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Review

Voltage clamp fluorometry: Combining fluorescence and electrophysiological methods to examine the structure–function of the Na^+/K^+-ATPase

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ABSTRACT

This paper summarizes our recent work investigating the conformational dynamics and structural arrangement of the Na^+/K^+-ATPase using voltage clamp fluorometry as well as the latest biochemical, biophysical and structural results from other laboratories. Our research has been focused on combining site-specific fluorophore labeling on the alpha, beta and/or gamma subunit with electrophysiological studies to investigate partial reactions of the ion pump by monitoring changes in fluorescence intensity following voltage pulses and/or solution exchange. As a consequence of these studies, we have been able to identify a residue on the beta subunit, which following labeling with tetramethylrhodamine-6-maleimide can be used as a reporter group to monitor the conformational state of the holoenzyme. Furthermore, we have been able to delineate distance constraints between the alpha, beta and gamma subunits and to examine the relative movements of these proteins during ion transport. Concurrent to this research, significant advancements have been made in understanding the molecular mechanism of the Na^+/K^+-ATPase. Thus, our research will be compared with the results from other groups and future experimental directions will be proposed.

1. Introduction

The Na^+/K^+-ATPase was first identified in 1957 and for this seminal discovery Jens Christian Skou was awarded the Nobel Prize in Chemistry in 1997 [1]. The reaction cycle of the Na^+/K^+-ATPase is described by the Albers–Post scheme (Fig. 1). In this scheme, the enzyme can reside in two principle conformations, E_1 and E_2 where, starting in E_1, three Na^+ ions bind on the cytoplasmic side for transport across the plasma membrane while two K^+ ions bind to extracellular sites when the protein is in the E_2 conformation for translocation into the cell. The overall reaction is electrogenic as there is a net movement of charge, three Na^+ ions for two K^+ ions. Furthermore, the main electrogenic events are the extracellular release/rebinding steps which are energetically coupled to the E_1P–E_2P conformational change [2,3].

The ion pump, which is expressed on the plasma membrane of most eukaryotic cells, is comprised of two obligatory subunits, alpha and beta, as well as one member of the FXYD family of proteins which is not required for function. The enzyme is a member of the P-type ATPase family, so called because each member of this family is phosphorylated at a highly conserved aspartate residue within the consensus sequence, DKTGTLT, upon ATP hydrolysis [4]. The alpha subunit comprises approximately 1000 amino acids and contains ligand (Na^+ and K^+), substrate (ATP) and inhibitor (digitoxin and ouabain) binding sites [5]. This subunit has ten transmembrane domains and three large cytosolic domains: the nucleotide binding site (N) which contains the ATP binding site, the activator domain (A) which acts as the activator of the gates that regulate binding and release of the transported cations and the phosphorylation domain (P) which contains the residue which is phosphorylated. Each of these domains undergoes a significant conformational change during the catalytic cycle which has been elegantly elucidated with a series of crystal structures of the Ca^{2+}-ATPase in both the E_1 and E_2 conformational states [6,7]. This conformational change is translated to the transmembrane domains during ion translocation and it has been demonstrated that the M5–M6 loop is involved in a functional rearrangement during the pumping cycle [8]. Finally, it should be noted that there are four isoforms of the alpha subunit in humans. The α1 isoform is expressed in the kidney and lung, while the α2 is the predominant isoform in skeletal muscle. The third isoform (α3) is present in brain and heart and the α4 isoform has been implicated in sperm motility.

In addition to the alpha subunit, the beta subunit is also required for function. This protein is approximately 300 amino acid long and contains multiple glycosylation sites as well as disulfide bridges which are highly resistant to reduction [9,10]. Interestingly, the beta subunit is required for proper trafficking of the holoenzyme to the plasma

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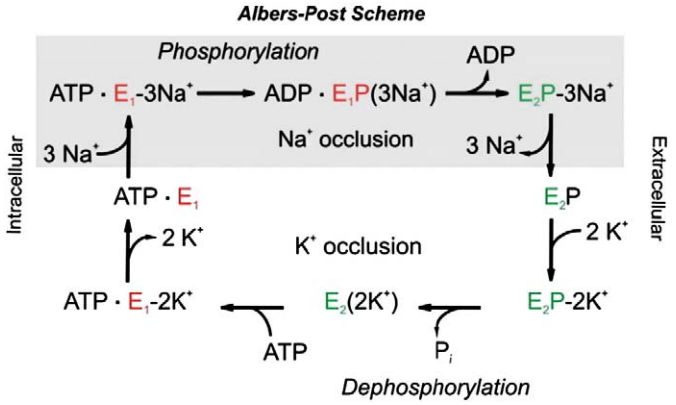

Fig. 1. Albers–Post scheme for the Na^+/K^+-ATPase reaction cycle. The enzyme can assume two major conformations: E_1 (red) where the ion binding sites face the cytoplasm and E_2 (green) where access to the cation binding sites is from the extracellular space. In the absence of extracellular K^+ and in the presence of Na^+, the enzyme is restricted to Na^+/Na^+ exchange conditions (grey box).

membrane [11]. That is, following heterologous expression of only the alpha subunit, this protein is localized in sub-cellular organelles and degraded [9]. The beta subunit is also recognized to modulate cation binding affinity [12]. There are three isoforms of the beta subunit (β1, β2 and β3) which are expressed differentially in tissues and it has been established that subtle sequence differences between isoforms result in functional differences [13].

In contrast to the alpha and beta subunits, the FXYD family of proteins is not required for function. There are seven members of the mammalian FXYD proteins which are less than 100 residues and modulate the apparent affinity for sodium, potassium and/or ATP [14]. As such an important physiological molecule, a wide variety of experimental techniques have been used to investigate the mechanism of this protein over the past 50 years. This includes purifying the enzyme from native species for subsequent biochemical and biophysical studies using fluorescence labeling [15,16]. The past few years have seen significant advances in understanding the mechanism of this enzyme, whether through the use of the experimental techniques described above or novel approaches. Some of the more recent work in these areas will be addressed in the following sections.

2. Mechanistic information on the ion pump

Although basic outline of the ion transport has been determined, as described by the Albers–Post scheme, much work has been done to elucidate the fine details of ion transport. Notably, Gadsby and co-workers have used the marine toxin palytoxin to examine the transmembrane pathway of ions through the Na^+ pump. Palytoxin binds to the Na^+/K^+-ATPase and initiates the formation of a non-selective cation channel. Thus, it has been possible to examine some partial reactions of the ion pump following application of ligands or following the application of thiol-specific reagents upon incorporation of cysteine residues on the ion pump [17–19]. As a function of these experiments, it has been determined that residues which reside in transmembrane domains 1, 2, 4 and 6 are involved in the cation pathway. Computational approaches have been combined with these results to describe the kinetics of phosphorylation and dephosphorylation of the ion pump in the presence of palytoxin [20].

During the past few years, the objective of our research has been to detect site-specific conformational changes in real time under physiological conditions by combining fluorescence and two electrode voltage clamp (voltage clamp fluorometry). In these experiments, two point mutations (Q111R and N122D) are utilized to confer a reduced ouabain sensitivity of the ion pump [21]. This enables selective inhibition of the endogenous Na^+/K^+-ATPase as it is expressed in *Xenopus* oocytes versus the heterologously expressed ion pump. Two further point mutations (C911S and C964A) were added in order to remove all free extracellular cysteine residues [22]. None of these mutations have an appreciable effect upon the kinetic parameters of the ion pump. Upon insertion of a cysteine residue at a specific site, it is then possible to site-specifically label the ion pump with tetramethyrhodamine-6-maleimide. It should be noted that the beta subunit contains additional cysteine residues. However, each of these residues forms disulfide bonds which are highly resistant to reduction and are not labeled with the fluorophore [9,10].

We utilized the information that the M5–M6 loop of the alpha subunit undergoes a conformational rearrangement during ion translocation to perform cysteine-scanning mutagenesis of this region followed by fluorophore labeling [8]. From this work, one residue (N790C) was identified which demonstrated changes in fluorescence intensity following changes in extracellular solution and/or membrane potential [23]. These experiments enabled the determination of spatially defined conformational changes of the fully functional Na^+/K^+-ATPase in real time and under physiological conditions. Furthermore, it was possible to determine the distribution of the two main conformational states under Na^+/Na^+ exchange conditions. Under these conditions the pump is restricted to the Na^+ translocating branch of the cycle (see Fig. 1) and shuttles in a voltage dependent manner almost exclusively between the E_1P and E_2P conformations as dephosphorylation under K^+-free conditions is very slow. Hyperpolarizing potentials drive the enzyme in a saturating fashion into the E_1P conformation and depolarizing potentials lead to accumulation of E_2P. Transient currents can be observed upon voltage jumps which are the product of electrogenic Na^+ re-uptake and Na^+ release. By measuring the fluorescence intensity and plotting this as a function of membrane potential, it was possible to determine the relative populations of holoenzyme in the E_1P and E_2P conformations. In addition, the kinetics of ion transport could be compared to the kinetics of the conformational changes. However, in these experiments it should be noted that the N790C mutation did affect the kinetic parameters of the ion pump. The value for $V_{0.5}$, which represents the membrane potential where there is an equal proportion of ion pumps in the E_1P and E_2P states, was more negative ($V_{0.5}$ of WT: ≈ -65 mV and N790C: -110 ± 9 mV) when compared to the wild type ion pump. This data suggests that the E_2P conformation was stabilized when compared to the wild type ion pump. In addition, the transient current kinetics of ion transport were slower (WT: ≈50–200 s^{-1} and N790C: ≈20 s^{-1}). Thus, the asparagine to cysteine mutagenesis and subsequent fluorescence labeling of the N790C residue affected the kinetic properties of the ion pump. Therefore, the next objective was to identify a construct which could be used as a reporter group for the ion pump, but where the kinetic parameters were not affected.

We therefore focused on the beta subunit of the ion pump. Following scanning cysteine mutagenesis of the sheep β subunit (isoform 1: $s\beta_1$), three residues were identified (S62C, F64C and K65C) which exhibited significant (greater than 5%) fluorescence changes in response to voltage steps under K^+-free conditions or concurrent with changes in extracellular ionic conditions that induce stationary Na^+/K^+ exchange currents (Fig. 2A).

The kinetics of the change in fluorescence intensity was distinct for each of these three constructs and therefore we will limit ourselves to the results for one construct, $s\beta_1$-S62C, in this review [24]. In order to compare fluorescence signals to certain partial reactions of the Albers–Post cycle, we performed voltage pulse experiments at high extracellular Na^+ concentrations (100 mM) in the absence of K^+. As mentioned previously, under these conditions the Na^+/K^+-ATPase carries out Na^+/Na^+ exchange [3]. We determined that the voltage dependence of displaced charge for this construct follows a Boltzmann distribution which reflects the distribution of the enzyme between E_1P and E_2P states (Fig. 2B). As the change in fluorescence intensity

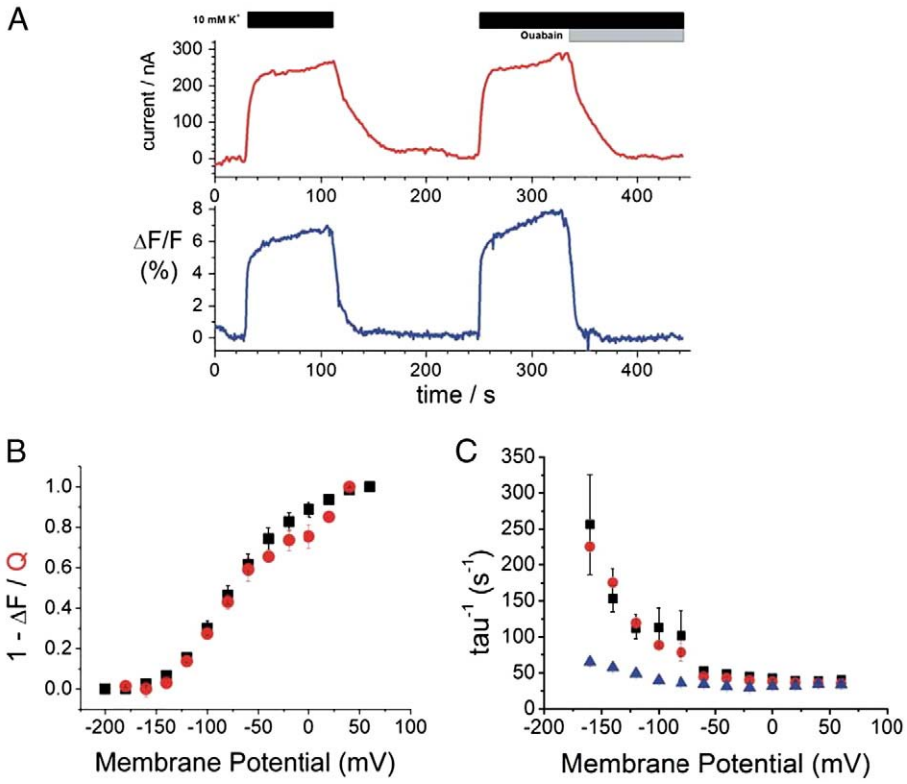

Fig. 2. (A) Parallel recording of pump current (top) and fluorescence changes (bottom) from an oocyte coinjected with the alpha subunit and F64C-beta subunit of the Na^+/K^+-ATPase in response to 10 mM K^+ and 10 mM ouabain at 0 mV holding potential. Voltage dependence (B) and kinetics (C) of voltage jump-induced fluorescence changes and comparison with properties of the corresponding transient charge movements under K^+-free (Na^+/Na^+) exchange conditions of the S62C construct. Panel B shows the voltage dependence of normalized fluorescence saturation values (■) obtained from monoexponential fits to the data and values for the translocated charge (●) obtained from integration of the transient currents recorded in parallel. Panel C shows the voltage dependence of reciprocal time constants obtained from monoexponential fits of the transient current traces before (■) and after (●) labeling of the oocytes with TMRM, together with reciprocal time constants from fits of fluorescence signals under K^+-free conditions (▲). Data are means ± SEM from six oocytes.

mirrored the Boltzmann distribution for the displaced charge, changes in fluorescence intensity could be directly used as a reporter group to determine the distribution of the enzyme in the E_1P or E_2P states.

In the case of the $s\beta_1$-S62C construct the reciprocal time constants of fluorescence changes and transient currents are both ~50 s^{-1} at potentials above 0 mV (Fig. 2C). This time constant is determined by extracellular Na^+ release, which is rate-limited by the $E_1P \rightarrow E_2P$ conformational transition. However, at hyperpolarizing potentials the reciprocal time constants of the transient currents have a stronger voltage dependence than the fluorescence changes. Under these conditions, the fast step, Na^+ re-binding, is measured by transient currents as this is a measurement of an electrogenic event. In contrast, the fluorescence measurements report the conformational transition $E_2P \rightarrow E_1P$ which is slower. However, there is a weak intrinsic voltage dependence in the fluorescence measurements [25]. In contrast to the alpha N790C construct, there were no functional effects due to the cysteine mutation or labeling with the fluorophore on the beta subunit. More specifically, the $V_{0.5}$ was not statistically different than the wild type enzyme (WT: −74 ± 12 mV and S62C: −80 ± 5 mV). In addition, z_q, which is the amount of charge translocated per translocating step was unchanged (WT: 0.59 ± 0.16 and S62C: 0.70 ± 0.10). Finally, neither the serine to cysteine mutation nor the fluorophore labeling changed the kinetic parameters of the ion pump (Fig. 2C). Thus, fluorophore labeling of the β subunit at this residue can be used to monitor the conformational state or transient current kinetics of the enzyme with a native (or mutant) α subunit. Furthermore, these results also enable an investigation of the K^+ branch of the reaction cycle. In general, it is difficult to examine the K^+ branch by purely electrophysiological methods due to the complicated redistribution of the reaction intermediates. However, it has been determined that kinetic parameters of the ion pump, such as the apparent binding affinity of external K^+, can be elucidated by voltage clamp fluorometry.

As a consequence of this research, it has been possible to examine the effects of N-linked glycosylation on the enzymatic properties of the ion pump [26]. In these experiments, it was determined that following asparagine to glutamine mutagenesis of all potential N-linked glycosylation sites on the beta subunit of the ion pump, no differences were observed in the ratio of the two main conformational states of the ion pump (E_1P or E_2P, see Fig. 1) under Na^+/Na^+ exchange conditions or the kinetics of ion transport when compared to the wild type ion pump [26]. This suggests that although N-linked glycosylation is important for a variety of cellular functions including proper folding, it is not essential for the Na^+/K^+-ATPase.

In a separate study, two highly conserved tyrosine residues in the transmembrane domain of the beta subunit were mutated to tryptophan residues, as it was previously observed that mutation of these residues affect the cation affinity of the holoenzyme [27]. In these experiments, it was observed that upon replacement, a shift towards the E_1P equilibrium and a decrease in the apparent affinity for extracellular K^+ were identified through voltage clamp fluorometry [28]. Interestingly, tryptophan substitutions on the M7 segment of the α subunit of the Na^+/K^+-ATPase resulted in a similar shift, suggesting that residues on both the alpha and beta subunits have a direct effect on the conformational equilibrium of the ion pump.

3. Structural information as revealed by voltage clamp fluorometry

Prior to December 2007, direct structural information on the Na^+ pump was limited. Although a number of homology models were

developed from the x-ray crystal structure of the Ca^{2+}-ATPase, these models provided no information with regard to the beta and FXYD subunits [6,7,29,30]. One approach to examine these proteins was through the expression and purification of multiple members of the FXYD family of proteins (FXYD1, FXYD3 and FXYD4) with subsequent analysis by NMR or circular dichroism [31]. More recently, the structure of FXYD1 was determined in micelles by NMR spectroscopy [32]. Some notable structural features could be elucidated from this structure. Conserved residues, which traverse the transmembrane helix in a groove were proposed to be the binding interface between the alpha and gamma subunits. It was also suggested that phosphorylation of this protein by protein kinase A increases the dynamics around the phosphorylated residue, Serine 68.

A second approach to obtaining structural information on the ion pump was through cryo-electron microscopy. These structures included data to 8 Å, and combined with a homology model of the Ca^{2+}-ATPase, it was possible to assign the large structural domains of the ion pump such as the A, N and P domains as well as the transmembrane domain but not to resolve more specific details of the ion pump [33,34].

In addition to monitoring the conformational state of the enzyme and the kinetics of ion transport through electrophysiological and fluorescence methods, our objective was to determine distance measurements between subunits of the holoenzyme in order to model the relative locations of the alpha, beta and gamma subunits as well as to determine the relative movement of the domains during ion translocation. Thus, constructs were made and expressed in *Xenopus* oocytes which contained a single cysteine construct on the alpha and beta subunits which could then be labeled with both a donor (fluorescein-5-maleimide, FM) and acceptor (tetramethylrhodmaine-6-maleimide, TMRM) fluorophore. These double cysteine constructs (αT309C/βS62C or αN790C/βS62C) were labeled at a FM to TMRM ratio of 1:4 to maximize the signal to noise ratio. To determine distances between two residues, the time dependence of donor bleaching was measured following labeling with the donor fluorophore in the presence or absence of the acceptor fluorophore. It has previously been determined that the observed photodestruction rate of the donor fluorophore depends on the donor cumulative excited lifetime [35]. On a single-molecule level, the donor, when not paired to an acceptor fluorophore, has a long excited state lifetime and therefore on the macroscopic scale photobleaches at a relatively rapid rate. The excited state lifetime of the donor fluorophore is shortened in the presence of an acceptor fluorophore due to energy transfer, which on a single-molecule level allows the donor to undergo more excitation cycles before photodestruction and which subsequently yields a slower macroscopic photobleaching. Furthermore, donor–acceptor pairs which are close together demonstrate a slower photodestruction rate of the donor fluorophore in the presence of the acceptor fluorophore as compared to a set of fluorophore-labeled residues which are further apart [36]. The difference between the rate of the irreversible photodestruction of the donor fluorophore in the presence and absence of the acceptor fluorophore can be used to calculate the distance between the two fluorophores using the Förster equation [35]. Similar methodology has been used to determine distance constraints for potassium channels [37,38].

The rate of decay of the donor fluorophore, in the presence or absence of an acceptor fluorophore, was therefore measured at a controlled membrane potential (−20 mV) to maintain a constant distance between subunits. It should be noted that no significant difference in donor photodestruction was observed for single-cysteine mutant constructs which were labeled with and without the acceptor fluorophore. This result suggests that the ion pump is monomeric as expressed on the surface of *Xenopus* oocytes. For the two sets of residues examined between the alpha and beta subunits, the distances between the residues were calculated from the rate of photodestruction using the Förster equation to be 65 ± 8 Å for αT309C/βS62C and 53 ± 3 Å for αN790C/βS62C [39].

Following the measurement of distance constraints, we were also interested in resolving the relative movement of the alpha and beta subunits during the E_2 to E_1 transition evoked by Na^+ and K^+ concentration changes. However, due to the relatively large error bars on the donor photodestruction measurements, only gross structural changes could be observed using this technique. Therefore we pursued an alternative strategy.

Previously, single-cysteine constructs labeled with a fluorophore were identified because the fluorescence intensity changes during the E_1 to E_2 transition and the fluorescence change is inhibited by high concentrations of ouabain [23,24]. In these experiments, the majority (~80%) of single-cysteine constructs investigated demonstrate no fluorescence changes during the E_1 to E_2 transition of the ion pump and these constructs have not been further studied [23,24]. The absence of fluorescence change for these constructs has two possible explanations: either the residue is not accessible to the extracellular solution and cannot be labeled or the residue is labeled with a fluorophore and there is no environmental change during the E_1 to E_2 transition. Thus, our strategy was to follow changes in fluorescence resonance energy transfer (FRET) at residues which could be labeled with a fluorophore but where the fluorescence intensity is insensitive to the E_1 to E_2 transition.

To identify residues which are labeled but insensitive to the E_1 to E_2 transition on both subunits, double-cysteine mutant constructs were expressed where one residue is known to bind a fluorophore (i.e., demonstrates fluorescence changes during solution exchange or voltage pulses which induce the E_2 to E_1 transition) and where it is unknown whether the second residue binds a fluorophore (i.e., no fluorescence changes during solution exchange or voltage pulses). To investigate FRET between these residues, the constructs were subsequently labeled with donor (FM) and acceptor (TMRM) fluorophores, at a 1:4 ratio, respectively. In these experiments, we were not measuring the relative movement of the two cysteine residues. Instead, one cysteine is used as a relay to transfer energy to the second residue. Only when both sites have a bound fluorophore will one be able to observe a fluorescence change which is the product of the environmental change of TMRM at one residue. It is irrelevant which of the two residues has a donor or acceptor fluorophore as each pairing will exhibit the same result. If, on the other hand, only one of the two residues is labeled, no change in fluorescence will be observed as we are exciting the donor fluorophore and measuring the intensity of the emission of the acceptor fluorophore. Using this methodology, it was determined that αL306C, αI788C and βP66C are accessible to fluorophore labeling but the fluorophores at these residues are insensitive to the E_1 to E_2 transition.

The relative movement of the alpha and beta subunits could therefore be analyzed using these residues. As the distances between the sets of residues, previously determined by irreversible donor photobleaching, are close to R_o, the distance corresponding to 50% efficiency for a specific donor–acceptor pair, this method is highly sensitive to changes in the distance between the two residues [35]. Following labeling with both the donor and acceptor fluorophores for the double-cysteine conformationally-insensitive constructs, an increase in acceptor intensity following solution exchange would indicate that the two residues are moving towards each other, while a decrease in acceptor intensity would indicate that the two residues are moving away from each other. No change in intensity would indicate that the relative distance of the two residues is constant.

Both double-cysteine constructs (αL306C/βP66C and αI788C/βP66C) which did not demonstrate changes in fluorescence intensity following solution exchange and/or membrane pulses were functional, and following the application of 10 mM K^+ a small increase in acceptor FRET intensity was observed. This result is directly attributable to the Na^+/K^+-ATPase as the FRET intensity change is

abolished following the addition of 10 mM ouabain. This data provides evidence that there is a conformational re-arrangement between the alpha subunit M3–M4 and M5–M6 loops and the transmembrane domain of the beta subunit where these domains move towards each other during the E_2 to E_1 transition.

4. The FXYD family of proteins

In contrast to the alpha and beta subunits of the ion pump, the FXYD family of proteins is not required for function. These proteins are single helix spanning proteins which have the consensus sequence FXYD (Phe-Xaa-Tyr-Asp), from which the name of the family is derived, and are from 60 to 160 amino acids in length. In humans, the FXYD proteins have been identified in a variety of tissues and have been shown to affect the apparent K^+ and Na^+ affinity as well as maximal velocity of the Na^+/K^+-ATPase (Table 1).

As the general function of these proteins has been established, more recent work has focused on examining more subtle effects on the holoenzyme. For example, some studies have examined the role of phosphorylation on protein function [40]. It was determined following heterologous expression in *Xenopus* oocytes, that phosphorylation of Ser68 on FXYD1 by protein kinase A increases the apparent Na^+ affinity. In contrast to this result, phosphorylation mediated by protein kinase C increases the maximal pump current of the ion pump. It is notable that the effect of protein kinase A is observed on both the α1/β1 and α2/β1 isoforms, while the effect of protein kinase C is only observed with the α2/β1 isoform. Thus, not only does the FXYD family member modulate the ion pump, but these proteins have different effects on different isoforms of the alpha subunit. Furthermore, this data should be taken in the context of the NMR structures described above where phosphorylation of FXYD1 increases the dynamics of the protein and therefore the apparent Na^+ affinity of both the α1 and α2 isoforms [32].

Our motivation with regard to the FXYD proteins was to gain insight into the location of the gamma subunit (FXYD2) relative to the alpha and beta subunits. Similar to our studies of the alpha and beta subunits, we were able to obtain distance constraints between the three subunits following heterologous expression in *Xenopus* oocytes (52 ± 3 Å for αI788C/β/γR19C, 66 ± 7 Å for α306C/β/γR19C and 55 ± 6 Å for α/βP66C/γR19C). This enabled us to model the relative location of the gamma subunit in comparison to the alpha and beta subunits as shown in Fig. 3 [41].

We also identified a residue on the γ subunit, R19C, which could be labeled with a fluorophore but did not demonstrate fluorescence changes upon changes in membrane potential and/or external solution exchange. Following this step, double cysteine constructs were expressed to determine whether there was a conformational rearrangement of the γ subunit when compared to the α or β subunits of the Na^+/K^+-ATPase.

Neither the αL306C/β/γR19C construct nor the αI788C/β/γR19C construct demonstrated a change in fluorescence intensity of the acceptor fluorophore following donor excitation upon addition of 10 mM KCl. In contrast, the α/βP66C/γR19C construct exhibited a significant change in fluorescence intensity of the acceptor fluorophore following donor excitation upon addition of 10 mM KCl. This fluorescence change is directly attributable to the Na^+/K^+-ATPase as the FRET intensity change is abolished following the addition of 10 mM ouabain.

Table 1
The expression and kinetic effects of the FXYD family of proteins.

	Common name	Protein expressed	Kinetic effects
FXYD1	Phospholemman	Heart	Lowered Na^+ affinity Lowered K^+ affinity
FXYD2	Gamma subunit	Kidney	Lowered Na^+ affinity
FXYD3	Mat-8	Stomach	Lowered Na^+ affinity Lowered K^+ affinity
FXYD4	CHIF	Kidney	Increased Na^+ affinity Lowered K^+ affinity
FXYD5	RIC, dysadherin	Kidney	
FXYD6	Phosphohippin	CNS	Lowered Na^+ affinity Lowered K^+ affinity
FXYD7		Brain	Lowered K^+ affinity

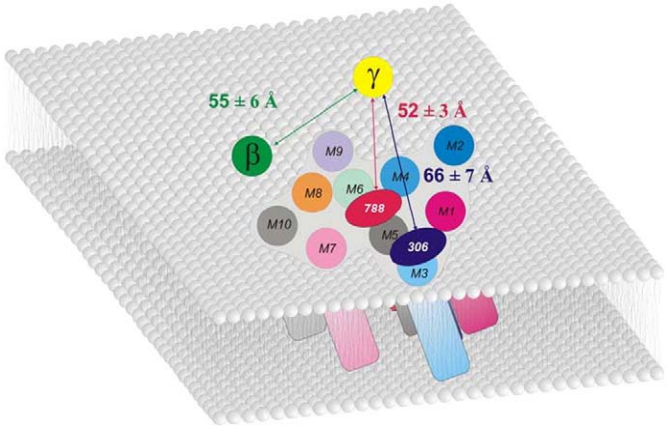

Fig. 3. Two-dimensional helix arrangement viewed from the surface of the cell, including ten transmembrane helices which form the alpha subunit (M1–M10), the transmembrane domains of the beta (in green) and the gamma (in yellow) subunits and the distance constraints determined in this study. Distance constraints were determined using residues αL306C (in blue), αI788C (in red), βP66C and γR19C [41].

By combining this data, a dynamic picture of each subunit emerges where the beta subunit moves relative to the alpha and gamma subunits during ion translocation. In contrast, the alpha and gamma subunits appear to have no significant relative distance change during ion translocation. This is consistent with the observation that no changes of fluorescence intensity were observed following fluorophore labeling of the gamma subunit and voltage pulses, suggesting that the environment of the gamma subunit does not change during the E_1 to E_2 transition.

5. X-ray crystallography of the Na^+/K^+-ATPase

In 2007, the x-ray structure of the Na^+/K^+-ATPase complex was published at a resolution of 3.5 Å (Fig. 4) [42]. The structure was obtained in the presence of two rubidium ions, which mimic potassium ions, in the transmembrane region of the alpha subunit and represents the ion-occluded E_2 conformation as described by the Albers–Post scheme (Fig. 1). The resolution is sufficiently high to identify residues which are in proximity to the rubidium ions (Glu327, Ser775, Asn776, Glu779 and Asp804). These residues could contribute to binding the rubidium, and therefore potassium ions.

In addition to the alpha subunit, and in contrast to the previous crystal structures of the SERCA Ca^{2+}-ATPase, which contains only the alpha subunit, the transmembrane domains of the beta and gamma subunits could be resolved in this crystal structure. Although the large cytosolic domain of the beta subunit could not be resolved, there is evidence that this domain covers the extracellular loops, M5–M6 and M7–M8. This could be an initial structural evidence that the beta subunit is involved in K^+ occlusion. Furthermore, the beta subunit is angled at 45° across the plasma membrane and makes contact with transmembrane domains seven and ten. The transmembrane domain of the gamma subunit has also been resolved and is located outside of the ninth transmembrane helix and not within the transmembrane 2 and 9 groove as has been described by both biochemical and electron microscopy experiments [27,33].

This initial crystal structure has already facilitated experimental approaches which were not previously readily apparent. Following the elucidation of the x-ray structure, a K^+ site was identified in the P

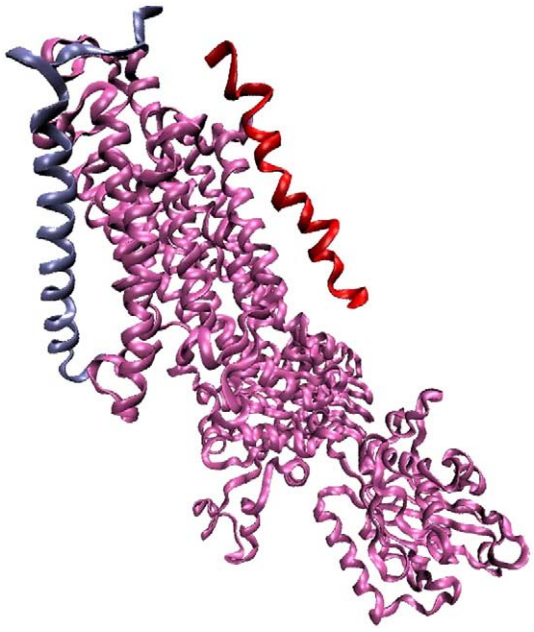

Fig. 4. X-ray crystal structure of the Na^+/K^+-ATPase determined to 3.5 Å [42]. The cytoplasmic side is down. The α-, β- and γ-subunits are coloured mauve, blue and red, respectively.

domain of the ion pump [43]. This is a non-transport site which, when occupied, increases E_2P dephosphorylation and stabilizes E_2. Furthermore, this data helps to provide further insight as to how cytoplasmic K^+ inhibits the ion pump at low ATP.

Although valuable information has been gained from the x-ray structure from Nissen et al., many more structures of the SERCA pump are available. Thus, an interest from some groups has been to combine structural data from the Ca^{2+}- and Na^+/K^+-ATPase to obtain more information about the movements of different domains of the Na^+ pump. On this front two residues have been identified, located in the extracellular loops M3–M4 and M7–M8 which inhibit the ion pump upon application of an oxidizing reagent (copper-phenanthroline). This suggests that these loops are in close proximity as the process is reversible after treatment with dithiothreitol [44]. Interestingly, residues on the M3–M4 loop have also been identified which following cysteine mutagenesis and fluorescence labeling, demonstrate changes in fluorescence intensity following voltage pulses and/or solution exchange [45].

6. Genetic and functional linkages to disease states

The well-studied Na^+/K^+-ATPase has only recently been implicated in certain disease states. Much of this work has been facilitated by functional analyses of mutants identified following sequencing efforts. An excellent paper which summarizes mutations which have been linked to familial hemiplegic migraine type two, sporadic hemiplegic migraine and rapid-onset dystonia parkinsonism as mapped on the Na^+/K^+-ATPase crystal structure has recently been published [46].

In addition to this extensive list of mutations, recent data has suggested that the ion pump is linked to additional diseases. For instance, FXYD6 is located on chromosome 11q23.3, which is in the region which has been linked to schizophrenia (11q22–24) [47,48]. Subsequent experiments have demonstrated that FXYD6 is present in highest amounts in the postnatal 3 week old brain and is still present in the adult brain. This suggests that the protein is important in postnatal development and the adult brain [49]. Most interestingly, the level of expression of FXYD6 was found to be depressed in a postmortem study of schizophrenia and bipolar disorder as compared to healthy subjects, suggesting a link between FXYD6 and schizophrenia (the Stanley Brain Collection, http://www.stanleyresearch.org/brain/). However, more recent studies have suggested that this link is not present in a Japanese population [50].

The expression level of the Na^+/K^+-ATPase has also been shown to be affected in the axon membrane of multiple sclerosis (MS) patients as the ion pump is reduced within chronic MS lesions [51]. More recently it was observed that, whereas in unaffected patients the ion pump is expressed uniformly along the axon, there is a higher level of expression of the enzyme along axons with acute MS lesions, but no detectable protein along the membrane in chronic MS lesions [52]. Whether there is a genetic component to this observation or if this is a cause or effect of the disease process itself is not known. However, this data suggests that one result of MS is that there is a change in the electrogenic machinery of the axons.

7. Summary and outlook

The past few years have been witness to an exciting convergence of functional, structural and physiological data and much work remains to be done in each of these areas. As mutations or phenotypic data is accumulated, this data can be modeled onto existing structures of the holoenzyme where biochemical and biophysical experiments can be performed to understand the physiological outcome of these changes in the wild type ion pump.

An important development has been the utilization of site-specific probes which can be used as a reporter group to monitor the conformational state and the kinetic parameters of the wild type and mutant ion pumps in situ during ion transport. These experiments have resulted in an effective methodology to investigate the influence of glycosylation on the ion pump as well as the importance of specific residues in stabilizing conformations of the Na^+ pump.

However recent results from the x-ray structure and biochemical and biophysical results are not entirely congruent. Whereas our experiments and those which employed site-directed mutagenesis on the alpha subunit suggested that the FXYD proteins are located in the M2–M9 groove, the x-ray structure shows this protein proximal to the M9 transmembrane domain [41,42,53]. This difference could simply be due to the error in the measurements that are intrinsic to the biochemical and biophysical experiments versus the high resolution structure or could demonstrate a dynamic interaction of the gamma subunit with the holoenzyme as has been described with the NMR experiments. Thus, although much has been learned over the past few years in regard to the mechanism of the Na^+/K^+-ATPase, there are plenty of open questions to investigate.

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