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RESEARCH ARTICLE

Physiological adaptation of an Antarctic Na^+/K^+-ATPase to the cold

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SUMMARY

Because enzymatic activity is strongly suppressed by the cold, polar poikilotherms face significant adaptive challenges. For example, at 0°C the catalytic activity of a typical enzyme from a temperate organism is reduced by more than 90%. Enzymes embedded in the plasma membrane, such as the Na^+/K^+-ATPase, may be even more susceptible to the cold because of thermal effects on the lipid bilayer. Accordingly, adaptive changes in response to the cold may include adjustments to the enzyme or the surrounding lipid environment, or synergistic changes to both. To assess the contribution of the enzyme itself, we cloned orthologous Na^+/K^+-ATPase α-subunits from an Antarctic (*Pareledone* sp.; –1.8°C) and a temperate octopus (*Octopus bimaculatus*; ~18°C), and compared their turnover rates and temperature sensitivities in a heterologous expression system. The primary sequences of the two pumps were found to be highly similar (97% identity), with most differences being conservative changes involving hydrophobic residues. The physiology of the pumps was studied using an electrophysiological approach in intact *Xenopus* oocytes. The voltage dependence of the pumps was equivalent. However, at room temperature the maximum turnover rate of the Antarctic pump was found to be 25% higher than that of the temperate pump. In addition, the Antarctic pump exhibited a lower temperature sensitivity, leading to significantly higher relative activity at lower temperatures. Orthologous Na^+/K^+ pumps were then isolated from two tropical and two Arctic octopus. The temperature sensitivities of these pumps closely matched those of the temperate and Antarctic pumps, respectively. Thus, reduced thermal sensitivity appears to be a common mechanism driving cold adaptation in the Na^+/K^+-ATPase.

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Keywords: Antarctica, ion channels, ion transporters, Na^+/K^+-ATPase, octopus, temperature adaptation.

INTRODUCTION

The extreme cold imposes significant constraints on cellular function, principally by reducing the activity of the enzymes important for cellular functions like ion homeostasis. As with all dynamic proteins, the function of transmembrane enzymes is temperature dependent but, in addition, they are further constrained by cold-induced changes to the lipid bilayer's viscosity and order. Therefore, for transmembrane proteins, adaptation could be at the level of the protein and/or the lipid bilayer in which it is embedded. Homeoviscous adaptation is the most common theory describing how membrane systems adapt to temperature. The theory states that the lipid membrane maintains a constant viscosity to compensate for physical factors, most notably temperature (Sinensky, 1974). This is accomplished by changing the degree of saturation of the phospholipid acyl chains, the composition of the phospholipid head groups or the cholesterol content of the bilayer. Because membrane-bound enzymes operate within the viscous drag of the lipid bilayer, homeoviscous adaptation could help mitigate the effects of temperature on their activity. Although in a wide variety of organisms it is well established that membranes do indeed undergo homeoviscous adaptation (Behan-Martin et al., 1993; Cossins and Prosser, 1978; Cossins and Prosser, 1982; Pernet et al., 2007; Sinensky, 1974), its effect on the embedded proteins is not clear (Raynard and Cossins, 1991). Further, the extent to which the membrane-embedded enzymes themselves adapt to the cold is even less well understood.

Do transmembrane proteins undergo specific changes to better respond to the cold? This is likely, given molecular adaptation has been shown for soluble enzymes, particularly those involved in metabolic pathways or proteolysis. Early studies using enzymes purified from the tissues of related organisms that have evolved in different thermal environments showed an increased catalytic efficiency for the cold orthologs (Asgeirsson and Bjarnason, 1991; Asgeirsson and Bjarnason, 1993; Dahlhoff and Somero, 1993; Dong and Somero, 2009; Fields and Somero, 1998; Genicot et al., 1988; Graves and Somero, 1982; Hochachka and Somero, 2002; Kawall et al., 2002; Simpson and Haard, 1984; Yancey and Siebenaller, 1987). This was accomplished by modification of the catalytic constant k_{cat}, the Michaelis–Menten constant K_m, or both. Molecular cloning studies have uncovered specific molecular changes underlying thermal adaptation. In general, cold adaptation requires a small number of amino acid substitutions that serve to reduce weak interactions within the enzyme, resulting in greater flexibility (Feller et al., 1997; Feller and Gerday, 1997; Fields and Houseman, 2004; Fields et al., 2006; Hochachka and Somero, 2002; Holland et al., 1997; Johns and Somero, 2004; Siddiqui and Cavicchioli, 2006; Somero, 2004). Active sites themselves remain invariant, and most changes target adjacent hinge regions, leading to greater

mobility of the catalytic center. It seems likely that membrane-bound enzymes would undergo similar adaptations.

To better understand adaptation in a transmembrane protein, we chose to focus on the Na^+/K^+-ATPase. This enzyme couples the energy derived from ATP hydrolysis to the transport of three Na^+ ions out of the cell, and two K^+ ions into the cell. It is invariably composed of an α- and a β-subunit (in vertebrates it has an additional regulatory γ-subunit), although the α-subunit contains all domains required for ion transport. Na^+/K^+-ATPase activity is crucial for maintaining the ion homeostasis that drives electrical signaling and solute transport. It also consumes a large proportion of a typical cell's energy (estimates range from 20 to 70% of total ATP depending on the preparation) (Baker and Connelly, 1966; Balaban et al., 1980; Gregg and Milligan, 1980a; Gregg and Milligan, 1980b; Gregg and Milligan, 1982a; Gregg and Milligan, 1982b; Milligan and McBride, 1985; Webster, 1981). Because of its central importance, it would be expected to be under strong selection to operate efficiently. Even though the overall cellular Na^+/K^+-ATPase activity from cold-adapted organisms is high (Raynard and Cossins, 1991; Schwarzbaum et al., 1992; Schwarzbaum et al., 1991; Willis et al., 1980), the underlying molecular mechanisms are not well understood. In an attempt to better understand how Na^+/K^+-ATPase activity is up-regulated, and to assess the contribution of direct changes to the protein's architecture, we cloned closely related orthologs of the α-subunit from two members of the family Octopodidae. *Octopus bimaculatus* (hereafter referred to as 'temperate') was collected adjacent to Catalina Island, Southern California, where the water temperature was approximately 18°C. The temperature range for this species is ~14–22°C. *Pareledone* sp. (hereafter referred to as 'Antarctic') was collected next to McMurdo Station, Antarctica, where the water temperature remains a constant –1.8°C throughout the year, and has done so for at least 14 million years as a result of the establishment of the circumpolar current over 30 million years ago, which isolated the continent and set in motion gradual cooling (Bo et al., 2009; Barker and Thomas, 2004). Using an electrophysiological approach, we compared the transport properties of these two recombinant enzymes in a common background, the *Xenopus* oocyte expression system. When compared at room temperature, the Antarctic Na^+/K^+-ATPase exhibited a slightly higher turnover rate than the temperate isoform. More striking, because of different thermal sensitivities, the discrepancy between the turnover rates grew steadily as the temperature was reduced, being 4 times greater at 10°C. Na^+/K^+-ATPase orthologs isolated from multiple cold- and warm-adapted species exhibited a similar segregation of thermal sensitivities.

MATERIALS AND METHODS

Sample collection

Pareledone sp., Robson 1932, was collected during January from below the ice in McMurdo station, Antarctica. The specimen collected was kindly provided by Dr Chris DeVries. *Octopus bimaculatus*, Verrill 1883, was collected by SCUBA diving from Wrigley Marine Lab, Two Harbors, Catalina Island, CA, USA. *Octopus defilippi*, Vérany 1851, was collected from Rio Grande, Puerto Rico. *Octopus digueti*, Perrier and Rochebrune 1894, was collected from San Lucas Cove, Santa Rosalia, Baja California Sur, Mexico. *Bathypolypus arcticus* (Prosch 1847) and *Benthoctopus piscatorum* (Verrill 1879) were collected off the north coast of the Svalbard archipelago, Norway. From all samples, the stellate ganglia were dissected, preserved in RNA later (Ambion, Austin, TX, USA) and frozen at –80°C.

Molecular cloning

Total RNA was extracted from each stellate ganglion using the RNAqueous-Micro kit (Ambion) and used as a template for cDNA synthesis with the SuperScript III first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Full-length Na^+/K^+-ATPase α-subunits were amplified by PCR using Taq polymerase (for *O. bimaculatus* and *Pareledone* sp.) or Phusion polymerase (*O. defilippi*, *O. digueti*, *B. arcticus* and *B. piscatorum*) and primers based on the UTRs of a *Loligo opalescens* Na^+/K^+-ATPase α-subunit clone [GenBank: EF467998 (Colina et al., 2007)]. To obtain a consensus sequence, multiple cDNA clones were sequenced to completion.

Functional expression in *Xenopus* oocytes

The coding region of the Na^+/K^+-ATPase α-subunit for each species was cloned into the *Xenopus* expression vector pBSTA (Liman et al., 1992) and used as a template for cRNA synthesis with T7 promotor-based kits (mMessage mMachine T7 Ultra, Ambion; or mScript mRNA production system, Epicentre, Madison, WI, USA). cRNA was also made for the native β-subunit of the squid giant axon system [GenBank: EF467996 (Colina et al., 2007)]. Oocytes were injected with 80 ng of an equimolar mix of α- and β-subunit cRNAs. Experiments were performed 3–4 days after injection. Oocytes were surgically removed and de-folliculated by an enzymatic treatment with collagenase. Stage V and VI oocytes were selected for injection. After injection, oocytes were maintained for 3–4 days at 18°C in ND-96 solution (in $mmol\,l^{-1}$: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 Hepes; pH 7.6). Immediately prior to recording, oocytes were loaded with Na^+ for 45 min in a Na^+-loading solution (in $mmol\,l^{-1}$: 100 Na-glutamate, 2.5 Na-citrate and 5 Hepes; pH 7.5) (Rakowski et al., 1991).

Experimental solutions for electrophysiological measurements

The extracellular solutions employed to evaluate pump function throughout this study were $5\,mmol\,l^{-1}$ K^+ solution (in $mmol\,l^{-1}$: 100 Na-glutamate, 5 K-glutamate, 5 $BaCl_2$, 2 $NiCl_2$, 5 Hepes, 2 $MgCl_2$ and 0.3 niflumic acid) and K^+-free solution (same as $5\,mmol\,l^{-1}$ K^+ solution except that $5\,mmol\,l^{-1}$ *N*-methyl D-glucamine was substituted for the $5\,mmol\,l^{-1}$ K^+). Ouabain was used at a concentration of $100\,\mu mol\,l^{-1}$ in both the $5\,mmol\,l^{-1}$ K^+ or K^+-free solutions. When using the 'cut-open oocyte Vaseline-gap' technique (see below), the internal solution contained ($mmol\,l^{-1}$): 80 Na-glutamate, 20 TEA-glutamate, 10 $MgSO_4$, 10 Hepes, 5 EGTA and 5 MgATP. To gain electrical access to the inside, oocytes were permeabilized with 0.2% (w/v) saponin in the internal solution. The pH of all experimental solutions was adjusted to 7.5 with *N*-methyl D-glucamine at room temperature and was determined to vary by 0.2 units between 30 and 5°C.

Na^+/K^+-ATPase expression levels

Expression levels were assessed as the total ouabain-sensitive current recorded from whole oocytes expressing either construct. Oocytes were clamped using a conventional two-microelectrode voltage clamp amplifier (GeneClamp 500B; Axon Instruments, Foster City, CA, USA). Analog signals were filtered at 1 kHz and digitized using an Innovative Integrations SBC6711 A/D converter (Simi Valley, CA, USA). GPATCH software was kindly provided by Dr Francisco Bezanilla. Oocytes were held at 0 mV, and pumps were fully activated with the $5\,mmol\,l^{-1}$ K^+ solution, followed by addition of ouabain. Endogenous pump expression was recorded from un-injected oocytes. All experiments to assess expression level were performed at room temperature (~22°C).

Voltage dependence and turnover rate studies

Voltage dependence and turnover rate experiments were performed using the 'cut-open oocyte Vaseline-gap' technique (Taglialatela et al., 1992). Oocytes were clamped with a Dagan CA-1B high-performance oocyte clamp (Dagan Corporation, Minneapolis, MN, USA), sampling exclusively from the animal pole to minimize the signal from endogenous pumps (Holmgren and Rakowski, 1994). In these experiments, oocytes were held at 0 mV and stepped to various potentials ranging from –200 to +50 mV with 10 mV increments. The length of the pulses was either 35 or 60 ms. An Innovative Integrations SBC6711 A/D board with GPATCH software was used to control voltage protocols and digitize analog signals. Records on a slow time base ('chart records') were collected using a MiniDigi 1A board and AxoScope 9.0 software (Axon Instruments). Signals were acquired at 100 kHz and filtered at 10 kHz. Intracellular voltage was measured with a 0.2–0.3 MΩ pipette filled with 1 mol l^{-1} NaCl, and bridges filled with 3 mol l^{-1} Na-MES (4-morpholineethanesulfonic acid sodium salt) in 3% (w/v) agarose. In all experiments, the stability of the recordings was assessed by time controls, obtained from the difference between current–voltage (*I–V*) relationships obtained under the same experimental conditions at equivalent time intervals. These experiments were performed at ~22°C.

The voltage dependence of the Na^+/K^+-ATPase current was evaluated by performing the voltage protocol in 5 mmol l^{-1} K^+ solutions before and after the addition of 100 µmol l^{-1} ouabain. The difference between these recordings yielded the ouabain-sensitive pump current. Steady-state current produced at each voltage was measured and plotted. The potential for half-maximal current (mid-point; mean ± s.e.m.) for each Na^+/K^+-ATPase construct was measured from the *I–V* plot for each experiment and then averaged. The significance of the mid-point values was tested using a two-population *t*-test with α=0.05.

In order to determine the maximum turnover rate of the pumps, both the total number of functional pumps and the current they produce were estimated in the same oocyte. Maximal pump current was measured at 0 mV following 5 mmol l^{-1} K^+ activation (at voltages greater than –10 mV the pump current reaches its maximum). The number of pumps was determined by measuring charge translocation under Na^+/Na^+ exchange (K^+-free conditions). Immediately after 5 mmol l^{-1} K^+ activation, extracellular K^+ was removed to constrain pumps to Na^+ translocation steps, and the voltage protocol was repeated before and after addition of ouabain. The difference between these recordings yielded ouabain-sensitive transient currents with a negligible steady-state component. The area underneath these transient currents represents the amount of charge moved by the Na^+/K^+-ATPase at each voltage. The distribution of charge along the voltage (Q_V) follows a Boltzmann function:

$$Q_V = Q_{\min} + Q_{\mathrm{tot}} / \{1 + \exp[z_{\mathrm{q}} (V_{\mathrm{q}} - V_{\mathrm{m}}) F / RT]\}\,, \quad (1)$$

where $Q_{\min}$ is the minimum charge moved at negative potentials, Q_{tot} is the total amount of charge moved, z_q is the apparent valency of the charge moved, V_q is the mid-point voltage, V_m is the membrane potential, F is the Faraday constant, R is the molar gas constant and T is temperature. A Boltzmann fit of the charge–voltage (*Q–V*) relationship provides a reasonable estimate of the total amount of charge moved. By comparing the total charge moved with other means of counting pumps, in both guinea pig cardiac myocytes and *Xenopus* oocytes, it has been shown that each pump moves the equivalent of one elementary charge (Holmgren and Rakowski, 1994; Nakao and Gadsby, 1986). Thus, maximal turnover rate becomes the forward pump current (I_p) at potentials more positive than 0 mV divided by Q_{tot}. Turnover rates (means ± s.e.m.) were determined for individual experiments and averaged afterwards. The significance of the differences observed in the calculated turnover rates was tested using a two-population *t*-test with α=0.05.

Temperature sensitivity of the Na^+/K^+-ATPase turnover rate

The temperature sensitivity of the pumps was studied in whole oocytes using two-microelectrode voltage clamp. Oocytes were held at 0 mV in a chamber where the temperature was controlled using Peltier devices. Pumps were activated with 5 mmol l^{-1} K^+ at 30°C. Once fully activated, the temperature was progressively reduced to ~5°C and then returned to 30°C. This protocol was then repeated in the presence of ouabain to assess leak currents. During current recordings, temperature was recorded simultaneously by placing a thermocouple immediately adjacent to the oocyte. The output of the thermocouple was calibrated and collected at 1 kHz on-line. The temperature-dependent leak was negligible compared with the pump current. For each experiment, the amount of ouabain-sensitive current at each temperature was normalized to the value at 22°C. Normalized values obtained from individual experiments were then averaged for multiple oocytes. Estimates of the amount of endogenous pump current at each temperature were made using the magnitude of the endogenous current in un-injected oocytes at 22°C and the temperature sensitivity of the endogenous current (see supplementary material Fig. S1). The endogenous values were then subtracted from the data for the clone-injected oocytes. These values (means ± s.e.m.) were converted to turnover rates based on turnover rates determined at 22°C. In some cases data were normalized to the current value at 28°C.

RESULTS

Molecular cloning of Na^+/K^+-ATPase orthologs

Our first objective was to clone orthologous Na^+/K^+-ATPase α-subunit cDNAs from the two octopus species. As a starting point for our cloning strategy, we used the cDNA sequence of a squid Na^+/K^+-ATPase α-subunit that is expressed in the giant axon system [GenBank: EF467998 (Colina et al., 2007)] to clone octopus Na^+/K^+-ATPase α-subunits from the most similar region of their anatomy. In squid of the genus *Loligo*, and some others, the cell bodies for the giant axon reside in the giant fiber lobe of the stellate ganglion. These cell bodies send out axons that fuse to form the giant fiber (Young, 1938). The stellate ganglion also contains numerous cell bodies that send out axons that do not fuse. These small fibers parallel the giant fiber. Octopus do not possess giant axons, only a bundle of small fibers that project towards the mantle. Accordingly, their stellate ganglion does not have a giant fiber lobe. To clone octopus Na^+/K^+-ATPase cDNAs, we used total stellate ganglion RNA as a template for cDNA synthesis. Using oligonucleotide primers complementary to the 5′ and 3′ untranslated regions of the squid cDNA (sp112: CTTACACTTAGAGATGGCG, and sp113: GACTAATATGGCACAATCCTC), full-length cDNAs were amplified from the octopus samples. Multiple clones were sequenced to determine a consensus. Bioinformatics searches showed that the translations of both sequences were similar to other Na^+/K^+-ATPase α-subunits. The two sequences were found to be highly similar, sharing 97% identity (Fig. 1). In length, they differ by one amino acid. Of the 35 positions that differ, 25 involve changes in hydrophobicity. Eight differences occur within the transmembrane spans, and 11 within the nucleotide-binding domain. The rest are scattered throughout the molecule. The two octopus clones are ~93% identical to the clone from *Loligo*.

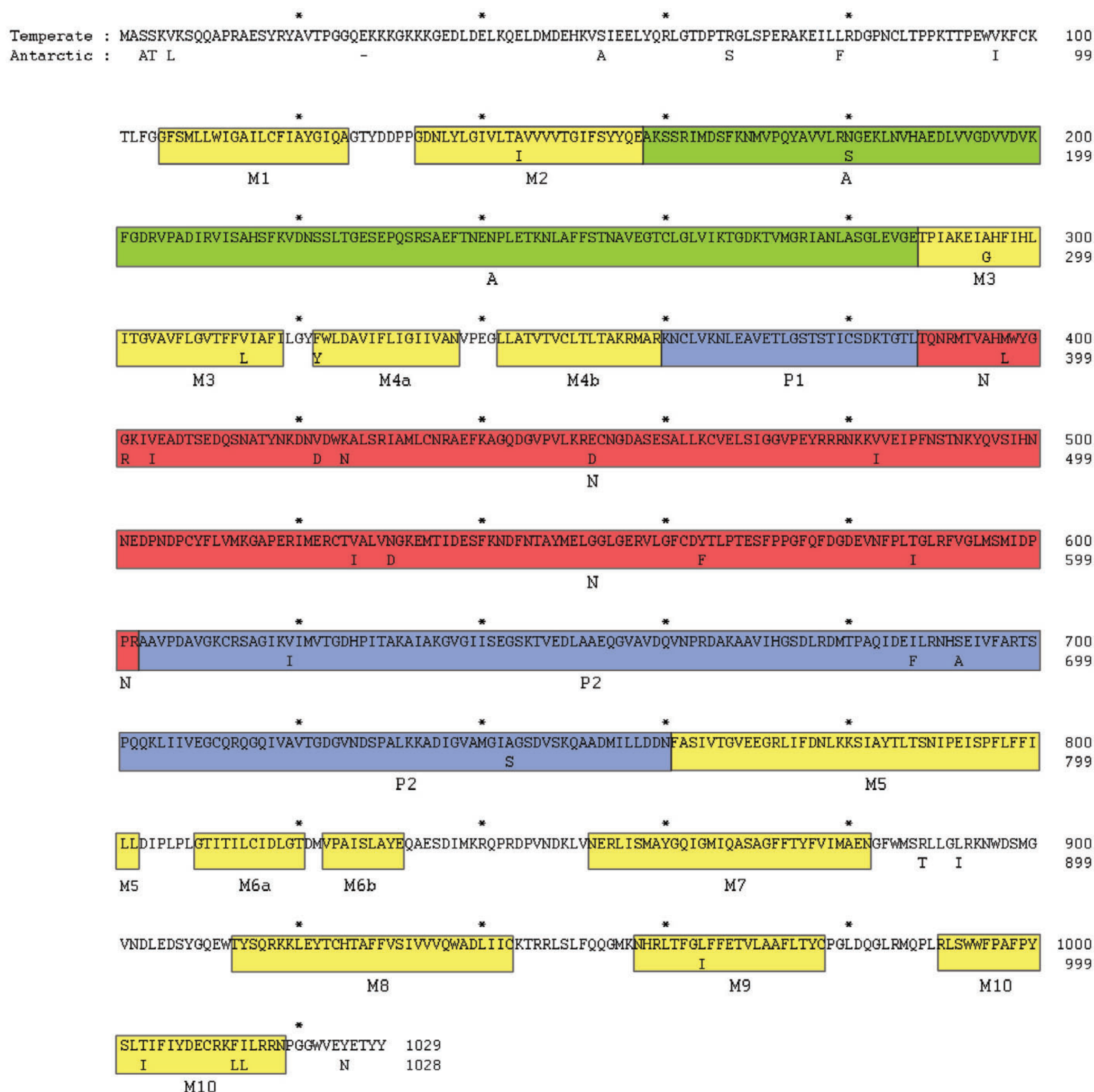

Fig. 1. Alignment of the predicted amino acid sequences for the Antarctic and temperate Na+/K+-ATPase. This figure shows the predicted amino acid sequence of the temperate Na+/K+-ATPase (top strand), and the residues that differ in the Antarctic Na+/K+-ATPase (bottom strand). Overall, they share 97% identity. The different domains of the enzyme are colored as follows: activation domain (A) in green; nucleotide-binding domain (N) in red; phosphorylation domain (P) in blue; and transmembrane spans (M1–M10) in yellow. GenBank accession numbers available from the authors on request.

Functional expression of octopus Na+/K+-ATPase α-subunits in *Xenopus* oocytes

Our primary goal was to compare the transport properties of the temperate and Antarctic octopus Na+/K+-ATPase α-subunit clones in a common background, in order to identify potential adaptive differences that arise from the pumps themselves. We chose an electrophysiological approach because it allowed us to conduct our studies in an intact cell that closely resembles native conditions. For example, native Na+/K+-ATPases are exposed to an asymmetric ionic distribution and a membrane potential, conditions that cannot be replicated with biochemical assays using pumps in membrane fragments. cRNA was synthesized from each clone and co-injected with a squid Na+/K+-ATPase β-subunit cRNA (Colina et al., 2007) into *Xenopus* oocytes. We chose to use a common β-subunit so that potential findings could be ascribed directly to the α-subunits. In addition, transport properties are largely determined by the α-subunits (Kaplan, 2002) and the squid β-subunit has the desirable property of not enhancing endogenous Na+/K+-ATPase levels in *Xenopus* oocytes (Colina et al., 2007).

To assess expression, oocytes were clamped at 0 mV and currents were activated with 5 mmol l^{-1} extracellular K^+ and then blocked with 100 μmol l^{-1} ouabain (Fig. 2). The ouabain-sensitive fraction was assumed to be mediated by the Na^+/K^+-ATPase. These experiments used a conventional two-microelectrode voltage clamp and therefore measured the current over the entire oocyte membrane. A first consideration was whether heterologously expressed pump currents were significantly greater than the small endogenous pump current in *Xenopus* oocytes (Eisner et al., 1987; Rakowski and Paxson, 1988). Fig. 2A shows examples of currents at 22°C from an un-injected oocyte and from oocytes injected with cRNA of either the Antarctic or temperate Na^+/K^+-ATPase. Clearly, the oocytes injected with cRNA express more ouabain-sensitive current. Analysis of multiple oocytes showed that the expression level of the Antarctic clone is 5 times, and that of the temperate clone 10 times, greater than the endogenous expression level (Fig. 2B). For some experiments we were able to further increase heterologous expression over background by using the 'cut-open oocyte Vaseline-gap' method (Taglialatela et al., 1992), which allowed us to record exclusively from the oocyte animal pole. Because endogenous Na^+/K^+-ATPase expression is twice as high in the vegetal pole, we effectively doubled the ratio of heterologous to endogenous expression (Colina et al., 2007) compared with experiments using the whole oocyte. This technique also changes the voltage very rapidly, a requirement for our measurements of voltage dependence and turnover rates.

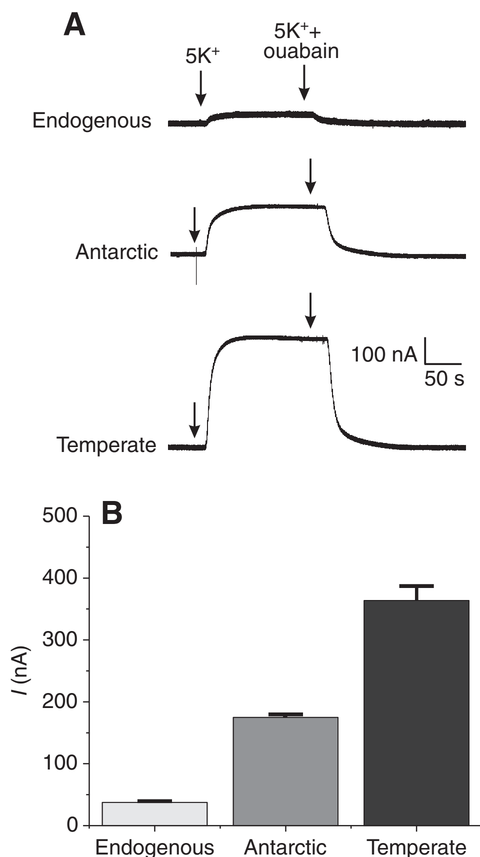

Fig. 2. Functional expression of octopus Na^+/K^+-ATPases in *Xenopus* oocytes. Recordings were made from whole oocytes using a conventional two-microelectrode voltage clamp. (A) Representative current traces for the endogenous, Antarctic and temperate Na^+/K^+-ATPases. The first arrow indicates when pump currents were activated with 5 mmol l^{-1} extracellular K^+ and the second arrow indicates when they were blocked with ouabain. Expression levels were taken as the ouabain-sensitive fraction of the current produced by activation with 5 mmol l^{-1} extracellular K^+. (B) The mean (±s.e.m.) expression levels were 37.4±2.2 nA (*N*=8) for the endogenous Na^+/K^+-ATPase, 174.8±5.0 nA (*N*=8) for the Antarctic Na^+/K^+-ATPase and 363.9±23.1 nA (*N*=10) for the temperate Na^+/K^+-ATPase.

Voltage dependence of octopus Na^+/K^+-ATPases

Na^+/K^+-ATPase transport is voltage dependent. Interestingly, at resting membrane potentials Na^+/K^+ pumps from a variety of organisms operate at about only half of their maximal capacity because of negative voltages inhibiting the Na^+ half of the transport cycle (Gadsby et al., 1985; Gadsby and Nakao, 1989; Gadsby et al., 1989; Goldshlegger et al., 1987; Nakao and Gadsby, 1986; Nakao and Gadsby, 1989; Rakowski and Paxson, 1988). When Na^+ ions leave their binding sites in the protein's core they are thought to exit through a narrow access channel that spans part of the transmembrane electric field (Gadsby et al., 1993; Hilgemann, 1994; Holmgren et al., 2000). High extracellular Na^+ concentrations and negative voltages tend to force Na^+ ions back to their binding sites, causing inhibition. Therefore, it is possible that the intrinsic voltage sensitivity could itself be a target for adaptation. Fig. 3A shows a chart record, recorded on a slow time scale, which illustrates our experimental approach. Using the 'cut-open oocyte Vaseline-gap' technique, oocytes were held at 0 mV in 5 mmol l^{-1} K^+ external solution to fully activate pump activity. *I–V* relationships were assessed by giving 60 ms pulses to voltages between –200 and +50 mV in 10 mV increments (rapid vertical current deflections). This protocol was repeated as a time control to assess the stability of the oocyte. Upon addition of ouabain to the external solution, the holding current decreased as the Na^+/K^+ pumps became inhibited. Once inhibition was complete, the *I–V* relationship protocol was repeated twice. Fig. 3B (middle) shows examples of ouabain-sensitive currents in response to voltage jumps from 0 to +40 mV or –140 mV. These pump currents were obtained from the difference between the current records before and after the application of ouabain. As can be seen, subtractions made in the same experimental conditions (time controls: 1–2 or 3–4) reveal minimal current, indicating that the preparation was stable over time. The difference before and after ouabain, however, shows robust currents composed of transient and steady-state components. The transient components are due to a voltage-dependent redistribution of Na^+ ions between the external solution and their binding sites (De Weer et al., 2001; Fendler et al., 1985; Gadsby et al., 1993; Hilgemann, 1994; Holmgren et al., 2000; Nakao and Gadsby, 1986). The steady-state component (vertical arrow in Fig. 3B) represents forward pump current at a particular voltage. Mean steady-state currents are plotted *vs* voltage in Fig. 3C. The voltage dependence was virtually identical for the two pumps (*Pareledone* sp. $V_{0.5}$=–127.0±3.0 mV; *O. bimaculatus* $V_{0.5}$=–127.0±5.0 mV, where $V_{0.5}$ is membrane potential at half-maximal current; *P*=0.99). Both reached a maximum at voltages greater than –10 mV.

Turnover rate of octopus Na^+/K^+-ATPases

The similarity between the voltage sensitivities of the two octopus Na^+/K^+-ATPases allowed us to directly compare the maximal turnover rates per pump under identical experimental conditions. To estimate the maximum turnover rate per pump, transport current and the number of pumps must be determined in the same experiment. Here again we used the 'cut-open oocyte Vaseline-gap' technique because it allowed us to control the intracellular solution. In the presence of saturating concentrations of ATP and Na^+ inside, and K^+ outside, and at a membrane potential of 0 mV, Na^+/K^+ pumps

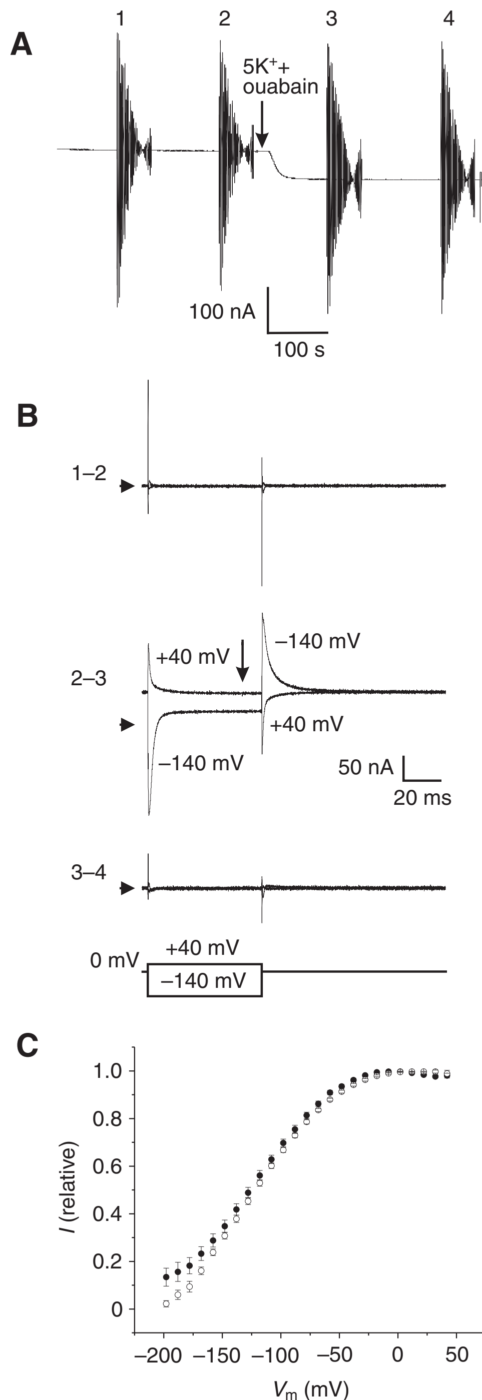

Fig. 3. Voltage dependence of the Antarctic and temperate Na^+/K^+-ATPases. (A) Chart recording illustrating the overall experiment used to test the voltage dependence of the Na^+/K^+-ATPases. *I–V* relationships were obtained in the presence of 5 mmol l^{-1} K^+, before and after ouabain treatment (1–4). The arrow indicates the application of 100 μmol l^{-1} ouabain. All experiments were performed at 22°C. (B) Ouabain-sensitive currents were obtained from the difference between the *I–V* patterns before and after ouabain addition (2–3). For simplicity, only currents generated in response to two selected voltages are shown. Ouabain-sensitive currents were bracketed by before and after ouabain time controls (1–2 and 3–4, respectively), subtraction of which shows no detectable current. Steady-state pump currents were measured at the vertical arrow. Horizontal arrowheads indicate the zero current level. (C) Mean *I–V* plot for the Antarctic (filled circles) and temperate (open circles) Na^+/K^+-ATPases. The ouabain-sensitive current (relative current) at each potential is plotted against voltage (membrane potential, V_m). The mid-point values (means ± s.e.m.) are identical for each construct (Antarctic, –127.0±3.0 mV, *N*=8; temperate, –127.0±5.0 mV, *N*=5; *P*=0.99). The maximum activity for both Na^+/K^+-ATPases reaches saturation at potentials above –10 mV.

will cycle around the scheme shown in Fig. 4A at their maximal velocity. The membrane current produced by pumps operating under these saturating substrate conditions can be determined by subtracting the membrane leak current in the absence of external K^+, a condition in which pumps are not able to complete the cycle (Fig. 4B; first 200 s of the experiment). To determine the number of underlying pumps, we maintained the oocyte in an extracellular solution that lacked K^+, a condition that restricts the pumps to distribute only among the states shaded in gray (Fig. 4A). These states represent binding/unbinding and occlusion/deocclusion of extracellular Na^+. Their occupancy can be manipulated by changing the membrane potential, where negative voltages favor the occluded state [$(Na_3)E_1$-P] and positive voltages favor the deoccluded state (P-E_2). The electrical signal associated with these transitions is obtained from the difference between the current records in the presence and absence of ouabain (Fig. 4B). Fig. 4C shows two examples of ouabain-sensitive currents (2–3) in response to the voltage protocols shown at the bottom. These transient currents originate because, in order to reach the extracellular milieu, Na^+ ions must traverse a portion of the electric field after leaving their binding sites, rendering these transitions voltage dependent. Therefore, a voltage step will rapidly change the local Na^+ concentration at the binding sites, causing a redistribution between the occluded and deoccluded states. As the system relaxes to the new equilibrium set by the voltage step, a transient current is produced with essentially no steady-state component because of the absence of external K^+. As can be visually appreciated, these transient currents are composed of more than one component, presumably representing the binding/unbinding and occlusion/deocclusion of different Na^+ ions. In cardiac myocytes and *Xenopus* oocytes (Holmgren and Rakowski, 1994; Nakao and Gadsby, 1986), the slow component of these transient currents has been successfully used to count the total number of pumps. This is achieved by integrating these currents to estimate the amount of charge moved at each voltage (Fig. 4D; charge moved upon return to 0 mV). The *Q–V* distribution follows a Boltzmann function with an apparent valency of 1, and the total amount of charge represents the total amount of pumps in the preparation. In the example shown in Fig. 4, the total amount of charge was 1506 pC, which corresponds to 9.4×10^9 pumps. In this experiment, the maximal current produced by these pumps was 35.6 nA, equivalent to 2.2×10^{11} cycles s^{-1} (in every cycle each pump transports a net +1 positive charge). Accordingly, for this oocyte we calculated the maximum turnover rate per pump to be 23.6 s^{-1}. Fig. 4E presents the mean maximum turnover rates for the Antarctic and temperate pumps recorded at 22°C. The turnover rate for the Antarctic pump (25.55±0.98 s^{-1}) is approximately 25% greater than that for the temperate pump (20.13±1.07 s^{-1}).

Temperature sensitivity of maximum turnover rate

At 22°C, which is close to the environmental temperature for the temperate octopus, the Antarctic Na^+/K^+-ATPase showed a small but significant enhancement of activity above that of the temperate

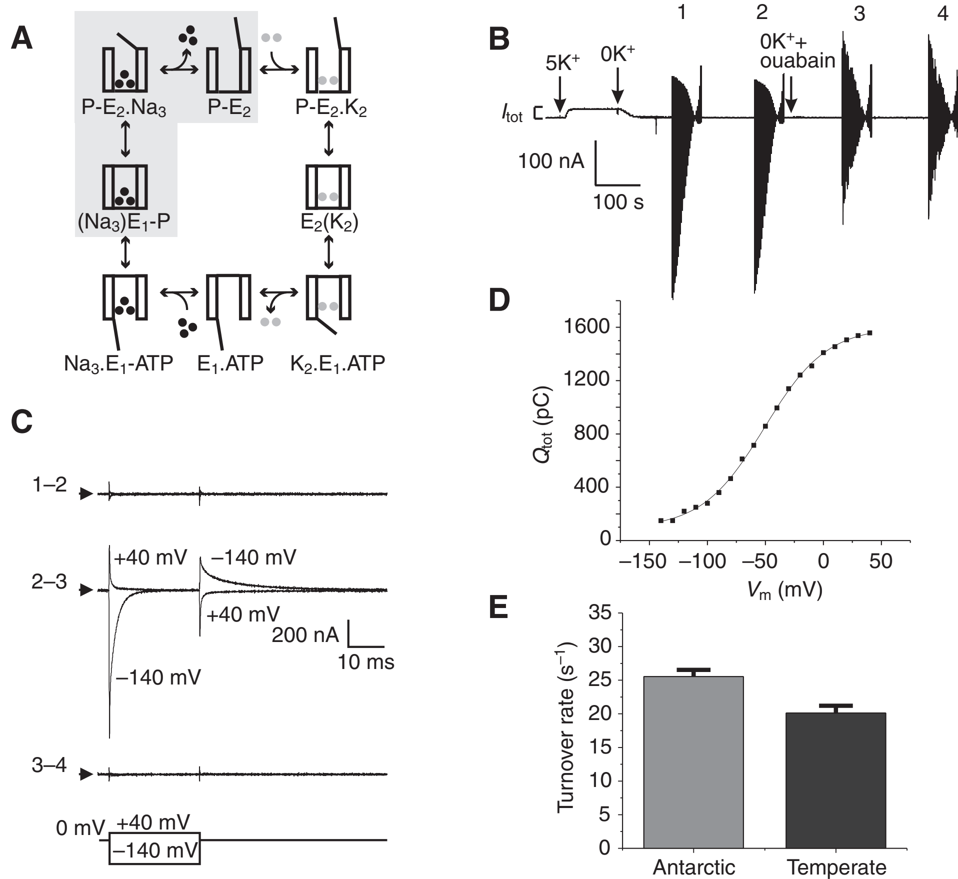

Fig. 4. Turnover rates of the Antarctic and temperate Na^+/K^+-ATPases. (A) Simplified Post–Albers model (Post et al., 1969; Albens, 1967) describing the transport cycle of the Na^+/K^+-ATPase. The conformational changes that export Na^+ ions (black circles) and import K^+ ions (gray circles) are induced by phosphorylation (P) and dephosphorylation reactions, respectively. These reactions force the enzyme to alternate exposure of the binding sites to the cytoplasm (E_1) or to the extracellular milieu (E_2). When deprived of extracellular K^+, the enzyme is constrained to Na^+ translocation steps. The states of the enzyme involved in Na^+ translocation are highlighted with a gray box. (B) Chart record illustrating the experimental approach used to measure the turnover rate of the pumps. The number of functional pumps and the total current (I_{tot}) they produce were measured from the same oocyte. Total pump current was considered to be the current produced by a 5 mmol l^{-1} K^+ activation. The number of pumps was determined by measuring charge translocation in the absence of extracellular K^+. *I–V* relationships were obtained before and after ouabain treatment (1–4). Experiments were performed at ~22°C. (C) Ouabain-sensitive transients (2–3) were bracketed by before and after ouabain time controls (1–2 and 3–4, respectively), which are essentially flat. Arrowheads indicate the zero current level. (D) Charge movement by the Na^+/K^+-ATPases. Charge movement was determined by integrating the ouabain-sensitive transients 0.5 ms after the offset of the voltage pulses (off transients). The voltage plot of charge movement was fitted to a Boltzmann distribution to determine total charge (Q_{tot}) moved by the pumps. The number of pumps was determined assuming one charge equivalent is moved per pump. (E) Turnover rates (means + s.e.m.) were calculated by dividing the number of ions transported per second by the total number of functional pumps. The Antarctic Na^+/K^+-ATPase turnover rate (25.55±0.98 s^{-1}, N=9) was significantly higher (P=0.002) than that of the temperate Na^+/K^+-ATPase (20.13±1.07 s^{-1}, N=10).

Na^+/K^+-ATPase. At –1.8°C, the environmental temperature for the Antarctic species, this difference could be substantially larger, depending on the temperature sensitivity of the two isoforms. We determined the thermal sensitivity for each pump over a temperature range between 5 and 30°C. For these experiments, we needed a relatively high expression level because signals were reduced at low temperatures, so we measured the Na^+/K^+ pump current from whole oocytes using a two-microelectrode voltage clamp protocol. Oocytes were held at 0 mV in a thermally controlled chamber. A thermocouple was placed immediately adjacent to the oocyte and temperature and current were recorded simultaneously. Fig. 5A shows an example of a typical experiment. Pump current was activated with 5 mmol l^{-1} K^+ starting at 30°C. The arrival of the 5 mmol l^{-1} K^+ solution caused a small downward deflection in the chamber temperature due to a slightly faster flow rate. Once currents were fully activated, and the temperature was stable at ~28°C, the flow of the solution was stopped. The chamber was then lowered to ~5°C and subsequently returned to 30°C while constantly monitoring the current. At the end of the run, ouabain was applied and the temperature cycle was repeated. Clearly, the vast majority of temperature-sensitive current is due to the Na^+/K^+-ATPase. The temperature-dependent leak current was negligible. At temperatures below 10°C, pump currents were too small to be measured with certainty. In separate experiments we also measured the temperature sensitivity of the endogenous pump current so that we could estimate its magnitude and subtract this from our data for the clones.

For both constructs, currents were strongly temperature dependent. Fig. 5B shows the temperature sensitivity of the maximum turnover rate (normalized to the maximum turnover rate estimated in Fig. 4) for the Antarctic and temperate Na^+/K^+-ATPases. They are quite different. Clearly, as temperature diminishes, the difference between the maximum turnover rates of the two pumps increases. At 10°C, the Antarctic Na^+/K^+-ATPase is ~4 times more active than the temperate ortholog. It should be

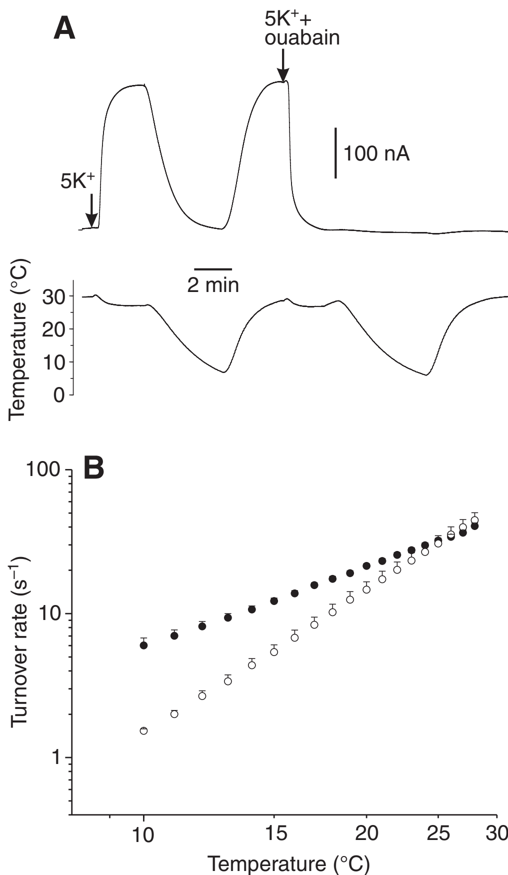

Fig. 5. Thermal sensitivity of the turnover rate of the Na^+/K^+-ATPases. (A) Measurement of the thermal sensitivity of each pump. After pumps were fully activated by 5 mmol l^{-1} K^+ (first arrow), the temperature was changed from 30 to 5°C and then returned to 30°C. This temperature cycle was then repeated in the presence of ouabain (second arrow) to assess the thermal sensitivity of the small leak current. Current and temperature were recorded simultaneously. (B) Thermal sensitivity of turnover rate. The relative proportion of current remaining after the decrease in temperature was measured at 1°C decrements, from 28 to 10°C, for both the Antarctic (filled circles) and temperate (open circles) Na^+/K^+-ATPase. These values (means ± s.e.m.) were then normalized to the turnover rates at 22°C calculated in Fig. 4. *N*=9 for the Antarctic Na^+/K^+-ATPase and *N*=10 for the temperate Na^+/K^+-ATPase.

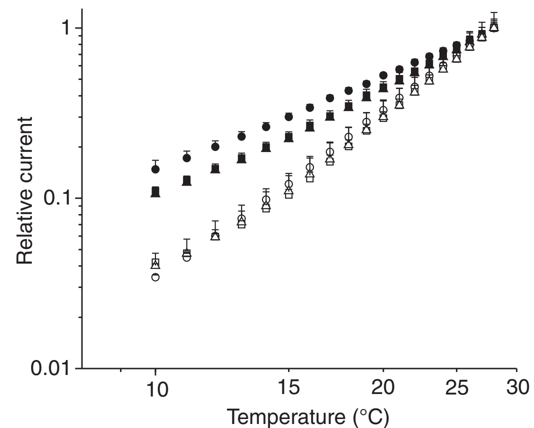

Fig. 6. Thermal sensitivity of cold-adapted and warm-adapted Na^+/K^+-ATPase orthologs. The symbols for warm-adapted clones are open and those for cold adapted clones are filled. Temperature sensitivity was determined as in Fig. 5, except that current values were normalized to the value at 28°C. The Na^+/K^+-ATPase orthologs studied were isolated from the following octopus species: *Pareledone* sp. (Antarctic, filled circles, *N*=9), *Bathypolypus arcticus* (Arctic, filled squares, *N*=5), *Benthoctopus piscatorum* (Arctic, filled triangles, *N*=6), *Octopus bimaculatus* (temperate, open circles, *N*=10), *Octopus defilippi* (tropical, open squares, *N*=6) and *Octopus digueti* (tropical, open triangles, *N*=5). The values are presented as means ± s.e.m.

noted that the temperature sensitivity of the endogenous oocyte Na^+/K^+-ATPase is virtually identical to that of the temperate pump (see supplementary material Fig. S1).

In comparative physiology, the use of only two species to draw conclusions about adaptive mechanisms is controversial (Garland and Adolph, 1994). To further support our findings regarding the mechanisms of cold adaptation, we isolated Na^+/K^+-ATPase orthologs from four additional octopus species. *Octopus defilippi* was captured from the north coast of Puerto Rico where the water was approximately 30°C. *Octopus digueti* was captured from a desert lagoon on the shores of the Gulf of California, from 35°C water. *Benthoctopus piscatorum* and *B. arcticus* were captured in a benthic trawl north of the Norwegian Svalbard archipelago from 0°C water. The amino acid sequences for these clones were highly similar (~95% identical; see supplementary material Fig. S2). All clones were expressed at high levels in *Xenopus* oocytes. Fig. 6 shows their normalized I_p temperature sensitivities plotted with those of the original temperate and Antarctic clones. Clearly, the temperature sensitivity of the Arctic pumps very closely resembles that of the Antarctic pump. The temperature sensitivities of all warm-adapted species are virtually identical.

DISCUSSION

In order to function properly, cells need to maintain a high intracellular K^+ concentration and to pump out excess Na^+. When faced with cold temperatures, ion homeostasis is challenged because the temperature sensitivity of Na^+ leaking into the cell is lower than that of the pumps that clear it (Hochachka, 1988). There are a variety of ways in which organisms that live in chronically cold conditions could adapt to this challenge. Perhaps the simplest mechanism would be to reduce the Na^+ leak so that an increase in pumping capacity would not be necessary (Schwarzbaum et al., 1991). Alternatively, a cell could augment transport by increasing the number of pumps (Schwarzbaum et al., 1991) or the turnover rate of individual pumps. The latter possibility could be accomplished by homeoviscous adaptation (Cossins et al., 1981; Raynard and Cossins, 1991; Schwarzbaum et al., 1992; Sinensky et al., 1979) or by direct modification of the Na^+/K^+-ATPase. This study focused on the second possibility: protein level differences between a temperate and an Antarctic Na^+/K^+-ATPase. We cloned, expressed and performed a comprehensive electrophysiological study of both orthologs using the *Xenopus* oocyte expression system and then verified our findings by isolating and expressing orthologs from four additional octopus species. Even though the lipid composition of *Xenopus* oocytes differs from that of octopus neurons, by using a common background we could assess the intrinsic response of the activity of these proteins to temperature. In this study we identified that the principal difference between cold- and warm-adapted pumps is that the former are less sensitive to temperature. This has important consequences for the turnover rate, particularly at cold temperatures.

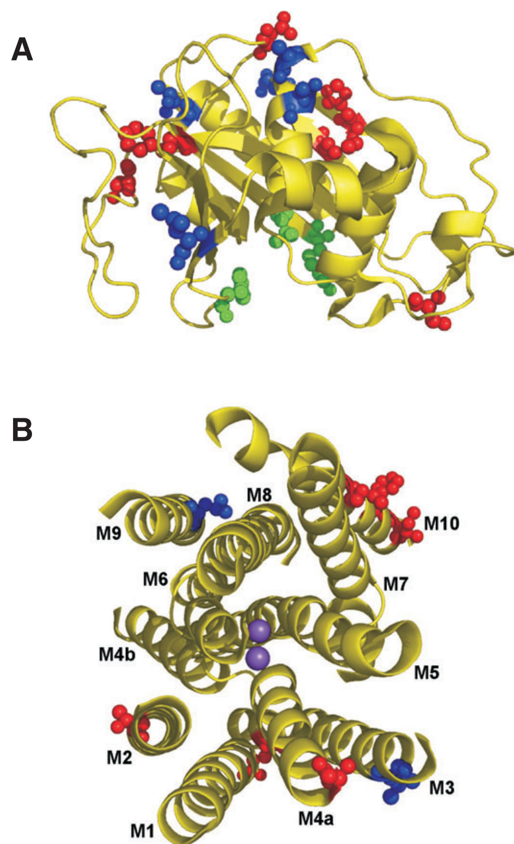

Fig. 7. Amino acid variations within the ATP-binding and transmembrane domains of the Antarctic and temperate Na^+/K^+-ATPases. The crystal structure of the shark rectal gland Na^+/K^+-ATPase (Protein Data Bank accession number 2ZXE) was used to map the amino acid positions within the ATP-binding domain (A) and transmembrane spans M1–M10 (B) that vary between the Antarctic and temperate orthologs. The amino acid side chains for the shark Na^+/K^+-ATPase at these positions are highlighted in red. Amino acid side chains highlighted in blue represent variable positions that correlate with the environmental temperature of all octopus species examined. The two purple spheres in B represent K^+ ions at their binding sites in the structure. Only a cartoon representation of the backbone for the transmembrane helices and ATP-binding domain is shown. For the variable positions given below, the numbers correspond to the amino acid position in the temperate Na^+/K^+-ATPase (see Fig. 1). The letters stand for the corresponding amino acid in the Antarctic, temperate and shark Na^+/K^+-ATPase, respectively. (A) Amino acid variations within the ATP-binding domain. The variable positions in the ATP-binding domain are: 397-LMM, 401-RGN, 404-IVH, 422-DVA, 425-NKS, 452-DES, 483-IVI, 526-IVT, 530-DNN, 564-FYF and 587-ITT. Variable positions that correlate with environmental temperature are: 401-RGN, 422-DVA, 425-NKS, 483-IVI and 526-IVT. Most substitutions within this domain are predicted to be accessible to the aqueous environment, and none are located within the ATP-binding pocket. Side chains relevant to ATP binding are highlighted in green. (B) Amino acid variations within transmembrane helices. The variable positions in transmembrane spans are: 144-IAT (M2); 295-GAE, 314-LVI (M3); 322-YFS (M4); 964-ILL (M9); and 1003-ITI, 1013-LFF, 1014-LII (M10). Variable positions that correlate with environmental temperature are: 314-LVI (M3) and 964-ILL (M9). Most substitutions in the transmembrane region were found to face the lipid environment.

Based on Fig. 5B, we predict that the Antarctic and temperate pumps would have equivalent turnover rates at temperatures greater than ~25°C. However, at 10°C, because of its lower thermal sensitivity, the Antarctic Na^+/K^+-ATPase activity is about 4 times greater than the temperate Na^+/K^+ activity. At its native temperature, we predict that this difference would be greater still. In spite of this, it is clear that there is not a complete compensation for the effects of temperature: at the native temperature, the turnover rate of the Antarctic Na^+/K^+-ATPase would be far lower than that of the temperate Na^+/K^+-ATPase. This seems reasonable. Not only would the ion leak be reduced by the cold, but other factors (e.g. increased Na^+/K^+-ATPase expression or homeoviscous changes to the lipid bilayer) could also play an important role. The cellular environments between a cephalopod nerve and a *Xenopus* oocyte are quite different, particularly with respect to the plasma membrane (Hill et al., 2005; Yamaguchi et al., 1987). Thus, the absolute turnover rates measured in this study could be different from those of the native pumps. However, a previous report on native Na^+/K^+-ATPases from *Octopus vulgaris* estimated turnover rates of 25–50 s^{-1} at 27°C. These values, obtained using a biochemical approach, agree well with our value of 35 s^{-1} at the same temperature (Turner et al., 2005).

In a number of systems it has been shown that psychrophilic enzymes have higher catalytic rates than warm-adapted orthologs (Asgeirsson and Bjarnason, 1991; Asgeirsson and Bjarnason, 1993; Fields and Somero, 1998; Genicot et al., 1988; Graves and Somero, 1982; Hochachka and Somero, 2002; Kawall et al., 2002; Simpson and Haard, 1984; Yancey and Siebenaller, 1987). Low and colleagues postulated that the higher activity results from specific mutations that lower the activation free energy of catalysis (Low et al., 1973). Further, they suggested that these mutations lower the activation free energy by reducing the activation enthalpy, leading to higher catalytic rates. At present, we cannot extract meaningful measurements of activation free energy and enthalpy from our data. This is because during the transport cycle the Na^+/K^+-ATPase undergoes at least 10 major conformational changes, all of which could have different temperature sensitivities. For example, when we make an Arrhenius plot of our turnover rate data, the relationship is not linear (data not shown) as is the case for cytosolic enzymes. We are presently working on electrophysiological methods to examine the rates of single transitions within the Na^+/K^+-ATPase transport cycle. These measurements should prove useful for more formal thermodynamic analyses, and will help shed light on whether membrane-bound enzymes, like the Na^+/K^+-ATPase, and cytosolic enzymes use similar strategies for temperature adaptation.

These data provide a foundation upon which to explore the mechanistic basis of an adaptive change in thermal sensitivity. The idea that cold adaptation is achieved by modifying the enzyme's temperature sensitivity is supported by both Arctic and Antarctic orthologs. There are only 35 differences between the primary structures of the Antarctic and temperate Na^+/K^+-ATPases. About one-third of these sites lie within the external surface of the ATP-binding domain (Fig. 7A), and many of the rest occur in transmembrane spans (Fig. 7B). All of these regions are known to undergo large-scale conformational rearrangements during the transport cycle (Morth et al., 2007; Ogawa et al., 2009; Shinoda et al., 2009; Sorensen et al., 2004; Toyoshima and Nomura, 2002; Toyoshima et al., 2004). The sequences from the Arctic and tropical orthologs give us additional resolution. When considering all the sequences presented in this study, eight variable positions correlate well with cold adaptation (see supplementary material Fig. S2). Of these, five are in the ATP-binding domain, two are in transmembrane spans and one is in an extracellular loop. None of the variable positions are located within the active site for ATP hydrolysis nor are they in the ion-binding sites. This finding is in agreement with sequence data from cold-adapted cytosolic enzymes where modifications leading to increased catalytic rates occur outside of the catalytic centers (see Hochachka and Somero, 2002).

Presumably, at least some of the octopus residues that scale with temperature are important for cold adaptation.

The thermal sensitivity of dynamic proteins is determined by the energetic landscapes between functional states that are shaped by protein–protein interactions and protein–substrate interactions. These interactions define the stability of a particular conformational state or the transition pathway between two states. Previous studies have shown that the temperature sensitivity of the Na^+/K^+-ATPase is not linear (Esmann and Skou, 1988; Friedrich et al., 1996; Glitsch and Pusch, 1984). In theory, the different thermal sensitivities of the Antarctic and temperate pumps could be explained by temperature-induced changes to their affinities for substrates (e.g. extracellular K^+ or intracellular Na^+ and ATP). However, we consider this unlikely because substrate concentrations were at saturating levels at room temperature, and previous studies on the Na^+/K^+-ATPase have shown that the cold increases the apparent affinity for extracellular K^+ and intracellular Na^+ (Ellory and Willis, 1982), and for ATP (Marjanovic and Willis, 1992). Furthermore, both the ATP-binding pocket and cation-binding sites of the Antarctic and temperate orthologs are conserved.

For membrane enzymes like the Na^+/K^+-ATPase, thermal sensitivity could also be influenced by interactions between the protein and the lipids of the cell membrane. Interestingly, almost all of the differences between the Antarctic and temperate pumps that occur in the transmembrane spans cause small changes in hydrophobicity at positions facing the lipid bilayer (Fig. 7B). Two of these positions correlate well with the thermal environment of all the species studied. In addition, the temperate and tropical Na^+/K^+-ATPases show very high thermal sensitivity at low temperatures. High temperature sensitivity at low temperatures has been noted before. A good example is action potential repolarization in squid axon, particularly in tropical species (Hodgkin and Katz, 1949; Rosenthal and Bezanilla, 2002). Such a marked difference between cold- and warm-adapted species at low temperatures may relate to how their enzymes withstand fluidity changes of the plasma membrane. Previous studies on native pumps have shown that the degree of acyl chain saturation within the plasma membrane correlates well with Na^+/K^+-ATPase activity (Else et al., 1996; Else and Wu, 1999). Other studies using reconstituted pumps have hypothesized that cholesterol makes a direct interaction, regulating the turnover rate in a manner that depends on acyl chain length and saturation (Cornelius, 1995; Cornelius, 2001; Cornelius, 2008). In this paper, we have shown that changes to a membrane protein make a contribution to thermal adaptation. A synergy between these changes and modifications to the bilayer could also play an important role. Because there are few differences between all pump orthologs that scale with temperature, the specific residues that underlie cold adaptation should be identifiable.

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