Department of Woman and Child Health, Pediatric Unit,
Astrid Lindgren Children's Hospital
Karolinska Institutet, Stockholm, Sweden

Imaging Studies of Cell Physiology with Particular Reference to Na,K-ATPase Function

Ronnie M Andersson

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"Do what you can, with what you have,
right where you are."
Theodore Roosevelt

To my family: my father Jerry and mother
Gudrun and my brothers and sisters

Abstract

The membrane protein Na,K-ATPase is well known for its critical function of transporting sodium out of the cell and potassium into the cell, thereby creating a fundamental electrochemical gradient upon which several other important cell functions are dependent. In the current study we have investigated novel aspects of Na,K-ATPase function in cell physiology. In order to study this, a considerable part of the present thesis has involved methodological development of different microscopic techniques.

It is well established that Na,K-ATPase is important for maintaining cell volume, membrane potential and reabsorption of electrolytes in the kidney. The new findings in this study are: 1) A role for Na,K-ATPase in the regulation of cell adhesion. Partial inhibition of Na,K-ATPase activity significantly reduced cell attachment to fibronectin. The results suggest that this effect is mediated by perturbation of normal Ca^{2+} signaling and a reduction of focal adhesion kinase activity. These findings indicate the importance of Na,K-ATPase during development and differentiation. 2) A major role of Na,K-ATPase activity in regulatory volume decrease (RVD). A direct link between Na,K-ATPase activity and the ability of COS-7 cells to perform RVD was demonstrated. With site directed mutagenesis of the α subunit of Na,K-ATPase it was demonstrated that Na,K-ATPase may have both negative and positive effects on the rate of RVD. Further knowledge about the interaction between ouabain and Na,K-ATPase was achieved by demonstrating that a Leu-799 to Cys substitution in the α subunit of rat Na,K-ATPase produced complete ouabain resistance. This residue belongs to the extracellular loop between transmembrane segment 5 and 6 of the enzyme which is of importance for ion occlusion and ion transport. The same mutant has been demonstrated to have a positive effect on the rate of RVD.

This thesis was also concerned with the rigorous use of fluorescence lifetime imaging to investigate intracellular pH. We made theoretical predictions

concerning sensitivity and noise which were supported by experimental results. Our study on the influence of probe binding on pH based fluorescent lifetime imaging indicates that the method is not a straightforward approach to measure pH in the absence of correction for the effect of probe binding. We find it likely that other fluorescent ion probes have similar probe-binding-sensitive fluorescence lifetimes. However, the overall effect is difficult to predict.

In conclusion, this thesis demonstrates that Na,K-ATPase is a key enzyme in a variety of important cell functions beyond those that were previously known. In addition to being the dynamic modulator of ion transport, Na,K-ATPase also serves a primary role in regulatory cell volume decrease and cell attachment.

Publications

This thesis is based on the following publications:

I **Andersson R.M,** Cheng S.X.J, and Aperia A
"Forskolin-induced down-regulation of Na^+,K^+-ATPase activity is not associated with internalization of the enzyme"
Acta Physiol Scand **16**: 39-46, 1998

II Belusa R, Aizman O, **Andersson R.M,** and Aperia A
"Changes in Na^+-K^+-ATPase activity influence cell attachment to fibronectin"
Am J Physiol Cell Physiol **282**: C302–C309, 2002

III **Andersson R.M,** Aizman O, Aperia A, and Brismar H.
"Modulation of Na^+,K^+-ATPase Activity is of Importance for RVD"
Submitted

IV **Andersson R.M,** Uhlén P, Brismar H, and Aizman O
"Functional Consequences of the Mutation L799C in the M5-M6 Segment of the Na^+,K^+-ATPase Catalytic Subunit"
Submitted

V Carlsson K, Liljeborg A, **Andersson R.M,** and Brismar, H
"Confocal pH imaging of microscopic specimens using fluorescence lifetimes and phase fluorometry: influence of parameter choice on system performance"
J Microscopy **199**, Pt 2: 106-114, August 2000

VI **Andersson R.M,** Carlsson K, Liljeborg A, and Brismar H.
"Characterization of Probe Binding and Comparison of Its Influence on Fluorescence Lifetime of Two pH-Sensitive Benzo[*c*]xanthene Dyes Using Intensity-Modulated Multiple-Wavelength Scanning Technique"
Anal Biochem **283,** 104–110, 2000

Abbreviations

ATP	Adenosine Triphosphate
Ca^{2+}	Calcium
CCD	Charged Couple Device
Da	Dalton
FAK	Focal Adhesion Kinase
FL	Fluorescence Lifetime
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
H^+	H_3O^+
IMS	Intensity-Modulated Multiple Wavelength Scanning
K^+	Potassium
LSCM	Laser Scanning Confocal Microscope
MAPK	Mitogen Activated Protein Kinase
Na,K-ATPase	Sodium, Potassium Adenosine-Triphosphatase
Na^+	Sodium
OEM	Electro Optic Modulator
PKA	Protein Kinase A
PKC	Protein Kinase C
PMT	Photo Multiplier Tube
RMS	Root mean square noise

RVD	Regulatory Volume Decrease
RVI	Regulatory Volume Increase
SNR	Signal-to-noise ratio

Contents

Abstract	i
Publications	iii
Abbreviations	iv
Contents	vi
Introduction	1
Na,K-ATPase	1
Structure-Function Relationship	1
Cation Binding	4
Ouabain Binding	4
Regulation	6
Physiological Role	7
Na,K-ATPase in Cell Volume Regulation	8
The Regulatory Volume Decrease Process	9
Na,K-ATPase and K^+ channels	10
Na,K-ATPase in Cell Attachment	12
Fluorescence – Spectral Properties	14
Fluorescence – Lifetime Properties	16
Fluorescence Microscopy	18
Confocal Microscopy	19
Ratiometric Measurements	21
Measurement of Fluorescence Lifetime	22
Noise	24
Detectors	24
Noise Analysis	24
Noise in Optical signal	24
Electronic Noise	26

Specific Aims 28

Materials and Methods 29

Materials 29

- Fluorescent Probes 29
- Cells 30

Methods 31

- How to Measure Na,K-ATPase Activity 31
- How to measure cell volume 32
- How to Measure Cell Attachment 32
- Measurements of Intracellular Ion Concentrations 33
- Ratiometric Measurements : Advantages and Disadvantages 33
- Lifetime Measurements: Adantages and Disadvantages 34

Results and Comments 36

- Ouabain and Na,K-ATPase Interactions (Paper I and IV) 36
- Na,K-ATPase in Cell Attachment (Paper II) 37
- Na,K-ATPase in Cell Volume Regulation (Paper III and IV) 38
- Methodological Aspects about Fluorescent Lifetime Imaging (Paper V and VI) 41

Discussion and Future Perspectives 44

Use of Fluorescent Lifetime Measurement in Cell Biology 48

Conclusion 49

Acknowledgements 51

References 53

Paper I – VI

Introduction

There are two related general areas in this thesis. One deals with physiological aspects of Na,K-ATPase, which is hypothesis driven experimental research. The other deals with methodological and technological development of microscopical methods, which is more applied technical studies. Rigorous assessment and understanding of the microscopical methods was essential for their proper application to the Na,K-ATPase studies in this thesis. Several of the Na,K-ATPase experiments, i.e. measurement of K^+ affinity of enzyme and rate of regulatory volume decrease (RVD), could not have been performed without use of advanced microscopical methods.

Na,K-ATPase

The Na, K-ATPase is an ubiquitous plasma membrane protein complex that belongs to the P-type family of ion motive ATPases. Under normal conditions, it couples the hydrolysis of one molecule of ATP to the exchange of three Na^+ for two K^+ ions, thus maintaining the normal gradient of these cations in all mammalian cells. This is of vital importance for cellular function and subserves activities such as volume regulation, cell attachment, pH maintenance, secondary active transport and generation.

Structure-Function Relationship

The Na,K-ATPase is a heterodimeric protein with a main catalytic α subunit (112 kDa) which is associated with a smaller glycosylated β subunit (55 kDa) (1). In the membrane, it appears to exist as a dimer $(\alpha/\beta)_2$.

There exists four isoforms of the α and three isoforms of the β subunit of the Na,K-ATPase. These isoforms are expressed in a tissue- and development-specific manner (2). Whereas the $\alpha1$ and $\beta1$ are ubiquitously expressed, the expression of $\alpha2$ and $\alpha3$, as well as $\beta2$ and $\beta3$ is more tissue-specific. The $\alpha2$ subunit is found mainly in skeletal muscle and heart, whereas $\alpha3$ is predominantly detected in neurons (2). Recently, a fourth type of α subunit was discovered in sperm (3).

It is generally agreed that the catalytic α subunit of Na,K-ATPase transverses the cell membrane ten times (M1-M10) as outlined in figure 1. Both the N- and C-termini are located in the cytosol. Most of the α subunit is located inside the cell in a major intracellular domain. The β subunit crosses the membrane only once; a small N-terminal segment is located in the cytoplasm, whereas C-terminus and most of the subunit is located outside the cell. In addition, a third subunit of Na,K-ATPase, known as γ subunit (7.5 kDa), has been identified (4); Therien, 2001 #1036]. The gamma subunit is a small single transmembrane protein that associates in approximately equimolar amounts with the α and β subunit of Na,K-ATPase. Recent studies indicate that the γ subunit may influence Na,K-ATPase by increasing the apparent affinity of the α subunit for ATP and shifting the conformational equilibrium towards the Na^+ binding state of the enzyme (5). The physiologic role of the γ subunit is not clear.

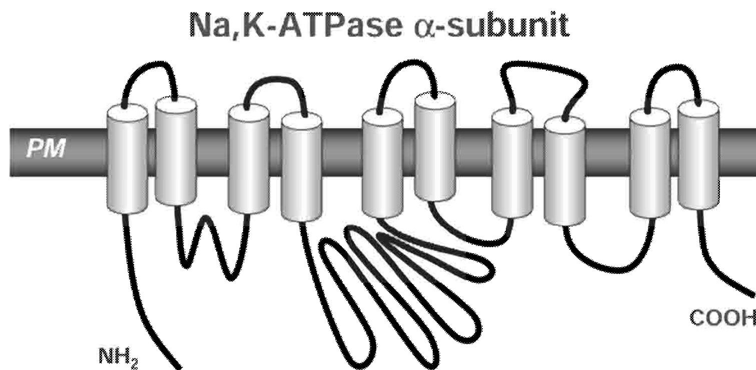

Fig. 1. Ten transmembrane spanning model of the α subunit of Na,K-ATPase.

All of the studies in this thesis addressing Na,K-ATPase (**paper I-IV**), are related to the function of the α subunit. This is because the α subunit contains all the major properties of the enzyme: ATP binding site, binding sites for Na^+ and K^+, the regulatory kinase phosphorylation sites and the binding site for ouabain (1).

The ATP binding site is located in the large cytoplasmic entity between M4 and M5. Regulatory phosphorylation sites, see figure 4, have been demonstrated both in the cytoplasmic loop between M8 andM9 near the C-terminus (protein kinase A) and in the beginning of the N-terminus side before the M1 segment (protein kinase C) (6-8). The regulatory site for protein kinase A is highly conserved throughout species and isoforms (8; 9), suggesting a general role for PKA in the regulation of sodium pump activity. The PKC phosphorylation site is unique for the rat Na,K-ATPase α1 subunit. For instance, endogenous α1 Na,K-ATPase in COS-7 cells used in **paper I** lacks this PKC phosphorylation site. There are, however, indications that more than one phosphorylation site exists and that these sites are present in other species (10).

Cation Binding

Of special interest is the M5-M6 segment, which is well conserved among different isoforms and species (11-13). Studies, mainly performed on sheep Na,K-ATPase, have indicated that this segment is important for ion occlusion, energy transduction, and ouabain affinity (14-20), although other amino acid residues in other parts of the subunit also have been identified to have similar functions (21; 22). The M5-M6 region contains four amino acid residues, Ser-775, Glu-779, Asp-804, and Asp-808, most likely involved in cation coordination and transport. The asparagine residues 804 and 808 are via their negatively charged carboxyl groups important for coordinating Na^+ and K^+ occlusion. In particular Ser-775 has been shown to be critical for K^+ interactions (23). When substituting the Ser with an Ala or a Cys, Na^+ dependence of the enzyme was normal, whereas that for K^+ was drastically reduced with a 34-fold decrease for Ala and a 13-fold decrease for Cys. Indeed, Burns et al. showed that not only was the apparent K^+ affinity affected by a change of a single amino acid, Leu-793 to Ala in sheep, but also the ouabain affinity was affected (24). In addition, **Paper IV** in this thesis reinforces that the M5-M6 segment is important for both K^+ and ouabain affinity of the enzyme. Both the apparent K^+ affinity and the ouabain affinity were decreased by substituting Leu-799 with Cys in the α1 subunit of rat Na,K-ATPase, a site which corresponds to Leu-793 in sheep.

Ouabain Binding

Ouabain, an analogue of digoxin found in the plant *Digitalis purpurea* , is a cardiac glycoside which has been used for centuries in the treatment of heart disease, see figure 2 for chemical structure. Both glycosides

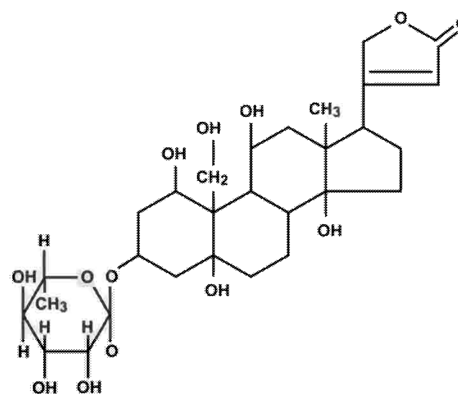

Fig. 2. Chemical structure of ouabain

have been shown to be very specific ligands with antagonistic effect on Na,K-ATPase. Radioactive [^{3}H]ouabain can therefore be used to assess the number of Na,K-ATPase molecules in the plasma membrane. We used this method in **paper I** where we studied whether phosphorylation of Na,K-ATPase may cause internalization of the enzyme. Any change in the number of Na,K-ATPase molecules present in the membrane will be detected as change in bound [^{3}H]ouabain. This approach is different from some other methods of quantifying the number of Na,K-ATPase units in that it only measures the number of active pump units.

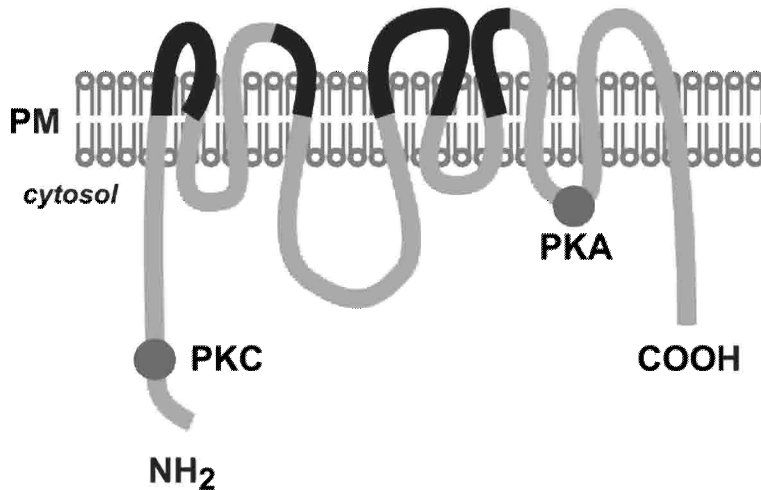

Fig. 3. Ouabain binding sites on α subunit of Na,K-ATPase.

The ouabain binding structure of Na,K-ATPase, figure 3, is quite complex in its arrangement (21; 22). The oubain binding pocket is believed to consist of the extracellular M1-M2 and M4 segments, and the M5-M6 and M7 segments of the α subunit.

Furthermore, different experimental approaches suggest that the M5-M6 segment may move during the catalytic cycle following phosphorylation [Arguello, 1991 #958; (19; 20). Consequently, the interaction of ouabain with the M5-M6

segment would be critical in terms of both binding and inhibition mechanisms. In **paper IV** we have mutated Leu-799 in the M5-M6 segment of rat α1 subunit into a Cys residue. This rendered the enzyme completely ouabain resistant.

Regulation

Due to the critical importance of Na,K-ATPase for cellular function, it is expected that the activity of Na,K-ATPase is not constant, but instead, precisely regulated, responding to changes in the ionic environment. This can be achieved by changing both sodium pump activity (short-term regulation) and the number of functioning units (long-term regulation) (25).

Here we focus on the short-term regulation of sodium pump activity, which is known to be mediated by a number of factors such as substrate availability (Na^+, K^+ and ATP), hormones and neurotransmitters, and interaction with cytoskeletal proteins. This regulation is accomplished within minutes, thus permitting the rapid adaptation of Na,K-ATPase to changing ionic conditions. Short-term regulation does not involve *de novo* protein synthesis, but instead is achieved through the modulation of kinetics of existing pumps by i.e. phosphorylation / dephopshorylation events that affect its turnover number (the number of ions transported per unit and time), its affinity for substrates and/or its abundance at the cell surface through translocation of existing units from an intracellular storage compartment. In regard to the last alternative, we showed in **Paper I** that translocation of Na,K-ATPase is not necessarily the regulating mechanism in COS-7 cells in response to phosphorylation of the enzyme. Two important phosphorylation sites in rat α1 subunit for PKA and PKC, respectively, can be seen in figure 4.

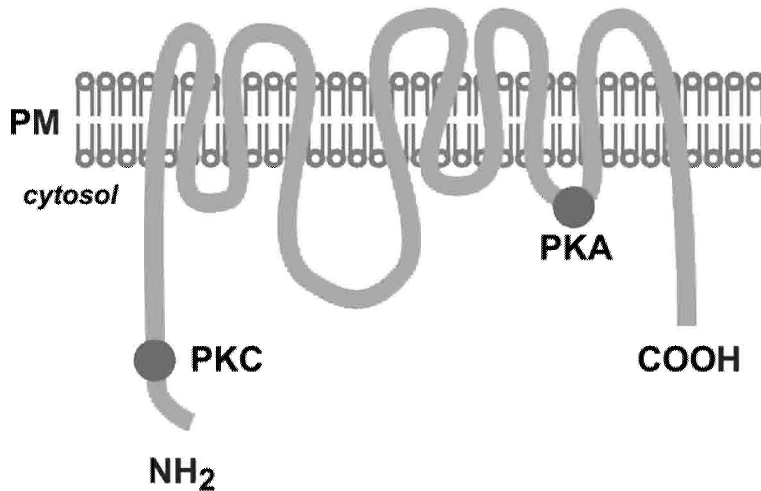

Fig. 4. Schematic drawing illustrating PKC and PKA phosphorylation sites on rat $\alpha 1$ subunit of Na,K-ATPase.

Physiological Role

Na,K-ATPase is an important enzyme for physiological functions, i.e. ion reabsorption in the kidney, muscle contraction, neuronal signaling, etc. In an overall perspective this is illustrated by the fact that Na,K-ATPase activity is responsible for approximately 30% of total oxygen consumption (26) and in some organs such as kidney and brain, this value can reach up to 80% (27).

As previously mentioned, the Na^+ gradient generated by Na,K-ATPase is used in many tissues to support the activity of different ion-exchange transport systems. In cardiac and skeletal muscle cells, Na,K-ATPase acts as an indirect regulator of muscle contraction and vascular constriction. Na^+/H^+ exchanger plays an important role in the regulation of pH_i. By being electrogenic, the Na,K-ATPase largely determines the resting membrane potential in cells. In excitable tissues, such as neurons and muscle cells, the pump is also involved in the generation of action potentials by its ability to rapidly restore the transmembrane ionic gradient. The Na^+ concentration gradient established by Na,K-ATPase is also used to

accomplish transport of some metabolites (sugars, amino acids) into the cell, figure 5.

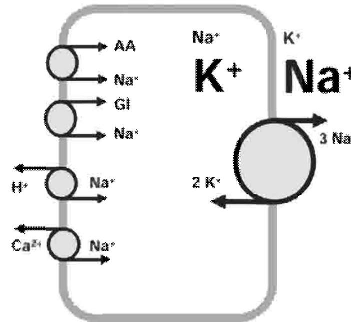

Fig. 5. Na,K-ATPase plays a key role in the maintenance of transmembrane Na^+, K^+ gradients and provides the driving force for Na^+-coupled transporters.

Na,K-ATPase inhibition and dissipation of the Na^+ gradient will lead to a decrease of nutrition supply and subsequently to inhibition of cell growth and proliferation (Lopina, 2001). Na,K-ATPase is also responsible for fluid movement across transporting epithelia. In the kidney, Na,K-ATPase plays a key role in regulating tubular Na^+ reabsorption. Na,K-ATPase generates the driving force that is essential for the reabsorption of a number of other ions and solutes, amino acids and sugars in the kidney. Na,K-ATPase is expressed in particularly high amounts in renal tissue.

Na,K-ATPase in Cell Volume Regulation

With only few exceptions (28), the membranes of animal cells are highly permeable to water, importantly facilitated by aquaporins (29-32). Any imbalance of intracellular and extracellular osmolarity is paralleled by respective water movement across cell membranes and subsequent alterations of cell volume. Most mammalian cells are surrounded by extracellular fluid with almost constant osmolarity. But even under these physiological steady-state conditions, the cell volume must be maintained constant by a “pump-leak mechanism”, by which the

osmotic pressure arising from the cytoplasmic impermeable solutes is counteracted by the activity of Na,K-ATPase requiring a constant expenditure of energy. Furthermore, even if cell volume were not threatened, mechanisms are still needed to alter cell volume, such as during hypertrophy and atrophy, apoptosis, and differentiation.

In addition to maintaining a constant cell volume at physiological steady state, most cells investigated have mechanisms that become activated after perturbations that affect cell volume , resulting in volume recovery processes. Swelling causes cells to activate transports systems for K^+, Cl^-, and certain organic molecules, resulting in a loss of osmolytes and the concomitant osmotically obligated loss of water (regulatory volume decrease, or RVD). On the other hand osmotic shrinkage can activate uptake systems for ions or trigger the expression of transporters for organic osmolytes. Shrunken cells can thereby increase their volume toward the normal level by net uptake of Na^+, Cl^-, and often K^+ as well, and concomitant uptake of water (regulatory volume increase, or RVI).

The role of Na,K-ATPase in these responses is incompletely understood. But there is evidence that total inhibition of Na,K-ATPase will abolish RVD in cells (33; 34). In several cell types, shrinkage (leading to RVI) has been observed to activate Na,K-ATPase, which ultimately will replace accumulated Na^+ with K^+ (35). Na,K-ATPase is considered to have a passive role in these processes. We show in **paper III** and **IV** that the activity of Na,K-ATPase does play an active role even in the early phase of RVD.

The Regulatory Volume Decrease Process

A number of different channels and transporters have been shown to be involved in RVD. However, the involvement of Na,K-ATPase needs further elucidation by actual experiments as well as understanding the context of RVD. Importantly, RVD involves reducing cell volume through extrusion of Cl^- and K^+ ions, followed by

water. A generalized scheme for the RVD process may be described as a net efflux of Cl^- and K^+ which lead to loss of water and restoration of the cell volume. When the cell swells Ca^{2+} enters through stretch-activated Ca^{2+} channels. The swelling itself and/or the Ca^{2+} opens various Cl^- and K^+ channels. With a few exceptions (36), the swelling-activated Cl^- channels observed are independent of $[Ca^{2+}]_i$. The cytoskeleton may be a more likely activator here (36). Many different types of K^+ channels have been reported to be activated by cell swelling including Ca^{2+}-activated maxi K channels (BK), Ca^{2+}-activated K^+ channels, stretch-activated K^+ channels, and voltage-dependent K^+ channels of different types (37). It has been described that the opening of K^+ channels follows that of Cl^- channels (38).In regard to K+ channels, ATP seems to play a key role in the initiation of the RVD response in several cell types (39-41). In Ehrlich ascites tumour cells addition of ATP evokes a Ca^{2+}-dependent ChTX-sensitive K^+ efflux (42), whereas the swelling induced K^+ current is known to be ChTX-insensitive. Thus, swelling of the cell in itself does not appear to affect ATPi in such a way that it leads to activation of swelling-activated K^+ current, which means that some other factor(s) is involved.

The consequence of a loss of Cl^- and K^+ via separate transport pathways has been shown to lead to a higher loss of K^+ compared to Cl^- to an extent which depends upon the cellular pH buffering capacity (37) . The reason for acidification in osmotically swollen cells is likely to result from recycling of Cl^- for HCO_3^-. This acidification of the cytoplasm activates the Na^+/ H^+ exchanger, which restores pH_i. However, the activation of the Na^+/ H^+ exchanger leads to an incomplete RVD by the uptake of osmotically active Na^+.

Na,K-ATPase and K^+ channels

The vast majority of K^+ is located in cells with only a very small fraction of total K^+ located in the extracellular space. The accumulation and high concentration of K^+ in cells depends on the coordinated activity of Na,K-ATPase and K^+-channels.

In renal tissue, Na,K-ATPase and K^+-channels are not only responsible for the proper distribution of K^+ but they also serve several additional cell functions. These include: (i) the generation and maintenance of negative cell potential, (ii) the regulation of cell volume, (iii) the recycling of K^+ across apical and basolateral membranes, (iv) the secretion of potassium in distal nephron segments, and (v) the control and regulation of contractility in mesangial and renal vascular cells.

There is evidence in renal proximal tubule cells that ATP sensitive K^+ channels link the basolateral K^+ conductance to the turnover rate of Na,K-ATPase (43-45). Thus, ATP_i is a coupling modulator of parallel Na,K-ATPase-K channel activity in renal proximal tubule. ATP sensitive K^+ channels are gated according to the availability of ATP_i. An increase in ATP_i closes the channel, whereas a decrease in ATP_i opens it. As the largest energy consumer in the renal proximal tubule cells, an increase in Na,K-ATPase activity can cause ATP levels to fall whereas it increases with enzyme inhibition. Subsequently, the extent of basolateral membrane K^+ recycling changes in parallel with Na,K-ATPase consumption of the negative channel modulator (ATP).

Recent studies have suggested a novel inwardly rectifying K^+ channel, Kir7.1, to be functionally coupled to Na,K-ATPase (46; 47). This channel exhibits very low single-channel conductance because the positively charged arginine residue in the pore region, conserved among other Kir family members, is replaced with a methionine (48). These unique pore properties of Kir 7.1 make it a strong candidate for the hypothetical low conductance K^+ channel that is functionally coupled to Na,K-ATPase by recycling K^+ (46; 47). However, it is unknown whether Kir 7.1 is an ATP sensitive channel. Other (but not all) Kir channels are ATP sensitive and this function may be mediated through a channel associated protein.

The functional relationship of Na,K-ATPase, the K^+-channel and the modulatory role of ATP_i provide important rationale in **paper III** and **IV** for

suggesting how Na,K-ATPase may play a more active role in RVD than previously thought.

Na,K-ATPase in Cell Attachment

Na,K-ATPase is an integral membrane protein and is well connected to the cytoskeleton through various cytoskeleton proteins, such as actin, ankyrin, adducin, and spectrin (49-53). It has been demonstrated that mutations in Na,K-ATPase may affect both cell morphology and its ability to attach to fibronectin (6). Further, in **paper II** we have linked the level of cell attachment to the degree of Na,K-ATPase activity.

There are several different cell-surface receptors that can mediate cell to extracellular matrix adhesion (54). Integrins are one of the most important (55). Since another ion-transporter, the Na^+/ H^+ exchanger, which possesses ankyrin binding sites, has been shown to be directly influenced by integrins (56; 57), it is possible that a similar regulatory relationship may occur between Na,K-ATPase and integrins.

Integrins are not simply adhesion receptors whose sole function is the mediation of physical transmembrane connections. Importantly, they also conduct both outside-in and inside-out signaling (58; 59). Activation of integrins, through binding to extracellular matrix proteins, leads to a rise in $[Ca^{2+}]_i$ and autophosphorylation of Focal Adhesion Kinase ($pp125^{FAK}$ or FAK) (60). How Na,K-ATPase activity influences cell attachment is not known. One can speculate that Na,K-ATPase activity influences levels of $[Ca^{2+}]_i$ during attachment, thereby affecting Ca^{2+} dependent processes for attachment.

Fluorescence

The use of fluorescent imaging techniques in the study of living biological systems has become an important experimental tool in modern biology.

Fluorescence, outlined in figure 6, is a property of some molecules to absorb light of certain wavelengths (i.e. photons with certain energy) and then after a short time interval, the fluorescence lifetime, to re-emit light at longer wavelengths (i.e. photons with lower energy).

Different fluorescent probes, ranging from small molecules to entire proteins, enable the detection and localization of biomolecular assemblies inside living cells or measurement of intracellular ion concentration with exquisite sensitivity and selectivity. New fluorescent probes continue to become available and the ways in which they are used are becoming increasingly creative. The use of antibodies is important to target fluorescent probes to specific structures within the cells. During the last decade, the use of green fluorescent protein (GFP) has provided new possibilities to visualize and follow the movement of proteins inside living cells.

Over recent years novel imaging technologies have been developed and older techniques refined. Commonly used imaging methods such as confocal and multiphoton microscopy, when combined with techniques such as fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM), provide powerful strategies for the study of molecular events in intact cells.

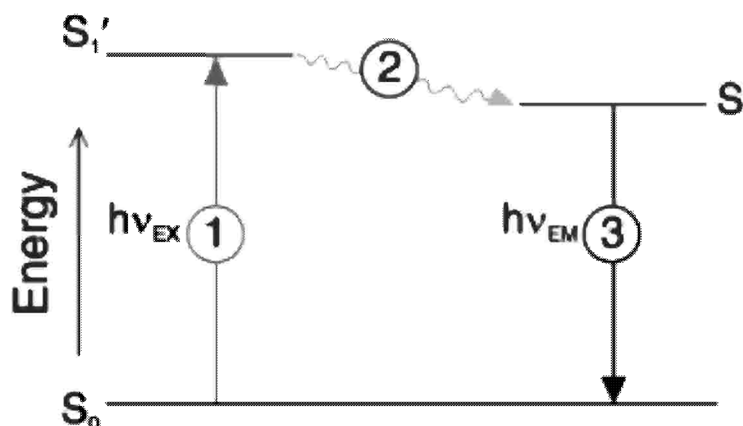

Fig. 6. Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. Excitation of fluorophore means that a photon of energy $h\nu_{EX}$ is absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. The energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates.

Fluorescence – Spectral Properties

The spectral properties of fluorescence are frequently used in immunolabeling studies where different antibodies are coupled to fluorophores with different excitation/emission spectra. By recording the fluorescence intensity in different wavelength bands the localization of several different proteins in the same sample can be determined. In this thesis the use of spectral information has been used in a different application to determine cell volume and intracellular ion concentrations.

Measured fluorescence intensity is quantitatively dependent on the concentration, extinction coefficient, and the fluorescence quantum yield of the probe and also on the excitation light intensity and the fluorescence detection efficiency of the measurement instrument.

In some biological studies, measurement of fluorescent intensity in a certain wavelength band as such is sufficient. This is the case in **paper III**, where we measured changes in cell volume by measuring the change in fluorescence intensity of the cytosolic probe calcein in a thin optical section by confocal microscopy.

There exist probes whose fluorescence intensity is sensitive to the concentration of certain ions. When using these probes it is important to check and compensate for potential artifacts from in leakage or uneven concentration of the probe. This limits their use in qualitative measurements. However there exist some probes whose spectral properties permit the use of ratio imaging. These probes often have an ion-bound and an ion-free state, each with separate spectral properties. In ratio imaging those properties are used to determine the amount of bound and free probe, i.e. the concentration of the ion. Two recordings with different excitation or emission spectra are performed. The ratio of the two measured values can then be used as an index of the actual ion concentration. This index can be related to the true ion concentration by a calibration measurement where the cells are filled with known concentrations of the measured ion. The requirement for the probes used in ratio measurements is that there is an isobestic point in their spectra, i.e. at a certain excitation or emission wavelength the probe is insensitive to differing ion concentrations. The measurements are typically done by measuring on either side of the isobestic point. For Fura-2 this corresponds to 340 and 380 nm, figure 7.

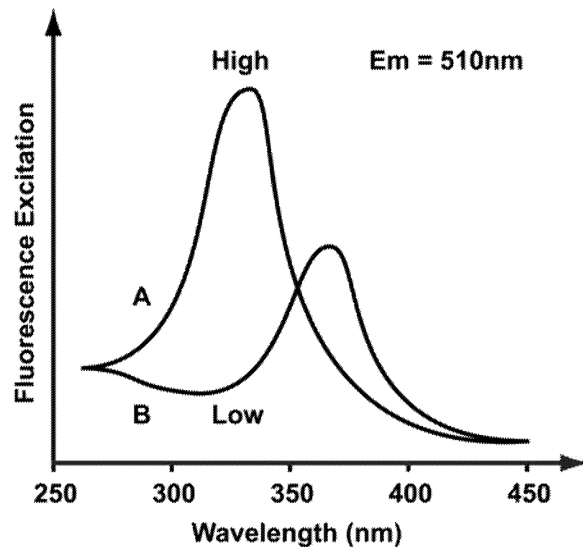

Fig. 7. Excitation spectra of Fura-2. High (A) represent Ca^{2+}-saturated Fura-2, while Low (B) represent Ca^{2+}-free Fura-2. Emission (Em) is detected at 510nm.

Fluorescence – Lifetime Properties

In addition to fluorescence intensity, fluorophores possess a property known as the fluorescence lifetime (FL) which is a measure of the decay rate of the fluorescence emission after the excitation radiation has ceased, figure 8. The decay of fluorescence lifetime is described by the equation $I(t)=I_0e^{-t/\tau}$, where I(t) is the intensity measured at time t, I_0 is the initial intensity immediately after the excitation pulse and τ is the fluorescence lifetime. Fluorescene lifetime is typically in the range of 3-10 ns for common fluorophores used in biology.

Changes in the chemical microenvironment can have drastic effects on the FL of a fluorophore even when there is only a minor effect on the fluorescence intensity (61). Thus, the two properties of a fluorophore, intensity and lifetime can give complementary information.

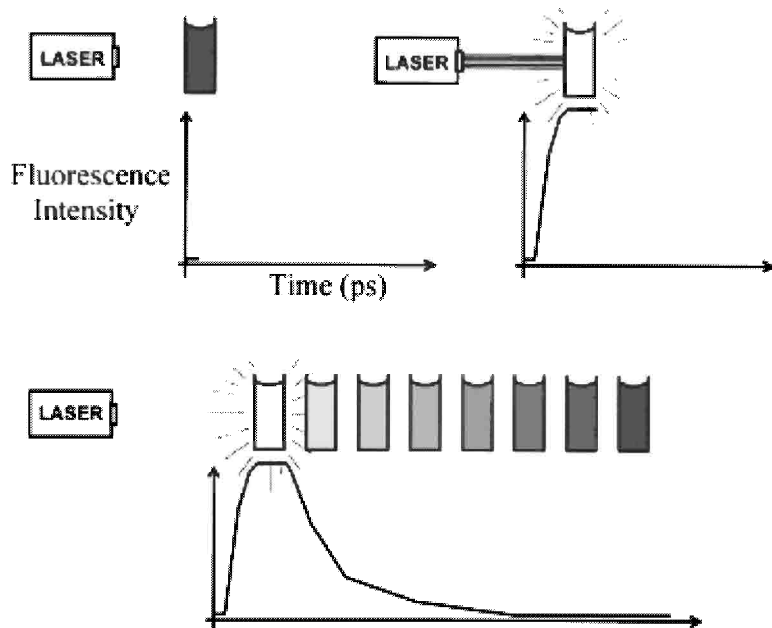

Fig. 8. Fluorescence lifetime. *Top left:* a vial containing a solution of a fluorophore is about to be illuminated by an excitation laser. *Top right:* upon excitation, fluorescent light is almost instantaneously emitted from the vial. *Bottom*: upon cessation of the excitation light, the fluorescence decays gradually with a characteristic decay profile. This is the FL of the fluorophore in this solution and this decay characteristic will change with the composition of the solution containing the fluorophore (e.g. Ca^{2+}, pH etc.) but not with the concentration of the fluorophore *per se.*

Fluorescence Microscopy

A fluorescence microscope, outlined in figure 9, is basically a conventional light microscope with added features and components that extend its capabilities. A conventional microscope uses light to illuminate the sample and produce a magnified image of the sample. A fluorescence microscope uses light of a narrow wavelength band and of high intensity to illuminate the sample. This light excites fluorescent dyes in the sample, which then emit light of a longer wavelength. A fluorescent microscope also produces a magnified image of the sample, but the image is based on the second light source (i.e. emitted) rather than from the light originally used to illuminate, and excite, the sample.

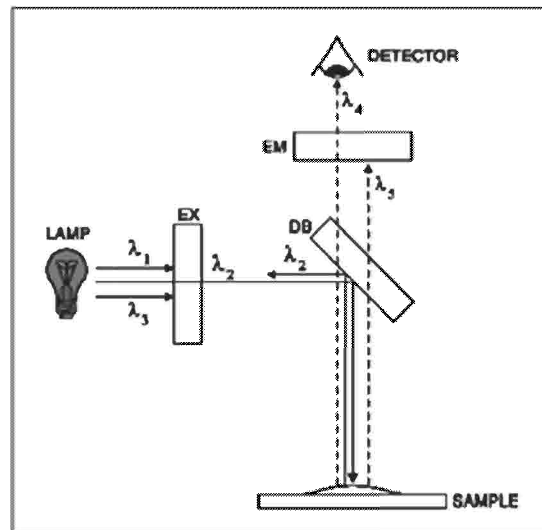

Fig. 9. Principle of the fluorescence microscope. The desired excitation wavelength (λ2) is selected from the spectral output of the lamp by the excitation filter (EX) and directed to the sample via the dichroic beamsplitter (DB). The beamsplitter separates emitted fluorescence (---) from scattered excitation light (—). The emission filter (EM) selectively transmits a portion of the emitted fluorescence (λ4) for detection and blocks other emission components (λ5).

Nearly all fluorescence microscopes use the objective lens to perform two functions, focus the excitation light on the sample and collect the emitted fluorescence. In order to excite fluorescent species in a sample, the optics of a fluorescent microscope must focus the excitation light on the sample to a greater extent than is achieved using the simple condenser lens system found in the illumination light path of a conventional microscope. This type of excitation-

emission configuration, in which both the excitation and emission light travel through the objective, is called epifluorescence. The key to the optics in an epifluorescence microscope is the separation of the excitation light from the fluorescence emission emanating from the sample. In order to obtain either an image of the emission without excessive background illumination, or a measurement of the fluorescence emission without background "noise", the optical elements used to separate these two light components must be very efficient.

In a fluorescence microscope, a dichroic mirror is used to separate the excitation and emission light paths. Within the objective, the excitation and emission wavelength share the same optics. In a fluorescence microscope, the dichroic mirror separates the light paths. The excitation light reflects off the surface of the dichroic mirror into the objective. The fluorescence emission passes through the dichroic to the eyepiece or detection system. The dichroic mirror reflects wavelengths of light below a certain wavelength and transmits wavelengths above this wavelength.

Two filters are used along with the dichroic mirror, an excitation and an emission filter. In order to select the excitation wavelength, an excitation filter is placed in the excitation path just prior to the dichroic mirror. In order to more specifically select the emission wavelength of the light emitted from the sample and to remove traces of excitation light, an emission filter is placed behind the dichroic mirror. In this position, the emission filter functions to both select the emission wavelength and to eliminate any trace of the wavelengths used for excitation.

Confocal Microscopy

The laser scanning confocal microscope (LSCM) is an essential tool for many biomedical imaging applications. The major imaging modes of the LSCM includes single optical sections, multiple wavelength images, three-dimensional

reconstructions, and living cell and tissue sequences. In this thesis LSCM was used in **paper III**, **V** and **VI**.

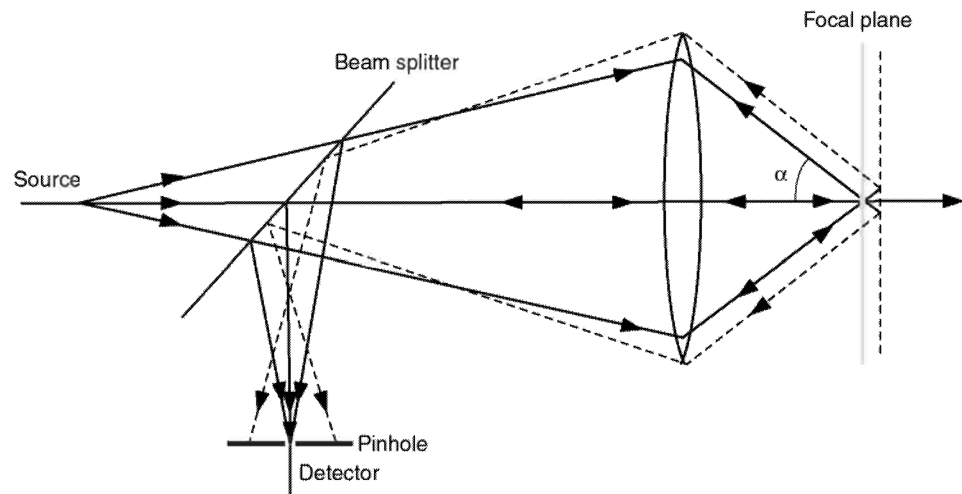

Fig. 10. Principle of the confocal microscope.

The confocal measurement principle ensures that light which does not originate from the focal plane of the microscope objective does not pass through the pinhole and consequently is not detected, figure 10. Light rays from below the focal plane come to a focus point well before reaching the detector pinhole, after which they diverge so that they are physically blocked from reaching the detector by the detector pinhole. Similarly, light from above the focal plane comes to a focus behind the detector pinhole, so that most of that light is prevented from reaching the detector by the edges of the pinhole. A 3D image can be processed by taking a series of confocal images at successive planes through the specimen and assembling them.

One major advantage of the confocal measurement principle is the high vertical and lateral resolution. A theoretical increase of nearly 32% lateral

diffraction limited resolution compared to conventional microscopy is achieved through the physical pinhole filtering.

Ratiometric Measurements

Ratiometric imaging is performed by recording two images of a sample with different settings for excitation or emission detection. The ratio image is formed by dividing the two images pixel by pixel. This kind of measurement is routinely done using epifluorescence microscopy, and was in this thesis done for measuring $[Ca^{2+}]_i$ and $[Na^{+}]_i$ in **paper II**,**III** and **IV** using the fluorescent probes Fura-2 and SBFI The images are recorded with a CCD camera coupled to an image intensifier and stored on a computer for data analysis.

Using confocal microscopy for ratiometric measurement is in theory possible, but fixed excitation wavelengths, which characterize the lasers that are used as excitation sources, limit the use of confocal microscopy in dual excitation ratiometric measurements.

Ratiometric measurements are not limited to simple ion concentration measurements. In **paper IV,** we describe a novel method of measuring the activity and apparent K^{+} affinity of Na,K-ATPase through measurement of $[Na^{+}]_i$. Hence, a higher level of interpretation can be obtained from ratiometric data with appropriate experimental conditions.

A typical preparation of cells for studying intracellular ion concentration, i.e. $[Na^{+}]_i$, is exemplified by the following. Cells grown on glass coverslips are then placed in physiological salt solution containing 5 μM membrane-permeable SBFI, Na^{+} sensitive fluorophore, for three hours and one hour with probenecid, 0.6 mM, at 31 °C in an atmosphere of 5% CO_2, - and 95% air. Probenecid is added to block the transport of SBFI out of the cells. Loading temperature was kept lower than

37 °C to minimize compartilization of the dye into vesicles. Finally, coverslips are mounted in a chamber and put on the microscope stage for investigation.

Measurement of Fluorescence Lifetime

Since fluorescence lifetime (FL) is usually measured in nanoseconds, special techniques are needed for its measurement. One of two methods measures the FL directly by illuminating the sample with a short pulse and measuring the fluorescence decay with a fast detector (time-domain measurements), as illustrated in figure 11. The second method, used in **paper V** and **VI,** is called frequency-domain measurement. The Intensity-Modulated Multiple Wavelength Scanning (IMS) Technique is such a method and involves a continuous source of excitation which is intensity modulated at high frequency (62-65). The resulting fluorescence is varying at the same frequency, but, because of the FL, there will be a phase shift in the emission compared to the excitation. By measuring the phase shift between excitation and emission the FL can be calculated. The experimental setup for the IMS technique is explained and illustrated in figure 11.

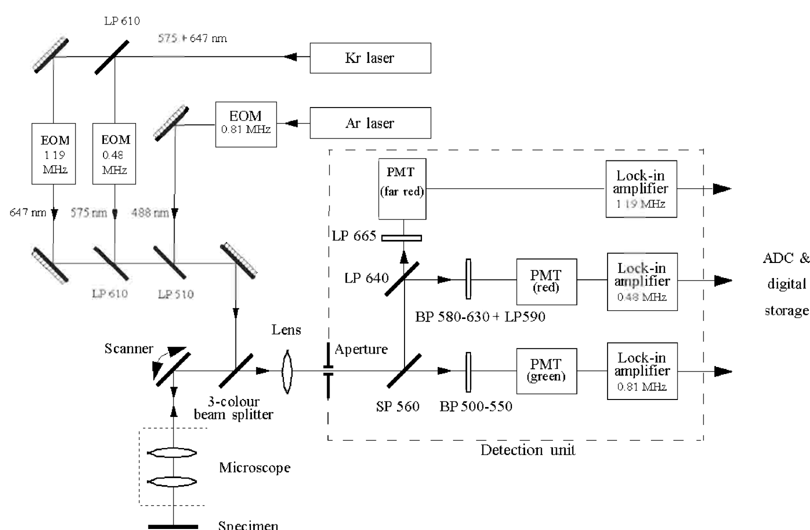

Fig. 11. The experimental setup for fluorescence lifetime imaging. Laser of choice (Kr/Ar laser) which illuminates the specimen is modulated by an electro-optic modulator (EOM). The excitation light enters the confocal microscope via photo-tube. The fluorescent light passes through a dichroic beam-splitter and the confocal aperture and reaches the selected photomultiplier tube (PMT) detector via a filter. The detector output signal enters the lock-in amplifier which is phase-locked to the modulating frequency. Here frequency and phase selective detection produces two output signals S_1 and S_2. The ratio S_1/S_2 is calibrated using a reference specimen of known values.

In **paper VI** we used the pH sensitive probe SNAFL together with FLIM to monitor pH inside living cells and evaluate the sensitivity of this probe to cellular components such as lipids and proteins. In a study performed by another group, FLIM was used to show that the acidification of *stratum corneum* does not occur via a uniform gradient, but through progressive accumulation of acidic mircrodomains, a process which involves the Na^+/H^+ antiporter (66). In general FLIM is a preferred technique together with FRET. This is particularly true when different GFP construct are used and where FLIM circumvents the spectral overlaps of the GFPs by measuring their fluorescence lifetime instead of fluorescence intensity (67; 68). There is no difference in preparing cells for either fluorescence lifetime or ratiometric measurement. Dye concentration is in the same range for both methods.

Noise

Detectors

Quantitative fluorescence measurement requires devices to detect the photons emitted by the probed specimen. For image acquisition a CCD-camera or a scanning device combined with a photomultiplier tube are commonly used. These are connected to a microscope, electronic equipment, and a computer. The recorded image consists of pixels that form a matrix where each pixel corresponds to a point measurement. CCD-cameras register all pixels in the image at once, while photomultiplier tubes require scanning devices and consequently register the pixels sequentially. Since the image is a collection of recorded data, measurements of different kinds can be performed on the image. Often pseudo colour maps are used to enhance the visibility of the image/measurement data.

Noise Analysis

Quantum noise represents the fundamental limit of the achievable signal-to-noise (SNR) ratio of an optical system, like the microscope. Examples of quantum noise are photon noise in an optical signal and shot noise in an electrical conductor or semiconductor. Noise analysis was in this thesis limited to the studies of fluorescence lifetime but the same principles apply to fluorescence intensity.

Noise in Optical signal

The noise problem in an optical signal arises from the fundamentally statistical nature of photon production. It cannot be assumed that, in a given pixel for two consecutive but independent observation intervals of length T, the same number of

photons would be observed, figure 12. Laws of quantum physics apply to photon production and therefore only an average number of photons can be observed within a given time interval. The spread in the results is described by a Poisson distribution.

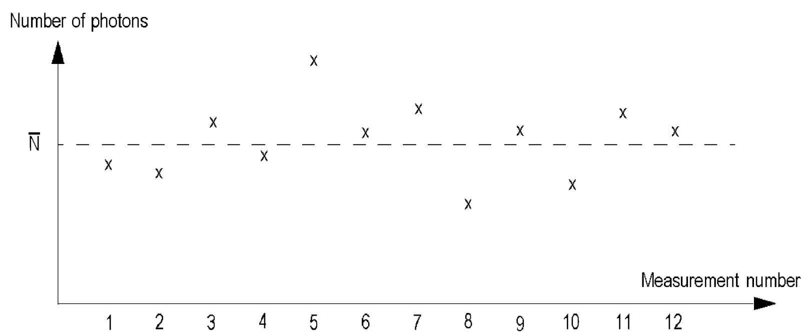

Fig. 12. Repeated measurements of photons in a detector will give varying photon numbers (N). Reprinted with permission from Dr. K. Carlsson, "Imaging Physics," KTH, 2002.

It is critical to understand that even if there were no other noise sources in the imaging chain, the statistical fluctuations associated with photon counting over a finite time interval T would still lead to a finite SNR.

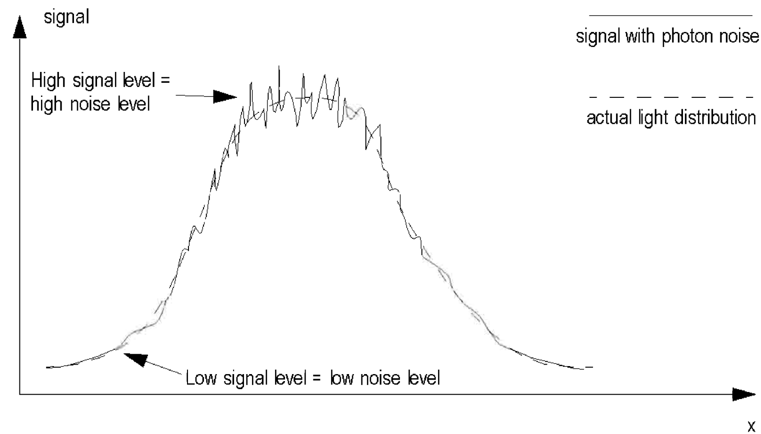

Fig. 13. Photon noise superimposed on actual light distribution. Reprinted with permission from Dr. K. Carlsson, "Imaging Physics," KTH, 2002.

Photon noise differs from many other types of noise in that it increases when the signal level increases, figure 13. However in relative terms, SNR becomes better as signal increases more than photon noise. For very bright signals, where the average number of photons exceeds 10^5, the noise fluctuations due to photon statistics can be ignored if the sensor has a sufficiently high saturation level. For fluoresence microscopy in general and confocal microscopy in particular, the number of photons is well below this limit and quantum photon noise is therefore important to consider in all measurements.

Electronic Noise

Besides photon quantum noise in the optical signal, there are also other sources of noise belonging to the detection system in the microscope, referred to as shot noise. This is the noise caused by random fluctuations in the motion of charge carriers in a conductor. Two sources of shot noise can be recognized in the microscope system: multiplication noise in the photo multiplier tube (PMT) and amplifier noise.

When thermal energy in the PMT is not negligible in comparison to the work function, a significant number of electrons are released from the cathode surface by thermal emission. These electrons are accelerated in the electric field and a so-called “dark current” is created. If the dark current was constant, substraction from the signal current would eliminate the problem. Unfortunately, the dark current will inevitably fluctuate due to the quantity of the current, being a current of electrons.

Specific Aims

1. To study various ouabain and Na,K-ATPase interactions
2. To evaluate the role of Na,K-ATPase activity in cell attachment
3. To study the role of Na,K-ATPase in regulatory volume decrease
4. To investigate the use of fluorescence lifetime properties of pH sensitive fluorophores in biological systems and evaluate the method's sensitivity and noise

Materials and Methods

For detailed description of all materials and methods used in this thesis, please refer to the original papers enclosed.

Materials

Fluorescent Probes

Several fluorescent probes have been used in this thesis (Table 1). All probes in this thesis are cell-permeant dyes since they are substituted with acetoxymethyl (AM) esters of their carboxyl groups. When inside the cells, the probes become cell-impermeable because intracellular esterases hydrolyze the ester bonds and carboxyl groups are reformed. However some probes "leak" out of the cell faster than others. This is because of so called ABC transporters, which actively transport molecules not belonging inside the cell. For some reason this may vary from probe to probe and from cell type to cell type. Probenecid is a chemical often used to block this outward transport and was used in **paper IV** when measuring $[Na^+]_i$ with the probe SBFI in COS-7 cells.

Loading concentrations of the probes are typically between 1 and 30 μM. However, loading time into cells is very much dependent on probe and cell type. Of the probes used in this thesis, calcein, **paper III** and **IV**, was the probe with the shortest loading time of 30 min and SBFI had the longest loading time of 3 to 4 hours. Various chemical agents must occasionally be used to get the probe into proper solution so that they can be taken up by the cell, i.e. we used pluronic acid to facilitate the entry of SBFI into cells.

Fluorescent Probe	*Concentration*	*Action*
Carboxy-SNAFL-1 AM Ester Acetate	30 μM	pH_i
Carboxy-SNAFL-2 AM Ester Acetate	30 μM	pH_i
Fura-2 AM Ester Acetate	2 μM	$[Ca^{2+}]_i$
Calcein AM Ester Acetate	5 μM	Cell volume
SBFI-AM Ester Acetate	5 μM	$[Na^{+}]_i$, (measure Na,K-ATPase activity and its apparent K^+ affinity)

Table 1. Fluorescent probes used in this thesis and their working concentrations.

Cells

In this thesis we mainly used COS-7 cells, a well established cell line derived from African green monkey kidney (69). These cells have become a recognized cell model for expression of various proteins, including that of rat or sheep Na,K-ATPase subunit isoforms (70; 71). COS-7 cells express a basic set of cellular receptors and signalling proteins which make them a good model for studying function and regulation of Na,K-ATPase. Since COS-7 cells are replateable without protease treatment, we found that these cells are particular useful in our cell attachment studies. A major shortcoming of COS-7 cells, but not of that much concern in this thesis, is that they are rather primitive and undifferentiated. One indication of this is that even though COS-7 cells are proximal tubule cells, they have lost their ability to differentiate their plasma membrane into an apical and a basolateral membrane .

Methods

The use of microscopical techniques has been of central importance in this thesis (see "Fluorescence"). Here, special focus is made on methods for studying Na,K-ATPase function and the advantages and disadvantages of ratiometric and lifetime imaging.

How to Measure Na,K-ATPase Activity

In this thesis (**paper I**, **II**, **III**, and **IV**) three different methods were employed to measure the activity of Na,K-ATPase, i.e. ATP hydrolysis, $^{86}Rb^{+}$ uptake, and K^{+} -sensitive Na^{+} efflux.

Each of the these methods determines Na,K-ATPase activity, but they do so by analyzing different aspects of the enzyme's activity. In the first two methods ouabain is used to measure Na,K-ATPase specific ATP hydrolysis or $^{86}Rb^{+}$ uptake. The third method is ouabain-independent and is a more indirect method of measuring Na,K-ATPase activity, based on the postulate that Na,K-ATPase is the only transporter to mediate a K^{+} -sensitive Na^{+} efflux from the cell. This method was developed as a modification of that of Borin (72), in order to evaluate the ouabain-insensitive mutant, L799C, of rat $\alpha 1$ subunit in **paper IV**. This method will be discussed in the next chapter. The three methods employed here belong to two different groups, i.e. ex vivo cell measurement (ATP hydrolysis) and in vivo cell measurement ($^{86}Rb^{+}$ uptake and K^{+} sensitive Na^{+} efflux).

The hydrolytic activity of Na,K-ATPase was determined on preparations of cell membranes under optimal conditions of Na^{+}, K^{+}, and ATP for the enzyme. This is a straightforward process of determining the Na,K-ATPase activity and changes in its kinetic properties at both saturating and non saturating conditions of sodium. However, the in vivo situation of the enzyme is completely lost, i.e. no difference in intra and extracellular ion concentrations, no membrane potential, and absence of soluble intracellular regulatory molecules of the enzyme. Another

limitation of this method is that it determines total Na,K-ATPase activity, which includes pumps inserted in the plasma membrane as well as those located intracellularly.

To avoid these limitations, $^{86}Rb^+$ uptake and K^+ sensitive Na^+ efflux are the techniques of choice. Between the two, $^{86}Rb^+$ uptake represents the best method to measure Na,K-ATPase activity under physiological conditions, due to the use of the specific inhibitor of the enzyme. However, the $^{86}Rb^+$ uptake does not permit discrimination between primary $^{86}Rb^+$ uptake (mediated by Na,K-ATPase) and secondary uptake (secondary to increased Na^+ entry). This can easily be resolved by the use of ATP hydrolysis where the Na^+ concentration is clamped.

How to measure cell volume

In **papers III** and **IV** involving studies of cell volume changes, RVD was detected as a change in calcein fluorescence intensity in a single focal plane through the center of cells with a LSCM (73). The calcein AM ester is a pH and calcium insensitive fluorescent dye, which easily penetrates cell membranes and is evenly distributed in the cytosol and nucleus of the cell. This method is straightforward in its application. Cells do not have to be removed from their solid support and brought into suspension; instead only a short incubation time with calcein is necessary. Cell volume measurements with LSCM are fast because changes in intensity are collected from a very thin optical section. This makes LSCM a favorably method compared to other methods.

How to Measure Cell Attachment

Cell attachment is the stage where cells adhere to a solid structure, i.e. in **paper III** this is a fibronectin coated glass plate. There are a variety of assays for the measurement of cell adhesion, including simple counting by hand, labelling of cells

(radioactive markers, fluorescent markers etc.), measuring proteins, nucleic acid content, and enzymatic reactions from attached cells.

In **paper III** we chose to label cells with [^{3}H]-thymidine. Thymidine is a building block of DNA, and [^{3}H]-thymidine will therefore be incorporated into DNA. By measuring radioactivity we can calculate how many cells attach to any surface. The advantage of this method is that thymidine is a naturally occurring molecule in cells as compared to a fluorescent probe which potentially could interfere with the attachment process itself. A second advantage is that measurement of radioactivity is very sensitive. A disadvantage of the method is the radioactivity itself, but tritium has low energy radiation and is used in very small amounts.

We measured the early phase, zero to 80 min, of the interaction between COS-7 cells and fibronectin, which minimizes the risk for involvement of unspecific interactions. We chose to work with COS-7 cells because of their high expression levels of fibronectin binding proteins, $\alpha5\beta1$ integrin, and that these cells do not require protease treatment to be replated.

Measurements of Intracellular Ion Concentrations

In this thesis intracellular Na^+, Ca^{2+} and pH have been measured with either ratiometeric or lifetime measurements. The technical details of these measurements are presented in the chapter "Fluorescence Microscopy". Here, I limit the discussion to advantages and disadvantages of each method.

Ratiometric Measurements : Advantages and Disadvantages

Ratiometric measurement of fluorescence will be independent of the amount of fluorophore in the microscopic light path, differences in cell thickness, presence of

organelles excluding the dye, variable dye loading, dye leakage, and dye photobleaching.

Cumbersome intracellular calibrations are necessary due to the fact that the fluorescence of probes is sensitive to cellular constituents like proteins and lipids.

In general fluorescent probes are unnatural chemical entities in the cell and their cellular toxicity is far from completely studied for all probes. There are inherent limitation of probes when it comes to measurement of ion concentration because fluorophores may buffer ions and thereby affect ion homeostasis.

Lifetime Measurements: Adantages and Disadvantages

Changes in chemical microenvironment may have drastic effects on the FL of a fluorophore even when there is only a minor effect on fluorescence wavelength (cf. ratiometric optical probes–i.e. fluorescence wavelength imaging) (74). Conversely, fluorophore concentration, fluorescence intensity, light path length and photobleaching do not affect the FL (74-77).

Multiple FLs, from distinct fluorophores, can be measured simultaneously with resolution from a single measurement using frequency-domain spectral analysis [(75; 78; 79). This means that multiple chemical components can be measured from a single image capture. A further unique property of the FLIM method described by Lakowicz et al. (77) is the ability to suppress the emission signal for any desired lifetime.

However, cumbersome intracellular calibrations are still necessary due to the fact that the FL of probes is not insensitive to cellular constituents like lipids and proteins. This is systematically studied in **paper VI** for the pH sensitive probes SNAFL-1 and SNAFL-2, both ex vivo (in capillaries) and in vivo (in cells).

Probes used for FL measurement are unnatural chemical entities, as for probes used in ratiometric measurement, and consequently FL probes have the same limitations as ratiometric ones.

Results and Comments

(References to figures,tables, and equations are found in the individual papers)

Ouabain and Na,K-ATPase Interactions (Paper I and IV)

In **paper I**, changes in the number of Na,K-ATPase molecules on the cell surface was assessed by saturating [^{3}H]ouabain binding conditions. Since endogenous Na,K-ATPase in COS cells is much more sensitive to ouabain (µM) than the expressed rat enzyme (mM), saturating ouabain conditions could not be achieved with the latter. The oubain binding process occurs quite rapidly, and at 15 minutes association and dissociation of ouabain have reached equilibrium (Figure 1a). Under present conditions, non-specific ouabain binding to the cells corresponds to only 1-2% of the total binding measured at the optimal concentration of 0.20 µM [^{3}H] ouabain used (Figure 1b).

We attempted to alter the number of pumps in the membrane through 2nd messenger stimulation, i.e. forskolin together with IBMX, OAG, and PDBu, which leads to activation of PKA and PKC with subsequent phosphorylation and inactivation of Na,K-ATPase (Figure 2a & Table 1). However, none of these treatments caused any measureable change in ouabain binding (Figure 2b, Figure 3 & Table 1) and it was only PKA activation that had an effect on endogenous Na,K-ATPase activity in COS-7 cells (Figure 1a). It is known that the PKA phosphorylation site on Na,K-ATPase α subunit is highly conserved between isoforms and species, whereas the PKC site is present is some species such as the rat, but not in others such as the monkey and human. We validated the ability of the method to measure internalization of Na,K-ATPase through ATP depletion. ATP depletion is well known to lead to internalization of the pump and it was demonstrated in COS-7 cells.

Further knowledge about the ouabain and Na,K-ATPase interaction was achieved in **paper IV**. Here, a single amino acid substitution at Leu-799 to Cys in the M5-M6 segment caused virtually complete ouabain resistance of the rat $\alpha 1$ subunit. This was confirmed by total ATP hydrolysis measurement and by measuring $[Na^+]_i$ in the presence of ouabain. Interestingly, the M5-M6 segment is quite conserved. Several of its amino acid residues are the same in all Na,K-ATPases' sequenced to date (11-13). The hairpin loop has been identified as important for ion occlusion, energy transduction, and ouabain affinity [Jewell-Motz, 1993 #957; Arguello, 1991 #958; Feng, 1994 #274; Feng, 1995 #959; Lutsenko, 1994 #960; Lutsenko, 1995 #948]. In addition, there is also experimental evidences that this domain may move during the catalytic cycle following phosphorylation (15; 19; 20). This consequently gives an interesting model how the interaction of ouabain with M5-M6 segment could be critical in terms of the binding and inhibition.

Na,K-ATPase in Cell Attachment (Paper II)

The purpose of **paper II** was to evaluate the physiological significance of the relationship between Na,K-ATPase activity and cell attachment. Studies were performed on untransfected COS-7 cells expressing endogenous Na,K-ATPase, COS-7 cells stably transfected with rat wild type $\alpha 1$ subunit of Na,K-ATPase or mutated (L799C – see **paper IV** for details) ouabain-resistant rat $\alpha 1$ subunit of Na,K-ATPase. COS-7 cells express high levels of $\alpha 5 \beta 1$ integrin (unpublished data from Dr. Roger Belusa), a major fibronectin receptor. In this study we demonstrate that partial inhibition of the activity of Na,K-ATPase has a dramatic influence on cell attachment to fibronectin. Using ouabain at concentrations that only partially inhibit Na,K-ATPase activity significantly decreased cell attachment to fibronectin (Figure 1). We observed a dose-dependent decrease in cell attachment in response to ouabain in untransfected cells and cells expressing wild type Na,K-ATPase, but

not in cells expressing ouabain-insensitive Na,K-ATPase (Figure 2). Lowering of the K^+ concentration in the extracellular medium inhibited the Na,K-ATPase activity and attenuated attachment in all three types of cells (Figure 3a). Reactivation of Na,K-ATPase by restoring the extracellular K^+ completely restored cell attachment (Figure 3b). Transient inhibition of Na,K-ATPase activity did not influence cell viability. Inhibition of Na^+ influx pathways did not have any significant effect on cell attachment. Na,K-ATPase is known to indirectly modulate $[Ca^{2+}]_i$ which itself plays an important role in the early phase of cell attachment. In control cells we observed a transient increase in $[Ca^{2+}]_i$ during the initial phase of attachment (Figure 4a). Inhibition of Na,K-ATPase activity in cells exposed to ouabain caused a sustained increase in $[Ca^{2+}]_i$ that obscured these Ca^{2+} transients (Figure 4b). Focal adhesion kinase is one of the key mediators during integrin-mediated cell adhesion to fibronectin (80). Autophosphorylation of FAK on tyrosine-397 is an initial step in its activation. The level of FAK autophosphorylation was determined by using an antibody against the phospho-397 FAK. We found that inhibition of Na,K-ATPase activity significantly reduces FAK autophosphorylation during the early phase of attachment (Figure 7). Cell surface integrin expression was not changed by Na,K-ATPase inhibition.

Na,K-ATPase in Cell Volume Regulation (Paper III and IV)

Na,K-ATPase function is recognized for its importance in long term cell volume regulation, preventing the cell from swelling since its cytoplasm is hyperosmotic compared to extracellular fluid. In **paper III** and **IV,** we explore the incompletely investigated role of Na,K-ATPase activity in short term cell volume regulation, i.e. RVD.

In **paper III**, we demonstrate through three independent methods that the activity of Na,K-ATPase is important for the rate of RVD in COS-7 cells. These cells were stably transfected with either wild type rat $\alpha 1$ subunit or a PKC site

deficient mutant, S23A, form of rat α1 subunit. The involvement of Na,K-ATPase activity in RVD was first studied by the dose-dependent effects of ouabain on RVD. Inhibition of WT Na,K-ATPase by ouabain was found to dose dependently attenuate the rate of RVD in response to changing osmolarity from 300 to 250 mOsm.

Next we observed an elevated ouabain sensitive Rb^+ uptake during RVD. During the first 2 min of RVD, the ouabain sensitive Rb^+ uptake was more than doubled, whereas there was no significant change in ouabain insensitive Rb^+ uptake.

As PKC is an important modulator of both RVD and Na,K-ATPase activity, we compared RVD in cells expressing either WT rat Na,K-ATPase or S23A rat Na,K-ATPase. The rate of RVD in cells expressing S23A rat Na,K-ATPase was only about 60% of the rate in cells expressing WT rat Na,K-ATPase.

To investigate the mechanism behind the difference in RVD rate between WT rat and S23A rat Na,K-ATPase expressing cells, we compared the Rb^+ uptake during the first 2 min of RVD. The hypoosmotic induced cell swelling revealed that WT rat Na,K-ATPase, but not S23A rat Na,K-ATPase has an elevated activity during RVD. This indicates both that the activity of Na,K-ATPase and the ability of the enzyme to be regulated by PKC phosphorylation plays an important role for RVD.

To better understand the unexpected result that phosphorylation of the Na,K-ATPase seems to promote RVD, we investigated the levels of $[Ca^{2+}]_i$ during RVD in both cell types. We did this because it has been shown that the effect of phosphorylation on the activity of Na,K-ATPase is dependent on $[Ca^{2+}]_i$. At low $[Ca^{2+}]_i$, phosphorylation results in a reduced Na,K-ATPase activity. At elevated $[Ca^{2+}]_i$, phosphorylation results instead in an increased Na,K-ATPase activity. We found that cells of both types responded with elevated $[Ca^{2+}]_i$ within a few seconds after cell swelling.

The findings that ouabain causes a dose dependent attenuation of RVD, that Na,K-ATPase mediated Rb^+ flux is increased during RVD, and that PKC mediated activation of Na,K-ATPase is important for the rate of RVD supports the concept that regulation of Na,K-ATPase activity plays an active role in RVD.

The purpose of **paper IV** was to investigate the M5-M6 segment of Na,K-ATPase and its influence on RVD. The main reason for this, as mentioned above, is that the M5-M6 segment contains several amino acid residues responsible for cation transport. We show here that mutation of the conserved residue Leu-799 to Cys in rat Na,K-ATPase confers not only ouabain insensitivity but also lowers the apparent affinity of K^+ for the enzyme. Because of the mutant's lack of sensitivity to ouabain we developed a novel method, modified from that of Borin's (81), to measure Na,K-ATPase dependent changes in intracellular sodium. We superfused cells with a solution that contained normal Na^+ but lacked K^+; this resulted in the inhibition of Na,K-ATPase and in a prompt increase in $[Na^+]_i$. Different concentrations of K^+ were reintroduced into the medium to activate Na,K-ATPase. The decrease in $[Na^+]_i$ after reintroduction of K^+ into the medium will to a high degree reflect Na,K-ATPase dependent Na^+ efflux. The rate of Na,K-ATPase activation was significantly slower for L799C compared to WT at all K^+ concentrations studied. In support of this result are data showing that $[Na^+]_i$ is almost two-fold higher in the mutant expressing cells than in WT expressing cells. This could not be attributed to a difference in the number of pumps, as assessed by Western Blots.

Measurements of total Rb^+ uptake showed significantly higher uptake in L799C than in WT cells when extracellular K^+ was 4.0 mM. Further, the barium-sensitive portion, related to K^+ channels, of total Rb^+ uptake was higher in cells expressing L799C Na,K-ATPase, 35.6 ± 2.7% (n=7) compared to cells expressing WT Na,K-ATPase, 19.5 ± 3.3% (n=7).

The physiological implication of the difference in apparent K^+ affinity and total Rb^+ uptake was clearly seen as a significantly higher rate of RVD in cells

expressing mutant Na,K-ATPase compared to cells expressing WT Na,K-ATPase. Interesting observations were seen in Rb^+ uptake experiments. During the first 30 seconds of RVD the mutant L799C had a lower ouabain sensitive Rb^+ uptake than the WT. But after 2 min of RVD their Rb^+ uptake was the same. This suggests that the Na,K-ATPase uptake profile of K^+ influences the RVD rate.

Methodological Aspects about Fluorescent Lifetime Imaging (Paper V and VI)

In **paper V** and **VI** we have investigated the performance of confocal pH imaging in cells and in capillaries when using phase fluorometry and fluorophores (SNAFL-1, SNAFL-2, and SNARF-1) with pH-dependent lifetimes. The lifetime-dependent phase shift in the fluorescent signal is detected by a lock-in amplifier, and converted into a pH value through calibration. In **paper V** we made a theoretical investigation of how the different system parameters, i.e. non-negative output signals, noise in pH measurements, and sensitivity to pH variations, will influence the results concerning sensitivity and noise. We noted that the angle set in the lock-in amplifier should, during practical image recording, be set in such a way (Equation 9) that the brightest parts of the two recorded images have similar intensities. This avoids pixel values close to zero, which generate high noise in the image.

Noise in the output signals from the lock-in amplifier will introduce a statistical variation in time of the ratio value. This noise will propagate into the pixel values of the pH image. The root mean square (RMS) noise in the pH measurements (σ_{pH}) is calculated from lifetime, the measured pH value, and the pH value which we would measure in the absence of noise (Equation 10). Equation 10 is transferred into equation 15 (SNARF-1) and 16 (SNAFL-2). If excitation light has 50% modulation and an average of 100 photons are detected per pixel, σ_{pH}, will be in the order of 1-1.5 pH units. Even if the number of photons is increased to

1000, which is large for confocal fluorescence microscopy, σ_{pH} will be nearly 0.5 pH units.

Optimum modulation frequency depends on fluorescence lifetime, and was found to be in the range of 30-110 MHz. In our set-up we were limited to the use of 23 MHz. This means that σ_{pH} will be larger by a factor of up to 2.8 compared with the optimum frequency choice (Figure 2). In order to render the noise value as low as possible a modulation frequency of 33 MHz should be chosen. For this frequency and the frequency used we have calculated the expected sensitivity values (Table 1) in different pH regions using equation 19. From this it was possible to calculate that for a pH change of 0.1 units, we can expect a change in an 8-bit digital ratio image of at least two to three units. This sensitivity should be quite sufficient considering the noise level in the measurement (0.5 pH units).

pH imaging of macrophages from rat lung gave an average pH_i of the entire cell of 8.11±0.08. This is higher than the normal pH of 7.2 considered to be found in the cytoplasm. Besides the higher value, the pH was also practically constant throughout the whole cell (Figure 5b). Possible explanations for this discrepancy were investigated in **paper VI.**

In this study we undertook a systematic study of the potential factors influencing the FL of SNAFL-1 and SNAFL-2 at different pH. We investigated the individual effects of lipids and proteins, and their combined effect. Even though the structural difference between SNAFL-1 and SNAFL-2 is only one chloride atom, their lifetime behaviors are opposite in regards to the effect of proteins (Figure 2). Increasing protein concentration at pH 6.0 had no change in FL for SNAFL-1 but overestimated pH values by more than two pH units for SNAFL-2. At pH 9.0 FL for SNAFL-1 was under estimated by 1 pH unit at maximum protein concentration used. SNAFL-2 showed only minor under estimations at increasing protein concentration pH 9.0.

Interestingly, lipids exerted the same influence on FL for both SNAFL-1 and SNAFL-2 (Figure 3). In general, the two lowest concentrations of lipids used had

minor effects on FL, However saturated solution of lipids caused large (two pH units) difference in FL at pH 6.0 but had significantly less influence on FL at pH 9.0. Notable was the small overestimation in FL of SNAFL-1 and the same small underestimation of FL for SNAFL-2 for all lipid concentrations studied.

The combined effects of lipids and proteins on FL for both probes showed the same tendency (Figure 4), i.e. large overestimation at pH 6.0 (between 1 and 2 pH units for SNAFL-1 and 2 to 3 pH units for SNAFL-2) and comparably small underestimates at pH 9.0 (between 0 and 1 pH unit for SNAFL-1 and 0 to 0.5 pH unit for SNAFL-2).

The effect on FL observed in cells mimicked that of the combined effect of lipids and proteins, but measured deviations from true pH were smaller (Figure 5). At pH 6.0 there was an overestimate of pH of 0 to 0.5 pH units for SNAFL-1 and 0 to 1.5 pH units for SNAFL-2. The underestimate at pH 9.0 was 0 to 0.3 pH units for SNAFL-1 and 0 to 0.1 pH units for SNAFL-2.

Discussion and Future Perspectives

Ouabain and ouabain like substances have been found in mammalian tissues and have started to receive attention as endogenous physiological regulators of Na,K-ATPase activity (82; 83). Endogenous ouabain is, like other steroid hormones, synthesized and released from the adrenals (84) and is also locally produced in other tissues, such as the hypothalamus (85; 86). The physiological role of endogenous ouabain has not been clearly defined which, in part, is due to the great species variation, even within mammals, in Na,K-ATPase sensitivity to ouabain. This difference in sensitivity is attributed to the primary structure of Na,K-ATPase and as outlined previously the complexity of ouabain binding is being resolved (21; 22). We demonstrate in this thesis that the M5-M6 segment of Na,K-ATPase is a key-structure in contributing to the binding of ouabain (Paper IV). Here we describe the highly ouabain resistant mutation of a single residue, Leu-799 to Cys, in the M5-M6 segment of rat α1 Na,K-ATPase. This M5-M6 segment has been targeted by a number of mutagenesis, truncation, and fusion-protein studies [Burns, 1993 #954; Feng, 1994 #274; Arguello, 1995 #953; Burns, 1996 #946; Blostein, 1997 #952; Lambrecht, 1998 #951; Peluffo, 2000 #955]. A plausible mechanism by which ouabain could inhibit Na,K-ATPase would be by the overlap in ouabain and cation binding sites found within the M5-M6 segment. This overlap led us to measure the L799C apparent K^+ affinity. Our results showed that the mutant enzyme had a lower apparent K^+ affinity and Vmax compared with WT Na,K-ATPase. Burns et al. showed the reverse effect on K^+ affinity when mutating the same residue in sheep into a Pro residue (24). Despite much biochemical evidence indicating that this segment is important for K^+ and ouabain binding, no major cell physiological consequences have been investigated. Importantly, it was noted in one study that cells with mutation of Ser-775 into Ala or Cys did not survive in medium containing normal potassium concentration(23). Another important

observation was that expression of L799C Na,K-ATPase resulted not only in a decreased affinity of Na,K-ATPase to K^+, but affected other K^+ transporters such as K^+ channels. We found that K^+ channels substantially contribute to the net K^+ transport in both WT and L799C cell types. However K^+ channel-mediated K^+ transport was 2-fold higher in cells expressing L799C Na,K-ATPase compared to cells expressing WT Na^+,K^+-ATPase. This may suggest that L799C cells express more K^+ channels or that these channels are more active in supporting greater K^+ outflux.

The process of regulatory volume decrease is particularly dependent on cellular potassium homeostasis. After cell swelling, one of the principle ions that is transported out of the cell is K^+. Na,K-ATPase is the main enzyme responsible for maintaining a high level of intracellular K^+, and is therefore likely involved in the RVD process. We have shown (**paper III**) that ouabain, in a dose-dependent manner, inhibits RVD in COS-7 cells transfected with WT rat $\alpha 1$ subunit of Na,K-ATPase. Further, we have also been able to link this involvement to the M5-M6 segment of the enzyme (**paper IV**). Of particular interest, from both studies, is the fact that ouabain-sensitive Rb^+ uptake is stimulated by cell swelling. Both WT and L799C mutant of rat $\alpha 1$ subunit show an up-regulation in their activity. Since K^+ channel-mediated K^+ transport was 2-fold higher in cells expressing L799C Na,K-ATPase compared to WT, this may explain the faster rate of RVD observed in L799C cells.

Interestingly, a PKC site deficient mutant of Na,K-ATPase, S23A, did not show this up-regulation during RVD. This could indicate that PKC is an important signal to Na,K-ATPase in the swelling process. These results suggest that a regulatory site of a target protein may be affecting RVD. How Na,K-ATPase actually contributes to the RVD process itself is uncertain. It should be noted that its basic function of transporting 3 Na^+ ions out and 2 K^+ ion into the cell, thereby fuelling K^+ channels, is in favor of the RVD process, but likely not enough to be significant. An interesting model, which remains to be proven, is a functional

coupling between Na,K-ATPase and K^+ channels under the RVD process. It is already known that intracellular ATP may coordinate the activity of these two transporters, where an activation of Na,K-ATPase leads to a decrease in $[ATP]_i$ and consequent activation of K^+ channels. This would explain how Na,K-ATPase could be an important component of RVD. Another plausible function of Na,K-ATPase of critical value to RVD could be that it promotes the activity of the Na^+/H^+ exchanger during the RVD process. This would counteract the acidification of the cytoplasm which takes place as HCO_3^- is exchanged for Cl^-. Thus, while both hypotheses consider known regulators of RVD, whether $[ATP]_i$ and pH_i exert their effect on RVD via Na,K-ATPase remains to be investigated in future studies.

Partial inhibition of Na,K-ATPase activity not only affects cell volume regulation, it also leads to a significant reduction in cell attachment to fibronectin (**paper II**). We suggest that this can be at least partially mediated by changes in $[Ca^{2+}]_i$ induced by Na,K-ATPase inhibition. However, the exact signaling mechanism remains to be elucidated. It is known that correct Ca^{2+} signaling is crucial for the initial steps of cell attachment (87). A transient increase in $[Ca^{2+}]_i$ is one of the earliest events during integrin-mediated cell adhesion and required for activation of adhesion complex proteins (integrin, FAK) and cytoskeleton rearrangement (87; 88). Therefore, the perturbations of this initial Ca^{2+} signal, observed as a result of Na,K-ATPase inhibition, may account for disturbances in cell attachment. We made an attempt to dissect the involvement of Ca^{2+} regulatory proteins (calmodulin, CaMK II and calcineurin) in the mechanism of impaired cell attachment induced by Na,K-ATPase inhibition. Inhibitors of Ca^{2+} regulatory proteins did not recover cell attachment after Na,K-ATPase inhibition, but rather caused a significant reduction in the basal level of attachment, thus suggesting that a normal physiological pattern of Ca^{2+} signaling is crucial for cell adhesion. The inhibition of Na,K-ATPase activity will not only influence $[Ca^{2+}]_i$ but also $[Na^+]_i$ and pH_i. Therefore, it is possible that additional factors may also contribute to the effect of Na,K-ATPase inhibition on cell attachment. There are some indications

that changes in pH_i may influence the cellular adhesion process (89). In our case however, the inhibition of the Na^+/H^+ exchanger did not have a significant effect on cell attachment. In addition, inhibition of Na^+ influx pathways had no significant effect on cell attachment. The activity and cell surface abundance of fibronectin receptor, $\alpha5\beta1$ integrin, is known to be regulated by Ca^{2+} (87). Since we did not find any changes in the cell surface expression of $\beta1$-integrin after Na,K-ATPase inhibition, it is very unlikely that the observed decrease in cell attachment was due to integrin internalization. At this point, we can not exclude that impaired activation of integrins on the cell surface and/or perturbation of the signal between the integrin and FAK are responsible for observed changes in cell adhesion. Interestingly, there is some evidence for the involvement of MAP kinase pathway in the regulation of cell-matrix adhesion (90). Since several groups have reported that even partial inhibition of Na,K-ATPase activity by ouabain activates MAPK pathway (91-93), this mechanism of regulation of cell attachment has to be considered in future studies. The novel role of Na,K-ATPase in the regulation of cell adhesion will broaden the importance for Na,K-ATPase during development. The activity of Na,K-ATPase can be modulated by a number of physiological and pharmacological factors, including hormones, endogenous ouabain and cytoskeleton proteins (94). It will be important to find out what effect these factors have on cell attachment.

Besides our findings of novel roles of Na,K-ATPase through ouabain, it has also been suggested that ouabain may act as a natriuretic hormone (95; 96), but unlike most other natriuretic factors, it may also have a vasoconstrictive effect (82). Evidence collected over several years indicates that cardiac glycosides also may act as anticancer agents (97-99). Indeed, there are strong indications that these substances influence the function and proliferation of multiple cancer cell types, both in vitro and under clinical settings (98; 99). According to recent studies, ouabain plays a role in growth, differentiation and apoptosis (91; 100; 101). The level of endogenous ouabain is known to be significantly increased in some clinical

conditions where extensive cell growth and differentiation is required. Consistent with these findings, high circulating levels of ouabain are found in pregnancy (102) and postnatally (Di Bartolo et al., 1995). Interestingly, it has been reported that endogenous ouabain levels are increased following nephrectomy (103), a condition that is associated with compensatory growth of the remaining kidney.

Use of Fluorescent Lifetime Measurement in Cell Biology

It was not until recently, in the beginning of the 1990s, that one could perform confocal fluorescence lifetime imaging (FLIM) microscopy. It was the improvements in high-speed lasers and the development of very sensitive, high-speed gated image detection devices that made this possible. However, as with most other new techniques, several years are required to fully understand the limitations and potential errors. **Paper V** and **VI** in this thesis were concerned with the proper use of FLIM to investigate intracellular pH. We made theoretical predictions concerning sensitivity and noise which were supported by experimental results. When scanning living cells the results were different from what was expected. Macrophages were chosen for pH imaging because of their well known production of vesicles called phagosomes which have low internal pH (4-6). In addition to these intracellular organelles, mitochondria and peroxisomes would be expected to provide a spatial pH difference inside the cell. However, our pH measurements in cells recorded a nearly homogeneous pH. Such homogeneous pH images of cells have been previously reported (104). A possible explanation for these results is that more than two fluorophore populations exist due to fluorophore interaction with, for example, proteins inside the cell (105). Our study (**paper VI**) on the influence of probe binding on pH based FL indicates that the method is not a straightforward approach to measure pH in the absence of correction for the effect of probe binding. We find it likely that other fluorescent ion probes have a probe-binding-sensitive fluorescence lifetime. However, this effect is difficult to predict.

We have shown in **paper VI** that the effect of probe binding is different even with small structural differences between fluorophores, such as between cSNAFL-1 and cSNAFL-2. Apart from pH, protein concentration has a major effect on the fluorescence lifetime for both cSNAFL-1 and cSNAFL-2. In general, lipids exerted less influence on fluorescence lifetime, independent of pH, compared to that of proteins. The similar results from the combined effects of lipids and proteins and the effects in cells on cSNAFL-1 and cSNAFL-2 indicate that the protein- and lipid-dense cytoplasm of cells alters the lifetime-based estimate of pH, producing a homogenous intracellular pH throughout the cell interior.

It is indeed interesting to see that other investigators have been able to address important biological problems with FLIM microscopy, as previously outlined in the introduction. In the case of pH, several groups have made significant observations on how NHE1 regulates epidermal pH and barrier function with the use of FLIM (66). In their study they use the pH sensitive fluorophore BCECF. They were able to retrieve life time images showing structures, however the noise level was rather high as also seen in our studies.

Conclusion

This thesis presents two novel aspects of Na,K-ATPase function. Here we demonstrate that Na,K-ATPase has an important role in both regulatory volume decrease and in cell attachment. Significantly, both of these functions have been elucidated by studying Na,K-ATPase and ouabain interactions.

A major role of Na,K-ATPase activity in regulatory volume decrease was established. We found a direct link between Na,K-ATPase activity and the ability of COS-7 cells to perform RVD. We demonstrated this through three independent methods involving measurement of RVD under direct inhibition of the Na,K-ATPase by various concentration of ouabain, ouabain sensitive Rb^+ uptake during

RVD, and RVD in a PKC-site deficient mutant (S23A) of Na,K-ATPase. All three methods supported the view that Na,K-ATPase activity is important for RVD.

Our study of the L799C mutant of Na,K-ATPase provided not only evidence for the involvement of the pump in RVD but also contributed to the understanding of the interaction between Na,K-ATPase and ouabain. We have strong evidence that the L799C mutation, located in the M5-M6 segment of the enzyme, is very important for ouabain binding. Further, we have also demonstrated that the mutant has a lower apparent affinity for K^+, indicating an overlap with respect to the ouabain and K^+ binding sites in the enzyme.

A role for Na, K-ATPase in the regulation of cell adhesion was demonstrated. We found that a decrease in Na, K-ATPase activity leads to decreased cell-extracellular matrix adhesion. This reduction in cell adhesion was mediated via changes in $[Ca^{2+}]_i$ and focal adhesion kinase activity. The changes in cell adhesion may have an effect on cell growth, proliferation and development.

From a methodological perspective, we have evaluated the use of fluorescent lifetime in assessing intracellular pH. We have shown that the fluorescent lifetime of probes, i.e. SNAFL-1 and SNAFL-2, are far from inert with respect to the presence of proteins and lipids in the cytoplasm. We have demonstrated this both in cells and in protein- and lipid solutions. Even small structural differences, as seen between SNAFL-1 and SNAFL-2, may have drastic effects on their sensitivity to cellular constituents. Thus there is no difference in use of intracellular calibration in traditional ratiometric measurement and fluorescent lifetime measurement as had earlier been suggested.

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