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Binding domain of oligomycin on Na^+,K^+-ATPase

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Abstract

Oligomycin inhibits Na^+,K^+-ATPase activity by stabilizing the Na^+ occlusion but not the K^+ occlusion. To locate the binding domain of oligomycin on Na^+,K^+-ATPase, the tryptic-digestion profile of Na^+,K^+-ATPase was compared with the profile of Na^+ occlusion within the digested Na^+,K^+-ATPase in the presence of oligomycin. The Na^+ occlusion profile is responsible for the digestion profile of the α-subunit, which is the catalytic subunit of the ATPase. The effect of oligomycin on chimeric Ca^{2+}-ATPase activity was examined. The chimera used, in which the 163 N-terminal amino acids of chicken sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 were replaced with the 200 N-terminal amino acids of the chicken Na^+,K^+-ATPase α1-subunit, partially retains the Na^+-dependent characteristics of Na^+,K^+-ATPase, because the chimeric Ca^{2+}-ATPase activity is activated by Na^+ but inhibited by ouabain, a specific inhibitor of Na^+,K^+-ATPase (Ishii, T., Lemas, M.V., Takeyasu, K., 1994, Proc. Natl. Acad. Sci. U. S. A., 91, 6103–6107). Oligomycin depressed the activation by Na^+ of the chimeric Ca^{2+}-ATPase activity. These findings suggest that the 200 N-terminal amino acids of the Na^+,K^+-ATPase α-subunit include a binding domain for oligomycin.

Keywords: Oligomycin; Na^+,K^+-ATPase; Ca^{2+}-ATPase; Na^+ occlusion; Chimeric ATPase

1. Introduction

Na^+,K^+-ATPase, one of the P-type ATPases, actively transports Na^+ across the plasma membranes from the inside to the outside of the cell, and K^+ in the reverse direction, using ATP as the driving force (Lingrel and Kuntzweiler, 1994). Na^+,K^+-ATPase is composed of α- and β-subunits in an equimolar ratio. The α-subunit, a catalytic protein, has 10 putative transmembrane segments (M1–M10). The β-subunit, a glycosylated protein, has one transmembrane segment. The chemical and electrical gradients formed by the ATPase are the essential driving forces for the Na^+-dependent system of amino acids and glucose absorption in the small intestine and are essential to the maintenance of the resting potential in nerve and muscle. This ATPase is also a receptor for cardiac steroids, e.g., ouabain (Lingrel and Kuntzweiler, 1994). Due to its physiological and pharmacological significance, numerous investigators have studied the reaction mechanism of Na^+,K^+-ATPase.

Oligomycin is a macrolide antibiotic that is well known as an inhibitor of the mitochondrial H^+-ATPase. This antibiotic also inhibits Na^+,K^+-ATPase activity (Pedersen and Carafoli, 1987; Homareda, 1999). Because it does not inhibit the formation of $NaE_1 \sim P$ (phosphorylated intermediate with high energy) and the dephosphorylation of E_2-P (phosphorylated intermediate with low energy), which are, respectively, the partial reactions in the complete ATP hydrolysis reaction mechanism of Na^+,K^+-ATPase, oligomycin has been believed to inhibit the sodium releasing step from $NaE_1 \sim P$ to $Na^+ + E_2$-P (Schuurmans Stekhoven and Bonting, 1981). Homareda et al. (Matsui and Homareda, 1982; Homareda and Matsui, 1982; Matsui and Homareda, 1988; Arato-Oshima et al., 1996) demonstrated that oligomycin stimulated Na^+, but not K^+, binding to the nonphosphorylated Na^+,K^+-ATPases. Esmann and Skou (1985) proposed that oligomycin stabilizes Na^+ occlusion within the ATPase molecule. At present, oligomycin is extensively used as a stabilizer of Na^+

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occlusion. The binding domain of oligomycin on Na^+,K^+-ATPase, however, is not yet identified. We attempted to identify it in this study. First, we examined Na^+ occlusion within the tryptic-digested ATPases in the presence of oligomycin to distinguish which of the α- and β-subunits has the binding domain. The Na^+ occlusion capacity was decreased with degradation of the α-subunit. Second, we examined the inhibitory effect of oligomycin on the ATPase activity of the chimeric Ca^{2+}-ATPase. [n/c]CC, the chimeric ATPase, in which the Met^1 to Asp^{162} sequence of chicken sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA-ATPase) is replaced by the Met^1 to Asp^{200} sequence of chicken Na^+,K^+-ATPase α1-subunit, exhibits SERCA-ATPase activity. This activity is stimulated by Na^+ but inhibited by ouabain and thapsigargin, which are specific inhibitors of Na^+,K^+-ATPase and SERCA-ATPase, respectively (Ishii and Takeyasu, 1993; Ishii et al., 1994). Oligomycin inhibited the Na^+-activated SERCA-ATPase activity of [n/c]CC. Based on the present data, we propose that the 200 N-terminal amino acids of the α-subunit include a binding domain for oligomycin.

2. Materials and methods

2.1. Materials

Oligomycin B, thapsigargin and ATP were purchased from Sigma (St. Louis, MO, USA). $^{22}NaCl$ and reagents for cell culture were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England) and Gibco BRL (Rockville, MD, USA), respectively. Second antibodies conjugated to peroxidase and staining kits for immunoblotting were purchased from Wako (Tokyo, Japan) and Vector Laboratories (Burlingame, CA, USA). Other reagents were purchased from Wako.

2.2. Tryptic digestion of Na^+,K^+-ATPase

Na^+,K^+-ATPase purified from canine kidney (Hayashi et al., 1977) was digested with trypsin for 0, 10 s, 1, 6 and 60 min in the presence of 10 mM RbCl, 1 mM ethylenediaminetetra acetic acid (EDTA) and 10 mM Tris–HCl (pH 7.4 at 37°C) according to the method of Capasso et al. (1992). To remove RbCl, the digested ATPase was incubated with 25 mM imidazole–HCl (pH 7.4 at 25°C) and 1 mM EDTA at 37°C for 30 min and then centrifuged at 40,000 rpm for 30 min (70.1 Ti rotor; Beckman, Fullerton, CA, USA) at 2°C. The precipitates were suspended in 1 mM EDTA for the measurement of $^{22}Na^+$ binding and ATPase activity or dissolved in 100 μl of 5% sodium dodecyl sulfate (SDS) for immunoblotting. The peptide fragments released into the supernatants were precipitated with trichloroacetic acid, washed with water and dissolved in 50 μl of 5% SDS.

2.3. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Schägger and Von Jagow (1987) with a 12% separating gel without glycerol.

2.4. Immunoblotting

Intact Na^+,K^+-ATPase or its trypsin-digested fragments were transferred from the SDS-PAGE gel to a nitrocellulose membrane, using a transblotting apparatus. The membrane was incubated with antiserum raised against α1- or β1-subunit (Homareda et al., 1993) or the 815–828 sequence of the α1-subunit, which links to M6 (gift of Prof. J.V. Møller). After incubation for 1 h at 37°C, the membranes were washed with 150 mM NaCl, 20 mM Tris–HCl (pH 7.4 at 37°C) (TBS) with 0.05% Tween 20, then TBS without the detergent and incubated with a second antibody conjugated to peroxidase for 1 h at 37°C. After washing, the antibody-bound peptides were visualized using a staining kit. An image analyzer (Model TIAS-200; ACI JAPAN, Tokyo, Japan) analyzed the staining patterns.

2.5. Na^+ binding

Reaction mixtures (0.1 ml) containing 30 μg intact or digested ATPase, 50 mM triethanolamine–HCl (pH 7.4 at 0°C), 100 μM $^{22}NaCl$ (specific radioactivity, 222 kBq/μmol), 2% ethyl alcohol and with or without 45 μM oligomycin B were prepared on ice. They were immediately centrifuged at 100,000 rpm for 20 min at 2°C (TLA 100.2 rotor; Beckman). The supernatants were aspirated off, and the inside walls of the tubes were carefully wiped. The pellets were dissolved in 0.1 ml 1 M NaOH and neutralized with 1 M HCl. The radioactivities of the solutions were measured using a liquid scintillation spectrophotometer. The procedures are described elsewhere in more detail (Matsui and Homareda, 1988). Oligomycin-stimulated Na^+ binding, which was regarded as the occluded Na^+, was calculated from the difference between the radioactivities of the pellets prepared in the presence and absence of oligomycin.

2.6. Expression of chicken chimeric cDNA by mouse LtK^+ β cells

The chimeric chicken cDNA called [n/c]CC, which encodes Met^1 to Asp^{200} of the chicken Na^+,K^+-ATPase α1-subunit and Ile^{163} to COOH of chicken SERCA-ATPase, was prepared as described elsewhere (Ishii and Takeyasu, 1993; Ishii et al., 1994). Introduction of the cDNA construct into mouse LtK^+ β cells (L cells) that had already been transfected with a cDNA encoding the chicken Na^+,K^+-ATPase β1-subunit, and culture of colonies positive to a monoclonal antibody, 5D2, which is specific to chicken SERCA-ATPase, were done as de-

scribed elsewhere (Takeyasu et al., 1988; Ishii and Takeyasu, 1993; Ishii et al., 1994).

2.7. Preparation of L cell microsomes

The microsomes were prepared as described elsewhere (Ishii and Takeyasu, 1993; Ishii et al., 1994) except that the cell homogenates were centrifuged at 3000 rpm for 10 min in a swing rotor (Model CD-50SN; Tomy Seiko, Tokyo, Japan) to remove cell debris before centrifugation at 20,000 rpm for 30 min (45 Ti rotor; Beckman).

2.8. Measurement of ATPase activities

2.8.1. Chimeric SERCA-ATPase activity

L cell microsomes containing chimeric SERCA-ATPase (30 μg) were suspended in 100 mM KCl, 3 mM $MgCl_2$, 125 μM $CaCl_2$, 125 μM EGTA, 50 mM imidazole–HCl (pH 7.4 at 37°C), 5 mM NaN_3, 80 mM choline chloride or NaCl, 1% ethyl alcohol, 0–10 μM oligomycin B and with or without 500 nM thapsigargin. Then ATP (final concentration, 1 mM) was added to start the hydrolysis reaction, and the mixture (0.5 ml) was incubated for 120 min at 37°C. The reaction was stopped by adding 50 μl of 50% trichloroacetic acid. After centrifugation for 2 min at 10,000 rpm in a microcentrifuge (Model MR-15A; Tomy Seiko), 450 μl of supernatant was used for Pi measurement (Fiske and Subbarow, 1925). SERCA-ATPase activity was calculated from the difference between ATPase activity measured in the absence and presence of thapsigargin at various oligomycin concentrations.

2.8.2. Sarcoplasmic reticulum Ca^{2+}-ATPase

Sarcoplasmic reticulum vesicles (15 μg, rabbit muscle) were suspended in 100 mM KCl or NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 1 mM ATP, 20 mM imidazole–HCl (pH 7.0 at 25°C), 1% ethyl alcohol, with or without 10 μM oligomycin B and with or without 1 μM thapsigargin. The reaction mixture (0.5 ml) was incubated for 30 min at 25°C. Then the amount of Pi liberated was measured (Fiske and Subbarow, 1925). Ca^{2+}-ATPase activity was calculated from the difference between the ATPase activity measured in the absence and presence of thapsigargin.

2.8.3. Plasma membrane Ca^{2+}-ATPase

The reaction mixture (0.5 ml) contained 30 μg wild-type L cell microsomes, 100 mM KCl, 3 mM $MgCl_2$, 1 mM ATP, 125 μM EGTA, 50 mM imidazole–HCl (pH 7.4 at 37°C), 5 mM NaN_3, 1% ethyl alcohol, with or without 10 μM oligomycin B and with or without 125 μM $CaCl_2$. The incubation was for 60 min at 37°C. Ca^{2+}-ATPase activity was calculated from the difference between the ATPase activity measured in the presence and absence of Ca^{2+}.

2.8.4. K^+-ATPase activity

The reaction mixture (0.5 ml) contained 15 μg gastric vesicles (hog stomach), 3 mM $MgCl_2$, 1 mM ATP, 50 mM 2-morpholinoethanesulfonic acid (Mes)–Tris (pH 5.5 at 37°C), 1% ethyl alcohol, with or without 10 μM oligomycin B and with or without 15 mM KCl. The mixture was incubated for 15 min at 37°C. K^+-ATPase activity was calculated from the difference between the ATPase activity measured in the presence and absence of K^+.

2.8.5. Na^+,K^+-ATPase activity

The reaction mixture (0.5 ml) contained 2 μg intact or digested ATPase, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM ATP, 50 mM imidazole–HCl (pH 7.4 at 37°C) and 1% ethyl alcohol, with or without 10 μM oligomycin B. The mixtures were incubated for 15 min at 37°C.

2.9. Determination of protein and oligomycin concentrations

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Oligomycin concentrations were determined with a spectrophotometer at 224 nm using a molecular absorption coefficient of 32,200 in methyl alcohol (Carter, 1986).

3. Results

3.1. Na^+ occlusion within Na^+,K^+-ATPase peptide fragments

Na^+,K^+-ATPase was digested with trypsin for 10 s, 1, 6 and 60 min at 37°C in the presence of Rb^+ according to the method of Capasso et al. (1992). Rb^+ was washed out from the digested ATPases because Rb^+ is a strong inhibitor of Na^+ binding (Matsui and Homareda, 1988). The amount of undigested α-subunit was equivalent to 27%, 2%, 2% and 7% of the control, respectively (Fig. 1, lanes 1–5). The immunoblotting pattern showed that the α-subunit was gradually degraded as the digestion time was increased, whereas the β-subunit was degraded stepwise (Fig. 1, lanes 6–10).

The 10-s digest produced no apparent reduction in oligomycin-induced Na^+ occlusion, though the remaining ATPase activity decreased to 35% of the control (Fig. 2). Therefore, the Na^+ occlusion by oligomycin does not require intact Na^+,K^+-ATPase. The 1-min digest reduced Na^+ occlusion to 40% of the control (Fig. 2) and the ATPase activity to 10% of the control. At the same time, we observed the release of fragments containing M5–M6 in the 1-min digest from the plasma membrane (Fig. 3).

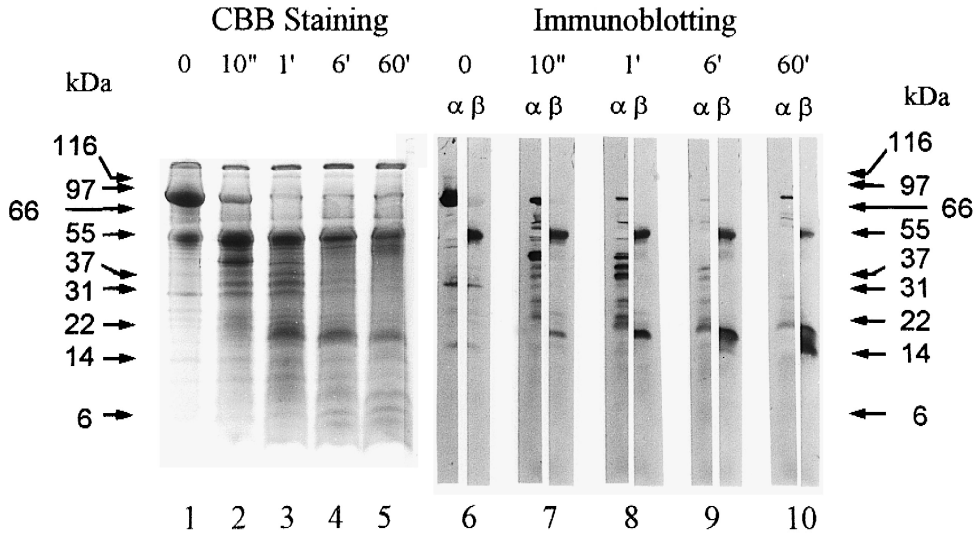

Fig. 1. SDS-PAGE and immunoblotting of intact and digested Na^+,K^+-ATPase using antisera raised against the α1- and β1-subunits. Intact Na^+,K^+-ATPase and its trypsin-digested peptide fragments (precipitate fractions containing 20 μg protein each) were fractionated by SDS-PAGE. The gel was cut into two parts between lanes 5 and 6. The gel fragment with lanes 1–5 was stained with Coomassie brilliant blue (CBB), and the rest of the gel was transblotted onto a nitrocellulose membrane. Lanes 6–10 were cut into two vertical strips each. The left-hand strips were reacted with the anti-α subunit serum, and the right-hand strips were reacted with the anti-β subunit serum. Then, all strips were reacted with the second antibody and visualized with a staining kit (Wako). The CBB staining pattern and the immunoblotting patterns were photographed and aligned to the same scale, as shown in the figure. Molecular masses and positions of standard proteins are indicated in kDa on the ordinates. 0, 10″, 1′, 6′ and 60′ represent Na^+,K^+-ATPase digested with trypsin for 0, 10 s, 1, 6 and 60 min, respectively.

This result was consistent with the finding by Lutsenko et al. (1995) that removal of Rb^+ from the digested Na^+,K^+-ATPase preparation induced the release of the M5–M6 fragment from the membranes. On the other hand, the M1–M4 fragment was not released (data not shown). These results showed that degradation of the original organization of transmembrane segments of the α-subunit was responsible for the reduction in Na^+ occlusion. Consequently, as shown in Figs. 1–3, the α-subunit includes a domain for the interaction of oligomycin with Na^+.

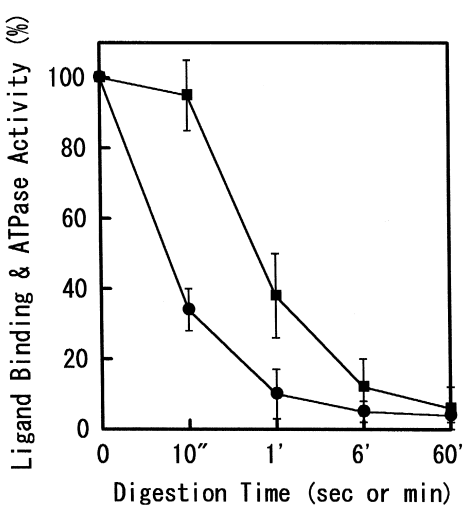

Fig. 2. ATPase activity and Na^+ occlusion of intact and digested Na^+,K^+-ATPase. The compositions of the reaction mixtures and the experimental procedures are described in Section 2. Oligomycin and ethyl alcohol are removed from the mixtures for ATPase activity. ATPase activity (●) and Na^+ occlusion (■) of the intact ATPase were 11.1 μmol min^{-1} mg^{-1} (mean of three determinations) and 3.1 nmol/mg (mean of four determinations), respectively, which were plotted as 100%. Plots and bars represent the means and S.D. ($n = 3$ or 4), respectively. 0, 10″, 1′, 6′ and 60′ represent Na^+,K^+-ATPase digestion time.

3.2. Effect of oligomycin on P-type ATPase activities

Prior to looking whether oligomycin inhibits chimeric SERCA-ATPase activity, we examined the effect of this antibiotic on wild-type SERCA-ATPase activity under various ligand conditions. The sarcoplasmic reticulum Ca^{2+}-ATPase activity in the absence of Na^+ and K^+ was not inhibited by 10 μM oligomycin, which can inhibit Na^+,K^+-ATPase activity by 75% (Table 1). Addition of 100 mM Na^+ or K^+, which activates sarcoplasmic reticulum Ca^{2+}-ATPase activity (The and Hasselbach, 1972) at 25°C rather than 37°C (data not shown), did not influence the oligomycin effect. This antibiotic, thus, does not affect the interaction of SERCA-ATPase with Ca^{2+}, Na^+ and K^+. In addition, oligomycin had little effect on plasma membrane Ca^{2+}-ATPase activity and gastric H^+,K^+-ATPase activity at an acidic pH (Table 1). Hongo et al. (1990) showed no effect of oligomycin on H^+,K^+-ATPase activity at a neutral pH. These results demonstrated that this antibiotic affected only the interaction of Na^+,K^+-ATPase with Na^+ in the typical P-type ATPases.

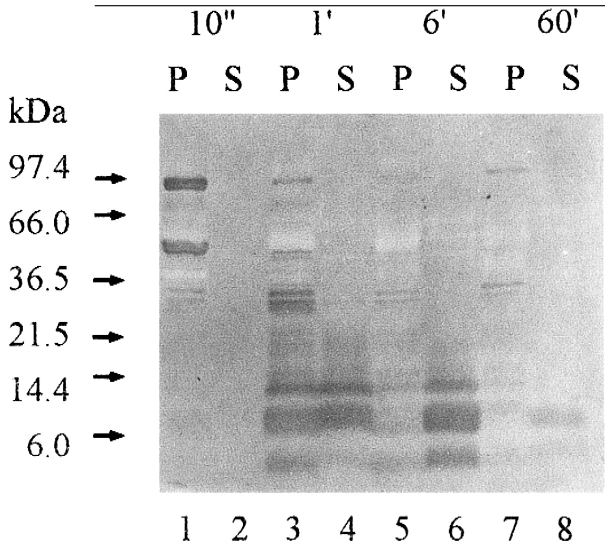

Fig. 3. Immunoblotting of digested ATPase using the peptide-directed antibody. The precipitate fractions (P) and the supernatant fractions (S) of the 10 s (10″), 1 min (1′), 6 min (6′) and 60 min (60′) digests were dissolved in 100 and 50 μl of 5% SDS, respectively, as described in Section 2. SDS-solutions of P, 1.2, 1.6, 1.7 and 2.4 μl of respective 10″, 1′, 6′ and 60′ digestions (containing 10 μg protein each) and SDS-solutions of S, 3.0, 4.0, 4.3, 6.0 μl of respective 10″, 1′, 6′ and 60′ digestions (proteins could not be determined), volume of the each being 2.5-fold that of the respective SDS-solution of P, were applied to SDS-PAGE and then transblotted onto a nitrocellulose membrane. The membrane was reacted with the antiserum raised against the 815–823 sequence of the α1-subunit, then the second antibody and visualized with a staining kit (Vector Laboratories). Molecular masses and positions of standard proteins are indicated in kDa on the ordinate.

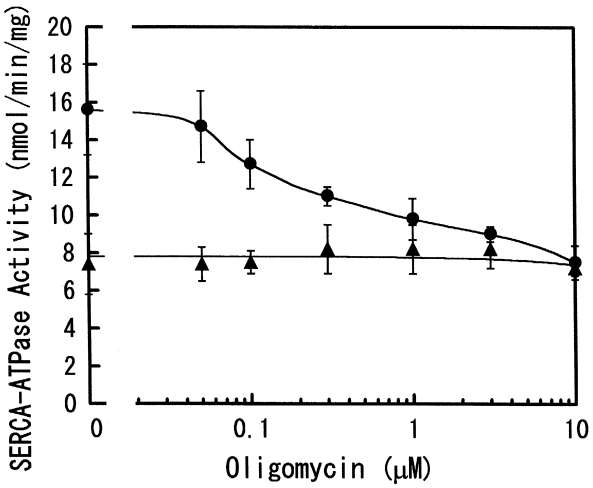

Fig. 4. Effect of oligomycin on chimeric SERCA-ATPase activity. The compositions of the reaction mixtures, which contained 80 mM NaCl (●) or choline chloride (▲), and the procedures are described in Section 2. Thapsigargin-inhibitable SERCA-ATPase activity was calculated from the difference between ATPase activity in the absence and presence of 500 nM thapsigargin at the oligomycin concentrations indicated on the horizontal line of the figure. Free Ca^{2+} concentration was calculated to be 2.3 μM in the reaction mixture containing 125 μM $CaCl_2$ and 125 μM EGTA (Goldstein, 1979). Plots and bars represent the means ± S.D. ($n = 3$–5).

3.3. Effect of oligomycin on the chimeric SERCA-ATPase activity

In the presence of 100 mM K^+, the SERCA-ATPase activity of [n/c]CC was doubled by the addition of 80 mM NaCl (Fig. 4) but was inhibited by thapsigargin and ouabain as previously shown (Ishii and Takeyasu, 1993; Ishii et al., 1994). Because the activation by Na^+ is not observed with the wild-type SERCA-ATPase and other chimeric SERCA-ATPases, the 200 N-terminal amino acids of [n/c]CC are considered to retain partially the Na^+-dependent characteristics of Na^+,K^+-ATPase (Ishii and Takeyasu, 1993; Ishii et al., 1994). We now found that oligomycin inhibited the Na^+-activated SERCA-ATPase activity of [n/c]CC. The oligomycin concentration that gave half-maximal inhibition was 0.2 μM (Fig. 4), which is one-tenth of the value for wild-type Na^+,K^+-ATPase activity (Arato-Oshima et al., 1996). However, the basal SERCA-ATPase activity, which was measured in the presence of 80 mM choline chloride instead of NaCl, was not inhibited by oligomycin. This antibiotic had no effect on

Table 1
Effects of oligomycin on typical P-type ATPase activities under the various conditions. Compositions of the reaction mixtures and procedures for the measurement of ATPase activities are described in Section 2. Values represent the means ± S.D. ($n = 3$)

ATPase	Ligands	pH	Temperature (°C)	ATPase activity 10 μM Oligomycin		(+)/(−) × 100
				(−)	(+)	
Sarcoplasmic reticulum				μmol min^{-1} mg^{-1}		
Ca^{2+}-ATPase	Ca^{2+}	7.0	25	0.18 ± 0.01	0.18 ± 0.01	100
	Ca^{2+}, Na^+	7.0	25	0.29 ± 0.02	0.28 ± 0.01	97
	Ca^{2+}, K^+	7.0	25	0.26 ± 0.01	0.28 ± 0.02	108
Plasma membrane				nmol min^{-1} mg^{-1}		
Ca^{2+}-ATPase	Ca^{2+}, K^+	7.4	37	22.4 ± 0.6	19.0 ± 1.2	85
				μmol min^{-1} mg^{-1}		
H^+,K^+-ATPase	K^+	5.5	37	0.15 ± 0.03	0.12 ± 0.02	80
Na^+,K^+ ATPase	Na^+, K^+	7.4	37	11.1 ± 0.02	2.80 ± 0.01	25

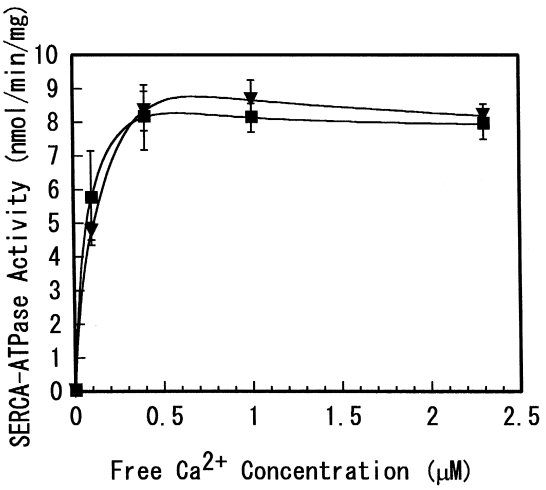

Fig. 5. Effect of oligomycin on the chimeric SERCA-ATPase activity at various concentrations of free Ca^{2+}. The reaction mixtures consisted of 30 μg microsomes containing chimeric SERCA-ATPase, 180 mM KCl, 3 mM $MgCl_2$, 50 mM imidazole–HCl (pH 7.4 at 37°C), 5 mM NaN_3, 1 mM ATP, 1% ethyl alcohol, with (▼) or without (■) 1 μM oligomycin, with or without 500 nM thapsigargin, 125 μM $CaCl_2$ and 125–190 μM EGTA. The procedures were described in Section 2. Thapsigargin-inhibitable SERCA-ATPase activity was calculated from the difference between ATPase activity in the absence and presence of thapsigargin at free Ca^{2+} concentrations indicated in the figure. Plots and bars represent the means ± S.D. ($n = 3–4$).

the Ca^{2+}-activation curve of the basal SERCA-ATPase activity, suggesting that oligomycin does not affect the affinity of the chimeric SERCA-ATPase for Ca^{2+} (Fig. 5). Thus, the present results suggest strongly that the 200 N-terminal amino acids of the Na^+,K^+-ATPase α-subunit include a binding domain for oligomycin.

4. Discussion

Oligomycin inhibits the mitochondrial H^+-ATPase in the F-type ATPases but not other F-type ATPases or the V-type ATPases (Pedersen and Carafoli, 1987; Homareda, 1999). The present data showed that the antibiotic affected only the interaction of Na^+,K^+-ATPase with Na^+ in the typical P-type ATPases (Table 1). It has been pointed out that, in the mitochondrial H^+-ATPase, the oligomycin sensitivity-conferring protein (OSCP) is not a true binding protein but that the F_0-portion includes the binding domain (Senior, 1979). However, it is unclear which component of the F_0-portion includes the binding domain.

To study the oligomycin binding of Na^+,K^+-ATPase is difficult. We have attempted to adapt the assay method used for $^{22}Na^+$ binding for use with [^{3}H]oligomycin binding to characterize the binding properties, but the low solubility of oligomycin in water caused highly non-specific binding to the plasma membrane. Next, we examined the covalent binding of oligomycin to the ATPase to identify the binding domain, but the structural modification of oligomycin for this purpose remains unsuccessful. In the present study, we used an indirect method coupled with the chimeric ATPase, because the thapsigargin-sensitive domain of sarcoplasmic reticulum Ca^{2+}-ATPase was located by this method (Nørregaard et al., 1994). We hypothesized that the 200 N-terminal amino acids of the Na^+,K^+-ATPase α-subunit include a binding domain for oligomycin. It is unknown why the oligomycin concentration that halved Na^+-activated SERCA-ATPase activity (0.2 μM) was one-tenth of the concentration that halved wild-type Na^+,K^+-ATPase activity (2 μM). The binding domain of oligomycin may be modified in the chimeric ATPase molecule.

Or et al. (1999) proposed a spatial organization of 10 transmembrane segments of the Na^+,K^+-ATPase α-subunit. In this model, M4 and M6 are located in the center of the α-subunit and are surrounded by the remaining segments. Because several laboratories (e.g., Pedersen et al., 1998; Vilsen and Andersen, 1998) have reported that some amino acids with acidic residues or oxygen-containing residues in M4–M6, e.g., Glu^{327}, Glu^{779}, Asp^{804}, Thr^{774} (pig α1-subunit), are essential for Na^+ and K^+ occlusion, M4–M6 would have crucial functions in the active transport of Na^+ and K^+. Vilsen and Andersen (1998) showed that substitution of Glu^{329} for Gln in M4 (rat α1-subunit) decreased the affinity for Na^+ required for the phosphorylation of the Na^+,K^+-ATPase α-subunit and that oligomycin restored the decreased Na^+ affinity. If oligomycin binds to some amino acids in the M4–M6, this would not explain why only Na^+ occlusion is stabilized by oligomycin, which has little effect on the conformational change of Na^+,K^+-ATPase (Arato-Oshima et al., 1996). Oligomycin, therefore, does not seem to interact directly with these amino acids. Because an extracellular loop between M7 and M8 associates with the β-subunit (Lemas et al., 1994), it is difficult to suppose that the M7–M8 domain interacts with oligomycin, which accesses to Na^+,K^+-ATPase from the extracellular side (Cornelius and Skou, 1985). Arato-Oshima et al. (1996) suggested that Na^+,K^+-ATPase possesses a single oligomycin binding site based on a kinetic study. Thus, it is likely that oligomycin interacts with the M1–M2 domain, which is contained in the 200 N-terminal amino acids of the Na^+,K^+-ATPase α-subunit, so that Na^+ occlusion is stabilized.

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