Na,K-ATPase regulation in skeletal muscle

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ABSTRACT

Skeletal muscle contains one of the largest and the most dynamic pools of Na,K-ATPase (NKA) in the body. Under resting conditions NKA in skeletal muscle operates at only a fraction of maximal pumping capacity, but it can be markedly activated when demands for ion transport increase, such as during exercise or following food intake. Given the size, capacity, and dynamic range of the NKA pool in skeletal muscle, its tight regulation is essential to maintain whole-body homeostasis as well as muscle function. To reconcile functional needs of systemic homeostasis with those of skeletal muscle, NKA is regulated in a coordinated manner by extrinsic stimuli, such as hormones and nerve-derived factors, as well as by local stimuli arising in skeletal muscle fibers, such as contractions and muscle energy status. These stimuli regulate NKA acutely by controlling its enzymatic activity and/or its distribution between plasma membrane and the intracellular storage compartment. They also regulate NKA chronically by controlling NKA gene expression, thus determining total NKA content in skeletal muscle and its maximal pumping capacity. This review focuses on molecular mechanisms that underlie regulation of NKA in skeletal muscle by major extrinsic and local stimuli. Special emphasis is given to stimuli and mechanisms linking regulation of NKA and energy metabolism in skeletal muscle, such as insulin and the energy-sensing AMP-activated protein kinase. Finally, the recently uncovered roles for glutathionylation, nitric oxide, and extracellular K⁺ in regulation of NKA in skeletal muscle are highlighted.
INTRODUCTION

Na, K-ATPase (NKA) is a transmembrane pump that transports three Na\(^+\) from the cell and two K\(^+\) into the cell against their concentration gradients (316). In animal cells NKA represents the only mechanism that counteracts uptake of extracellular Na\(^+\) and loss of intracellular K\(^+\) (300). NKA is therefore essential for cellular ion and water homeostasis, preservation of membrane potentials, and Na\(^+\)-coupled transport of various substances. In multicellular organisms, these basic cellular functions are integrated to serve the needs of specialized organs, including neuronal activity (70, 297) and muscle contraction (58), as well as whole-body homeostasis, such as maintaining water balance (300), and extracellular K\(^+\) concentrations (225). Participation of NKA at different levels of physiological organization requires precise coordination between its cellular, organ, and whole-body functions. The challenge of coordinating different NKA functions is particularly obvious in skeletal muscle, which contains one of the largest (55) and the most dynamic pools of NKA in the body (63, 250). To oppose Na\(^+\) influx and K\(^+\) efflux NKA activity in skeletal muscle increases upon transition from resting to an actively contracting state (58, 250). Such dynamic activation of NKA not only maintains muscle excitability and contractility (58), but also prevents excessive loss of intracellular K\(^+\), which could precipitate hyperkalemia and cardiac arrest (57, 61, 225). On the other hand, the NKA pool in skeletal muscle is so large (55) that its inappropriate activation could rapidly deplete the extracellular K\(^+\) pool and lead to equally dangerous hypokalemia (57, 61). Clearly, tight regulation of NKA is mandatory to maintain skeletal muscle function and whole-body homeostasis.

Regulation of active Na\(^+\)-K\(^+\) transport in skeletal muscle has been the focus of intense research efforts for more than 50 years. Just a few years after discovery of NKA in crab nerves in
1957 (316), isoproterenol (87) and high extracellular K\(^+\) concentrations (136) were shown to stimulate Na\(^+\) extrusion from skeletal muscle. Since these early pioneering works decades of research have provided detailed insights into the intricate network of physiological stimuli and signaling pathways that regulate NKA function in skeletal muscle. Comprehensive and detailed discussion of all historical and current aspects of NKA regulation in skeletal muscle is beyond the scope of this review (for detailed overview of research up to 2003 see (58)). Here we provide an overview of molecular mechanisms that underlie regulation of skeletal muscle NKA by major physiological stimuli, including hormones, nerve-derived factors, and muscle contractions. In particular, we focus on emerging aspects of NKA regulation in skeletal muscle and cover in depth developments that have taken place in the last 15 years. In addition, we highlight challenges and perspectives for future research.

NKA IN SKELETAL MUSCLE: STRUCTURE, FUNCTION, AND DISTRIBUTION

Isoforms of NKA α- and β-subunits in skeletal muscle

NKA is a heterodimer that comprises one α-subunit and one β-subunit. Na\(^+\) and K\(^+\) ions are pumped by the catalytic α-subunit, a 100-112 kDa type IIC P-ATPase, which exists in four isoforms (α1-4) (34). Skeletal muscle contains primarily α2-subunits (125, 133, 215, 267, 322) unlike most other tissues, where α1-subunits predominate (312). Indeed, according to different estimates α2-subunits constitute at least 60% and up to 75-90% of NKA pool in skeletal muscle (125, 133, 183, 267, 322). Besides α1- and α2-subunits skeletal muscle also contains α3-subunits (142, 243, 267, 277, 278, 354), which are especially abundant in sensory and motor neurons that innervate muscle spindles (86, 92, 271, 298). In skeletal muscle α3-subunits likely play an important role in motor and sensory neurons that innervate muscle spindles (92, 271), but their
physiological role in muscle fibers is uncertain. The transcript of the testis-specific α4-subunit (310) was also detected in human and murine skeletal muscle (173). However, the very low expression level (259) indicates that the α4-subunit is likely of minor quantitative and functional importance.

The β-subunit, a 35-60 kDa glycoprotein, exists in three isoforms (β1-3) (34). Skeletal muscle contains mainly β1- and β2-subunits (58), but the β3-subunit has also been detected (243, 278, 354). Although the β-subunit does not possess catalytic activity, it is essential for assembly, maturation, and function of α/β heterodimers (2, 115, 224). In addition, it modulates kinetic properties of α/β heterodimers (74) and probably affects responsiveness of α/β heterodimers to regulatory modifications, such as glutathionylation (154, 158).

**Kinetic characteristics and physiological functions of α/β heterodimers in skeletal muscle**

Heterodimers that comprise different α- and β-subunits have distinct kinetic characteristics, such as turnover number as well as affinities for Na⁺ ($K_{1/2,Na}$), K⁺ ($K_{1/2,K}$), and ATP ($K_{m,ATP}$) (34, 74, 82). Functional significance of all α-β combinations has not been determined, but some α/β heterodimers clearly perform distinct physiological functions. For instance, α1/β heterodimers are primarily responsible for maintenance of Na⁺ and K⁺ gradients in resting skeletal muscle. In contrast, α2/β heterodimers contribute little to NKA activity in resting skeletal muscle (48, 133, 134, 186, 187, 288), although they represent the bulk of NKA pool (125, 133, 267, 322). Activation of α2/β heterodimers occurs in response to stimulation with insulin or muscle contractions (140, 289). Without the α2-subunit skeletal muscle fatigues quickly, which translates into poor exercise performance (219, 289), underscoring the role of α2/β heterodimers for normal muscle contraction. Notably, up-regulation of the α1-subunit
cannot compensate for the loss of the α2-subunit (289), which clearly demonstrates that these subunits are functionally non-redundant. In summary, α1/β heterodimers play housekeeping functions, while α2/β heterodimers represent a large reserve NKA pool, which is able to dynamically adapt to fluctuating demands for Na+-K+ transport.

Kinetic characteristics and physiological functions of α/β heterodimers are highly likely correlated. For instance, rat and human α2/β heterodimers expressed in Sf-9 insect cells and *Xenopus* oocytes, respectively, have lower affinities for extracellular K+ ($K_{1/2,K} = 1.3-4.8$ mM) than α1/β heterodimers ($K_{1/2,K} = 0.92-1.9$ mM) (34, 74). Similarly, in mice apparent NKA affinity for K+ is markedly lower in membranes from wild-type muscles ($K_{1/2,K} \sim 3.6$ mM), which express predominantly α2/β heterodimers, than in membranes from the α2-deficient muscles ($K_{1/2,K} \sim 1.7$ mM) (82). Lower affinity for K+ makes α2/β heterodimers particularly suitable to operate under high K+ conditions. Notably, extracellular K+ concentrations are markedly higher during muscle contractions (reaching up to 10 mM or more), when activity of α2/β heterodimers predominates, than under resting conditions (~4 mM), when activity of α1/β heterodimers predominates (122, 163, 257). Thus, differences in K+ affinity of α2/β and α1/β heterodimers likely reflect physiological conditions under which these heterodimers normally operate.

**Distribution of α/β heterodimers in different types of muscle fibers**

The β-subunit displays clear fiber type-dependent distribution in humans and rats. In humans the β2-subunit is more abundant in glycolytic type IIa fibers, while expression of α- and β1-subunits is similar across different fiber types (354). One previous study found greater α2 expression in type II fibers (329), but the reason for the apparent discrepancy has not been determined. Like in humans, the β2-subunit predominates in rat glycolytic muscles (110, 141,
However, in contrast to human data, the rat β1-subunit also displays fiber type-dependent distribution. Indeed, in rats the β1-subunit predominates in oxidative muscles and is only lowly expressed in glycolytic muscles (110, 141, 152, 157, 183, 332, 337, 358). Interestingly, rat and human α/β2 heterodimers tend to have lower affinity for $K^+$ than α/β1 heterodimers expressed in Sf-9 cells or *Xenopus* oocytes (34, 74). Similarly, sarcolemmal giant vesicles from rat glycolytic muscles, which express primarily α/β2 heterodimers, have lower apparent NKA affinity for $K^+$ ($K_{1/2,K} \sim 3.1-4.6$ mM) than sarcolemmal giant vesicles from oxidative muscles ($K_{1/2,K} \sim 0.7-1.5$ mM), where α/β1 heterodimers predominate (152). These data suggest that asymmetric distribution of β-subunits in different types of muscle fibers might be functionally and physiologically important.

Subcellular distribution of α/β heterodimers in skeletal muscle

Functional differences between α1/β and α2/β heterodimers are paralleled by their asymmetrical subcellular localization. Surface sarcolemma is enriched in α1-subunits, but also contains α2-subunits (142, 196, 197, 289), especially in specialized domains, such as the neuromuscular junction (48, 134), costameres (348), and caveolae (185). The majority or at least a major fraction of α2-subunits resides in T-tubules (289), although their fractional distribution among surface sarcolemma, specialized membrane domains, and T-tubular membrane varies between muscle types (184). In contrast, α1-subunits are almost completely excluded from the T-tubules (196, 289, 348). Unlike α-subunits, which show a clear predilection for distinct membrane domains, both β1- and β2-subunits are localized in T-tubules, surface sarcolemma as well as in the intracellular compartment (140, 184, 197, 198).
Even in muscles devoid of α2-subunits, α1-subunits are almost entirely localized in the sarcolemma and not in the T-tubular system (289). This indicates that skeletal muscle fibers actively maintain an asymmetrical localization of α1- and α2-subunits. Proper localization of α1- and α2-subunits in specialized domains of sarcolemma as well as their asymmetrical distribution between sarcolemma and T-tubular system are controlled by distinct molecular mechanisms (348). To maintain composition of specialized membrane domains, muscle fibers require sophisticated machinery for protein synthesis and vesicle sorting that directs various proteins to their correct locations in the membrane. Although scant cytoplasm deceptively suggests that muscle fibers contain little else than bundles of actomyosin filaments enclosed in cylinders of sarcolemma, they possess fully developed endoplasmic reticulum, Golgi apparatus (165, 245), and extensive system of vesicles involved in membrane trafficking (194, 195, 336). This is not surprising since muscle fibers use as much as 17% of oxygen for protein synthesis, compared with 24% in liver and 3-6% in kidney, brain, and heart (297). Moreover, muscle fibers are secretory cells, which produce and release up to several hundred peptides and proteins (130) with plethora of paracrine and endocrine effects (275).

How this complex synthetic and protein sorting machinery participates in assembly and trafficking of heterogeneous NKA α/β heterodimers in skeletal muscle fibers has not been fully characterized. However, studies in Xenopus oocytes (2) and cultured Madin-Darby canine kidney (MDCK) cells (334, 335) have established that α/β heterodimers are assembled in 1:1 stoichiometry in endoplasmic reticulum and that failure to form a heterodimer leads to degradation of surplus subunits, thus highlighting an essential role for endoplasmic reticulum. Importantly, the α1-subunit is the preferred binding partner of the β1-subunit, while the α2-subunit is the preferred binding partner of the β2-subunit in MDCK cells and/or murine brain
(333), indicating that assembly of $\alpha/\beta$ heterodimers is isoform-selective. Mechanisms underlying assembly of $\alpha/\beta$ heterodimers and their preferential trafficking to distinct subcellular compartments in skeletal muscle have not been resolved. Interestingly, skeletal muscle seems to have surplus $\beta$-subunits (197), whose physiological role is uncertain. Furthermore, skeletal muscle contains $\alpha_2/\beta_1$ as well as $\alpha_1/\beta_2$ heterodimers (183). Thus, mechanisms underlying assembly and trafficking of $\alpha/\beta$ heterodimers are likely tissue-specific.

**NKA AND ENERGY METABOLISM IN SKELETAL MUSCLE**

NKA is intimately linked with energy metabolism in skeletal muscle. Notably, NKA is one of the major consumers of oxygen in skeletal muscle under resting conditions (297). Indeed, 5-10% of oxygen consumption in resting skeletal muscle is coupled to NKA activity. NKA is an even more significant consumer of oxygen in the kidney and the brain, where it uses as much as 40-70% and 40-60% of oxygen-coupled ATP, respectively (70, 297). At the whole-body level NKA uses 19-28% of ATP (297), two thirds of which is used by NKA in the kidney and the brain (314). Differences in oxygen-coupled ATP usage by NKA reflect, in part, distinct physiological characteristics of ion transport in skeletal muscle, kidney, and brain. Under resting conditions, 2-6% of total NKA capacity suffices to oppose passive $Na^+$ and $K^+$ leaks across sarcolemma (58, 63). Conversely, high NKA activity is constantly required to sustain high rate of tubular ion transport in kidney and signal transmission in brain (70, 297). In addition, skeletal muscle uses a major fraction of ATP to energize sarcoplasmic reticulum $Ca^{2+}$-ATPase, which is required to preserve $Ca^{2+}$ homeostasis, and actomyosin ATPase, which is required for contractions (325). Thus, fractional ATP utilization by NKA is 5-10% even in contracting
skeletal muscle, which consumes up to 10-25% of ATP for sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and 65-80% of ATP for actomyosin ATPase (297).

Furthermore, ATP usage by NKA in skeletal muscle is tightly coupled with glycolysis and lactate production. Estimation of oxygen-coupled ATP usage by NKA might therefore underestimate total ATP usage by NKA. For instance, Na\(^+\)-ionophore monensin, which stimulates NKA activation (59), increases lactate production in isolated rat extensor digitorum longus (EDL) and soleus muscles. This increase in lactate production is inhibited by ouabain (146), indicating that Na\(^+\)-stimulated NKA activation enhances the rate of glycolysis. This is also true vice versa: inhibition of glycolysis markedly increases intracellular Na\(^+\) concentrations and accelerates loss of force of contracting muscle (238, 265). In contrast, inhibition of oxidative phosphorylation or hypoxia does not alter intracellular Na\(^+\) concentrations or accelerate loss of muscle force (238, 265). Thus, glycolysis seems to be a major source of ATP for NKA in skeletal muscle. Consistent with this notion, ouabain reduces basal as well as epinephrine-stimulated NKA activity and lactate release from isolated rat EDL and soleus muscles (147, 265). Notably, epinephrine-stimulated NKA activation and lactate production might be important in patients with sepsis (endotoxemia). In healthy subjects infusion of endotoxin, which stimulates epinephrine secretion, decreases K\(^+\) loss and increases lactate release from the legs (43). Furthermore, infusion of ouabain reduces lactate production in skeletal muscle of patients in septic shock (201), which again suggests that NKA activation by epinephrine stimulates glycolysis. Clearly, ATP usage by NKA appears to be a major driver of lactate production in skeletal muscle.

By maintaining high intracellular K\(^+\) concentrations NKA enables normal function of K\(^+\)-dependent enzymes (269, 300), including glycolytic enzyme pyruvate kinase (166). Pyruvate
kinase, which catalyzes net formation of ATP in the glycolytic pathway by converting phosphoenolpyruvate to pyruvate, is absolutely dependent upon K⁺ (166, 269). Formation of ATP by pyruvate kinase appears to be particularly important to support NKA activity in T-tubules (91). Indeed, NKA function in T-tubules of skinned EDL muscle fibers cannot be maintained solely by providing ATP and creatine phosphate (91). Conversely, NKA function is increased by addition of phosphoenolpyruvate (91). Stimulation of NKA function by phosphoenolpyruvate suggests that local production of ATP by pyruvate kinase is important to energize NKA activity. Due to tight control of intracellular K⁺ concentrations, K⁺ is not regarded as an important physiological regulator of K⁺-activated enzymes in general (269). However, intracellular K⁺ concentrations can drop by as much as ~30-50 mM in contracting skeletal muscle (155), which might be sufficient to suppress the activity of pyruvate kinase. Thus, we can infer that activation of NKA during muscle contractions not only blunts the fall in intracellular K⁺ concentrations but also indirectly limits changes in pyruvate kinase activity, thus avoiding fluctuations in synthesis of ATP, which is required by NKA.

Finally, by maintaining low intracellular Na⁺ concentrations, NKA promotes Na⁺-coupled uptake of substrates, which play key roles in energy metabolism of skeletal muscle. For instance, uptake of creatine, which is required for synthesis of phosphocreatine, a high-energy compound used for rapid replenishment of ATP in skeletal muscle, is dependent upon Na⁺ gradient and NKA activity (263, 363). NKA also drives skeletal muscle uptake of carnitine (117), an essential component of the carnitine-palmitoyl transferase system, which delivers fatty acids for β-oxidation in mitochondrial matrix (320), and inorganic phosphate (1), which enables normal turnover of high-energy compounds like ATP and phosphocreatine. Finally, transmembrane Na⁺ gradient energizes uphill Na⁺-coupled uptake of amino acids like glutamine and alanine (81),
whose concentrations in skeletal muscle are 32- and 9-fold, respectively, higher than in plasma (27).

GENERAL PRINCIPLES OF NKA REGULATION IN SKELETAL MUSCLE

NKA is regulated at three levels: intrinsic activity of NKA, abundance of NKA in plasma membrane, and/or total content of NKA in skeletal muscle. Alterations in intrinsic activity and/or membrane abundance underpin acute adaptations to fluctuating demands for Na\(^+\) and K\(^+\) transport, such as during an acute bout of exercise or after a meal (25, 58, 61, 97). Alterations of total NKA content underpin adaptations to chronic demands for Na\(^+\) and K\(^+\) transport, such as chronic level of physical activity or dietary habits (47, 58, 113, 176, 231, 262).

Regulation of intrinsic activity of NKA

Intrinsic activity of NKA is regulated by at least three mechanisms. First, concentrations of substrates (Na\(^+\), K\(^+\), and ATP) regulate NKA directly by mass action. Second, protein-protein interactions between NKA and FXYD proteins regulate NKA by modulating its kinetic properties. Third, covalent modifications of NKA subunits, such as phosphorylation and glutathionylation regulate NKA by stimulating or suppressing its activity.

Regulation of NKA activity by Na\(^+\), K\(^+\), and ATP concentrations

Isolated NKA is dependent on Na\(^+\) and K\(^+\) ions, which stimulate its enzymatic activity directly (316). This mode of regulation is operative also under physiological conditions, where elevations in intracellular Na\(^+\) or extracellular K\(^+\) concentrations represent the most basic mechanism to increase NKA activity in skeletal muscle (58, 63, 82). NKA is also directly
dependent on concentrations of ATP (34, 316). However, ATP concentrations in skeletal muscle are normally kept within narrow limits (8). Taken together with relatively high affinity of NKA for ATP \( (K_{m,ATP} = 0.07-0.8\, \text{mM}) \) (34, 97, 346) this implies that ATP is not a limiting factor for NKA under most physiological conditions. Indeed, ATP concentrations are well above \( K_{m,ATP} \) even during extreme fatigue, when ATP concentrations can drop 80% (~1.2 mM) (8, 169).

**Modulation of kinetic properties by protein-protein interactions: phospholemman and other FXYD proteins**

Phospholemman (aka FXYD1) is a member of the FXYD family of small transmembrane regulators of ion transport, which act as tissue specific regulators of NKA (115, 323). Among the seven mammalian FXYDs (FXYD1-7), phospholemman is the most important regulator of NKA in skeletal muscle. It associates with \( \alpha_1 \)- and \( \alpha_2 \)-subunits (48, 134, 181, 293). Notably, some estimates suggest that as many as 30% of \( \alpha_1 \)- and \( \alpha_2 \)-subunits are associated with phospholemman in rat skeletal muscle (291). Association with phospholemman reversibly inhibits NKA activity (73). Disinhibition of NKA is achieved by phosphorylation of phospholemman at Ser\(^{63} \) and Ser\(^{68} \), which increases affinity for Na\(^+ \) (decreases \( K_{1/2,Na} \)), maximal ATPase activity \( (V_{\text{max}}) \) and/or maximal transport activity \( (I_{\text{max}}) \) (33, 73, 80, 124, 208). The increase in Na\(^+ \) affinity has been the most consistent finding upon phosphorylation of phospholemman, while increases in \( V_{\text{max}} \) and/or \( I_{\text{max}} \) have been somewhat more variable.

Phosphorylation of phospholemman is catalyzed by protein kinase C (PKC; at Ser\(^{63} \) and Ser\(^{68} \)) and protein kinase A (PKA; at Ser\(^{68} \)) (270, 345). PKA and/or PKC are activated by various hormones, including epinephrine and insulin (345), as well as by exercise (26, 330). Phospholemman therefore represents a direct molecular link between regulation of NKA in...
skeletal muscle and several major physiological stimuli. Notably, data from *Xenopus* oocyte
expression system indicate that phospholemman regulates different α/β heterodimers in distinct
ways (33, 38). PKA-stimulated phosphorylation of Ser^{68} increases Na\(^+\) affinity of α1/β1 and
α2/β1 heterodimers without altering \(I_{\text{max}}\). In contrast, PKC-stimulated phosphorylation of
phospholemman increases maximal transport activity of α2/β1 heterodimers, but does not alter
\(I_{\text{max}}\) of α1/β1 heterodimers (33). Similarly, in cardiac myocytes activation of PKA increases only
Na\(^+\) affinity of α1/β and α2/β heterodimers, while activation of PKC increases Na\(^+\) affinity of
α1/β and α2/β heterodimers as well as \(I_{\text{max}}\) of α2/β heterodimers (38). Taken together, these data
suggest that different patterns of phospholemman phosphorylation may have different
downstream effects. Moreover, phosphorylation of phospholemman may represent an entry point
to isoform-specific regulation of α/β heterodimers. Studies that investigated phospholemman
phosphorylation and expression in skeletal muscle are summarized in Table 1.

Skeletal muscle also expresses FXYD5 (37), which interacts with NKA and increases \(I_{\text{max}}\)
without altering affinity for Na\(^+\) or K\(^+\) (115, 213). Expression of FXYD5 in skeletal muscle
increases with physical inactivity (37). However, physiological role of FXD5 in skeletal muscle
NKA regulation remains unclear and requires further characterization.

*Modulation of kinetic properties by covalent modifications: phosphorylation and
 glutathionylation*

The α-subunit contains several phosphorylatable Ser, Thr, and Tyr residues in living cells
(5, 50-52) (Table 2) and is a target for several major kinases, including PKA (29, 53, 356), PKC
(29, 53), ERK1/2 (5), and cGMP-activated protein kinase (PKG) (109). In addition, AMPK
regulates phosphorylation of the α1-subunit indirectly (24). In response to phosphorylation of the
α-subunit the intrinsic activity of NKA can decrease, increase, or remain unaltered. For instance, phosphorylation of the α-subunit by PKA and PKC in the presence of Triton X-100 reduces activity of purified NKA from shark rectal gland (29). Conversely, in liposomal preparations PKA-induced phosphorylation increases activity of shark NKA, but does not alter activity of pig NKA (71). PKG phosphorylates the α1-subunit and increases activity of purified NKA from mammalian kidneys (109). Phosphorylation of the α-subunit by PKA, PKC, and/or PKG, which are activated in response to hormonal stimulation and/or muscle contractions (16, 26, 330, 345, 357), may thus directly regulate intrinsic NKA activity in some cases. However, phosphorylation of the α-subunit has another, more important, role in regulation of NKA; that is it regulates translocation of NKA from the intracellular compartment to the plasma membrane as well as its retrieval from the membrane back to the intracellular compartment (25). Indeed, phosphorylation of the α-subunit is able to affect its subcellular distribution without altering NKA activity (5, 51). Dynamic regulation of NKA membrane abundance represents a key aspect of NKA regulation in skeletal muscle and will be discussed separately. Studies that investigated phosphorylation of the α-subunit in skeletal muscle are summarized in Table 2.

Glutathionylation, which involves formation of a reversible covalent bond between glutathione and reactive thiol residues on α- and β-subunits, inactivates NKA (104, 154, 158, 211, 280). Interestingly, 4-9% of α-subunits and 15-20% of β-subunits are glutathionylated in rat skeletal muscle under resting condition (154). Similarly, more than 20% of α-subunits and 6-26% of β-subunits are glutathionylated in resting human skeletal muscle (158). These results suggest that a substantial fraction of the total NKA pool in skeletal muscle might be not only inactive, but also unavailable for immediate activation.
A note on the assessment of NKA activity in skeletal muscle

NKA is a Na\(^+\)-K\(^+\) pump and an enzyme (ATPase). NKA activity can therefore be assessed either by measuring its pumping or enzymatic activity. The ouabain-sensitive uptake of \(^{86}\text{Rb}^+\) is considered the gold standard for quantitative assessment of NKA pumping activity in cultured myotubes (5, 23, 24) or in isolated skeletal muscle (63). An alternative approach to assess Na\(^+\)-K\(^+\) ion flux is to measure ouabain-sensitive influx of \(^{42}\text{K}^+\) or efflux of \(^{22}\text{Na}^+\) (68). Finally, NKA is an electrogenic pump, which produces a net outward current (NKA pumps out 3 Na\(^+\) in exchange for 2 K\(^+\)). This property can be exploited to estimate NKA activity by measuring ouabain-sensitive changes in resting membrane potential (48, 64, 105, 134, 181, 186, 187) or by measuring pump current (28, 33, 73, 80, 274, 311). However, these approaches cannot be used to assess NKA function in muscle biopsy samples.

ATPase activity of NKA is determined in membrane fractions by estimating hydrolysis of ATP, a physiological NKA substrate. Hydrolysis of ATP is estimated in by measuring release of P\(_i\). This can be done radiometrically by using [\(\gamma^{32}\text{P}\)]ATP or by using a non-radioactive malachite-based Biomol Reagent (161, 183). To avoid challenges of sample purification, which may result in relatively low NKA yields, enzymatic activity of NKA can be assessed by measuring \(\text{K}^+\)-dependent phosphatase activity in muscle homogenates using artificial substrates p-nitrophenyl phosphate (the p-NPPase activity) (76, 142) or 3-\(\text{O}\)-methylfluorescein phosphate (the 3-O-MFPase activity) (111). The 3-O-MFPase was thought to be particularly useful due to its sensitivity (111).

However, estimation of NKA activity with the ATPase and the 3-O-MFPase methods has not always produced congruent results, especially regarding maximal NKA activity in skeletal muscle during exercise (161). Indeed, while the 3-O-MFPase method consistently indicated that
exercises reduces maximal NKA activity (226), the ATPase method showed an increase in
maximal NKA activity (161). Results obtained with these methods were thus subject to different
interpretations (40, 150). A possible reason for the discrepancy is methodological; the 3-O-
MFPase assay measures K⁺-dependent hydrolysis of an artificial substrate under Na⁺-free
conditions and therefore cannot detect changes in Na⁺ affinity (150, 161). This is a major
disadvantage, given that changes in Na⁺ affinity are regarded a major mechanism underlying
stimulation of NKA by muscle contractions as well as hormones (58). In addition, the 3-O-
MFPase assay has a bell-shaped concentration-activity curve (111), which might have affected
results (150, 161). However, a recent study showed that maximal NKA activity can be reduced
following exercise even if estimated by the ATPase method (138), possibly due to inactivation of
NKA by glutathionylation (158). Clearly, futures studies will have to examine these issues in
more detail, taking into account advantages, disadvantages, as well as possible artifacts of
different methods for the assessment of NKA activity in skeletal muscle.

Regulation of NKA abundance in plasma membrane

NKA translocation between subcellular compartments in skeletal muscle

The capacity for Na⁺-K⁺ transport at any given moment depends on the NKA abundance
in the plasma membrane. NKA is localized in membranes of T-tubular system and surface
sarcolemma, as well as in membranes of the intracellular compartment (140, 198, 266, 350).
Similarly, phospholemman is localized in surface sarcolemma as well as in internal membranes
(20, 152, 291). Membrane abundance of NKA can be altered acutely by redistribution of NKA
between the membrane and the intracellular compartment. Intracellular compartment thus
contains a reserve pool of NKA that can be rapidly recruited to the plasma membrane in response
to various stimuli, including insulin, muscle contractions, and AMPK activation (24, 25). Acute translocation of NKA subunits from the intracellular compartment to the plasma membrane, has been so far demonstrated in different experimental models, including cultured myotubes (5, 7, 24), isolated skeletal muscles (7, 25, 50), as well as in vivo studies in animals (140, 157) and humans (160). Importantly, translocation of NKA to and from the plasma membrane appears to be a universal mechanism, which was independently demonstrated in various types of non-muscle cells (49, 51, 52, 54). Studies that investigated NKA translocation in skeletal muscle are summarized in Table 3.

Recruitment of NKA to plasma membrane involves stimulation of exocytosis of intracellular NKA-containing vesicles and/or suppression of endocytosis of NKA (25). Dynamic redistribution of NKA between different compartments is similar to trafficking of other membrane proteins, such as translocation of the glucose transporter GLUT4 from the intracellular storage sites to the sarcolemma in response to insulin (90, 347), muscle contractions (301), and activation of AMPK (200). Importantly, despite obvious similarities, NKA and GLUT4 are not localized in the same intracellular vesicles (196), indicating membrane abundance of different membrane proteins is regulated by distinct regulatory pathways. In addition to NKA and GLUT4, dynamic translocation to and from the membrane has also been demonstrated for amino acid transporters (143), transferrin receptor (200), receptor for low density lipoproteins (LDL) (168), and even large macromolecular complexes like the nicotinic acetylcholine receptor (nAChR) (42, 222). Clearly, muscle fibers can dynamically regulate abundance of various membrane proteins by controlling their subcellular localization.

NKA translocation is regulated by phosphorylation of the α-subunit
Subcellular localization of NKA is regulated by phosphorylation of the α-subunit, which can promote exocytosis or endocytosis of the NKA heterodimer (25). Divergent physiological effects arise from species- and isoform-specific differences in phosphorylation sites (5, 51, 52). For instance, dopamine-induced PKC activation in rat proximal tubular cells increases phosphorylation of the α1-subunit at Ser^{18} and induces NKA endocytosis (51, 52). Conversely, activation of PKC is required for insulin-stimulated increase in membrane abundance of α1- and α2-subunits in human myotubes (5). Notably, human α1- and α2-subunits lack Ser^{18} (103) and are poor direct substrates for PKC (5), which explains how PKC activation can lead to different effects in human skeletal muscle and rat kidney cells. Notably, phospholemman can also be translocated acutely to the muscle membrane (152, 291), which presumably alters NKA activity. Mechanisms underlying translocation of phospholemman are unclear, but the available data suggest that phosphorylation of Ser^{68} is not involved (291).

Reconciliation of NKA translocation and [^{3}H]ouabain binding studies

Insulin- and contraction-induced translocation of NKA has been subject to different opinions (25, 60). However, despite past controversy, the balance of evidence now supports the existence of NKA translocation upon stimulation with insulin (5, 7, 50, 140, 196-198, 220) as well as during muscle contractions (157, 160, 185, 291, 303) (Table 3). NKA translocation was demonstrated in these studies using different methodological approaches, including immunoelectron microscopy, surface biotinylation followed by immunoblotting, and membrane fractionation followed by immunoblotting and/or measurement of NKA activity.

A major argument against the existence of NKA translocation was failure to observe an increase in [^{3}H]ouabain binding upon stimulation of insulin and/or electrical stimulation of
isolated muscles (68, 228, 241). $[^3]$Houabain binding is the gold standard to determine NKA content in skeletal muscle and other tissues (58, 59). Under standard protocol NKA content is determined by incubating skeletal muscle at $[^3]$Houabain at 30°C for prolonged periods of time (~120 min). Notably, ouabain triggers endocytosis of the $\alpha_1$- as well as the $\alpha_2$-subunit (179, 212). During incubation at 30°C membrane abundance of NKA therefore keeps changing due the ongoing endocytosis and exocytosis. While the ongoing NKA trafficking to and from the membrane does not affect determination of total NKA content, it precludes reliable estimation of membrane abundance of NKA. Prolonged $[^3]$Houabain binding at 30°C therefore reflects total NKA content rather than momentary membrane abundance of NKA. In contrast, if vesicle trafficking during incubation with $[^3]$Houabain is blocked by reducing temperature to 16-18°C, membrane abundance of NKA remains constant, which reveals insulin- and contraction-stimulated NKA translocation to the membrane (25). In sum, the seemingly opposing results of translocation studies with $[^3]$Houabain can be reconciled if we take into consideration that incubation at 30°C overestimates membrane abundance of NKA due to on-going traffic of NKA and thus obscures its translocation in response to physiological stimuli like insulin and muscle contractions.

Regulation of NKA content in skeletal muscle

Molecular mechanisms underlying regulation of NKA content

NKA content in skeletal muscle is regulated by ions (239), hormones (95, 209), physical activity (96, 119), and nutrition (113, 176, 262). The major underlying mechanism is transcriptional regulation of NKA gene expression, which has been recently reviewed in detail (206) and will not be discussed here. Other underlying mechanisms include regulation of
translation as well as protein stability. Epigenetic mechanisms are also likely important. Regulation of gene expression via epigenetic mechanisms includes methylation of cytosine residues in DNA and various posttranslational modifications of histones, such as acetylation, methylation, and ubiquitination. However, while these mechanisms have emerged as regulators of skeletal muscle adaptations to various physiological stimuli, such as exercise (21), epigenetic regulation of NKA expression in skeletal muscle has surprisingly not been thoroughly examined to date. This is even more surprising since the data from non-muscle cells and tissues suggest that epigenetic mechanisms regulate expression of α- and β-subunits as well as FXYDs (19, 190, 308). Indeed, hypomethylation of promoter and intragenic regions of the α4 gene in mouse sperm may explain why the α4-subunit is expressed specifically in sperm (190). In addition, promotor methylation reduces gene expression of the β1-subunit in renal cancer cells (308). Similarly, promotor methylation reduces expression of phospholemman, which likely explains regional distribution of phospholemman in mouse brain (19). These studies raise the question whether similar mechanisms might play a role in regulation of NKA in skeletal muscle. For instance, it would be important to determine whether epigenetic mechanism might contribute to asymmetric distribution of β1- and β2-subunits between glycolytic and oxidative muscle fibers.

Estimation of NKA content: [3H]ouabain binding vs. immunoblotting

The most precise and quantitative approach to determine total NKA content is [3H]ouabain binding (58, 59, 61). Importantly, estimation of concentration of [3H]ouabain binding sites allows calculation of theoretical maximal capacity of NKA in skeletal muscle (63). NKA content can also be assessed in a semi-quantitative manner by immunoblotting. Although immunoblotting cannot provide a quantitative estimate of total NKA content or maximal
theoretical NKA activity, it is useful for several reasons. First, it enables estimation of NKA subunits in skeletal muscle. This is important information given that α/β heterodimers display distinct kinetic and physiological properties (34, 74, 82, 183, 289). In addition, different α/β heterodimers are regulated by phospholemman and glutathionylation in distinct ways (33, 38, 154, 158). Second, when combined with immunoprecipitation immunoblotting is a particularly useful approach to analyze interactions between NKA and regulatory proteins, such as phospholemman. For instance, using this approach fractional association between phospholemman and NKA was obtained (291). Moreover, immunoprecipitation revealed that NKA and phospholemman interact with nAChR (134). Finally, immunoblotting is a powerful tool to assess regulatory posttranslational modifications of NKA, such as phosphorylation (5-7, 24, 50) and glutathionylation (154, 158). To conclude, immunoblotting clearly cannot provide an accurate estimate of total NKA content or maximal transport capacity like the [³H]ouabain binding. However, it provides valuable additional insights into NKA function in skeletal muscle.

**PHYSIOLOGICAL STIMULI REGULATING NKA IN SKELETAL MUSCLE**

NKA activity in skeletal muscle requires acute adjustments in two major physiological situations: physical activity and feeding. During physical activity NKA activation counteracts fluxes of K⁺ and Na⁺ across sarcolemma and thus maintains muscle contractility. In addition, by limiting K⁺ loss from contracting skeletal muscle, increased NKA activity helps to prevent or at least blunts exercise-induced hyperkalemia. After feeding NKA activation increases K⁺ uptake into skeletal muscle, which helps to accommodate K⁺ load ingested with a meal and prevents development of postprandial hyperkalemia. Stimuli which regulate NKA under these conditions can be divided into extrinsic, which include hormones (Figure 1) and nerve-derived factors
NKA activity is regulated primarily by extrinsic stimuli, such as insulin and possibly other hormones, which are secreted in response to nutrient ingestion. Conversely, local stimuli are the prime driver of NKA activation in contracting skeletal muscle. Nevertheless, extrinsic stimuli, such as catecholamines, can significantly enhance local adaptations and thus improve muscle function as well as whole-body homeostasis. Clearly, both types of stimuli play complementary roles in acute regulation of skeletal muscle NKA. In addition, they couple chronic levels of nutrient intake and physical activity to long-term regulation of NKA in skeletal muscle.

**Hormonal regulation of NKA**

**Regulation of NKA by insulin**

Insulin is a major acute regulator of NKA activity in skeletal muscle after a meal. In addition, it is one of chronic regulators of muscle NKA content. Insulin acutely increases NKA activity in skeletal muscle (67, 68, 93), which shifts K\(^+\) from the extracellular into the intracellular space and reduces extracellular K\(^+\) concentrations (78, 299). Actually, hypokalemia was described in insulin-treated experimental animals (39, 129) and patients (129) immediately after discovery of insulin in 1922. One of the most likely physiological roles of insulin-stimulated NKA activation and K\(^+\) uptake is to buffer postprandial excursions in plasma concentrations of K\(^+\) (97, 324). Although kidney plays a key role in long-term K\(^+\) homeostasis it is unable to rapidly excrete large K\(^+\) loads (225). Most of the ingested K\(^+\) load is therefore temporarily taken up by the skeletal muscle, which prevents hyperkalemia and enables kidney K\(^+\) excretion to take effect. Buffering of fluctuating K\(^+\) intake by the skeletal muscle is remarkably effective as plasma concentrations of K\(^+\) do not increase after ingestion of a K\(^+\)-containing meal,
although it takes 5 hours to excrete 75% of the ingested K⁺ load (286). Insulin-stimulated K⁺ uptake is important also clinically. On the one hand, injudicious use of insulin can cause profound hypokalemia (57, 116). On the other hand, K⁺-lowering effect of insulin can be exploited for treatment of some forms of hyperkalemia.

As well as regulating K⁺ concentrations after a meal, activation of NKA by insulin probably directly promotes Na⁺-coupled uptake of substrates, such as creatine (319), carnitine (320), inorganic phosphate (285), and amino acids (81, 143), or at least prevents an excessive increase in intracellular Na⁺ concentrations that would otherwise result from insulin-driven Na⁺-coupled transport (324). Insulin-like growth factor I (IGF-I) also activates NKA (89) and stimulates amino acid uptake via Na⁺-coupled transporters (139), thus underscoring the link between activation of NKA and Na⁺-coupled transport in skeletal muscle.

Insulin stimulates NKA in skeletal muscle acutely by at least two mechanisms (Figure 1). First, it increases intrinsic activity of NKA by elevating its affinity for intracellular Na⁺ (175, 214). This effect is readily explained by insulin-stimulated activation of PKC, which phosphorylates phospholemman at Ser⁶³ and Ser⁶⁸ (270, 345) and consequently suppresses its inhibitory effect on NKA (33, 38). Second, insulin induces phosphorylation and subsequent translocation of NKA heterodimers from the intracellular compartment to the plasma membrane as demonstrated in human and rat myotubes (5, 7, 139) as well as in rat hind limb muscles upon in vivo (140, 220) or ex vivo insulin treatment (50). This response is subunit-selective and insulin induces primarily translocation of the α₂-subunit (140, 220), although translocation of the α₁-subunit was also noted (7) (Table 3). Insulin-induced phosphorylation of the α₂-subunit occurs more rapidly and is more pronounced than phosphorylation of the α₁-subunit (50), which provides one possible mechanism for differences in translocation response.
Insulin-stimulated translocation of NKA in isolated skeletal muscle and cultured myotubes depends on atypical PKCs, while conventional or novel PKCs do not seem to be involved (5, 7). The PKC family comprises conventional PKCs (α-, βI-, βII- and γ-isoforms), which are activated by diacylglycerol/phorbol esters and Ca\(^{2+}\), novel PKCs (δ-, ε-, θ-, and η-isoforms), which are activated only by diacylglycerol/phorbol esters, and atypical PKCs (ζ-, ι/λ-isoforms), which are not regulated by diacylglycerol/phorbol esters and Ca\(^{2+}\) (246, 247). In cultured myotubes insulin activates atypical PKCs, which in turn lead to activation of ERK1/2 and phosphorylation of α1- and α2-subunits at Thr-Pro residues. Phosphorylation of Thr-Pro residues by ERK1/2 likely increases abundance of NKA in the plasma membrane by blocking its endocytosis, but does not alter its intrinsic activity (5, 25). Insulin also stimulates phosphorylation of Tyr residues in α-subunits (50), but these phosphorylations are not mediated by ERK1/2 (5) and their functional significance remains to be determined. Clearly, results obtained in cultured myotubes cannot be extrapolated to skeletal muscle under in vivo conditions without reservations. Still, these cells have been used successfully to investigate basic molecular physiology and pharmacology of skeletal muscle (304), demonstrating that molecular pathways are often conserved in cultured myotubes. Thus, while independent confirmation in other experimental models is required, these results nevertheless suggest that activation of atypical PKC/ERK1/2 pathway might explain also insulin-stimulated translocation of NKA in vivo.

In addition to acute regulation of NKA activity insulin controls gene expression of the different NKA subunits (206) and thereby determines total NKA content in skeletal muscle. Notably, in untreated diabetic subjects skeletal muscle NKA content is reduced, while its content increases upon insulin treatment (58). Furthermore, glucose intolerant men have reduced intracellular Na\(^{+}\)-to-K\(^{+}\) ratio in skeletal muscle (191), which demonstrates that insulin resistance
reduces NKA activity in skeletal muscle. Insulin is therefore one factor that determines NKA transport capacity in the long term. A corollary of these long-term effects is that insulin might affect exercise performance of skeletal muscle, although it is not important for acute activation of NKA during exercise. For instance, reduced capacity for NKA transport in insulin resistant diabetic muscle may lead to increased fatigability and lower exercise performance.

Regulation of NKA by C-peptide and amylin

In response to food intake, β-cells secrete not only insulin, but also C-peptide and amylin. Limited in vitro data suggest that these peptides might also be involved in NKA regulation in skeletal muscle. The physiological role of secreted C-peptide is not well defined; however, C-peptide supplementation ameliorates dysfunction of nerves, kidney, and retina in type 1 diabetes, indicating that C-peptide exerts direct physiological effects and that its deficiency leads to multiple abnormalities (343). One of major effects of C-peptide is regulation of NKA in various types of cells (108, 114, 264). Importantly, recent evidence shows that physiological concentrations of C-peptide (1 nM) stimulate $^{86}\text{Rb}^+$ uptake in cultured skeletal muscle cells, suggesting a role for C-peptide in regulation of NKA in skeletal muscle (188) (Figure 1). This result is at variance with an earlier study, in which supraphysiological concentrations of C-peptide (100 nM) failed to stimulate NKA in isolated skeletal muscle (56). Besides different experimental models, the discrepancy between these two studies might be due to the usage of different C-peptide concentrations. Indeed, C-peptide displays bell-shaped dose-response curve, thus its effects are most pronounced if C-peptide is used in concentrations, which are close to physiological range (361).
Amylin induces satiety, suppresses glucagon release, and slows gastric emptying (131). It exerts these effects via amylin receptors, which comprise calcitonin receptor (CT) and an accessory protein RAMP (RAMP1, 2, or 3) (131). Unlike the role of C-peptide, the physiological role of amylin is much better defined and its analogue, pramlintide, has already been successfully introduced into clinical practice for treatment of type 1 and type 2 diabetes (131). Amylin activates NKA in isolated skeletal muscle (56, 147) (Figure 1). However, these effects were achieved using amylin concentrations 10 nM or higher, while physiological amylin concentrations are in the lower picomolar range (3-25 pM) (131). Notably, physiological and pharmacological concentrations of amylin were noted to have divergent effects in skeletal muscle (362). It is therefore unclear whether amylin can stimulate NKA activation under physiological conditions. Clearly, physiological role of skeletal muscle NKA regulation by amylin as well as C-peptide needs further characterization.

Regulation of NKA by epinephrine and other catecholamines

Epinephrine and other endogenous catecholamines, norepinephrine and dopamine, are involved in the regulation of NKA in skeletal muscle and various other organs (30, 31, 58, 64, 65, 102, 105). Regulation of skeletal muscle NKA by catecholamines is particularly important during exercise (58), when activation of sympathetic nervous system increases plasma concentrations of epinephrine and norepinephrine (4, 44, 128). Catecholamine-stimulated NKA activity improves muscle function and delays fatigue. For instance, loss of force due to high extracellular K⁺ concentrations is markedly blunted by epinephrine and exogenous catecholamines, such as salbutamol (69). NKA activation by catecholamines also limits net K⁺ loss from contracting muscle fibers (8, 44, 58), thus blunting exercise-induced hyperkalemia.
Notably, plasma K\(^+\) concentrations during high intensity exercise can reach up to \(\sim 8\) mM (233). In addition to exercise, catecholamines stimulate NKA activation also under pathophysiological conditions such as sepsis (43, 201).

In skeletal muscle epinephrine and norepinephrine acutely stimulate NKA activity via \(\beta\) adrenoceptors (65) (Figure 1). Similarly, NKA activity is increased by treatment of muscle membranes with cAMP (162) as well as by treatment of isolated skeletal muscles with dibutyryl-cAMP (65), a membrane-penetrable analogue of cAMP. Activation of \(\beta\) adrenoceptors triggers the cAMP-PKA pathway, which increases phosphorylation of phospholemman at Ser\(^{68}\), as demonstrated in epinephrine-treated myocardium (270) and isolated rat diaphragm (345). Once phosphorylated at Ser\(^{68}\), phospholemman reduces \(K_{1/2,Na}\), thus increasing NKA activity (80, 145).

Among endogenous catecholamines epinephrine is the most potent stimulator of NKA in skeletal muscle (65, 167). The threshold for epinephrine-stimulated activation of NKA in isolated skeletal muscle is approximately 6 nM, while half-maximal stimulation is achieved by 13 nM (65). In contrast, 25-times higher concentrations of norepinephrine are required to achieve a comparable effect (65). This is consistent with rank order of potency of catecholamines (epinephrine>norepinephrine) (192, 207) for \(\beta_2\) adrenoceptor, the predominant \(\beta\) adrenoceptor in skeletal muscle (148, 174). Norepinephrine is a full \(\beta_2\) adrenoceptor agonist and, if used in high concentrations (~1 \(\mu\)M), is as effective activator of NKA as epinephrine (65). However, during exercise epinephrine and norepinephrine concentrations in plasma reach 2-5 nM and 10-20 nM, respectively (4, 44, 120, 128). Similarly, low nanomolar concentrations of norepinephrine were detected directly in skeletal muscle during direct sympathetic stimulation (355), muscle stretch (204), and exercise (235). Taken together, epinephrine is likely the major adrenergic regulator of NKA in skeletal muscle under most physiological conditions. Besides exercise and stress
response, stimulation of NKA by catecholamines is important also pharmacologically. For instance, pharmacological β2 adrenoceptor agonists, which are widely used for treatment of asthma and other conditions, may lead to clinically significant hypokalemia due to activation of NKA and the concomitant shift of extracellular K⁺ into skeletal muscle (57, 116).

Dopamine, which plays a major role in local regulation of NKA in kidney tubules (102), is only a weak partial β2 adrenoceptor agonist (207) and cannot stimulate NKA in skeletal muscle fully even at high concentrations (30 μM) (167). Thus, its physiological plasma concentrations (<0.65 nM) (112) imply dopamine is likely of minor importance for regulation of NKA via β2 adrenoceptors. Nevertheless, infusions of dopamine or its analogue dobutamine are able to induce hypokalemia (36, 318). Notably, antagonists of β2 receptors do not block dopamine-induced hypokalemia, indicating involvement of other receptors. Interestingly, dopamine receptors were detected in rat diaphragm (283) and human vastus lateralis muscle (352). Activation of dopamine receptors in kidney induces phosphorylation of Ser¹⁸ of the α₁-subunit and stimulates its endocytosis (51, 52, 102). Whether and how dopamine receptors in human skeletal muscle link to regulation of α₁- and α₂-subunits, which lack Ser¹⁸, has not been established.

Regulation of NKA by thyroxine

Thyroxine (T₄), in its physiologically active form T₃, is essential for normal growth and development as well as regulation of energy metabolism (236). In addition, T₃ has a major stimulatory effect on skeletal muscle NKA activity (58) (Figure 1). Stimulation of NKA activity in response to T₃ occurs by two separate mechanisms (58, 70, 236). First, T₃ stimulates intrinsic activity of NKA indirectly by increasing transmembrane leaks of Na⁺ and K⁺ (58, 236). Second,
T3 determines the content of NKA in skeletal muscle (95, 209). In human skeletal muscle T3 increases expression of NKA α2- and β1-subunits (282), while in rat it increases expression of α2- and β2-subunits (13). Depending on thyroid status total NKA content in skeletal muscle can vary between 100 pmol/g to 600 pmol/g in hypothyroidism and hyperthyroidism, respectively (58, 296). In hyperthyroid subjects total NKA content is increased almost 100% compared with healthy controls (296). This increase is completely reversed when euthyroid status is achieved with antithyroid drugs (295).

Stimulation of NKA activity in skeletal muscle was once thought to provide a major contribution to T3-induced energy expenditure; however, this effect is probably not of major quantitative importance (70), except perhaps in subjects with hyperthyroidism (236). Indeed, T3 increases energy expenditure primarily through other mechanisms, including up-regulation of sarcoplasmic reticulum Ca^{2+}-ATPase (236), which has been recently identified as a major site of muscle-based thermogenesis (15). Finally, it is important to note that in this context the increased NKA content and activity do not translate into improved muscle performance. Overstimulation with thyroid hormones alters different aspects of muscle function besides NKA, which results in myopathy and muscle weakness, thus demonstrating that increased NKA transport capacity does not always parallel increased muscle performance.

Regulation of NKA by calcitonin

Calcitonin is a peptide hormone secreted from parafollicular cells (C-cells) of thyroid gland. Traditionally calcitonin has been linked to regulation of calcium and phosphate homeostasis; however, its role in humans remains poorly defined (180). Like the closely related peptides the calcitonin-gene related peptide (CGRP) and amylin, calcitonin increases NKA
activity in isolated rat skeletal muscle (10). However, whether this effect is important physiologically is unclear. First, the lowest concentration of calcitonin that stimulates NKA in skeletal muscle is approximately 1 nM (10). In contrast, normal plasma concentrations of calcitonin are in the low picomolar range (72). Second, skeletal muscle is not a major target tissue for calcitonin and stimulation of NKA by pharmacological concentrations of calcitonin may result from activation of amylin receptors. Finally, hypersecretion of calcitonin by medullary thyroid carcinoma or surgical removal of thyroid gland without calcitonin supplementation do not result in clinically significant abnormalities in mineral metabolism. Still, calcitonin is used therapeutically and may perhaps affect NKA in skeletal muscle if given in pharmacological doses.

Regulation of NKA by glucocorticoids

Glucocorticoids are adrenal stress hormones with complex anti-inflammatory, immunosuppressive, and metabolic effects. Dexamethasone, a synthetic glucocorticoid, increases total content of NKA in human (84, 258) and rat (88, 292, 331) skeletal muscle (Figure 1). In human vastus lateralis muscle dexamethasone increases NKA α1-, α2-, β1- and β2-subunits (258), while in most rat muscles dexamethasone increases only α2- and β1-subunits (331). Importantly, increased NKA content after short-term dexamethasone treatment is paralleled by increase in maximal NKA activity (256) and improved muscle K⁺ homeostasis during low or moderate intensity exercise (258). However, dexamethasone reduces insulin-stimulated K⁺ uptake in rats, suggesting dexamethasone blunts NKA activation by insulin despite increased NKA content in skeletal muscle (294). Taken together, these data indicate that activation of NKA by exercise and insulin is regulated by distinct signaling events and that insulin-responsive
pathways are selectively impaired by dexamethasone. Interestingly, this resembles the situation in insulin resistant patients who have normal glucose uptake in response to exercise (172), although insulin-stimulated uptake of glucose in skeletal muscle is markedly reduced (79).

Finally, we have to note that major alterations of NKA content in skeletal muscle were observed only during exposure to pharmacological doses of glucocorticoids (58, 256, 258). In rat muscles NKA content is not altered significantly even after removal of adrenal gland (88), which results in severe deficiency of glucocorticoids. Thus, whether and how cortisol or other endogenous glucocorticoids regulate NKA in skeletal muscle under physiological conditions is uncertain. Still, regulation of NKA by glucocorticoids might be important in patients who are being treated with pharmacological doses of dexamethasone or other potent glucocorticoids. For instance, muscle NKA content is increased 61% in patients with chronic obstructive pulmonary disease (292) who were treated with high doses of various glucocorticoids (equivalent to ~43 mg prednisolone/day). In contrast, NKA content remained unaltered in patients with lung and kidney transplants who received lower doses of prednisolone (~5-20 mg/day) (227, 279), indicating that a certain threshold dose of glucocorticoids is required to increase NKA content in skeletal muscle. Indeed, NKA content in skeletal muscle increases with glucocorticoid intake (292). However, on the other hand, transplant patients have impaired exercise performance, which would tend to decrease muscle NKA content (227, 279). Thus, prednisolone might have prevented inactivity-induced decrease in muscle NKA content in these patients. Notably, a inhalation of low dose budesonide in healthy subjects increases muscle NKA content 17% in two weeks (137), indicating that even low doses of glucocorticoids may have a significant effect on NKA in skeletal muscle.
Regulation of NKA by aldosterone

Aldosterone, the major mineralocorticoid in terrestrial animals, is a key regulator of Na\(^+\) and K\(^+\) homeostasis (300). It plays a pivotal role in long-term regulation of electrolyte balance mainly in the kidney, where it stimulates Na\(^+\) reabsorption and K\(^+\) secretion by increasing NKA activity and ion conductance in tubular cells (102). Although skeletal muscle is not one of the classical aldosterone target tissues, novel evidence suggests that skeletal muscle expresses functional mineralocorticoid receptor (45) (Figure 1). Furthermore, patients aldosterone-secreting adrenal adenoma have increased content of α2- and β1-subunits as well as increased NKA activity in vastus lateralis muscle (Table 4) (281). In these patients NKA was assessed after aldosterone-induced hypokalemia had been corrected with oral potassium, indicating up-regulation of NKA in skeletal muscle may be a direct effect of aldosterone. Consistent with this notion, removal of adenoma, which normalized aldosterone concentrations, reduced expression of α2- and β1-subunits and NKA activity (281). An earlier study in rats suggested that aldosterone reduces NKA content in skeletal muscle (88). However, in rats, which developed aldosterone-induced hypokalemia, K\(^+\) was not supplemented (88), suggesting NKA content might have been reduced by K\(^+\) deficiency (262) and not directly by aldosterone (58). Notably, aldosterone increases expression of α1- and β1-subunits in cultured cardiomyocytes (144) as well as in vascular smooth muscle cells (244), consistent with the notion that aldosterone regulates NKA in muscle cells.

Regulation by endogenous cardiotonic steroids

The NKA α-subunit has an evolutionary-conserved site for cardiotonic steroids, such as ouabain, marinobufagenin, and digoxin (210). Endogenous cardiotonic steroids are physiological
NKA ligands and probably include endogenous ouabain or a closely related compound (14, 35, 306). Circulating endogenous cardiotonic steroids are likely synthesized in the adrenal gland (14, 35). In high concentrations, cardiotonic steroids bind to the α-subunit and inhibit NKA pumping activity. This effect has been exploited clinically to treat heart failure with exogenous cardiotonic steroids derived from foxglove extracts for centuries. Although largely superseded by novel therapies, they remain in clinical use. Predictably, intoxication with exogenous cardiotonic steroids leads to muscle weakness and hyperkalemia (199, 218, 351). Furthermore, even if they are within the therapeutic range, exercise-induced increases in plasma K⁺ concentrations are enhanced, indicating significant inhibition of skeletal muscle NKA and enhanced K⁺ loss from contracting muscles (260). In low concentrations, which are insufficient to inhibit NKA pumping, cardiotonic steroids activate NKA receptor complex, thus leading to activation of intracellular signaling pathways (11, 14). NKA receptor complex includes NKA, which does not possess intrinsic kinase activity, tyrosine kinase receptor for epidermal growth factor, as well as non-receptor tyrosine kinase Src (11, 14).

Existence of endogenous cardiotonic steroids suggests that they may represent an additional humoral mechanism for regulation of NKA in skeletal muscle (Figure 1). Two lines of evidence support this notion (Table 4). First, mice expressing the ouabain-resistant α2-subunit (α2R/R) have increased exercise performance (287). Given that exercise increases concentrations of ouabain-like compound (22), increased performance of α2R/R mice suggests that endogenous cardiotonic steroids regulate NKA during exercise. Notably, isolated EDL muscles from α2R/R mice and EDL muscles from wild-type mice infused with cardiotonic steroid-blocking antibody transport more ⁸⁶Rb⁺ after electrical stimulation than untreated wild-type EDL muscles (287). Second, in cultured human myotubes ouabain stimulates basal and insulin-stimulated glycogen
synthesis in concentrations which only slightly inhibit NKA activity (179). Notably, ouabain-stimulated glycogen synthesis is mediated via Src signaling pathway (179), thus indicating that NKA receptor complex might be involved in regulation of skeletal muscle glucose metabolism.

Neurogenic regulation of NKA

Regulation of NKA by acetylcholine

Acetylcholine, released from the motor neuron at the neuromuscular junction, binds to and opens nAChR channels in junctional sarcolemma (Figure 2). Opening of nAChR channels increases Na\(^+\) influx and K\(^+\) efflux and triggers the excitatory endplate potential in the muscle fiber (98). Once the threshold for opening voltage-gated Na\(^+\) channels is reached, excitatory endplate potential translates into the action potential and muscle contraction (99). This sequence of events represents the only physiological pathway for stimulation of muscle contractions. NKA enables efficient neuromuscular transmission by preserving excitability of junctional sarcolemma. Prolonged depolarization of sarcolemma inactivates voltage-gated Na\(^+\) channels and blocks neuromuscular transmission. Conversely, stimulation of NKA accelerates repolarization and restores excitability and contractility of skeletal muscle (217).

Interestingly, evidence suggests that acetylcholine exerts direct control over NKA activity at the neuromuscular junction (Table 4). Low concentrations of acetylcholine, which are insufficient to trigger massive opening of nAChR channels, selectively stimulate α2/β heterodimers (48, 134, 186) (Figure 2). Such concentrations of acetylcholine (~50 nM) are found in the synaptic cleft due to non-quantal acetylcholine release under resting conditions (254). In addition, low concentrations of acetylcholine remain in the synaptic cleft for some time following nerve activity. Thus, regulation of NKA by low concentrations of acetylcholine is
likely physiologically relevant. Notably, the enhanced electrogenic activity of $\alpha_2/\beta$ heterodimers leads to a more negative membrane potential in junctional than in extra-junctional membrane regions of the same muscle. These data suggest a mechanism by which nAChR activates NKA and thereby maintains the resting potential in the voltage range of slow inactivation of Na$^+$ channels, thus supporting membrane excitability during muscle use (134). Indeed, acetylcholine-induced hyperpolarization of sarcolemma would tend to increase the number of voltage-gated Na$^+$ channels that can be activated, which would in turn enhance the efficiency of neuromuscular transmission. Underlying mechanisms have not been fully established, but likely involve conformational change of nAChR to non-conducting desensitized state. Importantly, nAChR, NKA, and phospholemman form macromolecular complexes in the junctional sarcolemma (48, 134) (Figure 2). Thus, while low concentrations of acetylcholine or other nAChR agonists are not sufficient to open nAChR channel, they may produce conformational changes of nAChR that stimulate NKA activity through protein-protein interactions.

On the other hand, high acetylcholine concentrations (0.2-5 mM) markedly inhibit NKA in normal and denervated skeletal muscle ex vivo (309). Notably, acetylcholine was linked to inhibition of NKA also in cardiac sarcolemma (76). Although acetylcholine concentrations in the synaptic cleft can reach 0.3 mM during active neuromuscular transmission (189), it is degraded in less than 1 ms by the acetylcholine esterase (Figure 2), which restricts its diffusion outside the synaptic cleft. Thus, while acetylcholine might inhibit NKA in junctional sarcolemma during these very brief concentration peaks, this effect is most likely not important for regulation of extra-junctional NKA in skeletal muscle under physiological conditions.

Regulation of NKA by CGRP
CGRP is a well-established activator of NKA in skeletal muscle (10, 56, 216). In skeletal muscle CGRP is co-released with acetylcholine from the motor neuron at the neuromuscular junction (223, 338). An even more important source of CGRP in skeletal muscle are sensory nerve endings (302). In skeletal muscle CGRP may therefore regulate NKA at junctional as well as extra-junctional sites. CGRP triggers its effects by activating the CGRP receptors, which comprises calcitonin-like receptor (CLR) and an accessory protein RAMP1 (132). Treatment of diaphragm muscle with CGRP increases cAMP levels (326), thus suggesting CGRP stimulates NKA activation via the cAMP/PKA pathway (58). By stimulating NKA activity in isolated skeletal muscle exogenous CGRP counteracts force decline due to high extracellular concentration (10-12.5 mM) of K⁺ (10, 69, 216). Notably, capsaicin-stimulated release of endogenous CGRP from sensory nerve endings is apparently sufficient for this effect (216). Exogenous CGRP also promotes force recovery of isolated EDL muscle after fatiguing electrical stimulation (234) as well as after electroporation-induced injury to sarcolemma (66). Finally, electrical stimulation stimulates endogenous CGRP release and force recovery in K⁺-suppressed muscles ex vivo (253). Importantly, depletion of endogenous CGRP by muscle denervation or capsaicin pre-treatment impair this recovery, thus indicating regulation of skeletal muscle NKA by the endogenous CGRP is physiologically relevant (253).

Regulation of NKA by purines and agrin

Aside from acetylcholine and CGRP motor neuron secretes, ATP and other purines (313, 342), and agrin (255) (Figure 2). Purines may act as acute regulators of NKA activity (162, 346). Since they are also released by muscle fibers, they will be discussed under local regulation (see below). Agrin is a heparan-sulfate proteoglycan which plays major roles in development and
maintenance of the neuromuscular junction. In addition, agrin was shown to increase expression of NKA α1- and α2-subunits in cultured myotubes (164). In sum, it appears that motor neuron might control NKA activity and content at junctional sarcolemma through several distinct pathways.

Local regulation of NKA: stimuli arising in skeletal muscle fibers

Muscle contractions are the strongest stimulus for NKA activation. Indeed, direct stimulation of isolated skeletal muscle rapidly increases Na\(^+\) efflux 20-fold, thus reaching approximately 60% of theoretical maximal capacity for Na\(^+\)-K\(^+\) transport calculated from the number of \([^3]H\)ouabain binding sites (250). During maximal stimulation with Na\(^+\) loading, NKA activity in isolated skeletal muscle can reach up to 90% of theoretical total NKA capacity (63). Thus, skeletal muscle has a large capacity for NKA activity, but this functional reserve is only transiently called upon to maintain Na\(^+\) and K\(^+\) gradients during contractions (250). In contrast, acute stimulation with insulin, catecholamines, CGRP, and other neurohumoral factors increases basal NKA activity by up to 100% (10, 56, 64, 65, 105). Clearly, local stimuli, which arise in skeletal muscle fibers, are much more effective at stimulating NKA activation than extrinsic neurohumoral regulatory factors. Ouabain markedly accelerates loss of force of contracting skeletal muscle ex vivo (238, 251), which underlines the essential role for contraction-induced NKA activation. Contractions activate NKA through several mechanisms (Figure 3), including increased intracellular Na\(^+\) and extracellular K\(^+\) concentrations, altered kinetic properties of NKA, and increased membrane abundance of NKA. Additional mechanisms may include activation of AMPK, release of purines as well as nitric oxide (NO).
During muscle contractions intracellular concentrations of Na\(^+\) increase due to net influx of Na\(^+\) ions (155). Under physiological conditions intracellular Na\(^+\) concentration is not saturating. Thus, increased intracellular Na\(^+\) concentration stimulates NKA activity (63) (Figure 3). Notably, Na\(^+\)-loading of isolated skeletal muscle effectively blunts force decline due to high extracellular (10 mM) K\(^+\) concentrations (69), demonstrating physiological significance of Na\(^+\)-stimulated NKA activation. However, NKA activity in contracting muscle is markedly increased even with modest increases in intracellular Na\(^+\), indicating that Na\(^+\)-stimulated NKA activation is not the only underlying mechanism (94). For instance, muscle contractions also increase affinity of NKA for intracellular Na\(^+\) (46), which further stimulates its activity. Mechanisms by which contractions increase NKA affinity for Na\(^+\) are not firmly established, but likely involve phosphorylation of phospholemman by the conventional PKC α/β or via other contraction-triggered signaling pathways (26, 330). However, contractile activity does not always increase phosphorylation of phospholemman or affinity for Na\(^+\) (152, 219, 291, 330), indicating that other mechanisms may link contractions to activation of NKA.

Interstitial K\(^+\) concentrations can increase from ~4 mM at rest to over 10 mM during intense exercise due to net loss of K\(^+\) from contracting skeletal muscle (122, 163, 249). However, unlike intracellular Na\(^+\) concentrations, alterations of interstitial K\(^+\) concentrations were traditionally not regarded as an important stimulator of NKA activity. This assumption was based on estimates of K\(^+\) affinity (\(K_{1/2,K} \sim 0.8\) to 1.5 mM) (307), which indicated that most K\(^+\) binding sites are occupied already under resting conditions. Recent evidence, however, suggests that α2/β heterodimers in rat skeletal muscle have much lower apparent affinity for K\(^+\) (\(K_{1/2,K} \sim\)
3.6 mM) than previously thought (82). Lower K⁺ affinity for α2/β heterodimers is consistent with earlier data from different expression systems (34, 74). This means that extracellular K⁺ could stimulate α2/β heterodimers up to concentration of 40 mM (82). Due to diffusional limitations the highest K⁺ concentrations during muscle contractions are encountered in T-tubules, where the majority of α2/β heterodimers is localized. Extracellular K⁺ may therefore play a major role in activation of NKA in contracting skeletal muscle.

Regulation of NKA membrane abundance during muscle contractions

Membrane abundance of α2- and β1-subunits in vastus lateralis is increased following approximately 5 minutes of fatiguing knee-extensor exercise in healthy subjects (160). This increase is not paralleled by increased total muscle content of α2- and β1-subunits (160), indicating they were translocated from the intracellular compartment to the membrane. Low-intensity exercise in rats also increases membrane abundance of NKA subunits (157). Similarly, electrical stimulation of isolated skeletal muscles increases membrane abundance of NKA subunits (157, 185) as well as [³H]ouabain binding (25), which demonstrates that muscle contractions alone are sufficient to trigger translocation of NKA to muscle membrane. Underlying mechanisms have not been studied in detail, but contraction-activated signaling pathways provide the most plausible mechanism. For instance, contractions activate ERK1/2 and atypical PKC ζ/λ (25, 26, 276), both of which have been linked to translocation of NKA from the intracellular compartment to the sarcolemma in cultured skeletal muscle cells (5, 7) (Figure 3). One of key challenges for future research will be to examine whether these signaling pathways regulate translocation of NKA in contracting skeletal muscle under in vivo conditions. Overview of studies investigating contraction-induced NKA translocation is provided in Table 3.
Regulation of NKA by energy status: AMPK

Although ATP levels decrease in contracting skeletal muscle, ATP is likely not a major direct regulator of NKA in skeletal muscle. On the other hand, reductions in ATP concentrations are paralleled by much more pronounced increases in ADP and, especially, AMP concentrations (8, 127). Decreased ATP/ADP and ATP/AMP ratios activate AMPK, a cellular energy sensor (126). AMPK is involved in acute and chronic regulation of NKA in skeletal muscle (Table 4) (145, 185). Activation of AMPK was initially linked with endocytosis and acute suppression of NKA activity in epithelial cells (123). In addition, intraperitoneal application of AMPK activator AICAR in rats acutely reduced abundance of the α2-subunit in sarcolemmal giant vesicles from mixed hind limb muscles (185). These results suggested that AMPK acts as a negative regulator of NKA in skeletal muscle, which would be consistent with the role of AMPK as energy sensor that, once activated, suppresses energy-consuming processes and stimulates energy-generating metabolic pathways.

Conversely, sarcolemmal abundance of α2-subunits was not observed in skeletal muscles of rats, which received intravenous infusion of AMPK activator AICAR (360). AICAR infusion also reduced plasma K⁺ concentrations without affecting urinary K⁺ excretion (360), consistent NKA activation and redistribution of K⁺ from the extracellular to the intracellular compartment. Furthermore, AMPK activation acutely increases NKA activity in cultured rat L6 myotubes (24) as well as in MDCK kidney cells (9). Thus, it appears that acute AMPK activation in skeletal muscle might be linked to NKA activation rather than suppression. Notably, a recent study showed that activation of AMPK increases activity of sarcoplasmic reticulum Ca²⁺-ATPase in
vascular smooth muscle cells (305), which again demonstrates that AMPK does not necessarily suppress all energy-consuming processes.

AMPK activation in cultured L6 myotubes is associated with decreased phosphorylation of Ser^{18} and increased membrane abundance of the α1-subunit (24). Increased phosphorylation of Ser^{18} stimulates NKA endocytosis in kidney cells (51, 52), indicating activation of AMPK in L6 myotubes blocks endocytosis of the α1-subunit and thereby increases its membrane abundance and activity. Mechanism by which activation AMPK reduces Ser^{18} phosphorylation has not been fully elucidated, but it seems likely that AMPK stimulates dephosphorylation of Ser^{18} by activating protein phosphatase 2A (24). One in vivo study in rats found that sarcolemmal abundance of the α1-subunit remains unaltered one hour after intraperitoneal AICAR application (185), but this study did not examine phosphorylation status of the α1-subunit. Importantly, intravenous AICAR infusion reduces plasma K^+ concentrations only after exposures longer than one hour (360), suggesting that AICAR might increase membrane abundance of the α1-subunit only during more prolonged exposures.

Whether activation of AMPK acutely regulates membrane abundance of NKA in skeletal muscle obviously requires further assessment. However, data from chronic experiments support the role of AMPK in regulation of NKA in skeletal muscle. In mice treated with AICAR, phospholemman abundance was decreased, while its relative phosphorylation and NKA affinity for Na^+ were increased (145). Conversely, phospholemman abundance was increased in AMPK kinase-dead mice, although this increase was not accompanied by changes in its phosphorylation status or NKA affinity for Na^+ (145). Taken together, these data indicate that AMPK exerts chronic control over NKA in skeletal muscle by regulating phospholemman expression and phosphorylation.
Off-target effects of pharmacological AMPK activators are an important caveat when assessing the role of AMPK in the regulation of NKA. Indirect AMPK activators suppress energy metabolism, which activates AMPK but also triggers a plethora of cellular responses not linked to AMPK activation. Direct AMPK activators, which bind to AMPK and activate it directly, may therefore seem a better choice. However, even with direct AMPK activators off-target effects are an important consideration. Indeed, A-769662, a widely used direct AMPK activator, inhibits purified NKA (23). AMPK activators like AICAR, metformin, and rosiglitazone do not alter activity of purified NKA, thus demonstrating inhibition of NKA is not a general property of AMPK activators. Besides inhibiting NKA directly, A-769662 triggers NKA endocytosis in cultured L6 myotubes through an unknown mechanism that does not involve AMPK activation. Indeed, A-769662 reduces surface abundance of the α1-subunit even in murine skeletal muscle cells lacking functional AMPK (23). These effects clearly preclude use of A-769662 for the assessment of AMPK as the regulator of NKA function.

Regulation of NKA by NO

Contractions activate the muscle isoform of neuronal (type I) NO synthase (nNOSμ) (315), which markedly increases NO production (16). According to a very recent study, NO stimulates NKA activity in glycolytic muscles (Table 4) (153). Similarly, NO stimulates activation of NKA in cardiac myocytes (274). Interestingly, NO does not activate NKA in oxidative skeletal muscle, indicating regulation of NKA by NO is fiber type-specific (153). Failure to stimulate NKA activation in oxidative muscles is consistent with an earlier study, which examined effects of NO in isolated soleus muscle (237).
Stimulation of NKA in glycolytic muscles apparently requires the canonical NO signaling pathway, which involves activation of soluble guanylate cyclase, formation of cyclic guanosine monophosphate (cGMP) and subsequent activation of protein kinase G (PKG) (153) (Figure 3). Besides activating cGMP/PKG pathway, NO can modulate protein function in skeletal muscle through post-translational modifications, such as S-nitrosylation, S-glutathionylation and nitration of tyrosine residues. However, spermine NONOate, a NO donor, does not alter activity of NKA in isolated muscle membranes (153), indicating NO does not regulate NKA by producing such modifications. Finally, phospholemman does not seem to be involved in regulation of NKA by NO in skeletal muscle (153), unlike in heart, where NO activates NKA via PKCe-stimulated phosphorylation of phospholemman at Ser$^{63}$ and Ser$^{68}$ (274). Thus, the final common pathway for NO-stimulated NKA activation remains to be elucidated. Also, mechanisms underlying selective activation of NKA in glycolytic muscles will have to be determined. Interestingly, chronic inhibition of NOS reduced total NKA content in rat diaphragm (232), indicating NO signaling might regulate NKA not only acutely but also chronically. For more detailed discussion on regulation of NKA by NO see (284).

Regulation of NKA by purinergic signaling

Contracting muscle fibers release purines, such as ATP, ADP, and adenosine, into the extracellular space (106, 107, 203). An additional source of the purines is also the motor neuron at the neuromuscular junction (317). By activating purinergic and adenosine receptors purines may regulate different aspects of skeletal muscle function, including NKA activity (Table 4). Treatment of isolated rat soleus muscle with ATP increases NKA activity (41). Similarly, ATP and ADP increase its activity in isolated membranes from rat vastus lateralis and/or soleus
muscles (346). Increased NKA activity is paralleled by increased $V_{\text{max}}$ and affinity for Na$^+$ (reduced $K_{1/2,\text{Na}}$) (346). ATP- and ADP-induced increase in $V_{\text{max}}$ is likely mediated by several P2Y receptors, including P2Y$_1$, P2Y$_{2/4}$ and/or P2Y$_{13}$. While the exact mechanism by which activation of P2Y receptors increases $V_{\text{max}}$ has not been identified, inhibition of phospholipase C prevents ATP-induced NKA activation (41), thus strongly suggesting that phospholipase C provides the key link between P2Y receptors and NKA. ADP increases phosphorylation of phospholemman at Ser$^{68}$, which readily explains the increase in NKA affinity for Na$^+$. In contrast to ADP-induced increase in $V_{\text{max}}$, which requires P2Y receptors, increase in affinity for Na$^+$ does not seem to involve activation of these receptors (346).

Importantly, treatment with ATP and ADP blunts decline of force of isolated soleus muscle in the presence of high (10 mM) K$^+$ concentrations (41). ATP and ADP release from contracting muscles may therefore play a role in preventing or delaying fatigue during exercise. However, physiological role of purines in regulation of NKA is uncertain due to apparent species-specific differences of purine-mediated effects. Indeed, ADP apparently reduces NKA activity in muscle membranes from human vastus lateralis muscle (162), which suggests purines might act as negative regulators of NKA in human skeletal muscle. Differential expression of purinergic receptors is one possible reason for the apparent discrepancy between effects of purines in rat and human muscles (162). However, even in rat skeletal muscle purines might have a dual, stimulatory and suppressive, effect on NKA. Indeed, ADP increases phosphorylation of the α1-subunit as Ser$^{18}$ (346), which suggests that ADP might trigger its endocytosis. Clearly, the role of purines in regulation of NKA in skeletal muscle requires further characterization.
Oxidative stress and glutathionylation

Oxidative stress inhibits NKA in cardiac myocytes by promoting reversible glutathionylation of the α-subunit (280) as well as the β1-subunit (104). Glutathionylation of α- and β-subunits was detected in rat (154) and human (158) skeletal muscle (Table 4), but the β1-subunit appears to be particularly susceptible. Increase in glutathionylation of the β1-subunit reduces NKA activity, while a decrease in its glutathionylation has an opposite effect. Glutathionylation of the β1-subunit is stimulated by exercise and adrenergic signaling via the β2-adrenoceptors (158). Glutathionylation of NKA subunits has two important corollaries. First, basal glutathionylation of NKA subunits in rat and human skeletal muscle (154, 158) suggests that a significant fraction of NKA heterodimers, though expressed, might be unavailable for activation during exercise or upon stimulation with hormones. Notably, this would explain why NKA activity in electrically stimulated or Na⁺-loaded muscles has a plateau at 60-90% of maximal theoretical capacity (63, 250). Second, dynamic increase in glutathionylation of the β1-subunit during exercise and/or stimulation of β2 adrenoceptors suggests a mechanism by which exercise reduces NKA activity in skeletal muscle (158). Notably, N-acetylcysteine, an anti-oxidative agent, attenuates a reduction in NKA activity during prolonged cycling exercise (229). Taken together, current evidence suggests that oxidative stress may reduce NKA activity during prolonged exercise via glutathionylation.

Glutathionylation does not modify only NKA, but also other proteins involved in regulation of NKA. For instance, phospholemman can also be glutathionylated, which would tend to protect NKA from inactivation (32). Other notable targets of glutathionylation include PKA and PKC (272), which increase NKA activity by phosphorylating phospholemman. Inactivation of PKA and PKC would therefore tend to reduce NKA activity. On the other hand,
agonists of β2 adrenoceptors, which signal via PKA, increase glutathionylation of the β1-subunit (158). While physiological significance of most of these mechanisms will require independent confirmation in skeletal muscle, it is clear that relationship between oxidative stress, glutathionylation and NKA activity in skeletal muscle is complex. The final outcome therefore likely depends on constellation of several mechanisms that regulate NKA activity in parallel.

Regulation of NKA by pH

In resting skeletal muscle intracellular space is slightly more acidic (pH~7.2) than the interstitium (pH~7.4). Contracting skeletal muscles produces acid, thus reducing intracellular and interstitial pH. This reduction is especially marked in exhausting exercise, during which intracellular pH can drop from ~7.1-7.2 at rest to ~6.7-6.8 at exhaustion (18, 159). This pH reduction corresponds to almost a 3-fold increase in intracellular H⁺ concentration. Due to the subsequent H⁺ efflux via the Na⁺/H⁺ exchanger and H⁺/lactate symporter interstitial pH can also markedly decrease (151, 156). For instance, reductions from ~7.4 at rest to ~7.0 were measured during knee-extensor exercise (321). Reduction of pH is paralleled by accumulation of lactate in skeletal muscle, whose concentrations can increase up to 25-40 mmol/kg wet weight (18, 156) or ~60-100 mmol/kg dry weight (159).

Intracellular and extracellular pH can be reduced experimentally by exposing isolated skeletal muscle to acidic solutions, containing high concentrations of lactic acid or high concentrations of CO₂ (77, 182, 252). Under these conditions NKA activity remains unaltered, indicating that low pH or high lactate concentration do not directly regulate its activity. However, these approaches do not entirely reflect pH alterations during muscle contractions. Indeed, while exposure of isolated skeletal muscle to lactic acid or high CO₂ reduces intracellular
and extracellular pH, it almost completely abolishes transmembrane pH gradient (77, 182, 252). In contrast, pH gradient between the intracellular space and interstitium is increased in contracting skeletal muscle. If this situation is reproduced by exposing isolated skeletal muscle to sodium lactate rather than lactic acid, NKA is activated under low pH conditions (182). Activation of NKA under these conditions is secondary to increased Na$^+$ influx via Na$^+$/H$^+$ exchanger and/or Na$^+$/HCO$_3^-$ symporter. Indeed, inhibition of these transporters abolishes NKA activation by low pH (182). To summarize, low pH or high lactate concentrations do not regulate NKA directly. However, acidification of contracting muscle fibers leads to NKA activation indirectly via increased intracellular Na$^+$ concentrations.

A note on species, tissue, and fiber-type specificity of NKA regulation

Regulatory stimuli may have divergent effects on NKA in different tissues, species, and/or types of muscle fibers. This divergence is not surprising given the complex sequence of events which couple stimulus to its final downstream effect on NKA. For instance, a humoral factor first binds to a specific receptor, which transduces the stimulus by triggering intracellular signaling responses. These responses in turn modulate different aspects of NKA function, including its intrinsic activity, subcellular distribution, and gene expression. In continuation we provide three examples of divergent effects, which arise at the level of receptors, signaling pathways, and NKA.

Receptor diversity underlies activation or suppression of NKA activity by acetylcholine

In skeletal muscle low concentrations of acetylcholine or its analogue carbachol stimulate NKA $\alpha_2/\beta$ heterodimers via nAChR (48, 134, 186, 187), as described above (Regulation of NKA
by acetylcholine). In contrast, carbachol reduces NKA activity in cardiac sarcolemma (76). In cardiac sarcolemma inhibition of NKA was blocked by atropine, a muscarinic receptor antagonist, and pertussis toxin (76), which suggests involvement of cardiac muscarinic receptors. Activation of cardiac muscarinic receptors suppresses adenylate cyclase and the cAMP/PKA pathway. Suppression of cAMP/PKA pathway would tend to decrease phospholemman phosphorylation and thus decrease NKA activity in cardiac sarcolemma (80, 273). In summary, divergent effects of acetylcholine and/or carbachol in skeletal muscle and the heart can be explained by the involvement of different membrane receptors.

Signaling pathway diversity underlies AMPK-stimulated NKA endocytosis and exocytosis

Activation of AMPK stimulates NKA endocytosis in rat alveolar cells (123, 341), whereas it increases membrane abundance of NKA in rat L6 myotubes (24). Divergent result can be explained by intracellular signaling downstream of AMPK. Indeed, activation of AMPK by AICAR or hypoxia in rat alveolar epithelial cells activates atypical PKCζ (123), which would tend to increase phosphorylation of α-subunit Ser18 and thus its endocytosis (75). Conversely, AMPK activation by AICAR or A-769662 does not increase phosphorylation of PKCζ/λThr410/403 (24), indicating its activity remained unaltered. Furthermore, AMPK activation decreases phosphorylation of Ser18, as described above (Regulation of NKA by energy status: AMPK).

Target diversity underlies differential responsiveness of NKA to regulatory modifications

A major example of target diversity is the species-specific presence or absence of key phosphorylation sites in different isoforms of α-subunits (103). Functional consequences of these
differences are discussed in other part of this review and will not be repeated here. Recent
evidence suggests that isoforms of β-subunit may also importantly determine responsiveness of
NKA to regulatory modifications. For instance, while all major isoforms of NKA subunits can be
glutathionylated, the β1-subunit is apparently the most susceptible. In rats the β1-subunit is
expressed predominantly in oxidative muscles (110, 141, 152, 157, 183, 332, 337, 358),
suggesting glutathionylation might be more important for regulation of NKA in these muscles
than in glycolytic muscles. Consistent with this view, oxidized glutathione inhibits NKA activity
in muscle membranes from rat oxidative muscles more effectively than in membranes from rat
glycolytic muscles (154). In addition, treatment with dithiothreitol, a reducing agent which
should reduce basal glutathionylation, increases NKA $V_{max}$ only in muscle membranes from
oxidative muscles (154). In contrast to rat muscles, distribution of the β1-subunit is similar in
human oxidative and glycolytic fibers. Thus, glutathionylation is not expected to have
differential effects on NKA activity in different types of muscle fibers.

INTEGRATIVE REGULATION OF NKA: EXERCISE, NUTRITION, AND AGEING

Under physiological conditions various extrinsic and local stimuli do not exist in
isolation, but interact in complex ways to set NKA activity in skeletal muscle to meet systemic
and local needs for maintaining homeostasis. Furthermore, some stimuli may arise concurrently
from muscle fibers as well as extrinsic sources. For instance, purinergic signaling can be
activated by release of purines from motor neuron terminals (342) as well as from contracting
muscle fibers (135). Thus, NKA activity in skeletal muscle at any given moment represents an
integrated response to multiple regulatory pathways. This is especially true of exercise and
nutrition, which represent particularly complex stimuli for systems involved in maintaining
energy and ion homeostasis. Accordingly, exercise or nutrient intake set off a plethora of homeostatic responses, including those involved in acute and chronic regulation of NKA in skeletal muscle.

Regulation of NKA by exercise

Exercise regulates NKA acutely and chronically. An acute bout of exercise increases intrinsic activity of NKA (58) and induces NKA translocation to the sarcolemma (see Table 3). Increased NKA activity during exercise requires recruitment of α2/β heterodimers. Indeed, activation of α1/β heterodimers alone does not enable normal exercise performance (289). Activation of NKA is the final result of complex interactions of local alterations in contracting skeletal muscle as well as systemic adaptations that accompany exercise. For instance, elevated intracellular Na⁺ concentrations (58) and, probably, extracellular K⁺ concentrations (82) directly increase intrinsic NKA activity in contracting skeletal muscle. This increase can be further enhanced by exercise-induced phosphorylation of phospholemman at Ser⁶³, Ser⁶⁸, and/or Thr⁶⁹ (26, 328-330). Pattern of phospholemman phosphorylation apparently differs with intensity and/or type of exercise. Indeed, acute bout of one-legged cycling at moderate intensity increased phosphorylation of Ser⁶³ and Ser⁶⁸ (26), while acute cycling at high-intensity increased phosphorylation of Ser⁶⁸ and Thr⁶⁹ (328). During moderate intensity exercise phospholemman phosphorylation (Ser⁶⁸) was increased at 60 minutes, but not at 30 minutes (26). In contrast, phosphorylation of Ser⁶³ was increased already at 30 minutes (26). Thus, phosphorylation of phospholemman at different sites may play distinct roles depending on the amount as well as intensity of exercise. Notably, moderate intensity exercise increased phosphorylation of PKCα/βII Thr⁶³⁸/⁶⁴¹ (26), while the high-intensity exercise did not (328). Phosphorylation of
Ser\textsuperscript{63} is PKC-dependent (270, 345), which suggests that differences in exercise-induced
activation of PKC\textalpha/\textbeta II may explain why phosphorylation of Ser\textsuperscript{63} was increased only in
moderate-intensity exercise. Importantly, phosphorylation of phospholemman at different sites
alters kinetic characteristics of \textalpha/\textbeta heterodimers in distinct ways (33, 38). Thus, the pattern of
phospholemman phosphorylation in different types of exercise might be functionally important.

Phosphorylation of phospholemman during exercise is increased only in contracting
skeletal muscles (26), despite markedly increased plasma concentrations of epinephrine and
norepinephrine (4, 44, 128). This is true vice versa: neither electrically stimulated, nerve-evoked
contractions in vivo, nor fatiguing contractions of isolated skeletal muscles ex vivo increased
phosphorylation of phospholemman in mice (219). Failure to observe increased phosphorylation
of phospholemman during stimulated muscle contractions indirectly suggests that systemic
adaptations have a permissive effect on phosphorylation of phospholemman in contracting
skeletal muscles. Thus, while increased plasma concentrations of epinephrine and norepinephrine
during exercise are apparently not sufficient to stimulate phosphorylation of phospholemman in
resting muscles, they may selectively promote its phosphorylation in contracting skeletal muscle.
Selective effects of epinephrine in resting and contracting skeletal muscle are well established.
For instance, epinephrine significantly enhances glycogenolysis in contracting skeletal muscle
(101), although its effects on glycogenolysis in resting skeletal muscle are negligible (193).
However, although contractions seem to be required for exercise-induced phosphorylation of
phospholemman (26), a very recent study showed that acute hind limb suspension transiently
increases abundance of phospholemman as well as its phosphorylation at Ser\textsuperscript{63} and Ser\textsuperscript{68} (181).
Clearly, physical activity regulates phosphorylation of phospholemman in complex ways.
Exercise does not always induce phosphorylation of phospholemman in skeletal muscle 
(291, 330). Furthermore, recent evidence suggests that phosphorylation of phospholemman 
(Ser$^{63}$ or Ser$^{68}$) is not required for exercise-induced NKA activation (219). Thus, all types of 
exercise likely do not require phosphorylation of phospholemman for NKA activation in skeletal 
muscle. However, phospholemman may contribute to acute NKA regulation in other ways. For 
instance, exercise translocates phospholemman to muscle membrane, which increases 
association between phospholemman and α1-subunits (291). Phospholemman may therefore 
modulate NKA kinetic properties without alteration in its phosphorylation status. Furthermore, 
under conditions of oxidative stress or catecholamine stimulation, which both tend to increase 
glutathionylation of β1-subunits (158), phospholemman may protect NKA from inactivation by 
serving as an alternative target for glutathionylation (32).

Chronic level of physical activity determines muscle content of NKA (58) as well as 
phospholemman (113, 293, 328). In general, exercise training increases muscle NKA content 
(96, 119, 231), while physical inactivity reduces it (37, 83). Similarly, some types of exercise 
training increase phospholemman expression in skeletal muscle (328), while physical inactivity 
reduces it (37). NKA content is determined by the amount of physical activity as well as by its 
intensity; however, the correlation between these parameters is apparently complex. For instance, 
increased training intensity may lead to increased content of the α2-subunit despite reduced total 
amount of training (17). Training-induced increases in NKA content are associated with 
increased NKA transport function, as indicated by slower increase in the interstitial K$^{+}$ 
concentrations during muscle contractions, and improved exercise performance (249).

Effects of training on NKA expression are isoform-specific. For instance, exercise 
training increases expression of α1-, α2-, and/or β1-subunits (26, 249, 354). Conversely,
complete spinal cord injury, as an extreme example of chronic physical inactivity, reduces muscle expression of α1-, α2-, and β1-subunits, but not α3-, β2-, and β3-subunits (37). Expression of the α2-subunit, but not other subunits, is also markedly reduced in vastus lateralis muscle after severe knee-injury (277). In contrast, incomplete spinal cord injury is not associated with reduced expression of NKA subunits (37). Similarly, osteoarthritis does not produce alterations in [3H]ouabain binding site content (278), indicating that less extreme cases of physical inactivity are not associated with pronounced reductions in muscle NKA content. Expression of phospholemman is also reduced by complete spinal cord injury (37). In contrast, expression of FXYD5 is increased in these patients (37), thus strongly suggesting physical activity suppresses FXYD5 expression.

Exercise training may alter phospholemman abundance, phosphorylation status, or its association with NKA. However, effects of training on phospholemman have not been consistent, possibly due to different training protocols. For instance, while some studies showed increased phospholemman abundance (293, 328), others showed no change in response to exercise training (26, 113). Furthermore, in some studies training increased basal phosphorylation of phospholemman (Ser\(^{68}\)) (328), but did not alter it in other studies (26). Finally, in senescent rats training tends to increase association between phospholemman and the α2-subunit and to decrease its association with the α1-subunit (293). This result indicates that training may alter association between phospholemman and NKA in isoform-specific manner.

One of the challenges for future research will be to uncover molecular mechanisms that link the level of physical activity to NKA, phospholemman, and FXYD5 expression. Transcript as well as protein levels of NKA subunits in response to different types of exercise have been thoroughly examined (12, 118, 121, 240, 242), but the downstream signaling pathways and
effectors that induce gene expression are still largely obscure. Alterations in ion concentrations
represent one possible underlying mechanism. Indeed, increased extracellular $K^+$ or intracellular
$Ca^{2+}$ concentrations, which mimic conditions in contracting muscles, alter mRNA expression of
NKA subunits in isolated skeletal muscle (239). Interestingly, exercise-induced neurohumoral
responses, such as epinephrine and norepinephrine secretion, do not seem to enhance
transcriptional responses in exercising skeletal muscle (259). Also, chronic infusions of
terbutaline, a $\beta_2$-adrenergic agonist, do not alter muscle NKA content in rat muscle (292).
Available evidence therefore suggests that local responses in contracting skeletal muscle fibers
are the key driver of transcriptional responses. However, transcriptional responses to exercise do
not always reflect subsequent alterations in protein levels of NKA subunits (12, 242, 243). Regula-
tion of translation and/or protein stability by exercise is therefore also important.

Regulation of NKA by nutrition

Nutritional factors regulate NKA acutely and chronically. These regulatory effects are
exerted indirectly via hormones, such as insulin, or directly by nutrients and/or their metabolites.
A major example of acute regulation of NKA by nutrients is postprandial secretion of insulin,
which stimulates $K^+$ uptake in skeletal muscle (225, 324). Another interesting example is
ingestion of glucose or other carbohydrates during exercise. Notably, ingestion of glucose during
prolonged exercise increases time to fatigue as well as maximal in vitro NKA activity in skeletal
muscle (120). One possible explanation for this increase is glucose-induced insulin secretion.
Indeed, stimulation of NKA by insulin protects against force decline of contracting skeletal
muscle exposed to high $K^+$ concentrations ex vivo (62, 69). Notably, insulin blunts force decline
to a similar extent in the presence or absence of 5 mM glucose, which demonstrates that insulin-
stimulated glucose uptake is not required for this effect (69, 349). Activation of NKA by insulin is probably not of major physiological importance if exercise is performed in the postabsorptive state since insulin concentrations are low and decline progressively under these conditions (3). In contrast, insulin concentrations increase at least transiently if high glucose doses are ingested just before and/or during exercise (120, 149).

Whether this increase is sufficient to explain NKA activation is unclear for three reasons. First, although insulin concentrations are higher after glucose ingestion than in controls they are still relatively low and decline down to or even below fasting insulin levels as exercise progresses (100, 120, 149). Second, glucose ingestion lowers epinephrine levels (120), which would tend to reduce epinephrine-stimulated NKA activation. Third, insulin infusion in rats does not improve performance of electrically stimulated plantaris muscle if normoglycemia is maintained (170). In sum, insulin plays a major role in acute regulation of NKA in resting skeletal muscle during the postprandial period, but is most likely not important for acute regulation of NKA in contracting skeletal muscle. Finally, it has to be noted that insulin secretion is likely not the only mechanism by which glucose alters NKA activity in skeletal muscle. Indeed, acute exposure to high glucose concentrations (25 mM) reduces maximal NKA activity in isolated soleus muscle (50). This reduction is mediated by activation of conventional and/or novel PKCs (50). Similarly, high glucose inhibits NKA activity in isolated pancreatic islets (268).

Nutritional factors also exert chronic effects on skeletal muscle NKA content and function. One key nutritional factor is the total amount of ingested macronutrients (fats, protein, carbohydrates). Indeed, starvation reduces NKA content in skeletal muscle (176). Starvation reduces concentrations of T3 and insulin (221), which can directly explain why NKA content in
skeletal muscle is reduced (176). An additional explanation for reduced NKA content in
starvation might be provided by proteolysis and loss of muscle tissue. Another important
nutritional factor is the relative amount of individual macronutrients, such as fats. For instance,
while physical inactivity reduces expression and activity of NKA in skeletal muscle (37, 58,
113), high-fat diet in sedentary rats further reduces NKA content and activity, impairs insulin-
stimulated translocation of α2-subunits, and increases phospholemman expression (113). Thus, a
sedentary lifestyle, in combination with unhealthy diet, might synergistically disrupt NKA
function in skeletal muscle. Importantly, exercise training can normalize these parameters
despite the high-fat diet (113). Clearly, lifestyle factors, such as nutrition and exercise, interact in
regulation of skeletal muscle NKA content and function. Interestingly, some evidence suggests
that polymorphisms in the α2-subunit may determine utilization of macronutrients, as assessed
by respiratory quotient (171), response to exercise training (290), as well as susceptibility to
adverse effects of overfeeding (339). Thus, it seems that bidirectional interactions exist between
NKA and nutritional factors.

In addition to macronutrients, K⁺ intake is also important for regulation of NKA in
skeletal muscle. Dietary K⁺ overload up-regulates muscle NKA (58, 225). Conversely, chronic
K⁺ deficiency in rats reduces NKA content and activity in skeletal muscle (262). Consistent with
reduced NKA activity, skeletal muscle K⁺ content is also reduced (262). In contrast to skeletal
muscle, K⁺ content in most other tissues remains virtually constant over several weeks of K⁺
deficiency. These data demonstrate not only a major effect of K⁺ availability on skeletal muscle
NKA function, but also highlight they key role played by skeletal muscle in long-term K⁺
homeostasis. Indeed, alterations in NKA activity enable skeletal muscle to take up or release K⁺,
thus buffering fluctuations in extracellular K⁺ concentrations (225). While underlying
mechanisms remain to be determined, regulation of NKA content by $K^+$ implies existence of
humoral factors and/or a local $K^+$-sensing mechanisms that regulate NKA activity in response to
$K^+$ availability.

Effects of ageing

Ageing is characterized by loss of skeletal muscle mass and strength (age-related
sarcopenia) with the attendant alterations in protein expression, including NKA. In various
species, including rat, mouse, and guinea pig, ageing reduces NKA content in skeletal muscle,
thus leading to reduced capacity for ion pumping (58, 177). Interstitial $K^+$ concentrations in
contracting muscles increase more in aged rats than in young adult rats, demonstrating that
reduced NKA pumping capacity is functionally significant (205). In humans, results have been
less consistent. Studies initially demonstrated relatively small and statistically non-significant
reductions in NKA content with ageing (178, 261), or, recently, unchanged NKA content with
age (230). However, a recent study which compared middle-aged and old subjects, has suggested
that reductions of NKA content may occur relatively late in life, possibly not before 70 years of
age. Indeed, in this study NKA content in vastus lateralis muscle of subjects in their 70s was
~26% lower than in vastus lateralis muscle of subjects in their 50s and 60s (278). Importantly,
this might explain small and non-significant trends in earlier studies, some of which included
only people younger than 70 years.

Recent evidence also suggests that ageing alters expression pattern of NKA subunits. In
type I fibers, expression of $\alpha_1$- and $\beta_3$-subunits is increased, while expression of the $\beta_2$-subunit
is decreased. Similarly, in type II fibers, expression of the $\beta_3$-subunit is increased, while
expression of $\alpha_3$- and $\beta_2$-subunits is decreased (353). Interestingly, broadly similar trends were
observed in ageing rats. Indeed, expression of α1- and β3-subunits was increased in skeletal muscles of aged rats, while expression of the β2-subunit was decreased (248, 358). In contrast, an earlier human study showed that the α1-subunit remains unaltered, while the α2-subunit declines with ageing (230); however, in light of recent evidence demonstrating that GAPDH content in skeletal muscle is reduced with ageing (353), these data might need to be reinterpreted. Indeed, without using GAPDH as a loading control, expression of the α2-subunit is apparently similar between young and aged subjects (353).

Taken together, current evidence suggests that muscle NKA content in humans declines late in life, but to validate this notion future studies will need to include older age groups. Another challenge for future research will be to uncover mechanisms that lead to alterations in total NKA content and expression pattern of NKA subunits. Notably, evidence from rats suggest that age-related alterations are not simply a reflection of physical inactivity (248), thus indicating the existence of other ageing-related mechanisms. For instance, ageing affects type II fibers more than type I fibers (202), suggesting one mechanism why the β2-subunit, which is selectively expressed in type II fibers, is consistently reduced in aged skeletal muscle. Finally, it will be important to determine whether and how age-dependent alterations in NKA expression patterns affect skeletal muscle function.

**CONCLUSIONS AND PERSPECTIVES FOR FUTURE RESEARCH**

Recent research has added important new insights into NKA regulation in skeletal muscle. Notably, the list of mediators regulating NKA in skeletal muscle has expanded to include acetylcholine (48, 134, 181, 186), purines (162, 346), aldosterone (281), and most recently extracellular K⁺ (82), and NO (153). Novel mediators highlight the complexity of NKA
regulation in skeletal muscle and open important new avenues for future research. One of the key challenges will be to establish how this plethora of mediators acts in concert to tune NKA activity to the needs of whole-body homeostasis as well as to the needs of skeletal muscle. Another challenge will be to dissect molecular mechanisms that link these mediators to alterations of NKA function.

Although missing links remain, our knowledge of basic molecular mechanisms underpinning regulation of NKA in skeletal muscle has expanded markedly. For instance, phospholemman has been firmly established as a major NKA regulator, which responds to various physiological stimuli, including exercise (26, 291, 328-330), nutrition (113), and AMPK activation (145). Another important development has been identification of signaling pathways that link physiological stimuli, such as insulin and muscle contractions, to activation of NKA and/or its translocation to plasma membrane (5, 7, 24, 26, 330). A challenge for the future will be to uncover the gaps in the signaling network linking various stimuli, NKA, and phospholemman. For instance, AMPK increases activity and abundance $\alpha_1/\beta$ heterodimers in cultured myotubes (24), but the effectors that link AMPK activation to NKA stimulation remain to be uncovered. Similarly, molecular mechanisms that underpin selective NKA activation by NO in glycolytic muscles will have to be examined in more detail (153, 284).

Finally, studies during the last 15 years have clearly demonstrated that NKA $\alpha_1/\beta$ and $\alpha_2/\beta$ heterodimers play distinct physiological roles in skeletal muscle (48, 133, 134, 288, 289). Notably, a very recent study highlighted this distinction by demonstrating that unlike $\alpha_1/\beta$ heterodimers, activity of $\alpha_2/\beta$ heterodimers can be regulated by physiological alterations in K$^+$ concentrations (82). Similarly, skeletal muscle $\alpha/\beta_1$ and $\alpha/\beta_2$ heterodimers have been shown to differ in their enzymatic properties (183) and susceptibility to regulatory modifications like...
glutathionylation (154, 158). A challenge for the future will be to determine whether and how these differences affect muscle function. Last but not least, it will be important to uncover molecular mechanisms by which skeletal muscle regulates expression and assembly of α/β heterodimers. This is a key question, given their distinct functional characteristics. While regulatory mechanisms in skeletal are unknown at present, research in other tissues hints at the possibility that epigenetics might provide some of the answers.

We can conclude that despite its long history, NKA regulation in skeletal muscle remains a vital field of research, which will continue to provide answers to fundamental questions in skeletal muscle physiology. Ageing of the endocrine system may provide another mechanism. Furthermore, elucidation of novel regulatory mechanisms will open new avenues to modulate NKA and skeletal muscle function under pathophysiological conditions.

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**FIGURE LEGENDS**

**Figure 1. Regulation of NKA by hormones.** Insulin increases abundance of NKA in plasma membrane by activating ERK1/2, which phosphorylates NKA α-subunit and thereby stimulates its exocytosis or suppresses its endocytosis. Insulin and catecholamines (epinephrine and norepinephrine) activate PKC and PKA, respectively, which stimulates phosphorylation of phospholemman (FXYD1), and thereby increase intrinsic activity of NKA. Amylin, which also acts via the PKA pathway, may activate NKA by a similar mechanism. Amylin receptors, which comprise a calcitonin receptor (CTR) and an accessory protein RAMP, can also be activated by the calcitonin gene-related peptide (CGRP) and calcitonin (not shown). Membrane receptor and intracellular signaling pathways linking C-peptide to activation of NKA have not been established, but might involve PKCs and/or ERK1/2. T₃, cortisol, and aldosterone act on nuclear receptors and alter gene expression. Effects of T₃ on ion transport are not depicted. IRS: insulin receptor substrate, PI 3-kinase: phosphoinositide 3-kinase, PDK1: phosphoinositide-dependent protein kinase 1, TR: thyroid hormone receptor, GR: glucocorticoid receptor, MR: mineralocorticoid receptor, ATP1A: gene for NKA α-subunit, ATP1B: gene for NKA β subunit.

**Figure 2. Regulation of NKA by acetylcholine at the neuromuscular junction.** Low concentrations of acetylcholine (ACh) activate NKA α2 heterodimers via nicotinic acetylcholine receptors (nAChR) and hyperpolarize the junctional membrane. Hyperpolarization increases the number of closed voltage-gated Na⁺ channels that can be activated by subsequent depolarization and thereby enhances efficiency of the neuromuscular transmission. During active neuromuscular transmission high acetylcholine concentrations open nAChR channels, which
increases conductance for Na\(^+\) and K\(^+\), depolarizes junctional sarcolemma and finally triggers action potential by opening voltage-gated Na\(^+\) channels. As well as acetylcholine, motor neuron secretes ATP, calcitonin-gene related peptide (CGRP), and heparin-sulfate proteoglycan agrin. Inhibition of NKA by high concentrations of acetylcholine is not presented in this figure. Receptors for purines, CGRP, and agrin are not depicted. AChE: acetylcholine esterase.

**Figure 3. Regulation of NKA by local stimuli.** Generation of action potentials trigger muscle contractions and increase intracellular Na\(^+\) ([Na\(^+\)]\(_i\)) and extracellular K\(^+\) concentrations ([K\(^+\)]\(_o\)), which directly stimulates intrinsic activity of NKA. Reduced intracellular pH (pH\(_i\)) stimulates NKA indirectly by increasing net Na\(^+\) influx (only Na\(^+\)/H\(^+\) exchanger is depicted). Contractions increase abundance of NKA in plasma membrane by stimulating exocytosis or by suppressing endocytosis of NKA. Underlying molecular mechanisms probably involve activation of PKCs, ERK1/2, and/or AMPK. Activation of AMPK may stimulate NKA translocation to plasma membrane via protein phosphatase 2A (PP2A). In addition, contraction-induced activation of PKC may increase intrinsic NKA activity by stimulating phosphorylation of phospholemman. Nitric oxide (NO) and activation of PKG increase NKA activity in glycolytic fibers through an unknown mechanism. Purines, such as ATP and ADP, which are released during muscle contractions, may regulate NKA via purinergic receptors. Oxidative stress stimulates glutathionylation and NKA inactivation. Activation of β2 adrenergic receptors may enhance glutathionylation. ATP1A: gene for NKA α-subunit, ATP1B: gene for NKA β-subunit.
<table>
<thead>
<tr>
<th>Species, Muscle</th>
<th>Stimulus, procedure, or condition</th>
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<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, diaphragm</td>
<td>Insulin, PMA</td>
<td>Increased phosphorylation of PLM.</td>
<td>(344)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>Insulin</td>
<td>Increased phosphorylation of PLM Ser^{63} and Ser^{64}.</td>
<td>(345)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>Epinephrine, isoproterenol (ex vivo)</td>
<td>Increased phosphorylation of PLM Ser^{68}.</td>
<td>(345)</td>
</tr>
<tr>
<td>Mouse, mixed hind limb muscles</td>
<td>cAMP</td>
<td>Increased phosphorylation of PLM Ser^{68} and decreased $K_{i,2,Na}$.</td>
<td>(145)</td>
</tr>
<tr>
<td>Mouse, hind limb muscles</td>
<td>AICAR (in vivo 27 d)</td>
<td>Decreased total PLM expression, increases phosphorylation of PLM Ser^{68}. In AMPK kinase-dead mice total PLM was increased.</td>
<td>(145)</td>
</tr>
<tr>
<td>Rat, vastus lateralis and soleus</td>
<td>ADP (ex vivo)</td>
<td>Increased phosphorylation of PLM Ser^{68}.</td>
<td>(346)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>Nicotine (in vivo 21-31 d)</td>
<td>Increased phosphorylation of PKCα/βII Thr^{638/641}, PKCδ Thr^{605}, and PLM Ser^{68}.</td>
<td>(48)</td>
</tr>
<tr>
<td>Rat, EDL, gastrocnemius, soleus</td>
<td>SpNONOate</td>
<td>Did not alter phosphorylation of PLM Ser^{68}.</td>
<td>(153)</td>
</tr>
<tr>
<td>Mouse, EDL and soleus from (WT and PKCα KO)</td>
<td>Electrical stimulation</td>
<td>Increased phosphorylation of PLM Ser^{68} in WT soleus, decreases phosphorylation of PLM Ser^{68} in WT EDL. Phosphorylation of PLM Ser^{68} is markedly reduced and unresponsive to electrical stimulation in soleus and EDL from PKCα KO.</td>
<td>(330)</td>
</tr>
<tr>
<td>Rat, gastrocnemius, EDL</td>
<td>Ageing</td>
<td>Did not alter PLM expression. Increased association between PLM and α1 subunit.</td>
<td>(293)</td>
</tr>
<tr>
<td>Rat, gastrocnemius, EDL</td>
<td>Exercise training</td>
<td>Increased PLM expression in senescent rats. Increased association between PLM and α2-subunit.</td>
<td>(293)</td>
</tr>
<tr>
<td>Rat, gastrocnemius</td>
<td>HFD</td>
<td>Increased PLM abundance.</td>
<td>(113)</td>
</tr>
<tr>
<td>Rat, gastrocnemius</td>
<td>Exercise training</td>
<td>Increased membrane abundance of PLM and increased association between PLM and the α1-subunit. Did not alter phosphorylation of PLM Ser^{68}.</td>
<td>(291)</td>
</tr>
<tr>
<td>Rat, soleus, diaphragm</td>
<td>Exercise (acute)</td>
<td>Increased PLM expression and phosphorylation of PLM Ser^{63} and Ser^{68}.</td>
<td>(181)</td>
</tr>
<tr>
<td>Rat, soleus, diaphragm</td>
<td>Hind limb suspension (acute)</td>
<td>Tended to increase association between PLM and the α2-subunit.</td>
<td></td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise training</td>
<td>Increased phosphorylation of PKCαβ/IIII Thr^{638/641} in parallel with phosphorylation of PLM Ser^{63} and Ser^{68}.</td>
<td>(327)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise (acute)</td>
<td>Increased phosphorylation of PKCαβ/IIII Thr^{638/641} in parallel with phosphorylation of PLM Ser^{63} and Ser^{68}.</td>
<td>(26)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise training</td>
<td>Did not alter total PLM expression or PLM phosphorylation.</td>
<td>(26)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise (acute)</td>
<td>Increased phosphorylation of PLM Ser^{63} and Ser^{64}.</td>
<td>(330)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Spinal cord injury</td>
<td>Reduced total PLM expression and increased phosphorylation of PLM Ser^{68}.</td>
<td>(37)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise (acute)</td>
<td>Increased phosphorylation of PLM Ser^{63} in type II muscle fibers.</td>
<td>(329)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Terbutaline, exercise</td>
<td>Terbutaline tended to increase phosphorylation of PLM Ser^{68} in non-fatigued muscle. Exercise to fatigue increased phosphorylation of PLM Ser^{68}.</td>
<td>(138)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise (acute)</td>
<td>Did not increase phosphorylation of PKCαβ/IIII Thr^{638/641}</td>
<td>(328)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise training</td>
<td>Increased expression of total PLM as well as basal and exercise-induced phosphorylation of PLM Ser^{68} (but not Ser^{63} and Thr^{69}).</td>
<td>(328)</td>
</tr>
</tbody>
</table>

**Table 1. Phosphorylation and expression of phospholemman in skeletal muscle.** Abbreviations:
- AICAR: 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AMPK activator), IP: immunoprecipitation, HFD: high-fat diet, PKC: protein kinase C, PKCα KO: PKCα knock-out mice, PLM: phospholemman (FXYD1), PMA: phorbol 12-myristate 13-acetate (PKC activator), SpNONOate: spermine NONOate (NO donor).
<table>
<thead>
<tr>
<th>Species, Muscle/Cells</th>
<th>Stimulus, procedure, or condition</th>
<th>Major findings</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, soleus muscle</td>
<td>Insulin (ex vivo)</td>
<td>Increased Ser, Thr, and Tyr phosphorylation of α-subunit. Phosphorylation of Ser and Thr was, in part, dependent on atypical PKCs. Phosphorylation of Tyr was PKC-independent.</td>
<td>(50)</td>
</tr>
<tr>
<td>Rat, soleus muscle</td>
<td>PMA (ex vivo)</td>
<td>Increased Ser and Thr phosphorylation of α-subunit via conventional or novel PKs.</td>
<td>(50)</td>
</tr>
<tr>
<td>Rat, soleus muscle</td>
<td>High glucose (ex vivo)</td>
<td>Increased Ser and Thr phosphorylation of α-subunit via conventional or novel PKCs.</td>
<td>(50)</td>
</tr>
<tr>
<td>Rat, unspecified</td>
<td>c-Src (in vitro)</td>
<td>Increased phosphorylation of α-subunit in isolated muscle membranes.</td>
<td>(6)</td>
</tr>
<tr>
<td>Rat, soleus muscle</td>
<td>Electrical stimulation (in vivo)</td>
<td>Increased Tyr phosphorylation of α2-subunit, but did not alter Tyr phosphorylation of α1-subunit.</td>
<td>(303)</td>
</tr>
<tr>
<td>Rat, red and white gastrocnemius</td>
<td>Ageing</td>
<td>Decreased phosphorylation of α1-subunit (Ser18) in red gastrocnemius. Phosphorylation pattern differed between fiber types.</td>
<td>(359)</td>
</tr>
<tr>
<td>Rat, vastus lateralis or soleus</td>
<td>ADP (ex vivo)</td>
<td>Increased phosphorylation of PLM Ser68 and α1-subunit (Ser18).</td>
<td>(346)</td>
</tr>
<tr>
<td>Rat, cultured L6 myotubes</td>
<td>AICAR</td>
<td>Decreased phosphorylation of α1-subunit (Ser18).</td>
<td>(24)</td>
</tr>
<tr>
<td>Human, cultured myotubes</td>
<td>Insulin</td>
<td>Increased Ser and Thr phosphorylation of α-subunit via atypical PKCs and ERK1/2. Increased phosphorylation of Thr-Pro motif in α1- and α2-subunits via atypical PKCs and ERK1/2. Increased Tyr phosphorylation of α-subunit independent of ERK1/2.</td>
<td>(5)</td>
</tr>
<tr>
<td>Human, cultured myotubes</td>
<td>Ouabain</td>
<td>Increased Tyr phosphorylation of α1- and α2-subunits (via Src). Increased phosphorylation of Thr-Pro motif of α1- and α2-subunits via ERK1/2.</td>
<td>(179)</td>
</tr>
</tbody>
</table>

**Table 2. Phosphorylation of NKA in skeletal muscle.** Abbreviations: AICAR: 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AMPK activator), ERK1/2: extracellular signal-regulated kinase 1/2 (p44/42 MAPK), IP: immunoprecipitation, MEK1/2: kinase upstream of ERK1/2, ³²P release: hydrolysis of [γ³²P]ATP, PKC: protein kinase C, PLM: phospholemman, PMA: phorbol 12-myristate 13-acetate (PKC activator), c-Src: non-receptor tyrosine kinase c-Src, Thr-Pro motif: the threonine-proline.
<table>
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<th>Major findings</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, hind limb muscles</td>
<td>Insulin (in vivo)</td>
<td>Increased abundance of α2- and β1-subunits in plasma membrane (WB).</td>
<td>(140)</td>
</tr>
<tr>
<td>Rat, soleus, gastrocnemius, quadriceps</td>
<td>Insulin (in vivo)</td>
<td>Increased abundance of α2-subunits in plasma membrane</td>
<td>(220)</td>
</tr>
<tr>
<td>Rat, hind limb red and white muscles</td>
<td>Insulin (in vivo)</td>
<td>Increased NKA activity (3-0-MFPase) and abundance of α2- and β1-subunits in plasma membrane (WB)</td>
<td>(198)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Insulin (ex vivo)</td>
<td>Increased NKA activity (32P release) and abundance of the α2-subunit (WB) in plasma membrane.</td>
<td>(50)</td>
</tr>
<tr>
<td>Rat, hind limb muscles</td>
<td>Insulin (in vivo)</td>
<td>Increased membrane abundance of the α2-subunit (WB).</td>
<td>(7)</td>
</tr>
<tr>
<td>Rat, epitrochlearis</td>
<td>Insulin (ex vivo)</td>
<td>Increased membrane abundance of α1- and α2-subunits (biotinylation, WB).</td>
<td>(7)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Insulin (ex vivo)</td>
<td>Increased NKA activity (32P release) and abundance of the α2-subunit (WB).</td>
<td>(228)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>HFD + Insulin (ex vivo)</td>
<td>Increased membrane abundance of α1- and α2-subunits (surface biotinylation, WB).</td>
<td>(113)</td>
</tr>
<tr>
<td>Human, cultured myotubes</td>
<td>Insulin (in vitro)</td>
<td>Increased membrane abundance of α1- and α2-subunits (WB).</td>
<td>(7)</td>
</tr>
<tr>
<td>Human, cultured myotubes</td>
<td>Insulin (in vitro)</td>
<td>Increased NKA activity (32P release) (surface binding), while [3H]ouabain binding remained unaltered.</td>
<td>(5)</td>
</tr>
<tr>
<td>Rat, red and white hind limb muscles</td>
<td>Treadmill running</td>
<td>Increased abundance of α1- and α2-subunits in plasma membranes. Trend towards increase in β1-and β2-subunits in plasma membranes of red and white muscles, respectively.</td>
<td>(337)</td>
</tr>
<tr>
<td>Rat, oxidative and glycolytic hind limb muscles</td>
<td>Treadmill running</td>
<td>Increased membrane abundance of α1-, α2-, and β2-subunits in giant sarcolemmal vesicles (WB). Increased the β1-subunit only in oxidative muscles.</td>
<td>(157)</td>
</tr>
<tr>
<td>Rat, mixed hind limb muscles</td>
<td>Treadmill running</td>
<td>Increased [3H]ouabain binding in sarcolemmal giant vesicles.</td>
<td>(157)</td>
</tr>
<tr>
<td>Rat, soleus, EDL, soleus</td>
<td>Treadmill running</td>
<td>Did not increase ex vivo in vivo [3H]ouabain binding.</td>
<td>(241)</td>
</tr>
<tr>
<td>Rat, red gastrocnemius, soles, EDL, white vastus lateralis</td>
<td>Treadmill running</td>
<td>Increased sarcolemmal NKA V_{max} and K_{1/2,Na} in soleus, decreased K_{1/2,Na} in glycolytic muscles (3P release), increased sarcolemmal abundance of α2 subunit in glycolytic muscles, and PLM in glycolytic and oxidative muscles (WB). Increased abundance of α1-, α2-, and β1-subunits in giant sarcolemmal vesicles (WB).</td>
<td>(152)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Electrical stimulation (ex vivo)</td>
<td>Increased NKA V_{max} (3-O-MFPase) and abundance of α1- and α2-subunits (WB) in sarcolemmal/particulate membrane fraction.</td>
<td>(303)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Electrical stimulation (in vivo)</td>
<td>Increased NKA V_{max} (3P release) and abundance of α subunits and PLM in sarcolemmal giant vesicles.</td>
<td>(291)</td>
</tr>
<tr>
<td>Rat, mixed hind limb muscles</td>
<td>Treadmill running</td>
<td>Increased [3H]ouabain binding at 16-18°C but not at 30°C.</td>
<td>(185)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Electrical stimulation</td>
<td>Increased surface abundance of α2 subunit in soleus muscle (surface biotinylation, WB).</td>
<td>(25)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Electrical stimulation</td>
<td>Increased α2 abundance (WB) in outer membrane fraction and sarcolemmal giant vesicles. Increased NKA activity (3-O-MFPase) in sarcolemmal giant vesicles.</td>
<td>(185)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>One-legged knee extensor exercise</td>
<td>Increased abundance of α2- and β-subunits in giant sarcolemmal vesicles (WB).</td>
<td>(160)</td>
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**Table 3. Insulin- and contraction-induced translocation of NKA in skeletal muscle.** Abbreviations:

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<tbody>
<tr>
<td>Rat, soleus, EDL</td>
<td>Adrenalectomy</td>
<td>Did not alter [3H]ouabain binding significantly.</td>
<td>(88)</td>
</tr>
<tr>
<td>Rat, soleus, EDL, gastrocnemius, diaphragm</td>
<td>Aldosterone (chronic, in vivo)</td>
<td>Reduced [3H]ouabain binding, but this reduction might have been caused by the uncorrected aldosterone-induced hypokalemia.</td>
<td>(88)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Aldosterone (adrenalectomy)</td>
<td>NKA activity (3-O-MFase) and expression of α2- and β1-subunits (PCR, WB) were higher in patients with aldosterone-secreting adenoma than in healthy controls. Removal of adenoma reduced NKA activity (3-O-MFase) and expression of α2- and β1-subunits (PCR, WB). Hypokalemia was corrected before surgery.</td>
<td>(281)</td>
</tr>
<tr>
<td>Mice (α2**, WT), EDL</td>
<td>Electrical stimulation (ex vivo)</td>
<td>After electrical stimulation α2** EDL transported more 86Rb+ than WT EDL.</td>
<td>(287)</td>
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<tr>
<td>Human, cultured myotubes</td>
<td>Ouabain</td>
<td>Increased basal and insulin-stimulated glycogen production via NKA receptor complex and Src kinase.</td>
<td>(179)</td>
</tr>
<tr>
<td>Rabbit, gastrocnemius</td>
<td>ACh (ex vivo)</td>
<td>Inhibited NKA activity (P production in innervated (1-5 mM ACh) and denervated (0.2-3 mM ACh) muscles.</td>
<td>(309)</td>
</tr>
<tr>
<td>Mouse, gastrocnemius, diaphragm</td>
<td>ACh (ex vivo)</td>
<td>Increased NKA activity in gastrocnemius muscle and hyperpolarized diaphragm muscle fibers.</td>
<td>(85)</td>
</tr>
<tr>
<td>Mouse, diaphragm</td>
<td>ACh (ex vivo)</td>
<td>Hyperpolarized the endplate zone. Ouabain abolished surplus hyperpolarization of the endplate zone.</td>
<td>(254)</td>
</tr>
<tr>
<td>Rat, diaphragm muscle fibers</td>
<td>ACh (ex vivo)</td>
<td>Hyperpolarized diaphragm muscle fibers and increased contribution of the α2-subunit to membrane potential (RPM). Contribution of the α1-subunit was unaltered by ACh.</td>
<td>(187)</td>
</tr>
<tr>
<td>Rat, diaphragm muscle fibers</td>
<td>ACh (ex vivo)</td>
<td>Hyperpolarized sarcolemma by activating α2/β heterodimers (RPM) via nAChR.</td>
<td>(186)</td>
</tr>
<tr>
<td>Rat, diaphragm muscle fibers</td>
<td>ACh (ex vivo)</td>
<td>Activated NKA α2 heterodimers by promoting its interaction with nAChR in non-conducting, desensitized state.</td>
<td>(134)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>Nicotine (in vivo, chronic)</td>
<td>Decreased membrane abundance of the α2-subunit (WB), but increased its transport activity (RPM), possibly by activating PKC and increasing PLM phosphorylation.</td>
<td>(48)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>Nicotine (ex vivo)</td>
<td>Hyperpolarized junctional and extra-junctional membrane. Ouabain blocked these effects.</td>
<td>(181)</td>
</tr>
<tr>
<td>Rat, tibialis, gastrocnemius, quadriceps, mixed hindlimb muscles</td>
<td>AICAR (in vivo)</td>
<td>Reduced plasma K⁺ concentrations, but did not alter NKA activity (32Pi release) in gastrocnemius homogenates or abundance of the α2-subunit (WB) in plasma membranes from mixed muscles.</td>
<td>(360)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>AICAR (in vivo)</td>
<td>Reduced abundance of the α2-subunit in sarcolemmal giant vesicles.</td>
<td>(185)</td>
</tr>
<tr>
<td>Mouse, hind limb muscles</td>
<td>AICAR (in vivo, chronic)</td>
<td>Decreased PLM expression, increased relative PLM phosphorylation (Ser68), increased K_{Na,2}, (Biomol Green).</td>
<td>(145)</td>
</tr>
<tr>
<td>Rat, L6 myotubes</td>
<td>A-769662</td>
<td>Reduced membrane abundance of the α1-subunit via an AMP-independent mechanism. Inhibits NKA directly.</td>
<td>(23)</td>
</tr>
<tr>
<td>Rat, L6 myotubes</td>
<td>AICAR</td>
<td>Increased membrane abundance of the α1-subunit and transport activity of NKA (86Rb⁺ uptake) via AMPK activation.</td>
<td>(24)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>SNP (ex vivo)</td>
<td>Suppressed early postdenervation depolarization. This effect was blocked by DNQ and NO scavengers.</td>
<td>(340)</td>
</tr>
<tr>
<td>Rat, soleus</td>
<td>SNP, SNAP (ex vivo)</td>
<td>Did not alter intracellular Na⁺ content in isolated soleus.</td>
<td>(237)</td>
</tr>
<tr>
<td>Rat, diaphragm</td>
<td>L-NNA, hypoxia (chronic)</td>
<td>Decreased NKA content ([3H]ouabain) in control rats and especially in rats exposed to chronic hypoxia.</td>
<td>(232)</td>
</tr>
<tr>
<td>Rat, EDL, white vastus lateralis, soleus</td>
<td>SpNONOate, cGMP (in vitro)</td>
<td>Increased NKA activity (Biomol Green) in homogenates from EDL and/or white vastus lateralis muscles, but not in homogenates from soleus muscle.</td>
<td>(153)</td>
</tr>
<tr>
<td>Rat, soleus muscle</td>
<td>ATP (ex vivo)</td>
<td>Increased NKA activity (86Rb⁺ uptake) and blunted loss of force in the presence of 10 mM K⁺.</td>
<td>(41)</td>
</tr>
<tr>
<td>Rat, vastus lateralis and soleus</td>
<td>ADP (in vitro)</td>
<td>Increased V_{max} and decreased K_{0,5,Na} in isolated membranes (Biomol Green).</td>
<td>(346)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>ADP (in vitro)</td>
<td>Increased phosphorylation of PLM (Ser68) and the α1-subunit (Ser71).</td>
<td>(162)</td>
</tr>
<tr>
<td>Rat, glycolytic and oxidative muscles</td>
<td>GSSG (in vitro)</td>
<td>Increased glutathionylation of α-subunits (in membranes of both types of muscles) and β2-subunits (in membranes of glycolytic muscles). Inhibited NKA activity (Biomol Green) more effectively in membranes of oxidative muscles.</td>
<td>(154)</td>
</tr>
<tr>
<td>Human, vastus lateralis</td>
<td>Exercise, terbutaline</td>
<td>Increased glutathionylation of the β1-subunit synergistically. Glutathionylation of α-subunits and the β2-subunit remained unaltered. NKA subunits were glutathionylated under basal conditions.</td>
<td>(158)</td>
</tr>
</tbody>
</table>
Table 4. **Selected regulators of NKA in skeletal muscle.** Abbreviations: $\alpha^{2R/R}$: mice with mutant ouabain-resistant $\alpha$-subunit, A-769662: AMPK activator, ACh: acetylcholine, Biomol Green: malachite-based Biomol Green reagent for the assessment of ATPase activity, EDL: extensor digitorum longus, GSSG: oxidized glutathione, L-NNA: $N^\omega$-nitro-$L$-arginine (inhibitor of NO synthase), NAC: N-acetylcysteine, nAChR: nicotinic acetylcholine receptor, 3-O-MFPase: 3-0-methylfluorescein phosphatase assay, $^{32}$P release: hydrolysis of $[\gamma^{32}$P]ATP, PCR: polymerase chain reaction, PLM: phospholemman (FXYD1), RPM: measurement of resting membrane potential, SNP: sodium nitroprusside (NO donor), SNAP: S-nitroso-N-acetylpenicillamine (NO donor), SpNONOate: spermine NONOate (NO donor), Src: non-receptor tyrosine kinase, WB: Western Blot, WT: wild-type. Ex vivo refers to treatment of isolated skeletal muscle, in vitro refers to treatment of isolated membranes from skeletal muscles.
Figure 2

Motor neuron

Synaptic cleft

Muscle fiber

Voltage-gated Na⁺ channels (closed)

Voltage-gated Na⁺ channels (open)

FXYD1

Hyperpolarization

Depolarization (endplate potential)

Low [ACh]

High [ACh]

Acetate + choline

Na⁺

ACh

AChE

Action potential

ACh

ATP

CGRP

Agrin

nAChR (closed)

nAChR (open)

α2

β

nAChR

Na⁺

K⁺

Endplate potential