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Properties of silkworm Na^+/K^+-ATPase

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The high Na^+ and low K^+ concentrations in mammalian blood are maintained by Na^+/K^+-ATPase. In contrast, the K^+ concentration is higher than the Na^+ concentration in the hemolymph of the silkworm *Bombyx mori*, a Lepidopterous insect. Although Na^+/K^+-ATPase, therefore, appears not to be in silkworm, we confirmed the presence of Na^+/K^+-ATPase in nerve tissues of silkworm but not in skeletal muscle or the dorsal vessel. The enzymatic properties of silkworm Na^+/K^+-ATPase were characterized in detail and compared with those of dog Na^+/K^+-ATPase. Silkworm Na^+/K^+-ATPase had a much lower affinity for K^+ and a somewhat higher affinity for Na^+ than dog Na^+/K^+-ATPase. The optimal temperature of silkworm Na^+/K^+-ATPase activity was lower than that of dog Na^+/K^+-ATPase. The optimal Mg^{2+} concentration, pH and sensitivities to Ca^{2+} and ouabain, a specific inhibitor of Na^+/K^+-ATPase, of the two ATPases were identical. These results indicate that the enzymatic properties of the silkworm Na^+/K^+-ATPase are suitable for its growth, despite the differences between dog and silkworm Na^+/K^+-ATPases. Antisera raised against dog Na^+/K^+-ATPase recognized only the α-subunit of silkworm Na^+/K^+-ATPase.

Keywords: dog/Na^+/K^+-ATPase/nerve tissue/ouabain/silkworm.

Abbreviations: K^+-pNPPase, K^+-dependent *p*-nitrophenylphosphatase; Na^+/K^+-ATPase, Na^+/K^+-exchanging ATPase; EC 3.6.3.9; pNPP, *p*-nitrophenylphosphate; pNP, *p*-nitrophenol; PVDF, polyvinylidenedifluoride.

In mammals, the monovalent cation composition of blood is 140 mM NaCl and 4–5 mM KCl, while the cation composition in the cell is 10–20 mM NaCl and 150 mM KCl (*1*). Na^+/K^+-ATPase (Na^+/K^+-exchanging ATPase; EC 3.6.3.9) in the cell membranes actively transports Na^+ from the inside to the outside of the cell and K^+ is transported in the reverse direction using the energy from ATP hydrolysis, so that the asymmetric composition of Na^+ and K^+ is maintained across the cell membrane.

In insects, the monovalent cation composition in hemolymph varies greatly. A high concentration of Na^+ relative to K^+ is observed in omnivorous insects (*2*). For example, in the hemolymph of *Chiromonanus* members of the order Diptera, like the mosquito and fly, the monovalent cation composition was ~100 mM Na^+ and 2 mM K^+ (*3*), suggesting the presence of Na^+/K^+-ATPase. In contrast, the monovalent cation concentrations of the hemolymph of Lepidoptera such as the butterfly and moth, which are phytophagous insects, are low Na^+ and high K^+ (*2*). For example, the monovalent cation composition in hemolymph of the silkworm *Bombyx mori* was reported to be 14.6 mM NaCl and 46.1 mM KCl (*3*) or 12 mM NaCl and 62 mM KCl (*4*). Lepidoptera, therefore, appear to lack Na^+/K^+-ATPase. Moreover, Fitzgerald *et al.* (*5*) suggested that Na^+/K^+-ATPase was present in skeletal muscle cells of Dipterous but not in Lepidopterous insects. On the other hand, Vaughan and Jungreis (*6*, *7*) demonstrated that nerve tissues of three Lepidopterous insects contained ouabain-sensitive Na^+/K^+-ATPase, although these insects were resistant to cardiac glycosides *in vivo* (*7*, *8*). It is believed that neurons of insects are separated from the hemolymph by the blood–brain barrier system, which surrounds the neurons and limits the movement of water-soluble ions between hemolymph and the neuronal surface (*2*, *4*, *9–12*) so that the Na^+/K^+-ATPase of Lepidopterous insects is active in their nerve tissues (*2*). Despite these reports, the enzymatic properties of insect Na^+/K^+-ATPase have not been examined in detail. Therefore, we investigated Na^+/K^+-ATPase of the silkworm *B. mori*, a Lepidopterous insect. We confirmed that Na^+/K^+-ATPase was present in nerve tissue, but not in skeletal muscle or the dorsal vessel, which functions as the heart, of silkworm larva using immunochemical and biochemical techniques. The enzymatic properties of silkworm Na^+/K^+-ATPase were characterized and compared with the properties of canine kidney Na^+/K^+-ATPase. The silkworm Na^+/K^+-ATPase showed a much lower affinity for K^+ and a lower optimal temperature than dog Na^+/K^+-ATPase, while the optimal pH, optimal Mg^{2+} concentration and sensitivities to Ca^{2+} and ouabain of the two enzymes differed little.

Na^+/K^+-ATPase is known to be composed of α-subunit of 100 kDa and β-subunit of 50 kDa (*9*, *13*, *14*). Antibodies raised against canine kidney Na^+/K^+-ATPase recognized the α-subunit but not the β-subunit of silkworm Na^+/K^+-ATPase.

Materials and Methods

Materials

Silkworm larvae in the fifth instar feeding stage were purchased from Zen-noh (Tokyo, Japan). $ATP(Na)_2$ purchased from Roche

Diagnostics GmbH (Penzberg, Germany) was converted to the sodium-free ATP form using an ion-exchange column and neutralized with imidazole. Ditris salt of *p*-nitrophenylphosphate (pNPP) was purchased from ICN Biomedicals Inc., (Aurora, OH, USA). $EDTA(H)_4$ purchased from Dojin Laboratories Ltd (Kumamoto, Japan) was recrystallized and neutralized with imidazole. Complete mini (protease inhibitor cocktail tablet) was purchased from Roche Diagnostics GmbH. Oxalic acid from Wako Pure Chemicals Industries Ltd (Osaka, Japan) was neutralized with imidazole. Other reagents were purchased from Wako Pure Chemicals Ltd and Sigma Chemical Company (St Louis, MO, USA).

Methods

Protein assay. Protein was measured using a 2D Quant kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Preparation of microsomes. Fifth instar feeding silkworm larvae were reared at 22–24°C with an artificial diet and frozen at −80°C in plastic test tubes just before spinning the cocoon. Body walls of the silkworm were peeled off from the dorsal and abdominal sides in a semi-frozen condition at near 0°C. Under a stereomicroscope, nerve tissues were isolated from the abdominal side. Skeletal muscles were peeled from the body wall of the dorsal side, to which skeletal muscles are attached. The dorsal vessel was isolated from the dorsal side. These tissues were suspended separately in an ice-cold preparation solution (5 mM DTT, 5 mM EDTA, 10 mM oxalic acid, 250 mM sucrose, protease inhibitors and 50 mM imidazole-HCl, pH 7.4, at 25°C), minced with scissors, homogenized with a polytron and a glass homogenizer and centrifuged at 2,500*g* (Hitachi himac CF15D2, Tokyo) for 20 min at 5°C. Supernatants were centrifuged at 100,000*g* (Beckman-Coulter, Brea, CA, USA) for 20 min at 5°C. Precipitates were suspended in the preparation solution without protease inhibitors and stored in liquid nitrogen.

Canine kidney Na^+/K^+-ATPase was purified as described elsewhere (*15*). More than 95% of total activity was ouabain-sensitive.

Assay of Na^+/K^+-ATPase activity. Reaction mixture A to measure silkworm Na^+/K^+-ATPase activity was composed of 4 μg microsomes, 10 mM NaCl, 30 mM KCl and a common ligand mix (4 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP and 50 mM imidazole-HCl, pH 7.4, at 25°C) with or without 0.1 mM ouabain in 500 μl. Reaction mixture B to measure canine kidney Na^+/K^+-ATPase activity was composed of 0.5 μg purified enzyme, 100 mM NaCl, 10 mM KCl and the common ligand mix in 500 μl. The ATPase reaction was started by addition of ATP, incubated for 1 h at 37°C and stopped by addition of 1 ml of molybdate-H_2SO_4. Liberated Pi was measured with the method of Fiske and Subbarow (*16*). For silkworm ATPase activity, the difference between the activities in the absence and presence of ouabain (the ouabain-sensitive ATPase activity) or the difference between the activities in the absence and presence of Na^+ and K^+ (the Na^+ and K^+-activated ATPase activity) was defined as Na^+/K^+-ATPase activity. For canine kidney Na^+/K^+-ATPase activity, the Na^+ and K^+-activated ATPase activity was defined as Na^+/K^+-ATPase activity. The ouabain-insensitive ATPase activity was ignored.

Assay of K^+-pNPPase activity. The reaction mixture was composed of 5 μg microsomes or 0.5 μg purified enzyme, 0–300 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM pNPP and 50 mM imidazole-HCl (pH 7.4 at 25°C) in 500 μl. The reaction was started by addition of pNPP, incubated for 1 h at 37°C and stopped by addition of 1 ml 1 N NaOH. The liberated *p*-nitrophenol (pNP) was measured with a spectrophotometer at 440 nm. The difference between the activities in the absence and presence of KCl was defined as K^+-dependent *p*-nitrophenylphosphatase (K^+-pNPPase) activity.

Immunoblotting analysis. Twenty micrograms each of nerve, muscle and dorsal vessel microsomes of silkworm and 0.1 μg of canine kidney Na^+/K^+-ATPase were applied to SDS–PAGE with 10.5% polyacrylamide gel and then blotted onto a polyvinylidenedifluoride (PVDF) membrane as described elsewhere (*17*). The membrane was incubated in a blocking solution overnight at 4°C, incubated in the mixture of 1,000-fold diluted anti-α antiserum and 500-fold diluted anti-β antiserum for 1 h at room temperature and then incubated for 10 min in 10,000-fold diluted second antibody coupled to a fluorescent intermediate using the ECL Plus Western Blotting Detection System (GE Healthcare). Chemiluminescence derived from the second antibody was visualized using an image reader with a CCD camera (LAS-3000UVmini, Fujifilm, Tokyo).

Anti-α and anti-β sera were prepared using α- and β-subunits purified from the SDS-solubilized canine kidney Na^+/K^+-ATPase as described elsewhere (*18*).

Results

Damage to the body wall changes the hemolymph of silkworms from colourless to dark brown. To prevent this, we used DTT as a reducing reagent and oxalic acid and EDTA as chelators for copper ions, an essential co-factor for tyrosinase activity, which catalyses the melanin-type pigmentation (*19*). Of the tested reagents, DTT was most effective to prevent the pigmentation.

Localization of Na^+/K^+-ATPase

Microsomes containing ~15, 20 and 20 μg protein were prepared from nerve tissue, skeletal muscle and dorsal vessel, respectively, of one silkworm larva. The Na^+/K^+-ATPase activity of silkworm microsomes was measured in reaction mixture B containing 100 mM NaCl and 10 mM KCl for measuring mammalian Na^+/K^+-ATPase activity (Fig. 1). Microsomes of nerve tissues showed ouabain-sensitive ATPase activity, whereas microsomes of muscles and dorsal vessels little showed ATPase activity.

Figure 2 shows the results of immunoblotting analysis using the antisera raised against the α- and β-subunits of canine kidney Na^+/K^+-ATPase. The α-subunit was clearly observed in nerve tissues but not in dorsal vessels. The α-subunit observed faintly in muscles appeared to be due to the nerve fibres contaminating muscle microsomes (lane 3 of Fig. 2). On the other hand, the β-subunit was not observed in any tissue.

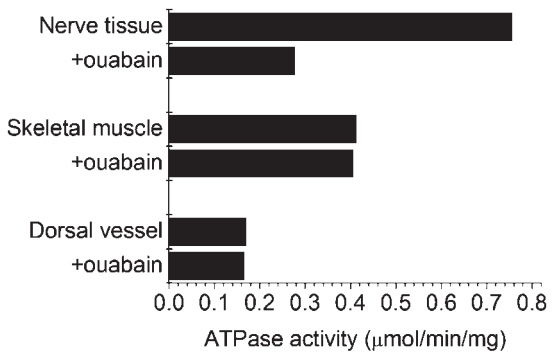

Fig. 1 ATPase activities of microsomes prepared from nerve tissue, skeletal muscle and dorsal vessel of silkworm. The reaction mixture was composed of 4 μg (nerve tissue) or 10 μg (skeletal muscle and dorsal vessel) of microsomes, 100 mM NaCl, 10 mM KCl and common ligands (4 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 50 mM imidazole-HCl, pH 7.4, at 25°C) with or without 1 mM ouabain in 500 μl, as described in 'Materials and Methods' section. Bars represent the average of two different determinations.

Enzymatic characteristics of silkworm Na^+/K^+-ATPase

Affinities for Na^+ and K^+. In experiments for determining the affinities of silkworm Na^+/K^+-ATPase for Na^+ and K^+ and the optimal $Na^+:K^+$ ratio for ATPase activity, the Na^+ and K^+-activated ATPase activity was defined as Na^+/K^+-ATPase activity instead of the ouabain-sensitive ATPase activity because the ouabain-sensitivity of the ATPase activity decreased in the presence of Na^+ and K^+ concentrations at >100 mM (data not shown).

At a constant concentration of 10 mM KCl, which is close to the K^+ concentration in mammalian blood, the NaCl concentration giving the maximal silkworm Na^+/K^+-ATPase activity was lower than that giving maximal canine kidney Na^+/K^+-ATPase activity (Fig. 3), suggesting that the affinities for Na^+ and/or K^+ of silkworm Na^+/K^+-ATPase were different from those of canine kidney Na^+/K^+-ATPase activity. Ai *et al*. (*20*) demonstrated that the optimal composition of physiological saline for maintaining the heart beat of Lepidopterous insects was 12–28 mM NaCl, 32–16 mM KCl ($[Na^+]+[K^+]=44$), 9 mM $CaCl_2$, 18 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 1.5 mM Na_2HPO_4 and 175 mM sucrose, pH 6.5. We referred to this ion composition to determine the optimal cation composition for silkworm Na^+/K^+-ATPase activity, except for $CaCl_2$, an inhibitor of Na^+/K^+-ATPase activity as described later and phosphate buffer, which interfered with the liberated Pi assay system.

At a constant concentration of 30 mM KCl, the Na^+ and K^+-activated ATPase activity increased with increasing NaCl concentrations up to 100 mM (Fig. 4). The NaCl concentration giving the half-maximal activity was 6.0 mM for silkworm Na^+/K^+-ATPase (Fig. 4A), which was about one-half the value (14 mM) for canine kidney Na^+/K^+-ATPase (Fig. 4B).

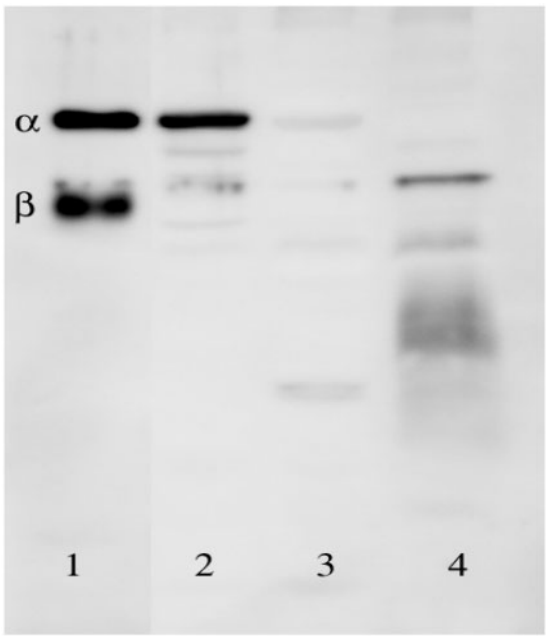

Fig. 2 Immunoblotting of microsomes from various tissues of silkworm and dog Na^+/K^+-ATPase. Microsomes (20 μg) and 0.1 μg canine kidney Na^+/K^+-ATPase were applied to SDS–PAGE with 10.5% acrylamide gel, blotted onto a PVDF membrane, incubated with first and second antibodies and visualized using a CCD camera, as described in 'Materials and Methods' section. Lanes 1, 2, 3 and 4 represent dog Na^+/K^+-ATPase, nerve tissue, skeletal muscle and dorsal vessel, respectively.

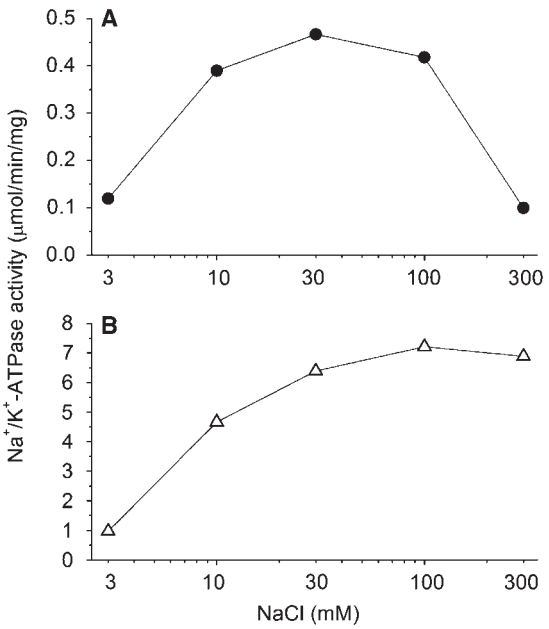

Fig. 3 Affinity of silkworm and dog Na^+/K^+-ATPases for Na^+ in the presence of 10 mM KCl. The reaction mixture was composed of 4 μg nerve tissue microsomes (A) or 0.5 μg canine kidney Na^+/K^+-ATPase (B), 0–300 mM NaCl, 10 mM KCl and common ligands in 500 μl. The Na^+ and K^+-activated ATPase was defined as Na^+/K^+-ATPase activity, as described in 'Materials and Methods' section. Symbols represent the average of two different determinations.

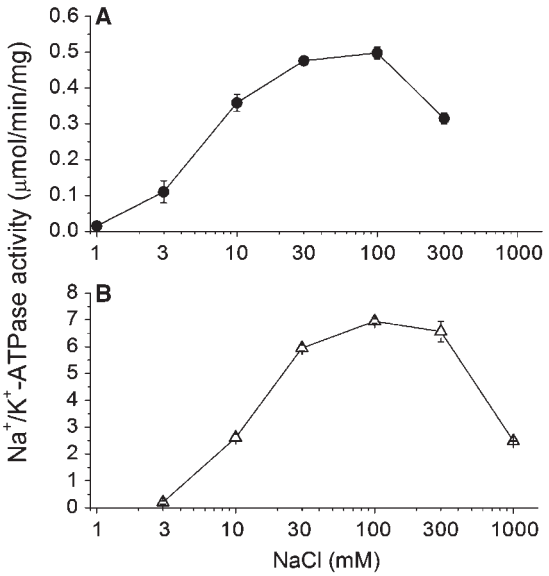

Fig. 4 Affinity of silkworm and dog Na^+/K^+-ATPases for Na^+ in the presence of 30 mM KCl. The reaction mixture was composed of 4 μg nerve tissue microsomes (A) or 0.5 μg canine kidney Na^+/K^+-ATPase (B), 0–1000 mM NaCl, 30 mM KCl and common ligands in 500 μl. The Na^+ and K^+-activated ATPase was defined as Na^+/K^+-ATPase activity. Symbols and bars represent the average ± SD from three different determinations.

At a constant concentration of 10 mM NaCl, silkworm Na^+/K^+-ATPase activity increased with increasing KCl concentrations up to 30 mM (Fig. 5A). The KCl concentration giving the half-maximal activity was 1.2 mM for silkworm Na^+/K^+-ATPase (Fig. 5A). This value was six times the value (0.2 mM) shown by canine kidney Na^+/K^+-ATPase (Fig. 5B). The low affinity of silkworm Na^+/K^+-ATPase for K^+ was also observed in K^+-pNPPase activity (Fig. 6). The KCl concentration giving the half-maximal activity was 6.0 mM for silkworm, which is 10 times the value (0.64 mM) for dog (Fig. 6).

The $Na^+:K^+$ ratios giving the maximal activity were 1:3–3:1 for silkworm and 1:1–10:1 for dog (Figs 4 and 5). The optimal $Na^+:K^+$ ratio for silkworm Na^+/K^+-ATPase was within the $Na^+:K^+$ ratio in physiological saline reported by Ai *et al*. (*20*).

Effects of Mg^{2+} and Ca^{2+}. The $MgCl_2$ concentration giving the maximal ATPase activity was determined at constant concentrations of 10 mM NaCl and 30 mM KCl for silkworm Na^+/K^+-ATPase and at constant concentrations of 100 mM NaCl and 10 mM KCl for canine kidney Na^+/K^+-ATPase (Fig. 7). The optimal concentration of $MgCl_2$ was 3 mM for both Na^+/K^+-ATPases. The maximal Mg^{2+}-ATPase activity of silkworm, which was measured in a reaction mixture excluding Na^+ and K^+, was also observed at 3 mM $MgCl_2$ (data not shown).

$CaCl_2$ up to 300 µM did not affect either Na^+/K^+-ATPase activity, but $CaCl_2$ at >1 mM inhibited the ATPase activities (Fig. 8).

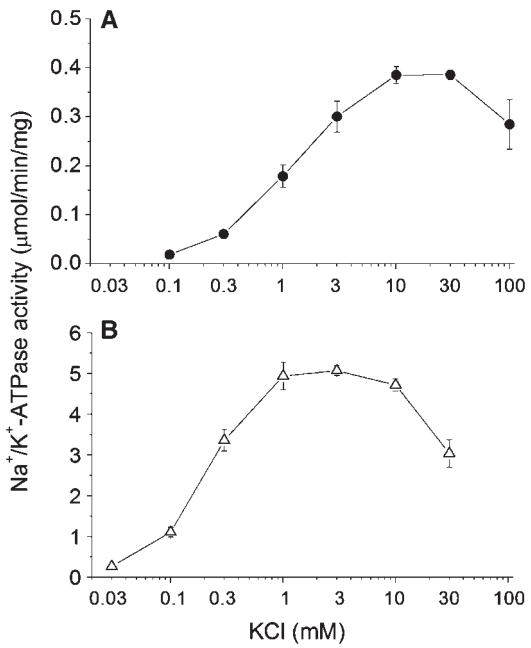

Fig. 5 Affinity of silkworm and dog Na^+/K^+-ATPases for K^+ in the presence of 10 mM NaCl. The reaction mixture was composed of 4 µg nerve tissue microsomes (A) or 0.5 µg canine kidney Na^+/K^+-ATPase (B), 10 mM NaCl, 0–100 mM KCl and common ligands in 500 µl. The Na^+ and K^+-activated ATPase was defined as Na^+/K^+-ATPase activity. Symbols and bars represent the average ± SD from three different determinations.

Ouabain sensitivity. The ouabain sensitivity of silkworm Na^+/K^+-ATPase activity at constant concentrations of 10 mM NaCl and 30 mM KCl was compared with that of canine kidney at constant concentrations of 100 mM NaCl and 10 mM KCl (Fig. 9). Ouabain up to 1 mM decreased the silkworm ATPase activity to 42% of the maximal ATPase activity. The Mg^{2+}-ATPase activity corresponded to 43% of the maximal ATPase activity (Fig. 9). This result indicated the absence of ouabain-resistant Na^+/K^+-ATPase and that ~60% of total ATPases in the nerve tissues microsomes was ouabain-sensitive Na^+/K^+-ATPase. The

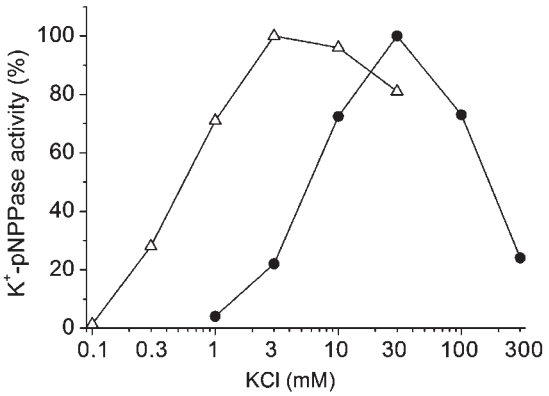

Fig. 6 K^+-pNPPase activites of silkworm and dog Na^+/K^+-ATPase. The reaction mixture was composed of 5 µg nerve tissue microsomes (filled circle) or 0.5 µg canine kidney Na^+/K^+- ATPase (open triangle), 0–300 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM pNPP and 50 mM imidazole-HCl (pH 7.4 at 25°C) in 500 µl. The difference between the activities in the absence and presence of KCl was defined as K^+-pNPPase activity. K^+-pNPPase activities were expressed as a percentage of the respective maximal activities, which were 0.18 and 1.8 µmol pNP/min/mg of silkworm and dog Na^+/K^+-ATPase, respectively. Symbols represent the average of two different determinations.

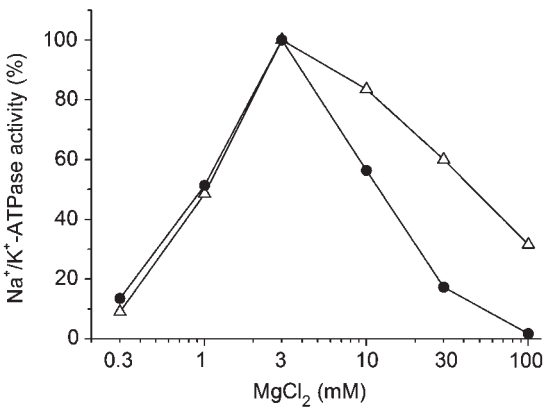

Fig. 7 Effect of Mg^{2+} on silkworm and dog Na^+/K^+-ATPase activities. The reaction mixture for silkworm ATPase (filled circle) comprised 4 µg nerve tissue microsomes, 1mM EDTA, 1 mM ATP, 50 mM imidazole-HCl, pH 7.4, at 25°C, 0–100 mM $MgCl_2$, 10 mM NaCl and 30 mM KCl. The reaction mixture for dog Na^+/K^+-ATPase activity (open triangle) comprised 0.5 µg canine kidney Na^+/K^+-ATPase, 1 mM EDTA, 1 mM ATP, 50 mM imidazole-HCl, pH 7.4, at 25°C, 0–100 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl. The Na^+ and K^+-activated ATPase was defined as Na^+/K^+-ATPase activity. The activities were expressed as a percentage of the respective maximal activities. Symbols represent the average of two different determinations.

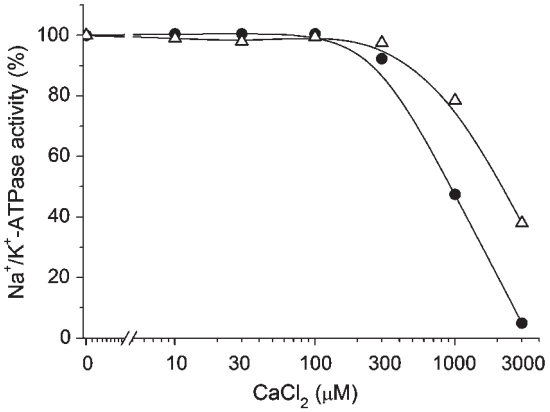

Fig. 8 Effect of $CaCl_2$ on silkworm and dog Na^+/K^+-ATPase activities. Reaction mixtures A and B with 0–3,000 μM $CaCl_2$ were used to measure silkworm (filled circle) and dog (open triangle) Na^+/K^+-ATPase activity, respectively, as described in 'Materials and Methods' section. Ouabain-sensitive ATPase activities were defined as Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activities were expressed as a percentage of the activity of silkworm and dog in the absence of $CaCl_2$. Symbols represent the average of two different determinations.

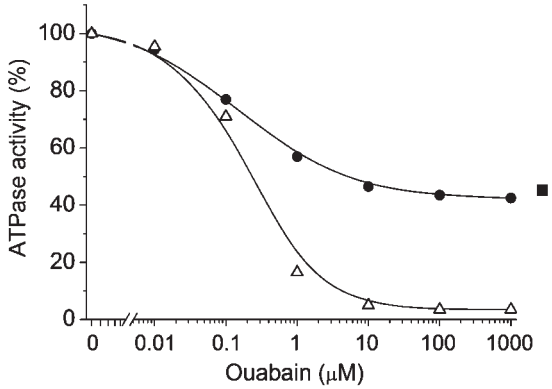

Fig. 9 Ouabain-sensitivity of silkworm and dog Na^+/K^+-ATPase activities. Reaction mixtures A and B with 0–1,000 μM ouabain were used to measure silkworm (filled circle) and dog (open triangle) ATPase activity, respectively, as described in Materials and Methods. Filled square represents silkworm Mg^{2+}-ATPase activity, which were measured in reaction mixture A without NaCl, KCl and ouabain. ATPase activities were expressed as a percentage of the activity of silkworm and dog in the absence of ouabain. Symbols represent the average of two different determinations.

ouabain concentration giving the half-maximal inhibition was 0.2 μM under the incubation conditions used. This value is consistent with the concentration (0.25 μM) for canine kidney Na^+/K^+-ATPase activity. The inhibition curve of silkworm Na^+/K^+-ATPase activity showed a single phase like the curve of canine kidney Na^+/K^+-ATPase (Fig. 9). Therefore, silkworm Na^+/K^+-ATPase has an isoform of the α-subunit, which is highly sensitive to ouabain.

Optimal pH. The effect of pH on silkworm and dog Na^+/K^+-ATPase activities was measured in the range 6.1–8.1 at constant concentrations of 10 mM NaCl and 30 mM KCl and at constant concentrations of 100 mM NaCl and 10 mM KCl, respectively (Fig. 10). The pH giving the maximal Na^+/K^+-ATPase activity was 7.5 for both ATPases. More than 90% of the maximal activity of silkworm Na^+/K^+-ATPase remained in the pH range around 7.0. The remaining activities were higher than the activities of canine kidney ATPase. Therefore, silkworm Na^+/K^+-ATPase was more active than canine kidney Na^+/K^+-ATPase in this pH range.

Optimal temperature. The effect of temperature on silkworm and dog Na^+/K^+-ATPase activities was measured from 15°C to 45°C at constant concentrations of 10 mM NaCl and 30 mM KCl and at constant concentrations of 100 mM NaCl and 10 mM KCl, respectively (Fig. 11). The temperature giving the maximal activity for silkworm Na^+/K^+-ATPase was ~35°C, although the ouabain-insensitive ATPase activity increased up to 45°C (data not shown). The ouabain-sensitive ATPase activity of the enzyme pre-incubated at 45°C for 30 min was not recovered by a shift in temperature to 35°C (Table I). On the other hand, the temperature giving the maximal activity for canine kidney Na^+/K^+-ATPase was 45°C or higher (Fig. 11). When the

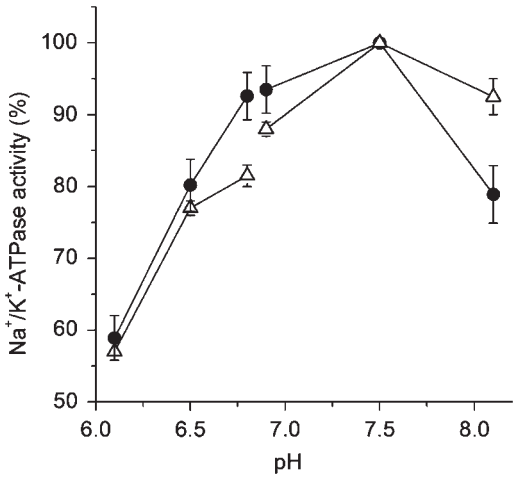

Fig. 10 Effect of pH on silkworm and dog Na^+/K^+-ATPase activities. Reaction mixtures A and B without buffer were used to measure silkworm (filled circle) and dog (open triangle) Na^+/K^+-ATPase activity, respectively, as described in 'Materials and Methods' section. The pH values from 6.1 to 6.8 at 37°C were adjusted with 50 mM Bis-HCl. The pH values from 6.9 to 8.1 at 37°C were adjusted with 50 mM triethanolamine-HCl. Ouabain-sensitive ATPase activities were defined as Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activities were expressed as a percentage of the ATPase activity of silkworm and dog at pH 7.5. Symbols and bars represent the average ± SD from three different determinations.

activity at 35°C was assumed to be 100%, the remaining activities of silkworm ATPase in the range from 15 to 35°C were higher than the remaining activities of canine kidney ATPase. Therefore, silkworm Na^+/K^+-ATPase was more active than canine kidney ATPase in this range.

Discussion

We demonstrated the enzymatic properties of Na^+/K^+-ATPase of the silkworm *B. mori*, a Lepidopterous insect. The properties differed somewhat from those

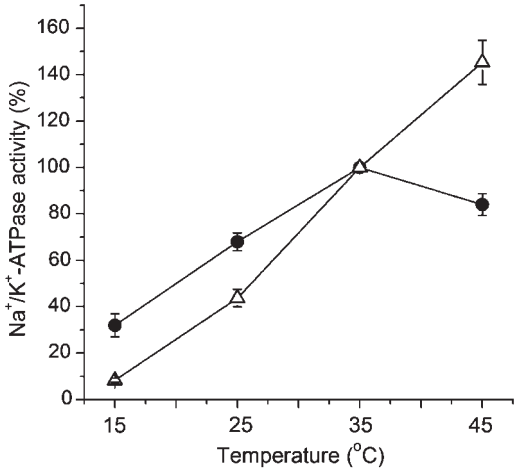

Fig. 11 Effect of temperature on silkworm and dog Na^+/K^+-ATPase activities. Reaction mixtures A and B without buffer were used to measure silkworm (filled circle) and dog (open triangle) Na^+/K^+-ATPase activity, respectively, as described in 'Materials and Methods' section. The pH values of reaction mixtures at 15, 25, 35 and 45°C were adjusted to 7.5 with 50 mM imidazole-HCl. Ouabain-sensitive ATPase activities were defined as Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activities were expressed as a percentage of the ATPase activity of silkworm and dog at 35°C. Symbols and bars represent the average ± SD from three different determinations.

Table I. Temperature sensitivity of silkworm Na^+/K^+-ATPase.

Temperature (°C) at			Na^+/K^+-ATPase activity	
Expt.	Pre-incubation	Incubation	nmol/min/mg	(%)
1	35	35	359 ± 28	(100)
2	45	35	228 ± 12	(64)
3	45	45	217 ± 1	(60)

Reaction mixture A containing 50 mM imidazole-HCl (pH 7.5 at 45°C) was pre-incubated at 35°C (Expt 1) and 45°C (Expts 2 and 3) for 30 min. Then, reaction mixture was shifted to 35°C in Expt 2. The ATPase reaction was started by addition of ATP and incubated at 35°C (Expts 1 and 2) and 45°C (Expt 2) for 30 min. Ouabain-sensitive ATPase was defined as Na^+/K^+-ATPase activity ($n = 3$).

of the dog, a mammal. The affinity for K^+ of silkworm Na^+/K^+-ATPase was one-sixth the affinity for K^+ of canine kidney Na^+/K^+-ATPase, whereas the affinity for Na^+ of silkworm Na^+/K^+-ATPase was two times the affinity for Na^+ of dog Na^+/K^+-ATPase under the ligand conditions tested (Figs 4 and 5). The low affinity for K^+ of silkworm Na^+/K^+-ATPase was supported by the difference in the K^+ concentrations required for activation of the two K^+-pNPPase activities (Fig. 6). The optimal $Na^+ : K^+$ ratio for silkworm Na^+/K^+-ATPase activity was 1 : 3–3 : 1, which was different from the $Na^+ : K^+$ ratio of 1 : 1–10 : 1 for dog Na^+/K^+-ATPase, due to the lower affinity for K^+ of silkworm Na^+/K^+-ATPase (Figs 4 and 5). The ratio of 1 : 3–3 : 1 was within the $Na^+ : K^+$ ratio of silkworm hemolymph, i.e. 14.6 mM Na^+ and 46.1 mM K^+ (*3*) and the $Na^+ : K^+$ ratio of the physiological saline buffer reported by Ai *et al.* (*20*), *i.e.* 12–28 mM NaCl and 32–16 mM KCl ($[Na^+] + [K^+] = 44$). Therefore, the Na^+ and K^+ concentrations in the blood–brain barrier, which surrounds insect nerve, do not seem to be greatly different from those in hemolymph. The optimal Mg^{2+} concentration was 3 mM for both the silkworm and canine Na^+/K^+-ATPases (Fig. 7). Because the Mg^{2+} concentrations in silkworm hemolymph are reported to be 101 mM (*3*), this result suggests that the Mg^{2+} concentration in the barrier is much lower than that in the hemolymph. The sensitivity of silkworm Na^+/K^+-ATPase activity to $CaCl_2$ was similar to the sensitivity of dog Na^+/K^+-ATPase activity (Fig. 8). $CaCl_2$ at >1 mM inhibited both Na^+/K^+-ATPase activities. Because the Ca^{2+} concentrations in silkworm hemolymph are reported to be 24.5 mM (*3*), this result also suggests that the Ca^{2+} concentration in the barrier is much lower than in the hemolymph. The blood–brain barrier is believed to limit the movements of water-soluble ions and small molecules between the hemolymph and neuronal surface in insects (*2*, *4*, *10*, *11*, *21*). The present data, however, suggest that the barrier completely limits movement of divalent cations but loosely limits that of monovalent cations, although the ionic composition in the barrier is unclear.

The ouabain concentration giving the half-maximal inhibition of silkworm Na^+/K^+-ATPase activity was 0.2 μM in the presence of 10 mM NaCl and 30 mM KCl. This value is close to the 0.25 μM for canine kidney Na^+/K^+-ATPase activity in the presence of 100 mM NaCl and 10 mM KCl under the incubation conditions used (Fig. 9). The inhibition curve was single phase (Fig. 9). Therefore, silkworm was suggested to have a kind of α-subunit isoform with high-sensitivity to ouabain, as shown in other insect Na^+/K^+-ATPases (*9*). Because silkworm Na^+/K^+-ATPase is highly sensitive to ouabain in the presence of a high K^+ concentration *in vitro* (Fig. 9), the resistance of phytophagous insects to ouabain *in vivo* (*6*, *7*) should be not due to antagonizing of ouabain binding to Na^+/K^+-ATPase by high K^+ concentration (*6*, *7*) but due to other mechanisms (*22*).

The optimal pH for silkworm Na^+/K^+-ATPase activity was near 7.5, consistent with that for dog Na^+/K^+-ATPase activity (Fig. 10). Silkworm Na^+/K^+-ATPase activity maintained >90% of the maximal activity in the pH range around 7.0. Because the pH of silkworm hemolymph is reported to be 6.5–6.7 (*23*, *24*), the present result is comparable to the pH values of hemolymph.

The optimal temperature for silkworm Na^+/K^+-ATPase activity was near 35°C, which is lower than the optimal temperature (45°C or higher) for dog Na^+/K^+-ATPase activity (Fig. 11). The decrease in the ATPase activity at 45°C was irreversible (Table I). The body temperature of dog, which is a homoiothermal animal, is maintained at ~38°C. Silkworm larvae are reared at 22–28°C. Since silkworm is a poikilothermal animal, its body temperature must be close to the ambient temperature. Therefore, the difference between the optimal temperatures for silkworm and dog Na^+/K^+-ATPase activities may correspond to the difference between the body temperatures for silkworm and dog.

The enzymatic properties of silkworm Na^+/K^+-ATPase demonstrated in this article led to a conclusion

that the properties are suitable for the growth of the silkworm, although they were somewhat different from those of mammalian Na^+/K^+-ATPase. The different properties of the two ATPases should directly correlate with the difference in the two ATPase structures. We failed to detect the β-subunit by immunoblotting analysis (Fig. 2) and did not find any information on the structure of silkworm Na^+/K^+-ATPase. Thus, we cannot discuss the difference of the enzymatic properties between silkworm and dog at molecular level in this article. Despite the failure to detect the β-subunit, we assume the β-subunit to be present because (i) the molecular size of the silkworm α-subunit is consistent with that of dog α-subunit (Fig. 2), (ii) Na^+/K^+-ATPase without the β-subunit has not been detected yet and (iii) the partially identified sequences for fruit fly, brine shrimp and mosquito β-subunits are reported in the database of silkworm (*25*). Sun and Salvaterra (*26*) have demonstrated that two nervous system-specific glycoproteins from the adult fruit fly *Drosophila*, a Dipterous insect, which were recognized by anti-horseradish peroxidase antibody, were homologous to the β-subunit of Na^+/K^+-ATPase. Their amino acid sequences had ~46% similarity and identity with mammalian β-subunits. The amino acid sequence of the silkworm Na^+/K^+-ATPase β-subunit probably also has a low identity with those of mammalian β-subunits. It is proposed that the β-subunit plays a critical role in K^+ binding in mammals (*27–30*). Therefore, sequencing the β-subunit amino acids might give an answer for the low affinity for K^+ of silkworm Na^+/K^+-ATPase.

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Conflict of interest

None declared.

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