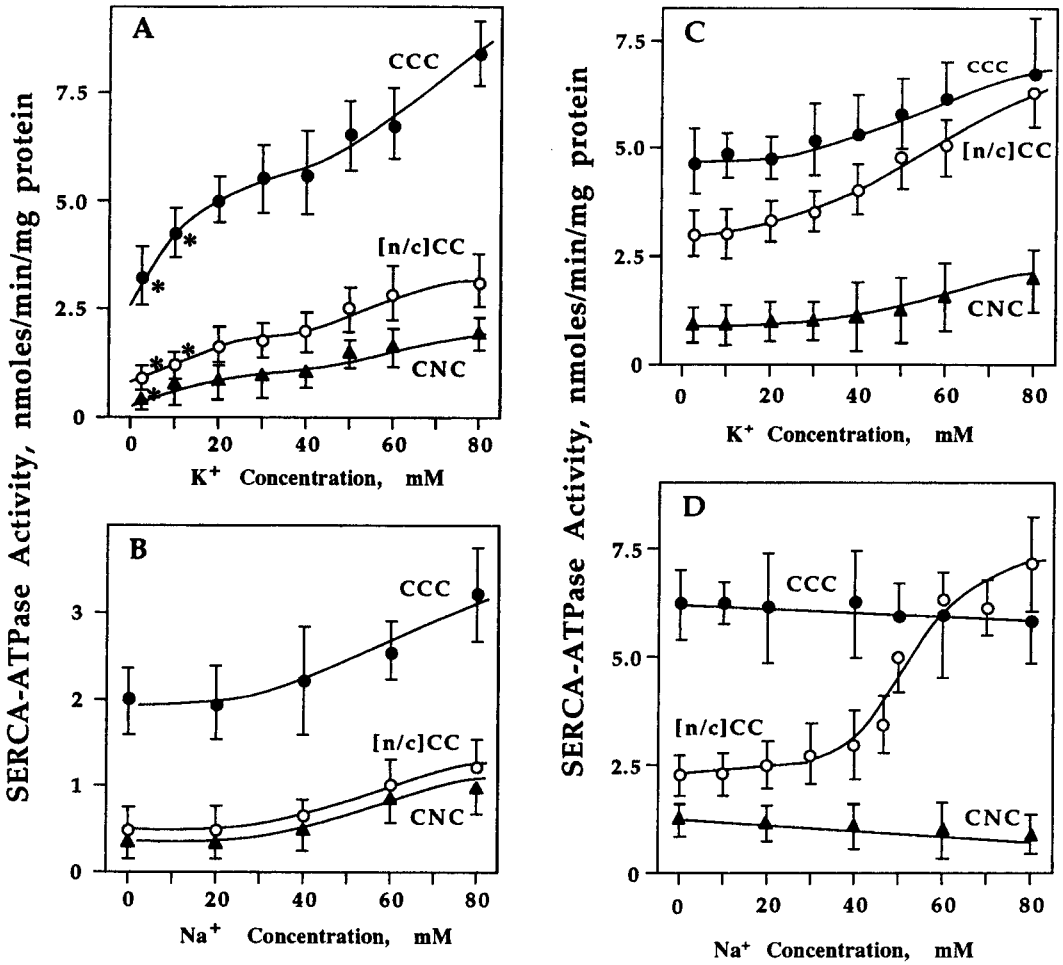

FIGURE 9: Search for high-affinity sites for K^+. Thapsigargin- and Ca^{2+}-sensitive (SERCA-) ATPase activities of CCC, [n/c]CC, and CNC were measured at 5 μM free Ca^{2+} and different concentrations of Na^+ and/or K^+. All data points were obtained by subtracting the mouse endogenous SERCA-ATPase activity (Figure 3B) from the total SERCA-ATPase activity of transfected cells: (A) effect of K^+ in the absence of Na^+, (B) effect of Na^+ in the absence of K^+, (C) effect of K^+ in the presence of 100 mM Na^+, and (D) effect of Na^+ in the presence of 100 mM K^+. In A and B, the total ionic strength was kept at 180 mM with KCl (or NaCl) plus choline chloride. An asterisk (*) represents the degree of significance of differences ($p < 0.05$) compared to the activity at 40 mM K^+ or Na^+. In C and D, the total ionic strength was kept constant at 180 mM with 100 mM KCl (or NaCl) and 80 mM NaCl (or KCl) plus choline chloride (Jewell & Lingrel, 1991). The SERCA-ATPase activities of all constructs tested here were stimulated in a two-step fashion by K^+ in the absence of 100 mM Na^+ (A) but stimulated in a single-step fashion in the presence of Na^+ (C). The SERCA-ATPase activity of [n/c]CC was markedly stimulated by Na^+ in the presence of 100 mM K^+, due to the presence of the Na^+-sensor [[n/] (Ishii et al., 1994)] (D) but not in the absence of K^+ (B). Addition of 4 mM ouabain to the assay systems did not influence the effects of Na^+/K^+ on the CCC and CNC activities (data not shown) but did inhibit the SERCA-ATPase activity of [n/c]CC which is known as a Na^+-, ouabain-, Ca^{2+}-, and thapsigargin-sensitive ATPase (Ishii et al., 1994). All curves were fitted by eye to the data points (mean ± standard deviation) obtained from four to six separate measurements.

Na^+,K^+-ATPase α1 subunit apparently removes the lower-affinity activation of SERCA-ATPase activity by K^+. Thus, in conclusion, the SERCA-ATPase possesses at least two different sites for activation by K^+: one at the carboxy terminus with a lower affinity and the other within M3 and M4 and/or M5 and M6 regions with a higher affinity.

It is important to note that K^+ binding to the high-affinity sites can be competed for by Na^+ (Figures 2 and 3). Recent molecular biological analyses employing site-directed mutagenesis have suggested that charged amino acids within the transmembrane regions, M4−M6, of the SERCA-ATPase are critical for Ca^{2+}-sensitive ATPase activity (Andersen & Vilsen, 1992, 1994, 1995; Clarke et al., 1989). Furthermore, the most recent similar experiments on the sheep Na^+,K^+-ATPase α subunit have shown that Glu327 in M4 and Ser775 in M5 control the K^+ sensitivity of the enzyme activity (Kuntzweiler et al., 1995; Arguello & Lingrel, 1995). These membranous regions might be critical for the ion dependence of all P-type ATPases in general.

The Carboxy-Terminal Region of the Na^+,K^+-ATPase α1 Subunit Contains Distinct Domains for Assembly with the β Subunit (Asn894−Ala919) and for K^+ Activation (Phe920−Tyr1021). Chemical modification of Glu in M9 of the Na^+,K^+-ATPase α subunit with *N,N′*-dicyclohexylcarbodiimide (DCCD) has suggested the importance of this residue in ^{86}Rb occlusion (Goldshleger et al., 1992). Although the precise interpretation of this type of result is difficult, it seems that the carboxy terminal regions may be involved in determining ion binding. Indeed, a 19 kDa tryptic fragment of the carboxy terminus of the Na^+,K^+-ATPase α1 subunit binds $^{86}Rb^+$ with high affinity (Goldshleger et al., 1992; Capasso et al., 1992). This 19 kDa fragment includes the 161 amino acids of sodium pump present in chimera CC-[c/n/n]. This is consistent with the idea that the apparent loss of the low-affinity stimulation by K^+ seen in CC[c/n/n] is due to the replacement of this responsible domain, [c/c/c], with the high-affinity domain of the Na^+,K^+-ATPase α subunit, [c/n/n].

A line of evidence suggests that assembly of the Na^+,K^+-ATPase α subunit with different types of β subunit isoforms can modulate the effects of K^+ on the α subunit (Eakle et al., 1992, 1994; Jaisser et al., 1992; Jaunin et al., 1993). A recent report has indicated that the $^{86}Rb^+$-occlusion ability of the 19 kDa fragment of the α-subunit can be affected by the integrity of the β subunit (Lutsenko & Kaplan, 1993). The extracellular 26 amino acids at the carboxy terminus (within the 19 kDa fragment) of the α subunit have been identified as the major domain for α/β subunit assembly (Lemas et al., 1994). Therefore, it was critical to determine whether the K^+-sensitive region is the same as the assembly domain. Our experiments demonstrated that the sites for the K^+ stimulation and the assembly with the β subunit are distinct (Figure 6) and that the K^+-sensitive region [a stretch of 102 amino acids (Phe920−Tyr1021)] resides adjacent to the assembly domain (Asn894−Ala919) within the carboxy-terminal region of the Na^+,K^+-ATPase α subunit.

It is intriguing that chimera CC[c/n/c] requires the Na^+,K^+-ATPase β subunit for its SERCA-ATPase activity (Figures 7 and 8). Assembly with the β subunit is required for the Na^+,K^+-ATPase α subunit for stabilization and maturation in ER membranes, and transportation to the plasma membrane (Fambrough et al., 1994; Geering, 1991; Takeyasu et al., 1989). A very limited region (26 amino acid residues) of the catalytic α subunit might be sufficient to assure the structural (and thus functional) integrity of chimera CC[c/n/c] through assembly with the Na^+,K^+-ATPase β subunit. Further studies are required to determine what roles the Na^+,K^+-ATPase β subunit plays.

ACKNOWLEDGMENT

This work was completed during the tenure of K.T. as an Established Investigator of the American Heart Association (1992−1996).

REFERENCES

Albers, R. W. (1967) *Annu. Rev. Biochem. 36*, 727−756.
Andersen, J. P., & Vilsen, B. (1992) *J. Biol. Chem. 267*, 19383−19387.
Andersen, J. P., & Vilsen, B. (1994) *J. Biol. Chem. 269*, 15931−15936.
Andersen, J. P., & Vilsen, B. (1995) *FEBS Lett. 359*, 101−106.
Arguello, J. M., & Lingrel, J. B. (1995) *J. Biol. Chem. 270*, 22764−22771.
Bodemann, H. H., & Hoffman, J. F. (1976) *J. Gen. Physiol. 67*, 497−525.
Capasso, J. M., Hoving, S., Tal, D. M., Goldshleger, R., & Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1150−1158.
Clarke, D. M., Loo, T. W., Inesi, G., & MacLennan, D. H. (1989) *Nature 339*, 476−478.
Dean, R. B. (1941) *Biol. Symp. 3*, 331−348.
De Meis, L., & Vianna, A. (1979) *Annu. Rev. Biochem. 48*, 275−292.
Eakle, K. A., Kim, K. S., Kabalin, M. A., & Farley, R. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2834−2838.
Eakle, K. A., Kabalin, M. A., Wang, S.-G., & Farley, R. A. (1994) *J. Biol. Chem. 269*, 6550−6557.
Ebashi, S., & Lipman, F. (1962) *J. Cell Biol. 14*, 389−400.
Fambrough, D. M., & Bayne, E. K. (1983) *J. Biol. Chem. 258*, 3926−3935.
Fambrough, D. M., Lemas, M. V., Hamrick, M., Emerick, M., Renaud, K. J., Inman, E. M., Hwang, B., & Takeyasu, K. (1994) *Am. J. Physiol. 266 (Cell Physiol. 35)*, C579−C589.
Geering, K. (1991) *FEBS Lett. 285*, 189−193.
Goldshleger, R., Tal, D. M., Moorman, J., Stein, W. D., & Karlish, S. J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6911−6915.
Hansen, O. (1984) *Pharmacol. Rev. 36*, 143−163.
Hasselbach, W., & Makinose, M. (1961) *Biochem. Z. 333*, 518−528.
Horiuchi, K. (1986) *J. Physiol. 373*, 1−23.
Inesi, G., & De Meis, L. (1989) *J. Biol. Chem. 264*, 5929−5936.
Ishii, T., & Takeyasu, K. (1993) *Proc. Natl. Acad. Sci. USA 90*, 8881−8885.
Ishii, T., Lemas, M. V., & Takeyasu, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6103−6107.
Jaisser, F., Canessa, C. M., Horisberger, J.-D., & Rossier, B. C. (1992) *J. Biol. Chem. 267*, 16895−16903.
Jaunin, P., Jaisser, F., Beggah, A. T., Takeyasu, K., Mangeat, P., Rossier, B. C., Horisberger, J.-D., & Geering, K. (1993) *J. Cell Biol. 123*, 1751−1759.
Jewell, E. A., & Lingrel, J. B. (1991) *J. Biol. Chem. 266*, 16925−16930.
Jorgensen, P. L., & Andersen, J. P. (1988) *J. Membr. Biol. 103*, 95−120.
Kaprielian, Z., & Fambrough, D. M. (1987) *Dev. Biol. 124*, 490−503.
Karin, N. J., Kaprielian, Z., & Fambrough, D. M. (1989) *Mol. Cell. Biol. 9*, 1978−1986.
Kijima, Y., Ogunbunmi, E., & Fleischer, S. (1991) *J. Biol. Chem. 266*, 22912−22918.
Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., & Lingrel, J. B. (1995) *J. Biol. Chem. 270*, 2993−3000.
Lemas, M. V., Takeyasu, K., & Fambrough, D. M. (1992) *J. Biol. Chem. 267*, 20987−20991.
Lemas, M. V., Hamrick, M., Takeyasu, K., & Fambrough, D. M. (1994) *J. Biol. Chem. 269*, 8255−8259.
Lutsenko, S., & Kaplan, J. H. (1993) *Biochemistry 32*, 6737−6743.
Lytton, J., Westlin, M., & Hanley, M. R. (1991) *J. Biol. Chem. 266*, 17067−17071.
Matthews, I., Sharma, R. P., Lee, A. G., & East, J. M. (1990) *J. Biol. Chem. 265*, 18737−18740.
Meisnner, G. (1975) *Biochim. Biophys. Acta 241*, 365−378.
Paul, J. K., Nettikadan, S. R., Ganjeizadeh, M., Yamaguchi, M., & Takeyasu, K. (1994) *FEBS Lett. 346*, 289−294.
Post, R. L., & Jolly, P. C. (1957) *Biochim. Biophys. Acta 25*, 118−128.
Post, R. L., Hegyvary, C., & Kume, S. (1972) *J. Biol. Chem. 247*, 6530−5640.
Sagara, Y., Fernandez-Belda, F., Meise, L. D., & Inesi, G. (1992) *J. Biol. Chem. 267*, 12606−12613.
Sasaki, T., Inui, M., Kimura, Y., Kuzuya, T., & Tada, M. (1992) *J. Biol. Chem. 267*, 1674−1679.
Shigekawa, M., Dougherty, J. P., & Katz, A. M. (1978) *J. Biol. Chem. 253*, 1442−1450.
Skou, J. C. (1957) *Biochim. Biophys. Acta 23*, 394−401.
Stults, N. L., Asta, L. M., & Lee, Y. C. (1989) *Anal. Biochem. 180*, 114−119.
Takeyasu, K., Tamkun, M. M., Siegel, N., & Fambrough, D. M. (1987) *J. Biol. Chem. 262*, 10733−10740.
Takeyasu, K., Tamkun, M. M., Renaud, K. J., & Fambrough, D. M. (1988) *J. Biol. Chem. 263*, 4347−4354.
Takeyasu, K., Renaud, K. J., Taormino, J., Wolitzky, B. A., Barnstein, A., Tamkun, M. M., & Fambrough, D. M. (1989) *Curr. Top. Membr. and Transp. 34*, 143−164.
Zhang, Z., Sumbilla, C., Lewis, D., Summers, S., Klein, M. G., & Inesi, G. (1995) *J. Biol. Chem. 270*, 16283−16290.

BI960644C