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Review article

Redox-dependent regulation of the Na^+–K^+ pump: New twists to an old target for treatment of heart failure ☆

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ABSTRACT

By the time it was appreciated that the positive inotropic effect of cardiac glycosides is due to inhibition of the membrane Na^+–K^+ pump, glycosides had been used for treatment of heart failure on an empiric basis for ~200 years. The subsequent documentation of their lack of clinical efficacy and possible harmful effect largely coincided with the discovery that a raised Na^+ concentration in cardiac myocytes plays an important role in the electromechanical phenotype of heart failure syndromes. Consistent with this, efficacious pharmacological treatments for heart failure have been found to stimulate the Na^+–K^+ pump, effectively the only export route for intracellular Na^+ in the heart failure. A paradigm has emerged that implicates pump inhibition in the raised Na^+ levels in heart failure. It invokes protein kinase-dependent activation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and glutathionylation, a reversible oxidative modification, of the Na^+–K^+ pump molecular complex that inhibits its activity. Since treatments of proven efficacy reverse the oxidative Na^+–K^+ pump inhibition, the pump retains its status as a key pharmacological target in heart failure. Its role as a target is well integrated with the paradigms of neurohormonal abnormalities, raised myocardial oxidative stress and energy deficiency implicated in the pathophysiology of the failing heart. We propose that targeting oxidative inhibition of the pump is useful for the exploration of future treatment strategies. This article is part of a Special Issue entitled "Na^+ Regulation in Cardiac Myocytes".

Contents

Abbreviations: NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; $[Na^+]_i$, intracellular Na^+ concentration; I_p, electrogenic Na^+–K^+ pump current; ACE, angiotensin converting enzyme; AR, adrenergic receptor; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; GSH, glutathione; Ang II, angiotensin II; Grx1, glutaredoxin 1; cAMP, cyclic adenosine monophosphate, πGST, π isoform of glutathione S-transferase; sGC, soluble guanylyl cyclase; PKG, protein kinase G; PP2A, protein phosphatase 2A; $ONOO^-$, peroxynitrite.

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1. Introduction

The Na^+–K^+ pump has justifiably been referred to as "the oldest pump" [1]. It was the first of the family of P-type ATPases to be discovered [2] and it had been a therapeutic target for treatment of heart failure with cardiac glycosides for almost 200 years [3] before it was appreciated that the glycosides cause Na^+–K^+ pump inhibition [4]. The glycoside-induced increase in the intracellular Na^+ concentration ($[Na^+]_i$) causes an increase in $[Ca^{2+}]_i$ via reduced net Na^+–Ca^{2+} exchange-mediated Ca^{2+} export. The increase in $[Ca^{2+}]_i$ then enhances contractility [5]. The demonstration of ouabain bound to the Na^+–K^+ pump molecular complex in its three-dimensional crystal structure [6,7] would have completed a perfect bench-to-bedside integration of molecular structure and function with one of the most classical pharmacological paradigms known. However, when efficacy was finally examined in a placebo-controlled trial in heart failure, this perfect integration was challenged by the bedside reality: there was no effect of digoxin on overall survival [8] and even a decrease in some patient subgroups [9]. A recent study raised serious doubts about the safety of digoxin when used for control of ventricular rate in atrial fibrillation [10], the main indication for which it is still commonly used. In addition to therapeutic use of cardiac glycosides, one of these, ouabain, is secreted endogenously and implicated in the pathogenesis of hypertension. The complex mechanisms proposed for this have been comprehensively reviewed recently [11]. An increase in the synthesis of endogenous ouabain has also been reported in heart failure but it seems unlikely that this has significant effects on the heart as reviewed [12].

While the Na^+–K^+ pump has lost its status as a useful target for treatment of heart failure with cardiac glycosides, it remains critically important for newer treatments. Here we review the Na^+–K^+ pump's role in current evidence-based treatments, how this role may be integrated with molecular and cellular mechanisms for the pathogenesis of the heart failure syndrome and how the relationship between treatment efficacy and effects of treatments on the Na^+–K^+ pump has led to a paradigm of redox-dependent regulation of pump activity.

2. Intracellular Na^+ and the Na^+–K^+ pump in heart failure

Many studies have shown that $[Na^+]_i$ is raised in the myocardium in heart failure and this is believed to contribute to the clinical manifestations of contractile abnormalities and arrhythmias [13,14]. These adverse effects occur in part because Na^+–Ca^{2+} exchange increases cytosolic Ca^{2+}. Ca^{2+}-induced diastolic Ca^{2+} release from the sarcoplasmic reticulum then reduces the amount available for release in systole [13,15]. Raised $[Na^+]_i$ is also thought to contribute to the heart failure phenotype by reducing mitochondrial Ca^{2+} uptake which in turn increases production of reactive oxygen species [16]. An inhibitory oxidative modification of mitochondrial ATP synthase [17] then reduces energy supply [18] (Fig. 1).

Raised $[Na^+]_i$ can result from enhanced Na^+ influx. Of pathways implicated in heart failure, the late Na^+ current has attracted much recent attention. Ca^{2+}/calmodulin activated by reactive oxygen species augments the current, and an increase in $[Na^+]_i$ from this source can contribute to diastolic $[Ca^{2+}]_i$ accumulation. Augmentation of the late Na^+ current may also contribute to prolongation of the action potential duration and to arrhythmogenesis [19]. Targeting the current therapeutically in heart failure is under clinical investigation [20].

Raised $[Na^+]_i$ can also result from reduced efflux mediated by the Na^+–K^+ pump, effectively the only export route for Na^+. Most studies examining the myocardial Na^+–K^+ pump in heart failure have reported reduced activity. The relative contribution of enhanced influx versus reduced efflux to the increase in $[Na^+]_i$ and to abnormalities in $[Ca^{2+}]_i$ and cardiac electrophysiology has recently been evaluated quantitatively using a mathematical model. A decrease of pump activity was the most important contributor to an increase in $[Na^+]_i$ and abnormalities of Ca^{2+} handling and action potentials [21]. Since decreased electrogenic Na^+–K^+ pump current (I_p) precedes a reduction in sarcoplasmic Ca^{2+} content and cytoplasmic Ca^{2+} transients in myocytes from guinea pigs with heart failure [22], pump inhibition may be a primary abnormality.

2.1. The Na^+–K^+ pump as a contemporary pharmacological target in heart failure

In view of the potential role of Na^+–K^+ pump abnormalities in heart failure it is of interest to consider the relationship between outcomes of clinical trials and the effect we have found the trial treatments have on the Na^+–K^+ pump in cardiac myocytes. Such an approach is effectively an exercise in reverse engineering, useful for understanding basic mechanisms of the heart failure syndrome [23]. Unless indicated, we have administered the treatments to rabbits in vivo and then studied the Na^+–K^+ pump in myocytes ex vivo. Most studies were performed in normal rabbits, indicating that the results can be attributed to a primary pharmacological action rather than to a treatment-induced improvement of underlying pathology via independent mechanisms. Measurements of I_p in cardiac myocytes were performed using standardized criteria [24] in accordance with those originally described by Gadsby et al. [25,26].

The most commonly used evidence-based treatments for human heart failure are based on the "neurohormonal hypothesis" [27] and antagonise activation of the renin–aldosterone–angiotensin system or adrenergic hyperactivity [23]. Treatment of rabbits with the angiotensin converting enzyme (ACE) inhibitor captopril, increased I_p in voltage clamped myocytes studied ex vivo and correspondingly decreased

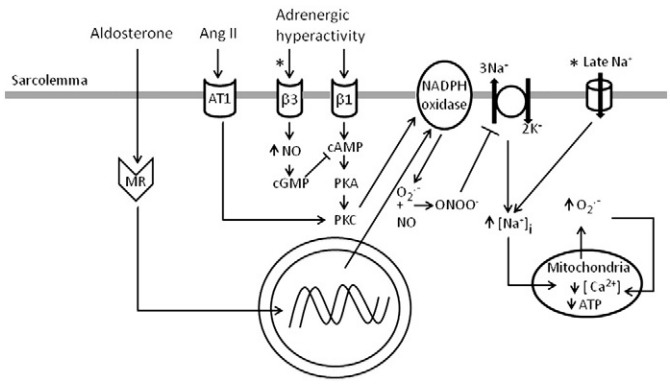

Fig. 1. Neurohormonal abnormalities, cytosolic Na^+, oxidative stress and energy metabolism in heart failure. Neurohormones activate NADPH oxidase via genomic and non-genomic pathways. Superoxide ($O_2^{\bullet-}$) inhibits the Na^+–K^+ pump and activates the late Na^+ current. Reduced export and enhanced influx increases $[Na^+]_i$ which, in turn, reduces mitochondrial $[Ca^{2+}]$ and increases mitochondrial $O_2^{\bullet-}$ synthesis. Oxidative modification and inhibition of ATP synthase reduces ATP synthesis. β_3 AR activation may counteract effects of the other receptors, in part by reducing cAMP levels in critical microdomains.

$[Na^+]_i$ measured in excised ventricular trabeculae [28]. There was a similar effect on I_p in a disease model when a decrease of I_p in myocytes from rabbits with alloxan-induced diabetes was reversed by treatment with the angiotensin receptor antagonist losartan [29]. Angiotensin promotes synthesis of aldosterone that may have harmful effects, in part because it upregulates NADPH oxidase [30]. Consistent with clinical efficacy of the aldosterone receptor antagonist spironolactone, treatment with spironolactone reversed a decrease in I_p and a corresponding increase in $[Na^+]_i$ caused by in vivo administration of aldosterone mimicking serum levels in heart failure [31]. Treatment of normal rabbits [32] with the β_1 adrenergic receptor (β_1 AR) antagonist metoprolol, increased I_p and abolished a decrease in I_p in myocytes from rabbits with heart failure [33]. Nitric oxide (NO) donors are not widely used in heart failure but nevertheless are of proven benefit [34]. We have not examined in vivo effects of NO donors, but in vitro exposure of voltage clamped myocytes increases I_p [24].

In vitro activation of β_3 adrenergic receptors increases I_p of cardiac myocytes [35] as does in vivo activation with an agonist [36]. Treatment with a β_3 AR agonist in vivo also reverses a decrease in I_p in myocytes isolated from rabbits with diabetes, a condition predisposing to heart failure [36]. As expected from activation of the Na^+–K^+ pump, acute intravenous administration of a β_3 AR agonist has opposite effects on cardiac performance in sheep with and without heart failure, consistent with the known differential effects on excitation–contraction coupling with changes in $[Na^+]_i$ from low- and high baseline levels [35]. The β_3 AR is up-regulated in human heart failure. This has widely been considered maladaptive. However, when seen in the light of the β_3 AR-dependent Na^+–K^+ pump activation, we believe human studies actually suggest β_3 AR agonists might be beneficial, although the evidence is indirect as reviewed [37]. We are currently examining the effect of treatment with a β_3 AR agonist on the Na^+–K^+ pump and on clinical features in rabbits with heart failure. A human clinical trial is planned [38].

The role of the Na^+–K^+ pump, and by inference $[Na^+]_i$, in heart failure can also be implicated from the effect on I_p of treatments that have turned out to be harmful. Amiodarone increases mortality in patients with class III heart failure [39] and I_p of myocytes isolated from rabbits given the drug is reduced [40]. The β_1 AR partial agonist Xamotarol increases mortality in human heart failure [41] and the drug is expected to activate adenylyl cyclase-dependent signalling that inhibits I_p [42], at least when activation occurs in vitro.

In summary, there is a robust relationship between clinical efficacy of treatments of human heart failure and the effects such treatments have on the Na^+–K^+ pump when studied in rabbit cardiac myocytes. The pump therefore retains its status as a therapeutic target in heart failure that it first earned from interaction with cardiac glycosides. However, contrary to the original paradigm, stimulation and not inhibition of the pump is beneficial.

3. Protein kinase-dependent signalling and the Na^+–K^+ pump as a treatment target

It is widely reported that phosphorylation of the FXYD1 protein that associates closely with the α/β pump heterodimer (Fig. 2A) stimulates the Na^+–K^+ pump in cardiac myocytes as reviewed [43]. However such an effect of FXYD1 phosphorylation cannot be immediately reconciled with hyperphosphorylation of FXYD1 [44] and reports of Na^+–K^+ pump inhibition [22,33] in heart failure, nor does stimulation of the pump mediated by FXYD1 phosphorylation readily explain the increase in I_p induced by treatment with β_1 AR blockers and ACE inhibitors [28,32,33] and the clinically beneficial effects of these drugs [23] despite

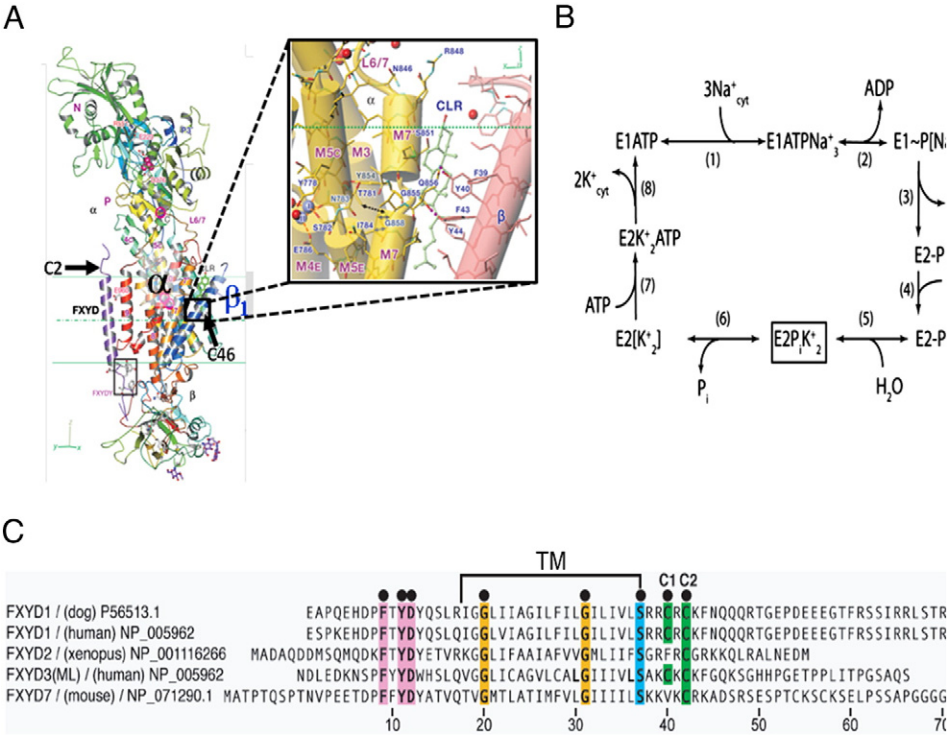

Fig. 2. Structure and catalytic cycle of the Na^+–K^+ pump. A. Three-dimensional structure of α/β/FXYD complex in a conformation analogous to $E2PiK_2^+$. Transmembrane domains are between the 2 unbroken green lines. The β subunit and the FXYD proteins are single-transmembrane spanning while 10 helices of the α subunit span the membrane. Reactive cysteine residues in β_1 subunit (C46) and FXYD (C2) are indicated. The expanded section illustrates proximity of the glutathionylation site to hydrogen bonds between β- and α subunits (broken lines). B. Albers-Post scheme for Na^+–K^+ pump catalytic cycle. When 3 Na^+ ions have been bound to the E1 conformation (1) the cytoplasmic access gate is closed and locked with phosphorylation of the α subunit (2), causing occlusion of Na^+ ($[Na^+]_3$) within the molecule. A gate opens to the outside and Na^+ is released (3) when its binding affinity decreases with E1P → E2P change. K^+ is bound (4), the gate is closed and the resultant conformational change of the pump stimulates its dephosphorylation (6). The $E2PiK_2^+$ product state of dephosphorylation (in box) is the conformation for which the three-dimensional crystal structure is known. C. Sequence alignment of FXYD1-3 and 7. Numbering corresponds to the FXYD1 sequence and begins at 1 after the signal peptide (not shown). Conserved residues are marked with filled circles with conserved cysteine residues labelled C1 and C2. TM indicates the transmembrane domain.

the known harmful effects of raised $[Na^+]_i$ [13,14] in heart failure: β_1 ARs- and angiotensin II (Ang II) receptors are coupled to activation of protein kinase A (PKA) and -C (PKC), thus in vivo treatment with β_1 AR blockers or ACE inhibitors should reduce PKA- and PKC activities by reducing adrenergic activity and levels of Ang II in the myocardium. This should also reduce phosphorylation of FXYD1. We have confirmed this experimentally in normal rabbits [32]. If FXYD1 phosphorylation were to stimulate Na^+–K^+ pump activity, β_1 AR blockers and ACE inhibitors should therefore accentuate harmful effects of the raised $[Na^+]_i$ in heart failure.

3.1. Protein kinase-dependent redox signalling and the Na^+–K^+ pump

Since phosphorylation of the Na^+–K^+ pump molecular complex cannot readily account for effects of the two best documented and most commonly used treatment modalities in heart failure on Na^+–K^+ pump function we have examined if oxidative posttranslational modifications might play a role. Oxidative modifications can affect structure and function of proteins in a manner analogous to phosphorylation [45] and seemed a plausible alternative because heart failure is associated with increased myocardial oxidative stress [46] and because chemical oxidants can inhibit Na^+–K^+ ATPase in membrane fragments [47] and pump activity in cardiac myocytes [48]. Of the oxidative modifications, a disulphide bond between cysteine residues on the cytosolic tripeptide glutathione (GSH) and a protein is of particular interest because it is stable, yet reversible [45].

We examined if receptor-coupled activation of oxidative signalling and glutathionylation of the Na^+–K^+ pump contribute to pump regulation. Exposure of myocytes to Ang II increased the co-immunoprecipitation of the membranous $p22^{phox}$ subunit of NADPH oxidase with the cytosolic $p47^{phox}$ subunit in myocyte lysate consistent with the translocation of $p47^{phox}$ to the cell membrane that is required for activation of NADPH oxidase [49]. It also increased co-immunoprecipitation of the Na^+–K^+ pump molecular complex with $p47^{phox}$ while it decreased I_p. The decrease in I_p was abolished by blocking translocation of $p47^{phox}$, and hence NADPH oxidase activation, and by blocking εPKC activation [49]. These results are consistent with PKC-dependent phosphorylation of $p47^{phox}$ necessary for its translocation. The Ang II-induced activation of oxidative signalling was associated with glutathionylation of the β_1 subunit of the Na^+–K^+ pump [50]. A decrease in β_1 subunit glutathionylation after treatment with an ACE inhibitor suggests that Ang II has the same effect in vivo [32]. Mutational studies of Na^+–K^+ pumps expressed in *Xenopus* oocytes identified cysteine 46 (C46) as the reactive residue in the β_1 subunit [50] and, consistent with the NADPH oxidase-dependence of Ang II-induced inhibition of I_p in cardiac myocytes [49], there was a causal relationship between β_1 subunit glutathionylation and pump inhibition [50].

We have also examined if β_1 AR-dependent signalling causes downstream oxidative modification of the Na^+–K^+ pump. In in vitro studies we used forskolin to activate adenylyl cyclase that is coupled to the β_1 AR rather than a receptor agonist because of the imperfect selectivity of the available agonists. Forskolin activated NADPH oxidase via PKA- and PKC-dependent pathways and inhibited I_p of cardiac myocytes [42]. It also induced glutathionylation of the β_1 Na^+–K^+ pump subunit and a decrease in I_p that was abolished by inhibition of PKA, εPKC or NADPH oxidase [42]. Consistent with these results, in vivo β_1 AR blockade in normal rabbits inhibited εPKC and NADPH oxidase activation, reduced β_1 Na^+–K^+ pump subunit glutathionylation and increased I_p of cardiac myocytes [32]. The β_1 AR blockade also decreased β_1 pump subunit glutathionylation and increased I_p in rabbits with heart failure [33].

The β_1 Na^+–K^+ pump subunit is glutathionylated at baseline and, in contrast to effects mediated by β_1 AR-dependent signalling, the NO-dependent pathways coupled to the β_3 AR cause a decrease in the β_1 subunit glutathionylation and an increase in I_p with in vitro [35] as well as with in vivo activation of the receptor [36]. NO-dependent signalling can occur via nitrosylation of target cysteine residues [51] and Yukasev et al. [52] have quoted us as having reported that nitrosylation is an intermediate step in glutathionylation of the β_1 Na^+–K^+ pump subunit. If nitrosylation of the β_1 subunit were to account for the β_3 AR- and NO-dependent stimulation one would have to assume the effect of nitrosylation on Na^+–K^+ pump function is opposite to that of glutathionylation. However, we have never reported nitrosylation of the β_1 subunit, and we have previously shown that the "classical" [51] soluble guanylyl cyclase/cGMP/PKG dependent pathway can account for NO-dependent Na^+–K^+ pump stimulation. The pump stimulation is okadaic acid- sensitive implicating activation of protein phosphatase in the stimulation [24]. Phosphatase-mediated dephosphorylation of the $p47^{phox}$ subunit has been implicated in inhibition of NADPH oxidase in neuotrophils [53] and the balance between PKC-dependent phosphorylation and protein phosphatase-mediated dephosphorylation was suggested to determine NADPH oxidase activity [54]. We are currently examining if protein phosphatase-dependent dephosphorylation of $p47^{phox}$ can account for the effect of okadaic acid-sensitive activation of the classical pathway on Na^+–K^+ pump activity in cardiac cells. Such activation in combination with a β_3 AR-dependent reduction of cyclic adenosine monophosphate (cAMP) levels in critical microdomains [55], might relieve oxidative inhibition of the Na^+–K^+ pump and hence contribute to β_3 AR-dependent pump stimulation. The role the classical NO-dependent pathway may have in Na^+–K^+ pump stimulation is summarized in Fig. 3.

3.2. FXYD proteins and redox-dependent Na^+–K^+ pump regulation

While phosphorylation of FXYD1 is implicated in regulation of cardiac myocyte Na^+–K^+ pump, functional phosphorylation sites on FXYD2-7 have not been firmly demonstrated. However, two cysteine residues in the cytoplasmic terminal, named C1 and C2 in Fig. 2C, are conserved in the 7-member mammalian family. While most cysteine residues in proteins do not undergo oxidative modifications, C1 and C2 are good candidates because they are mostly flanked by the basic amino acids lysine and arginine. FXYD1, native to cardiac myocytes, and other FXYD proteins that we expressed in *Xenopus* oocytes were susceptible to glutathionylation. Mutagenesis identified C2 but not C1 as reactive, with reactivity of C2 depending on flanking basic amino acids. The three dimensional structure suggested proximity to basic amino acids in the α subunit might account for differences

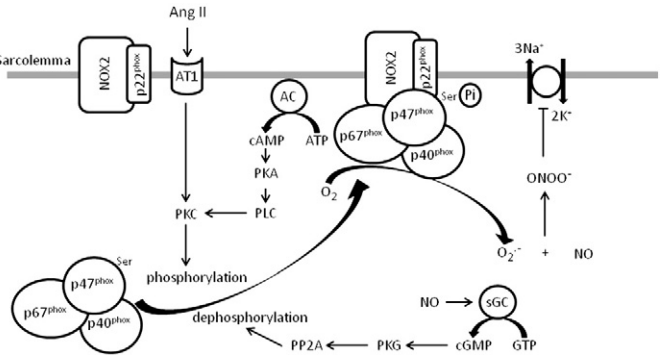

Fig. 3. Scheme proposed for nitric oxide-dependent Na^+–K^+ pump regulation. Superoxide ($O_2^{\bullet -}$) is synthesised when receptor-dependent protein kinase Cs phosphorylates $p47^{phox}$ and activates NADPH oxidase. Reaction of $O_2^{\bullet -}$ with NO leads to the formation of the biologically highly reactive species $ONOO^-$. This promotes glutathionylation-induced Na^+–K^+ pump inhibition. However, when NO activates a sGC/cGMP/PKG/PP2A-dependent pathway, PP2A dephosphorylates $p47^{phox}$ and inactivates NADPH oxidase, allowing reversal of glutathionylation of the pump and relief from inhibition. Spatial dependence of NO concentrations relative to NADPH oxidase and the Na^+–K^+ pump and/or a differential NO concentration-dependence of its effects to form $ONOO^-$ or activate sGC might determine whether NO increases or decreases pump activity.

in reactivity between C1 and C2 [56]. A reactive cysteine in the C2 position of FXYD proteins was critical for reversal of glutathionylation of C46 of the β_1 subunit and Na^+–K^+ pump inhibition induced by chemical oxidants or exposure of myocytes to Ang II. Results obtained in *Xenopus* oocytes expressing FXYD proteins with- and without a reactive C2 independently supported this conclusion (see Bibert et al., for details) [56]. Of importance for receptor-coupled signalling, a decrease from baseline C46 glutathionylation and an increase in I_p induced by a β_3 AR agonist was also dependent on a reactive C2 [56].

As discussed previously [57], glutathionylation of PKA and PKC can inhibit activity of the kinases and an oxidant signal might therefore inhibit Na^+–K^+ pump activity by decreasing the phosphorylation of FXYD1 that is maintained by constitutively active protein kinases. However, co-expression of FXYD1 with α_1/β_1 subunits in *Xenopus* oocytes prevents a decrease in I_p induced by an oxidant signal that otherwise occurs when only α_1/β_1 subunits are expressed. This effect is eliminated when the reactive C2 in the wild-type FXYD1 is mutated to a non-reactive amino acid residue while leaving phosphorylation sites on FXYD1 intact [56]. The decrease in I_p is also eliminated when the reactive C46 in the β_1 subunit is mutated to a non-reactive residue or if α_1 subunits are co-expressed with β_2- or β_3 subunits that do not have a reactive cysteine residue [50]. Redox-sensitivity of protein kinases cannot account for these results in *Xenopus* oocytes. Oxidative inhibition of protein kinases also cannot account for Na^+–K^+ pump inhibition we have attributed to pathways that are coupled to the β_1 AR [42] and Ang II receptors [49] in cardiac myocytes because in vivo treatments with a β_1 AR antagonist or an ACE inhibitor increase I_p while the treatments decrease activities of PKA and PKC. As expected from the decrease in protein kinase activities, treatment with the β_1 AR antagonist decreased phosphorylation of FXYD1. The effect of the catalytic subunit of PKA included in patch pipette solutions to decrease I_p [32] independently supports the conclusion that the PKA-dependent Na^+–K^+ pump inhibition we report is not secondary to oxidation-induced inhibition of PKA and a decrease in phosphorylation of FXYD1.

3.3. Glutathionylation of the α Na^+–K^+ pump subunit

We have been unable to identify glutathionylation of the α_1 Na^+–K^+ pump subunit in cardiac myocytes [42,50,56] and, while we found that oxidant stress decreases I_p of *Xenopus* oocytes when α_1 subunits are co-expressed with wild-type β_1 subunits, the absence of any decrease in I_p when α_1 subunits are co-expressed with C46-mutated β_1 subunits [50] indicates that no functional effect could be attributed to glutathionylation of the α_1 subunits in our experiments. In contrast, Petrushenko et al. [58] and Yakushev et al. [52] have recently reported that several cysteine residues on the α_1 subunit are susceptible to glutathionylation. Glutathionylation in Na^+–K^+ ATPase-enriched membrane fragments, detected under baseline conditions, was enhanced with exposure to oxidised GSH. The exposure decreased Na^+–K^+ ATPase activity but, as pointed out by the authors, "removal of basal glutathionylation by DTT (dithiothretiol) was not followed by an alteration of the Na^+–K^+ ATPase activity". These results contrast the strong correlation between an increase in I_p and a decrease in β_1 subunit glutathionylation from baseline that occurs when the β_3 AR is activated in cardiac myocytes [35]. A causal relationship between glutathionylation of the α_1 subunit and Na^+–K^+ ATPase activity remains to be established. Mutation of the implicated cysteine residues would be essential for this. It would also be important to establish if receptor-coupled signalling alters α_1 subunit glutathionylation.

Petrushenko et al. [58] proposed that hypoxia is a physiological stimulus that induces regulatory S-glutathionylation of the α_1 Na^+–K^+ pump subunit in rat myocardium and an associated decrease in Na^+–K^+ ATPase activity. However, functional effects attributed to α_1 subunit glutathionylation were only evident at an ATP concentration <500 μM [58], an unlikely concentration under physiological conditions and also not expected to be encountered with the modest decrease in the ATP concentration that occurs in heart failure [59]. Yakushev et al. [52] referred to a hypoxia-induced decrease in Na^+–K^+ ATPase activity of 20% that we had attributed to glutathionylation of C46 in the β_1 subunit and compared it with a much more extensive inhibition of activity known to occur in ischemic heart. This comparison is invalid. We have reported on the effect of myocardial infarction on glutathionylation of the β_1 subunit [50] but not on the effect of hypoxia on glutathionylation, and we have not reported on any effect of infarction or hypoxia on Na^+–K^+ pump function.

4. Structural changes during the Na^+–K^+ pump cycle and glutathionylation-dependent function

4.1. Susceptibility of C46 in β_1 Na^+–K^+ pump subunit to glutathionylation

Since GSH is hydrophilic and strictly cytosolic, glutathionylation of C46 is counterintuitive in view of its location in the transmembrane segment (Fig. 2A), with its sulfhydryl group facing the lipid bulk phase. The three dimensional structure that indicates this location is known in only one of the Na^+–K^+ pump's conformations and we subsequently showed that susceptibility to glutathionylation of C46 depends on the conformational states the pump undergoes in its catalytic cycle (Fig. 2B) [60]. The β subunit forms many contacts with transmembrane segments 7 (αM7) and 10 of the α subunit [61] with polar residues lining the interface between the subunits from the cytoplasm to C46 [62] and, using molecular dynamics simulations, Thøgersen and Nissen [62] demonstrated that minor structural changes in the pump molecular complex are likely to cause a membrane deformation that yields a hydrophilic environment for C46. This might explain the conformation-dependence of access for GSH.

There are no neighbouring basic amino acids in the primary sequence of the β_1 subunit that would reduce pKa of the sulfhydryl group to promote glutathionylation of C46. However, a cluster of 4 arginines and one lysine near the C terminus of αM10 is ~15 Å from the side chain of C46 in the known crystal structure [61] and might move in response to Na^+ binding. Such movement and membrane deformation allowing access of the sulfhydryl group of C46 might provide an environment promoting glutathionylation of C46. Correlation between conformation-dependent access for trypsin to digest the β_1 subunit and the C terminus of αM10 [63] would seem consistent with such speculations.

Speculations about changes in pKa of C46 during the catalytic cycle are based on the tacit assumption that glutathionylation must always be accounted for by physicochemical properties of the glutathionylated cysteine residue. However, in intact cells, glutathionylation of proteins can be catalysed by glutathione S-transferase (GST) [64], and we have preliminary data indicating that exposing Na^+–K^+ ATPase-enriched membrane fragments to the π isoform of GST facilitates glutathionylation of the β_1 subunit (unpublished). Similarly, deglutathionylation is not necessarily only described in physicochemical terms. Deglutathionylation of proteins is selectively catalysed by glutaredoxin 1 (Grx1). Grx1 co-immunoprecipitates with FXYD1 and the β_1 pump subunit in cardiac myocyte lysate [56] and addition of recombinant Grx1 to the lysate reverses β_1 subunit glutathionylation induced by oxidative stress [56]. When included in patch pipette solutions, recombinant Grx1 also counteracted oxidative stress-induced inhibition of I_p [50]. We have recently found that translocation of Grx1 may contribute to the in vivo deglutathionylation that occurs with blockade of the β_1 AR [32]. A balance between opposing effects of πGST and Grx1 may be important in determining the level of glutathionylation of the Na^+–K^+ pump in a manner reminiscent of the roles kinases and phosphatases have in determining phosphorylation of proteins. Differential access of πGST and Grx1 to the Na^+–K^+ pump in its different conformations may contribute to conformation-dependence of glutathionylation in cells.

4.2. Glutathionylation and integrity of the Na^+–K^+ pump molecular complex

The ~305 Da negatively charged GSH adduct may weaken the interaction of tyrosines 40 and 44 of the β subunit with αM7 (Fig. 2A) [61], reminiscent of the effect mutation the tyrosines have on αM7/β_1 interaction [65]. Consistent with this, glutathionylation decreases the α/β co-immunoprecipitation. A disruption of the α/β heterodimer with glutathionylation is also supported by its increased sensitivity to trypsin digestion, in particular the sensitivity of the β subunit [60].

Assuming co-immunoprecipitation reflects a direct physical interaction, a decrease in FXYD1/α_1- and an increase in FXYD1/β_1 subunit co-immunoprecipitation with oxidative stress can also be viewed in structural terms. The signature motif of FXYD10 (non-mammalian FXYD1 homologue) in shark rectal gland Na^+–K^+ ATPase forms a network of hydrogen bonds to α and β subunits extracellularly, while it forms only a single hydrogen bond to α in the transmembrane segment [61]. Positively charged basic amino acids near the cytosol-membrane interface may stabilize FXYD/α interaction because of the electrostatic attraction they share to negative charges at the inner membrane leaflet. Electrostatic switch theory for interaction of proteins with membranes [66] suggests such stabilization might be disrupted when the C2-equivalent of FXYD proteins acquires the negatively charged GSH adduct. However, interaction of the extracellular FXYD motif with the β subunit should remain unaffected, shifting the relative strength of association of FXYD proteins from the α- towards the β subunit as suggested by the co-immunoprecipitation experiments.

Even with structural changes that occur during the Na^+–K^+ pump cycle, the large distance between C46 in the β_1 subunit and C2 in FXYD proteins (Fig. 2A) precludes simple disulfide exchange between the cysteine residues as a mechanism for their functional interaction, and a more complicated scheme needs to be invoked. Interaction of the Na^+–K^+ pump molecular complex with πGST and Grx1 as possible candidate partners can be involved in such a scheme. Grx1 activation may occur when conformational changes in proteins or multimeric protein complexes allow access for it to target disulfide bonds [67], and conformation-dependence of co-immunoprecipitation of Grx1 with the β_1 subunit of Na^+–K^+ ATPase [60] is consistent with conformation-dependence of Grx1-mediated de-glutathionylation. With interaction of Grx1 and possibly πGST with cysteine residues in the C2 position of FXYD proteins and C46 in the β_1 subunit in structural conformations corresponding to different sub-states of the pump's catalytic cycle (Fig. 2B), a large number of schemes for C2/C46 interaction become possible.

4.3. Glutathionylation and Na^+_i/K^+_i-dependence of Na^+–K^+ pump turnover

A monensin-induced increase in $[Na^+]_i$ renders the β_1 Na^+–K^+ pump subunit resistant to glutathionylation in intact myocytes [60], and an Ang II-induced increase in oxidative stress inhibits I_p of voltage clamped myocytes when $[Na^+]$ in patch pipette solutions is near physiological intracellular levels but not when it is high or when pipette solutions are K^+-free [60]. The in vivo relevance of this is highlighted by the dependence of an increase in I_p on $[K^+]$ in pipette solutions when myocytes are studied ex vivo after treatment of rabbits with an ACE-inhibitor [68]. Corresponding results have been obtained in diabetes, known to be associated with oxidative stress. Diabetes induced experimentally in rabbits caused a decrease in I_p that was dependent on the pipette $[K^+]$ as was reversal of the decrease when the rabbits had been treated with an Ang II receptor antagonist [29].

The dependence of oxidative Na^+–K^+ pump inhibition on $[Na^+]_i$ and $[K^+]_i$ is consistent with the susceptibility of the β_1 subunit to glutathionylation in different conformational states of the pump. Binding of Na^+ occurs to Na^+–K^+ pump species in the E1 conformation (Fig. 2B), a confirmation that is highly susceptible to glutathionylation [60]. Since Na^+ binds in competition with K^+, kinetically incompetent, susceptible E1 species that have bound K^+ accumulate when $[K^+]_i$ is high while a high $[Na^+]_i$ has the opposite effect, i.e. it is expected to decrease the abundance of E1 species and hence decrease glutathionylation. Such a dependence of glutathionylation on $[Na^+]_i$ and $[K^+]_i$ has important consequences for pump function.

Glutathionylation-dependent Na^+–K^+ pump inhibition could become self-amplifying if an increase in $[Na^+]_i$ were to increase oxidative stress (Fig. 1). However, the increase in the $[Na^+]_i$: $[K^+]_i$ ratio with pump inhibition should reduce susceptibility to glutathionylation and hence eliminate the risk of self-amplifying pump inhibition abolishing all function during oxidative stress. Although less abundantly expressed than pumps with β_1 subunits, pumps with β_2 or β_3 subunits should provide some additional back-up function because these subunits are not susceptible to glutathionylation [50]. $[Na^+]_i$- and $[K^+]_i$-dependence of β_1 subunit glutathionylation is also expected to mediate receptor-coupled, protein kinase-dependent regulation of Na^+–K^+ pump function in a manner that might traditionally have been attributed to effects on ligand binding sites. For example, the Ang II-induced pump inhibition at low- but not high $[Na^+]_i$ [60] we referred to above that might have been due to effects of Ang II-dependent signalling on Na^+ binding can also be accounted for by the inverse relationship between $[Na^+]_i$ and the susceptibility of C46 in β_1 subunits to glutathionylation. This relationship would effectively mimic a change in the pump's Na^+ affinity.

5. Summary and perspectives

The idea that inhibition of the Na^+–K^+ pump is desirable in heart failure became untenable when it was recognized that cardiac glycosides are ineffective and that raised $[Na^+]_i$ is harmful. However, effects of current evidence-based treatments on oxidative modification and function of Na^+–K^+ pump are highly compatible with the neurohormonal hypothesis. Since receptor-coupled signalling targeted in heart failure activates NADPH oxidase or up-regulates components of it, the effects are also compatible with the firmly established role of oxidative stress in the pathogenesis of heart failure. Neurohormone-mediated oxidative inhibition of the pump can also be integrated with the role raised $[Na^+]_i$ has in mitochondrial production of ROS and uncoupling from ATP synthesis (Fig. 1) in a scheme that readily integrates major current paradigms in the pathophysiology of heart failure syndrome.

The relationship between outcomes of clinical trials in heart failure and effects the treatments have on the Na^+–K^+ pump and oxidative signalling pathways that regulate it indicate that the pump is an important treatment target. However, in contrast to the role classically assigned to the Na^+–K^+ pump when targeted with cardiac glycosides, we propose the newer evidence-based treatments target the pump indirectly via the effect they have on the pathways that modulate oxidative modifications of it.

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Conflict of interest

The authors have nothing to disclose.

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