

# Quantitative calculation of the role of the $\text{Na}^+, \text{K}^+$ -ATPase in thermogenesis

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## A B S T R A C T

The  $\text{Na}^+, \text{K}^+$  ATPase is accepted as an important source of heat generation (thermogenesis) in animals. Based on information gained on the kinetics of the enzyme's partial reactions we consider via computer simulation whether modifications to the function of the combined  $\text{Na}^+, \text{K}^+$  ATPase/plasma membrane complex system could lead to an increased body temperature, either through the course of evolution or during an individual's lifespan. The enzyme's kinetics must be considered because it is the rate of heat generation which determines body temperature, not simply the amount of heat per enzymatic cycle. The results obtained indicate that a decrease in thermodynamic efficiency of the  $\text{Na}^+, \text{K}^+$  ATPase, which could come about by  $\text{Na}^+$  substituting for  $\text{K}^+$  on the enzyme's extracellular face, could not account for increased thermogenesis. The only feasible mechanisms are an increase in the enzyme's expression level or an increase in its ion pumping activity. The major source of  $\text{Na}^+, \text{K}^+$  ATPase related thermogenesis (72% of heat production) is found to derive from passive  $\text{Na}^+$  diffusion into the cell, which counterbalances outward  $\text{Na}^+$  pumping to maintain a constant  $\text{Na}^+$  concentration gradient across the membrane. A simultaneous increase in both  $\text{Na}^+, \text{K}^+$  ATPase activity and the membrane's passive  $\text{Na}^+$  permeability could promote a higher body temperature.

### Keywords:

Sodium pump  
Heat generation  
Albers-Post cycle  
Transport pathways  
Kinetic simulations  
Power output

## 1. Introduction

In general there are two major theories or contributions to non shivering heat generation in animals. One involves ATP synthesis; the other involves ATP utilisation. ATP synthesis occurs to the greatest extent during aerobic respiration in the mitochondria, where energy released in the electron transport chain is stored in the form of an  $\text{H}^+$  gradient across the inner mitochondrial membrane. If this membrane is leaky towards  $\text{H}^+$  ions, rather than the  $\text{H}^+$  ions passing through the ATP synthase, coupling  $\text{H}^+$  diffusion to ATP synthesis, some of the  $\text{H}^+$  ions can pass across the membrane, releasing energy in the form of heat. If this process was not counteracted in any way, the  $\text{H}^+$  gradient across the inner mitochondrial membrane would be partially dissipated, and the ability of the mitochondria to synthesise ATP would be compromised. Therefore, any increase in the  $\text{H}^+$  leak across the membrane must be compensated for by an increase in  $\text{H}^+$  pumping out of the internal mitochondrial matrix by the protein complexes of the electron transport chain (i.e., the NADH CoQ reductase, CoQ cytochrome c reductase and cytochrome c oxidase), either by an increase in their expression levels or their intrinsic activities.

This mechanism of heat generation is thought to occur particularly within the mitochondria of cells of brown adipose tissue [1,2], whose inner mitochondrial membranes contain uncoupler proteins which enhance the passive  $\text{H}^+$  permeability. However, brown adipose tissue is not present at all in birds and only in very small amounts in adult humans [1]. Therefore, the development of brown adipose tissue as a means by which endothermy (i.e. warmbloodedness) could have evolved seems untenable in birds and its contribution to the constant high body temperature of adult humans seems likely to be negligible, although recruitment of brown adipose tissue in adult humans as an adaptive mechanism under conditions of prolonged cold exposure (e.g. hours or more) during an individual's lifespan cannot be excluded. Nevertheless, regardless of the role of brown adipose tissue, a basal proton leak across the inner mitochondrial membrane in the cells of all tissues (except of course tissues whose cells don't contain mitochondria, e.g. red blood cells), is thought to make an approximately 20% contribution to heat generation in mammals [3].

The second major source of heat generation involves ATP utilisation. ATP is the major biological energy source, driving countless biological reactions. If the energy released by ATP hydrolysis exceeds that required to drive a biological reaction, any excess energy is released as heat. Direct evidence for heat production within a living cell caused by the activity of the  $\text{Ca}^{2+}$  ATPase in the endoplasmic

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reticulum of a HeLa cell has been reported by Suzuki et al. [4], in support of earlier work indicating a role of the  $\text{Ca}^{2+}$  ATPase in thermo genesis [5]. Recent theoretical calculations [6,7] have indicated that the thermodynamic efficiency of the  $\text{Ca}^{2+}$  ATPase from sarcoplasmic reticulum is far below 100%, i.e., a significant amount of the energy released by ATP hydrolysis is dissipated as heat rather than being used for ion pumping. De Meis et al. [5] have provided evidence that the  $\text{Ca}^{2+}$  ATPase can vary the relative amounts of energy it uses for the work of ion pumping and converts into heat by varying the amount of enzyme passing through an alternative catalytic pathway in which ATP is hydrolysed but no  $\text{Ca}^{2+}$  ions are transported. However, because the  $\text{Na}^+, \text{K}^+$  ATPase utilizes more ATP than any other animal enzyme [3], we concentrate here on its heat generation, rather than that of its related enzyme, the  $\text{Ca}^{2+}$  ATPase. As pointed out by de Meis et al. [5], there is no experimental data available showing whether or not the  $\text{Na}^+, \text{K}^+$  ATPase is able to vary the amount of heat it produces or work it does. However, regardless of whether or not a mechanism exists for varying thermodynamic efficiency, the decisive point is how fast heat is generated. Body temperature is influenced by the power output in the form of heat, not simply the amount of heat produced per enzymatic cycle. Therefore, in any consideration of mechanisms of changing body temperature the kinetics of thermogenesis is the crucial factor. For this reason we will present detailed kinetic modelling of  $\text{Na}^+, \text{K}^+$  ATPase activity based on information obtained on the enzyme's partial reactions. However, it is insufficient to consider only the heat released directly by the  $\text{Na}^+, \text{K}^+$  ATPase through ATP hydrolysis. As  $\text{Na}^+$  and  $\text{K}^+$  ions diffuse passively across the plasma membrane in the directions opposite to their pumping direction, thus setting up a steady state, much of the energy of ATP hydrolysis which was stored in the  $\text{Na}^+$  and  $\text{K}^+$  electrochemical potential gradients created by the  $\text{Na}^+, \text{K}^+$  ATPase is re released as heat. Thus, both the direct and indirect sources of heat through ion pumping and ion diffusion need to be considered together.

The idea that  $\text{Na}^+, \text{K}^+$  ATPase activity is an important determinant of an animal's ability to generate heat has been around since the 1970s [8–10]. The important point is that only now, because of the accumulated information on the kinetics of the enzyme's partial reactions, has a quantification of the actual amount of heat it generates under physiological conditions become possible. In principle an increase in the turnover of the  $\text{Na}^+, \text{K}^+$  ATPase's kinetic cycle, a decrease in its thermodynamic efficiency (i.e., the proportion of energy utilised per pump cycle in ion pumping relative to the proportion released as heat) or an increase in its expression within the plasma membrane could all lead to an increased rate of heat generation. Here we will consider via computer simulations whether a change in thermodynamic efficiency of the enzyme could have contributed to the evolution of endothermy. Within the enzyme's mechanism a change in thermodynamic efficiency could, in principle, easily come about by a change in the specificities of the enzyme's ion binding sites for  $\text{Na}^+$  and  $\text{K}^+$ , in particular an increased degree of competition from  $\text{Na}^+$  for the occupation of  $\text{K}^+$  binding sites on the phosphorylated form of the protein. In fact voltage clamp measurements carried out by Peluffo et al. [11] have shown that substitution of the amino acid glutamate for alanine at position 779 on the  $\alpha$  subunit of the enzyme dramatically increases the level of electrogenic  $\text{Na}^+$   $\text{Na}^+$  exchange activity relative to  $\text{Na}^+$   $\text{K}^+$  exchange activity. However, whether a change in the relative amounts of  $\text{Na}^+$   $\text{Na}^+$  and  $\text{Na}^+$   $\text{K}^+$  exchange is a feasible mechanism whereby an increase in heat generation could come about depends crucially on the kinetics of the pump cycle.

## 2. Materials and methods

### 2.1. Pump turnover simulations

Computer simulations of the steady state  $\text{Na}^+, \text{K}^+$  ATPase turnover were performed using the commercially available program Berkeley Madonna 8.0 and the variable step size Rosenbrock integration method

for stiff systems of differential equations. The simulations yielded the time course of the concentration of each enzyme intermediate involved and the steady state flux through each of the enzyme's parallel transport pathways. For the purposes of simulations, each enzyme intermediate was normalized to a unitary concentration and the enzyme was assumed arbitrarily to be initially totally in the E1 state. To determine the steady state flux through each pathway, each simulation was carried out until the distribution between the different enzyme states no longer changed and the fluxes reached constant values.

## 3. Results

### 3.1. Heat generation by $\text{Na}^+, \text{K}^+$ and $\text{Na}^+, \text{Na}^+$ exchange

The mechanism of the  $\text{Na}^+, \text{K}^+$  ATPase is generally described by the Albers Post scheme (see Fig. 1), which involves a sequence of  $\text{Na}^+$  and  $\text{K}^+$  binding steps, enzyme conformational changes, ATP phosphorylation and dephosphorylation. One of the binding sites on the E1 state of the enzyme, which opens towards the cytoplasm, is very specific for  $\text{Na}^+$  and cannot be replaced by  $\text{K}^+$ . However, in the E2P state competition between extracellular  $\text{Na}^+$  and  $\text{K}^+$  ions for two of the enzyme's binding sites occurs and it is theoretically possible for the phosphorylated enzyme to be dephosphorylated in the presence of  $\text{Na}^+$  rather than  $\text{K}^+$ . If this occurs, the pump cycle could transport  $3\text{Na}^+$  ions out of the cell and  $2\text{Na}^+$  in, giving a net transport of  $1\text{Na}^+$  out, rather than the more usual mode of transport involving  $3\text{Na}^+$  being transported out and  $2\text{K}^+$  ions in per ATP hydrolysed. The different transport modes have significantly different thermodynamic efficiencies, i.e. they produce different amounts of heat per ATP hydrolysed.

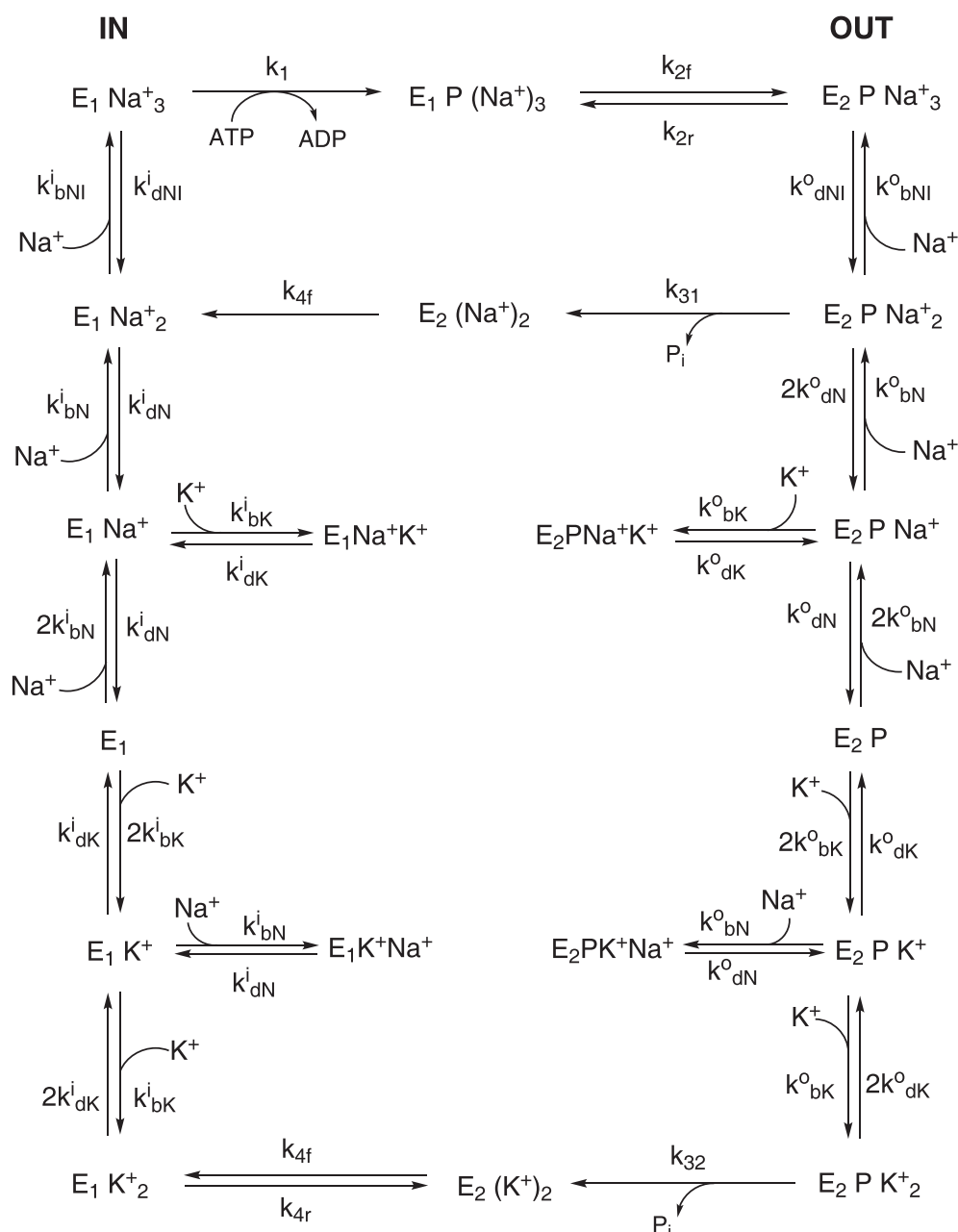
In the usual  $3\text{Na}^+/2\text{K}^+$  exchange mode of the  $\text{Na}^+, \text{K}^+$  ATPase, based on a consideration of the electrochemical potential gradients for both ions across the plasma membrane it can be shown that the work done by the enzyme in ion pumping is given by,

$$w_{\text{Na},\text{K}} = RT \left( 3 \ln \frac{c_{\text{Na}}^o}{c_{\text{Na}}^i} - 2 \ln \frac{c_{\text{K}}^o}{c_{\text{K}}^i} \right) - FV_m \quad (1)$$

where  $R$  is the ideal gas constant,  $T$  is the absolute temperature,  $c_{\text{Na}}^o$ ,  $c_{\text{Na}}^i$ ,  $c_{\text{K}}^o$  and  $c_{\text{K}}^i$  represent the  $\text{Na}^+$  and  $\text{K}^+$  concentrations either inside (in the cytoplasm) or outside the cell,  $F$  is Faraday's constant and  $V_m$  is the transmembrane electrical potential, defined as the electrical potential inside minus that outside the cell. If  $\text{Na}^+$  ions substitute for  $\text{K}^+$  on the phosphorylated form of the enzyme, such that the enzyme undergoes a  $3\text{Na}^+/2\text{Na}^+$  exchange (also known as  $\text{Na}^+$  ATPase activity), the work done by the enzyme in ion pumping is in this case:

$$w_{\text{Na},\text{Na}} = RT \ln \frac{c_{\text{Na}}^o}{c_{\text{Na}}^i} - FV_m \quad (2)$$

Typical values of the  $\text{Na}^+$  and  $\text{K}^+$  concentrations are  $c_{\text{Na}}^o = 140$  mM,  $c_{\text{Na}}^i = 15$  mM,  $c_{\text{K}}^o = 4$  mM and  $c_{\text{K}}^i = 120$  mM [12]. However, in order to calculate  $w_{\text{Na},\text{K}}$  and  $w_{\text{Na},\text{Na}}$  one also needs to know the value of  $V_m$ , which depends not only on the  $\text{Na}^+$  and  $\text{K}^+$  concentrations on each side of the membrane, but also on the distribution of  $\text{Cl}^-$  ions. In the case of many animal cells (red blood cells being a notable exception) typical  $\text{Cl}^-$  concentrations are  $c_{\text{Cl}}^o = 116$  mM and  $c_{\text{Cl}}^i = 4$  mM [13]. Typical permeabilities of the three ions for mammalian cells under resting conditions are  $P_{\text{K}} = 5 \times 10^{-7}$  cm s $^{-1}$ ,  $P_{\text{Na}} = 5 \times 10^{-9}$  cm s $^{-1}$  and  $P_{\text{Cl}} = 1 \times 10^{-8}$  cm s $^{-1}$  [13]. Inserting these values of the permeabilities as well as the concentrations of each of the three ions into the Goldman Hodgkin Katz equation one arrives at a value for  $V_m$  of  $-79$  mV at  $24^\circ\text{C}$ . We have chosen to do the calculation at  $24^\circ\text{C}$  because much of the kinetic data on the  $\text{Na}^+, \text{K}^+$  ATPase which we will use later was obtained at this temperature. If the calculation is



**Fig. 1.** Albers-Post scheme describing the Na<sup>+</sup>,K<sup>+</sup>-ATPase catalytic mechanism. The reactions drawn horizontally are: (1) Phosphorylation by ATP and occlusion of Na<sup>+</sup> within the protein, (2) Conformational change of the phosphorylated protein, deoccluding the Na<sup>+</sup> ions and giving them access to the extracellular medium, (3) Occlusion of K<sup>+</sup> within the protein and dephosphorylation, and (4) Conformational change of the unphosphorylated protein, deoccluding the bound ions and giving them access to the cytoplasm. Reactions 1 and 2 are coupled to reactions 3 and 4 by a series of vertically drawn ion binding and dissociation reactions on the cytoplasmic face of the protein (superscript i, on the left) and on the extracellular face (superscript o, on the right). Two possible enzymatic pathways are considered, with 3Na<sup>+</sup> ions being pumped from the cytoplasm to the extracellular medium in exchange for either 2Na<sup>+</sup> ions (Na<sup>+</sup>/Na<sup>+</sup> exchange mode) or 2K<sup>+</sup> ions (Na<sup>+</sup>/K<sup>+</sup> exchange mode).

performed at the normal human body temperature of 37 °C, this results in only a 4% increase in  $V_m$ , assuming no change in the relative values of the permeability coefficients of the ions. Utilizing the value of  $V_m$  just calculated and the Na<sup>+</sup> and K<sup>+</sup> concentrations given above, values for  $w_{Na,K}$  and  $w_{Na,Na}$  can be calculated to be 41.0 kJ mol<sup>-1</sup> and 13.1 kJ mol<sup>-1</sup>, respectively, at 24 °C. The permeabilities and ion concentrations of mammalian cells may vary from species to species and tissue to tissue. Therefore, if values of these parameters are available for a specific tissue of a specific species, the calculations of  $w_{Na,K}$  and  $w_{Na,Na}$  could be repeated if desired. For a given species this would allow the relative contributions of different tissues to thermogenesis to be determined. However, because muscle cells require a relatively large amount of expressed Na<sup>+</sup>,K<sup>+</sup> ATPase to pump out Na<sup>+</sup> ions

which flow into the cytoplasm through voltage gated Na<sup>+</sup> channels at each action potential and because muscle is a significant component of soft body mass, it appears likely that muscle tissue would be a likely major contributor to Na<sup>+</sup>,K<sup>+</sup> ATPase related heat generation.

Before continuing, it is worthwhile to point out that the energy released by ATP hydrolysis initially does work by causing conformational changes of the protein. However, regardless of which enzyme state one takes as the starting conformation, after each enzymatic cycle the enzyme reverts to its initial state. Hence the only net work done is that of ion pumping and any heat generated by the conformational changes is included in the amount of heat released by ATP hydrolysis itself.

Now, in order to determine the amount of heat released in each catalytic cycle of the Na<sup>+</sup>,K<sup>+</sup> ATPase in the Na<sup>+</sup>,K<sup>+</sup> and Na<sup>+</sup>,Na<sup>+</sup>

exchange modes one needs to know how much total energy is released via ATP hydrolysis. The maximum energy available for the work of ion pumping (i.e. the free energy of ATP hydrolysis,  $\Delta G$ ) is given by,

$$\Delta G = RT \ln \left( \frac{c_{ADP} c_P}{c_{ATP} K_h} \right) \quad (3)$$

where  $c_{ADP}$ ,  $c_{ATP}$  and  $c_P$  are the concentrations of ADP, ATP and inorganic phosphate within the cytoplasm of the cell, respectively, and  $K_h$  is the equilibrium constant for the ATP hydrolysis reaction based on a standard state of 1 M [14]. According to Lauger [14], typical values for the concentrations are  $c_{ADP} = 0.1$  mM,  $c_{ATP} = 5$  mM and  $c_P = 5$  mM and the value of  $K_h$  at 25 °C is approximately  $4 \times 10^5$ . Based on these values one can calculate a value for  $\Delta G$  from Eq. (3) of  $-54.6$  kJ mol<sup>-1</sup>. This value agrees quite well with the value of  $-52$  kJ mol<sup>-1</sup> estimated by Freeman et al. [15] and is also quite close to the enthalpy change,  $\Delta H$ , accompanying ATP hydrolysis by the Na<sup>+</sup>,K<sup>+</sup> ATPase, which has been determined experimentally using the technique of isothermal titration microcalorimetry to be  $-51 (\pm 1)$  kJ mol<sup>-1</sup> [16]. This indicates that the entropy change ( $\Delta S$ ) accompanying ATP hydrolysis and the proportion of energy released by ATP hydrolysis that cannot be harnessed to do the work of ion pumping ( $T\Delta S$ ) are both relatively minor.

According to the first law of thermodynamics, energy can be transferred in only two ways, as heat or work. Therefore, the amount of heat,  $q$ , released by ATP hydrolysis per catalytic cycle of the Na<sup>+</sup>,K<sup>+</sup> ATPase in the Na<sup>+</sup>,K<sup>+</sup> and Na<sup>+</sup>,Na<sup>+</sup> exchange modes can be calculated by subtracting the work,  $w$ , done in ion pumping from the free energy available from ATP hydrolysis. This gives the following two values:

$$q_{Na,K} = -\Delta G - w_{Na,K} = 54.6 - 41.0 = 13.6 \text{ kJ mol}^{-1}$$

$$q_{Na,Na} = -\Delta G - w_{Na,Na} = 54.6 - 13.1 = 41.5 \text{ kJ mol}^{-1}$$

Thus, approximately three times as much heat is released by the Na<sup>+</sup>,K<sup>+</sup> ATPase in the Na<sup>+</sup>,Na<sup>+</sup> exchange mode relative to the Na<sup>+</sup>,K<sup>+</sup> exchange mode.

However, body temperature depends on the rate of heat production (i.e. the power output), not simply on the amount of heat produced by a biochemical reaction. Therefore, for any change in Na<sup>+</sup>,K<sup>+</sup> ATPase function to cause a higher body temperature it is important that the rate of heat production increase. Hence we need to consider the turnovers of the Na<sup>+</sup>,K<sup>+</sup> and Na<sup>+</sup>,Na<sup>+</sup> exchange modes under physiological conditions, not just the amount of heat per turn over produced.

### 3.2. Frequency of parallel transport pathways

The rate with which the enzyme cycles within the Na<sup>+</sup>,K<sup>+</sup> or Na<sup>+</sup>,Na<sup>+</sup> exchange modes depends in part on the degree of occupation of the E2P state of the enzyme by K<sup>+</sup> or Na<sup>+</sup>. To carry out 3Na<sup>+</sup>/2K<sup>+</sup> pumping the enzyme must reach the E2PK<sup>+</sup><sub>2</sub> state, whereas to carry out 3Na<sup>+</sup>/2Na<sup>+</sup> pumping the enzyme must reach the E2PNa<sup>+</sup><sub>2</sub> state. These enzyme states are linked via a series of coupled ion binding/dissociation reactions (see Fig. 1). On the basis of experimental data [17–19] we have assumed in this scheme that the enzyme possesses one transport site which is specific for Na<sup>+</sup> and two sites which can be occupied by Na<sup>+</sup> or K<sup>+</sup> with no interaction between them. Furthermore, because stopped flow investigations of mammalian kidney Na<sup>+</sup>,K<sup>+</sup> ATPase [20,21] suggest that the occlusion (i.e. entrapment within the protein matrix) of two K<sup>+</sup> ions from the extracellular medium occurs simultaneously, we have excluded the possibility of occlusion pathways with only a single extracellularly bound ion. We have also excluded the possibility that the enzyme cycles back from the E2P to the E1 state with neither Na<sup>+</sup> nor K<sup>+</sup> bound

to the transport sites, i.e., we assume that dephosphorylation of the enzyme is dependent on a conformational change stimulated by the occlusion of ions. This is based on measurements of pH dependence of phosphorylation by inorganic phosphate [22], which suggest that in the absence of both Na<sup>+</sup> and K<sup>+</sup> ions dephosphorylation only occurs after occlusion of 2H<sup>+</sup> ions within the protein. At a physiological pH of 7.4, however, the degree of occupation of E2P by H<sup>+</sup> ions can be expected to be extremely low. Thus, we only consider two competing cycles: 3Na<sup>+</sup>/2K<sup>+</sup> and 3Na<sup>+</sup>/2Na<sup>+</sup> exchange.

In our model we don't explicitly include the kinetics of ATP binding. The normal physiological concentration of ATP of 5 mM far exceeds the dissociation of ATP to its catalytic site of approximately 8 μM [12]. Therefore, we assume that the enzyme is always 100% saturated by ATP. Calculations at lower levels of ATP would require the value of the rate constant for phosphorylation by ATP,  $k_1$ , to be reduced by the fractional degree of saturation.

The degrees of occupation of the ion binding sites of the different E2P enzyme states shown in Fig. 1 depend on the extracellular Na<sup>+</sup> and K<sup>+</sup> concentrations and the magnitudes of the forward and backward rate constants of the entire mechanism. Because the dissociation of Na<sup>+</sup> from its binding sites has been shown in intact cells to produce a measurable current [17,23–29], the rate constants for Na<sup>+</sup> dissociation and re binding depend, furthermore, on the value of the electrical membrane potential. Fortunately, sufficient experimental data from measurements on both purified Na<sup>+</sup>,K<sup>+</sup> ATPase and the enzyme in intact cells is now available to allow the flux through the Na<sup>+</sup>,K<sup>+</sup> and Na<sup>+</sup>,Na<sup>+</sup> transport pathways to be calculated.

The effects of the transmembrane electrical potential difference  $V$  (defined as electrical potential in minus electrical potential out) on the ion binding and dissociation reactions can be taken into account by multiplying the relevant rate constants at a membrane potential of 0 Volts by Boltzmann expressions, given by  $\exp(\alpha FV/RT)$  (see Supplementary Material for the exact equations). The numerical factor  $\alpha$ , termed a dielectric coefficient, is the fraction of the total transmembrane potential difference that influences the electrogenic reaction concerned. Sometimes the dielectric coefficients are approximated as the fractional distance across the membrane over which an ion is transported during an electrogenic reaction [30,31]. The dielectric coefficient values of  $-0.65$  and  $+0.65$  for release and re binding of the first Na<sup>+</sup> ion from the E2PNa<sup>+</sup><sub>3</sub> state is derived from electrical measurements on the effects of the membrane potential on extracellular Na<sup>+</sup> binding [24,28,32]. A value of 0.185 was chosen for the two step Na<sup>+</sup> release from the nonspecific sites to balance the dielectric coefficient of  $-0.185$  (or  $-0.37$  in total for the two ions) determined by Rakowski et al. [33] for extracellular K<sup>+</sup> binding and yield an overall transfer of one positive charge into the extracellular medium.

The entire coupled series of differential rate equations necessary to simulate the kinetics of the Na<sup>+</sup>,K<sup>+</sup> ATPase and calculate the fluxes through the 3Na<sup>+</sup>/2K<sup>+</sup> and 3Na<sup>+</sup>/2Na<sup>+</sup> pathways are given in the Supplementary Material. All of the values of the rate constants used in the simulations are also given in the Supplementary Material in Table S1. The values were all determined from measurements on the most abundant  $\alpha_1\beta_1$  isoform of the Na<sup>+</sup>,K<sup>+</sup> ATPase. Thus, all of the calculations which follow refer to this isoform.

Based on the typical physiological concentrations of Na<sup>+</sup> and K<sup>+</sup> on each side of the membrane given in the previous section and the membrane potential of  $-79$  mV, one can calculate based on the scheme shown in Fig. 1 and the mathematical model described in detail in the Supplementary Material that the fluxes through the 3Na<sup>+</sup>/2K<sup>+</sup> and 3Na<sup>+</sup>/2Na<sup>+</sup> pathways would be  $0.724$  s<sup>-1</sup> and  $0.002$  s<sup>-1</sup>, respectively. This indicates that under physiological conditions 99.7% of the cycles would follow the 3Na<sup>+</sup>/2K<sup>+</sup> pathway. Hence, at least for the mammalian  $\alpha_1\beta_1$  form of the enzyme one is justified in neglecting the 3Na<sup>+</sup>/2Na<sup>+</sup> pathway totally.

There are two reasons why the  $3\text{Na}^+/2\text{K}^+$  pathway is so dominant under physiological conditions. Firstly, the binding affinity of  $\text{K}^+$  to the E2P state is much higher than that of  $\text{Na}^+$ . Secondly, as well as this thermodynamic reason there is also an important kinetic factor. The rate constant for ion occlusion and dephosphorylation leading to the E2 state depends very strongly on which ion is occluded by E2P. In particular it is known that the  $\text{K}^+$  ion is far more effective than  $\text{Na}^+$  in stimulating dephosphorylation, i.e., the maximum turnover of  $3\text{Na}^+/2\text{K}^+$  activity has been reported to be approximately 17 fold higher than that of  $3\text{Na}^+/2\text{Na}^+$  activity [34]. Therefore, under physiological conditions  $\text{K}^+$  occlusion and dephosphorylation of  $\text{E2PK}^+_2$  tends to siphon off enzyme from the entire pool of enzyme in E2P states, which are all in rapid equilibrium with one another on the timescale of  $\text{K}^+$  occlusion by E2P. This effectively increases the proportion of enzyme following the  $3\text{Na}^+/2\text{K}^+$  cycle even further and decreases the proportion of enzyme following the  $3\text{Na}^+/2\text{Na}^+$  cycle.

### 3.3. Power output of the $\text{Na}^+,\text{K}^+$ ATPase

For the body temperature of an animal to increase via mutations in the  $\text{Na}^+,\text{K}^+$  ATPase structure or by post translational modifications it is important that the rate of heat production by the enzyme,  $dq/dt$ , increase, i.e. there must be an increase in its power output. An increase in the amount of heat produced per mole of hydrolysed ATP is insufficient for an increase in body temperature. The power output of an enzymatic cycle can simply be calculated by multiplying the flux through the pathway by the heat produced per cycle.

In the last section we already calculated the expected flux through the  $3\text{Na}^+/2\text{K}^+$  pathway of  $0.724 \text{ s}^{-1}$ . In Section 3.1 we calculated a heat generation per mole of ATP hydrolysed,  $q_{\text{Na},\text{K}}$ , of  $13.6 \text{ kJ mol}^{-1}$ . Multiplying these two numbers together yields a power output,  $dq_{3\text{Na}/2\text{K}}/dt$ , of  $9.85 \text{ kJ mol}^{-1} \text{ s}^{-1}$  (or  $\text{kW mol}^{-1}$ ). In contrast, if we artificially block the occlusion pathway of  $\text{K}^+$  by setting the rate constant for this reaction to 0, thus forcing the enzyme through the  $3\text{Na}^+/2\text{Na}^+$  pathway, simulations yield a flux of only  $0.0086 \text{ s}^{-1}$ . Multiplying this value by the heat generated per mole of ATP hydrolysed in this pathway,  $q_{\text{Na},\text{Na}}$ , (calculated in Section 3.1) of  $41.5 \text{ kJ mol}^{-1}$  yields a power output,  $dq_{3\text{Na}/2\text{Na}}/dt$ , of only  $0.36 \text{ kJ mol}^{-1} \text{ s}^{-1}$ . Thus, even though the heat output per cycle is greater in the  $\text{Na}^+,\text{Na}^+$  exchange mode, forcing more enzyme through this pathway instead of through the  $\text{Na}^+,\text{K}^+$  pathway actually decreases the power output of the enzyme.

The conclusion from these calculations is that a higher power output by the  $\text{Na}^+,\text{K}^+$  ATPase and a higher body temperature would not be expected if the sites on E2P became less selective towards  $\text{K}^+$ . In contrast, an increase in their selectivity towards  $\text{K}^+$  should lead to a slight increase in body temperature. This is consistent with the finding here that the mammalian kidney enzyme passes almost exclusively through the  $3\text{Na}^+/2\text{K}^+$  pathway under physiological conditions. When sufficient partial reaction kinetic data is available on the  $\text{Na}^+,\text{K}^+$  ATPase for a cold blooded animal, it would be interesting to repeat these calculations for that enzyme to see if significant differences in power output between endothermic and ectothermic animals would be expected.

### 3.4. Passive ion fluxes

A further important contribution to heat generation, linked to the activity of the  $\text{Na}^+,\text{K}^+$  ATPase, would be expected to arise from passive ion fluxes across the plasma membrane. For any cell in a steady state, active ion pumping across the membrane by the  $\text{Na}^+,\text{K}^+$  ATPase is exactly compensated for by passive ion fluxes across the plasma membrane. If ion pumping alone occurred, the  $\text{Na}^+$  concentration within the cytoplasm would continually fall and the  $\text{K}^+$  concentration within the cytoplasm would continually rise. Therefore, there must be passive leaks of  $\text{Na}^+$  ions back into the cytoplasm and of  $\text{K}^+$  out of the cell. In fact, even in the absence of the  $\text{Na}^+,\text{K}^+$  ATPase there would be a driving

force for accumulation of  $\text{Na}^+$  ions in the cytoplasm due to the equilibrium Donnan effect, arising from the neutralisation of negatively charged macromolecules within the cytoplasm. A major role of the  $\text{Na}^+,\text{K}^+$  ATPase is to prevent this accumulation of  $\text{Na}^+$  ions which would otherwise lead to an osmotic swelling of the cell.

The major source of passive  $\text{Na}^+$  diffusion into cells is thought to arise from the  $\text{Na}^+/\text{H}^+$  antiporter [35,36]. This transporter utilizes the energy stored in the  $\text{Na}^+$  concentration gradient to actively extrude  $\text{H}^+$  ions out of the cell and prevent the environment of the cytoplasm from becoming too acidic. Therefore, in the first instance passive diffusion of  $\text{Na}^+$  into the cell doesn't just release heat; it also does work. However, if the pH gradient across the plasma membrane is to be maintained at a constant level, there must also be a passive flux of  $\text{H}^+$  ions back into the cell and this must occur at the same rate, so that the work done of  $\text{H}^+$  extrusion is re-released as heat. The same applies for any other  $\text{Na}^+$  coupled transport system. Therefore, regardless of the mechanism of passive  $\text{Na}^+$  diffusion into the cell, ultimately all of the energy stored in the  $\text{Na}^+$  concentration gradient across the membrane is released as heat and at the same rate as  $\text{Na}^+$  is pumped out by the  $\text{Na}^+,\text{K}^+$  ATPase.

Based on the electrochemical potential differences of  $\text{Na}^+$  and  $\text{K}^+$  across the plasma membrane, the heat produced per mole of ion due to passive fluxes would be given by:

$$q_{\text{Na},p} = RT \ln \frac{c_{\text{Na}}^i}{c_{\text{Na}}^o} + FV_m \quad (4)$$

$$q_{\text{K},p} = RT \ln \frac{c_{\text{K}}^o}{c_{\text{K}}^i} - FV_m \quad (5)$$

The  $\text{K}^+$  distribution across the membrane is much closer to equilibrium than that of  $\text{Na}^+$ . Therefore, one would expect much less heat generation from  $\text{K}^+$  diffusion than from  $\text{Na}^+$ . This prediction is borne out by quantitative calculation. According to the typical physiological ion concentrations and membrane potential given earlier, Eqs. (4) and (5) yield values of  $q_{\text{Na},p} = -13.1 \text{ kJ mol}^{-1}$  and  $q_{\text{K},p} = -0.8 \text{ kJ mol}^{-1}$ . Now considering the major  $3\text{Na}^+/2\text{K}^+$  cycling mode of the  $\text{Na}^+,\text{K}^+$  ATPase, because of the enzyme's ion stoichiometry the passive flux of  $\text{Na}^+$  ions must be 3 times that of the enzyme turnover and that of  $\text{K}^+$  must be twice that of the turnover. Utilizing the  $3\text{Na}^+/2\text{K}^+$  cycling turnover of  $0.724 \text{ s}^{-1}$  at physiological concentrations and membrane potential calculated in the previous section yields passive  $\text{Na}^+$  and  $\text{K}^+$  fluxes of  $2.17 \text{ ions (ATPase molecule)}^{-1} \text{ s}^{-1}$  and  $1.45 \text{ ions (ATPase molecule)}^{-1} \text{ s}^{-1}$ . Multiplying these values by the values just calculated for the heats produced per mole of ion yields power outputs of  $28 \text{ kJ (mol of ATPase)}^{-1} \text{ s}^{-1}$  for  $\text{Na}^+$  and  $1.2 \text{ kJ (mol of ATPase)}^{-1} \text{ s}^{-1}$  for  $\text{K}^+$ . Adding both of these to the power output of  $3\text{Na}^+/2\text{K}^+$  cycling of the  $\text{Na}^+,\text{K}^+$  ATPase itself ( $9.85 \text{ kJ (mol of ATPase)}^{-1} \text{ s}^{-1}$ ) yields a total power output of  $39.4 \text{ kJ (mol ATPase)}^{-1} \text{ s}^{-1}$ . By far the largest contribution to the power output associated with  $\text{Na}^+,\text{K}^+$  ATPase activity (72%) is due to passive diffusion of  $\text{Na}^+$  into the cell balanced by outward  $\text{Na}^+$  pumping of the enzyme to maintain a constant  $\text{Na}^+$  concentration gradient across the plasma membrane.

If the number of active  $\text{Na}^+,\text{K}^+$  ATPase molecules within the plasma membrane of a cell is known, the cell's total power output associated with  $\text{Na}^+,\text{K}^+$  ATPase activity can be estimated. In the case of human red blood cells it is thought that the membrane contains only 250 copies of the protein [37]. Using the total power output per mole of ATPase just calculated one can estimate the total  $\text{Na}^+,\text{K}^+$  ATPase related power output of a human red blood cell to be  $1.6 \times 10^{-17} \text{ W}$ . In other types of cells for which ion transport is a much more essential part of their physiological activity, e.g. kidney cells, nerve cells and muscle cells, the level of  $\text{Na}^+,\text{K}^+$  ATPase expression is much higher and hence the  $\text{Na}^+,\text{K}^+$  ATPase related power output would be much greater. For example, it has been estimated

that cultured cardiac myocytes contain approximately  $2 \times 10^6$  copies of the protein [38]. Assuming that all of the expressed  $\text{Na}^+, \text{K}^+$  ATPase is 100% active and the same ADP/ATP concentration ratio in the cell, this corresponds to a total  $\text{Na}^+, \text{K}^+$  ATPase related power output of  $1.3 \times 10^{-13}$  W, i.e. approximately 8000 times greater than that of a red blood cell. In myocytes actively undergoing contraction and relaxation, one would expect the degree of expression of the  $\text{Na}^+, \text{K}^+$  ATPase and its related power output to exceed that of red blood cells by even more.

#### 4. Discussion

The calculations presented here are in accord with the general view that the  $\text{Na}^+, \text{K}^+$  ATPase is an important source of heat production in animals. The kinetic simulations indicate that a decrease in the thermodynamic efficiency of the enzyme, i.e., the fraction of energy it uses for the work of ion pumping relative to the total energy available from ATP hydrolysis, is unlikely to be a mechanism whereby body temperature could be increased. The reason for this is that, at least for mammalian  $\alpha_1\beta_1$   $\text{Na}^+, \text{K}^+$  ATPase, cycles in which  $\text{Na}^+$  substitutes partially or totally for  $\text{K}^+$  as the ion which binds to the E2P state of the enzyme and stimulates dephosphorylation are much slower than those in which E2P is saturated by  $2\text{K}^+$  ions. Therefore, although more heat per cycle would be produced by replacing  $\text{K}^+$  by  $\text{Na}^+$ , the slower rate of ATP hydrolysis would lead to a lower rate of heat production, i.e., a lower power output, and hence a lower body temperature.

Quantitatively the major origin of thermogenesis related to  $\text{Na}^+, \text{K}^+$  ATPase action can be attributed to the passive diffusion of  $\text{Na}^+$  into the cell, which balances  $\text{Na}^+$  extrusion by the  $\text{Na}^+, \text{K}^+$  ATPase and maintains a constant  $\text{Na}^+$  concentration gradient across the plasma membrane. This passive  $\text{Na}^+$  diffusion accounts for 72% of  $\text{Na}^+, \text{K}^+$  ATPase related thermogenesis. In comparison, direct release of heat by the  $\text{Na}^+, \text{K}^+$  ATPase accounts for 25% and passive  $\text{K}^+$  diffusion accounts for 3% of the total  $\text{Na}^+, \text{K}^+$  ATPase related heat production.

Based on the calculations presented here it appears that increased thermogenesis by  $\text{Na}^+, \text{K}^+$  ATPase activity could only be produced via an increase in the expression level of the enzyme within a cell or by an increase in the turnover rate of individual enzyme molecules. An increase in turnover could be produced in the course of evolution through mutations to the enzyme's structure or, within the timespan of an individual animal's existence (e.g. under conditions of cold stress), through either a regulatory post translational modification or the activation of pump molecules stored in a dormant state (e.g. aggregated within the membrane). Whichever way an increase in  $\text{Na}^+, \text{K}^+$  ATPase activity is achieved, it must occur in parallel with an increase in the plasma membrane's permeability towards  $\text{Na}^+$  and  $\text{K}^+$  to prevent any changes in the concentration gradients of these ions across the membrane. In agreement with this finding, there seems to be clear evidence that in certain tissues (heart, skeletal muscle, fat, liver and kidney)  $\text{Na}^+, \text{K}^+$  ATPase expression is enhanced by the action of thyroid hormone, a known regulator of metabolic activity [39–41].

To isolate effects which could lead an increase in turnover of a  $\text{Na}^+, \text{K}^+$  ATPase molecule and hence an increase in power output, we have carried out kinetic simulations of its pump cycle in which we have modified all of its rate constants and substrate concentrations independently by 10%. The results of those calculations are shown in Table 1. Similar calculations were previously performed by Kong and Clarke [12], but there the effect of the physiological membrane potential was not taken into account. In the case of variations of the rate constants, because the heat generated by each  $\text{Na}^+, \text{K}^+$  ATPase cycle remains constant under those conditions and because  $\text{Na}^+$  and  $\text{K}^+$  pumping are exactly compensated by passive transport processes of both ions across the membrane, the percentage changes in turnover exactly equal the percentage changes in total

power output, i.e., from  $\text{Na}^+, \text{K}^+$  ATPase pumping plus passive ion fluxes.

The results in Table 1 show that the turnover of the enzyme is particularly sensitive to the cytoplasmic  $\text{Na}^+$  concentration. The reason for this is that under physiological conditions the E1 state is far from being saturated by  $\text{Na}^+$ , which makes the ATP phosphorylation reaction leading from  $\text{E1Na}^+_3$  to  $\text{E1P}(\text{Na}^+)_3$  a major rate determining step of the enzyme cycle. The low level of saturation of E1 by  $\text{Na}^+$  is partly due to the relatively low cytoplasmic  $\text{Na}^+$  concentration of 15 mM and in part due to competition from cytoplasmic  $\text{K}^+$ , which is present at the much higher concentration of 120 mM.

An increase in the cytoplasmic  $\text{Na}^+$  concentration might seem, therefore, to be a mechanism whereby  $\text{Na}^+, \text{K}^+$  ATPase activity could be stimulated. Hence, its potential as a way of enhancing heat generation should be considered. The numerical simulation method used here shows that if a rapid change in the cytoplasmic  $\text{Na}^+$  concentration did occur, the  $\text{Na}^+, \text{K}^+$  ATPase could readjust its activity and reach a new steady state in less than one second. An increase in cytoplasmic  $\text{Na}^+$  concentration could come about via an increase in the passive permeability of the plasma membrane to  $\text{Na}^+$ . Because the  $\text{Na}^+, \text{K}^+$  ATPase is not saturated by cytoplasmic  $\text{Na}^+$ , an influx of  $\text{Na}^+$  would increase the outward  $\text{Na}^+$  pumping rate, thus causing the cytoplasmic  $\text{Na}^+$  concentration to drop again, but not to its initial level. In the absence of any changes in expression level of the  $\text{Na}^+, \text{K}^+$  ATPase or structural mutation, any increase in the membrane's passive permeability towards  $\text{Na}^+$  must cause a net increase in the steady state level of  $\text{Na}^+$  within the cell. This would upset the osmotic balance across the plasma membrane and be accompanied by an increase in cell water content and hence an increase in cell volume. An impairment of the ability to regulate cell volume is well known for red blood cells with membrane transport defects [42]. Therefore, an increase in the plasma membrane's  $\text{Na}^+$  permeability by itself is not a viable mechanism for increasing thermogenesis.

**Table 1**  
Effects of  $\pm 10\%$  variations in the substrate concentrations, membrane potential, and rate constants on the turnover number of the enzyme.\*

Parameter varied	Percentage changes in turnover	
	+10%	10%
$[\text{Na}^+]_i$	+21.6	20.8
$[\text{Na}^+]_o$	2.97	+2.82
$[\text{K}^+]_i$	13.7	+16.8
$[\text{K}^+]_o$	+0.23	0.35
$V_m$	+21.2	18.5
$k_1$	+7.45	6.14
$k_{2f, v \ o}$	+1.24	1.47
$k_{2r, v \ o}$	1.28	+1.32
$k_{32}$	+0.121	0.147
$k_{4f}$	+6.07	6.54
$k_{4r}$	5.85	+6.63
$k_{bN1, v \ o}^p$	+6.63	7.07
$k_{dN1, v \ o}^p$	6.22	+7.11
$k_{bN}^p$	+10.5	14.5
$k_{dN}^p$	12.1	+14.3
$k_{bK}^p$	13.5	+16.8
$k_{dK}^p$	+14.9	14.9
$k_{bN1, v \ o}^p$	1.66	+1.72
$k_{dN1, v \ o}^p$	+1.56	1.85
$k_{bN, v \ o}^p$	1.21	+1.20
$k_{dN, v \ o}^p$	+2.49	3.10
$k_{bK, v \ o}^p$	+0.245	0.331
$k_{dK, v \ o}^p$	0.187	+0.160

\* The percentage changes in turnover are relative to a value of  $0.724 \text{ s}^{-1}$ , which was calculated using physiological substrate concentrations of  $[\text{Na}^+]_i = 15 \text{ mM}$ ,  $[\text{Na}^+]_o = 140 \text{ mM}$ ,  $[\text{K}^+]_i = 120 \text{ mM}$ ,  $[\text{K}^+]_o = 4 \text{ mM}$ ,  $[\text{ATP}]_i = 5 \text{ mM}$ ,  $V_m = 79 \text{ mV}$  and the values of the rate constants given in Table S1 of the Supplementary Material. The reactions associated with each of the rate constants are defined in Fig. 1.

The results in Table 1 also show that the trans membrane potential,  $V_m$ , affects  $\text{Na}^+/\text{K}^+$  ATPase turnover. This is because of the  $3\text{Na}^+ : 2\text{K}^+$  pumping stoichiometry of the enzyme, i.e., it produces a net outward current across the membrane. However, under steady state conditions  $V_m$  is determined by the permeabilities of the membrane to all ions and their concentration gradients across the membrane. Hence any change in the steady state value of  $V_m$  would have to come about by changes in ion concentrations and permeabilities. For the same reasons as just described for the cytoplasmic  $\text{Na}^+$  concentration, changes in  $V_m$  are, thus, not a viable mechanism for increasing thermogenesis. Transient changes in  $V_m$  are crucial to the function of excitable cells, such as muscle and nerve cells, and these would be expected to produce transient changes in cell power output. However, with the exception of heart muscle cells, the duration of the transient changes in  $V_m$  (the action potential) would normally be negligible relative to the time the cell spends at its resting potential. Furthermore, as stated in the Introduction, we are considering here non shivering thermogenesis, i.e., heat generation without exposure to cold stress and without the need for any enhanced muscular activity.

The results given in Table 1 indicate that the most effective viable way of increasing heat output via a modification to  $\text{Na}^+/\text{K}^+$  ATPase function would be to increase its cytoplasmic selectivity towards  $\text{Na}^+$  ions relative to  $\text{K}^+$  ions. For example, a 10% increase in the rate constant for cytoplasmic  $\text{Na}^+$  binding to the nonspecific transport sites (i.e., a increase in  $k_{bN}^i$  of 10%) would be expected to increase total heat output by 10.5%, whereas an increase in the rate constant for competing  $\text{K}^+$  binding from the cytoplasm to the same sites (i.e., a increase in  $k_{bK}^i$  of 10%) would be expected to decrease total heat output by 13.5%. Such changes could potentially come about in the course of evolution through the mutation of amino acid residues associated with ion binding. In terms of a single animal's existence a post translational regulatory modification of the  $\text{Na}^+/\text{K}^+$  ATPase affecting ion binding could potentially bring about a temporary increase in heat output. However, in both cases it is important to bear in mind that a constant  $\text{Na}^+$  concentration gradient across the plasma membrane needs to be maintained. Any increase in the active pumping of  $\text{Na}^+$  out of the cell must occur in parallel with an increase in the passive  $\text{Na}^+$  flux into the cell and they must exactly compensate one another.

Although an increase in heat production by the combined effects of the  $\text{Na}^+/\text{K}^+$  ATPase and passive  $\text{Na}^+$  diffusion would not be expected to affect any secondary transport processes relying on the  $\text{Na}^+$  electrochemical potential gradient across the membrane, it is worthwhile to mention that excessive heat production could have pathological consequences in some tissues. A notable example is the heart where, in addition to its nutritional role, the blood supply acts as a "radiator" cooling the heart muscle [43]. In heart failure an increase in the temperature of blood leaving the heart has been observed. In accord with the effects of passive  $\text{Na}^+$  diffusion on heat production described here, a possible origin of the increased heat production of the failing heart could be an increase in  $\text{Na}^+$  permeability of the sarcolemma due to the sustained open state of  $\text{Na}^+$  channels following oxidative damage [44].

Finally, it is important to mention that a "leaky membrane" hypothesis as a mechanism whereby heat generation by the  $\text{Na}^+/\text{K}^+$  ATPase could be enhanced has previously been suggested by Else and Hulbert [45,46]. The results presented here indicate that this hypothesis is quantitatively consistent with what is now known about the kinetics of the enzyme's partial reactions. However, the term "leaky membrane" hypothesis would seem to imply that an increase in the membrane's passive permeability alone can explain increased thermogenesis. As explained above, this is not the case. Passive inward  $\text{Na}^+$  flux and active outward  $\text{Na}^+$  pumping are intimately coupled. The  $\text{Na}^+/\text{K}^+$  ATPase produces the  $\text{Na}^+$  concentration gradient across the membrane which drives the passive inward  $\text{Na}^+$  flux. If the kinetics of one is changed without the other, this will lead to changes in the  $\text{Na}^+$  concentration gradient and pathological consequences.

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