Quantification of Na^+,K^+ pumps and their transport rate in skeletal muscle: Functional significance

Torben Clausen

Department of Biomedicine, Aarhus University, DK-8000 Aarhus C, Denmark

During excitation, muscle cells gain Na^+ and lose K^+, leading to a rise in extracellular K^+ ($[K^+]_o$), depolarization, and loss of excitability. Recent studies support the idea that these events are important causes of muscle fatigue and that full use of the Na^+,K^+-ATPase (also known as the Na^+,K^+ pump) is often essential for adequate clearance of extracellular K^+. As a result of their electrogenic action, Na^+,K^+ pumps also help reverse depolarization arising during excitation, hyperkalemia, and anoxia, or from cell damage resulting from exercise, rhabdomyolysis, or muscle diseases. The ability to evaluate Na^+,K^+-pump function and the capacity of the Na^+,K^+ pumps to fill these needs require quantification of the total content of Na^+,K^+ pumps in skeletal muscle. Inhibition of Na^+,K^+-pump activity, or a decrease in their content, reduces muscle contractility. Conversely, stimulation of the Na^+,K^+-pump transport rate or increasing the content of Na^+,K^+ pumps enhances muscle excitability and contractility. Measurements of [^{3}H] ouabain binding to skeletal muscle in vivo or in vitro have enabled the reproducible quantification of the total content of Na^+,K^+ pumps in molar units in various animal species, and in both healthy people and individuals with various diseases. In contrast, measurements of 3-O-methylfluorescein phosphatase activity associated with the Na^+,K^+-ATPase may show inconsistent results. Measurements of Na^+ and K^+ fluxes in intact isolated muscles show that, after Na^+ loading or intense excitation, all the Na^+,K^+ pumps are functional, allowing calculation of the maximum Na^+,K^+-pumping capacity, expressed in molar units/g muscle/min. The activity and content of Na^+,K^+ pumps are regulated by exercise, inactivity, K^+ deficiency, fasting, age, and several hormones and pharmaceuticals. Studies on the α-subunit isoforms of the Na^+,K^+-ATPase have detected a relative increase in their number in response to exercise and the glucocorticoid dexamethasone but have not involved their quantification in molar units. Determination of ATPase activity in homogenates and plasma membranes obtained from muscle has shown ouabain-suppressible stimulatory effects of Na^+ and K^+.

Introduction: Transport and content of Na^+ and K^+ in skeletal muscle

The Na^+,K^+-ATPase (also known as the Na^+,K^+ pump) is the major translator of metabolic energy in the form of ATP to electrical and chemical gradients for the two most common ions in the body. These gradients enable the generation of action potentials, which are essential for muscle cell function. Evaluation of the physiological and clinical significance of the Na^+,K^+ pumps requires measuring the transmembrane fluxes of Na^+ and K^+ in intact muscles or cultured muscle cells. The simplest approach involves incubating intact muscles isolated from small animals in temperature-controlled and oxygenated buffers with electrolyte and glucose concentration comparable to that normally present in blood plasma. Initial studies used cut hemi- or quarter-diaphragm muscles from rats, mice, or guinea pigs for incubation because these muscles were considered thin enough to allow adequate oxygenation under these conditions (Gemmill, 1940). However, such preparations have numerous cut muscle ends, allowing large passive movements of Na^+ and K^+ and free access of Ca^{2+} to the cell interior. This unavoidably boosts the energy required for active transport of Na^+, K^+, and Ca^{2+}, and leads to impaired cell survival. Thus, in cut muscles, the components of O_2 consumption and ^{42}K uptake attributable to the Na^+,K^+ pump (i.e., the fraction suppressible by the cardiac glycoside ouabain, which binds to and inhibits the Na^+,K^+-ATPase) have been severely overestimated. (The ouabain-suppressible components of O_2 consumption or ^{42}K uptake are measured in isolated muscles incubated without or with ouabain and calculated as the difference.) Such overestimation led to the assumption that in skeletal muscle, the Na^+,K^+ pumps mediate a large fraction of total energy turnover, suggesting that a major part of the thermogenic action of thyroid hormone is caused by an increased rate of active Na^+,K^+ transport (Asano et al., 1976). In contrast, in intact resting muscle preparations, only 2–10% of the total energy turnover is used for active Na^+,K^+ transport (Creese, 1968; for details, see Clausen et al., 1991). Even during maximum contractile work in human muscles, only a small fraction (2%) (Medbø and Sejersted, 1990) of

Correspondence to Torben Clausen: tc@fi.au.dk

Abbreviations used in this paper: 3-O-MFPase, 3-O-methylfluorescein phosphatase; CGRP, calcitonin gene–related peptide; EDL, extensor digitorum longus.

The Rockefeller University Press
J. Gen. Physiol. Vol. 142 No. 4 327–345
www.jgp.org/cgi/doi/10.1085/jgp.201310980

total energy release is used for the Na^+,K^+ pumps. Thus, in skeletal muscle, the thermogenic action of the Na^+,K^+ pumps is modest.

For the analysis of Na^+,K^+ transport in skeletal muscle, isolated intact limb muscles are used, primarily mammalian soleus, extensor digitorum longus (EDL), extensor digitorum brevis, or epitrochlearis muscles. These preparations can survive during incubation for many hours and can undergo repeated excitation. More recently, the isolated rat sternohyoid muscle, which also offers thin dimensions, cellular integrity, and simple handling, has been introduced (Mu et al., 2011).

Measurement of muscle Na^+ and K^+ content requires extraction. In the past, this was done by digesting the muscle preparation in nitric acid, a sometimes risky procedure. More recently, this approach has been superseded by homogenization of the tissue in 0.3 M trichloroacetic acid, followed by centrifugation to sediment the proteins (Kohn and Clausen, 1971). The clear supernatant may then be diluted for flame photometric determination of Na^+ and K^+ or counting of the isotopes ^{22}Na or ^{42}K. Because ^{42}K has a short half-life (12.5 h) and is of limited availability, the K^+ analogue ^{86}Rb is often used as a tracer for K^+. For the quantification of Na^+,K^+ pump–mediated (i.e., ouabain-suppressible) transport of K^+, ^{86}Rb gives the same results as ^{42}K (Clausen et al., 1987; Dørup and Clausen, 1994). For other flux measurements, however, the results obtained with ^{86}Rb differ appreciably from those obtained with ^{42}K. For example, the fractional loss of ^{86}Rb from intact resting rat soleus muscles is only 45% of that measured using ^{42}K. Moreover, two agents shown to stimulate the Na^+,K^+ pumps in isolated rat soleus muscle, salbutamol (Clausen and Flatman, 1977) and rat calcitonin gene–related peptide (CGRP) (Andersen and Clausen, 1993), both induce a highly significant stimulation of ^{86}Rb efflux from the same muscle (Dørup and Clausen, 1994). In contrast, these same agents induced a rapid but transient (20-min duration) inhibition of the fractional loss of ^{42}K, indicating that a large fraction of the ^{42}K lost from the cells is reaccumulated as a result of stimulation of the Na^+,K^+ pumps (Andersen and Clausen, 1993; Dørup and Clausen, 1994). Finally, in skeletal muscle, bumetanide, an inhibitor of the NaK2Cl cotransporter, produces no inhibition of ^{42}K uptake but clear-cut inhibition of ^{86}Rb uptake (see Tables 1 and 2 in Dørup and Clausen, 1994). Thus, results obtained with ^{86}Rb must be verified with ^{42}K or flame photometric measurements of changes in intracellular Na^+ and K^+ content. The activity of the Na^+,K^+ pump depends on ATP supplied by glycolysis or oxidative phosphorylation. Excitability of isolated rat soleus or EDL muscles can be maintained in the presence of the electron transport inhibitor cyanide or during anoxia (Murphy and Clausen, 2007; Fredsted et al., 2012), whereas contractions are markedly suppressed by 2-deoxyglucose, which interferes with the production of glycolytic ATP. Thus, in keeping with the studies of Glitsch (2001), these data suggest that glycolytic ATP is a primary source of energy for the Na^+,K^+ pumps (see also Okamoto et al., 2001). More focused studies showed that Na^+,K^+-pump function is not adequately supported when cytoplasmic ATP is at the normal resting concentration of ∼8 mM, but that the addition of as little as 1 mM phosphoenol pyruvate produces a marked increase in Na^+,K^+-pump function that is supported by endogenous pyruvate kinase bound within the t-tubular triad (Dutka and Lamb, 2007). In anoxic rat EDL muscles, contractility could be restored by stimulating the Na^+,K^+ pumps with the β_2 agonists salbutamol or terbutaline, effects that were abolished by ouabain or 2-deoxyglucose (Fredsted et al., 2012). Glycolytic ATP furnishes energy for contractile activity under the critical condition of anoxia. Because the Na^+,K^+ pumps are essential for the maintenance of excitability and contractions, it would not be surprising if they were also kept going on glycolytic ATP.

The Na^+,K^+-ATPase and why it should be quantified

The Na^+,K^+ pump, which is identical to the membrane-bound Na^+,K^+-ATPase discovered by Skou (1965), has been found in most skeletal muscle types from many species. As illustrated in Fig. 1, the Na^+,K^+-pump molecule comprises a catalytic α subunit, a β subunit involved in its translocation to the plasma membrane, and a regulatory subunit (FXYD; in skeletal muscle named FXYD1 or phospholemman). The Na^+,K^+ pumps in skeletal muscle are subject to acute activation or inhibition of their transport rate as well as long-term regulation of their content (expressed in molar units, usually as pmol per gram wet weight) (Clausen, 2003). In human skeletal muscle, the content of Na^+,K^+ pumps measured using [^{3}H]ouabain binding is ∼300 pmol/g wet wt (Clausen, 2003). In an adult human subject weighing 70 kg, the muscles will weigh around 28 kg and therefore contain a total of 28,000 × 300 pmol = 8.4 µmoles of Na^+,K^+ pumps. Muscles represent the largest single pool of cells in the human body, containing the largest pool of K^+ (2,600 mmol; Clausen, 2010) and one of the largest pools of Na^+,K^+ pumps (Clausen, 1998). Many diseases are associated with anomalies in the content of Na^+,K^+ pumps in skeletal muscle (Clausen, 1998, 2003), and during the last decades, the number of published articles on Na^+,K^+ pumps in skeletal muscle has increased appreciably. The capacity of the Na^+,K^+ pumps in muscles is crucial for the clearance of extracellular K^+ at rest or during exercise, and details of the transport capacity are required for clinical evaluation of the risk of developing hypo- or hyperkalemia (for clinical examples and details, see Clausen, 1998, 2003, 2010; Sejersted and Sjøgaard, 2000). Moreover, this information is key to understanding the functional significance of physiological changes or pathological anomalies in the content of Na^+,K^+ pumps in

skeletal muscle. The Na^+,K^+-pump capacity may be calculated by multiplying the number of Na^+,K^+ pumps by their turnover number (determined with the Na^+,K^+-ATPase from ox brain at 37°C as 8,000 molecules of ATP split per Na^+,K^+-ATPase molecule per min; Plesner and Plesner, 1981) and by the number of K^+ ions transported per cycle, which is normally 2. From this information it can be calculated that the total transport capacity for K^+ of the pool of Na^+,K^+ pumps in human skeletal muscle amounts to 2 × 8,000 × 8.4 µmoles/min = 134 mmoles/min. Provided that all the Na^+,K^+ pumps in all muscles are operating at maximum speed (which rarely happens), they could clear all extracellular K^+ in the human body (56 mmoles, calculated by multiplying the extracellular water space [14 liters] by the content of K^+ [4 mmol/liter] in 25 s; Clausen, 2010). The present Review was prompted by the ongoing problems of Na^+,K^+-pump quantification and recovery, how they can be solved, and what insight may be gained by using more specific methods and adequate quantitative analysis. The early development of the field has been discussed previously (Ewart and Klip, 1995; Sejersted and Sjøgaard, 2000; Clausen, 2003). This Review focuses on the most relevant publications appearing during the last 10 years; to cover the background, it includes references back to the discovery of the Na,K pump in 1957 (Skou, 1957).

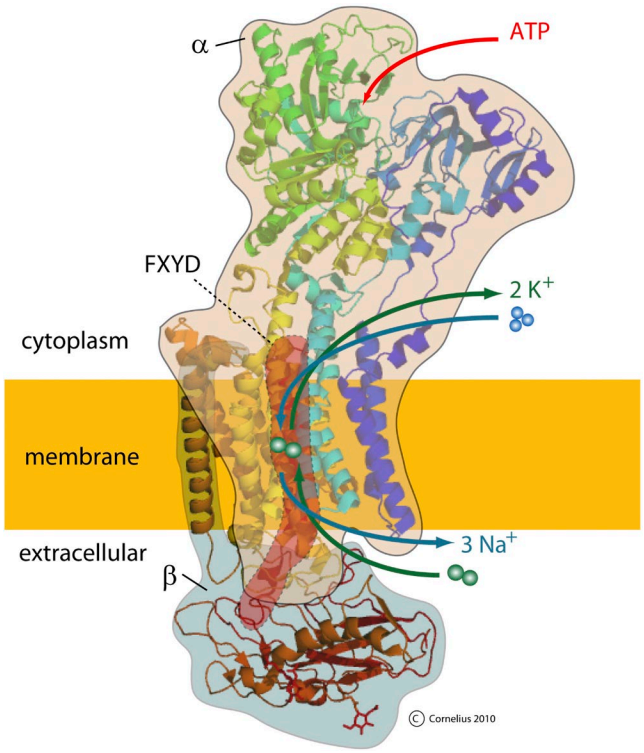

Figure 1. The molecular structure of Na^+,K^+-ATPase. The figure is based upon the crystal structure of the homologous Na^+,K^+-ATPase in the E_2P_2K conformation (Shinoda et al., 2009) and drawn by Flemming Cornelius (Aarhus University, Aarhus, Denmark). The Na^+,K^+ pump comprises an α and a β subunit, a glycoprotein that participates in the translocation of the molecule from the cell interior to its correct position in the lipid bilayer of the plasma membrane. A regulatory subunit (FXYD) is also shown. During each transport cycle of the Na^+,K^+ pump, one ATP molecule is bound to the cytoplasmic site of the α subunit; its hydrolysis provides energy for the active transport of Na^+ and K^+. The transmembrane domain consists of 10 transmembrane helices and contains the binding sites for three Na^+ or two K^+ ions, respectively, which pass sequentially through the same cavity in the molecule during each transport cycle.

Problems in assessing Na^+,K^+-ATPase activity and recovery. When the classical assay for membrane-bound ATPase activity stimulated by Na^+ and K^+ (Skou, 1965) and blocked by ouabain is used with crude muscle homogenates, there is an overwhelming background of other ATPases. Therefore, during the early phase of Na^+,K^+-pump investigation (1957–1977), many research groups were primarily interested in the detection and purification of the Na^+,K^+-ATPase. The differential centrifugation procedures developed during that period often discarded a major part of the enzyme activity present in homogenates of the intact tissue to eliminate other ATP-splitting cellular components, such as myosin, Ca^{2+}-activated ATPase, and unspecified Mg^{2+}-activated ATPase, and obtain a pure enzyme. Therefore, accurate information about Na^+,K^+-ATPase recovery was not always available, and these methods were not suitable for quantification of the total content of Na^+,K^+ pumps in the intact tissue. Moreover, the low recovery of Na^+,K^+-ATPase activity often obtained raised doubts as to whether the Na^+,K^+-ATPase in the samples tested was representative of the entire population of enzyme molecules present in the starting tissue. These problems were analyzed in a review, which found that, among 12 papers, only 3 obtained a recovery >5% of the Na^+,K^+-ATPase present in the starting material and, in 9 of these papers, recovery was in the range of 0.2 to 3.5% (Hansen and Clausen, 1988). To obtain better purification of the Na^+,K^+-ATPase in sarcolemma, giant vesicles were later produced from the sarcolemmal membranes of rat hind-limb muscles. However, they only contained 0.3% of the total content of Na^+,K^+ pumps present per gram of muscle (Juel et al., 2001). Thus, it is difficult to evaluate to what extent down-regulation of the content of Na^+,K^+ pumps might reduce Na^+,K^+ pump–mediated K^+ uptake in skeletal muscle and thereby impair the clearance of K^+ from extracellular space and plasma.

The Na^+,K^+-ATPase activity has been measured in plasma membranes prepared from various rat muscles. In total membranes, Na^+ (0–80 mM) and K^+ (0–10 mM) were shown to activate ouabain-suppressible ATPase (Juel, 2009). Surprisingly, V_{max} for Na^+ given as µmoles of ATP split per milligram protein per hour was higher in total membranes than in the sarcolemmal membranes, which might be expected to show a higher value, because of partial purification of the Na^+,K^+-ATPase. In total

membranes, as well as in plasma membranes, 30 min of running gave only a minor increase in V_{max}, which was only significant in a few instances, suggesting only modest translocation of the Na^+,K^+ pumps from an intracellular pool of endosomal membranes to the plasma membrane (see the section below, Translocation of Na^+,K^+ pumps compared..., for alternatives to translocation) (Juel, 2009). Because of a lack of accurate information about plasma membrane recovery, however, these observations could not readily be translated to pmol of Na^+,K^+ pumps per gram wet weight.

3-O-methylfluorescein phosphatase (3-O-MFPase) assay. Using 3-O-methylfluorescein phosphate as a substrate, K^+-dependent and ouabain-suppressible phosphatase activity has been measured in a fluorimetric assay for the Na^+,K^+-ATPase in skeletal muscle (Nørgaard et al., 1984). Early studies on rat skeletal muscle showed good agreement between the 3-O-MFPase activity of crude homogenates and the number of [^{3}H]ouabain-binding sites measured in the same intact muscles or biopsies thereof (Hansen and Clausen, 1988), suggesting that this assay provided a reliable measure of Na^+,K^+-ATPase activity. Moreover, the 3-O-MFPase activity showed similar decreases with aging or K^+ depletion of the rats as the [^{3}H]ouabain-binding capacity. However, studies on biopsies of human vastus lateralis muscle showed that exercise to fatigue reduced 3-O-MFPase activity by 13% ($P < 0.001$), whereas [^{3}H]ouabain binding remained constant (Leppik et al., 2004). Several other studies have also reported activity-dependent decreases in 3-O-MFPase activity in muscle: Another study from the same laboratory showed that acute exercise induced a 22% drop in the 3-O-MFPase activity of human vastus lateralis muscle (McKenna et al., 2006). In human vastus lateralis muscle, 16 h of heavy intermittent cycle exercise induced a 15% reduction in 3-O-MFPase (Green et al., 2007). In rats, running exercise induced a 12% decrease in 3-O-MFPase in all muscles (Fowles et al., 2002). However, other studies have not. For instance, electrical stimulation of the isolated rat soleus muscle for 15 or 90 min induced a 40 or 53% increase in 3-O-MFPase activity of crude homogenate, respectively (Sandiford et al., 2005). In keeping with this, giant vesicles representing sarcolemmal membrane obtained from rat muscles after 3 min of running exercise showed a 37% increase in 3-O-MFPase activity (Kristensen et al., 2008). Moreover, a full Na^+,K^+-ATPase assay showed that 30 min of treadmill running increased V_{max} by 12 to 39% in sarcolemmal membranes from rat hind-limb muscles (Juel, 2009). In contrast, high frequency stimulation of isolated rat EDL muscles caused no significant change in the content of 3-O-MFPase (Goodman et al., 2009). These discrepancies indicate that measurements of 3-O-MFPase provide inconsistent information about the content of Na^+,K^+-ATPase in skeletal muscle. Moreover, as pointed out by Juel (2009), the 3-O-MFPase cannot be used to measure Na^+-dependent activation. Therefore, further use of the 3-O-MFPase assay requires closer analysis of the causes of these discrepancies.

[^{3}H]Ouabain binding. [^{3}H]Ouabain and other labeled cardiac glycosides bind stoichiometrically to the extracellular surface of the α subunit of the Na^+,K^+ pump (one drug molecule per Na^+,K^+-ATPase molecule). Therefore, incubation of tissues, cells, or plasma membranes with [^{3}H]ouabain enables the quantification of the content of Na^+,K^+-ATPase in molar units by liquid scintillation counting of extracts of the tissue, cells, or membranes (Clausen and Hansen, 1974, 1977; Hansen and Clausen, 1988). Because K^+ interferes markedly with the binding of cardiac glycosides, studies of [^{3}H]ouabain binding to intact rat, mouse, or guinea pig muscles are performed using K^+-free (KR) buffer (Clausen and Hansen, 1974). [^{3}H]Ouabain binding is saturable and reversible without showing [^{3}H]ouabain penetration into the intracellular space. In isolated soleus muscle obtained from 4-wk-old rats, [^{3}H]ouabain binding amounts to 720 pmol/g wet wt, corresponding to 3,350 molecules of Na^+,K^+-ATPase per μ^2 of sarcolemma (not including t-tubules). When injected intraperitoneally, [^{3}H]ouabain binds rapidly to the outer surface of the muscle cells, showing saturation and the same content of [^{3}H]ouabain-binding sites per gram tissue wet weight as measured in intact muscles incubated with [^{3}H]ouabain in vitro (Clausen et al., 1982; Murphy et al., 2008). The [^{3}H]ouabain binding in vivo is faster than in vitro, reaching saturation in 20 min, partly because of the higher temperature and better access to the muscle cells via the capillaries. When bound to the outer surface of intact muscles, [^{3}H]ouabain is rapidly displaced by the addition of an excess of unlabeled ouabain during a subsequent wash performed both after binding in vitro and in vivo, indicating that the [^{3}H]ouabain is not internalized into the cytoplasm (Clausen and Hansen, 1974; Clausen et al., 1982).

It has been estimated that, in rat skeletal muscle, most of the Na^+,K^+ pumps (around 80%) are the α_2-subunit isoform, which has high affinity for [^{3}H]ouabain. A minor fraction (around 20%) is the α_1 isoform, which has a lower affinity for [^{3}H]ouabain (Hansen, 2001) and therefore may not be detected at the concentration of [^{3}H]ouabain used in the standard assay for quantification of [^{3}H]ouabain-binding sites (10^{-6} to 5×10^{-6} M). However, these values for α_1 and α_2 isoforms depend on the antibody used and its interaction with newly synthesized α subunits and are therefore inconclusive (for detailed discussion, see Clausen, 2003). Another study in which relative subunit composition was determined with antibodies indicated that in mouse EDL muscle, the α_2 subunit accounted for 87% of the total amount of the α subunit and the α_1 subunit for 13% (He et al., 2001).

More importantly, measurements of the maximum rate of ouabain-suppressible active ^{86}Rb uptake in Na^+-loaded rat soleus muscles have given values in good agreement with those obtained in the standard [^{3}H]ouabain-binding assay, indicating that this assay quantifiably measures the majority of the total content of functional Na^+,K^+ pumps (Clausen et al., 1987).

The phosphate analogue vanadate (VO_4) binds to the intracellular surface of the Na^+,K^+ pumps and thereby facilitates [^{3}H]ouabain binding to the outer surface of the Na^+,K^+ pumps in the plasma membranes (Hansen and Clausen, 1988). This enabled development of an assay in which cut muscle tissue segments are incubated with [^{3}H]ouabain in a Tris buffer–containing Tris vanadate (Nørgaard et al., 1983). Because the muscle segments are cut, vanadate gains ready access to the cytoplasm and can bind to the inner surface of the Na^+,K^+-ATPase. This assay gives the same values for [^{3}H]ouabain binding as measurements of [^{3}H]ouabain binding in intact muscles incubated in K^+-free KR. A comparison of the binding kinetics of [^{3}H]ouabain obtained using the vanadate-facilitated assay showed that the α_1-, α_2-, and α_3-subunit isoforms of the pumps in human tissues have similar affinity for [^{3}H]ouabain (Wang et al., 2001). This indicates that the vanadate-facilitated [^{3}H]ouabain-binding assay quantifies the sum of the three Na^+,K^+-pump isoforms present in human muscle biopsies (for details, see Clausen, 2003). Another major advantage of the vanadate-facilitated [^{3}H]ouabain-binding assay with small (2–5-mg) cut muscle specimens is that it can be used to measure Na^+,K^+ pumps in frozen muscle samples. Storage in the freezer for at least 4 yr causes no change in the content of [^{3}H]ouabain-binding sites of human muscle samples, and samples may be sent on dry ice by air freight. The assay is rapid, efficient, and inexpensive, and the results are reproducible. 17 studies on human skeletal muscle biopsies performed in seven different laboratories obtained similar values for the content of [^{3}H]ouabain-binding sites of human skeletal muscle samples (in the range of 243 to 425 pmol/g wet wt; see Table 1 in Clausen, 2003). More recent measurements of [^{3}H]ouabain-binding sites in human vastus lateralis muscle performed in four different laboratories showed results in the same range (326 ± 30 pmol/g wet wt [Nordsborg et al., 2005]; 272 ± 10 pmol/g wet wt [Green et al., 2004]; 240 ± 10 pmol/g wet wt [Boon et al., 2012]; 350 ± 108 pmol/g wet wt [McKenna et al., 2012]). [^{3}H]ouabain binds to both the outer surface of sarcolemma and the inner surface of the t-tubular lumen. Lau et al. (1979) showed that t-tubules from rabbit muscle bind [^{3}H]ouabain with the same affinity (dissociation constant around 5×10^{-8} M) and capacity (700 pmol/g wet wt) as the intact rat soleus muscle (Clausen and Hansen, 1974).

The rate of [^{3}H]ouabain binding depends on the rate of active Na^+,K^+ transport (Clausen and Hansen, 1977; Andersen and Clausen, 1993; Clausen, 2000, 2003). [^{3}H]Ouabain binding requires a certain configuration of the binding site on the extracellular portion of the α subunit that occurs only once during each Na^+,K^+-pumping cycle (Schwartz et al., 1975; Clausen and Hansen, 1977). Hence, an increased rate of cycling augments the chances for the [^{3}H]ouabain molecule to bind. Therefore, an increase in the rate of [^{3}H]ouabain binding likely indicates that the Na^+,K^+-pumping rate is augmented.

Regulation of the Na^+,K^+ pumps in skeletal muscle

Regulation of the Na^+,K^+ pumps evaluated by measurement of Na^+ and K^+ fluxes and [^{3}H]ouabain binding. Short-term regulation of Na^+,K^+-pump activity. The Na^+ ionophore monensin (10^{-5} M) induces a graded increase in Na^+ influx in skeletal muscle, enabling the evaluation of the effects of increased intracellular Na^+ on the rate of active Na^+,K^+ transport (measured as ouabain-suppressible ^{42}K or ^{86}Rb influx) and of possible changes in pump affinity for intracellular Na^+ (Buchanan et al., 2002). The Na^+,K^+ pumps undergo short-term and long-term regulation, often defined as acute changes in transport activity (e.g., μmoles of Na^+ extruded per gram wet weight per minute) or slower and more long-lasting changes in the content of Na^+,K^+ pumps (expressed as pmol of [^{3}H]ouabain bound per gram wet weight), respectively. In skeletal muscle, the most common cause of an acute increase in Na^+,K^+-pump activity is excitation-induced rise in intracellular Na^+ caused by increased Na^+ influx. This and around 20 other conditions or agents shown to stimulate the Na^+,K^+ pump in skeletal muscle are listed in Table 1. The earliest measurements of Na^+ influx in single frog semitendinosus muscle fibers showed a value of 11 nmol/g wet wt/action potential (Hodgkin and Horowicz, 1959). In close agreement with this value, intact rat EDL muscle stimulated for 5 s at 90 Hz to produce isometric tetanic contraction showed a Na^+ influx of 12 nmol/g wet wt/action potential and a K^+ efflux of 10 nmol/g wet wt/action potential, close to the 1:1 exchange of Na^+ and K^+ causing the action potential (Clausen et al., 2004). The excitation-induced increase in intracellular Na^+ primarily takes place at the inner surface of the plasma membrane, which is close enough to the Na^+,K^+ pumps to induce prompt activation of their transport activity. An early increase in Na^+,K^+-pump activity may also involve an excitation-dependent increase in Na^+,K^+-pump affinity for $[Na^+]_i$ that is independent of the rise in intracellular Na^+ per se (Buchanan et al., 2002). Thus, as shown in Fig. 2, electrical stimulation induces a leftward shift of the curve relating intracellular Na^+ to Na^+,K^+ pump–mediated ^{86}Rb uptake. Electrical stimulation of rat soleus for 10 s at 60 Hz at 30°C induced an immediate 58% increase in intracellular Na^+ content. During subsequent rest at 30°C, re-extrusion of Na^+ was complete in 2 min, and this was followed by a statistically significant undershoot in $[Na^+]_i$

($P < 0.001$) (Everts and Clausen, 1994). During the rapid early decrease in Na^+ content, it can be assumed that activity of the Na^+,K^+ pumps is stimulated 15-fold, even though $[Na^+]_i$ has decreased from its peak value down to the range found in resting muscle. The excitation-induced increase in Na^+ affinity was also detected by a highly significant ($P < 0.001$–0.05) decrease in intracellular Na^+ of ~25% that lasted up to 30 min after 60 s of electrical stimulation of isolated rat soleus muscles. Similar poststimulatory undershoots in $[Na^+]_i$ was seen in rat EDL muscle. It was blocked by ouabain or cooling to 0°C and was assumed to be mediated by activation of the Na^+,K^+ pumps by CGRP released from nerves in the muscles. Thus, reducing CGRP content by capsaicin or by prior denervation of the muscles prevented both excitation-induced force recovery and the drop in intracellular Na^+ (Nielsen and Clausen, 1997). This provides further evidence that activity-dependent stimulation of the Na^+,K^+ pump in muscle is not solely a function of the rise in $[Na^+]_i$ but might be caused by a mechanism similar to that in the electric organ of *Narcine brasiliensis*, where electrical stimulation markedly augments the activity of the Na^+,K^+-ATPase within fractions of a second despite a minimal change in intracellular Na^+ (Blum et al., 1990).

The slowly hydrolyzed cholinergic antagonist carbachol induces a long-lasting activation of the nicotinic acetylcholine receptors. Our experiments showed that in isolated rat soleus muscle, carbachol augments Na^+ influx leading to depolarization, increased intracellular Na^+, and loss of force (Macdonald et al., 2005). This reduction in force is significantly ($P < 0.05$) restored by stimulating the Na^+,K^+ pumps with epinephrine, salbutamol, or CGRP. All these effects are likely to be indirect, mediated by cAMP generated by stimulation of the adenylate cyclase. CGRP is found in sensory nerve endings from which it may be released by capsaicin or electrical stimulation to stimulate the Na^+,K^+ pumps in the muscle cells (Nielsen et al., 1998). Thus, CGRP release and the ensuing stimulation of pump activity may contribute to the above-mentioned long-lasting drop in intracellular Na^+ caused by electrical stimulation. Because the excitation-induced force recovery seen at increased $[K^+]_o$ is suppressed by the CGRP analogue CGRP-(8–37), which acts as a competitive CGRP antagonist, it is likely mediated by local release of CGRP from nerve endings in the muscle (Macdonald et al., 2008). In the isolated rat soleus muscle, repeated electrical stimulation caused hyperpolarization (11 mV) and increased amplitude of the action potentials. These effects were abolished by ouabain, cooling, or omission of K^+ from the buffer, suggesting that they resulted from of the electrogenic Na^+,K^+ pumps (Hicks and McComas, 1989) (see below in The electrogenic action of the Na^+,K^+ pumps).

As listed in Table 1, ~20 conditions or agents induce acute activation of the Na^+,K^+ pumps. cAMP (and agents that increase intracellular cAMP) not only increases the ouabain-suppressible uptake of ^{42}K or ^{86}Rb, but it also decreases intracellular Na^+ concentration. The decrease in intracellular Na^+ concentration, which is often maintained as long as the stimulating agent is present, may reach 60% below the control value (Clausen and Flatman, 1977). Like electrical stimulation (Fig. 2),

TABLE 1

Acute stimulation of the Na^+,K^+ pumps in skeletal muscle

Stimulating hormones, agents, and conditions	Mechanisms of action
Epinephrine, norepinephrine	Stimulate generation of cAMP, which in turn activates the Na^+,K^+ pumps via PKA, increasing the affinity of the Na^+K^+ pumps for Na^+
Isoproterenol, salbutamol, salmeterol	
Other β_2 agonists	
β_3 agonists	
Calcitonins	Stimulate generation of cAMP
CGRP	"
Amylin	"
cAMP, dibutyryl cAMP	Activates Na^+,K^+ pumps via PKA
Theophylline	Inhibits phosphodiesterase A, which degrades cAMP. This leads to intracellular accumulation of cAMP. Theophylline is a degradation product of caffeine.
Insulin, insulin-like growth factor I	Both act via the insulin receptors, increasing the affinity of Na^+,K^+ pumps for Na^+
Monensin	A Na^+ ionophore that increases $[Na^+]_i$, which directly stimulates the Na^+,K^+ pumps
Veratridine	Augments the Na^+ influx per action potential, thereby increasing $[Na^+]_i$
Excitation	Augments $[Na^+]$ influx, thereby increasing $[Na^+]_i$
Increasing temperature	When temperature increases 10°C, the rate of Na^+,K^+ pumping increases 2.3-fold
ATP, ADP	Stimulate the Na^+,K^+ pumps via purinergic receptors

salbutamol causes a leftward shift of the curve relating intracellular Na^+ to Na^+,K^+ pump–mediated uptake of ^{86}Rb in rat soleus muscle (Buchanan et al., 2002). The rapid stimulating effect of the above-mentioned agents on the Na^+,K^+ pumps, their mechanisms, and the physiological importance have been described in detail (Clausen, 2003). The stimulatory effects of these agents have been exploited in the treatment of hyperkalemia and the ensuing muscular weakness or paralysis. Thus, salbutamol was introduced for the treatment of paralytic attacks in patients with hyperkalemic periodic paralysis (Wang and Clausen, 1976). In muscles isolated from knock-in mice with the same genetic anomaly, stimulation of the Na^+,K^+ pumps with salbutamol or CGRP restored muscle force (Clausen et al., 2011). These observations suggest that the beneficial effect of mild exercise on severe weakness seen during attacks of hyperkalemic periodic paralysis is likely related to the stimulatory effect of locally released CGRP on the Na^+,K^+ pumps.

In rat soleus muscle, ATP induces a twofold increase in Na^+,K^+ pump–mediated ^{86}Rb uptake (Broch-Lips et al., 2010). ATP induces a marked recovery of force and M-waves (the sum of action potentials as recorded from the surface of the muscle) in muscles inhibited by increasing $[K^+]_o$, indicating that ATP-induced stimulation of the Na^+,K^+ pumps enhances excitability. Similar effects are exerted by ADP and are mediated by purinergic receptors. These effects are of particular interest during intense exercise, where local release of ATP or ADP from the muscle cells may reduce the inhibitory effect on excitability of the concomitant increase in $[K^+]_o$ (Broch-Lips et al., 2010). The importance of the electrogenic action of the Na^+,K^+ pumps is also evident from the observation that at increased $[K^+]_o$, where M-wave amplitude and area are decreased, stimulation with salbutamol or ATP restores the M-waves (Overgaard et al., 1999; Broch-Lips et al., 2010). In conclusion, the short-term increase in Na^+,K^+-pump activity in response to excitation has at least two components: (1) a direct stimulatory effect of $[Na^+]_i$ on the Na^+,K^+ pump; and (2) an increase in the affinity of the Na^+,K^+ pump for Na^+, potentiating the stimulating effect of $[Na^+]_i$. This seems to be mediated by cAMP, protein kinases, or ATP, and may involve release of a local hormone (CGRP). For more details, see below in Molecular mechanisms of pump regulation.

Long-term regulation of Na^+,K^+-pump content. Long-term increases in the muscle content of Na^+,K^+ pumps is most commonly seen after training. As measured using [^{3}H] ouabain binding, such an increase has been observed in nine different species and in numerous studies on human and rat muscle (see Table 2 in Clausen, 2003). The relative increase observed in the different studies depends on the duration and intensity of the training and ranges from 14 to 46%. There is a great deal of evidence for such changes in Na^+,K^+-pump content, which can also be induced by electrical stimulation. For instance, the content of [^{3}H]ouabain-binding sites in the tibialis anterior muscle of the rabbit was more than doubled after 20 d of electrical stimulation in vivo. Furthermore, it was correlated to the amplitude of M-waves ($P < 0.01$; $r = 0.8$) (Hicks et al., 1997). In pigs in which shivering is induced by lowered temperature, [^{3}H]ouabain binding is increased by 58–84% after weeks (for details, see Clausen, 2003). Running 100 km in 10.7 h produced a significant ($P < 0.05$) 13% increase in the content of [^{3}H]ouabain-binding sites in human vastus lateralis muscle (Clausen, 2003). Cycling exercise for 3 d increased the content of [^{3}H]ouabain-binding sites in human vastus lateralis muscle by 8% ($P < 0.05$) (Green et al., 2004). A recent study showed that rats performing voluntary free wheel running covered a distance of 13 km/day. After 8 wk, their soleus muscles contained 22% more [^{3}H]ouabain-binding sites than those from untrained controls. Moreover, the soleus muscle of the

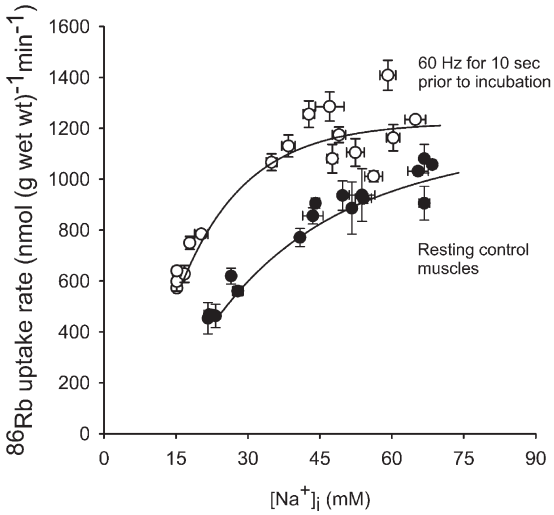

Figure 2. Electrical stimulation affects the relationship between $[Na^+]_i$ and $^{86}Rb^+$ uptake rate. Rat soleus muscles were mounted isometrically on electrodes and were stimulated for 10 s at 60 Hz. After a 2-min rest, they were transferred into solutions containing $^{86}Rb^+$, incubated for 2 min before being washed to remove extracellular ^{86}Rb and Na^+, and blotted, and intracellular $^{86}Rb^+$ and $[Na^+]$ were determined. $[Na^+]_i$ was manipulated by preincubating in buffer in which the Na^+ content had been reduced or in standard KR buffer containing the Na^+ ionophore monensin. ●, resting muscles; ○, muscles stimulated. Each point represents the mean ± SEM of measurements performed on three muscles. The curves were fitted using a computer program. Reprinted with permission from *The Journal of Physiology* (Buchanan et al., 2002).

trained rats showed considerably better contractile tolerance to increased $[K^+]_o$ (9 mM) than those from untrained controls (Broch-Lips et al., 2011). In rats exposed to hypoxia for 6 wk (causing chronic stimulation of respiration), the content of [^{3}H]ouabain-binding sites in the diaphragm was increased by 24% and the contractile endurance of the muscle was significantly augmented (McMorrow et al., 2011).

Conversely, a decrease in the muscle content of Na^+,K^+ pumps is seen during reduced activity or immobilization. In rats, guinea pigs, sheep, and humans, immobilization reduced the content of [^{3}H]ouabain-binding sites by 20–27%, changes that were reversible after mobilization (Clausen, 2003). Thus, after 2 wk of decreased mobility, the content of [^{3}H]ouabain-binding sites in guinea pig gastrocnemius muscles was 258 ± 13 pmol/g wet wt (25% below that of the contra-lateral freely mobile muscle; $P < 0.02$). After 3 wk of reduced mobility and 3 wk of training by running, the content of [^{3}H]ouabain-binding sites in the gastrocnemius muscle of these animals increased by 57%, whereas those that had not been immobilized reached 498 ± 25 pmol/g wet wt, which is 93% higher than that of the muscles with 2 wk of reduced mobility (Leivseth et al., 1992). This indicates that the regulatory range of Na^+,K^+-pump content in muscle represents roughly a doubling from the immobilized to the trained muscle. These results are particularly notable because, compared with rats or mice, all the Na^+,K^+ pumps in guinea pig muscle have high affinity for [^{3}H]ouabain, indicating complete occupancy of the [^{3}H]ouabain-binding sites.

Immobilization induced by denervation, a plaster cast, or tenotomy (tendon release) reduced the contents of [^{3}H]ouabain-binding sites in the skeletal muscles of mice, rats, guinea pigs, and sheep (Clausen, 2003; Clausen et al., 1982). In keeping with this, in human subjects complete spinal cord injury and partial denervation caused 58% reduction in the content of [^{3}H]ouabain-binding sites in the vastus lateralis muscle (Ditor et al., 2004). A more recent study confirmed this observation, showing ∼50% reduction in the content of [^{3}H]ouabain-binding sites in skeletal muscles of patients with complete cervical spinal cord injury (Boon et al., 2012). The mechanisms of exercise-related increases or decreases in the content of Na^+,K^+ pumps was explored in chick embryo leg muscle using monoclonal antibodies for estimating the relative changes in the number of Na^+,K^+ pumps. Increased intracellular Na^+ induced by activating the Na^+ channels with veratridine induced a 60–100% increase in the content of Na^+,K^+ pumps. Conversely, inhibition of the Na^+ channels with tetrodotoxin blocked this effect and induced a rapid decrease (Fambrough et al., 1987).

Hormonal regulation of Na^+,K^+-pump content. Thyroid hormone exerts the most potent hormonal stimulation of the synthesis of Na^+,K^+ pumps in skeletal muscle (Asano et al., 1976; for details, see Clausen, 2003). In rats, daily subcutaneous injection of thyroid hormone increases the content of [^{3}H]ouabain-binding sites by 75 pmol/g muscle wet wt/d (Everts and Clausen, 1988). The content of [^{3}H]ouabain-binding sites in the human vastus lateralis muscle varied from 100 pmol/g wet wt to 550 pmol/g wet wt in hypothyroid, euthyroid, and hyperthyroid subjects, with close linear correlation to free T_4 index ($r = 0.87$; $P < 0.001$) (Clausen, 2003). [^{3}H]ouabain binding in biopsies from vastus lateralis muscle of nine hyperthyroid patients was 89% higher value than in those form euthyroid controls. This increase in [^{3}H]ouabain binding, the concomitant increase in energy expenditure, and plasma thyroid hormone concentration were all restored to the control levels by the standard treatment of hyperthyroidism with the anti-thyroid drug methimazole, which inhibits the synthesis of thyroid hormone (Riis et al., 2005).

During fasting, the plasma concentration of thyroid hormones decreases (Spencer et al., 1983). In rats, total fasting for 72 h led to an ∼50% decrease in the content of [^{3}H]ouabain-binding sites in plasma membranes obtained from soleus muscle (Swann, 1984). 3 wk on reduced caloric intake caused a 45–53% decrease in rat plasma thyroid hormone concentration and a 25% decrease in the content of [^{3}H]ouabain-binding sites in the skeletal muscles (see Clausen, 2003). Because reduced caloric intake also decreases muscle mass, K^+ clearance from the extracellular space is likely to be further impaired. Worldwide, a starvation-induced decrease in Na^+,K^+-pump content is probably the most common muscle Na^+,K^+-pump disorder in human subjects, causing reduced tolerance to the hyperkalemia arising during K^+ ingestion and intensive work, leading to impaired physical performance. However, there is no information available about the effects of starvation on the content of Na^+,K^+ pumps in human skeletal muscle. The increase in Na^+,K^+-pump content in rat skeletal muscle induced by injection of thyroid hormone is preceded by an increase in the content of Na^+ channels in the muscles measured using ^{3}H-labeled saxitoxin as well as increased intracellular Na^+ measured by flame photometry (Clausen, 2003). Increased Na^+ influx in resting soleus muscle also induces an increase in the Na^+,K^+-pump content in knock-in mice with hyperkalemic periodic paralysis (Clausen et al., 2011). These observations are in keeping with the stimulatory effect of increased intracellular Na^+ on the synthesis of Na^+,K^+ pumps in cultured muscle cells (Fambrough et al., 1987). Adrenal steroids also influence the content of Na^+,K^+ pumps in skeletal muscle. Thus, in 36 patients treated for chronic obstructive lung disease with the glucocorticoid dexamethasone, the content of [^{3}H]ouabain-binding sites in needle biopsies of the vastus lateralis muscle was 31% higher than in 23 age- and sex-matched control subjects

($P < 0.001$) (Ravn and Dørup, 1997). In rats, 7–14 d of continuous infusion of dexamethasone via osmotic mini-pumps induced a 27–42% increase ($P < 0.001$–0.01) in the content of [^{3}H]ouabain-binding sites in soleus, EDL, gastrocnemius, and diaphragm muscles. In contrast, acute stimulation of Na^+,K^+-pump activity by infusion of the β_2 agonist terbutaline produced no significant change in [^{3}H]ouabain binding. A more detailed study (Dørup and Clausen, 1997) showed that the up-regulation of Na^+,K^+ pumps induced by dexamethasone could not be attributed to a mineralocorticoid action. Indeed, the mineralocorticoid aldosterone induced a decrease in the content of [^{3}H]ouabain-binding sites in rat skeletal muscle, which was closely correlated to a concomitant reduction in the content of K^+ in the muscles, induced by aldosterone stimulation of renal K^+ excretion This is in keeping with the down-regulation of Na^+,K^+ pumps in skeletal muscle observed in rats maintained on K^+-deficient fodder and in patients developing K^+ deficiency during treatment with diuretics (Clausen, 1998). In rat skeletal muscles, dexamethasone infusion for 14 d induced a relative rise in Na^+,K^+-ATPase α_2-subunit isoform of 53–78%, and in mRNA for the α_2 subunit a 6.5-fold increase was found (Thompson et al., 2001). In young human subjects, the ingestion of dexamethasone (2 mg twice daily for 5 d) increased the content of [^{3}H]ouabain-binding sites by 18% in the vastus lateralis muscle ($P < 0.001$) and by 24% in the deltoid muscle ($P < 0.01$). In the same subjects, the content of 3-O-MFPase in vastus lateralis and deltoid muscles increased by 14 and 18%, respectively ($P < 0.05$) (Nordsborg et al., 2005). Another study by the same group showed that the same dose of dexamethasone induced 17% relative increase in α_1- and α_2-subunit expression ($P < 0.05$) in vastus lateralis muscle, a significantly lower exercise-induced net K^+ release from the thigh muscles and a borderline significant prolongation of time to exhaustion ($P = 0.07$) (Nordsborg et al., 2008).

In rats made diabetic by streptozotocin pretreatment, the content of [^{3}H]ouabain-binding sites in skeletal muscle was reduced by 24% in soleus and 48% in EDL (Schmidt et al., 1994). These changes were completely restored by insulin treatment (Kjeldsen et al., 1987).

Translocation of Na^+,K^+ pumps compared with other types of activation. In skeletal muscle, the Na^+,K^+-pump molecules are located in the sarcolemma (primarily the α_1-subunit isoform) and in the t-tubular membranes (primarily the α_2-subunit isoform) (Marette et al., 1993; Williams et al., 2001; Cougnon et al., 2002; Radzyukevich et al., 2013). This localization seems to be strategic, reflecting a particular need for transport of Na^+ and K^+ across the t-tubular membranes during and after work. Thus, a large fraction of the Na^+,K^+ exchange takes place via the t-tubular membranes, and because of the small volume of the t-tubules, the intra-tubular concentration of K^+ is likely to reach high levels (36.6 mM in 1 s of stimulation at 100 Hz, sufficient to cause severe interference with excitability; Kirsch et al., 1977). A recent simulation study on single rat EDL muscle fibers showed that stimulation at 30 Hz increased the K^+ concentration in the t-tubules to a plateau of 14 mM within 1 s (Fraser et al., 2011). Therefore, during intense work, there is an urgent need for increased active transport of K^+ out of the t-tubular lumen and into the cytoplasm. The most efficient way of meeting this demand would be augmented affinity of the Na^+,K^+ pumps for Na^+ and/or K^+. Alternatively, the entire Na^+,K^+-pump molecule might move to positions in the plasma membrane where there is more ready access to Na^+ or K^+. It has long been known that insulin induces a 1.7-fold increase in the binding of [^{3}H]ouabain in frog sartorius muscle (Erlij and Grinstein, 1976) that was proposed to reflect an "unmasking" of an intracellular pool of inactive Na^+,K^+ pumps. Furthermore, Tsakiridis et al. (1996) showed that in rats, 60 min of treadmill running induced a significant relative increase (43–94%) in the α_2 polypeptide of the Na^+,K^+-ATPase (now termed the α_2-subunit isoform) detected by immunoblotting of plasma membranes prepared from hind-limb muscles. This was interpreted as indicating that exercise might induce translocation from an intracellular pool of endosomal membranes to the plasma membrane. Surprisingly, however, there was no concomitant decrease in the α_2 polypeptide detectable in the intracellular pool, and there was no information about the recovery of the Na^+,K^+ pumps in the plasma membranes (Tsakiridis et al., 1996). A later study used differential centrifugation of a whole homogenate to separate sarcolemmal membranes from endosomal membranes (Sandiford et al., 2005). After electrical stimulation in vivo via the nerves, the maximum 3-O-MFPase activity in the sarcolemmal fraction had increased by ~40%. In the same fraction, the α_2 subunit had increased by 38–40%, and in the endosomal fraction, the α_2 subunit had decreased by 42%, in keeping with a translocation of Na^+,K^+ pumps from the endosomal membranes to the sarcolemmal membranes. However, in a more recent study, Na^+,K^+ pumps could barely be detected in the intracellular pool of membranes (the putative source for the translocation of Na^+,K^+ pumps to the plasma membrane) (Zheng et al., 2008). In rats, 60 min of treadmill running induced a 29% increase in [^{3}H]ouabain labeling of sarcolemmal giant vesicles obtained from mixed hind-limb muscles, as well as 19–32% increases in the contents of the $\alpha_{1\text{-}2}$ and $\beta_{1\text{-}2}$ subunits (Juel et al., 2001). The increase in subunits was reversible with a half-life of 20 min, but there is no information about the reversibility of the [^{3}H]ouabain binding. However, as mentioned in the paper (Juel et al., 2001), the content of Na^+,K^+ pumps in the vesicular membranes measured as [^{3}H]ouabain-binding sites was only 0.3% of the total content of Na^+,K^+ pumps in rat hind-limb muscles.

Therefore, it could not be concluded that the samples of Na^+,K^+ pumps in the giant vesicles were representative of the entire pool of Na^+,K^+ pumps in the exercising muscles, raising doubts as to whether translocation of Na^+,K^+ pumps had taken place.

Virtually all the evidence for translocation of Na^+,K^+ pumps has been obtained using membrane fractions isolated from muscle homogenates. In contrast, studies on intact muscles or cut muscle specimens in vitro or intact muscles in vivo have consistently failed to detect translocation. Thus, measurements of [^{3}H]ouabain binding to intact rat soleus muscles performed under equilibrium conditions showed no effect of insulin or other conditions known to stimulate the activity of the Na^+,K^+ pumps (electrical stimulation, epinephrine, insulin-like growth factor I, and amylin; see also Table 1) (Clausen and Hansen, 1977; Dørup and Clausen, 1995; Clausen, 2003; McKenna et al., 2003; Murphy et al., 2008). It cannot be excluded, therefore, that the 70% "unmasking" or "translocation" of Na^+,K^+ pumps by insulin (Erlij and Grinstein, 1976) reflects the increase in the [^{3}H]ouabain binding taking place before binding equilibrium has been reached (see the section above, [^{3}H]Ouabain binding). This is usually caused by inadequate saturation of the ouabain-binding sites with [^{3}H]ouabain (Clausen and Hansen, 1977). Translocation has often been proposed as the cause of Na^+,K^+-pump stimulation induced by electrical stimulation or exercise. This hypothesis was tested by comparing effects of electrical stimulation on Na^+ extrusion and [^{3}H]ouabain binding in isolated rat soleus. Electrical stimulation in vitro at 120 Hz for 10 s caused a rapid 70% rise in intracellular Na^+, followed by a subsequent 18-fold increase in net Na^+ extrusion from rat soleus muscle but no significant change in the content of [^{3}H]ouabain-binding sites (McKenna et al., 2003). A later study showed that after 10–60 min of running exercise, there were highly significant increases (18–80%; $P < 0.001$– 0.01) in the Na^+ content of intact rat soleus muscles in vivo but no significant change in [^{3}H]ouabain binding (Murphy et al., 2008). In isolated rat EDL muscles, 15 s of 60-Hz stimulation caused a 7.3-fold increase in net Na^+ extrusion (Clausen, 2011). Another study on rat EDL showed that a 10-s stimulation at 60 Hz induced a fivefold increase in Na^+,K^+-pump activity (Nielsen and Clausen, 1997). It seems unlikely that such pronounced increases in Na^+ extrusion mediated by the Na^+,K^+ pumps could be accounted for by the relatively modest translocation of Na^+,K^+ pumps or binding of [^{3}H]ouabain (no change detectable) described above.

Either in vivo or in vitro administration of salbutamol augments K^+ content and reduces Na^+ content in rat soleus muscle (Murphy et al., 2008), reflecting increased affinity for intracellular Na^+ (Buchanan et al., 2002) probably caused by phosphorylation of FXYD1. Conversely, inhibition of the Na^+,K^+ pumps with ouabain reduces intracellular K^+ and increases intracellular Na^+ in vivo (Murphy et al., 2008).

Measurement of maximum binding capacity for [^{3}H]ouabain in isolated rat soleus (0.72 nmol/g wet wt) would predict that if all Na^+,K^+ pumps were operating at full speed, the theoretical maximum K^+ uptake should reach 0.72 nmol × 8,900 = 6,408 nmol/g/min at 30°C, which is corrected for temperature using the observed Q_{10} of 2.3 for the rate of Na^+,K^+ pumping (Clausen and Kohn, 1977). Are such high values seen in the intact soleus muscle? When isolated rat soleus muscles are loaded with Na^+ by preincubation in K^+-free KR buffer without Ca^{2+} or Mg^{2+}, intracellular Na^+ reaches 126 mM (Clausen et al., 1987). When these muscles are subsequently incubated for 3 min in KR buffer containing ^{42}K or ^{86}Rb and between 5 and 130 mM K^+, the ouabain-suppressible rates of ^{42}K or ^{86}Rb uptake can be measured and the results plotted in an Eadie–Hofstee plot (Fig. 4 in Clausen et al., 1987). The maximum rates of ^{42}K and ^{86}Rb uptake determined from this plot reach 6,150 nmol/g wet wt/min, corresponding to 96% of the above-mentioned theoretical maximum K^+ uptake at 30°C. When the same Na^+-loading procedure was used, the maximum rate of ouabain-suppressible ^{86}Rb uptake was correlated ($r = 0.95$; $P < 0.001$) to the content of [^{3}H]ouabain-binding sites over a wide range of values obtained by varying thyroid status, age, or K^+ depletion (Fig. 5 in the present paper). These high ^{86}Rb uptake values were not suppressed by Ba^{2+} or the local anesthetic tetracaine, indicating that they were mediated by the Na^+,K^+ pumps and not by K^+ channels (Clausen et al., 1987).

In conclusion, translocation of Na^+,K^+ pumps happens in skeletal muscle, but it is difficult to define and quantify. As a mechanism for stimulation of the Na^+,K^+ pumps it is too slow to explain the documented evidence for the acute Na^+,K^+-pump activation, which may reach the theoretical maximum in less than 1 min.

Subunit isoforms of the Na^+,K^+-ATPase

In the mammalian brain, the Na^+,K^+-ATPase exists in two distinct molecular forms that differ with respect to affinity for ouabain (Sweadner, 1979). Similarly, two subunit isoforms of the enzyme, α_1 and α_2, were detected in rat skeletal muscle (Tsakiridis et al., 1996). The functional Na^+,K^+ pump is composed of one of four catalytic α subunits (α_1–α_4) and one of three structural β subunits (β_1–β_3). In skeletal muscle, a third subunit, FXYD1, is coexpressed with the α subunits and involved in the regulation of Na^+,K^+-pump activity (Fig. 3).

At present, the subunit isoforms of the Na^+,K^+-ATPase as detected by immunoblotting cannot be quantified and given in molar units for the contents per gram muscle wet weight (Tsakiridis et al., 1996; Clausen, 2003). A recent study (McKenna et al., 2012) indicates that in human vastus lateralis muscle, there is a relative decrease of −24% in the abundance of the α_2 subunit from 24 to

67 yr of age ($P < 0.05$). This decrease was associated with a 36% reduction in muscle strength and peak O_2 consumption. However, the same study showed no significant age-dependent change in the content of [^{3}H]ouabain-binding sites (Table1). In human subjects, 6–9 wk of speed endurance training caused no change in the abundance of α_1 subunit in vastus lateralis muscle but a 68% increase in the α_2-subunit isoform of Na^+,K^+-ATPase (Bangsbo et al., 2009). More recent studies on human vastus lateralis show that 10 d of training increases the abundance of α_1 and α_2 subunit by 113 and 49%, respectively (Benziane et al., 2011). In rat hind-limb muscle, 60 min of treadmill running (20 m/min) induces a 55–64% increase in the abundance of the Na^+,K^+-ATPase α_1 subunit and a 43–94% increase in that of the α_2 subunit (Tsakiridis et al., 1996).

In the isolated rat EDL muscle, the mechanisms for the excitation-induced up-regulation of mRNA for the $\alpha_{1\text{-}3}$ subunits were examined. Three bouts of electrical stimulation (60 Hz for10 s) had no immediate effect on the abundance of the mRNA-encoding α_1–α_3-subunit isoforms (Murphy et al., 2006). However, after 3 h of poststimulatory rest, there were 223, 621, and 892% increases in the abundance of the mRNA-encoding subunits α_1, α_2, and α_3, respectively. However, in rat EDL muscle, even massive increases (598–769%) in intracellular Na^+ induced by pretreatment with ouabain, veratridine, or monensin produced no significant change in the mRNA encoding any of the subunit isoforms. Increasing intracellular Ca^{2+} by preincubation with the Ca^{2+} ionophore A23187 produced no increase in the mRNA for the two major subunit isoforms (α_1 and α_2). Thus, a combination of multiple signals seems to be required to recapitulate the increase in abundance of the mRNAs that trigger the increase in synthesis of Na^+,K^+ pumps induced by exercise. In conclusion, the abundance of the α_1 and α_2 subunits seems to respond to training and age, but the observed relative changes show considerable variation and cannot yet be "translated" to molar units for Na^+,K^+ pumps per gram muscle tissue. As shown in Table 2, the relative changes in the abundance of α_2 in skeletal muscle induced by electrical stimulation, heavy cycle training, chronic obstructive lung disease, aging, or nicotine pretreatment are considerably more pronounced than in the content of [^{3}H]ouabain-binding sites measured in the same muscle samples. In contrast, the five other comparisons in Table 1 show good agreement between the relative changes in α_2 subunit and in [^{3}H]ouabain-binding sites. Thus, it is difficult to decide which of these estimates provides functionally or clinically relevant information about the capacity for clearing extracellular K^+ during exercise or K^+ loading.

A recent analysis concludes that the α_2 isozyme represents the major pool of Na^+,K^+-ATPase of skeletal muscle, essential for contractility and the prevention of fatigue. In a knockout mouse completely lacking the α_2 isozyme, loss of force and increased fatigability was seen (Radzyukevich et al., 2013). These defects could be reproduced in isolated muscles from control animals by selectively inhibiting the α_2 subunit with 5 μM ouabain.

Molecular mechanisms of pump regulation. Acute regulation of Na^+,K^+-ATPase by hormones. Insulin has a hypokalemic action, prompting its use in the therapy of hyperkalemia (Harrop and Benedict, 1924) and inspiring studies with isolated rat muscles that showed that insulin increases the uptake of K^+ and the extrusion of Na^+ (Creese, 1968), leading to reduced intracellular Na^+ and hyperpolarization (Zierler and Rabinowitz, 1964). Because the effects of insulin on Na^+,K^+ fluxes and membrane potential are suppressed by ouabain, they are likely caused by stimulation of the Na^+,K^+ pumps (Clausen and Kohn, 1977, Clausen, 2003). The mechanism seems to reflect increased affinity for Na^+ on the inner surface of the

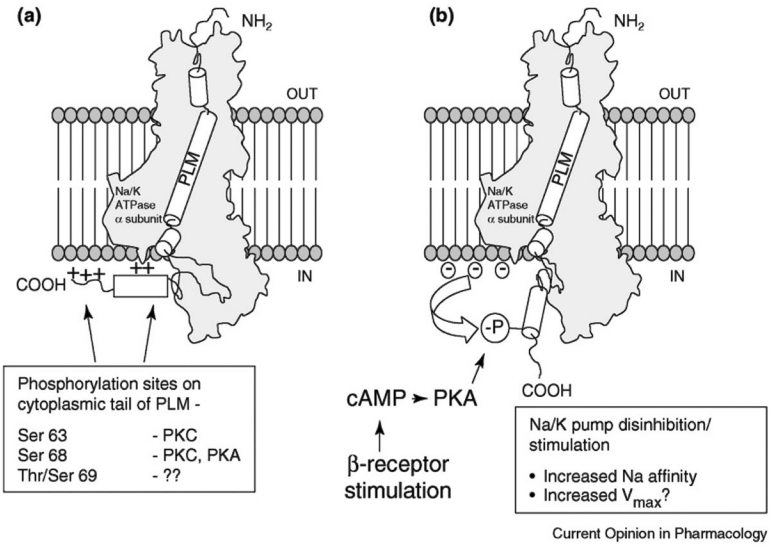

Figure 3. Hypothetical relationship between phospholemman (PLM or FXYD1) and the Na^+/K^+ATPase α subunit indicating possible effects of PLM phosphorylation on their interaction. The PLM cytoplasmic tail can be phosphorylated at Ser63, Ser68, and Thr69 (Ser69 in mouse in panel a). Phosphorylation at any or all of these sites (or, for example, at the site depicted as undergoing phosphorylation by PKA in panel b) may alter orientation of the cytoplasmic tail, thereby affecting its interaction with the Na^+/K^+ ATPase α subunit to increase ion transport. Reprinted with permission from *Current Opinion in Pharmacology* (Shattock, 2009).

Na^+,K^+ pumps (Clausen, 2003). As discussed in the section above on translocation (Translocation of Na^+,K^+ pumps compared...), the stimulating action of insulin on the Na^+,K^+ pumps may not be attributed to translocation of Na^+,K^+ pumps. Studies on cultured rat muscle cells indicate that the stimulatory effect of insulin on ^{86}Rb uptake is abolished by ouabain and reduced by phorbol ester, indicating that it is mediated by Na^+,K^+ pumps and caused by activation of PKC (Sampson et al., 1994).

Similarly, the hypokalemic action of catecholamines inspired studies with isolated muscles demonstrating stimulatory effects of epinephrine and norepinephrine on the uptake of K^+ and the net extrusion of Na^+ (Clausen and Flatman, 1977). Because of the 3:2 exchange of Na^+ versus K^+, the Na^+,K^+ pump is electrogenic and its stimulation leads to hyperpolarization. Detailed studies showed that this stimulation depended on a β_2 adrenoceptor–mediated activation of adenylate cyclase increasing the cytoplasmic concentration of cAMP (Clausen and Flatman, 1977; Clausen, 2003). cAMP, via PKA, induces phosphorylation of FXYD1serine68, causing increased Na^+ affinity of the Na^+,K^+ pumps (Shattock, 2009) and ensuing reduction of intracellular Na^+. A similar sequence of events explain the cAMP-mediated, Na^+,K^+ pump–stimulatory action of other hormones and pharmaceuticals (including calcitonin, CGRP, amylin, isoprenaline, and theophylline; see Table 1). Part of the mechanisms for the action of catecholamines and cAMP on the activation of the Na^+,K^+ pumps in skeletal muscle has been described previously (Clausen, 1998, and Fig. 6 in Clausen, 2003). More detailed molecular insight is discussed by Shattock (2009) and in this paper.

Na^+,K^+-ATPase regulation by auxiliary proteins. The Na^+,K^+ pumps are subject to regulation by a series of proteins. The β subunit (Fig. 1) is a glycoprotein required for the transfer of the entire Na,K-ATPase enzyme molecule from its site of synthesis in the endoplasmic reticulum to its site of insertion in the plasma membrane. The γ subunit, originally identified in the kidney, augments the affinity of the myocardial Na^+,K^+-ATPase for Na^+ (Bibert et al., 2008). In mammals, there is a family of at least seven regulatory proteins termed FXYD ("fix-it") that are associated with the Na^+,K^+-ATPase in a tissue-specific way (Mahmmoud et al., 2000; Cornelius and Mahmmoud, 2003). The first FXYD identified, called FXYD1 and also known as phospholemman, was originally found in myocardial cells (Palmer et al., 1991). FXYD1 is a major substrate for PKA and PKC and was later found to regulate the Na^+,K^+-ATPase in heart and skeletal muscle (Shattock, 2009). Increased cAMP activates cAMP-dependent kinase (PKA), which, in turn, phosphorylates serine-68 on FXYD1 (Fig. 3). This disinhibits the Na^+,K^+ pumps and stimulates their activity by raising V_{max} and the sensitivity of the pumps to intracellular Na^+. Phosphorylation of FXYD increases the maximal Na^+,K^+-pump current of α_2/β isozymes (Bibert et al., 2008). cAMP seems to function as a common signaling molecule acting via PKA to augment the affinity of the Na^+,K^+ pump for $[Na^+]_i$ in muscle (Shattock, 2009). In addition to Ser68, FXYD can also be phosphorylated at Ser63 (by PKC) and at Thr69 (by an unidentified kinase).

In the human vastus lateralis muscle, 30 s of intense exercise clearly increased phosphorylation of FXYD (Thomassen et al., 2011). Another study demonstrated that 60 min of acute exercise increases phosphorylation of FXYD from human vastus lateralis by 75% (Benziane et al., 2011). In soleus muscle of wild-type mice, 10 min of electrical stimulation caused a 59% increase in phosphorylation of FXYD. This phosphorylation was not seen in PKCα knockout mice and therefore seems to depend on PKC (Thomassen et al., 2011).

Physiology and pathophysiology of the Na^+,K^+-ATPase in skeletal muscle

The electrogenic action of the Na^+,K^+ pumps. Because each Na^+,K^+-pump molecule in the plasma membrane

TABLE 2

Relative changes in [^{3}H]ouabain binding and the α_2 subunit of Na^+,K^+-ATPase in skeletal muscle

References, muscle preparation, treatment	[^{3}H]Ouabain binding	α_2-subunit abundance
Thompson et al., 2001, rats treated for 14 d with dexamethasone	+22–48% ($P < 0.05$)	+53% ($P < 0.05$)
Juel et al, 2001, 1 h treadmill running 150-200 g rats, giant vesicles	+29% ($P < 0.05$)	+32% ($P < 0.05$)
Green et al., 2004, 6 d submaximal cycling	+13% ($P < 0.05$)	+9% ($P < 0.05$)
Sandiford et al., 2005, electrical stimulation of rat soleus	+16–21% ($P < 0.05$)	+38% ($P < 0.05$)
Green et al., 2007, 16 h heavy intermittent cycling	+8% ($P < 0.05$)	+26% ($P < 0.05$)
Green et al., 2008, 3 d submaximal cycling	+12% ($P < 0.05$)	+42% ($P < 0.05$)
Green et al., 2009, chronic obstructive lung disease	−6% (NS)	+12% ($P < 0.05$)
McKenna et al., 2012, young compared to aged subjects	−0.7% (NS)	−24% ($P < 0.05$)
Boon et al., 2012, spinal cord injury in human subject	−47% ($P < 0.05$)	−52% ($P < 0.05$)
Chibalin et al., 2012, nicotine pretreatment in 190 g rats	0% (NS)	−25% ($P < 0.05$)

Data were obtained from 10 different publications listed in the references of this paper. Each of them was selected for reporting the results of measurements of the content of [^{3}H]ouabain-binding sites as well as α_2-subunit abundance in the same muscle. Experimental details are given in the cited articles. The relative changes induced by the listed factors are given in percentages. Biopsies from human subjects were taken from the vastus lateralis muscle. The rat muscles were obtained from the hind limbs.

exchanges three Na^+ ions for two K^+ ions in each cycle, each cycle leads to a net extrusion of one positive charge. Measurements on isolated rat soleus muscle show that the resting ouabain-suppressible efflux of ^{22}Na at 30°C is 0.287 µmol/g wet wt/min, and the concomitant influx of ^{42}K is 0.196 µmol/g wet wt/min (Clausen and Kohn, 1977). Thus, the ratio between Na^+,K^+ pump–mediated Na^+ efflux and K^+ influx is 0.287/0.196 = 1.46, close to the 3:2 ratio. This electrogenic action of the Na^+,K^+ pumps is consistent with the observation that, in rat soleus muscle, blocking the Na^+,K^+ pumps with ouabain causes a depolarization of ~10 mV in 10 min (Clausen and Flatman, 1977). Conversely, acute stimulation of the rate of active Na^+,K^+ transport by epinephrine in isolated rat soleus muscle causes a hyperpolarization of ~8 mV, which is abolished by ouabain. The β_2 agonist salbutamol induces a similar effect in vitro, and when given intravenously, it causes 15-mV hyperpolarization in rat soleus (Clausen and Flatman, 1980). The following agents, which stimulate active Na^+,K^+ transport, all induce significant hyperpolarization in rat, mouse, or guinea pig soleus: epinephrine, norepinephrine, isoprenaline, salbutamol, dbcAMP, phenylephrine, rat, human or salmon calcitonin, CGRP, and insulin (Clausen and Flatman, 1977, 1987; Andersen and Clausen, 1993). Rat soleus muscles can be loaded with Na^+ by a 90-min preincubation in K^+-free KR. When subsequently transferred to KR with normal K^+, the rate of ^{22}Na efflux is more than doubled. This effect is blocked by ouabain and associated with an ~10-mV hyperpolarization (Clausen and Flatman, 1987). These observations indicate that the electrogenic action of hormonal Na^+,K^+-pump stimulation can be mimicked by Na^+ loading.

The Na^+,K^+ pumps compensate functional defects caused by plasma membrane leakage

Apart from the channel-mediated selective Na^+ and K^+ leaks that take place during excitation, the plasma membrane may develop nonspecific leaks caused by loss of integrity during intense work, anoxia, physical damage, electroporation, excessive swelling, rhabdomyolysis, or various muscle diseases. The response to such leaks and the possible role of the Na^+,K^+ pumps in compensating the ensuing functional disorders have been examined using electroporation of isolated rat soleus and EDL muscles (Clausen and Gissel, 2005). In these studies, muscles were mounted in an electroporation cuvette and exposed to short-lasting (0.1-ms) pulses of an electrical field of 100–800 V/cm across the muscles. This induces rapid formation of pores in the plasma membrane, increasing its permeability, but not in the membranes of intracellular organelles. This allows reversible influx of Na^+, loss of K^+ and excitability, release of intracellular proteins, and penetration of extracellular markers into the cytoplasm (Clausen and Gissel, 2005). As shown in Fig. 4, eight electroporation pulses of 500 V/cm induces immediate complete loss of tetanic force, which in 200 min is followed by spontaneous recovery to around 30% of the force measured before electroporation. The initial phase of this recovery is considerably (183–433%) enhanced by stimulating the Na^+,K^+ pumps with salbutamol, rat CGRP, or dibutyryl cAMP. Both the spontaneous 30% force recovery and the 50% force recovery induced by salbutamol were abolished by ouabain, indicating that they were caused by Na^+,K^+-pump stimulation. The electroporation caused a depolarization from −70 to around −20 mV, followed by a partial spontaneous recovery. Salbutamol (10^{-5} M) further improved the repolarization by 15 mV (Clausen and Gissel, 2005). In isolated rat EDL muscles, 30–60 min of intermittent stimulation caused a drop in tetanic force to 12% of the initial level, followed by slow spontaneous recovery to 20–25% of the initial force level. This was associated with 11–15-mV depolarization and marked loss of the intracellular protein lactic acid dehydrogenase. Subsequent stimulation of the Na^+,K^+ pumps with salbutamol

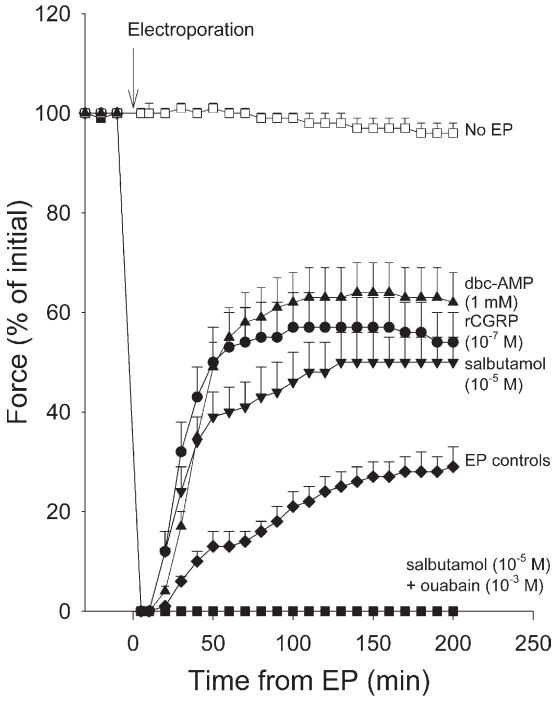

Figure 4. Effects of salbutamol, rat CGRP, dbcAMP, and ouabain on the response of tetanic force to electroporation (EP). Force was measured in rat soleus muscles mounted for isometric contractions in KR buffer at 30°C using 2-s pulse trains of 60 Hz. Trains were repeated three times to obtain an initial determination of tetanic force, and data are presented as a percentage of this value. After transfer to an electroporation cuvette, muscles either underwent electroporation (eight pulses of 500 V/cm and 0.1-ms duration) or were left without electroporation. Force was subsequently recorded at the indicated intervals in buffer without additions (EP controls) or in buffer containing salbutamol (10^{-5} M), rat CGRP (10^{-7} M), dbcAMP (1 mM), or salbutamol (10^{-5} M) plus ouabain (10^{-3} M). Each point represents the mean of observations on 4–13 muscles, with bars denoting SEM. Reprinted with permission from *Acta Physiologica* (Clausen and Gissel, 2005).

restored membrane potential to normal level. Salbutamol, epinephrine, rat CGRP, and dibutyryl cAMP all induced a significant increase (40–90%) in the force recovery after intermittent stimulation (Mikkelsen et al., 2006). In EDL muscle, anoxia caused massive reductions in the content of phosphocreatine and ATP and a marked loss of force, which was clearly reduced by the two β_2 agonists salbutamol and terbutaline. The force recovery seen after reoxygenation was markedly improved by salbutamol, the long-acting β_2 agonist salmeterol, theophylline (a phosphodiesterase inhibitor that inhibits the breakdown of cAMP), and dibutyryl cAMP (causing the following relative increases in force: 55–262%). The salbutamol-induced increase in force recovery could be related to partial restoration of excitability. In anoxic muscles, salbutamol had no effect on phosphocreatine or ATP but decreased intracellular Na^+ concentration and increased ^{86}Rb uptake and K^+ content, indicating that the mechanism is cAMP-mediated stimulation of the electrogenic Na^+,K^+ pumps (Fredsted et al., 2012) and cannot be related to recovery of energy status. Thus, the Na^+,K^+ pumps may restore excitability even when energy status is low. This interpretation is in keeping with a detailed analysis in isolated mouse soleus and EDL muscles, suggesting that impaired excitability is the main contributor to severe fatigue, discounting anoxia as the major contributor to fatigue in isolated muscles (Cairns et al., 2009).

Functional significance of Na^+,K^+ pumps. The evidence described above indicates that the content of Na^+,K^+ pumps measured using [^{3}H]ouabain binding represents Na^+,K^+ pumps, which may all become operational when activated by intracellular Na^+ loading and increased extracellular K^+, by hormones or excitation. Is it possible to induce a rapid activation of all the Na^+,K^+ pumps in the intact muscle? In rat soleus muscle, electrical stimulation at 120 Hz for 10 s increases intracellular Na^+ to ~50 mM (Nielsen and Clausen, 1997). When the muscles were subsequently allowed to rest in standard KR at 30°C, the net efflux of Na^+ recorded over the first 30 s was shown to reach 9,000 nmol/g wet wt/min, 97% of the theoretical maximum Na^+ transport rate of 9,300 nmol/g wet wt/min (Fig. 5 in Nielsen and Clausen, 1997). This indicates that during intense exercise, most likely to produce a high degree of Na^+,K^+-pump use, a 97% utilization has been documented. Apparently, it requires the combination of large increases in intracellular Na^+ and extracellular K^+, which, as described above, can be mimicked by Na^+ loading and subsequent incubation at high $[K^+]_o$ (Clausen et al., 1987). In rat soleus, 60-Hz stimulation induces a total efflux of K^+ of 5,580 nmol/g wet wt/min (Clausen et al., 2004). Only full activation of all Na^+,K^+ pumps would enable K^+ reuptake sufficient to allow extracellular K^+ clearance to keep pace with excitation-induced loss of K^+. As already noted, the theoretical maximum Na^+,K^+ pump–mediated K^+ uptake in rat soleus should reach 6,408 nmol K^+/g wet wt/min, slightly exceeding the above-mentioned total loss of K^+. Such large increases in active Na^+,K^+ transport would appear energetically unlikely. However, as stated previously, both in resting and working muscles, the energy requirement of the Na^+,K^+ pumps only amounts to 2–10% of the total energy turnover. This evaluation provides an example of the importance of accurate quantification of the Na^+,K^+ pumps and their energy requirements.

This information raises the question of whether such combinations of high $[Na^+]_i$ and $[K^+]_o$ occur in working intact muscle in vitro or in vivo. Several studies indicate that isolated muscles and muscle fibers, when stimulated at resting length, take up substantial amounts of Na^+ and release nearly equimolar amounts of K^+ (Hodgkin

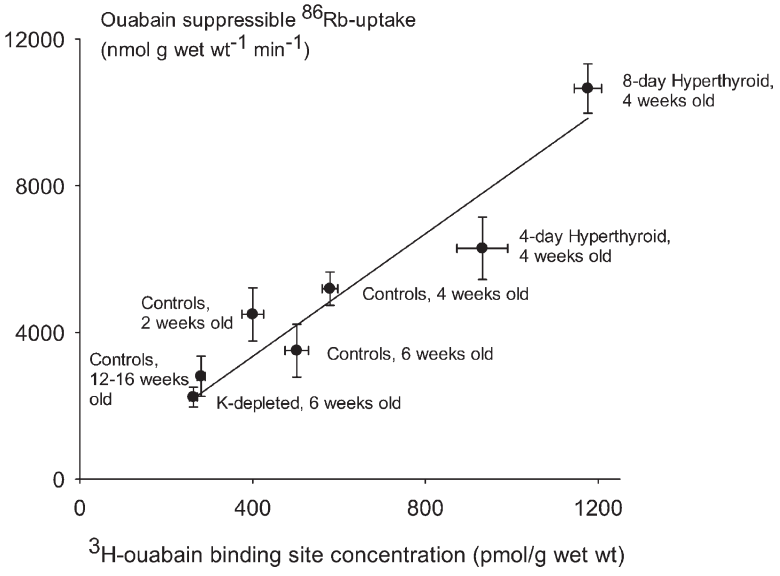

Figure 5. Correlation between ouabain-suppressible ^{86}Rb uptake and [^{3}H]ouabain-binding site content in soleus muscles of rats with K^+ deficiency and varying age or thyroid status. Ouabain-suppressible ^{86}Rb uptake was measured as described in Clausen et al. (1987). [^{3}H]Ouabain-binding site content was determined on muscle samples using the vanadate-facilitated assay. After correction for nonspecific uptake, [^{3}H]ouabain binding was corrected for radioisotopic purity, incomplete saturation, and loss of specifically bound [^{3}H]ouabain. The line was constructed using linear-regression analysis of unweighted values ($r = 0.95$; $P < 0.001$). Reprinted with permission from *The Journal of Physiology* (Clausen et al., 1987).

and Horowicz, 1959; Clausen, 2008b, 2011, 2013; Clausen et al., 2004). In vitro experiments show that when the amount of K^+ released from the cells (μmoles/gram wet weight as measured by flame photometry) is divided by the extracellular space (milliliter/gram wet weight), it can be calculated that in isolated soleus and EDL muscles, $[K^+]_o$ reaches levels of 20 or 50 mM, respectively, sufficient to suppress excitability (Clausen, 2008a). More detailed studies showed that in isolated EDL muscles, 60 s of stimulation at 20 Hz increases $[K^+]_o$ to around 45 mM, leading to loss of excitability (Clausen, 2011). Recent in vitro and in vivo studies show that during direct or indirect electrical stimulation (at 5 Hz for 300 s or 60 Hz for 60 s) of rat EDL muscles, the mean extracellular K^+ in the stimulated muscles may reach up to 50–70 mM (Clausen, 2013). This was combined with an appreciable decrease in $[Na^+]_o$ (46 mM), causing further loss of force. This provides new evidence that in the intact organism, excitation-induced rise in $[K^+]_o$ and decrease in $[Na^+]_o$ can be major causes of muscle fatigue. Moreover, as described above, the Na^+,K^+ pumps available in the muscles may fully use their maximum transport capacity to counterbalance the excitation-induced rise in $[K^+]_o$. An important function of the Na^+,K^+ pumps is to protect muscle excitability against increases in $[K^+]_o$. When exposed to 10–15 mM K^+, contractility of rat soleus or EDL muscles is reduced or lost. Force may rapidly be restored by stimulating the Na^+,K^+ pumps with salbutamol or insulin (Clausen, 2003; Clausen and Nielsen, 2007).

The excitation-induced rise in $[K^+]_o$ depends on muscle fiber type (slow-twitch or fast-twitch fibers) and the frequency of contractions. In isolated rat EDL muscle (predominantly fast-twitch fibers), the excitation-induced influx of Na^+ and release of K^+ per action potential is, respectively, 6.5- and 6.6-fold larger than in soleus (predominantly slow-twitch fibers) (Clausen et al., 2004). When stimulated continuously at 60 Hz, the rate of force decline over the first 20 s is 5.9-fold faster in EDL than in soleus muscle. When tested using continuous stimulation in the frequency range of 10 to 200 Hz, the rates of force decline in rat soleus and EDL muscles are closely correlated ($r^2 = 0.93$; $P < 0.002$ and 0.99; $P < 0.01$, respectively) to the excitation-induced measured increase in $[K^+]_o$ (Clausen, 2008b). These observations indicate that the faster rate of force decline and fatigability seen in fast-twitch muscles as compared with slow-twitch muscles is caused by the much larger excitation-induced passive Na^+,K^+ fluxes. In view of this difference, leading to a larger work-induced increase in intracellular Na^+, it would be expected that the content of Na^+,K^+ pumps in EDL would undergo long-term up-regulation to a level considerably over and above that seen in soleus. However, measurements of [^{3}H]ouabain-binding sites in vitro and in vivo show that rat EDL only contains 20–30% more Na^+,K^+ pumps per gram wet weight than soleus (Clausen et al., 1982; Murphy et al., 2008).

Control soleus muscle, ouabain-pretreated soleus, or soleus from K^+-deficient rats show a graded reduction in functional Na^+,K^+ pumps from 756 pmol/g wet wt down to 110 pmol/g wet wt. The content of [^{3}H]ouabain-binding sites is significantly correlated ($r^2 = 0.88$; $P < 0.013$) to the rate of force decline developing over 30 s of continuous stimulation at 60 Hz (Nielsen and Clausen, 1996). This illustrates the crucial role of the Na^+,K^+-pump capacity in maintaining contractile endurance. In keeping with this, K^+ deficiency in human subjects, which reduces the content of Na^+,K^+ pumps, causes reduced grip strength and fatigue (Clausen, 1998, 2003). Conversely, 19 studies have documented that exercise training leads to an increase in the content of Na^+,K^+ pumps measured as [^{3}H]ouabain binding (Clausen, 2003). This up-regulation improved the clearance of plasma K^+ during exercise, a mechanism for reducing fatigue (McKenna et al., 1993). During contractile activity, the K^+ released from the muscle cells is normally cleared via the circulation. When the blood vessels are compressed because of static contractions when carrying heavy burdens (Barcroft and Millen, 1939), this clearance is reduced or abolished. Therefore, extracellular K^+ may undergo further increase, and its clearance will depend on the transport capacity and activity of the Na^+,K^+ pumps. Through a similar mechanism, deficient circulation may lead to reduction of contractile force.

Conclusions

Recent studies indicate that in skeletal muscle, excitation-induced net gain of Na^+ and loss of K^+ are considerably larger than reported previously and represent important causes of fatigue. This implies that to counterbalance these rapid passive Na^+,K^+ fluxes, working muscles have to make full use of the transport capacity of their Na^+,K^+ pumps. This capacity can be quantified using [^{3}H]ouabain binding and expressed in picomoles of Na^+,K^+ pumps per gram muscle wet weight, offering values that can be confirmed by measurements of maximum ouabain-suppressible fluxes of Na^+ and K^+. This allows quantification of the capacity to clear extracellular K^+ in patients with reduced content of Na^+,K^+ pumps in their muscles. Numerous studies have shown that the content of Na^+,K^+ pumps in skeletal muscles can be quantified, documenting the up-regulation of the content of Na^+,K^+ pumps induced by exercise and hormones as well as the down-regulation seen in various diseases associated with reduced physical performance or fatigue. Assays based on immunoblotting provide relative changes in abundance that cannot yet be translated into molar units. The activity of the Na^+,K^+ pumps may increase rapidly (by electrical stimulation

up to 20-fold in 10 s) or by Na^+ loading, insulin, catecholamines, calcitonins, amylin, and theophylline.

I wish to thank Flemming Cornelius for valuable help and advice with the text and Fig. 1. I thank Marianne Stürup-Johansen for preparing illustrations and retrieval of literature.

This project was supported by grants from The Lundbeck Foundation (J. Nr. R31-A2536), The Karen Elise Jensen Foundation (J. Nr. 22401 8/04/05), and the Velux Foundation (J. Nr. 30118).

Elizabeth M. Adler served as editor.

Submitted: 22 February 2013
Accepted: 30 August 2013

REFERENCES

Andersen, S.L.V., and T. Clausen. 1993. Calcitonin gene-related peptide stimulates active Na^+-K^+ transport in rat soleus muscle. *Am. J. Physiol.* 264:C419–C429.

Asano, Y.M., U.A. Liberman, and I.S. Edelman. 1976. Thyroid thermogenesis. Relationships between Na^+-dependent respiration and Na^+ + K^+-adenosine triphosphatase activity in rat skeletal muscle. *J. Clin. Invest.* 57:368–379. http://dx.doi.org/10.1172/JCI108288

Bangsbo, J., T.P. Gunnarsson, J. Wendell, L. Nybo, and M. Thomassen. 2009. Reduced volume and increased training intensity elevate muscle Na^+-K^+ pump α_2-subunit expression as well as short- and long-term work capacity in humans. *J. Appl. Physiol.* 107:1771–1780. http://dx.doi.org/10.1152/japplphysiol.00358.2009

Barcroft, H., and J.L. Millen. 1939. The blood flow through muscle during sustained contraction. *J. Physiol.* 97:17–31.

Benziane, B., U. Widegren, S. Pirkmajer, J. Henriksson, N.K. Stepto, and A.V. Chibalin. 2011. Effect of exercise and training on phospholemman phosphorylation in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 301:E456–E466. http://dx.doi.org/10.1152/ajpendo.00533.2010

Bibert, S., S. Roy, D. Schaer, J.-D. Horisberger, and K. Geering. 2008. Phosphorylation of phospholemman (FXYD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *J. Biol. Chem.* 283:476–486. http://dx.doi.org/10.1074/jbc.M705830200

Blum, H., S. Nioka, and R.G. Johnson Jr. 1990. Activation of the Na^+, K^+-ATPase in Narcine brasiliensis. *Proc. Natl. Acad. Sci. USA.* 87:1247–1251. http://dx.doi.org/10.1073/pnas.87.3.1247

Boon, H., E. Kostovski, S. Pirkmajer, M. Song, I. Lubarski, P.O. Iversen, N. Hjeltnes, U. Widegren, and A.V. Chibalin. 2012. Influence of chronic and acute spinal cord injury on skeletal muscle Na^+-K^+-ATPase and phospholemman expression in humans. *Am. J. Physiol. Endocrinol. Metab.* 302:E864–E871. http://dx.doi.org/10.1152/ajpendo.00625.2011

Broch-Lips, M., T.H. Pedersen, and O.B. Nielsen. 2010. Effect of purinergic receptor activation on Na^+-K^+ pump activity, excitability, and function in depolarized skeletal muscle. *Am. J. Physiol. Cell Physiol.* 298:C1438–C1444. http://dx.doi.org/10.1152/ajpcell.00361.2009

Broch-Lips, M., F. de Paoli, T.H. Pedersen, K. Overgaard, and O.B. Nielsen. 2011. Effects of 8 wk of voluntary unloaded wheel running on K^+ tolerance and excitability of soleus muscles in rat. *J. Appl. Physiol.* 111:212–220. http://dx.doi.org/10.1152/japplphysiol.00687.2010

Buchanan, R., O.B. Nielsen, and T. Clausen. 2002. Excitation- and β_2-agonist-induced activation of the Na^+-K^+ pump in rat soleus muscle. *J. Physiol.* 545:229–240. http://dx.doi.org/10.1113/jphysiol.2002.023325

Cairns, S.P., A.J. Taberner, and D.S. Loiselle. 2009. Changes of surface and t-tubular membrane excitability during fatigue with repeated tetani in isolated mouse fast- and slow-twitch muscle. *J. Appl. Physiol.* 106:101–112. http://dx.doi.org/10.1152/japplphysiol.90878.2008

Chibalin, A.V., J.A. Heiny, B. Benziane, A.V. Prokofiev, A.V. Vasiliev, V.V. Kravtsova, and I.I. Krivoi. 2012. Chronic nicotine modifies skeletal muscle Na,K-ATPase activity through its interaction with the nicotinic acetylcholine receptor and phospholemman. *PLoS ONE.* 7:e33719. http://dx.doi.org/10.1371/journal.pone.0033719

Clausen, T. 1998. Clinical and therapeutic significance of the Na^+,K^+ pump. *Clin. Sci.* 95:3–17. http://dx.doi.org/10.1042/CS19970254

Clausen, T. 2000. Effects of amylin and other peptide hormones on Na^+-K^+ transport and contractility in rat skeletal muscle. *J. Physiol.* 527:121–130.

Clausen, T. 2003. Na^+-K^+ pump regulation and skeletal muscle contractility. *Physiol. Rev.* 83:1269–1324.

Clausen, T. 2008a. Clearance of extracellular K^+ during muscle contraction—roles of membrane transport and diffusion. *J. Gen. Physiol.* 131:473–481. http://dx.doi.org/10.1085/jgp.200809971

Clausen, T. 2008b. Role of Na^+,K^+-pumps and transmembrane Na^+,K^+-distribution in muscle function. The FEPS lecture — Bratislava 2007. *Acta Physiol. (Oxf.).* 192:339–349. http://dx.doi.org/10.1111/j.1748-1716.2007.01798.x

Clausen, T. 2010. Hormonal and pharmacological modification of plasma potassium homeostasis. *Fundam. Clin. Pharmacol.* 24:595–605. http://dx.doi.org/10.1111/j.1472-8206.2010.00859.x

Clausen, T. 2011. In isolated skeletal muscle, excitation may increase extracellular K^+ 10-fold; how can contractility be maintained? *Exp. Physiol.* 96:356–368.

Clausen, T. 2013. Excitation-induced exchange of Na^+, K^+, and Cl^- in rat EDL muscle in vitro and in vivo: Physiology and pathophysiology. *J. Gen. Physiol.* 141:179–192. http://dx.doi.org/10.1085/jgp.201210892

Clausen, T., and J.A. Flatman. 1977. The effect of catecholamines on Na-K transport and membrane potential in rat soleus muscle. *J. Physiol.* 270:383–414.

Clausen, T., and J.A. Flatman. 1980. β_2-adrenoceptors mediate the stimulating effect of adrenaline on active electrogenic Na-K-transport in rat soleus muscle. *Br. J. Pharmacol.* 68:749–755. http://dx.doi.org/10.1111/j.1476-5381.1980.tb10868.x

Clausen, T., and J.A. Flatman. 1987. Effects of insulin and epinephrine on Na^+-K^+ and glucose transport in soleus muscle. *Am. J. Physiol.* 252:E492–E499.

Clausen, T., and H. Gissel. 2005. Role of Na,K pumps in restoring contractility following loss of cell membrane integrity in rat skeletal muscle. *Acta Physiol. Scand.* 183:263–271. http://dx.doi.org/10.1111/j.1365-201X.2004.01394.x

Clausen, T., and O. Hansen. 1974. Ouabain binding and Na^+,K^+ transport in rat muscle cells and adipocytes. *Biochim. Biophys. Acta.* 345:387–404. http://dx.doi.org/10.1016/0005-2736(74)90200-4

Clausen, T., and O. Hansen. 1977. Active Na-K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. *J. Physiol.* 270:415–430.

Clausen, T., and P.G. Kohn. 1977. The effect of insulin on the transport of sodium and potassium in rat soleus muscle. *J. Physiol.* 265:19–42.

Clausen, T., and O.B. Nielsen. 2007. Potassium, Na^+,K^+-pumps and fatigue in rat muscle. *J. Physiol.* 584:295–304. http://dx.doi.org/10.1113/jphysiol.2007.136044

Clausen, T., O. Hansen, K. Kjeldsen, and A. Nørgaard. 1982. Effect of age, potassium depletion and denervation on specific displaceable [^{3}H]ouabain binding in rat skeletal muscle in vivo. *J. Physiol.* 333:367–381.

Clausen, T., M.E. Everts, and K. Kjeldsen. 1987. Quantification of the maximum capacity for active sodium-potassium transport in rat skeletal muscle. *J. Physiol.* 388:163–181.

Clausen, T., C. Van Hardeveld, and M.E. Everts. 1991. Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol. Rev.* 71:733–774.

Clausen, T., K. Overgaard, and O.B. Nielsen. 2004. Evidence that the Na^+-K^+ leak/pump ratio contributes to the difference in endurance between fast- and slow-twitch muscles. *Acta Physiol. Scand.* 180: 209–216. http://dx.doi.org/10.1111/j.0001-6772.2003.01251.x

Clausen, T., O.B. Nielsen, J.D. Clausen, T.H. Pedersen, and L.J. Hayward. 2011. Na^+,K^+-pump stimulation improves contractility in isolated muscles of mice with hyperkalemic periodic paralysis. *J. Gen. Physiol.* 138:117–130. http://dx.doi.org/10.1085/jgp.201010586

Cornelius, F., and Y.A. Mahmmoud. 2003. Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol. Sci.* 18:119–124.

Cougnon, M.H., A.E. Moseley, T.L. Radzyukevich, J.B. Lingrel, and J.A. Heiny. 2002. Na,K-ATPase α- and β-isoform expression in developing skeletal muscles: α_2 correlates with t-tubule formation. *Pflugers Arch.* 445:123–131. http://dx.doi.org/10.1007/s00424-002-0898-6

Creese, R. 1968. Sodium fluxes in diaphragm muscle and the effects of insulin and serum proteins. *J. Physiol.* 197:255–278.

Ditor, D.S., S. Hamilton, M.A. Tarnopolsky, H.J. Green, B.C. Craven, G. Parise, and A.L. Hicks. 2004. Na^+,K^+-ATPase concentration and fiber type distribution after spinal cord injury. *Muscle Nerve.* 29:38–45. http://dx.doi.org/10.1002/mus.10534

Dørup, I., and T. Clausen. 1994. ^{86}Rb is not a reliable tracer for potassium in skeletal muscle. *Biochem. J.* 302:745–751.

Dørup, I., and T. Clausen. 1995. Insulin-like growth factor I stimulates active Na^+-K^+ transport in rat soleus muscle. *Am. J. Physiol.* 268:E849–E857.

Dørup, I., and T. Clausen. 1997. Effects of adrenal steroids on the concentration of Na^+-K^+ pumps in rat skeletal muscle. *J. Endocrinol.* 152:49–57. http://dx.doi.org/10.1677/joe.0.1520049

Dutka, T.L., and G.D. Lamb. 2007. Na^+-K^+ pumps in the transverse tubular system of skeletal muscle fibers preferentially use ATP from glycolysis. *Am. J. Physiol. Cell Physiol.* 293:C967–C977. http://dx.doi.org/10.1152/ajpcell.00132.2007

Erlij, D., and S. Grinstein. 1976. The number of sodium ion pumping sites in skeletal muscle and its modification by insulin. *J. Physiol.* 259:13–31.

Everts, M.E., and T. Clausen. 1988. Effects of thyroid hormone on Na^+-K^+ transport in resting and stimulated rat skeletal muscle. *Am. J. Physiol.* 255:E604–E612.

Everts, M.E., and T. Clausen. 1994. Excitation-induced activation of the Na^+-K^+ pump in rat skeletal muscle. *Am. J. Physiol.* 266:C925–C934.

Ewart, H.S., and A. Klip. 1995. Hormonal regulation of the Na^+-K^+-ATPase: mechanisms underlying rapid and sustained changes in pump activity. *Am. J. Physiol.* 269:C295–C311.

Fambrough, D.M., B.A. Wolitzky, M.M. Tamkun, and K. Takeyasu. 1987. Regulation of the sodium pump in excitable cells. *Kidney Int. Suppl.* 23:S97–S116.

Fowles, J.R., H.J. Green, J.D. Schertzer, and A.R. Tupling. 2002. Reduced activity of muscle Na^+-K^+-ATPase after prolonged running in rats. *J. Appl. Physiol.* 93:1703–1708.

Fraser, J.A., C.L.H. Huang, and T.H. Pedersen. 2011. Relationships between resting conductances, excitability, and t-system ionic homeostasis in skeletal muscle. *J. Gen. Physiol.* 138:95–116. http://dx.doi.org/10.1085/jgp.201110617

Fredsted, A., H. Gissel, N. Ortenblad, and T. Clausen. 2012. Effects of β_2-agonists on force during and following anoxia in rat extensor digitorum longus muscle. *J. Appl. Physiol.* 112:2057–2067. http://dx.doi.org/10.1152/japplphysiol.01558.2011

Gemmill, C.L. 1940. The effect of insulin on the glycogen content of isolated muscles. *Bull. Johns Hopkins Hosp.* 66:232–244.

Glitsch, H.G. 2001. Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol. Rev.* 81:1791–1826.

Goodman, C.A., A. Hayes, and M.J. McKenna. 2009. Dissociation between force and maximal Na^+, K^+-ATPase activity in rat fast-twitch skeletal muscle with fatiguing in vitro stimulation. *Eur. J. Appl. Physiol.* 105:575–583. http://dx.doi.org/10.1007/s00421-008-0937-x

Green, H.J., D.J. Barr, J.R. Fowles, S.D. Sandiford, and J. Ouyang. 2004. Malleability of human skeletal muscle Na^+-K^+-ATPase pump with short-term training. *J. Appl. Physiol.* 97:143–148. http://dx.doi.org/10.1152/japplphysiol.00559.2003

Green, H.J., T.A. Duhamel, G.P. Holloway, J.W. Moule, J. Ouyang, D. Ranney, and A.R. Tupling. 2007. Muscle Na^+-K^+-ATPase response during 16 h of heavy intermittent cycle exercise. *Am. J. Physiol. Endocrinol. Metab.* 293:E523–E530. http://dx.doi.org/10.1152/ajpendo.00004.2007

Green, H.J., T.A. Duhamel, R.D. Stewart, A.R. Tupling, and J. Ouyang. 2008. Dissociation between changes in muscle Na^+-K^+-ATPase isoform abundance and activity with consecutive days of exercise and recovery. *Am. J. Physiol. Endocrinol. Metab.* 294:E761–E767. http://dx.doi.org/10.1152/ajpendo.00751.2007

Green, H.J., M.E. Burnett, C.L. D'Arsigny, K.A. Webb, I. McBride, J. Ouyang, and D.E. O'Donnell. 2009. Vastus lateralis Na^+-K^+-ATPase activity, protein, and isoform distribution in chronic obstructive pulmonary disease. *Muscle Nerve.* 40:62–68. http://dx.doi.org/10.1002/mus.21296

Hansen, O. 2001. The α_1 isoform of Na^+,K^+-ATPase in rat soleus and extensor digitorum longus. *Acta Physiol. Scand.* 173:335–341. http://dx.doi.org/10.1046/j.1365-201X.2001.00910.x

Hansen, O., and T. Clausen. 1988. Quantitative determination of Na^+-K^+-ATPase and other sarcolemmal components in muscle cells. *Am. J. Physiol.* 254:C1–C7.

Harrop, G.A., and E.M. Benedict. 1924. The participation of inorganic substances in carbohydrate metabolism. *J. Biol. Chem.* 59:683–697.

He, S., D.A. Shelly, A.E. Moseley, P.F. James, J.H. James, R.J. Paul, and J.B. Lingrel. 2001. The α_1- and α_2-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281:R917–R925.

Hicks, A., and A.J. McComas. 1989. Increased sodium pump activity following repetitive stimulation of rat soleus muscles. *J. Physiol.* 414:337–349.

Hicks, A., K. Ohlendieck, S.O. Göpel, and D. Pette. 1997. Early functional and biochemical adaptations to low-frequency stimulation of rabbit fast-twitch muscle. *Am. J. Physiol.* 273:C297–C305.

Hodgkin, A.L., and P. Horowicz. 1959. Movements of Na and K in single muscle fibres. *J. Physiol.* 145:405–432.

Juel, C. 2009. Na^+-K^+-ATPase in rat skeletal muscle: muscle fiber-specific differences in exercise-induced changes in ion affinity and maximal activity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296: R125–R132. http://dx.doi.org/10.1152/ajpregu.90760.2008

Juel, C., L. Grunnet, M. Holse, S. Kenworthy, V. Sommer, and T. Wulff. 2001. Reversibility of exercise-induced translocation of Na^+-K^+ pump subunits to the plasma membrane in rat skeletal muscle. *Pflugers Arch.* 443:212–217. http://dx.doi.org/10.1007/s004240100674

Kirsch, G.E., R.A. Nichols, and S. Nakajima. 1977. Delayed rectification in the transverse tubules: origin of the late after-potential in frog skeletal muscle. *J. Gen. Physiol.* 70:1–21. http://dx.doi.org/10.1085/jgp.70.1.1

Kjeldsen, K., H. Braendgaard, P. Sidenius, J.S. Larsen, and A. Nørgaard. 1987. Diabetes decreases Na^+-K^+ pump concentration in skeletal muscles, heart ventricular muscle, and peripheral nerves of rat. *Diabetes.* 36:842–848. http://dx.doi.org/10.2337/diabetes.36.7.842

Kohn, P.G., and T. Clausen. 1971. The relationship between the transport of glucose and cations across cell membranes in isolated tissues. VI. The effect of insulin, ouabain, and metabolic inhibitors on the transport of 3-O-methylglucose and glucose in rat soleus muscles. *Biochim. Biophys. Acta.* 225:277–290. http://dx.doi.org/10.1016/0005-2736(71)90221-5

Kristensen, M., M.K. Rasmussen, and C. Juel. 2008. Na^+-K^+ pump location and translocation during muscle contraction in rat skeletal muscle. *Pflugers Arch.* 456:979–989. http://dx.doi.org/10.1007/s00424-008-0449-x

Lau, Y.H., A.H. Caswell, M. Garcia, and L. Letellier. 1979. Ouabain binding and coupled sodium, potassium, and chloride transport in isolated transverse tubules of skeletal muscle. *J. Gen. Physiol.* 74:335–349. http://dx.doi.org/10.1085/jgp.74.3.335

Leivseth, G., T. Clausen, M.E. Everts, and E. Bjordal. 1992. Effects of reduced joint mobility and training on Na,K-ATPase and Ca-ATPase in skeletal muscle. *Muscle Nerve.* 15:843–849. http://dx.doi.org/10.1002/mus.880150714

Leppik, J.A., R.J. Aughey, I. Medved, I. Fairweather, M.F. Carey, and M.J. McKenna. 2004. Prolonged exercise to fatigue in humans impairs skeletal muscle Na^+-K^+-ATPase activity, sarcoplasmic reticulum Ca^{2+} release, and Ca^{2+} uptake. *J. Appl. Physiol.* 97:1414–1423. http://dx.doi.org/10.1152/japplphysiol.00964.2003

Macdonald, W.A., O.B. Nielsen, and T. Clausen. 2005. Na^+-K^+ pump stimulation restores carbacholine-induced loss of excitability and contractility in rat skeletal muscle. *J. Physiol.* 563:459–469. http://dx.doi.org/10.1113/jphysiol.2004.080390

Macdonald, W.A., O.B. Nielsen, and T. Clausen. 2008. Effects of calcitonin gene-related peptide on rat soleus muscle excitability: mechanisms and physiological significance. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295:R1214–R1223. http://dx.doi.org/10.1152/ajpregu.00893.2007

Mahmmoud, Y.A., H. Vorum, and F. Cornelius. 2000. Identification of a phospholemman-like protein from shark rectal glands. Evidence for indirect regulation of Na,K-ATPase by protein kinase c via a novel member of the FXYDY family. *J. Biol. Chem.* 275:35969–35977. http://dx.doi.org/10.1074/jbc.M005168200

Marette, A., J. Krischer, L. Lavoie, C. Ackerley, J.-L. Carpentier, and A. Klip. 1993. Insulin increases the Na^+-K^+-ATPase α_2-subunit in the surface of rat skeletal muscle: morphological evidence. *Am. J. Physiol.* 265:C1716–C1722.

McKenna, M.J., T.A. Schmidt, M. Hargreaves, L. Cameron, S.L. Skinner, and K. Kjeldsen. 1993. Sprint training increases human skeletal muscle Na^+-K^+-ATPase concentration and improves K^+ regulation. *J. Appl. Physiol.* 75:173–180.

McKenna, M.J., H. Gissel, and T. Clausen. 2003. Effects of electrical stimulation and insulin on Na^+-K^+-ATPase ([^{3}H]ouabain binding) in rat skeletal muscle. *J. Physiol.* 547:567–580. http://dx.doi.org/10.1113/jphysiol.2003.034512

McKenna, M.J., I. Medved, C.A. Goodman, M.J. Brown, A.R. Bjorksten, K.T. Murphy, A.C. Petersen, S. Sostaric, and X. Gong. 2006. N-acetylcysteine attenuates the decline in muscle Na^+,K^+-pump activity and delays fatigue during prolonged exercise in humans. *J. Physiol.* 576:279–288. http://dx.doi.org/10.1113/jphysiol.2006.115352

McKenna, M.J., B.D. Perry, F.R. Serpiello, M.K. Caldow, P. Levinger, D. Cameron-Smith, and I. Levinger. 2012. Unchanged [^{3}H]ouabain binding site content but reduced Na^+-K^+ pump α_2-protein abundance in skeletal muscle in older adults. *J. Appl. Physiol.* 113:1505–1511. http://dx.doi.org/10.1152/japplphysiol.01032.2011

McMorrow, C.A., A. Fredsted, J. Carberry, R.A. O'Connell, A. Bradford, J.F. Jones, and K.D. O'Halloran. 2011. Chronic hypoxia increases rat diaphragm muscle endurance and sodium-potassium ATPase pump content. *Eur. Respir. J.* 37:1474–1481. http://dx.doi.org/10.1183/09031936.00079810

Medbø, J.I., and O.M. Sejersted. 1990. Plasma potassium changes with high intensity exercise. *J. Physiol.* 421:105–122.

Mikkelsen, U.R., H. Gissel, A. Fredsted, and T. Clausen. 2006. Excitation-induced cell damage and β_2-adrenoceptor agonist stimulated force recovery in rat skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290:R265–R272. http://dx.doi.org/10.1152/ajpregu.00392.2005

Mu, L., S. Sobotka, and H. Su. 2011. Nerve-muscle-endplate band grafting: a new technique for muscle reinnervation. *Neurosurgery.* 69:ons208–ons224.

Murphy, K.T., and T. Clausen. 2007. The importance of limitations in aerobic metabolism, glycolysis, and membrane excitability for the development of high-frequency fatigue in isolated rat soleus muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292:R2001–R2011. http://dx.doi.org/10.1152/ajpregu.00714.2006

Murphy, K.T., W.A. Macdonald, M.J. McKenna, and T. Clausen. 2006. Ionic mechanisms of excitation-induced regulation of Na^+-K^+-ATPase mRNA expression in isolated rat EDL muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290:R1397–R1406. http://dx.doi.org/10.1152/ajpregu.00707.2005

Murphy, K.T., O.B. Nielsen, and T. Clausen. 2008. Analysis of exercise-induced Na^+-K^+ exchange in rat skeletal muscle in vivo. *Exp. Physiol.* 93:1249–1262. http://dx.doi.org/10.1113/expphysiol.2008.042457

Nielsen, O.B., and T. Clausen. 1996. The significance of active Na^+,K^+ transport in the maintenance of contractility in rat skeletal muscle. *Acta Physiol. Scand.* 157:199–209. http://dx.doi.org/10.1046/j.1365-201X.1996.d01-748.x

Nielsen, O.B., and T. Clausen. 1997. Regulation of Na^+-K^+ pump activity in contracting rat muscle. *J. Physiol.* 503:571–581. http://dx.doi.org/10.1111/j.1469-7793.1997.571bg.x

Nielsen, O.B., L. Hilsted, and T. Clausen. 1998. Excitation-induced force recovery in potassium-inhibited rat soleus muscle. *J. Physiol.* 512:819–829. http://dx.doi.org/10.1111/j.1469-7793.1998.819bd.x

Nordsborg, N., C. Goodmann, M.J. McKenna, and J. Bangsbo. 2005. Dexamethasone up-regulates skeletal muscle maximal Na^+,K^+ pump activity by muscle group specific mechanisms in humans. *J. Physiol.* 567:583–589. http://dx.doi.org/10.1113/jphysiol.2005.087031

Nordsborg, N., J. Ovesen, M. Thomassen, M. Zangenberg, C. Jøns, F.M. Iaia, J.J. Nielsen, and J. Bangsbo. 2008. Effect of dexamethasone on skeletal muscle Na^+,K^+ pump subunit specific expression and K^+ homeostasis during exercise in humans. *J. Physiol.* 586:1447–1459. http://dx.doi.org/10.1113/jphysiol.2007.143073

Nørgaard, A., K. Kjeldsen, O. Hansen, and T. Clausen. 1983. A simple and rapid method for the determination of the number of ^{3}H-ouabain binding sites in biopsies of skeletal muscle. *Biochem. Biophys. Res. Commun.* 111:319–325. http://dx.doi.org/10.1016/S0006-291X(83)80154-5

Nørgaard, A., K. Kjeldsen, and O. Hansen. 1984. (Na^+ + K^+)-ATPase activity of crude homogenates of rat skeletal muscle as estimated from their K^+-dependent 3-O-methylfluorescein phosphatase activity. *Biochim. Biophys. Acta.* 770:203–209. http://dx.doi.org/10.1016/0005-2736(84)90131-7

Okamoto, K., W. Wang, J. Rounds, E.A. Chambers, and D.O. Jacobs. 2001. ATP from glycolysis is required for normal sodium homeostasis in resting fast-twitch rodent skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 281:E479–E488.

Overgaard, K., O.B. Nielsen, J.A. Flatman, and T. Clausen. 1999. Relations between excitability and contractility in rat soleus muscle: role of the Na^+-K^+ pump and Na^+/K^+ gradients. *J. Physiol.* 518:215–225. http://dx.doi.org/10.1111/j.1469-7793.1999.0215r.x

Palmer, C.J., B.T. Scott, and L.R. Jones. 1991. Purification and complete sequence determination of the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C in myocardium. *J. Biol. Chem.* 266:11126–11130.

Plesner, I.W., and L. Plesner. 1981. The steady-state kinetic mechanism of ATP hydrolysis catalyzed by membrane-bound (Na^+ + K^+)-ATPase from ox brain. *Biochim. Biophys. Acta.* 648:231–246. http://dx.doi.org/10.1016/0005-2736(81)90039-0

Radzyukevich, T.L., J.C. Neumann, T.N. Rindler, N. Oshiro, D.J. Goldhamer, J.B. Lingrel, and J.A. Heiny. 2013. Tissue-specific role of the Na,K-ATPase α_2 isozyme in skeletal muscle. *J. Biol. Chem.* 288:1226–1237. http://dx.doi.org/10.1074/jbc.M112.424663

Ravn, H.B., and I. Dørup. 1997. The concentration of sodium, potassium pumps in chronic obstructive lung disease (COLD) patients: the impact of magnesium depletion and steroid treatment. *J. Intern. Med.* 241:23–29. http://dx.doi.org/10.1046/j.1365-2796.1997.69891000.x

Riis, A.L.D., J.O.L. Jørgensen, N. Møller, J. Weeke, and T. Clausen. 2005. Hyperthyroidism and cation pumps in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 288:E1265–E1269. http://dx.doi.org/10.1152/ajpendo.00533.2004

Sampson, S.R., C. Brodie, and S.V. Alboim. 1994. Role of protein kinase C in insulin activation of the Na-K pump in cultured skeletal muscle. *Am. J. Physiol.* 266:C751–C758.

Sandiford, S.D.E., H.J. Green, and J. Ouyang. 2005. Mechanisms underlying increases in rat soleus Na^+-K^+-ATPase activity by induced contractions. *J. Appl. Physiol.* 99:2222–2232. http://dx.doi.org/10.1152/japplphysiol.00577.2005

Schmidt, T.A., S. Hasselbalch, P.A. Farrell, H. Vestergaard, and K. Kjeldsen. 1994. Human and rodent muscle Na^+-K^+-ATPase in diabetes related to insulin, starvation, and training. *J. Appl. Physiol.* 76:2140–2146.

Schwartz, A., G.E. Lindenmayer, and J.C. Allen. 1975. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.* 27:3–134.

Sejersted, O.M., and G. Sjøgaard. 2000. Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol. Rev.* 80:1411–1481.

Shattock, M.J. 2009. Phospholemman: its role in normal cardiac physiology and potential as a druggable target in disease. *Curr. Opin. Pharmacol.* 9:160–166. http://dx.doi.org/10.1016/j.coph.2008.12.015

Shinoda, T., H. Ogawa, F. Cornelius, and C. Toyoshima. 2009. Crystal structure of the sodium-potassium pump at 2.4 A resolution. *Nature.* 459:446–450. http://dx.doi.org/10.1038/nature07939

Skou, J.C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta.* 23:394–401. http://dx.doi.org/10.1016/0006-3002(57)90343-8

Skou, J.C. 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.* 45:596–617.

Spencer, C.A., S.M.C. Lum, J.F. Wilber, E.M. Kaptein, and J.T. Nicoloff. 1983. Dynamics of serum thyrotropin and thyroid hormone changes in fasting. *J. Clin. Endocrinol. Metab.* 56:883–888. http://dx.doi.org/10.1210/jcem-56-5-883

Swann, A.C. 1984. Caloric intake and Na^+-K^+-ATPase: differential regulation by α_1- and β-noradrenergic receptors. *Am. J. Physiol.* 247:R449–R455.

Sweadner, K.J. 1979. Two molecular forms of (Na^+ + K^+)-stimulated ATPase in brain. Separation, and difference in affinity for strophanthidin. *J. Biol. Chem.* 254:6060–6067.

Thomassen, M., A.J. Rose, T.E. Jensen, S.J. Maarbjerg, L. Bune, M. Leitges, E.A. Richter, J. Bangsbo, and N.B. Nordsborg. 2011. Protein kinase Cα activity is important for contraction-induced FXYD1 phosphorylation in skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301:R1808–R1814. http://dx.doi.org/10.1152/ajpregu.00066.2011

Thompson, C.B., I. Dørup, J. Ahn, P.K.K. Leong, and A.A. McDonough. 2001. Glucocorticoids increase sodium pump α_2- and β_1-subunit abundance and mRNA in rat skeletal muscle. *Am. J. Physiol. Cell Physiol.* 280:C509–C516.

Tsakiridis, T., P.P.C. Wong, Z. Liu, C.D. Rodgers, M. Vranic, and A. Klip. 1996. Exercise increases the plasma membrane content of the Na^+-K^+ pump and its mRNA in rat skeletal muscles. *J. Appl. Physiol.* 80:699–705.

Wang, J., J.B. Velotta, A.A. McDonough, and R.A. Farley. 2001. All human Na^+-K^+-ATPase α-subunit isoforms have a similar affinity for cardiac glycosides. *Am. J. Physiol. Cell Physiol.* 281:C1336–C1343.

Wang, P., and T. Clausen. 1976. Treatment of attacks in hyperkalaemic familial periodic paralysis by inhalation of salbutamol. *Lancet.* 1:221–223. http://dx.doi.org/10.1016/S0140-6736(76)91340-4

Williams, M.W., W.G. Resneck, T. Kaysser, J.A. Ursitti, C.S. Birkenmeier, J.E. Barker, and R.J. Bloch. 2001. Na,K-ATPase in skeletal muscle: two populations of β-spectrin control localization in the sarcolemma but not partitioning between the sarcolemma and the transverse tubules. *J. Cell Sci.* 114:751–762.

Zheng, D., A. Perianayagam, D.H. Lee, M.D. Brannan, L.E. Yang, D. Tellalian, P. Chen, K. Lemieux, A. Marette, J.H. Youn, and A.A. McDonough. 2008. AMPK activation with AICAR provokes an acute fall in plasma [K^+]. *Am. J. Physiol. Cell Physiol.* 294:C126–C135. http://dx.doi.org/10.1152/ajpcell.00464.2007

Zierler, K.L., and D. Rabinowitz. 1964. Effect of very small concentrations of insulin on forearm metabolism. Persistence of its action on potassium and free fatty acids without its effect on glucose. *J. Clin. Invest.* 43:950–962. http://dx.doi.org/10.1172/JCI104981