Microfluorimetry of epithelial cells: lifetime-based sensing of Na\(^+\) concentration and the effects of chemical hypoxia.

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Biomedische Wetenschappen aan het Limburgs Universitair Centrum, te verdedigen door

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2000
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ABBREVIATIONS

ADP  adenosine diphosphate
AM   acetoxymethyl ester (form of a fluorescent indicator)
AMP  adenosine monophosphate
ATP  adenosine triphosphate
BAPTA 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BCECF 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
BSA  bovine serum albumin
\([\text{Ca}^{2+}]_i\) intracellular (cytoplasmic) free Ca\(^{2+}\) concentration
CCD  charge coupled device
CCT  cortical collecting tubules
CN\(^-\) cyanide
CW   continuous wave
DMEM Dulbecco's modified Eagle's medium
DMSO dimethyl sulfoxide
DOG  2-deoxyglucose
EDTA ethylenediamine-N,N,N',N'-tetraacetic acid
EGTA ethyleneglycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ENaC epithelial Na\(^+\) channel
ER   endoplasmic reticulum
FCCP carbonyl cyanide p-trifluoromethoxy-phenylhydrazone
FLIM fluorescence lifetime imaging microscopy
HEPES N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
IP\(_3\)  inositol (1,4,5) trisphosphate
IP\(_3\)R IP\(_3\) receptor
JC-1  5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethylbenzimidazolylcarbocyanine iodide
K\(_d\) ground-state dissociation constant
MDCK Madin-Darby canine kidney cells
\([\text{Mg}^{2+}]_i\) intracellular Mg\(^{2+}\) concentration
MgG  Magnesium Green
MOPS 4-morpholinepropanesulfonic acid
\([\text{Na}^+]_i\) intracellular Na\(^+\) concentration
NAD(P)H nicotinamide adenine dinucleotide (phosphate)
NHE Na\(^+\)/H\(^+\) exchanger
NMDG N-methyl-D-glucamine
pH\(_i\) intracellular pH
PMCA plasma membrane Ca\(^{2+}\) pump
RF   radiofrequency
ROC  receptor operated Ca\(^{2+}\) channels
SBFI sodium binding benzofuran isophthalate
SD   standard deviation
SEM  standard error of the mean
SERCA sarco/endoplasmic Ca\(^{2+}\) ATPase
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>SMOC</td>
<td>second messenger operated Ca^{2+} channels</td>
</tr>
<tr>
<td>SOC</td>
<td>store operated Ca^{2+} channels</td>
</tr>
<tr>
<td>TAL</td>
<td>thick ascending limb</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>trp</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VOC</td>
<td>voltage operated Ca^{2+} channels</td>
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<tr>
<td>ΔΨ</td>
<td>mitochondrial membrane potential</td>
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CHAPTER I

GENERAL INTRODUCTION
REGULATION OF INTRACELLULAR IONS WITH A REGULATORY FUNCTION IN EPITHELIAL CELLS

The importance of intracellular ions in the cellular physiology and pathology of epithelial cells is generally recognized. Under resting conditions, cytosolic ion concentrations are closely regulated by intracellular buffers and membrane transport systems. Under a variety of physiological as well as pathophysiological conditions, they are expected to change. Because intracellular ions are involved in the regulation of numerous cell functions, including the transepithelial transport, control of metabolism, mediation of signal transduction, cell proliferation and cell death, changes in their concentration are likely to have diverse consequences.

Calcium
Calcium ions are a general signal in both life and death (see Fig. 1). Although elevations in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (from a resting level of 50-200 nM to ≤1 μM) can act as a signal, prolonged increases in [Ca\(^{2+}\)]\(_i\) (to more than 1 μM) can be lethal (Berridge et al., 1998). The universality of Ca\(^{2+}\) as a signaling molecule can be invoked at two levels. On the first level, Ca\(^{2+}\) ions are used as a messenger by almost all cell types in the evolutionary ladder, from prokaryotic to eukaryotic cells. At the other level, that of a single, individual cell, Ca\(^{2+}\) signals trigger the activation or are involved, at one stage or another, in a wide variety of cellular processes. For example, Ca\(^{2+}\) triggers life at fertilization (Raz & Schalge, 1998; Hogben et al., 1998) and regulates the progression through each control point of the cell cycle (for a review, see Whitaker & Patel, 1990). Ca\(^{2+}\) controls the development and differentiation of cells into specialized types (Whitfield et al., 1995). It mediates the subsequent activity of these cells and, finally, Ca\(^{2+}\) is invariably involved in cell death (Trump & Berezovsky, 1995).

The basis of Ca\(^{2+}\) signal generation consists in the existence of a large electrochemical gradient across both the plasma membrane and the intracellular membranes delimiting the so-called Ca\(^{2+}\) stores. Currently, three main intracellular Ca\(^{2+}\) stores are recognized: the endoplasmic reticulum (ER), the mitochondria and the nucleus. The nuclear Ca\(^{2+}\) pool does not appear to be significantly involved in the generation or shaping of cellular calcium signals (Brini et al., 1994). The common view is that the nucleus, with its large pores allowing communication between nuclear matrix and the cytoplasm, is more the target for Ca\(^{2+}\) signaling rather than its source. The mitochondrial pool has been seen for many years mainly as a back-up system for removing excessive cytoplasmic Ca\(^{2+}\). However, several recent studies point out the fact that mitochondria could play a much more active regulatory role in Ca\(^{2+}\) signaling. They are able not only to accumulate excess of Ca\(^{2+}\) entering the cell during periods of intense cellular activity, but can also release the stored Ca\(^{2+}\) after the cessation of stimulation and thus prolong the Ca\(^{2+}\) signal (see Duchen, 1999). Nonetheless, mitochondria act as a rather passive intracellular Ca\(^{2+}\) store, which has to be preloaded with Ca\(^{2+}\) in order to become a Ca\(^{2+}\) source. Thus, the main intracellular source for initiating Ca\(^{2+}\) signaling remains the endoplasmic reticulum (Fig. 1).
The electrochemical gradient of Ca²⁺ across the plasma membrane is mainly maintained by high affinity ATP-dependent Ca²⁺ pumps (the plasma membrane Ca²⁺ ATPase - PMCA; Guerini et al., 1998). PMCAs transport Ca²⁺ in exchange for H⁺ with a stoichiometry of 1:1 for each ATP molecule hydrolyzed. PMCAs are expressed as products of four different genes: PMCA1, -2, -3 and -4. PMCA1 and PMCA4 are expressed in all tissues in large and comparable amounts and are referred to as housekeeping genes. PMCA2 gene is expressed in a tissue-specific manner, mainly in brain, whereas PMCA3 expression is limited to the adult brain and skeletal muscle. These pumps have 10 transmembrane domains (TMDs) and 3 large cytoplasmic-loops. Ca²⁺ binding sites are found in four of the TMDs (4,5,6 and 8) whereas the ATP binding site and the phosphorylation site are contained by the second cytoplasmic loop, placed between the TMDs 4 and 5.

Ca²⁺ can also be extruded from epithelial cells by the Na⁺/Ca²⁺ exchanger (Friedman, 1998), which utilizes the prevalent Na⁺ gradient to transport Ca²⁺ (see below). Accumulation of cytoplasmic Ca²⁺ into the ER is accomplished through the
Chapter I

sarco/endoplasmic Ca\(^{2+}\) ATPases (SERCA pumps; MacLennan et al., 1997). SERCA pumps are typical of the class of P-type ATPases, which form a phosphoprotein intermediate and undergo conformational changes during the course of ATP hydrolysis. Three differentially expressed genes encode SERCA proteins. SERCA1a and -1b are expressed in fast-twitch skeletal muscle. SERCA2a is the cardiac/slow-twitch isoform, whereas SERCA2b, with a C-terminal extension, is expressed in smooth muscle and non-muscle tissues. SERCA3 is expressed in a limited set of non-muscle tissues, including epithelial cells, and its knockout is not lethal (MacLennan et al., 1997). The current model of SERCA pumps is based on a 14 Å structure obtained by cryoelectron microscopy (Toyoshima et al., 1993). A large cytoplasmic head is linked to the transmembrane domain by a narrow stalk. The cytosolic head of SERCA is divided in two domains: the transduction domain and the catalytic domain, which includes both the phosphorylation site and the ATP-binding site. It has been proposed (MacLennan et al., 1997) that SERCA1a and SERCA2a have 10 TMDs, with TMDs 4, 5, 6 and 8 contributing to Ca\(^{2+}\) binding. An 11 transmembrane helix model has been suggested for SERCA2b (Lytton & MacLennan, 1988).

Ca\(^{2+}\) signals have two sources: Ca\(^{2+}\) release from the ER and Ca\(^{2+}\) entry from the external solution. In epithelial cells, Ca\(^{2+}\) release occurs through the inositol (1,4,5) trisphosphate receptor (IP\(_3\)R) channels. These channels are activated by an increase in the cytoplasmic inositol (1,4,5) trisphosphate (IP\(_3\)) concentration. Ca\(^{2+}\) release through IP\(_3\)R channels also depends on \([\text{Ca}^{2+}]_i\). An increase in \([\text{Ca}^{2+}]_i\) (in the 100-400 nM range) has an activating effect on IP\(_3\)R and so enhances the release of Ca\(^{2+}\) (calcium induced Ca\(^{2+}\) release). However, higher \([\text{Ca}^{2+}]_i\) inhibits further Ca\(^{2+}\) release (Tshipamba et al., 1993). IP\(_3\)R channels exist as tetramers, with the C-terminal region co-operating to form the Ca\(^{2+}\) channel. IP\(_3\)R isoforms are encoded by a multigene family, consisting of three distinct genes: IP\(_3\)R1 (present mainly in brain), IP\(_3\)R2 (predominant in liver and skeletal muscle) and IP\(_3\)R3 (abundant in epithelial and secretory cells). IP\(_3\)R channels have a large N-terminal cytoplasmic domain, which contains at its end the IP\(_3\) binding site.

Extracellular Ca\(^{2+}\) can enter the cells (Fig. 1) through voltage operated Ca\(^{2+}\) channels (VOC) (Catterall, 1995), receptor operated Ca\(^{2+}\) channels (ROC) (Singer-Lahat et al., 1996), second messenger operated Ca\(^{2+}\) channels (SMOC) (Vaca & Kunze, 1995) or store operated Ca\(^{2+}\) channels (SOC) (Berridge, 1995; Parekh & Penner, 1997). VOCs are highly Ca\(^{2+}\) selective channels that open upon membrane depolarization and are expressed mainly in excitable cells. ROCs are opened by the binding of a ligand to a receptor channel. An example of ROC is the Ca\(^{2+}\) channel activated by extracellular ATP in lacrimal cells (Benham & Tsien, 1987). SMOCs are activated by an intracellular messenger such as Ca\(^{2+}\), IP\(_3\), inositol (1,3,4,5) tetrakisphosphate or diacylglycerol. SOC channels are stimulated after Ca\(^{2+}\) release from the stores in order to maintain a prolonged elevation of \([\text{Ca}^{2+}]_i\) and to replenish the stores. The signaling mechanism used to activate these channels is still unknown. SOC channels are assumed to be encoded by a gene family called trp-family (trp stands for transient receptor potential). So far, at least 4 human isoforms are known. All trp's are characterized by six transmembrane spanning helixes and a putative pore region.
between the TMDs 4 and 5. Limited homology of the last four TMD segments to those of voltage operated Na\(^+\) and Ca\(^{2+}\) channels is found for all trp channels (Zhu & Birnbaumer, 1998). There is a clear difference among trp proteins concerning the mechanism of activation. Although it has been clearly shown that trp1 channels are opened by store depletion, no convincing evidence has thus far been obtained to show that trp3 is store operated. In fact, experimental results tend to indicate that they are not stimulated by store depletion (Nilius, 1998; Zhu et al., 1998).

Ca\(^{2+}\) channels from the apical membrane of the distal tubules of the nephron play a key role in Ca\(^{2+}\) reabsorption in the kidney. These channels are activated by the parathyroid hormone (Bacskaï & Friedman, 1990) and share some common properties (related to the ionic selectivity and pharmacological properties) with the L-type channels recorded in excitable tissues although they are not activated by membrane depolarization (Poujol et al., 1995). The channel has been recently cloned (Hoenderop et al., 1999). It has 730 amino acids and contains six putative membrane-spanning domains with an additional hydrophobic stretch predicted to be the pore region. This epithelial Ca\(^{2+}\) channel resembles the trp channels with respect to its predicted topology but shares less than 30% sequence homology with these channels (Hoenderop et al., 1999).

Protons

Epithelial cells maintain their intracellular pH (pH\(_i\)) within a narrow range (7.0-7.4). Generally, pH\(_i\) is about 1 pH unit higher than it would be expected from the electrochemical equilibrium of H\(^+\) across the plasma membrane. Intracellular buffers and acid-base transport systems regulate pH\(_i\). The role of intracellular buffering in pH\(_i\) regulation is to moderate large variations in pH\(_i\); they cannot prevent a change in pH\(_i\) but they can merely reduce its magnitude. Furthermore, buffering mechanisms cannot return pH\(_i\) towards its initial value following an acid or alkali load. This recovery is brought about by transport of acid and/or base across the cell membrane. There are several mechanisms of acid extrusion or loading in epithelial cells (reviewed by Boron, 1992). Probably the most widespread is the Na\(^+\)/H\(^+\) exchanger. The antiporter exchanges intracellular H\(^+\) for extracellular Na\(^+\) in a 1:1 stoichiometry and is driven by the large electrochemical gradient of Na\(^+\). Intracellular acidification activates the exchanger whereas a decrease in the extracellular pH inhibits its activity. Amiloride and its derivatives inhibit the Na\(^+\)/H\(^+\) exchanger activity by competing with Na\(^+\) for binding to the same external site (Moolenaar, 1986). Five isoforms of Na\(^+\)/H\(^+\) exchangers (NHEs) have been identified so far. The putative protein structure is virtually identical in all isoforms, predicting an N-terminal membrane spanning domain that translocates Na\(^+\) and H\(^+\) and confers allosteric regulation by H\(^+\), and a large cytoplasmic C-terminus that provides regulatory control. The ubiquitous NHE-1 isoform is found in many cell types and is involved in pH\(_i\) and cell volume regulation. The other four isoforms are mainly present in epithelial cells.

Another transport system involved in pH\(_i\) regulation is the Na\(^+\)-dependent Cl\(^-\)/HCO\(_3^-\) exchanger (Boyartsky et al., 1988). This antiporter has an absolute requirement for external Na\(^+\), external HCO\(_3^-\) or a related species and internal Cl\(^-\). Removing any one
of these three ions from the appropriate side of the membrane completely blocks acid extrusion. The precise identity of the species transported and the stoichiometry of the exchanger are not known. A Na⁺-independent Cl⁻/HCO₃⁻ exchanger that mediates the exchange of external Cl⁻ for internal HCO₃⁻ in a 1:1 ratio has also been observed (Boyarisky et al., 1988). Present in the basolateral membrane of several epithelia, including the renal proximal tubule, is an electrogenic Na⁺/HCO₃⁻ cotransporter (Boron & Boullpaep, 1983). In epithelia, the cotransporter normally mediates a net efflux of Na⁺ and HCO₃⁻. An electrogenic H⁺ pump is present in the distal nephron of the kidney as well as in the urinary bladder of the reptile (Al-Awqati, 1983). The H⁺ pump is capable of generating a large H⁺ gradient across the membrane (about 3 pH units in the absence of a membrane potential (Boron, 1992)). On the other hand, when compared to other H⁺ transporters, H⁺ pumps have a relatively low transport rate. It has been shown (Selvaggio et al., 1988) that the H⁺ pump contributes to the recovery of pH₄ after an acid load in collecting duct cells. Present in the parietal cells of the stomach (Sachs et al., 1989) and in cultured renal cells (Lang et al., 1990) is an ATP-driven pump that extrudes H⁺ in exchange for K⁺ (H⁺/K⁺ pump). Like the Na⁺/K⁺ pump (see below), the H⁺/K⁺ pump has two units: α and β. These subunits have been cloned and both are similar to the corresponding subunits of the Na⁺/K⁺ pump.

Many cellular processes are sensitive to pH₄. The most obvious way pH₄ affects the cell metabolism is via regulation of enzymes activity. This results in the regulation of protein synthesis as well as in the control of energy production and utilization. Changes in pH₄ have been shown to play an important role in the initiation of cell cycle and proliferation (Epel, 1990; LaPointe & Batlle, 1994). pH₄ modulates the activity of many ion channels, including the epithelial Na⁺ channels (Zeiske et al., 1999).

**Sodium**

In most cells, intracellular sodium concentration ([Na⁺]ᵢ) is highly regulated. Whereas cells are bathed in extracellular fluids that contain high [Na⁺] (more than 100 mM), [Na⁺]ᵢ is maintained at much lower levels, 10-25 mM in epithelial cells (Breyer & Fredin, 1996). The chemical driving force for Na⁺ entry into the cells is complemented by the inside negative membrane potential across the plasma membrane, resulting in a large electrochemical gradient. This Na⁺ gradient is maintained by the Na⁺/K⁺ pump, which utilizes the energy derived from ATP hydrolysis to exchange intracellular Na⁺ with extracellular K⁺. The Na⁺/K⁺ pump also maintains a nonequilibrium distribution of K⁺: intracellular K⁺ concentration is in the range of 100-130 mM whereas the external K⁺ is generally between 1 and 10 mM. The Na⁺/K⁺ pump has two protein subunits, α and β. The α subunit is a nonglycosylated polypeptide with a molecular weight of about 120 000 and the β polypeptide is a transmembrane glycoprotein and has a molecular weight of 50 000 (Lodish et al., 1995). The α subunit has the binding sites for Na⁺, K⁺, ATP and ouabain whereas the β subunit is essential for normal targeting and correct insertion of the α subunit into the cell membrane. Three isoforms of the α subunit and three of the β subunit are known. Of the α isoforms, α₁ is ubiquitous. It appears to be a "housekeeping" isoform in most tissues, but is expressed
at high levels in epithelia with high rates of solute transport (for example, renal tubular cells). The other isoforms have a more restricted distribution: \( \alpha_2 \) is found in muscle, adipose tissue and brain and \( \alpha_3 \) is expressed in neural tissue and heart. It has been shown that there are several functional differences between the \( \alpha \) isoforms. The apparent dissociation constant \( K_m \) for \( Na^+ \) of the rat \( \alpha_1 \), \( \alpha_2 \) and \( \alpha_3 \) isoforms transfected in HeLa cells are 12, 22 and 33 mM, respectively (Zahler et al., 1997). Because the \( K_m \) for \( Na^+ \) is an important determinant of [\( Na^+ \)] and therefore of the transmembrane \( Na^+ \) gradient, the expression of \( \alpha_3 \) isoform in a tubular cell would probably dramatically change the driving force for transcellular \( Na^+ \) transport (Aperia, 1995). The importance of the \( Na^+/K^+ \) pump in the kidney is showed by the fact that it uses about 25% from the ATP produced by renal cells (Lodish et al., 1995).

One of the main routes of \( Na^+ \) entry into epithelial cells is via the amiloride-sensitive epithelial \( Na^+ \) channels (ENaC). The basic unit of these channels is composed of three homologous but nonidentical subunits termed \( \alpha \), \( \beta \) and \( \gamma \) (Canessa et al., 1993). It is important to note that the \( \alpha \) subunit alone can form fully functional amiloride-sensitive \( Na^+ \) channels, whereas the \( \beta \) and \( \gamma \) subunits, individually or in combination, cannot. The presence of the \( \beta \) and \( \gamma \) subunits, however, leads to a greater expression of amiloride-sensitive \( Na^+ \) conductance, possibly by increasing the surface delivery of ENaC.

Many cells utilize the energy stored in the \( Na^+ \) gradient to couple the unfavorable transmembrane solute flow to \( Na^+ \) transport (the so-called secondary active transport). Examples include nutrient (sugars, amino acids) and neurotransmitter uptake. Such cotransport systems are essential for the reabsorption of organic osmolytes in proximal tubules of the kidney.

\( Na^+ \) gradient is related to the [\( Ca^{2+} \)] and \( pH \), via the \( Na^+/Ca^{2+} \) and \( Na^+/H^+ \) exchangers, respectively. The \( Na^+/Ca^{2+} \) exchanger extrudes one \( Ca^{2+} \) ion in exchange for three \( Na^+ \) ions (the normal mode of the antiporter). In conditions of cellular \( Na^+ \) load, due to, for example, ischemia or hypoxia, the \( Na^+/Ca^{2+} \) exchanger could operate in the reverse mode, thus resulting in \( Ca^{2+} \) overload (Haigney et al., 1992; Schroder et al., 1999). The \( Na^+/Ca^{2+} \) exchangers comprise a family of three genes (Blaustein & Lederer, 1999): NCX1, NCX2 and NCX3. The NCX1-coded \( Na^+/Ca^{2+} \) exchanger consists in 11 transmembrane-spanning sequences. A large intracellular loop follows the first 5 TMDs (Nicoll et al., 1990). The intracellular loop is thought to be involved in the modulation of the \( Na^+/Ca^{2+} \) exchanger function by intracellular kinases and ions, such as \( Na^+ \), \( Ca^{2+} \) and \( H^+ \). It also represents the locus where a complex pattern of isoform diversity is generated by the alternative splicing of the gene's primary transcript. Same overall organization of \( Na^+/Ca^{2+} \) exchanger protein is found in products of the other two genes, NCX2 and NCX3. A \( K^+ \) dependent \( Na^+/Ca^{2+} \) exchanger has been found in the vertebrate eye (Cervetto et al., 1989). Functionally, the retinal rod exchanger has a coupling ratio of 4 \( Na^+ \) to 1 \( Ca^{2+} \) plus 1 \( K^+ \). The gene (NCKX1) that codes for the rod \( K^+ \) dependent \( Na^+/Ca^{2+} \) exchanger was found to be virtually unrelated to the cardiac type \( Na^+/Ca^{2+} \) exchanger, except for two features: first, both proteins exhibit \( Na^+ \)-dependent \( Ca^{2+} \) transport and second, the general topologies of the proteins were
similar. Recently, a second $K^+$ dependent $Na^+/Ca^{2+}$ exchanger, NCKX2, was cloned from rat brain (Tsoi et al., 1998). The overall structure of this protein is very similar to both NCX1 and NCKX1.

The $Na^+/H^+$ exchanger plays an important role not only in pH homeostasis, as already discussed, but also in the regulation of cell volume. It has been shown that cell shrinkage activates the exchanger (for a review, see Demareux & Grinstein, 1994). Though the stoichiometry of the antiporter is 1:1, the exchange of $Na^+$ for $H^+$ results in net osmotic gain for two reasons: while $Na^+$ moves inwards and $H^+$ outwards, the intracellular concentration (activity) of $H^+$ is simultaneously compensated by dissociation of intracellular buffers. In addition, $pH$ increases during shrinkage and, as a consequence, the cytoplasmic concentration of $HCO_3^-$ rises. In these conditions, the $Cl^-/HCO_3^-$ exchanger is activated. Together, the parallel stimulation of these two antiporters promotes uptake of NaCl, coupled to the inflow of osmotic water. The activation of $Na^+/H^+$ exchanger in shrunken cells is most likely due to a shift in the alkaline direction in the $pH_i$-dependence of the antiporter activity (Demareux & Grinstein, 1994). A second $Na^+$ transport mechanism involved in regulatory volume increase of shrunken cells is the $Na^+/K^+/2Cl^-$ cotransporter. Four osmolytes are translocated inwards during each turnover of the symporter.

Cytoplasmic $Na^+$ is also important for $Na^+$ and $Ca^{2+}$ homeostasis in the mitochondria. As Fig. 2 shows, $Ca^{2+}$ efflux from the mitochondrial matrix occurs through an electroneutral $Na^+/Ca^{2+}$ exchanger (Gunter et al., 1994). $Na^+$ ions entering the mitochondria in exchange for $Ca^{2+}$ are subsequently extruded via the $Na^+/H^+$ exchanger.

The activity of many $Na^+$-coupled solute transporters can be modulated by changes in substrate concentrations or by allosteric modification (e.g., phosphorylation). Furthermore, the activity of the $Na^+/K^+$ pump is regulated by receptor-mediated intracellular signals. Such regulatory pathways include activation of cyclic AMP-dependent protein kinase or protein kinase C (Bertorello & Katz, 1995). The energy state of the cell is another modulator of the pump activity. Thus, $[Na^+]_i$ might be expected to change under a variety of physiological or pathophysiological conditions. Because of the critical role of the $Na^+$ gradient, such changes are likely to have diverse physiological consequences.

**Magnesium**

Cytosolic free Mg$^{2+}$ concentration ([Mg$^{2+}$]) is of the order of 0.5 mM (see London, 1991), lower than the concentration predicted from its electrochemical equilibrium. The mechanisms by which [Mg$^{2+}$]$_i$ is regulated are still poorly understood. A $Na^+/Mg^{2+}$ exchanger exists in the plasma membrane of many cells and contributes to the regulation of resting [Mg$^{2+}$]$_i$ (Zhang & Melvin, 1995; Tashiro & Konishi, 1997). The transport might be either electroneutral (Zhang & Melvin, 1995) or electrogenic (Tashiro & Konishi, 1997). The existence of ATP driven Mg$^{2+}$ pumps has also been suggested (Beyenbach, 1990). Free ionized Mg$^{2+}$ represents only a small part (less than 10%) of the total Mg$^{2+}$ content of the cells. The main intracellular Mg$^{2+}$ chelator
is ATP: the dissociation constant of Mg-ATP$^{2-}$ is 40 to 90 μM (Gupta & Gupta, 1987). Free Mg$^{2+}$ is involved in several cellular functions, including DNA transcription and protein synthesis (Cameron & Smith, 1989). Mg$^{2+}$ also participates as a cofactor in numerous enzymatic reactions (Garfinkel & Garfinkel, 1985). Mg$^{2+}$ regulates several ion transport systems, including the IP$_3$R Ca$^{2+}$ channels in the membrane of the (sarco)endoplasmic reticulum (Volpe & Vezu, 1993), Na$^+$ and K$^+$ channels (Bara et al., 1993).

**ION HOMEOSTASIS IN RENAL CELLS DURING HYPOXIA/ISCHEMIA**

Ischemia in vivo is characterized by decreased blood flow with subsequent hypoxia and limitation of substrate supply, particularly glucose. Although glycolysis may be stimulated initially, it typically is self-limited in vivo because of glycogen and glucose depletion and reduced pH$_i$, which inhibits critical enzymes, e.g. phospho-fructokinase. In the ischemic kidney in vivo, glycolysis is inhibited after about one hour by lack of substrate, lower pH$_i$ and accumulation of glycolysis end products, i.e. lactate (Kahng et al., 1978). Distinct segments from the nephron manifest different sensitivities to ischemia, with the cortical collecting tubules (CCT) being more resistant than the S$_1$ segment of the proximal tubule and the thick ascending limb of Henle's loop (TAL) (Wilson & Schrier, 1986).

Impairment of ion homeostasis is one of the earliest events that follow ATP depletion during hypoxia/ischemia and is suggested to be closely linked to the cellular injury. This is especially important in the renal tubules because these cells are actively involved in ion reabsorption and secretion. One of the main activities of the renal tubular cells is the reabsorption of filtered Na$^+$, which provides the driving force for the reabsorption of water and for the coupled transport of organic solutes. Na$^+$ reabsorption is an active transport process, mediated by the basolaterally located Na$^+/K^+$ ATPase and driven by cellular ATP. Energy deprivation will reduce the activity of the pump, leading to accumulation of intracellular Na$^+$. As the transmembrane Na$^+$ gradient is coupled to the intracellular pH and Ca$^{2+}$ homeostasis via the Na$^+$/H$^+$ and Na$^+$/Ca$^{2+}$ antiporters, a rise in [Na$^+$]$_i$ will result in perturbations of pH$_i$ and [Ca$^{2+}$]. Furthermore, pH$_i$ and [Ca$^{2+}$] can be affected directly by ATP depletion through inhibition of H$^+$ and Ca$^{2+}$ pumps, respectively.

Intracellular acidosis is a prominent feature during hypoxic or ischemic insults in many tissues, including the kidney and was proved to have a protective effect (Weinberg, 1991; Weinberg et al, 1991; Edelstein et al., 1996). The mechanisms behind the protection offered by lowering the pH$_i$ are largely unknown. It has been suggested (Edelstein et al., 1996) that the cytoprotective effects of low pH$_i$ during hypoxia in rat proximal tubules are mediated, at least in part, by inhibition of calpain activity. Weinberg et al. (1994) showed that the protective effects of intracellular acidosis and glycine are complementary. Acidic pH$_i$ could also protect the cells during hypoxia/ischemia by preventing the onset of mitochondrial permeability transition (Lemasters et al., 1997).
On the contrary, Ca\(^{2+}\) overload can contribute to cell toxicity during hypoxia/ischemia. The issue of whether elevated [Ca\(^{2+}\)] plays a major role in the ischemic injury of renal cells has been a matter of debate for some time (Weinberg et al., 1991; Smith et al., 1992; Kribben et al., 1994; Peters et al., 1996; Weinberg et al., 1997). Contradictory results have been reported, depending on the cellular model used or on the modality of inducing ischemia.

Oxygen deprivation due to hypoxia/ischemia inhibits the respiratory chain in the mitochondrial inner membrane (Fig. 2). In normal conditions, this chain couples the electron transport to O\(_2\) to the extrusion of H\(^+\) from the matrix. This results in a potential difference between the matrix and the cytoplasm (Fig. 2). The mitochondrial membrane potential (\(\Delta\Psi\)) can reach values as high as -150 to -180 mV, with the matrix more negative than the cytoplasm. \(\Delta\Psi\) is linked to several mitochondrial functions, including ATP synthesis, import of mitochondrial proteins, Ca\(^{2+}\) homeostasis and metabolic transport. With inhibition of oxidative phosphorylation \(\Delta\Psi\) is expected to collapse. However, it has been suggested (Duchen & Biscoe, 1992; Di

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**Figure 2.** Schematic representation of the main physiological processes within the mitochondria. The supply of substrate to the tricarboxylic acid cycle (TCA cycle) promotes the reduction of NAD\(^+\) to NADH and of FAD to FADH\(_2\). As these are re-oxidized, they supply electrons to the respiratory chain. In the process of electron transfer to O\(_2\), protons are translocated across the inner mitochondrial membrane, generating a potential gradient of -150 to -180 mV. ATP synthesis takes place at a separate site, the ATP synthase (\(F_1F_0\)-ATP synthase). The enzyme is driven by the downhill movement of H\(^+\) and phosphorylates ADP, producing ATP, which is transported out of the matrix by the adenine nucleotide translocase (ANT) in exchange for ADP. ANT is a part of the mitochondrial permeability transition pore (MPTP) (it has been shown separately for clarity). Ca\(^{2+}\) accumulation into mitochondria occurs via Ca\(^{2+}\) uniporter and is driven by \(\Delta\Psi\). Ca\(^{2+}\) is extruded from the matrix by an electroneutral Na\(^+\)/Ca\(^{2+}\) exchanger. The various inhibitors of these processes are indicated by

- RuR - Ruthenium Red; CsA - cyclosporine A (Adapted from Duchen, 1999).
Lisa et al., 1995; Leyssens et al., 1996) that ΔΨ can be preserved in the absence of electron transport by the F₁F₀ pump operating in the reverse mode, at the expense of the cellular ATP. Provided that hypoxia/ischemia is not sustained overlong, this may protect the cells by allowing Ca²⁺ accumulation into mitochondria (Silverman, 1993), thus avoiding a presumably damaging Ca²⁺ overload of the cytoplasm.

Several in vitro renal models have been employed in an attempt to uncover the cellular events that occur during energy depletion in renal cells. These include freshly isolated renal tubules, primary renal cell cultures and renal cell lines. The renal cell lines have some advantages: 1) they are highly reproducible; 2) they are long-lived, which allows for long-term studies; 3) their cellular properties are well studied and defined; 4) they are amenable to both biochemical and histological evaluation. In cultured renal cells, ischemia can be simulated by inhibition of both oxidative phosphorylation and glycolysis (Canfield et al., 1991; Mandel et al., 1993; Doctor et al., 1994). Respiration can be blocked either by the removal of oxygen (real hypoxia) or by the addition of mitochondrial inhibitors (chemical hypoxia). Glycolysis is generally inhibited through the removal of glucose and/or addition of 2-deoxyglucose or iodoacetic acid.

Madin-Darby canine kidney (MDCK) cells, a permanent cell line derived from dog kidney, are among the best characterized cultured renal epithelial cells. They form monolayers with a characteristic polarized epithelial morphology: brush-border, apical cell-to-cell junction and lateral spaces. Although the exact source of these cells is unknown, MDCK cells display several properties of the distal tubular cells: hormonal profile with a cAMP response to vasopressin, oxytocin, glucagon, prostaglandin and epinephrine; high activity of the Na⁺/K⁺-ATPase; the expression of a furosemide sensitive Na⁺/K⁺/2Cl⁻ cotransporter. A further support for the distal origin of these cells is provided by the fact that monoclonal antibodies prepared against MDCK cells bind to the thick ascending limb, distal convoluted tubule and cortical collecting duct of the dog kidney (Rindler & Saier, 1981). There are two strains of this cell line (Richardson et al., 1981), strain I derived from an early passage (60-70) and strain II from later passages (100-110). The most distinctive difference between these strains is their electrical resistance. Strain I forms tight epithelia with resistance over 3000 Ω cm², whereas strain II produces leaky epithelia (100 Ω cm²). If monolayers are grown on impermeable culture dishes, ion and fluid transport from the apical to the basolateral membrane results in the formation of domes, i.e. fluid-containing blisters between the basolateral membrane and the culture dish. MDCK cells manifest many morphological similarities with the mammalian cortical collecting tubules (Valentich, 1981). Fig. 3 illustrates the transport systems identified in MDCK cells (reviewed by Lang et al., 1990). Na⁺ entry across the apical membrane occurs through a Na⁺/H⁺ exchanger and Na⁺ extrusion at the basolateral side involves a Na⁺/K⁺-ATPase. K⁺ thus accumulated leaves the cell mainly via a K⁺ conductance at the basolateral membrane. Cl⁻ enters the cell via a Na⁺/K⁺/2Cl⁻ cotransporter located at the basolateral membrane and exits through a Cl⁻ channel at the apical side. In addition to Na⁺/H⁺ exchanger, H⁺ secretion at the apical side may also involve a K⁺/H⁺ pump.
Figure 3. Synopsis of transport systems identified in MDCK cells. Indicated are also the inhibitors of these transport systems. (Adapted from Lang et al., 1990)

MEASUREMENT OF INTRACELLULAR ION CONCENTRATIONS USING FLUORESCENT INDICATORS

Several techniques are available for measuring intracellular ion concentrations. These include atomic absorption spectroscopy, flame photometry, radioactive ion analogs, nuclear magnetic resonance and the use of ion sensitive microelectrodes. Only nuclear magnetic resonance and the ion selective microelectrodes measure the concentration of free ionized ions. All the other methods evaluate the total content of a certain ion within the cells. However, the quantity of interest is the concentration of free ions and this might differ substantially from the total cellular content of the respective ion. Except for the ion sensitive microelectrodes, these methods provide information concerning the average ion concentration of cell populations, but do not address the issue of heterogeneity within populations. The use of ion sensitive microelectrodes allows measurements of ion activities in single cells, but is limited to relatively large cells, which can withstand electrode penetration.

The development of fluorescent ion indicators allows the non-destructive monitoring of intracellular ion concentrations even in small intact cells. Most fluorescent probes are now available in an acetoxymethyl ester (AM) form, which passes readily across cell membranes. Once inside the cytoplasm, non-specific esterases hydrolyze it to its free acid form. As the plasma membrane is not permeable to this form of the probe, it is trapped and concentrated inside. At the concentrations normally found in cells (50–100 μM), the cytotoxicity of fluorescent probes is generally negligible. Fluorescence techniques enable the measurements of ion concentrations in populations of cells as well as at the single cell level, thereby permitting the evaluation of intercellular variability. Imaging techniques provide the ability to spatially resolve ion
concentrations within single cells. Rapid (on the sub-second time scale) changes in ion concentration (e.g. Ca$^{2+}$ transients or oscillations induced by cell activation) can be easily detected.

Changes in the fluorescence of the indicator upon ion binding can take several forms (Haugland & Johnson, 1999): (1) an increase or decrease in the fluorescence intensity of the indicator with little change in either the absorption or fluorescence spectrum (e.g. the Na$^+$-sensitive probe Sodium Green, the Mg$^{2+}$ indicator Magnesium Green or the Cl$^-$-sensitive probe 6-methoxy-N-(3-sulphopropyl)quinolinium (SPQ)); (2) a shift of the absorption, and therefore of the fluorescence excitation spectrum, with little shift in the emission maximum (e.g. Fura-2, SBFI and BCECF) or (3) a shift in both the excitation and emission spectra (as for the Ca$^{2+}$ indicator Indo-1 and the pH probes SNARF and SNAFL). The fluorescence intensity depends both on the indicator concentration and the ion concentration. Indicators that undergo an excitation or emission wavelength shift upon ion binding have particular advantages for measurements in living cells. For these indicators, the ratio of fluorescence intensities measured at two wavelengths can be used to determine the ion concentration. This ratio depends on the ion concentration, but is independent of the indicator concentration. Consequently, the use of the ratiometric method reduces problems related to an unequal loading of the dye, photobleaching, leakage of the indicator from intact cells and changes in the cell volume. In situ calibration can be performed by exposing the cells to various extracellular concentrations of the ion under study in the presence of specific ionophores. Such calibration is more difficult with indicators that are not amenable to ratiometric detection. As an alternative to intensity measurements in the case of non-ratiometric probes, the lifetime of the excited state can be used (Lakowicz et al., 1992), because it does not depend on the probe concentration and therefore has the same advantages as the fluorescence ratio.

The behavior of fluorescent ion indicators in cells may considerably depend on the presence of other ions, pH, ionic strength, viscosity and protein binding. For example, all Mg$^{2+}$-sensitive fluorescent indicators also show a significant sensitivity to Ca$^{2+}$ whereas Na$^+$-sensitive indicators bind also K$^+$, although with a lower affinity (Haugland, 1996). Furthermore, all ion indicators are somewhat sensitive to pH. Spectral changes upon binding to proteins have been well documented for many probes, including Fura-2 (Konishi et al., 1988; Keating & Wensel, 1991; Bancel et al., 1992a; Arner et al., 1998) and Indo-1 (Ikenouchi et al., 1991; Bancel et al., 1992b). Consequently, one might expect differences between the fluorescent characteristics of these indicators inside the cells and those exhibited in buffer solutions. Such differences have been reported for most of the fluorescent indicators currently available (Negulescu & Machen, 1990; Keating & Wensel, 1991; Baartscheer et al., 1997; Arner et al., 1998). Although the mechanisms responsible for these differences are important for the use of the ion indicators in cells, there are only a few detailed studies aimed at uncovering these mechanisms.
OBJECTIVES

Fluorescent ion indicators are powerful tools for measuring intracellular ion concentrations. However, their behavior in the cytoplasm is generally different from that in simple aqueous solution. Such differences have been reported for the Na⁺ indicators Sodium Green and SBFI (Harootunian et al., 1989; Negulescu & Machen, 1990; Borzak et al., 1992; Baartscheer et al., 1997; Haugland & Johnson, 1999) but no systematic study of the causes leading to them has been performed up till now. Consequently, the first objective of this thesis is to investigate the factors that affect the behavior of Sodium Green (Chapter III) and SBFI (Chapter IV) in HeLa cells. To this aim, steady-state and time-resolved fluorescence measurements are performed in buffer solutions with various compositions as well as in HeLa cells. The effect of binding to proteins, viscosity and presence of K⁺ ions are studied. We also investigate the possibility of using the lifetime of these probes as a [Na⁺]ᵢ sensor. As the lifetime does not depend on the total concentration of the indicator (Szmacinski & Lakowicz, 1994), this would offer a real advantage especially in the case of Sodium Green, which is not a ratiometric indicator.

Because the impairment of ion homeostasis is one of the earliest events that follow the onset of ischemia, the second objective of this thesis was to investigate the time course of changes in the energy metabolism and intracellular ion concentrations in renal cells during ischemia (Chapters V and VI). We used MDCK cells and chemical hypoxia (induced by cyanide and 2-deoxyglucose) as an in vitro model of ischemia. We selected MDCK cells due to their high resistance to ATP depletion (Sheridan et al., 1993; Wiegele et al., 1998; Feldenberg et al., 1999). It has been shown that MDCK cells display signs of necrotic death after more than 6 hours of chemical hypoxia (Sheridan et al., 1993; Wiegele et al., 1998). Consequently, they are appropriate models for studying the cellular processes that occur in the surviving renal cells during energy depletion under hypoxia/ischemia. Chapter V investigates the correlation between the ATP content, pHᵢ, [Mg²⁺]ᵢ and mitochondrial function whereas Chapter VI is dedicated to the dynamics of [Ca²⁺]ᵢ and [Na⁺]ᵢ during chemical hypoxia and washout.
CHAPTER II

MATERIALS AND METHODS
CELL CULTURE

Two cell lines were used in this study, MDCK and HeLa S₃. MDCK cells (low passage number) were obtained from Dr. H. De Smedt (Laboratory of Physiology, Leuven, Belgium). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12, supplemented with 10% fetal calf serum, 14 mM L-glutamine, 25 mM NaHCO₃, 100 U/ml penicillin and 100 μg/ml streptomycin. The glucose concentration in the culture medium was 10 mM. Cells were maintained in a humidified 5% CO₂ atmosphere at 37°C. The medium was renewed every three-four days. Cells were subcultured for serial passage and experimental use from confluent monolayers grown in 25-cm² culture flasks. Cell detachment was realized by incubating the cells with trypsin-EDTA for 20 min at 37°C. When cultured on permeable supports, the cells form confluent monolayers with a resistance (400-500 Ωcm²) intermediary between the resistance of strain I (high resistance) and strain II (low resistance). For experiments, 10⁵-2x10⁶ cells (diluted in 0.5 ml culture medium) were seeded onto round glass coverslips with a diameter of 24 mm. The coverslips were placed in six-well plates (Nunc, Roskilde, Denmark) with a diameter of 35 mm. The cells were allowed to settle for 30 minutes at room temperature. Afterwards, 4 ml culture medium was added to each coverslip and the cells were grown to confluence over 3-7 days as described above.

HeLa S₃ cells were grown in DMEM supplemented with 10% fetal calf serum, antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and non-essential amino acids. The cells were incubated at 37°C in a 5% CO₂ atmosphere. They were subcultured using the same protocol as for MDCK cells. HeLa cells on coverslips were used for experiments when they were 2-6 days old.

FLUORESCENCE IMAGING MICROSCOPY

Most of the experiments reported in this study were performed using fluorescence techniques and fluorescent indicators. Ion sensitive probes were utilized to determine [Ca²⁺], [Na⁺], pH, [Mg²⁺], and ΔΨ as indicated in Table 1.

Cells are cultured on round glass coverslips with a diameter of 24 mm, as described above. For fluorescence measurements, the coverslips are mounted into a holder and placed on the stage of an inverted epifluorescence microscope Zeiss Axiovert 100 (Jena, Germany) (Fig. 1). The cells are washed several times with a normal saline solution (see the Solutions and Chemicals section) before measuring the background signal at the wavelengths used for fluorescence experiments. Afterwards, the cells are loaded with an appropriate fluorescent indicator (see below). After loading, they are again washed several times with normal saline.

Steady-state fluorescence measurements are performed using the experimental set-up shown in Fig. 1. Fluorescence is elicited by illumination with a XBO 75 W/2 OFR xenon lamp (Osram, Berlin-München, Germany). The excitation filters are inserted into a computer controlled Lambda 10-2 optical filter changer (Sutter Instrument Company, Novato, CA), which allows fast alternation between different excitation
Table 1. Overview of the fluorescent indicators used in this study.

<table>
<thead>
<tr>
<th>Ion studied</th>
<th>Ion indicator</th>
<th>Method</th>
<th>Mode of detection</th>
<th>$\lambda_{ex}$ (nm)</th>
<th>$\lambda_{em}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>Fura-2</td>
<td>Steady-state</td>
<td>Dual excitation ratio</td>
<td>340/380</td>
<td>535</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>SBFI</td>
<td>Steady-state</td>
<td>Dual excitation ratio</td>
<td>340/380</td>
<td>535</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time-resolved</td>
<td>Frequency domain</td>
<td>363</td>
<td>535</td>
</tr>
<tr>
<td>Sodium Green</td>
<td></td>
<td>Steady-state</td>
<td>Fluorescence intensity</td>
<td>495</td>
<td>535</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time-resolved</td>
<td>Frequency domain</td>
<td>488</td>
<td>535</td>
</tr>
<tr>
<td>H$^+$</td>
<td>BCECF</td>
<td>Steady-state</td>
<td>Dual excitation ratio</td>
<td>495/440</td>
<td>535</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium Green</td>
<td>Steady-state</td>
<td>Fluorescence intensity</td>
<td>495</td>
<td>535</td>
</tr>
<tr>
<td>$\Delta\Psi$</td>
<td>JC-1</td>
<td>Steady-state</td>
<td>Dual emission ratio</td>
<td>495</td>
<td>590/535</td>
</tr>
</tbody>
</table>

Figure 1. Schematic representation of the experimental set-up for fluorescence imaging experiments.

filters. All optical filters and dichroic mirrors were obtained from Chroma Technology Corp. (Brattleboro, VT). The excitation light is directed to the sample by a dichroic mirror and a Zeiss objective LD Achromplan (40x/0.6 corr.). The fluorescence collected by the objective is transmitted through the dichroic mirror and a bandpass emission filter to a Quantix CCD camera (Photometrix, Tucson, Arizona). The camera is equipped with a Kodak KAF 1400 CCD (grade 2, MPP) with 1317x1035 pixels and
cooled to \(-25^\circ\text{C}\) by a thermoelectric cooler. The acquisition of image sequences (or pairs of images, in the case of ratiometric indicators) is controlled by a home-made program which uses the V for Windows software (Digital Optics, Auckland, New Zealand). To avoid bleaching of the probe and photodamage of the cells, the illumination is restricted to the periods when the images are taken. Neutral density filters are inserted in the excitation pathway for intensity control. The signals are obtained by integrating spatially pixels over the cells. The background image is automatically subtracted, pixel by pixel, from the image of the loaded cells.

Fig. 2 top shows the bright field image of a confluent monolayer of MDCK cells. The excitation ratio image of the same cells loaded with BCECF is shown in the bottom panel of Fig. 2. To obtain this image, the fluorescence intensity images due to excitation at 495 and 440 nm were divided pixel by pixel. The analysis of individual cells from the same monolayer showed little variability in the resting intracellular ion concentrations or in the changes produced by different factors. Consequently, for most of the experiments we used the average intensity or ratio over the whole image for further calculations.

**Intracellular Na\(^+\) concentration** in HeLa cells was measured using the fluorescent indicators Sodium Green and SBFI whereas only SBFI was used for MDCK cells. Sodium Green was loaded into HeLa cells by incubation with the membrane permeant (tetraacacetate) form of the dye (10 µM, from a 4 mM stock solution in DMSO) for two hours, at room temperature, in the presence of the non-ionic surfactant Pluronic F-127 (0.05% w/v, added to the loading buffer from a 25% w/v stock solution in DMSO). SBFI was loaded under the same conditions (10 µM, from a 5 mM stock solution in DMSO, in the presence of 0.05% w/v Pluronic F-127) but for a longer period (4 hours) in both HeLa and MDCK cells. Loading at room temperature is known to reduce compartmentalization of the fluorescent indicators (Negulescu et al., 1990). The imaging experiments showed that HeLa cells did not contain brightly fluorescent spots that are indicative of dye compartmentalization into organelles. Some spots have been observed for SBFI in MDCK cells. However, digitonin (0.25 mg/ml) released 70-75% of the cellular SBFI, suggesting that the indicator is also mainly located in the cytoplasm of MDCK cells.

Excitation of Sodium Green was done through a 495/10 nm bandpass excitation filter and the fluorescence was recorded using a dichroic mirror type 72100 (>500 nm longpass filter) and a 535/50 nm bandpass emission filter. SBFI was used in dual excitation ratiometric mode. The 340/10 nm and 380/10 nm bandpass filters were used for excitation whereas the emission was detected using the same combination of dichroic mirror and emission filter as for Sodium Green. For both SBFI and Sodium Green, data collection time for an image was 5 seconds. A neutral density filter of 1.0 was used to attenuate the excitation light for Sodium Green. No such filter was used with SBFI because the transmission of UV light by the optics of the microscope is reduced.

*In vivo* calibration of the fluorescence signals of Sodium Green and SBFI in HeLa cells was accomplished by exposing the cells to various extracellular [Na\(^+\)] in the
Figure 2. Top - Bright field image of a confluent monolayer of MDCK cells grown on a glass coverslip. Bottom - The pseudocolor image of BCECF ratio from the same cells (in control conditions). A darker blue indicates a lower ratio (and thus a lower pH). MDCK cells were loaded with BCECF (2 μM BCECF-AM for 30 minutes at room temperature) and the fluorescence was excited alternately at 495 and 440 nm. Intensity images were divided pixel by pixel to obtain the ratio image.
presence of the ionophores gramicidin D (10 µM, from a 2 mM stock solution in ethanol), monensin (15 µM, from a 7.5 mM stock solution in DMSO) and nigericin (10 µM, from a 13 mM stock solution in ethanol). The solutions with various [Na⁺] were prepared by mixing in different proportions two solutions of equal ionic strength and osmolality. One buffer contained 160 mM Na⁺ (30 mM NaCl, 130 mM Na gluconate) and no K⁺, while the other one had 160 mM K⁺ (30 mM KCl, 130 mM K gluconate) and was Na⁺ free. Both calibration buffers also contained 1 mM CaCl₂, 1.2 mM MgSO₄, 32 mM HEPES and 10 mM glucose and the pH was adjusted to 7.1 with TRIS (Zahler et al., 1997).

For the experiments with SBFI, a calibration curve was derived according to the procedure described by Zahler et al. (1997). The ratio, r, of the fluorescence signal of SBFI due to excitation at 340 nm (F₃₄₀) over that at 380 nm (F₃₈₀) was normalized as follows:

\[ \bar{r} = \frac{r - r_0}{r_{90} - r_0} \]  \hspace{1cm} (1)

where \( r_0 \) and \( r_{90} \) are the fluorescence ratios \( F_{340}/F_{380} \) at 0 and 90 mM Na⁺, respectively. The experimental points were fitted according to:

\[ \bar{r} = \frac{K_0[Na]}{K_1 + [Na]} \]  \hspace{1cm} (2)

where \( K_0 \) and \( K_1 \) are fitting parameters.

The same procedure was used to calibrate the excitation ratios of SBFI in MDCK cells. In this case, only gramicidin D (10 µM) was used to equilibrate the intracellular and extracellular Na⁺ concentrations. One of the solutions used to obtain various [Na⁺] contained 30 mM NaCl and 115 mM Na⁺ gluconate and the other contained 30 mM KCl and 115 mM K⁺ gluconate. Both solutions also contained 1.5 mM CaCl₂, 1 mM MgSO₄, 10 mM HEPES, 5.5 mM glucose and are titrated to pH=7.4 with TRIS.

**Intracellular Ca²⁺ concentration** was monitored in MDCK cells using the fluorescent probe Fura-2. The cells were loaded with Fura-2 by incubation with the membrane permeant acetoxymethyl ester form of the dye (10 µM, from a 5 mM stock solution in DMSO) for 2 hours at room temperature in the presence of 0.05% w/v Pluronic F-127. The imaging experiments showed that Fura-2 loaded cells did not contain brightly fluorescent spots that are indicative of dye compartmentalization into organelles. Because the fluorophores of Fura-2 and SBFI are similar, the experiments with Fura-2 were performed using the same combination of excitation and emission filters as with SBFI. Data collection time for an image was 5 seconds.

Fura-2 was calibrated *in vivo* at the end of each experiment, according to the equation derived by Grynkiewicz et al. (1985): \[ [Ca^{2+}]_{i} = K_{d} R_{max} (r - r_{min})/(r_{max} - r) \], where \( K_d \) is the dissociation constant of Fura-2 for Ca²⁺ of 135 nM at room temperature (Vanden Bergh et al., 1995), \( R_{max} \) is the maximum fluorescence intensity due to excitation at 380 nm (in the absence of Ca²⁺) divided by the minimum fluorescence intensity at 380 nm (in the presence of saturating Ca²⁺), \( r \) is the \( F_{340}/F_{380} \) fluorescence ratio, \( r_{max} \) and
$r_{\text{min}}$ are the $F_{495}/F_{440}$ fluorescence ratios in the presence of saturating Ca$^{2+}$ and in the absence of Ca$^{2+}$, respectively. $r_{\text{max}}$ was obtained by permeabilizing the cells with Ca$^{2+}$ ionophore ionomycin (10 μM, added from a 1mM stock solution in ethanol), in the presence of 1.5 mM extracellular Ca$^{2+}$. To obtain the minimum ratio, $r_{\text{min}}$, the cells were exposed to a Ca$^{2+}$ free solution (containing 10 mM EGTA) with 10 μM ionomycin and were further loaded with BAPTA-AM (10 μM, from a stock solution of 10 mM in DMSO) to buffer intracellular Ca$^{2+}$.

**Intracellular pH** was monitored in MDCK cells by using the fluorescent probe BCECF. The cells were loaded with BCECF by incubation with the membrane permeable AM form of the dye (2 μM, from a 1 mM stock solution in DMSO) for 30 minutes at room temperature. The measurements were performed in dual excitation ratiometric mode, with excitation at 495 and 440 nm (with a bandwidth of 10 nm). The emission was collected through a >500 nm long-pass dichroic mirror and a 535/50 nm bandpass emission filter. Data collection time for an image was 1 second and a neutral density filter of 1.0 was inserted in the excitation pathway. A calibration of BCECF excitation ratios $F_{495}/F_{440}$ in terms of pH$_i$ was performed at the end of each experiment using the method of Thomas et al. (1979). The cells were exposed to 13 μM nigericin and a high K$^+$ solution of the following composition: 145 mM KCl, 2 mM CaCl$_2$ and 10 mM HEPES. The pH of this solution was set to five different values in the range 6.6-7.6 with TRIS. A plot of the ratio $F_{495}/F_{440}$ against pH$_i$ resulted in a linear calibration curve. Figure 3 shows a typical calibration curve in a BCECF experiment.

![Figure 3. Typical calibration curve for the excitation ratio of BCECF in terms of pH.](image)

The changes in the **intracellular Mg$^{2+}$ concentration** in MDCK cells were followed using the fluorescent indicator Magnesium Green (MgG). The fluorescence intensity of MgG increases with increasing [Mg$^{2+}$], and the $K_d$ of the Mg$^{2+}$-MgG complex is $=1$ mM (Haugland, 1996). MDCK cells were loaded with 10 μM MgG in the AM form (added from a 4 mM stock solution in DMSO), in the presence of 0.05% w/v Pluronic F-127, for 2 hours at room temperature. After washing out the extracellular indicator, the cells were incubated in normal saline solution for 20 minutes, to allow the complete de-esterification of the indicator. MgG is a single-excitation, single-emission indicator. The probe was excited at 495 nm (10 nm bandwidth) and fluorescence was
collected using the same dichroic mirror and emission filter as for BCECF. Data collection time for an image was 5 seconds and a neutral density filter of 1.0 was inserted in the excitation pathway. Because the calibration of the MgG signal is extremely difficult (Leyssens et al., 1996), the results are presented as a percentage change compared with control.

**Mitochondrial membrane potential** was evaluated using the potentiometric indicator JC-1 (5,5',6,6' - tetrachloro - 1,1',3,3' - tetra - ethylbenzimidazolylcarbocyanine iodide). JC-1 is a lipophilic cation, thus it partitions into mitochondria according to the Nernst potential. JC-1 exists as a monomer at low concentrations and as aggregates, called J-aggregates, at high concentrations, i.e. in mitochondria with a high membrane potential (Reers et al., 1991). JC-1 in its monomeric form emits fluorescence with a maximum intensity at 530 nm (green fluorescence) when excited at 490 nm, while the maximum intensity of the fluorescence emitted by J-aggregates occurs at 590 nm (red fluorescence). Depolarization of the mitochondrial inner membrane leads to a redistribution of the dye with dissociation of J-aggregates (Reers et al., 1991; Cossarizza et al., 1993; Di Lisa et al., 1995; Reers et al., 1995). Consequently, the ratio of the fluorescence emission at 590 nm over that at 530 nm decreases upon depolarization of the mitochondrial membrane. It has been shown that the membrane potential reported by JC-1 is not significantly affected by changes in pH at pH values above pK_a=6.45 (Reers et al., 1991). MDCK cells were loaded with 10 μM JC-1 (from a 10 mM stock solution in DMSO) for 30 minutes at 37°C. Dual-emission ratiometric measurements were performed by manually changing the emission cube. The excitation was done through a 10 nm bandpass filter centred at 495 nm. The fluorescence emitted by the JC-1 monomers was collected through a >500 nm long-pass dichroic mirror and a 535/50 nm bandpass emission filter. The emission cube used to detect the J-aggregates consisted of a >560 nm long-pass dichroic mirror and a 590/55 nm emission filter. Data collection time for an image was 5 seconds and a neutral density filter of 1.0 was inserted in the excitation pathway. The mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP, 10 μM) was added at the end of each experiment (from a 10 mM stock solution in ethanol) to determine the JC-1 emission ratio associated with a collapsed ΔΨ.

For **NAD(P)H autofluorescence measurements**, MDCK cells were excited via a 360/40 nm excitation filter. The emission was transmitted to the camera through a >380 nm long-pass dichroic mirror and a 450/50 nm emission filter. Data collection time for an image was 5 seconds. At the end of each experiment, the cells were lysed with 0.1 % Triton X-100 to evaluate the background signal. The results are expressed as a percentage change compared with control. The signal-to-noise ratio in these experiments was 2 to 3.

**TIME-RESOLVED FLUORESCENCE MEASUREMENTS**

The fluorescence lifetime of a fluorophore represents the average amount of time the molecule remains in the excited state prior to its return to the ground state. The decay times of most fluorescent indicators are in the range of 1-10 ns. There are two widely
used methods to measure the fluorescence lifetimes: the pulse method in the time domain and the phase-modulation method in the frequency domain (for a general overview, see Lakowicz, 1983). In the pulse method the sample is excited with a brief pulse of light and the time-dependent decay of fluorescence intensity is measured (Fig. 4).

![Figure 4. Simulated fluorescence decay (time domain data) of a mono-exponential component.]

In the phase-modulation (or frequency domain) method, which was used to obtain the time-resolved data presented in this thesis, the sample is excited with sinusoidally modulated light (Fig. 5):

\[ E(t) = E_0 [1 + M_E \sin(\omega t + \Phi_E)] \]  

(3)

where \( M_E \) is the ratio of the AC over DC component of the excitation light \( (M_E = b/a, \ \text{Fig. 5}) \) and \( \omega \) is the angular frequency \( (\omega = 2\pi v, \ \text{with} \ v \ \text{the modulation frequency of the excitation light}) \). This modulation frequency ranges from a few to several hundred MHz for detecting fluorescence lifetimes. The optical frequency of the light is on the order of \( 10^{14} \) to \( 10^{15} \) Hz. The fluorescence emission will be modulated at the same frequency as the excitation. Because of the finite lifetime of the excited state, the

![Figure 5. Principle of the phase-modulation method for time-resolved fluorescence measurements.]

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modulated emission is delayed in phase by an angle $\Phi$ relative to the excitation. Furthermore, the emission is less modulated (demodulated) relative to the excitation (Eq. 4):

$$F(t) = F_0[1 + M_F \sin(\omega t + \Phi_F)]$$  \hspace{1cm} (4)

where $M_F = B/A$. The observable quantities in this method are the phase shift $\Phi = \Phi_F - \Phi_E$ and the demodulation $m = \frac{M_F}{M_E} \leq 1$.

Fig. 6 shows simulated data in the frequency domain for a mono-exponential decay. The phase shift increases and the modulation decreases as the lifetime increases. The modulation frequency must be comparable to the decay rate ($=1/\tau$) to obtain measurable values of $\Phi$ and $m$. For a given lifetime, increasing the modulation frequency results in larger phase shifts and greater degrees of demodulation. At low frequency the phase angle is close to zero and the modulation is about 1. At high frequencies, the phase shift approaches $90^\circ$ and the modulation is practically zero. Best results are obtained when the measurements are performed around the crossing point (e.g. $\approx200$ MHz for a decay time of 1 ns, according to Fig. 6). At frequencies lower than the crossing point, phase data are more accurate whereas at higher frequencies the modulation is determined more precisely.

![Figure 6. Simulated frequency domain data of the fluorescence decay of a mono-exponential component. Thin lines – the phase angle; thick lines – the modulation](image)

All the information content in a single fluorescence decay experiment is contained in the impulse response function (for the time domain data) or the transfer function (for the frequency domain data) of the sample. The impulse response function and transfer function are directly related to each other through the Fourier transform. The fluorescence impulse response function $f(t)$ is given by:

$$f(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i)$$

with $\alpha_i$ the pre-exponential factor associated with the decay time $\tau_i$. The frequency domain transfer function in complex notation (the Fourier transform of Eq. 5) is:
\[
f(\omega) = \sum_{i=1}^{n} \frac{\alpha_i \tau_i}{1 + j \omega \tau_i}
\]

For a mono-exponential decay, the phase shift and the demodulation of the fluorescence signal with respect to the excitation light are given by (Gratton et al., 1984a):

\[
\tan \Phi = \omega \tau, \quad m = \frac{1}{\sqrt{1 + \omega^2 \tau^2}}
\]

In the case of a multi-exponential decay, \( \Phi \) and \( m \) are given as follows (Lakowicz, 1983):

\[
\tan \Phi = \frac{N}{D}, \quad m = \frac{1}{\sqrt{N^2 + D^2}}
\]

with:

\[
N = \sum_{i=1}^{n} \frac{\omega \alpha_i \tau_i^2}{1 + \omega^2 \tau_i^2}, \quad D = \sum_{i=1}^{n} \frac{\alpha_i \tau_i}{\sum_{i=1}^{n} \alpha_i \tau_i}
\]

The fractional intensity of species \( "i" \), \( f_i \), can be calculated as:

\[
f_i = \frac{\alpha_i \tau_i}{\sum_{i=1}^{n} \alpha_i \tau_i}
\]

The fluorescence intensity decay is furthermore characterized by the average decay time \( <\tau> \) defined by

\[
<\tau> = \frac{\sum_{i=1}^{n} \frac{\alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i}}{\sum_{i=1}^{n} f_i \tau_i}
\]

For the frequency-domain method, a reference measurement is made to correct for the instrument effects on detector and excitation modulation and phase settings (Barbieri et al., 1989). Either scattered light or a homogeneous fluorophore solution with a single exponential decay with known lifetime can be used for the reference measurement. The use of a reference compound that is excited and emits fluorescence at the same wavelengths as the sample has the advantage of avoiding possible artifacts due to color and geometric effects in the photomultiplier. In practice, these effects can be minimized by a proper detector choice and detector orientation. If a fluorescent reference compound with the lifetime \( \tau_R \) is used, the phase shift \( \Phi_S \) and demodulation \( m_s \) of the sample are calculated using the expressions:

\[
\Phi_S = \Phi_{obs} + \tan^{-1}(\omega \tau_R), \quad m_s = m_{obs} \frac{1}{\sqrt{1 + \omega^2 \tau_R^2}}
\]

where \( \Phi_{obs} \) is the observed phase difference between the sample and the reference compound.

We used the frequency domain method to record the time-resolved data of Sodium Green and SBFI in various buffer solutions and in HeLa cells. The experimental set-up (Fig. 7) is based on the same fluorescence microscope as used for steady-state.
Figure 7. Schematic diagram of the experimental set-up for lifetime measurements in the frequency domain. Ar⁺-laser, CW Argon ion laser; BSD, beam stirring device; PIN, pinhole; TWP, two-way polarizer; PC, Pockels cell; M, 100% reflection mirror; L1, L2, lenses; RND, reflecting neutral density filter; RM, removable mirror; FCH-1, FCH-2, computer controlled filter wheels; EXF, excitation filter; DM, dichroic mirror; OBJ, objective; EMF, emission filter; P, polarizer; PMT-S, PMT-R, sample and reference photomultipliers; CCD, CCD camera; FH, filter holder; HV, high voltage supply; SG-M, SG-S, master and slave RF signal generators; RFA-1, RFA-2, RF amplifiers; SAM, synchronous acquisition module; Xe, xenon lamp; HF, heat filter.

measurements. For time-resolved measurements, the excitation light is provided by a water cooled CW Argon ion laser type 2020-03 (Spectra Physics, Mountain View, CA). A beam steering device (BSD) directs the laser light towards the optical modulator, composed of a two-way polarizer (TWP), a Pockels cell (PC, composed of two hygroscopic KDP crystals) and a 100% reflecting mirror (M) (ISS, Urbana Champaign, IL). Within the Pockels cell, the light intensity is sinusoidally modulated at a selected frequency in the appropriate RF range (5±300 MHz). Three pinholes (PIN) are placed in the optical path. The first pinhole sets the diameter of the incoming light beam, the second one selects a properly modulated light spot and the third pinhole controls the beam in front of the microscope. Modulated light is divided into two parts by a reflecting neutral density filter (RND) (Newport Corp., Irvine, CA): 99.9 % of the light is directed towards the sample and 0.1 % from the excitation light hits the reference photomultiplier, PMT-R (R 928, Hamamatsu, Joko-cho Japan). A removable mirror (RM) allows either the laser beam (for time-resolved measurements) or the light from the xenon lamp (for steady-state measurements) to enter into the microscope. Accurate collinear alignment of the laser excitation beam with the optical axis of the microscope is maintained with a 1 mm pinhole placed at the rear entry into
the microscope. Inside the microscope, the beam is reflected by a >500 nm long-pass dichroic mirror (DM) into the objective (OBJ). The objective focuses the beam onto the sample. The diameter of the exciting beam in the sample plane can be changed down to a limiting value of about 1 μm by the lens L2. The emission collected by the objective is transmitted through the dichroic mirror, the emission filter (EMF, the 535/50 nm bandpass filter), a film polarizer (P) oriented at the magic angle (with respect to the polarization of the excitation beam) and detected by the sample photomultiplier, PMT-S (R 3896, Hamamatsu, Joko-cho Japan). The photomultipliers were selected for a good frequency response. The gain of both photomultipliers is modulated at a frequency that differs by a cross-correlation frequency (400 Hz) from that used for the excitation light modulation. The modulation signals are generated by a pair of phase-locked signal generators (SG-M, SG-S) model 2023 (Marconi, Stevenage, UK) with further amplification for proper modulation levels using ENI (Rochester, NY) RF amplifiers 525LA (25 W for light intensity modulation, RFA-1) and 604L (4W for photomultiplier gain modulation, RFA-2). Digital data collection occurs with an ISS-A2D interface card (ISS, Urbana-Champaign, IL). A synchronous acquisition module (SAM) triggers the collection cycles of the card. The SAM unit also establishes the phase-locked loop between master and slave synthesizers. Measurements are performed in a so-called fast scan mode. In this mode, the frequency response of the sample is measured first over the selected frequency range followed by a measurement of the frequency response of the fluorescent reference compound. Data are collected to a phase and modulation accuracy of 0.2° and 0.004, respectively, to a maximum of 40 iterations. Each iteration collected 400 waveforms. At the cross-correlation frequency of 400 Hz used in all experiments, this means a data acquisition time of 1 second for each iteration. A fast Fourier transform (FFT) routine extracts the phase angle and demodulation values from the folded waveform signal (Feddersen et al., 1989). The instrument is completely computer controlled using a standard GPIB card, an ISS-A2D data acquisition card and software from ISS (Urbana Champaign, IL). The program calculates and stores in a file the phase shift and the demodulation factor of the sample at each modulation frequency.

The 488 nm line of the Argon ion laser is used to excite Sodium Green and the 363 nm line is used for SBF1. In all experiments, data were recorded at 12 modulation frequencies between 5 and 300 MHz. The reference compound used at both excitation wavelengths is Erythrosin B in distilled water (τ_R=90 ps) (Boens et al., 1990). The recording time for one curve (the sample and the reference compound) is about 6 minutes.

**Single frequency measurements**

The standard procedure described above requires the recording of the frequency response of the sample to be alternated with that of the reference compound. In this way, it is not possible to perform repeated measurements on the same group of cells (we cannot return, after measuring the reference, to exactly the same spot on the coverslip). To allow the monitoring in time of the fluorescence emitted by the same group of cells, some measurements were performed using a single modulation frequency (160 MHz). We measured repeatedly the phase shift of the fluorescence
signal of the sample with respect to the excitation light, $\Phi_s$, whereas the phase shift of the reference compound was determined only at the end of the experiment. These measurements require a very stable system over long periods of time (tens of minutes or even hours). To correct for possible instabilities of the system, after measuring $\Phi_s$, we determined the phase shift, $\Phi_{sc}$, of the light scattered by the coverslip. For these measurements, the emission filter and a filter that allows the scattered light to enter into the PMT-S (488/10 nm bandpass filter) were inserted in a filter wheel (FCH-2) (Sutter Instrument Company, Novato, CA). With a stable excitation, $\Phi_{sc}$ was constant during the experiment. Whenever $\Phi_{sc}$ varied, the changes in $\Phi_{sc}$ were used to correct the values of $\Phi_s$. The following expression was used to calculate the corrected phase shift of the sample at the $i$th point ($\Phi_{sc}^{cor}(i)$):

$$\Phi_{s}^{cor}(i) = \Phi_s(i) - \delta(i), \quad \delta(i) = \Phi_{sc}(i) - \Phi_{sc}(N)$$ (13)

where $\Phi_{sc}(i)$ and $\Phi_{sc}(N)$ are the phase shift of the scattered light at the $i$th point and at the end of the experiment (just before measuring the response of the reference compound), respectively.

**Analysis of time-resolved fluorescence data**

Frequency-domain data were analyzed in terms of a multi-exponential decay using software from Globals Unlimited (Urbana Champaign, IL). The quality of the fit was judged by the reduced $\chi^2$, i.e. the $\chi^2$ normalized to the degrees of freedom in the fitting procedure (Gratton & Linneman, 1984):

$$\chi^2 = \sum_{i=1}^{N} \sum_{j=1}^{n} \left[ \left( \Phi_{m}^{ij} - \Phi_{c}^{ij} \right)^2 \right] \left( \frac{\sigma_{\phi}^{ij}}{\sigma_{\phi}^i} + \frac{\left( M_{m}^{ij} - M_{c}^{ij} \right)^2}{\sigma_{M}^{ij}} \right) / \left( 2n - f - 1 \right)$$ (14)

where $\Phi_{m}^{ij}, M_{m}^{ij}$ are the measured phase angle and demodulation in the $i$th experiment at the $j$th frequency, respectively, $\Phi_{c}^{ij}, M_{c}^{ij}$ are the corresponding calculated values, $\sigma_{\phi}^{ij}, \sigma_{M}^{ij}$ are the standard deviations of the phase angle and demodulation in the $i$th experiment at the $j$th frequency, respectively, $N$ is the total number of experiments analyzed together, $n(i)$ is the number of frequencies in the $i$th experiment, $n$ is the total number of phase-modulation pairs recorded ($n = \sum_{i=1}^{N} n(i)$) and $f$ is the number of fitting parameters. Minimization of $\chi^2$ is performed using the Marquardt-Levenberg nonlinear least square algorithm.

For the analysis of the series of curves recorded in a buffer solution with the same composition or in HeLa cells at various $[Na^+]$, we used the following protocol: the frequency domain data at each $[Na^+]$ were analyzed separately. A preliminary set of models (sum of $k$ exponentials) was selected, based on the value of $\chi^2$ and the random distribution of residuals. The values of the decay times obtained at various $[Na^+]$ were compared. If the decay times did not change significantly with $[Na^+]$ (the differences were within the confidence intervals calculated using the exhaustive search in the
Globals program, see Beechem et al., 1991) and no trend was observed, data at various [Na⁺] were analyzed together with the decay times linked over [Na⁺] and the pre-exponential factors freely adjustable. The most likely models resulting from single curve analysis were tested. Criteria for selecting the global model were again the value of χ² and the random distribution of residuals. The simultaneous analysis of multiple fluorescence decay experiments (known as global analysis) has proved very useful for the accurate recovery of closely spaced exponential decaying functions (Beechem et al., 1983; Jameson et al., 1984; Gratton et al., 1984a; Gratton et al., 1984b).

The analyses reported here used only phase data, because in the frequency range investigated, phase angle values are in the sensitive range (5-70°) therefore they are more accurate than the modulation data. This is the case especially for SBFI, due to its shorter average decay time (see also the comment above Fig. 6). Similar photophysical models were revealed by the analysis of both phase and modulation data but the values of the reduced χ² were higher. The experimental accuracy of 0.2° was used as the standard deviation for the phase angle. For the experiments in buffer the indicated uncertainties are standard deviations obtained from one-dimensional error analysis (Beechem et al., 1991). The uncertainties for the experiments in cells are standard errors.

Assuming a 1:1 stoichiometry of Na⁺ binding to Sodium Green, an apparent dissociation constant, K_d^{app}, of the Na⁺-Sodium Green complex was derived from the relative phase angle (the actual phase angle divided by the phase angle in the absence of Na⁺, \( \Phi / \Phi_0 \)) at the modulation frequency of 160 MHz, using the relation:

\[
\frac{\Phi}{\Phi_0} = 1 + \frac{p[Na^+]}{K_d^{app} + [Na^+]} \tag{15}
\]

where p and K_d^{app} are fitting parameters.

The phase angle at the modulation frequency of 160 MHz was used to obtain a calibration curve for Sodium Green similar to that obtained from ratiometric measurements of SBFI (Eq. 2). The normalized phase angle is defined as:

\[
\Phi^* = \frac{\Phi - \Phi_0}{\Phi_{90} - \Phi_0} \tag{16}
\]

where \( \Phi_0 \) and \( \Phi_{90} \) are the phase angles of Sodium Green at 0 and 90 mM Na⁺, respectively.

**SPECTROFLUORIMETRY**

Excitation and emission spectra of Sodium Green and SBFI in various buffer solutions were recorded on a Perkin Elmer LS-5B Luminescence Spectrometer (Beaconsfield, United Kingdom).

**FLAME PHOTOMETRY**

In some solutions, [Na⁺] was determined with a flame photometer model K701-A from Eppendorf Geratebau (Hamburg, Germany).
MEASUREMENT OF THE CELLULAR ATP CONTENT
Confluent monolayers of MDCK cells grown on glass coverslips were washed with
normal saline solution and incubated with hypoxic solution (followed or not by
washout) for various periods of time. Control cells were incubated in normal saline
solution. ATP measurements were performed with a luciferin-luciferase based assay
kit (Molecular Probes, Eugene, OR). The reaction buffer contained 150 μg ml⁻¹
luciferin, 1.25 μg ml⁻¹ luciferase, 5 mM MgSO₄, 1 mM dithiothreitol (DTT), 25 mM
Tricine, 0.1 mM EDTA and 0.1 mM azide, pH=7.8. The cells were solubilized in 100
μl of somatic cell ATP-releasing agent (Sigma, St Louis, MO) for 30 seconds. 50 μl of
cell extract was added to 450 μl reaction buffer. ATP levels were measured with a
luminometer type 1250 from Wallac (Turku, Finland). Calibration was performed with
several standard ATP solutions in the concentration range 10⁻⁸⁻¹⁰⁻⁵ M. The results are
expressed as a percentage change compared with control.

All measurements reported in this thesis were carried out at room temperature.

STATISTICS
All reported data are expressed as means ± S.E.M. and the number of experiments is
indicated. To express the variability between different experiments in terms of the
standard deviation, SD can be calculated using the expression:

\[ SD = \sqrt{n} \text{ S.E.M.} \]  

(17)

where n is the number of experiments. Statistical discriminations were performed with
Student’s unpaired two-tailed t test. We also used the F test to compare variances. The
differences in means and variances were considered significant if P<0.05. Where
necessary, the Welch’s correction for unequal variances was applied to the t test.

SOLUTIONS AND CHEMICALS
The normal saline solution used for HeLa cells contained (in mM): 150 NaCl, 5 KCl, 1
CaCl₂, 1.2 MgSO₄, 32 HEPES, 10 glucose and 2 NaH₂PO₄ (pH=7.4). In K⁺ or Na⁺ free
solutions, these ions were substituted by equimolar N-methyl-D-glucamine (NMDG).
Twice distilled water was used in all solutions.

MDCK cells were bathed in a normal saline solution containing (in mM): 140 NaCl, 5
KCl, 1.5 CaCl₂ 1 MgSO₄, 10 HEPES and 5.5 glucose; pH adjusted to 7.4 with TRIS.
Chemical hypoxia was accomplished with a “hypoxic solution” containing (in mM):
135 NaCl, 5 KCl, 1.5 CaCl₂ 1 MgSO₄, 10 HEPES, 10 2-deoxyglucose (DOG) and 2.5
NaCN (pH=7.4). On washout, 7.5 mM Na⁺ pyruvate substituted glucose in the saline
solution (with a reduction in NaCl to maintain constant osmolality). In some
experiments, CaCl₂ and MgSO₄ were omitted from the bath solution and replaced by
0.5 mM EGTA (the Ca²⁺ and Mg²⁺ free solution). Na⁺ was substituted by equimolar
NMDG in Na⁺ free solutions.

The fluorescence intensity decays of Sodium Green free acid were measured in
HEPES buffer (10 mM) with or without 5% w/v bovine serum albumin (BSA). The
pH of the buffer was adjusted to 7.2 with TRIS. The final concentration of Sodium
Green in buffer solutions was 1 μM. Steady-state and time-resolved measurements of SBFI free acid were performed in MOPS buffer (10 mM) at pH=7.2 (adjusted with TRIS or NaOH). The concentration of SBFI in buffer solutions was 5 μM. Unless otherwise stated, various [Na⁺] were obtained by adding NaCl. In some experiments the sum of Na⁺ and K⁺ concentrations was kept constant to 145 mM.

All fluorescent indicators used in this work were from Molecular Probes, Inc. (Eugene, OR). Pluronic F-127 and BAPTA-AM were also from Molecular Probes. Gramicidin D, monensin, nigericin, FCCP, ionomycin, thapsigargin, verapamil and BSA (fraction V) were obtained from Sigma (St. Louis, MO). Erythrosin B and oligomycin (mixture of types A, B and C) were purchased from Acros Organics (Geel, Belgium). All the other chemicals were of analytical grade.
CHAPTER III

Fluorescence Lifetime Microscopy of the Na\(^+\) Indicator Sodium Green in HeLa Cells.
ABSTRACT
This study investigates the usefulness of lifetime measurements of Sodium Green for evaluating intracellular Na⁺ concentration in HeLa cells. Frequency domain lifetime measurements are performed in HeLa cells and in different buffer solutions (with and without K⁺ and bovine serum albumin). In all cases, the fluorescence decays of Sodium Green are multiexponential, with decay times independent of [Na⁺]. Three relaxation times are found in the various buffer solutions. Binding of the indicator to albumin results in an increase in the long and intermediate decay times. For Sodium Green inside HeLa cells, the intensity decay can be approximated by a bi-exponential. The ratio of the fractional intensity of the long decay time (τ₂=2.4±0.2 ns) to that of the short component (τ₁=0.4±0.1 ns) increases with [Na⁺]. These changes are significantly less pronounced in cells as compared with the buffer solutions. Similar values for the resting [Na⁺]ᵢ were estimated from lifetime measurements of Sodium Green and from ratiometric measurements using SBFI. Alternatively, [Na⁺]ᵢ can be monitored by measuring only the phase angle at the modulation frequency of 160 MHz. The usefulness of this latter approach is demonstrated by following the changes in [Na⁺]ᵢ induced by reversible inhibition of the Na⁺/K⁺ pump.

INTRODUCTION
In most cells intracellular sodium concentration is highly regulated. Resting [Na⁺]ᵢ is in the range of 5-20 mM, whereas [Na⁺] in the extracellular fluids is several times higher (more than 100 mM). This large electrochemical gradient of Na⁺ is maintained by the Na⁺/K⁺ pump, which utilizes the energy derived from ATP hydrolysis to exchange intracellular Na⁺ with extracellular K⁺. Na⁺ gradient is very important in excitable cells, where it provides the basis for action potentials and synaptic depolarization. Many cells utilize the energy stored in the Na⁺ gradient to couple the unfavorable transmembrane solute flow to Na⁺ transport (the so-called secondary active transport). Examples include nutrient (sugars, amino acids) and neurotransmitter uptake, acid extrusion via the Na⁺/H⁺ exchanger and Ca²⁺ transport through Na⁺/Ca²⁺ exchanger. Cytoplasmic Na⁺ is also important for Na⁺ and Ca²⁺ homeostasis in the mitochondria. Because the activity of the Na⁺/K⁺ pump and of many Na⁺-coupled solute transporters can be modulated by various factors, [Na⁺]ᵢ might be expected to change under a variety of physiological or pathophysiological conditions. Because of the critical role of the Na⁺ gradient, such changes are likely to have diverse physiological consequences. Thus, knowledge of [Na⁺]ᵢ is important in understanding various cellular mechanisms.

The use of Na⁺ sensitive fluorescent dyes allows the non-invasive monitoring of [Na⁺]ᵢ. The most widely used Na⁺ sensitive probe is sodium binding benzofuran isophthalate (SBFI). This indicator is generally used in dual excitation ratiometric mode. The ratio of the fluorescence signal due to excitation at 340 nm over that at 380 nm increases with [Na⁺]. Consequently, SBFI has the obvious advantages associated with a signal independent of dye concentration inside the cells: the ratio signal is not affected by dye leakage or changes in cell volume. However, due to its excitation in the UV range of the spectrum, SBFI cannot be used with several agents. A well known example of such an agent is amiloride, an inhibitor of epithelial Na⁺ channels, which is
also excited in the UV. Another problem consists in cellular autofluorescence, which is mainly attributed to NAD(P)H and has an excitation maximum near 360 nm. The excitation wavelengths do not allow the use of SBFI with a standard confocal microscope.

The relatively new Na⁺-sensitive fluorescent indicator, Sodium Green, can be excited with visible light, thus circumventing many of the disadvantages of SBFI. The Na⁺ binding site of Sodium Green is a crown ether, the same as for SBFI. Sodium Green has two identical fluorophores, based on analogs of fluorescein. As compared to SBFI, Sodium Green shows greater selectivity for Na⁺ than K⁺ (≈1 fold versus ≈18 fold) and a higher quantum yield (0.2 versus 0.08) in Na⁺ containing solutions (Haugland, 1996). The ground-state dissociation constant Kₐ is about 6 mM in K⁺ free solution and 21 mM in solutions with a combined Na⁺ and K⁺ concentration of 135 mM (Haugland, 1996). The fluorescence intensity of Sodium Green increases with increasing [Na⁺]. However, the dye does not exhibit spectral shifts upon ion binding so that the ratiometric approach cannot be used. Therefore only the changes in fluorescence intensity can be used to monitor [Na⁺]. This is a disadvantage as changes in fluorescence intensity might not be related to the ion under investigation. In these conditions, time-resolved measurements of Sodium Green, which are mostly independent of the probe concentration (Szmacinski & Lakowicz, 1994), can provide an important tool for accurate monitoring of [Na⁺]. Furthermore, the precise nature of the fluorescence decay can reveal details about the interactions of the fluorophore with its environment. As the behavior of the indicator in cells may considerably depend on the presence of other ions, pH, ionic strength, viscosity and protein binding, time-resolved measurements could provide useful information about the effect of these factors on the intracellular behavior of Sodium Green. The indicator was reported (Szmacinski & Lakowicz, 1997a) to be an excellent lifetime-sensitive Na⁺ probe in buffer solutions: the average lifetime increases more than two times going from the free form of the dye to the Na⁺-bound form.

This study investigates the applicability of lifetime measurements of Sodium Green for evaluating [Na⁺] in HeLa cells. It was previously reported that HeLa cells stain well with this indicator (Amorino & Fox, 1995). Lifetime measurements are performed in the frequency domain (Clegg et al., 1992; Szmacinski & Lakowicz 1994; French et al., 1998) using a fluorescence microscope and a CW Argon ion laser the light of which is modulated by a Pockels cell. The fluorescence intensity decays of Sodium Green loaded into HeLa cells are compared with those recorded in buffer solutions at various [Na⁺], with and without K⁺ and BSA. Calibration is performed in vivo by using Na⁺ ionophores. The analysis of the fluorescence intensity decay provides useful information about the behavior of the dye inside the cells, but for more practical approaches it is sufficient to measure only the phase angle at a single modulation frequency. Recent reports have already shown the usefulness of single modulation frequency measurements for lifetime imaging of cells loaded with the calcium indicator Quin-2 (Lakowicz et al., 1994) and for deriving the fluorescence lifetime in single cells with a flow cytometer (Yu et al., 1998). Preliminary accounts of these results have been reported and published in abstract form (Despa et al., 1998).
RESULTS

Steady-state measurements of Sodium Green in various buffer solutions
The ground-state dissociation constant, $K_d$, of the Na$^+$·Sodium Green complex was determined from a Hill plot using the integrated emission spectra with excitation at 488 nm. The fluorimetric titration indicated a 1:1 stoichiometry (slope=0.92±0.04) and yielded a value of 9±1 mM for $K_d$. This value compares well with the value reported by Haugland (1996) (6.0 mM) and by Szmucinski & Lakowicz (1997a) (8.4 mM). It has been indicated that the value of the dissociation constant depends considerably on the actual composition of the solutions (Haugland, 1996).

The presence of BSA (5% w/v) in the buffer solutions resulted in a red shift of about 10 nm in the emission maximum and a decrease in the fluorescence intensity. In the presence of BSA, the Hill plot revealed that the binding of Na$^+$ to Sodium Green had a 1:1 stoichiometry (slope=1.0±0.1) and the $K_d$ was higher than in the absence of the protein (30±8 mM). We observed that the increase in fluorescence intensity with [Na$^+$] was attenuated in the presence of BSA whereas the maximum intensity in the absence of Na$^+$ remained basically unchanged.

Time-resolved fluorescence measurements of Sodium Green in various buffer solutions
The fluorescence intensity decays of Sodium Green in buffer solutions containing various [Na$^+$] are shown in Fig. 1. Single curve analysis of these decays revealed that Sodium Green is characterized by three decay times that are independent of [Na$^+$]. Consequently, the decays were analyzed using a tri-exponential global analysis. The discrete decay times ($\tau_i$) were linked over different [Na$^+$] while the corresponding fractional intensities ($f_i$) were freely adjustable.

Table 1 summarizes the results of this analysis. The fractional intensity associated with the short decay time ($\tau_1=0.251±0.005$ ns) decreased with increasing [Na$^+$], the contribution of the long component ($\tau_2=2.90±0.02$ ns) increased with increasing [Na$^+$] while the fractional intensity of the intermediate component ($\tau_2=0.74±0.01$ ns) had no pronounced sensitivity with respect to changes in Na$^+$. For [Na$^+$] ≥ 50 mM, the fluorescence intensity decays were found to be bi-exponential, the contribution of the short component being negligible. Table 1 also shows that the average lifetime $<\tau>$ increased with [Na$^+$].

The sum of Na$^+$ and K$^+$ concentrations is kept relatively constant in biological cells. Therefore, the measurements were repeated in buffer solutions with various [Na$^+$] but with [Na$^+$]+[K$^+$]=145 mM (approximately the physiological value). In this case, the decay curves of Sodium Green could also be analyzed globally with three decay times (Table 2). The recovered decay times ($\tau_1=0.22±0.01$ ns, $\tau_2=0.69±0.01$ ns and $\tau_3=2.80±0.03$ ns) were comparable with those obtained in the absence of K$^+$ and the fractional intensities behaved in a similar manner. The contribution of the intermediate component was somewhat higher than in the absence of K$^+$. 

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To further mimic the conditions inside the cytoplasm, where a large amount of proteins is present (about 200-300 mg/ml (Alberts et al., 1986; Luby-Phelps, 1994)), the measurements were repeated in the presence of 5% (w/v) BSA, both with and without K⁺. Three decay times were found. The decay times were also independent of [Na⁺] so that the global tri-exponential analysis was used to determine the changes in fractional intensities with [Na⁺]. Global analysis results are presented in Table 3 (for buffer solutions without K⁺) and Table 4 (for buffer solutions where [Na⁺]+[K⁺]=145 mM). The intermediate and the long decay time were in these cases longer than in the absence of proteins: τ₂=1.20±0.02 ns and τ₃=3.70±0.06 ns in the absence of K⁺ and τ₂=1.58±0.02 ns, τ₃=3.91±0.07 ns when [Na⁺]+[K⁺]=145 mM. Similar to previous
Table 1. Global tri-exponential intensity decay analysis of Sodium Green in buffer solutions in the presence of various Na⁺ concentrations.*

<table>
<thead>
<tr>
<th>[Na⁺] (mM)</th>
<th>f₁</th>
<th>f₂</th>
<th>f₃</th>
<th>&lt;τ&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.11</td>
<td>0.36</td>
<td>1.25</td>
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<td>0.13</td>
<td>0.47</td>
<td>1.55</td>
</tr>
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<td>0.10</td>
<td>0.57</td>
<td>1.80</td>
</tr>
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<td>0.11</td>
<td>0.67</td>
<td>2.09</td>
</tr>
<tr>
<td>5</td>
<td>0.08</td>
<td>0.22</td>
<td>0.70</td>
<td>2.20</td>
</tr>
<tr>
<td>10</td>
<td>0.06</td>
<td>0.18</td>
<td>0.76</td>
<td>2.34</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.17</td>
<td>0.78</td>
<td>2.41</td>
</tr>
<tr>
<td>30</td>
<td>0.04</td>
<td>0.17</td>
<td>0.79</td>
<td>2.43</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>0.22</td>
<td>0.78</td>
<td>2.43</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>0.22</td>
<td>0.78</td>
<td>2.42</td>
</tr>
</tbody>
</table>

* Global decay times are: $\tau_1=0.251 \pm 0.005$ ns, $\tau_2=0.74 \pm 0.01$ ns, $\tau_3=2.90 \pm 0.02$ ns, $\chi^2=2.1$. In all tables, the uncertainties in the fractional intensities (standard deviations obtained from the one-dimensional error analysis) are of the order of the last significant digit.

Table 2. Global tri-exponential intensity decay analysis of Sodium Green in buffer solutions in the presence of different Na⁺ concentrations with $[Na⁺]+[K⁺]=145$ mM.*

<table>
<thead>
<tr>
<th>[Na⁺] (mM)</th>
<th>f₁</th>
<th>f₂</th>
<th>f₃</th>
<th>&lt;τ&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.32</td>
<td>0.37</td>
<td>0.31</td>
<td>1.19</td>
</tr>
<tr>
<td>2.5</td>
<td>0.21</td>
<td>0.30</td>
<td>0.49</td>
<td>1.63</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>0.30</td>
<td>0.55</td>
<td>1.79</td>
</tr>
<tr>
<td>10</td>
<td>0.10</td>
<td>0.27</td>
<td>0.63</td>
<td>1.98</td>
</tr>
<tr>
<td>20</td>
<td>0.08</td>
<td>0.22</td>
<td>0.70</td>
<td>2.14</td>
</tr>
<tr>
<td>30</td>
<td>0.05</td>
<td>0.21</td>
<td>0.74</td>
<td>2.24</td>
</tr>
<tr>
<td>50</td>
<td>0.02</td>
<td>0.21</td>
<td>0.77</td>
<td>2.30</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>0.22</td>
<td>0.78</td>
<td>2.34</td>
</tr>
</tbody>
</table>

* Global decay times are: $\tau_1=0.22 \pm 0.01$ ns, $\tau_2=0.69 \pm 0.01$ ns, $\tau_3=2.80 \pm 0.03$ ns, $\chi^2=2.6$.

Table 3. Global tri-exponential intensity decay analysis of Sodium Green in buffer solutions containing 5% (w/v) BSA, in the presence of various Na⁺ concentrations.*

<table>
<thead>
<tr>
<th>[Na⁺] (mM)</th>
<th>f₁</th>
<th>f₂</th>
<th>f₃</th>
<th>&lt;τ&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.47</td>
<td>0.23</td>
<td>0.30</td>
<td>1.47</td>
</tr>
<tr>
<td>2.5</td>
<td>0.34</td>
<td>0.29</td>
<td>0.37</td>
<td>1.80</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
<td>0.34</td>
<td>0.36</td>
<td>1.79</td>
</tr>
<tr>
<td>10</td>
<td>0.27</td>
<td>0.29</td>
<td>0.44</td>
<td>2.05</td>
</tr>
<tr>
<td>20</td>
<td>0.21</td>
<td>0.30</td>
<td>0.49</td>
<td>2.20</td>
</tr>
<tr>
<td>30</td>
<td>0.17</td>
<td>0.30</td>
<td>0.53</td>
<td>2.36</td>
</tr>
<tr>
<td>50</td>
<td>0.14</td>
<td>0.25</td>
<td>0.61</td>
<td>2.60</td>
</tr>
<tr>
<td>100</td>
<td>0.09</td>
<td>0.23</td>
<td>0.68</td>
<td>2.81</td>
</tr>
</tbody>
</table>

* Global decay times are: $\tau_1=0.205 \pm 0.004$ ns, $\tau_2=1.20 \pm 0.02$ ns, $\tau_3=3.70 \pm 0.06$ ns, $\chi^2=2.3$. 
Table 4. Global tri-exponential intensity decay analysis of Sodium Green in buffer solutions containing 5% (w/v) BSA, in the presence of different [Na\(^+\)] with [Na\(^+\)]+[K\(^+\)] = 145 mM. *

<table>
<thead>
<tr>
<th>[Na(^+)] (mM)</th>
<th>f(_1)</th>
<th>f(_2)</th>
<th>f(_3)</th>
<th>&lt;(\tau)&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.31</td>
<td>0.37</td>
<td>0.32</td>
<td>1.92</td>
</tr>
<tr>
<td>2.5</td>
<td>0.27</td>
<td>0.35</td>
<td>0.38</td>
<td>2.12</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>0.34</td>
<td>0.41</td>
<td>2.23</td>
</tr>
<tr>
<td>10</td>
<td>0.22</td>
<td>0.32</td>
<td>0.46</td>
<td>2.39</td>
</tr>
<tr>
<td>20</td>
<td>0.19</td>
<td>0.31</td>
<td>0.50</td>
<td>2.52</td>
</tr>
<tr>
<td>30</td>
<td>0.15</td>
<td>0.33</td>
<td>0.52</td>
<td>2.60</td>
</tr>
<tr>
<td>50</td>
<td>0.14</td>
<td>0.30</td>
<td>0.56</td>
<td>2.72</td>
</tr>
<tr>
<td>100</td>
<td>0.11</td>
<td>0.30</td>
<td>0.59</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* Global decay times are: \(\tau_1 = 0.299 \pm 0.004\) ns, \(\tau_2 = 1.58 \pm 0.02\) ns, \(\tau_3 = 3.91 \pm 0.07\) ns, \(\chi^2 = 2.3\).

cases, the fractional intensity of the short component was found to decrease with increasing [Na\(^+\)] while the relative contribution of the long component increased with increasing [Na\(^+\)]. The contribution of the intermediate component was insensitive to variations in [Na\(^+\)]. The average lifetime increased with [Na\(^+\)] in all cases.

**Time-resolved fluorescence measurements of Sodium Green in HeLa cells**

The intensity decays of unloaded cells were investigated first. A weak autofluorescence was observed, which accounted for less than 2% of the total fluorescence intensity. Because of this very low signal, the frequency domain data of the autofluorescence could not be determined accurately. In these conditions it was better not to perform corrections (Reinhart et al., 1991).

The fluorescence intensity decays of Sodium Green loaded into HeLa cells exhibited a relatively small variability between different cells. Frequency domain data for five different regions from the same coverslip are shown in Fig. 2.

![Figure 2](image-url).

**Figure 2.** Frequency-domain data (phase angle) of the fluorescence decay of Sodium Green trapped inside the cytoplasm of HeLa cells. The decays were recorded from 5 different groups of cells (containing 2 - 5 cells) from the same coverslip at resting Na\(^+\) concentration.
After recording the intensity decay under physiological conditions (at natural [Na\(^+\)]), an in vivo calibration was performed to investigate the Na\(^+\)-sensitivity of the dye inside the cells. Cells were exposed to different [Na\(^+\)] in the presence of the ionophores gramicidin D, monensin and nigericin (as described in Chapter II). Five different decay curves were recorded at each [Na\(^+\)], each time cells from different regions of the coverslip being studied. The average of the phase angles of Sodium Green at each [Na\(^+\)], (for different cells from the same coverslip) versus the modulation frequency, is shown in Fig. 3.

![Graph showing phase angle of Sodium Green against frequency for different Na\(^+\) concentrations](image)

**Figure 3.** Frequency-domain data (phase angle) of the fluorescence intensity decay of Sodium Green in HeLa cells at various [Na\(^+\)]. The data represent the averages for five different groups of cells from the same coverslip.

Time-resolved data collected under the same [Na\(^+\)], were analyzed with a multi-exponential model. Similar to the behavior of the dye in buffer solutions, the decay times appeared to be independent of [Na\(^+\)]. Consequently, a global multi-exponential decay analysis was performed with the decay times linked over all the data sets. A set of 25 decay curves were analyzed together, five for each [Na\(^+\)]. The tri- and bi-exponential global analyses yielded comparable \(\chi^2\) values (\(\chi^2=7.8\) for bi-exponential as compared with \(\chi^2=4.0\) for tri-exponential analysis). The results of a representative experiment are shown in Table 5. Variability in the recovered decay times over different coverslips was less than 10%. Table 5 shows that the fractional intensity of the long component (2.30±0.03 ns) increased with increasing [Na\(^+\)] and the contribution of the short decay time (0.40±0.01 ns) decreased accordingly. However, these changes were significantly less pronounced as compared with the corresponding changes observed in buffer solutions, even in the presence of BSA. Nevertheless, as Fig. 4 points out, the ratio of the fractional intensities of the long and short decay times (\(f_2/f_1\)) was still sensitive enough to [Na\(^+\)] to allow calibration. This ratio increased linearly with intracellular Na\(^+\) concentration over the 0-90 mM concentration range. This calibration curve can be used to evaluate the natural [Na\(^+\)].
Table 5. Global bi-exponential intensity decay analysis of Sodium Green in HeLa cells at different Na⁺ concentrations.*

<table>
<thead>
<tr>
<th>[Na⁺] (mM)</th>
<th>f₁</th>
<th>f₂</th>
<th>&lt;τ&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.28 ± 0.01</td>
<td>0.72 ± 0.01</td>
<td>1.76 ± 0.01</td>
</tr>
<tr>
<td>15</td>
<td>0.25 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>1.82 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>0.22 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>1.88 ± 0.02</td>
</tr>
<tr>
<td>45</td>
<td>0.20 ± 0.01</td>
<td>0.80 ± 0.01</td>
<td>1.92 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>0.16 ± 0.01</td>
<td>0.84 ± 0.01</td>
<td>2.00 ± 0.02</td>
</tr>
</tbody>
</table>

*Global decay times are: τ₁=0.40 ± 0.01 ns, τ₂=2.30 ± 0.03 ns, χ²=7.8. Five different decay curves were recorded at each [Na⁺]. Shown are the mean ± S.E.M.

Figure 4. The [Na⁺]₀ dependence of the ratio of the fractional intensities f₂ and f₁ corresponding to the long and the short decay time, respectively. The data represent the mean ± S.E.M. for five different groups of cells from the same coverslip.

The fractional intensities f₁ and f₂ are determined by a bi-exponential analysis of the decays obtained from cells in the various physiological conditions where the decay times are fixed to the values obtained from the analysis of the calibration decays. Using this procedure we found that the resting [Na⁺]₀ was 14±4 mM (mean±S.E.M. over 5 calibration experiments).

The analysis of the fluorescence intensity decay of Sodium Green at various [Na⁺]₀ is important for the characterization of the dye behavior inside the cells. When only the determination of [Na⁺]₀ is important, a more practical approach is to monitor the phase angle at a single modulation frequency. As can be seen in Fig. 3, the phase angle increased with increasing [Na⁺] for modulation frequencies higher than ~60 MHz. Within the frequency range investigated here, the maximum sensitivity of the phase angle to [Na⁺] was found for a modulation frequency around 160 MHz. Fig. 5 shows the relative phase angle, Φ/Φ₀ of Sodium Green in different buffer solutions as well as in HeLa cells at this modulation frequency. The presence of K⁺ reduced the sensitivity of the indicator to Na⁺. When [Na⁺]+[K⁺]=145 mM, the phase angle increased 1.9 times within the concentration range 0-100 mM, while the corresponding increase in the absence of K⁺ was 2.2 times. The presence of BSA induced a further decrease in the Na⁺ sensitivity of the indicator. When both K⁺ and BSA were present, there was only a 1.4 fold increase in the phase angle accompanying an increase in [Na⁺] from 0 to 100 mM. Inside the cells, only a 1.2 fold increase with [Na⁺] was observed.
Figure 5. \( [Na^+] \) dependence of the relative phase angle, \( \Phi/\Phi_0 \) (Eq. 15 in Chapter II), exhibited by Sodium Green at the modulation frequency of 160 MHz in various buffer solutions and in HeLa cells. (●) simple aqueous buffer; (■) buffer solution with \( [Na^+] + [K^+] = 145 \) mM; (○) buffer solution with 5% BSA; (□) buffer solution with BSA (5%), and K+ (so that \( [Na^+] + [K^+] = 145 \) mM); (×) HeLa cell.

Assuming a 1:1 stoichiometry of Na⁺ binding to Sodium Green, as indicated by the spectral measurements, an apparent dissociation constant, \( K_d^{app} \), was derived from the phase angle data at 160 MHz (see Eq. 15 in Chapter II). The \( K_d^{app} \) values obtained for the various buffer solutions and for HeLa cells are shown in Table 6.

Table 6. The apparent dissociation constant, \( K_d^{app} \), of the Na⁺–Sodium Green complex, derived from the phase angle at the modulation frequency of 160 MHz. *

<table>
<thead>
<tr>
<th>Condition</th>
<th>( K_d^{app} ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple buffer solution</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Buffer solution, ( [Na^+] + [K^+] = 145 ) mM</td>
<td>9.2 ± 0.8</td>
</tr>
<tr>
<td>Buffer solution with 5% BSA</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Buffer solution with 5% BSA and ( [Na^+] + [K^+] = 145 ) mM</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>56 ± 11 (n=6)</td>
</tr>
</tbody>
</table>

* For the experiments in buffer the indicated uncertainties are standard deviations obtained from the fitting procedure. The value reported for HeLa cells represents the mean ± S.E.M. over \( n \) experiments.

Fig. 6 shows a representative calibration curve for HeLa cells in terms of the normalized phase angle, \( \Phi \) (Eq. 16 in Chapter II), at the modulation frequency of 160 MHz. The natural \( [Na^+]_i \), can also be obtained from this curve. The value of natural \( [Na^+]_i \), obtained from this simplified approach (12±3 mM, \( n=5 \)) compared very well...
with the value determined from the ratio $f_2/f_1$ (14±4 mM, n=5, mean ± S.E.M. over n calibration experiments).

![Figure 6. Calibration curve of $[\text{Na}^+]_i$ in HeLa cells in terms of normalized phase angle (Eq. 16 in Chapter II), at the modulation frequency of 160 MHz. The points represent the mean ± S.E.M. for five different groups of cells from the same coverslip.]

The ability of the phase shift of Sodium Green fluorescence at the modulation frequency of 160 MHz to follow the time course of $[\text{Na}^+]_i$ under physiological conditions was tested. Changes in $[\text{Na}^+]_i$ were induced by blocking the Na$^+/K^+$ pump (cells were bathed with a K$^+$ free solution) followed by reactivation of the pump in the absence of extracellular Na$^+$ (a condition known to induce a high Na$^+$ efflux). An in vivo calibration has been performed at the end of each experiment. Fig. 7 shows the results of a representative experiment: the changes in the phase angle (Fig. 7A) and the corresponding variations of the $[\text{Na}^+]_i$ (Fig. 7B). $[\text{Na}^+]_i$ increased from 10 to 33 mM during a 40 minutes exposure to K$^+$ free solution. Reversal of the transmembrane Na$^+$ gradient resulted in a decrease of $[\text{Na}^+]_i$ to values close to the control in about 30 minutes.

![Figure 7. (A) Time course of the changes in the corrected phase angle of the fluorescence signal at the modulation frequency of 160 MHz of Sodium Green in HeLa cells induced by inhibition and reactivation of the Na$^+/K^+$ pump. The Na$^+/K^+$ pump was inhibited by excluding K$^+$ from the bathing solution. Reactivation of the pump (by adding back K$^+$) was done in the absence of external Na$^+$ to induce a fast efflux of Na$^+$ from the cells. (B) The corresponding changes in $[\text{Na}^+]_i$, derived after calibration of the Sodium Green signal.]
Steady-state measurements of Sodium Green and SBFI in HeLa cells

We used fluorescence imaging microscopy to measure the fluorescence intensity of Sodium Green in HeLa cells. The changes in Sodium Green intensity with [Na\(^+\)], during a calibration procedure were minimal (not shown), so that it was impossible to calculate the resting [Na\(^+\)]\(_i\). Under these conditions, we could not compare the steady-state and time-resolved methods. Consequently, we performed ratiometric measurements with SBFI and the results were compared with those provided by frequency-domain microscopy of Sodium Green. A typical steady-state experiment where SBFI was used to investigate the effect of the Na\(^+\)/K\(^+\) pump on the [Na\(^+\)]\(_i\) is shown in Fig. 8A. Where indicated, the cells were bathed in a solution without either K\(^+\) or Na\(^+\). An in vivo calibration was performed at the end of the experiment, as described in Chapter II. The fluorescence excitation ratio F\(_{340}/F_{380}\) closely followed [Na\(^+\)]. Fig. 8B shows a typical calibration curve (see Chapter II). The natural [Na\(^+\)]\(_i\) determined from this type of experiments was 16 ± 3 mM (n=6).

**Figure 8.** Monitoring of [Na\(^+\)]\(_i\) in HeLa cells by SBFI. (A) Changes in the SBFI excitation ratio F\(_{340}/F_{380}\) upon inhibition (0 mM K\(^+\), 140 mM Na\(^+\) solution) followed by reactivation (5 mM K\(^+\), 0 mM Na\(^+\) solution) of the Na\(^+\)/K\(^+\) pump. An in vivo calibration was performed at the end of the experiment; (B) The normalized ratio (Eq. 1 in Chapter II) as a function of [Na\(^+\)].

Fig. 9 shows the changes in [Na\(^+\)]\(_i\) observed during inhibition and re-activation of the Na\(^+\)/K\(^+\) pump, as indicated by the SBFI ratio. The time course of the changes in [Na\(^+\)]\(_i\) was essentially the same as that observed with Sodium Green.

**Figure 9.** Effect of blocking the Na\(^+\)/K\(^+\) pump (in the absence of K\(^+\) from the bathing solution) and Na\(^+\) efflux following the reactivation of the pump (by adding back K\(^+\)) in the absence of external Na\(^+\) on [Na\(^+\)]\(_i\) in HeLa cells, as assessed by ratiometric measurements with SBFI (see Fig. 8).
DISCUSSION

The fluorescence intensity decay of Sodium Green in buffer solutions with different compositions (in the absence of K\(^+\) or with [Na\(^+\)]+[K\(^+\)]=145 mM, with or without BSA) and at various Na\(^+\) concentrations can be described by a tri-exponential. In all cases, the relaxation times are independent of [Na\(^+\)]. Consequently, the decay curves recorded in each type of buffer solution are analyzed with a global tri-exponential model. In all these buffer solutions, the fractional intensity of the short decay time is found to decrease with increasing Na\(^+\) concentration, so that this component can be formally associated with a Na\(^+\)-free form of the indicator. Similarly, the long component can be associated with a Na\(^+\)Na\(_2\)Sodium Green complex, because its contribution to the fluorescence decay increases with increasing [Na\(^+\)]. The amplitude of the intermediate component is less sensitive to [Na\(^+\)]. We should emphasize that this is only an empirical description and that the photophysics of Sodium Green is certainly more complicated, since we found three decay times even in the absence of Na\(^+\). The independence of the relaxation times on [Na\(^+\)] suggests that the rate of the Na\(^+\)Na\(_2\)Sodium Green complex formation in the excited state is essentially negligible for [Na\(^+\)] up to 100 mM (the maximum [Na\(^+\)] investigated here).

The results are in good agreement with those previously reported by Szmacinski & Lakowicz (1997a). These authors also found that in buffer solution, in the absence of K\(^+\) and proteins, Sodium Green exhibits a tri-exponential decay with the decay times independent of [Na\(^+\)] and similar changes in the fractional intensities with increasing [Na\(^+\)]. The decay times recovered by Szmacinski & Lakowicz (1997a) are somewhat shorter than the values found here. These differences could be due to the different composition of the buffer solution.

The average lifetime of Sodium Green in simple aqueous solution is found to increase from 1.25 ns to 2.42 ns when [Na\(^+\)] increased from 0 to 100 mM. For a similar concentration range, Szmacinski & Lakowicz (1997a) found an increase from 1.13 ns to 2.39 ns. An increase in the average fluorescence relaxation time upon cation binding has also been observed for SBFI (Szmacinski and Lakowicz, 1997b; see Chapter IV), Quin-2 (Hirshfield et al., 1993; Hirshfield et al., 1996), Calcium Green (Hirshfield et al., 1993; Lakowicz et al., 1992) and Fura-2 (Van den Bergh et al., 1995; Arner et al., 1998). Although the decay times are similar, the average lifetime of Sodium Green is shorter in the presence of K\(^+\) (so that [Na\(^+\)]+[K\(^+\)]=145 mM) for [Na\(^+\)] from 0 to 100 mM. Szmacinski & Lakowicz (1997a) associated a decay time of 0.69 ns with the K\(^+\)bound form of Sodium Green, which is shorter than the relaxation time of the Na\(^+\)bound form (=2.5 ns). We also found that the fractional intensity corresponding to the intermediate component (0.69 ns) is larger in the presence of K\(^+\). Thus, one may conclude that the decrease in the average lifetime results from the competition between Na\(^+\) and K\(^+\) for binding to the crown ether of Sodium Green.

The presence of 5% w/v BSA in the buffer solutions (in the absence or presence of K\(^+\)) induces a significant shift in the intermediate and long decay times of Sodium Green towards higher values. The average lifetime in the absence of Na\(^+\) and at high [Na\(^+\)]:
(≥50 mM) is longer than in simple buffer solutions. An increase in average fluorescence decay time upon protein binding has also been reported for Fura-2 (Konishi et al., 1988, Keating & Wensel, 1991; Arner et al., 1998) and Quin-2 (Hirschfield et al., 1996) and SBFI (see Chapter IV). Sodium Green maintains its sensitivity to [Na⁺] in the presence of both K⁺ and BSA. The average lifetime increases from 1.92 ns to 2.83 ns with an increase in [Na⁺] from 0 to 100 mM. This is in contrast with the case of SBFI (see Chapter IV), where the changes in average lifetime with [Na⁺] are inhibited by the presence of BSA (5% w/v). We observed (see Chapter IV) that binding of SBFI to BSA results in a new species characterized by an additional decay time, which is considerably longer than those corresponding to free SBFI and Na⁺⊂SBFI complex. However, no new component has to be introduced to describe the fluorescence decay of Sodium Green in buffer solutions with BSA. We suggest (see Chapter IV) that SBFI binds with high affinity to hydrophobic sites of BSA, because the presence of protein resulted in an increase in fluorescence intensity, a blue shift in the emission spectra and an increase in the average lifetime. These changes were more pronounced in the absence of Na⁺, indicating that mainly the free form of SBFI binds to BSA. With Sodium Green, we observe that the increase in fluorescence intensity with [Na⁺] is attenuated in the presence of BSA whereas the maximum intensity in the absence of Na⁺ remains basically unchanged. The emission spectra are red shifted. All these data suggest that the binding of Sodium Green to BSA is relatively weak and it has a small influence on its photophysical behavior. It is likely that the presence of BSA mostly affects the ground state dissociation constant of the indicator. Indeed, we found that both \( K_d \) (steady-state measurements) and \( K_{d,app} \) (lifetime measurements) are higher in the presence of BSA.

A red shift in the emission spectra upon adding albumin to the buffer solution has been also observed for fluorescein (Meisingset & Steen, 1981) and the fluorescein-based pH indicator BCECF (Plasek et al., 1996).

An apparent dissociation constant, \( K_{d,app} \), was derived from the phase data at the modulation frequency of 160 MHz. \( K_{d,app} \) is different from the true dissociation constant because the phase angle is not proportional to the concentration of either the free or the Na⁺-bound form of the dye. However, it is a useful parameter describing the concentration range associated with phase angle sensitivity to [Na⁺]. The value obtained for Sodium Green in buffer solution without K⁺ and proteins (2.2±0.2 mM) is identical to that previously reported by Szmacinski & Lakowicz (1997a) for the phase angle at the modulation frequency of 151.8 MHz. Under more physiological conditions where [Na⁺]+[K⁺]=145 mM, the value of \( K_{d,app} \) is ≈4.5 times higher (9.2±0.8 mM), which also compares well to that obtained by Szmacinski & Lakowicz (1997a) (8.3±1.4 mM). Szmacinski & Lakowicz (1997a) performed the measurements by keeping the combined ionic concentrations of Na⁺ and K⁺ at 135 mM. It has been reported (Haugland, 1996) that the true \( K_d \) of Na⁺⊂Sodium Green complex, derived from the fluorescence intensity, increases 3.5 times in the presence of K⁺ ([Na⁺]+[K⁺]=145 mM).
Some of the problems associated with the use of the fluorescent indicators in cells are compartmentalization of the dye into organelles, incomplete ester hydrolysis and dye leakage. The experiments (including the loading) were performed at room temperature and not at 37° C. This condition is known to reduce both leakage and compartmentalization of many fluorescent indicators (Negulescu et al., 1990). Steady-state imaging experiments show that the cells loaded with Sodium Green do not contain brightly fluorescent spots that are indicative of dye compartmentalization into organelles. An incomplete de-esterification of Sodium Green is unlikely to affect significantly the fluorescence intensities in cells because the ester form is not fluorescent (Haugland, 1996).

An in vivo calibration has been performed to determine the sensitivity of the fluorescence intensity decay to [Na⁺]. Similar to the situation found in the various buffer solutions, the decay times of Sodium Green in HeLa cells are independent of Na⁺ concentration. The intensity decay can be approximated by a bi-exponential. The relative contribution of the long decay time to the fluorescence decay is found to increase with [Na⁺], at the expense of the contribution of the short component. However, these changes are less pronounced in HeLa cells as compared with the corresponding changes observed in buffer solutions. This is not surprising considering that in HeLa cells we observe minimal changes in Sodium Green intensity with [Na⁺], so that the calibration is not possible. It has been reported that in some cell types, the Na⁺ dependent increase in the fluorescence intensity of Sodium Green is severely attenuated, probably as a result of binding to proteins (Haugland & Johnson, 1999). The intensity measurements in cells are also affected by other factors, such as dye leakage and changes in cell volume. Generally, these factors do not influence frequency domain data. It has been suggested (Harootunian et al., 1989) that the differences in the spectral properties of ion indicators in cells as compared to buffer solution are due to a greater microviscosity of the cytoplasm. However, the cytoplasm of living cells has a fluid phase viscosity about that of water (Luby-Phelps et al., 1993) or only a few times greater than water (Luby-Phelps, 1994; Luby-Phelps et al., 1986).

The reduced sensitivity of Sodium Green to [Na⁺] in HeLa cells is also seen in the small changes of the average lifetime: it increases from 1.76 ns to 2.00 ns when [Na⁺] changes from 0 to 90 mM. Nevertheless, the ratio between the fractional intensities of the long and short decay times is still sensitive enough to allow calibration in terms of [Na⁺]. This ratio is found to increase linearly with intracellular Na⁺ concentration over the concentration range 0-90 mM.

The analysis of the fluorescence intensity decay of Sodium Green provides useful information about the behavior of the indicator inside the cells. However, the recording of these decays is on the one hand time-consuming and on the other hand it requires to move the objective from the sample to the reference compound. In this way, it is not possible to follow the time course of [Na⁺], in the same group of cells (one cannot return the objective to exactly the same spot on the sample coverslip). Although Sodium Green is more photostable than its precursor, fluorescein (Szmacinski & Lakowicz, 1997a), repeated measurements of the whole frequency
response of the probe on the same location will finally result in photobleaching or phototransformation. To allow the monitoring of the Sodium Green signal in the same group of cells, we measured the phase angle in cells at a single modulation frequency during the whole experiment. The phase shift of the reference compound was measured only at the end of the experiment. We used the light scattered by the coverslip as a local reference, which allowed us to correct the phase angle of the sample for possible instabilities of the system.

The maximum sensitivity of the phase angle to \([Na^+]) is found at modulation frequencies near 160 MHz. Consequently, this frequency was selected for single frequency measurements. At 160 MHz, the changes in the phase angle with \([Na^+]) are substantial in buffer solution. The sensitivity to \([Na^+]) drops considerably in the presence of \(K^+ ([Na^+]+[K^+]=145 \text{ mM}) and BSA (5\% w/v), while a further decrease is observed in HeLa cells. \(K_d^{opp}\) in HeLa cells is also considerably higher than the values found in various buffer solutions. The higher \(K_d^{opp}\) and the reduced sensitivity to \([Na^+]) inside the cytoplasm, where large amounts of \(K^+\) and proteins are present, are consistent with the results corresponding changes observed in buffer solutions containing \(K^+\) and BSA.

The resting \([Na^+]), derived from the phase angle of Sodium Green at the modulation frequency of 160 MHz compares well with the value determined using the ratio of the fractional intensities of the long and short decay times and to the value obtained from ratiometric measurements with SBFI. This shows the applicability of single frequency measurements for monitoring \([Na^+])\). The use of fractional intensities ratio has the further disadvantage of requiring additional calculations. The resting \([Na^+]) obtained in this study is close to the values previously reported for HeLa cells by Zahler et al. (1997) (19±5 mM) using ratiometric measurements with SBFI, and by Amorino & Fox (1995) (20 mM), who used Sodium Green with flow-cytometry. These authors performed the measurements at 37 °C while here all the experiments were done at room temperature. This could explain the small differences. For both the frequency domain measurements with Sodium Green and the ratiometric measurements with SBFI we found a relatively high variability in the calibration curves. Therefore, the calibration has to be performed at the end of each experiment.

The ability of the phase angle at the modulation frequency of 160 MHz to follow the physiologically induced changes in \([Na^+]) is investigated. \([Na^+]) is varied by a reversible inhibition of the \(Na^+/K^+\) pump. The same effect is evaluated by the ratiometric method using SBFI. Both methods reveal an about three-fold increase in \([Na^+]) when the cells are exposed to a \(K^+\) free solution for 40 minutes. The \(Na^+\) efflux, following the rise of extracellular \(K^+\) to 5 mM in the absence of external \(Na^+\), returns \([Na^+]) to values close to normal in 30 minutes. These results are comparable with those obtained by Zahler et al. (1997). The phase angle data exhibit somewhat larger fluctuations as compared to the excitation ratios of SBFI. This can be rationalized on the basis of the inherent averaging properties of time-integrated methods. Furthermore, the SBFI ratio is averaged over 40-50 cells while only 2-5 cells are investigated by time-resolved measurement with Sodium Green.
CONCLUSIONS

This report shows that although the fluorescence decay of Sodium Green is significantly less sensitive to [Na\(^+\)] in HeLa cells as compared to simple buffer solutions, the decay of the indicator can be used to follow changes in [Na\(^+\)]. Time-resolved measurements provide information about the behavior of the dye in the cells. However, when only the determination of [Na\(^+\)]\(_i\) is important, a more practical approach is to monitor the phase angle at a single modulation frequency. We found that the phase angle at 160 MHz can be used to monitor the increase in [Na\(^+\)]\(_i\) upon inhibition of the Na\(^+\)/K\(^+\) pump. This suggests that Sodium Green can be used with fluorescence lifetime imaging microscopy (FLIM) to detect physiological changes in [Na\(^+\)].
CHAPTER IV

Fluorescence Lifetime Microscopy of the Na\(^+\) Indicator SBFI in HeLa Cells.
ABSTRACT
The behavior of the Na$^+$ indicator sodium binding benzofuran isophthalate (SBFI) is studied in HeLa cells by time-resolved fluorescence microscopy. The fluorescence relaxation of SBFI in HeLa cells can be described by a tri-exponential for [Na$^+$] between 0 and 90 mM. Changes in [Na$^+$] affect neither the fluorescence relaxation times (0.21 ± 0.05 ns, 0.60 ± 0.03 ns and 2.7 ± 0.1 ns) nor the average decay time (2.2 ± 0.1 ns). The pre-exponential factor of the shortest decay time is negative. However, the ratio of the fluorescence excitation signal at 340 nm to that at 380 nm increases with [Na$^+$]. To elucidate the behavior of SBFI in cells experiments are performed on SBFI in buffer at various concentrations of sodium, potassium and bovine serum albumin (BSA) and at various viscosities. The fluorescence decay is tri-exponential only in the presence of BSA. The relaxation times are independent of [Na$^+$] and [BSA]. The pre-exponential factor of the shortest decay time is negative from a certain [BSA] on which depends on [Na$^+$]. The data indicate that interactions with intracellular components rather than microviscosity influence the SBFI behavior in cells. A model is suggested in which the fluorescence intensities are mainly determined by the signals from the Na$^+$⊂SBFI and SBFI⊂proteins complexes.

INTRODUCTION
Sodium binding benzofuran isophthalate is a widely used fluorescent probe to monitor [Na$^+$] (Minta & Tsien, 1989; Robertson & Foskett, 1995; Rose & Ransom, 1997). SBFI contains a crown ether which forms the binding site for Na$^+$. SBFI has two fluorophores that are benzofurans linked to isophthalate groups. Although the fluorescence quantum yield of free SBFI (0.045) and of the Na$^+$⊂SBFI (0.083) complex are rather low (Minta & Tsien, 1989) the probe has some suitable characteristics. Na$^+$ binds to SBFI in a 1:1 stoichiometry and the ground-state dissociation constant $K_d$ is in the range 4.2 mM – 7.4 mM (Minta & Tsien, 1989; Kowalczyk et al., 1997). The selectivity of Na$^+$ over K$^+$ is about 20 and the effective $K_d$ in the presence of K$^+$ is about 17 mM (Minta & Tsien, 1989). The indicator is generally used in dual excitation ratiometric mode. The ratio of the fluorescence signal due to excitation at 340 nm over that at 380 nm increases with [Na$^+$]. When the emission is recorded at 510 nm, SBFI in pure buffer has an isoexcitation point at ~ 360 nm (Negulescu & Machen, 1990), although a value of 375 nm has also been reported (Robertson & Foskett, 1995).

The photophysics of SBFI in simple buffer solution was recently described (Kowalczyk et al., 1997). The fluorescence intensity decays of the indicator were analyzed in terms of an intermolecular two-state excited-state process. It was found that the rate of complex formation in the excited-state reaction is negligible for [Na$^+$] up to 1 M.

Many investigators have reported that the intracellular spectra of SBFI are different from those recorded in buffer solutions. In Jurkat cells, the excitation maxima of SBFI were shifted to slightly longer wavelengths and the fluorescence signal of the intracellular dye appeared to be considerably higher than in aqueous solutions.
(Harootunian et al., 1989). These effects were explained by the larger microviscosity of cytoplasm compared to buffer solutions. A higher fluorescence signal of SBFI was also reported in myocytes (Baartscheer et al., 1997). The increase was more pronounced in the absence of Na⁺. The effect seemed to be due to the binding of the indicator to proteins because when proteins from lysed myocytes were added to a buffer solution, the characteristics of SBFI excitation spectra became similar to those of the intracellular spectra. In ventricular myocytes, the isoeexcitation point of the probe was blue shifted by approximately 25 nm (Borzak et al., 1992). The most important difference between SBFI spectra obtained in vitro and in rabbit gastric parietal cells was that the entire spectrum decreases in intensity as [Na⁺]ᵢ increases (Negulescu & Machen, 1990). Besides viscosity and protein binding, the presence of K⁺ ions could also affect the fluorescence characteristics of SBFI (Negulescu & Machen, 1990).

Therefore it can be expected that the behavior of the indicator inside a biological cell is more complicated than in simple buffer solutions. The purpose of this work is to examine the behavior of SBFI in different environments. The effects of binding to proteins, viscosity and presence of K⁺ ions are investigated. Steady-state and time-resolved fluorescence measurements are performed on SBFI in buffer solutions with various compositions as well as in HeLa cells. Steady-state measurements comprise spectrofluorimetry and imaging microscopy. The time-resolved fluorescence measurements are performed in the frequency domain using a fluorescence microscope and a harmonically modulated CW Argon laser.

RESULTS

Steady-state measurements of SBFI in buffer solutions
The ground-state dissociation constant K₅ of the Na⁺·SBFI complex can be determined fluorimetrically for physiologically relevant [Na⁺] because the excited-state reaction does not interfere (Kowalczyk et al., 1997). Emission spectra of SBFI in buffer solutions with various [Na⁺] but without K⁺ were collected using excitation at 363 nm. The fluorimetric titration, based on the integrated spectra, yielded a value of 9.1 ± 1.2 mM (corresponding pK₅=2.0 ± 0.06). This value compares well with the value reported by Minta & Tsien (1989) (pK₅=2.13), by Kowalczyk et al. (1997) (pK₅=2.38) and by Haugland & Johnson (1999) (pK₅=2.42). It has been indicated that the value of the dissociation constant depends considerably on the actual composition of the solutions (Haugland, 1996).

Time-resolved fluorescence measurements of SBFI in buffer solutions
The fluorescence intensity decays of SBFI in buffer solutions with various Na⁺ concentrations in the physiological range were recorded in the frequency domain (Fig. 1). From the variation of the phase angle with [Na⁺] it can be deduced that the average relaxation time increases with increasing [Na⁺]. The analysis of the decay curves revealed that the decays are bi-exponential, with decay times virtually independent of [Na⁺] in the concentration range 0-145 mM. This means that the complex formation in the excited-state is negligible for the investigated concentration
range (Kowalczyk et al., 1997). Consequently, the decays were analyzed simultaneously with a bi-exponential expression with the decay times being linked over $[\text{Na}^+]$. The changes in the pre-exponential factors with $[\text{Na}^+]$ are shown in Table 1. The resulting decay times are $\tau_s = 0.142 \pm 0.005$ ns and $\tau_l = 0.567 \pm 0.005$ ns. The intensity decay is almost monoexponential in the nominally Na$^+$ free solution and the decay time is given by $\tau_S$. The ratio $\alpha_l/\left(\alpha_S + \alpha_l\right)$ increases with increasing $[\text{Na}^+]$ in correspondence with the increase of the average decay time with $[\text{Na}^+]$ (Fig. 2).

The sum of Na$^+$ and K$^+$ concentrations is kept relatively constant in biological cells. Therefore, the measurements were repeated in buffer solutions with various $[\text{Na}^+]$ but with $[\text{Na}^+] + [\text{K}^+] = 145$ mM (approximately the physiological value). A good fit was obtained with a global bi-exponential model, where the decay times were linked over $[\text{Na}^+]$. The decay times found ($\tau_s = 0.17 \pm 0.02$ ns and $\tau_l = 0.530 \pm 0.006$ ns) are comparable with those obtained in the absence of K$^+$. However, the changes induced
Table 1. Global bi-exponential analysis of the fluorescence decay SBFI in buffer solutions in the presence of various Na\(^+\) concentrations.*

<table>
<thead>
<tr>
<th>[Na(^+)] (mM)</th>
<th>(\alpha_S)</th>
<th>(\alpha_L)</th>
<th>(&lt;\tau&gt;) (ns)</th>
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<tbody>
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<td>0.20</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>3</td>
<td>0.865</td>
<td>0.135</td>
<td>0.305</td>
</tr>
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<td>5</td>
<td>0.819</td>
<td>0.181</td>
<td>0.341</td>
</tr>
<tr>
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<td>0.360</td>
</tr>
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<td>0.393</td>
</tr>
<tr>
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<td>0.31</td>
<td>0.42</td>
</tr>
<tr>
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<td>0.65</td>
<td>0.35</td>
<td>0.43</td>
</tr>
<tr>
<td>145</td>
<td>0.63</td>
<td>0.37</td>
<td>0.44</td>
</tr>
</tbody>
</table>

*Global decay times are: \(\tau_S = 0.142 \pm 0.005\) ns, \(\tau_L = 0.567 \pm 0.005\) ns, \(\chi^2 = 1.2\).

In all tables, the uncertainties in the pre-exponential factors (standard deviations obtained from the one-dimensional error analysis) are of the order of the last significant digit.

by [Na\(^+\)] in the pre-exponential amplitudes are less pronounced than in the absence of K\(^+\) (Fig. 2). A six fold increase in \(\alpha_S/(\alpha_S + \alpha_L)\) was observed when [Na\(^+\)] increased from 0 to 145 mM in the absence of K\(^+\). When [Na\(^+\)]+145 mM the increase was only 2.3 times. A tri-exponential analysis yielded no substantial improvement in the quality of the fit. Furthermore, the values of two decay times were very close to each other.

![Figure 2](image)

Figure 2. The dependence of the pre-exponential factor associated with the long decay time on [Na\(^+\)]. The measurements were performed in buffer solutions with various [Na\(^+\)] and without K\(^+\) (■) or in buffer solutions where Na\(^+\) is exchanged for K\(^+\) such that [Na\(^+\)]+145 mM (□).

It was previously reported that the fluorescence decays of SBFI in buffer solutions can be analyzed in terms of a two-state excited-state reaction (Kowalczyk et al., 1997). The rate constants of the intermolecular two-state excited-state reaction can be recovered if the decay times change with [Na\(^+\)] (e.g. Beechem et al., 1985; Ameloot et al., 1991). This requires [Na\(^+\)] much higher than physiologically relevant [Na\(^+\)] (Kowalczyk et al., 1997). Minta & Tsien (1989) suggested that high concentrations of Cl\(^-\) might quench SBFI fluorescence, an effect that was not observed with acetate.
Fluorescence decays of the indicator were recorded in buffer solutions containing [Na\(^+\)] up to 4 M. Na\(^+\) was added to the buffer solution either as NaCl or sodium acetate. In both cases, the decays appeared to be bi-exponential (results not shown). The decay times did not vary significantly with [Na\(^+\)] and did not exhibit a definite trend.

Harootunian et al. (1989) explained the different behavior of SBFI in cells as compared with buffer solutions by the larger microviscosity of the cytoplasm. The effect of viscosity on the intensity decays of SBFI was investigated by adding various concentrations of choline chloride (up to 4 M) to the buffer solutions. The viscosity of the buffer solution with 4 M choline chloride was similar to that of a solution containing 1 M sucrose (=3.2 \(10^3\) Pa s, (Weast & Astle, 1978)). These experiments were done on a different batch of SBFI as for the other experiments. We found that the fluorescence relaxation of SBFI slightly depends on the batch received. It has been indicated that for the membrane permeant form of SBFI different loading efficiencies and sensitivities are possible for different batches (Negulescu & Machen, 1990). Despite the fact that at low viscosity the intensity decay of SBFI in a buffer solution without Na\(^+\) is almost mono-exponential, a pronounced bi-exponential behavior was noticed at higher viscosity (Fig. 3A). A similar effect was observed when viscosity was increased by adding 1 M sucrose (not shown). This suggests that the effect seen with choline-chloride is due to changes in viscosity and it is not a direct effect of choline (or changes in ionic strength – see below). Both the pre-exponential amplitudes and the decay times depend on viscosity. A similar effect of choline chloride was noticed in the presence of 135 mM Na\(^+\) (Fig. 3B). The presence of Na\(^+\) led to higher values for the long relaxation time while similar values were obtained for the short decay time. In both cases, the long decay time increases with increasing choline concentration, while the short one is independent of choline concentration.

**Figure 3.** The decay times (A) and the pre-exponential associated with the long decay time (B) of SBFI in buffer solutions with various concentrations of choline chloride. The measurements were performed both in the absence of Na\(^+\) (open symbols) and in the presence of 135 mM Na\(^+\) (filled symbols).
Steady-state measurements of SBFI in the presence of albumin

To mimic the conditions inside the cytoplasm, where a large amount of proteins is present (about 200-300 mg/ml (Alberts et al., 1986; Luby-Phelps, 1994)), the fluorescent characteristics of SBFI in the presence of BSA were investigated. Fig. 4 shows the emission spectra of SBFI in buffer solutions containing various concentration of BSA, both in the absence of Na\(^+\) and in the presence of a saturating Na\(^+\) concentration (145 mM). Although such a high [Na\(^+\)] is not reached inside the cytoplasm, the in vitro measurements at this [Na\(^+\)] allow one to investigate the effects of proteins on the Na\(^+\)-bound form of the indicator. The spectra were corrected for the autofluorescence of BSA and the inner filter effect. The presence of protein induced a blue shift of about 25 nm in the spectra and an increase in the fluorescence signal. This increase was more pronounced in the absence of Na\(^+\).

![Emission spectra of SBFI in buffer solutions with various concentrations of BSA, both in the absence (thin lines) and in the presence of 145 mM Na\(^+\) (thick lines). The spectra are corrected for BSA autofluorescence and inner filter effect. Fluorescence was excited at 363 nm.](image)

The binding of BSA to the indicator was evaluated by performing a fluorescence titration at [SBFI] = 5 \(\mu\)M in the absence and the presence of 145 mM Na\(^+\) (Fig. 5). There was a clear effect of Na\(^+\). In the presence of Na\(^+\) the range of changes in the fluorescence intensity was smaller. The half of the plateau value was reached at 82 \(\pm\) 6 \(\mu\)M and 2.4 \(\pm\) 0.4 \(\mu\)M in the presence and the absence of Na\(^+\), respectively.

Excitation spectra of SBFI in the presence of 750 \(\mu\)M (5% w/v) BSA were collected at several [Na\(^+\)] (not shown). The spectra were red shifted as compared with the excitation spectra in simple buffer solutions and no isoexcitation point was detected. The red shift in the excitation peak decreased with increasing Na\(^+\) concentration.

SBFI is generally used as a dual excitation ratiometric probe. The ratio of the fluorescence intensity due to excitation at 340 nm (F\(_{340}\)) to that at 380 nm (F\(_{380}\)) and recorded at 510-530 nm increases with increasing [Na\(^+\)]. The Na\(^+\)-sensitivity of the indicator in a protein rich environment was investigated by ratiometric measurements in solutions containing 750 \(\mu\)M (5% w/v) BSA and various [Na\(^+\)] (Fig. 6). A 1.5 times
Figure 5. The dependence of the total fluorescence intensity (integrated emission spectra with excitation at 363 nm) of SBFI in buffer solutions on BSA concentration. The measurements were performed in the absence Na\(^+\) (□) and in the presence of 145 mM Na\(^+\) (■).

Figure 6. The steady-state excitation ratio \(F_{340}/F_{380}\) of SBFI in buffer solutions with 750 \(\mu\)M BSA (5% w/v) as a function of [Na\(^+\)]. The ratio increases 1.5 times when [Na\(^+\)] was increased from 0 to 145 mM.

increase in the ratio \(F_{340}/F_{380}\) was induced by increasing [Na\(^+\)] from 0 to 145 mM. A calibration curve can be derived using the equation suggested by Gryniewicz et al. (1985).

Effect of binding to albumin on the fluorescence relaxation of SBFI

The effect of BSA on the fluorescence intensity decay of SBFI was investigated up to 1.5 mM (10% w/v). Pure BSA solutions were found to be slightly fluorescent at the wavelengths used (\(\lambda_{ex} = 363\) nm, \(\lambda_{em} = 535\) nm). Over the whole range of protein concentrations investigated, the BSA autofluorescence accounted for less than 1% from the fluorescence intensity. In these conditions, no corrections were performed (Reinhart et al., 1991).

The frequency domain data of the intensity decay curves of SBFI in buffer solutions with various BSA concentrations in the absence of Na\(^+\) and in the presence of 145 mM Na\(^+\) are shown in Fig. 7A and 7B, respectively. In the absence of Na\(^+\) and at low [BSA] the fluorescence relaxation was well described by a bi-exponential decay. Three relaxation times were required at higher [BSA]. In the presence of 145 mM Na\(^+\)
Figure 7. Frequency-domain data (phase angle) of the fluorescence decay of SBFI in buffer solutions with various concentrations of BSA in the absence of Na⁺ (A) and in the presence of 145 mM Na⁺ (B).

three relaxation times were required for an adequate description at all [BSA]. The values of these relaxation times were quite comparable with those obtained in the absence of Na⁺. A global analysis of the two data sets was performed in terms of a tri-exponential decay by linking the relaxation times over various [BSA] and [Na⁺]. The results are shown in Tables 2A and 2B. In the absence of Na⁺ the pre-exponential of the shortest decay time became negative from 15 μM (0.1%) BSA on. The average decay time increased with [BSA] and became constant when [BSA] ≥ 15 μM. Because of the relatively high contribution of the intermediate relaxation time the nominally Na⁺ free solutions containing large amounts of BSA were checked by flame photometry. It was found that the 750 μM (5%) and 1.5 mM (10% w/v) BSA solutions
Table 2A. Global tri-exponential intensity decay analysis of SBFI in buffer solutions with various amounts of BSA and no Na\(^+\).*

<table>
<thead>
<tr>
<th>[BSA] (µM)</th>
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<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
<th>(&lt;\tau&gt;) (ns)</th>
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</table>

Table 2B. Global tri-exponential intensity decay analysis of SBFI in buffer solutions with various amounts of BSA and 145 mM Na\(^+\).*

<table>
<thead>
<tr>
<th>[BSA] (µM)</th>
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<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
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<tr>
<td>750</td>
<td>-1</td>
<td>1.54</td>
<td>3.04</td>
<td>3.15</td>
</tr>
<tr>
<td>1500</td>
<td>-1</td>
<td>1.45</td>
<td>2.87</td>
<td>3.17</td>
</tr>
</tbody>
</table>

* The intensity decays of SBFI in buffer solutions with various concentrations of BSA and with or without Na\(^+\) were analyzed with a global tri-exponential model, with decay times linked over [BSA] and [Na\(^+\)]. Global decay times are: \(\tau_1=0.215\pm0.005\) ns, \(\tau_2=0.60\pm0.01\) ns, \(\tau_3=3.32\pm0.03\) ns, \(\chi^2=4.1\). Whenever the pre-exponential factor associated with the shortest decay time became negative, the pre-exponentials were normalized so that \(\alpha_1 = -1\).

contained Na\(^+\) in the millimolar range (≈3 mM and 5 mM, respectively). At [Na\(^+\)]=145 mM the pre-exponential factor associated with the shortest decay time was negative starting from 150 µM (1% w/v) BSA. The ratio of the pre-exponential of the longest decay time to that of the next shorter decay time increased with [BSA]. In the presence of Na\(^+\), the average decay time became constant when [BSA] ≥ 750 µM (5% w/v). The variation of the average decay time with [BSA] in the absence and presence of 145 mM Na\(^+\) is reflected in the phase angle at the modulation frequency of 160 MHz. Most features of the results shown in Fig. 8 are in complete agreement with those of Fig. 5, which was based on steady-state measurements. However, in contrast to the results for
the steady-state measurements the plateau values of the phase angle at high [BSA] are equal without and with 145 mM Na⁺.

**Figure 8.** The changes with BSA concentration in the phase angle at the modulation frequency of 160 MHz. The frequency-domain measurements were performed in buffer solutions in the absence of Na⁺ (□) and in solutions containing 145 mM Na⁺ (■).

We also investigated the Na⁺-sensitivity of the fluorescence decays of SBFI in the presence of 750 μM (5% w/v) BSA. This concentration approaches the physiological cytoplasmic protein content (Alberts et al., 1986; Luby-Phelps, 1994). The intensity decays recorded in the frequency domain at various [Na⁺] are shown in Fig. 9. It is obvious that the presence of the protein practically abolished the sensitivity of the phase angle to Na⁺. The time-resolved data were combined with the other experiments in the presence of BSA and globally analyzed according to a tri-exponential model. The relaxation times were essentially the same as those obtained for the previous analyses in the presence of BSA: 0.194±0.005 ns, 0.567±0.009 ns and 3.25±0.02 ns.

**Figure 9.** Frequency-domain data (phase angle) of the fluorescence decay of SBFI in buffer solutions with 750 μM (5% w/v) BSA, at various Na⁺ concentrations.
negative pre-exponential is found for the shortest decay time at all [Na\(^+\)] in the presence of 750 \(\mu\)M BSA (Table 3). The ratios of the positive pre-exponential factors are comparable with those obtained from Table 2B for 145 mM Na\(^+\) and 750 \(\mu\)M BSA. The average fluorescence relaxation time was independent of [Na\(^+\)] and equal to 3.1 ns.

**Table 3.** Global intensity decay analysis of SBFI in buffer with 750 \(\mu\)M (5% w/v) BSA and various Na\(^+\) concentrations.\(^*\)

<table>
<thead>
<tr>
<th>[Na(^+)] (mM)</th>
<th>(\alpha_1)</th>
<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
<th>(&lt;\tau&gt;) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-1</td>
<td>0.85</td>
<td>1.40</td>
<td>3.11</td>
</tr>
<tr>
<td>5</td>
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<td>2.59</td>
<td>3.11</td>
</tr>
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<td>2.07</td>
<td>5.57</td>
<td>3.12</td>
</tr>
<tr>
<td>25</td>
<td>-1</td>
<td>0.96</td>
<td>1.54</td>
<td>3.09</td>
</tr>
<tr>
<td>50</td>
<td>-1</td>
<td>1.04</td>
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</tr>
<tr>
<td>145</td>
<td>-1</td>
<td>1.40</td>
<td>2.93</td>
<td>3.10</td>
</tr>
</tbody>
</table>

\(^*\) The intensity decays of SBFI in buffer solutions with various concentrations of BSA and Na\(^+\) (data from Tables 2-3) were analyzed with a global tri-exponential model, with decay times linked over [BSA] and [Na\(^+\)]. Global decay times are: \(\tau_1 = 0.194 \pm 0.005\) ns, \(\tau_2 = 0.567 \pm 0.009\) ns, \(\tau_3 = 3.25 \pm 0.02\) ns, \(\chi^2 = 3.5\). The pre-exponentials were normalized so that the negative pre-exponential factor associated with the shortest decay is -1.

**Steady-state measurements of SBFI in HeLa cells**

SBFI was loaded into HeLa cells by incubating the cells with the membrane permeable AM form as described in Chapter II. Fluorescence images were collected using excitation at 340 nm and 380 nm. A typical in vivo calibration experiment for SBFI in HeLa cells is shown in Fig. 10. The cells were bathed with calibration solutions of various [Na\(^+\)] in the presence of the ionophores gramicidin D, monensin and nigericin (see Chapter II). The signals were obtained by integrating pixels over the cells. The fluorescence excitation ratio \(F_{340}/F_{380}\) correlates with the [Na\(^+\)] in the bathing solution. The ratio rose 1.6 times when the extracellular [Na\(^+\)] increased from 0 to 90 mM. Fig. 10 also shows that the calibration process is reversible because the ratios corresponding to zero Na\(^+\) at the beginning and the end of the calibration procedure are the same. From the type of experiments shown in Fig. 10, one can derive the resting intracellular [Na\(^+\)]. The value obtained was 16±3 mM (mean±SE, n=6).

**Fluorescence decay of SBFI in HeLa cells**

At the wavelengths used in this study, the cellular autofluorescence is attributed to coenzymes and flavins (Schneckenburger et al., 1996). The intensity decays of unloaded cells were investigated first. Due to the weak autofluorescence (accounting for less than 2% from the total fluorescence intensity), the values for the phase and the modulation of the background signal could not be determined accurately. In these conditions it was better not to perform corrections (Reinhart et al., 1991).
The fluorescence decays measured in the frequency domain of SBFI trapped inside the HeLa cells are shown in Fig. 11. Similar to the case where BSA was added to the buffer solutions, the phase angle (as an indicator of the average lifetime) has significantly higher values than in simple aqueous buffer solutions over the whole modulation frequency range.

An in vivo calibration was performed by exposing the cells to different extracellular [Na⁺] in the presence of the ionophores gramicidin D, monensin and nigericin. Five different decays were recorded at each [Na⁺], each time cells from different regions of the coverslip being studied. The time-resolved data collected at similar [Na⁺] were
analyzed with a multi-exponential model. Similar to the behavior of the dye in buffer solutions, the decay times appear to be independent of [Na\(^+\)]. Consequently, the global decay analysis was performed where the decay times were linked over all the data sets. 25 decays were analyzed together, five at each [Na\(^+\)]. The best fitting was obtained with a tri-exponential global analysis. This procedure was repeated for cells on four different coverslips. The decay times resulting from the global tri-exponential analyses are: \(\tau_1 = 0.21 \pm 0.05 \text{ ns, } \tau_2 = 0.60 \pm 0.03 \text{ ns, } \tau_3 = 2.7 \pm 0.1 \text{ ns (mean \pm SE, } n=4)\). Table 4 shows the pre-exponential amplitudes at various [Na\(^+\)], obtained from cells on a single coverslip. No significant change with [Na\(^+\)] is noticed. A negative pre-exponential amplitude is obtained for the shortest decay time at all [Na\(^+\)].

**Table 4. Global tri-exponential analysis of the intensity decays of SBFI inside the cytoplasm of HeLa cells.**

<table>
<thead>
<tr>
<th>[Na(^+)] (mM)</th>
<th>(\alpha_1)</th>
<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
<th>(&lt;\tau&gt;) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-1</td>
<td>0.84</td>
<td>0.38</td>
<td>2.05</td>
</tr>
<tr>
<td>15</td>
<td>-1</td>
<td>0.82</td>
<td>0.40</td>
<td>2.08</td>
</tr>
<tr>
<td>30</td>
<td>-1</td>
<td>0.92</td>
<td>0.53</td>
<td>2.11</td>
</tr>
<tr>
<td>45</td>
<td>-1</td>
<td>0.95</td>
<td>0.57</td>
<td>2.12</td>
</tr>
<tr>
<td>90</td>
<td>-1</td>
<td>1.03</td>
<td>0.65</td>
<td>2.12</td>
</tr>
</tbody>
</table>

* The global decay times are: \(\tau_1 = 0.157 \pm 0.005 \text{ ns, } \tau_2 = 0.64 \pm 0.01 \text{ ns, } \tau_3 = 2.53 \pm 0.02 \text{ ns, } \chi^2 = 3\). The pre-exponentials were normalized so that the negative pre-exponential factor associated with the shortest decay is -1.

**DISCUSSION**

**SBFI in buffer solutions with various Na\(^+\) concentrations**

The fluorescence decay of SBFI in buffer solutions at various [Na\(^+\)] can be described by a bi-exponential decay law. For the concentration range 0–145 mM, the relaxation times are independent of [Na\(^+\)]. The values of the relaxation times are \(\tau_1 = 0.142 \pm 0.005 \text{ ns and } \tau_2 = 0.567 \pm 0.005 \text{ ns}\). These values correspond very well with those obtained by Kowalczyk et al. (1997) (0.193±0.001 ns and 0.577±0.001 ns) using the time-correlated single-photon counting technique. When Na\(^+\) is exchanged for K\(^+\) such that [Na\(^+\)]+[K\(^+\)] = 145 mM almost identical decay times are obtained: 0.17±0.02 ns and 0.530±0.006 ns. The decay times are also independent of ionic strength as this was not kept constant in the time-resolved experiments. A similar result was obtained by Kowalczyk et al. (1997). This supports the finding of Minta & Tsiens (1989) that large increases in ionic strength have relatively minor effects on SBFI fluorescence. It has been suggested that this can be due to the fact that the crown ether, which is the binding site for Na\(^+\), is uncharged (Robertson & Foskett, 1995).

The relative pre-exponential factor \(\alpha_2/(\alpha_2+\alpha_4)\) increases with increasing [Na\(^+\)] and exhibits a saturating behavior. The range of changes in this parameter is smaller in the presence of K\(^+\), indicating an effect of K\(^+\) on the SBFI affinity for Na\(^+\). This could be expected because of the higher K\(_d\) observed in the presence of K\(^+\) (Minta & Tsiens, 1989). The variation of \(\alpha_4/(\alpha_2+\alpha_4)\) implies that the average lifetime also increases
with [Na\(^+\)]. In the absence of K\(^+\) the average lifetime increases 2.2 fold, which is comparable with the value of 1.7 obtained by Szmacinski et al. (1997b) using phase and modulation fluorimetry. However, Kowalczyk et al. (1997) found that \(\alpha_L/(\alpha_S+\alpha_L)\) decreases with increasing [Na\(^+\)] and that the average decay time is practically independent of [Na\(^+\)] (in the range 1.6 - 135 mM) and is about 0.52 ns. In their experiments a synchrotron source was used to obtain 330 nm and 340 nm as excitation wavelengths. We used an excitation wavelength of 363 nm and we verified that this was not an iso-excitation point. Szmacinski et al. (1997b) indicate that the lifetime sensitivity of SBFI for Na\(^+\) can be chosen by changing the excitation wavelength. An increase in the average fluorescence relaxation time upon cation binding has also been observed for Sodium Green (Szmacinski et al., 1997a; Despa et al., 1998), Quin-2 (Hirshfield et al., 1993; Hirshfield et al., 1996), Calcium Green (Hirshfield et al., 1993; Lakowicz et al., 1992) and Fura-2 (Van den Bergh et al., 1995; Arner et al., 1998).

Kowalczyk et al. (1997) modeled the photophysics of SBFI in terms of a two-state excited-state model. The two species in the excited state are the excited forms of the indicator without and with Na\(^+\). It is believed that there are only two spectroscopically distinct forms of the indicator although SBFI contains two chromophores. Kowalczyk et al. (1997) found that the rate of the complex formation in the excited state is essentially negligible for [Na\(^+\)] up to 1 M. Our data support this because the fluorescence relaxation times are independent of [Na\(^+\)] within the same range of [Na\(^+\)]. It is likely that there is an excited-state reaction occurring because our data show that for [Na\(^+\)] well above \(K_d\) the fluorescence relaxation is still bi-exponential. A mono-exponential decay can be expected for high [Na\(^+\)] if there is only a ground-state equilibrium.

The fluorescence relaxation of SBFI depends on the viscosity of the solvent. The fluorescence decay becomes a pronounced bi-exponential in the absence of Na\(^+\) when the viscosity is increased. The relaxation time \(\tau_L\) and the ratio \(\alpha_L/(\alpha_S+\alpha_L)\) increase with viscosity. The shorter decay time does not depend on viscosity. When the viscosity is increased in the presence of 135 mM Na\(^+\), the longer decay time and the ratio \(\alpha_L/(\alpha_S+\alpha_L)\) take even higher values. However, an increase in viscosity reduces the rate of complex formation. The pre-exponential factors in the fluorescence relaxation depend not only on the equilibrium in the ground state but also on the characteristics of the photophysical processes (e.g. Ameloot et al., 1991). Apparently, these characteristics change such that \(\alpha_L/(\alpha_S+\alpha_L)\) can still increase with [Na\(^+\)]. This means that the sensitivity of SBFI for sodium remains at higher viscosities. The effects observed by increasing the viscosity make that the two-state excited-state model has to be refined.

Harootunian et al. (1989) found that the intensity of the excitation spectra of SBFI increase with increasing viscosity. A similar effect has been found for other benzofuran based fluorescent indicators such as Fura-2 (Konishi et al., 1988; Poenie, 1990) and Quin-2 (Poenie, 1990). Benzofuran itself is not fluorescent in the visible but becomes so when linked to other chemical groups. It is known for a long time that a
more rigid molecular structure favors high fluorescence efficiency ( Förster, 1951). One could therefore suggest that the reduced mobility of the chromophoric part of SBFI by increased viscosity may lead to an enhanced fluorescence. Such an effect has been observed for the dialkyl amino groups on rhodamine B (Drexhage, 1977). The different electron distribution of the free and the bound form makes the shorter decay time apparently independent of viscosity. It is unlikely that the increase of \( \tau_0 \) can be ascribed to reduced oxygen quenching because the relaxation time is rather short. Furthermore, the two relaxation times are of the same order of magnitude so that a possible oxygen quenching effect would have been observed for both.

**Effect of binding to albumin on the fluorescence relaxation of SBFI**

The binding of the fluorescent indicators to proteins has been well documented for SBFO (Chatton & Spring, 1995), Fura-2 (Konishi et al., 1988; Keating & Wensel, 1991; Bancel et al., 1992a; Armer et al., 1998), Indo-1 (Ikenouchi et al., 1991; Bancel et al., 1992b), Quin-2 (Hirschfield et al., 1996), BCECF (Plasek et al., 1996), C.SNARF-1 (Srivastava & Krishnamoorthy, 1997; Vecer et al., 1998) and C.SNAFL-1 (Sanders et al., 1995; Vecer et al., 1998). However, most reports focus on the spectral changes upon binding and not on time-resolved fluorescence measurements. It has been reported (Chatton & Spring, 1995) that 0.1% BSA induces a shift from 376 to 360 nm in the isosbestic point for Na\(^+\) of SBFO, a Na\(^+\) - indicator which is closely related to SBFI. No effect of the ionic strength (up to 160 mM) was noticed, either in the presence or in the absence of BSA (Chatton & Spring, 1995). Baertscheer et al. (1997) found that when proteins from lysed myocytes are added to buffer solutions, the peak of SBFI excitation shifts about 10 nm to longer wavelengths and the fluorescence signal increases. Addition of 0.35% albumin had the same effects (Baartscheer et al., 1997). We also found that BSA (750 \( \mu \)M) induces a red shift in the excitation spectra of SBFI and this shift becomes less pronounced with increasing [Na\(^+\)].

Our results show that 15 \( \mu \)M (0.1\%) albumin induces a blue shift of about 25 nm in the emission spectra of SBFI. A blue shift in the emission spectrum has also been reported for Fura-2 bound to aldolase (Konishi et al., 1988) and Quin-2 bound to human serum albumin (Hirschfield et al., 1996). The presence of albumin produces an increase in the fluorescence signal of SBFI in a dose-dependent manner. An increase in fluorescence intensity upon protein binding has been described for SBFI (Baartscheer et al., 1997), Fura-2 (Konishi et al., 1988; Keating & Wensel, 1991) and Quin-2 (Hirschfield et al., 1996). The increase in fluorescence intensity and the blue shift in the emission spectrum suggest that the indicator is bound to a more hydrophobic environment. In general a decrease in solvent polarity of the environment of the probe reduces the rate of nonradiative decay. Hirschfield et al. (1996) were able to demonstrate that Quin-2 binds to two binding sites on human serum albumin. These binding sites contain mostly hydrophobic residues, which shield ligands from the aqueous environment (Hirschfield et al., 1996).

The increase in fluorescence intensity upon binding of SBFI to BSA is less pronounced in the presence of Na\(^+\), in accordance with the results obtained by
Baartscheer et al. (1997). These results indicate that the affinity of SBFI for albumin decreases with increasing Na⁺. A decrease in intensity in the presence of the cation has also been described for Fura-2 (Konishi et al., 1988; Keating & Wensel, 1991) and Quin-2 (Hirshfield et al., 1996). From our spectral observations of SBFI in the presence of BSA one can conclude that there is indeed binding of SBFI to BSA and that the affinity of SBFI for BSA depends on [Na⁺]. The spectral measurements and the titrations with BSA show that the affinity decreases with increasing [Na⁺].

The time-resolved measurements indicate that the average decay time of SBFI increases with [BSA] in the absence and the presence of Na⁺ until a maximum value is reached. In the presence of 145 mM Na⁺ ten times higher [BSA] is needed for this saturation to occur. An increase in average fluorescence decay time upon protein binding has also been reported for Fura-2 (Konishi et al., 1988; Keating & Wensel, 1991; Arner et al., 1998) and Quin-2 (Hirshfield et al., 1996). At low [BSA], the intensity decays of SBFI in the absence of Na⁺ are well described by a bi-exponential. Three relaxation times are required to describe the fluorescence relaxation of SBFI in the absence of Na⁺ but with high [BSA]. Three decay times are also needed in the presence of both BSA and Na⁺. The relaxation times are independent of [BSA] and [Na⁺]. This indicates that the rates of association in the excited-state are negligible with respect to the other rates. Only the pre-exponentials exhibit concentration dependence. The pre-exponential of the shortest decay time becomes negative from a certain [BSA] on. In the presence of Na⁺ a higher [BSA] is required before a negative pre-exponential can be observed. The [BSA] where the negative pre-exponential appears corresponds with the value at which the average decay time becomes constant.

The time-resolved observations can be rationalized as follows. In the presence of BSA and Na⁺ four molecular species can be expected: free SBFI and the complexes Na⁺SBFI, SBFI c BSA and (Na⁺SBFI) c BSA. However, only three relaxation times are observed. The two shorter relaxation times correspond with the relaxation times observed in the absence of BSA, so they are associated with free SBFI and Na⁺SBFI. The longest relaxation time is then to be associated with the binding to BSA. Because Na⁺ reduces the affinity of SBFI for BSA, it is unlikely that the longest relaxation time can be assigned to (Na⁺SBFI) c BSA but rather corresponds with SBFI c BSA. It is possible that the complex (Na⁺SBFI) c BSA exists and that binding to BSA is very weak or does not influence its photophysical behavior. We think we can rule out the possibility that the fourth relaxation time is too short to be measured with our instrumentation. Because the fluorescence intensity increases upon binding to BSA longer relaxation times can be expected rather. The fact that the pre-exponential of the shortest decay time becomes negative at sufficiently high [BSA] supports the model that mainly the free SBFI binds to BSA. A negative pre-exponential is indicative for a photophysical system in which an excited-state species is mainly created by an excited-state process. Because the relaxation times do not depend on [BSA] and [Na⁺] there exists, apart from deactivation, only excited-state processes with sufficiently high rates from the complexes towards free SBFI and not vice versa. This “feeding” of the free SBFI by the excited complexes leads to negative pre-exponentials. These negative pre-exponentials can show up more pronouncedly when
the excited SBFI is not generated substantially through direct excitation of the ground state form. In the presence of BSA and Na⁺, SBFI can form several complexes. If these bindings are relatively strong the concentration of free SBFI in the ground state will be relatively small. Without BSA a negative pre-exponential is not observed for the shortest decay time even at 145 mM Na⁺. This means that the weight of the SBFI:BSA complex in generating the negative pre-exponentials is more important. The negative pre-exponential is then mainly due to the dissociation of the SBFI:BSA complex in the excited state. In the presence of Na⁺ less SBFI binds to BSA because of formation of Na⁺:SBFI. This explains our finding that a higher [BSA] is required to observe the negative pre-exponential in the presence of Na⁺.

We suggest Scheme I (where the asterisk indicates the excited state) for the reaction of SBFI in the presence of BSA and Na⁺. It is implicitly assumed in this model that the different sites of BSA to which free SBFI can bind are spectroscopically indistinguishable. The intensity decays of the complexes are assumed to be mono-exponential. The expression for the pre-exponential factors and the relaxation times for this model are given in the Appendix. The pre-exponentials depend on the rates of the excited-state processes and on spectral absorption and emission factors (Beechem et al., 1985; Ameloot et al., 1991). For certain combinations of these parameters the pre-exponential associated with the shortest relaxation time becomes negative (see A7-9). The ratio of the pre-exponentials of the longest relaxation time to that of the next shorter one will increase with increasing BSA. This is indeed observed at 145 mM Na⁺ (see table 2B).

\[
\begin{align*}
\text{Na}^+ \text{SBFI}^* & \xrightarrow{k_{12}} \text{SBFI}^* \xrightarrow{k_{13}} \text{SBFI} \subset \text{BSA}^* \\
& \downarrow k_{e2} \quad \downarrow k_{e1} \quad \downarrow k_{e0} \\
2^* & \xrightarrow{k_{21}} 1^* \xleftarrow{k_{12}} 3^* \\
& \downarrow k_{e2} \quad \downarrow k_{e1} \quad \downarrow k_{e3}
\end{align*}
\]

Scheme 1

It was also found for Quin-2 (Hirshfield et al., 1996) and for Indo-1 (Bancel et al., 1992b) that mainly the free form of the indicator binds to proteins. Since the presence of calcium reduces the interaction between Quin-2 and human serum albumin and the affinity of Ca²⁺ for albumin is much less than that for Quin-2, Hirshfield et al. (1996) suggest that the carboxyl groups of Quin-2 are involved in the binding. These carboxyl groups are responsible for the binding of calcium to the indicator. A similar mechanism has been suggested for the protein binding of Indo-1 (Bancel et al., 1992b) and for Fura-2 (Hirshfield et al., 1996). Although we don't know how SBFI binds to BSA, it is likely that both fluorophores of SBFI are "buried" inside hydrophobic sites.
of BSA. Otherwise we would expect that binding to BSA will have less pronounced shift in the spectra. Upon dissociation free SBFI without Na⁺ will be generated. This dissociation process during the excited state leads eventually to a negative pre-exponential in the fluorescence decay at sufficiently high [BSA].

The average relaxation time of SBFI recorded in buffer solutions with 750 μM (5% w/v) BSA and various [Na⁺] did not depend on [Na⁺] in the range 0 - 145 mM. This result is consistent with the saturation noticed in BSA titration experiments, both with and without 145 mM Na⁺. Despite the invariability of the average relaxation time and the normalized pre-exponential factors, the fluorescence excitation ratio F₂₄⁰/F₃₈₀ is still sensitive to Na⁺. In the frequency-domain lifetime experiments only relative pre-exponential factors can be determined. Therefore the different behavior of the steady-state fluorescence intensities and the time-resolved data has to be explained by the initial value of the fluorescence relaxation. It is possible that the normalized pre-exponentials and the initial value exhibit a different Na⁺ dependence (See Appendix). The fact that the normalized pre-exponential factors are practically [Na⁺] independent implies that the ground state equilibrium must be shifted to the protein complex. If the equilibrium would be towards the Na⁺-SBFI complex a more pronounced dependence on Na⁺ would have been observed.

**SBFI in HeLa cells**

Some of the problems associated with the use of fluorescent indicators in cells are compartmentalization of the dye into organelles, incomplete ester hydrolysis and dye leakage. The experiments (including the loading) were performed at room temperature and not at 37°C. This condition is known to reduce both compartmentalization and leakage of the dye (Wong & Foskett, 1991; Negulescu et al., 1990). The steady-state imaging experiments showed that the cells did not contain brightly fluorescent spots that are indicative of dye compartmentalization into organelles. An incomplete de-esterification of SBFI is unlikely to affect significantly the fluorescence intensities in cells because the ester has a much lower fluorescence intensity than the free dye (Haugland, 1996). Moreover, the value of 16 ± 3 mM derived for the resting [Na⁺] from steady-state experiments compares well with the values obtained for HeLa cells by Zahler et al. (1997) (19 mM), using also SBFI, and by Amorino & Fox (1995) (20 mM) who used the fluorescent indicator Sodium Green. Harootunian et al. (1989) showed that dye released from cells had properties essentially identical to never esterified free acid. This indicates that incomplete hydrolysis is probably not a problem for SBFI in HeLa cells. The K_d for the Na⁺-SBFI complex in cells is found to be 26.6 mM (Ito et al., 1997) and 22.5 mM (Baartscheer et al., 1997), which is higher than in buffer without protein.

It was found in Jurkat cells that the excitation maxima of SBFI were shifted to slightly longer wavelengths and the fluorescence signal of the intracellular dye appeared to be considerably higher than in aqueous solutions (Harootunian et al., 1989). These effects were explained by the greater microviscosity of cytoplasm compared to buffer solutions. Increasing solution viscosity by addition of 1.75 M sucrose (resulting in a viscosity of about 15 10⁻³ Pa s, (Weast & Astle, 1978)) enhanced quantum efficiency
and slightly shifted the excitation peak of SBFI in vitro (Harootunian et al., 1989). Baartsoecheer et al. (1997) showed that the increase in the fluorescence signal of SBFI in cardiac myocytes is more pronounced in the absence of Na⁺. Negulescu & Machen (1990) found that the most dramatic difference between SBFI spectra in vitro and in vivo was that the entire spectrum decreases in intensity as [Na⁺] increases. The decreases are larger at 380 nm than at 340 nm so that the ratio F₃₈₀/F₃₄₀ increases with increasing [Na⁺]. Negulescu & Machen (1990) suggested that the so-called apparent quenching of fluorescence at high [Na⁺] could be related to changes in viscosity or ionic strength. However, the cytoplasm of living cells has a fluid phase viscosity about that of water (Luby-Phelps et al., 1993) or only a few times greater than water (Luby-Phelps et al., 1986; Luby-Phelps, 1994). Robertson & Foskett (1995) suggested that cellular effects on SBFI are of a different nature than viscosity effects. Changes in ionic strength can probably not explain the behavior of SBFI in cells as SBFI was found to be not too sensitive to changes in ionic strength (Minta & Tsien, 1989). Baartsoecheer et al. (1997) suggested that binding to proteins can explain the observed spectral shifts and the increase in fluorescence. The cytoplasmic protein concentration may be as high as 200-300 mg/ml (Alberts et al., 1986; Luby-Phelps, 1994), although it is not known how much of this protein is in solution in the fluid phase.

Our steady-state and time-resolved experiments of SBFI in cells and in the various buffers indicate that the binding to protein rather than changes in microviscosity explains the observed behavior of SBFI in cells. The observed increase in fluorescence intensity in cells as compared with buffer solutions and the decrease with increasing [Na⁺] are both elegantly explained by binding to proteins. The fluorescence decays of SBFI in HeLa cells and in solutions containing a sufficiently high [BSA] are both tri-exponential. The characteristics of the relaxation curves in HeLa cells are practically the same as those observed for SBFI in BSA solutions. The values of the relaxation times are practically the same. The normalized pre-exponentials are rather insensitive to [Na⁺] both in HeLa cells and in buffer solutions with 750 µM BSA. The pre-exponential of the shortest decay time is negative in cells and in BSA solutions at sufficiently high concentration of the protein. The value of the protein concentration at which the negative pre-exponential starts to appear depends on [Na⁺]. A higher protein concentration is required in the presence of Na⁺. It is also unlikely that the three relaxation times of SBFI in cells are related to incomplete hydrolysis of the membrane permeable form of SBFI (see above). From our experiments in buffer without BSA we know that an increase in viscosity will not change the bi-exponential decay into a tri-exponential relaxation. An increased viscosity cannot explain the observed decrease of the fluorescence signal with increasing [Na⁺].

CONCLUSION

This report shows that the behavior of SBFI in HeLa cells cannot be rationalized in terms of viscosity effects but rather by the binding of SBFI to intracellular components. The observed steady-state fluorescence intensities of SBFI in cells merely reflect the fractions of the SBFI bound to intracellular components and of the Na⁺-SBFI complex. The fluorescence relaxation times and the normalized pre-
exponential factors of the decay of SBFI in HeLa cells are essentially independent of intracellular [Na\(^+\)]. This means that SBFI cannot be used for fluorescence lifetime imaging techniques applied to cells. It has been suggested by Srivastava & Krishnamoorthy (1997) that fluorescence relaxation time measurements can be used to discriminate the fraction of the indicator bound to intracellular components. Our results show that this is not possible for SBFI. Although in practice SBFI has to be used under steady-state conditions, time-resolved measurements provide information about the behavior of the dye in the cell.

**APPENDIX**

The concentration of the excited species appearing in Scheme I will be denoted below as \(x_i^*(t)\), \(i=1,2,3\). The time dependence of \(x_i^*(t)\) can be calculated from a set of coupled differential equations and the values at time zero, \(x_i^*(0) = b_i\), \(i = 1,2,3\) (see e.g. Beechem et al., 1985; Ameloot et al., 1991). The values for \(b_i\) are determined by the ground-state equilibrium. After delta excitation \(x_i^*(t)\) are given by:

\[
x_i^*(t) = b_i \exp(-k_{0i}t) + \frac{b_2k_{ij}}{k_{0i}-S_2}\left[\exp(-S_2t) - \exp(-k_{0i}t)\right]
+ \frac{b_3k_{ij}}{k_{0i}-S_3}\left[\exp(-S_3t) - \exp(-k_{0i}t)\right]
\]

\[A.1-3\]

\[x_1^*(t) = b_2 \exp(-S_2t)\]

\[x_2^*(t) = b_3 \exp(-S_3t)\]

where the coefficients \(k_{ij}, k_{0j} > 0\), are the apparent rate constants of transfer of species \(j\) to species \(i\). The subscript 0 denotes the ground state of the considered species. \(S_1\) and \(S_2\) are defined as:

\[S_2 = k_{0i} + k_{12}\]

\[S_3 = k_{0i} + k_{13}\]

The observed fluorescence relaxation \(f(t)\) is then given by:

\[f(t) = \sum_{i=1}^{3} c_i x_i^*(t)\]

\[A.6\]

where the factor \(c_i\) is the fluorescence emission contribution of species \(i\) at the wavelength of observation. The factor \(c_i\) depends on the radiative deactivation rate of species \(i\) and the fraction of the emission spectrum that is monitored (Beechem et al., 1985; Ameloot et al., 1991). The factor \(c_i\) does not depend on concentration.
The pre-exponential factors $\alpha_i$ in Eq 1 associated with the decay times $\tau_1 = \frac{1}{k_{o1}}$, $\tau_2 = \frac{1}{S_2}$, $\tau_3 = \frac{1}{S_3}$ (assumed in the ranking $\tau_1 < \tau_2 < \tau_3$) are given by:

$$\alpha_1 = c_1(\frac{b_1 k_{12}}{k_{o1} - S_2} - \frac{b_1 k_{12}}{k_{o1} - S_3})$$

$$\alpha_2 = c_2 b_2$$

$$\alpha_3 = c_3 b_3$$

A.7-9

The fluorescence relaxation $f(t)$ can be written as

$$f(t) = f(0) \sum_{i=1}^{3} \hat{\alpha}_i \exp(-t / \tau_i)$$

A.10

with the fluorescence at time zero

$$f(0) = \sum_{i=1}^{3} c_i b_i$$

A.11

and the re-normalized pre-exponential factors

$$\hat{\alpha}_i = \frac{\alpha_i}{\sum_{i=1}^{3} \alpha_i}$$

A.12

The steady-state emission intensity $F_s$ is then given by:

$$F_s = f(0) \sum_{i=1}^{3} \hat{\alpha}_i \tau_i$$

A.13

The concentration dependence of $f(0)$ and the second factor in Eq. A.13 can be different. Even if the second factor is practically concentration independent $F_s$ can exhibit concentration dependence.
CHAPTER V

The relationship between ATP content, intracellular pH and mitochondrial state in MDCK cells exposed to chemical hypoxia
Chapter V

ABSTRACT
The relationship between the energy state and the mitochondrial function in conditions of chemical hypoxia (cyanide and 2-deoxyglucose) is investigated in confluent monolayers of MDCK cells. The time-course of changes in the intracellular pH, Mg\(^{2+}\) concentration, mitochondrial membrane potential and the cellular redox state was monitored by fluorescence imaging microscopy. Cellular ATP content was determined with a chemiluminescent assay.

Following the onset of chemical hypoxia, a profound ATP depletion and intracellular acidosis developed in about 15 minutes, with a similar time course. This suggests that ATP hydrolysis is the main source of protons in conditions of metabolic inhibition in MDCK cells. A partial recovery in both parameters was noticed on washout. Chemical hypoxia produced an increase in [Mg\(^{2+}\)], most likely due to the release of Mg\(^{2+}\) from Mg-ATP\(^{2-}\). However, the rise in [Mg\(^{2+}\)], did not follow exactly the changes in cellular ATP content, suggesting that part of the free Mg\(^{2+}\) resulting from ATP hydrolysis is buffered by other intracellular components.

ΔΨ decreased but was not completely dissipated during chemical hypoxia. Inhibition of the mitochondrial ATP synthase with oligomycin slowed down the ATP depletion and accelerated the mitochondrial depolarization. In the presence of oligomycin, ΔΨ was significantly lower throughout the application of the metabolic inhibitors. Thus, ΔΨ is partially maintained during chemical hypoxia by the mitochondrial ATP synthase, which operates in the reverse mode. Chemical hypoxia produced an immediate reduction in the cellular redox state, as assessed by an increase in the NAD(P)H autofluorescence. Afterwards, the NAD(P)H signal decreased slowly during a 60 minutes application of the metabolic inhibitors. An immediate drop in the cellular autofluorescence occurred on washout.

INTRODUCTION

During a limited period of hypoxia/ischemia, changes in the renal cell function may be fully reversible. However, longer exposure to ischemic conditions leads to irreversible changes in cell structure and function, which finally end with cell death. We are interested to investigate the cellular events that occur during hypoxia/ischemia in sublethally injured epithelial cells from the nephron, since the regeneration of the kidney after an ischemic insult is based on the capacity of surviving cells to recover and to proliferate.

ATP depletion and acidosis are prominent features during hypoxic or ischemic insults in many tissues, including the kidney. While ATP depletion is considered the central mediator of ischemic cellular injury, acidosis was proved to have a protective effect (Weinberg, 1991; Weinberg et al., 1991; Edelstein et al., 1996). ATP depletion is generally followed by impairment of ion homeostasis, alterations in the cytoskeletal structure and function and loss of polarity. Acidification of hypoxic tissue results from glycolytic lactate production, ATP hydrolysis and CO\(_2\) accumulation.
Direct and continuous measurements of cellular ATP content can be accomplished in living cells using the luciferin-luciferase based assay but require microinjection of luciferase. This is not easy to realize in monolayers of epithelial cells. The use of luciferin-luciferase method in living cells (in vivo) has some other problems including the low sensitivity to ATP in the millimolar range and the pH dependence of the signal (Leyssens et al., 1996). The affinity of ATP for Mg\(^{2+}\) is about tenfold greater than that of ADP (Veloso et al., 1973), consequently ATP hydrolysis results in a rise in intracellular free Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)). An increase in [Mg\(^{2+}\)]\(_i\) was found to accompany ATP depletion in cardiac myocytes (Silverman et al., 1994; Leyssens et al., 1996; Budinger et al., 1998), hepatocytes (Harman et al., 1990) and opossum kidney cells (Li et al., 1993). The measurements of [Mg\(^{2+}\)]\(_i\) can give an indirect measure of the changes in ATP content in living cells. Different from the ATP level, the time course of [Mg\(^{2+}\)]\(_i\) can be easily followed in cells in confluent monolayers by fluorescence microscopy. In these conditions, a combination of ATP measurements in cell extracts and [Mg\(^{2+}\)]\(_i\) measurements in vivo may provide both quantitative data and the time course of ATP depletion in metabolically challenged cells. Apart from reflecting the changes in ATP content, free Mg\(^{2+}\) is involved in several cellular functions, including DNA transcription and protein synthesis (Cameron & Smith, 1989). Mg\(^{2+}\) also participates as a cofactor in numerous enzymatic reactions (Garfinkel & Garfinkel, 1985). The consequences of changes of free Mg\(^{2+}\) on cell function are mostly unknown.

Mitochondria are increasingly recognized as an important target of toxicity in hypoxia/ischemia. However, the role of mitochondrial dysfunction in cell injury is not fully understood. It has been suggested (Duchen & Biscoe, 1992; Di Lisa et al., 1995; Leyssens et al., 1996) that in conditions of hypoxia/ischemia, mitochondria could turn from ATP producers into major consumers of the cellular ATP, in an attempt to preserve the proton electrochemical gradient across the mitochondrial inner membrane. While accelerating the ATP depletion, the maintenance of the mitochondrial membrane potential (ΔΨ) allows Ca\(^{2+}\) accumulation into mitochondria, thus avoiding a presumably damaging Ca\(^{2+}\) overload of the cytoplasm (Silverman, 1993). Provided that hypoxia/ischemia is not sustained overlong, this mitochondrial Ca\(^{2+}\) accumulation can occur without the irretrievable damage to mitochondria. Considering the importance of mitochondria in the energy metabolism of the cells, measurements of the mitochondrial redox state, reflected by changes in NAD(P)H autofluorescence, and of the mitochondrial membrane potential can give valuable information about mitochondrial function in conditions of hypoxia/ischemia.

In the present study, the correlation between ATP content, pH\(_i\), [Mg\(^{2+}\)]\(_i\), and mitochondrial function during chemical hypoxia (induced by cyanide and 2-deoxyglucose (DOG)) and washout is investigated in MDCK cells. MDCK is a cell line derived from dog kidney and the cells express many similarities with the mammalian cortical collecting tubules (CCT) (Valentich, 1981). CCT are more resistant to hypoxia/ischemia than the S3 segment of the proximal tubule and the thick ascending limb of Henle's loop (Wilson & Schrier, 1986). It has been shown that MDCK cells are particularly resistant to ATP depletion (Sheridan et al., 1993; Wiegele
et al., 1998; Feldenberg et al., 1999). The cells displayed signs of necrotic death after more than 6 hours of chemical hypoxia (Sheridan et al., 1993; Wiegele et al., 1998). This characteristic makes MDCK cells appropriate in vitro models for studying the cellular processes that occur in the surviving distal tubular cells during ischemia. We should however mention that although the study of renal cell cultures provides valuable insights into the mechanisms determining the cellular behavior in stress conditions, a direct extrapolation of the results from a cultured cell line to the in vivo situation should be done with caution.

RESULTS

Time course of ATP depletion, intracellular acidosis and [Mg²⁺], during chemical hypoxia.

ATP depletion and acidosis are important hallmarks of renal ischemia. Consequently, the time course of changes in ATP content and pHᵢ were monitored in confluent monolayers of MDCK cells exposed to chemical hypoxia. Inhibition of both oxidative phosphorylation (2.5 mM CN⁻) and glycolysis (10 mM DOG in the absence of glucose) produced a decrease in the cellular ATP level to 5.4 ± 1.6 % from control (n=3) in 20 minutes (Fig. 1). The metabolic inhibitors were withdrawn after 60 minutes and pyruvate was added to provide a substrate for mitochondria. The activation of mitochondrial ATP production was reflected in a partial recovery of ATP content (mean increase to 36 ± 10 %, n=3). When glucose was present in the bathing solution, CN⁻ did not affect the ATP level for 60 minutes (Fig. 1).

Figure 1. Effect of chemical hypoxia on ATP content of MDCK cells. The cells were exposed to the mitochondrial inhibitor CN⁻ (2.5 mM) in the presence of either 5.5 mM glucose (○) or 10 mM DOG (●) for 60 minutes (chemical hypoxia). Since DOG irreversibly inhibits glycolysis, pyruvate was added to the bath solution during washout as a substrate for mitochondria. Shown are the mean ± S.E.M. values from 3 monolayers.
The effect of chemical hypoxia on pH$_i$ was assessed using the fluorescent probe BCECF. The application of CN$^-$ and DOG resulted in an intracellular acidification from pH=$7.38 \pm 0.03$ to pH=$6.78 \pm 0.02$ (n=5) in about 15 minutes (Fig. 2). During the following 45 minutes of chemical hypoxia pH$_i$ continued to decrease slowly to 6.71 $\pm$ 0.03. A partial recovery was observed on washout: pH$_i$ increased slowly and stabilized at 6.95 $\pm$ 0.05 in about 60 minutes. Inhibition of oxidative phosphorylation in the presence of glucose produced a fast but smaller acidification (to pH=$7.15\pm0.04$, n=5), as shown in Fig. 2. No significant change occurred on washout.

![Figure 2](image)

**Figure 2.** Time course of intracellular acidification induced by chemical hypoxia in MDCK cells. Inhibition of both mitochondrial and glycolytic metabolisms using 2.5 mM CN$^-$ and 10 mM DOG (■) produced a profound decrease in pH$_i$. On washout, pH$_i$ partially recovered. A smaller effect was induced by the application of CN$^-$ in the presence of 5.5 mM glucose (□). The values shown are the mean±S.E.M. from 5 separate experiments.

ATP is predominantly present in the cells as Mg-ATP$^{2-}$. The affinity of Mg$^{2+}$ for ATP is more than one order of magnitude greater than for other adenine nucleotides or inorganic phosphate (Veloso et al., 1973). Accordingly, ATP depletion is expected to result in an increase in [Mg$^{2+}$]. The Mg$^{2+}$-sensitive probe Magnesium Green was used in this study to monitor [Mg$^{2+}$], in confluent monolayers of MDCK cells exposed to chemical hypoxia. The fluorescence intensity of the indicator started to increase after application of metabolic inhibitors (CN$^-$ and DOG) and stabilised at 127±3 % (n=6) as compared with control after 20-25 minutes (Fig. 3). The increase in [Mg$^{2+}$]$_i$ was reversible since on washout the MgG signal returned to the control level in about 20 minutes. Cyanide had no effect on [Mg$^{2+}$]$_i$ when glucose was present (Fig. 3).

One of the problems associated with the use of Mg$^{2+}$-sensitive fluorescent indicators is that they all show a significant sensitivity to Ca$^{2+}$ (Haugland, 1996). Consequently, the changes in MgG signal must be interpreted with caution under conditions in which
Figure 3. Changes in $[\text{Mg}^{2+}]$ during chemical hypoxia followed by washout in MDCK cells. In the presence of glucose (5.5 mM), $\text{CN}^{-}$ had no effect on MgG signal ($\Delta$). The application of $\text{CN}^{-}$ and DOG ($\blacktriangle$) resulted in a reversible increase in MgG fluorescence. The values shown are the mean $\pm$ S.E.M. from 6 separate experiments.

$[\text{Ca}^{2+}]_i$ is known to increase. In MDCK cells, chemical hypoxia ($\text{CN}^{-}$ and DOG) produced a transient increase in $[\text{Ca}^{2+}]_i$ in the presence of 1.5 mM external Ca$^{2+}$ whereas no effect was noticed in Ca$^{2+}$ and Mg$^{2+}$ free conditions (see Chapter VI). To check whether the changes in $[\text{Ca}^{2+}]_i$ affect the fluorescence of MgG during chemical hypoxia, the experiments were repeated in the absence of external Ca$^{2+}$ and Mg$^{2+}$. As Fig. 4 shows, the changes in fluorescence intensity do not depend on external Ca$^{2+}$ and Mg$^{2+}$, so that the interference of intracellular Ca$^{2+}$ with MgG signal can be excluded.

Figure 4. Effect of removal of external Ca$^{2+}$ and Mg$^{2+}$ on the changes induced by $\text{CN}^{-}$ and DOG on MgG signal. The effect of chemical hypoxia (2.5 mM $\text{CN}^{-}$ and 10 mM DOG) on MgG fluorescence was identical in the presence of 1.5 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ in the bathing solution ($\blacktriangle$) and in the absence of these cations ($\Delta$). Shown are the mean $\pm$ S.E.M. values from 6 different monolayers.
Changes in $\Delta \Psi$ during chemical hypoxia. Role of the mitochondrial ATP synthase in determining the rate of cellular ATP depletion.

It has been suggested that mitochondrial ATP synthase operates in the reverse mode in conditions of metabolic inhibition (Duchen & Biscoe, 1992; Di Lisa et al., 1995; Leyssens et al., 1996). More precisely, it hydrolyses ATP and this process is coupled to the pumping of $\text{H}^+$ from the matrix. In these conditions, $\Delta \Psi$ is, at least partially, preserved in the absence of electron transport while the rate of ATP depletion is increased. To investigate whether such a mechanism operates in MDCK cells exposed to chemical hypoxia, $\Delta \Psi$ was evaluated using the fluorescent indicator JC-1. In monolayers of MDCK cells, large variations in dye loading efficiency were recorded among cells. Furthermore, various levels of J-aggregates fluorescence were found in mitochondria from the same cell (not shown), suggesting that mitochondria within the same cell do not have identical membrane potential. Similar heterogeneities for MDCK cells have been recently reported (Diaz et al., 1999). The sensitivity of the indicator was tested with the protonophore FCCP (10 $\mu$M), applied in the presence of 10 mM DOG to inhibit glycolysis (Fig. 5). In these conditions a complete depolarization of the mitochondrial inner membrane is expected. FCCP produced a fast decrease in the signal at 590 nm (Fig. 5A), associated with the disaggregation of J-aggregates, and an increase in the fluorescence intensity at 530 nm (Fig. 5B). This later result cannot be explained by changes of the JC-1 concentration in the matrix, suggesting an interaction of the probe with mitochondrial membrane. Similar response of JC-1 to de-energizing conditions has been reported in other cells (Cossarizza et al., 1993; Di Lisa et al., 1995; Leyssens et al., 1996). Fig. 5C shows the effect of FCCP on the emission ratio $F_{590}/F_{335}$ of JC-1. In the following, we used this parameter as a measure of $\Delta \Psi$.

Metabolic inhibition might result in depolarization of the plasma membrane. As a lipophilic cation, the accumulation of JC-1 in living cells depends on the plasma membrane potential. To investigate the effect of the plasma membrane potential on JC-1 emission ratio, the membrane was depolarized with 10 $\mu$M gramicidin D (not shown). Gramicidin had no significant effect on the fluorescence of JC-1, thus ruling out an interference of the plasma membrane potential with the measurements of $\Delta \Psi$.

A representative experiment where JC-1 emission ratio was used to assess the effect of chemical hypoxia on $\Delta \Psi$ is shown in Fig. 6. The metabolic inhibitors (CN$^-$ and DOG) were applied for 60 minutes. Chemical hypoxia was followed by washout (for 30 minutes). FCCP (10 $\mu$M) was added at the end of the experiment. As Fig. 6 shows, the application of FCCP resulted in an immediate drop in the fluorescence ratio to a level significantly lower than the minimum level reached in the presence of CN$^-$ and DOG.

To compare experiments with different control values of the JC-1 emission ratio, the results are presented as a percentage change compared with control, assuming that 10 $\mu$M FCCP (added at the end of each experiment) completely abolishes the
Figure 5. Effect of FCCP (10 \mu M) on the JC-1 fluorescence in MDCK cells. Glycolysis was inhibited with 10 mM DOG. A) The mitochondrial uncoupler FCCP produced a fast decrease in the fluorescence at 590 nm, indicating the disaggregation of J-aggregates. B) The signal at 535 nm increased upon the application of FCCP, suggesting an interaction of JC-1 with the mitochondrial membrane. C) The emission ratio $F_{590}/F_{535}$ of JC-1 dropped immediately after the application of the uncoupler, indicating the collapse of $\Delta \Psi$. 

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mitochondrial membrane potential. The following expression was used to calculate the percentage of the changes in $F_{590}/F_{535}$ ($\overline{R}$) as compared with control:

$$\overline{R} = \frac{R - R_{\text{FCCP}}}{R_{\text{control}} - R_{\text{FCCP}}} \times 100$$  \hspace{1cm} (1)

where $R_{\text{control}}$ is the ratio in control conditions and $R_{\text{FCCP}}$ is the JC-1 emission ratio after adding FCCP.

Fig. 7 shows the time course of changes in $\Delta \Psi$ following the application of 2.5 mM CN$^-$ and 10 mM DOG. $\Delta \Psi$ dropped to $26 \pm 6\%$ (n=6) from the control value during the first 25 minutes of chemical hypoxia. This decrease was followed by a partial recovery of $\Delta \Psi$ (to $48 \pm 5\%$ from control) during the next 35 minutes, in the continuous presence of the metabolic inhibitors. No significant change in $\Delta \Psi$ was noticed on washout (with pyruvate added as a substrate for mitochondria). The experiments were repeated in the presence of oligomycin, a well known inhibitor of mitochondrial ATP synthase. Oligomycin (20 μg ml$^{-1}$) was added to the bath solution 10 minutes before the metabolic inhibitors and did not induce any significant change in $\Delta \Psi$. In the presence of oligomycin, chemical hypoxia produced a faster and more pronounced drop in $\Delta \Psi$ (Fig. 7): after 6 minutes, the ratio $\overline{R}$ decreased to $18 \pm 2\%$ (n=6) from the control level, compared to $57 \pm 10\%$ in the absence of oligomycin. No significant recovery occurred as long as the metabolic inhibitors and oligomycin were present. On washout, the mitochondrial inner membrane repolarized rapidly to the same level as in cells which were not exposed to oligomycin. The inhibition of the mitochondrial ATP synthase led to significantly lower $\Delta \Psi$ throughout the application of CN$^-$ and DOG (60 minutes).
Figure 7. Effect of chemical hypoxia (2.5 mM CN⁻ and 10 mM DOG) on ΔΨ in MDCK cells, as assessed by the ratio \( \frac{R}{\text{Washout}} \) (Eq. 1). The experiments were performed in the absence (●) or in the presence (○) of 20 µg ml⁻¹ oligomycin. The drug was added to the bath solution 10 minutes before the metabolic inhibitors. After 60 minutes of chemical hypoxia, the metabolic inhibitors and oligomycin were removed and 7.5 mM pyruvate was added to provide a substrate for mitochondria. The values shown are the mean ± S.E.M. from 6 separate experiments.

To further test the hypothesis that mitochondrial ATP synthase operates in the reverse mode during chemical hypoxia, we compared the effects of CN⁻ and DOG on ATP level, \([\text{Mg}^{2+}]_i\), and \(\text{pH}_i\) in the presence and in the absence of oligomycin (20 µg ml⁻¹). Oligomycin per se had no effect on cellular ATP or on MgG signal for up to 60 minutes (results not shown), but induced a decrease in \(\text{pH}_i\) (mean decrease was 0.26 ± 0.04 pH units, n=5). As Fig. 8A shows, the rate of ATP depletion due to metabolic inhibitors was significantly lower in the presence of oligomycin. However, after 20 minutes of chemical hypoxia the ATP level was independent of the presence of oligomycin in the bath solution. Despite a different time course in cellular ATP content, the changes in MgG fluorescence during chemical hypoxia were not significantly affected by oligomycin (Fig. 8B). The presence of oligomycin together with the metabolic inhibitors did not result in a different intracellular acidification either (Fig. 8C). Oligomycin seems to block irreversibly the ATP synthase, since the cells were not able to restore either the ATP pools (Fig. 8A), \([\text{Mg}^{2+}]_i\) (Fig. 8B) or \(\text{pH}_i\) (Fig. 8C) during 60 minutes of reperfusion. However, the mitochondrial membrane potential recovers on washout in cells treated with oligomycin, suggesting that oxidative phosphorylation restarts.
Figure 8. The influence of oligomycin (20 μg ml⁻¹) on the changes in ATP content, [Mg²⁺]ᵢ, and pHᵢ induced by chemical hypoxia. A) Oligomycin (〇) reduced the rate of ATP depletion during chemical hypoxia (●) but did not affect the final ATP level. The data are the mean±S.E.M. from 3 separate experiments. B) The rise in MgG fluorescence during chemical hypoxia (▲) was not significantly altered by the presence of oligomycin (△). The values shown are the mean ± S.E.M. from 6 separate experiments. C) In the presence of oligomycin (□), chemical hypoxia produced a similar intracellular acidification as in the absence of the drug (■), although oligomycin per se induced a decrease in pHᵢ. The data are the mean ± S.E.M. from 5 separate experiments.
We showed above that the mitochondrial uncoupler FCCP induces an immediate depolarization of the mitochondrial inner membrane. Accordingly, the effect of FCCP (10 μM) and DOG (10 mM) on cellular ATP content, [Mg2+], and pH_i was investigated (Fig. 9). Following the application of FCCP and DOG, the cellular ATP level dropped to 6.0 ± 1.8 % from control (n=3) in 15 minutes (Fig. 9A) and the effect was not reversed on washout. Fig. 9B shows the changes in MgG signal upon the application of FCCP and DOG. MgG fluorescence rose faster and to significantly higher levels than in the presence of CN− and DOG (140 ± 4 %, n=5, as compared with 127 ± 3%, n=6, P=0.026). On washout, the fluorescence intensity decreased to 119 ± 2% as compared with control in about 30 minutes. In the presence of FCCP and DOG, pH_i decreased to 6.77 ± 0.06 (n=4) in three minutes (Fig. 9C). In the same time interval, CN− and DOG acidified the cells to only pH_i = 7.22 ± 0.02 (n=5) (Fig. 2). However, the final level of intracellular acidification was similar in the two cases.

Changes in the cellular redox state during chemical hypoxia

Mitochondrial NADH is the primary electron source for the electron transport chain in oxidative phosphorylation. Consequently, one of the earliest processes following inhibition of the respiration will consist in a shift of the mitochondrial NADH/NAD+ redox state towards the reduced form NADH. This reduction will be reflected by an increase in NAD(P)H autofluorescence. Indeed, the application of CN− and DOG produced an immediate rise (mean increase to 151±7 % from control, n=6) in the cellular autofluorescence (Fig. 10A). This increase was followed by a slow decrease in the signal, so that after one hour of chemical hypoxia the NAD(P)H autofluorescence was 106 ± 8 % from control. The washout of metabolic inhibitors, with pyruvate added as a substrate, resulted in an immediate drop of the NAD(P)H autofluorescence to a level well below that observed under control conditions (55 ± 6 %). Qualitatively, a similar behaviour of NAD(P)H autofluorescence was observed when azide (5 mM) was used instead of cyanide (azide blocks the respiratory chain at the same site as cyanide) (results not shown). The application of CN− in the presence of glucose (5.5 mM) had similar effects (Fig. 10B). However, on washout, with glucose as substrate, the decrease in NAD(P)H autofluorescence was significantly less than the decrease observed after changing from CN− and DOG solution to the pyruvate solution (mean decrease to 87 ± 3 % from control, n=6, as compared with 55 ± 6 %). Inhibition of glycolysis with 10 mM DOG produced a slow and gradual decrease in NAD(P)H autofluorescence (Fig. 10C), so that after 60 minutes the signal was 86 ± 2 % from control (n=6). A further decrease to 63 ± 3 % was noticed upon switching to the pyruvate solution.
Figure 9. Effect of the mitochondrial uncoupler FCCP (10 μM) and 10 mM DOG on ATP content, [Mg²⁺], and pHᵢ in MDCK cells. A) FCCP and DOG produced an ATP depletion comparable with the effect of CN⁻ and DOG. Shown are the mean ± S.E.M. values from 3 different monolayers. B) The MgG signal raised faster and to higher levels as compared to the application of CN⁻ and DOG. The data are the mean ± S.E.M. from 5 separate experiments. C) FCCP and DOG produced a faster acidification than the combination of CN⁻ and DOG, although the final level was similar. The data are the mean ± S.E.M. from 4 separate experiments.
Figure 10. Effect of metabolic inhibitors on NAD(P)H autofluorescence in MDCK cells. Inhibition of both oxidative phosphorylation (2.5 mM CN⁻) and glycolysis (10 mM DOG) (A) or only of oxidative phosphorylation (B) resulted in an immediate increase in NAD(P)H autofluorescence, followed by a slow decrease during 60 minutes of the treatment. On washout, with pyruvate (7.5 mM) added as a substrate, the NAD(P)H signal decreased to a significantly lower value than the control (A). When glucose (5.5 mM) was present during the washout (B), the NAD(P)H autofluorescence returned to a level only slightly lower than the control. Inhibition of glycolysis (C) resulted in a slow decrease in NAD(P)H autofluorescence. A further drop was noticed upon the exchange of DOG with 7.5 mM pyruvate. The values shown are the mean ± S.E.M. from 6 separate experiments.
DISCUSSION

ATP depletion and intracellular acidification in MDCK cells during chemical hypoxia

This study investigates the changes in energy metabolism during chemical hypoxia (2.5 mM CN\(^-\) and 10 mM DOG in the absence of glucose) in MDCK cells. This is considered an in vitro model of the reversibly injured cells from the distal tubules of ischemic kidney. We found that chemical hypoxia produces a profound ATP depletion in MDCK cells (to less than 10% of the control) in about 20 minutes. This result agrees with the previously published data (Sheridan et al., 1993; Doctor et al., 1994; Feldenberg et al., 1999). Feldenberg et al. (1999) found that inhibition of both glycolysis and oxidative phosphorylation in MDCK cells resulted in a very rapid and profound depletion of ATP levels to <4% of controls. However, the cells displayed signs of necrotic death within 6 hours of treatment. A similar time course for the necrotic pathway of cell death in MDCK cells has been reported by Wiegele et al. (1998). Signs of apoptosis were observed at early stages only in a small proportion of cells (Wiegele et al., 1998). Consequently, in this study MDCK cells were exposed to chemical hypoxia for 60 minutes. On washout, with pyruvate added as a substrate for mitochondria, the cellular ATP content recovered partially. The only partial recovery may be explained by the absence of glucose from the recovery solution (because DOG is thought to block irreversibly the glycolysis), so the glycolytic metabolism is absent in these conditions. The recovery in the ATP content suggests that the cells are not lethally injured. The full or partial recovery observed with the other parameters investigated in this and in the next Chapter (pH, [Mg\(^{2+}\])\(_i\), \(\Delta\Psi\), cellular redox state, [Ca\(^{2+}\])\(_i\), and [Na\(^+\])\(_i\)) points toward the same conclusion, and so does the fact that no sudden loss of fluorescent indicators from the cells was observed. Imaging experiments indicate little variability between the behavior of single cells.

A profound intracellular acidification (0.6 pH units) developed in parallel with the drop in cellular ATP content. The decrease of pH\(_i\) has a similar time-course as the ATP depletion, suggesting that the hydrolysis of ATP is the main source of H\(^+\). The other possible sources of H\(^+\) during ischemia, glycolytic lactate production and CO\(_2\) accumulation, can be excluded here since glycolysis was inhibited and the experiments were performed in an open bath. We cannot rule out an impairment of H\(^+\) extrusion mechanisms due to low ATP concentration.

The high resistance of MDCK cells to a severe reduction in the cellular ATP content is well documented (Sheridan et al., 1993; Lieberthal et al., 1997) but the mechanisms responsible for this effect are largely unknown. One can speculate that the rapid and profound intracellular acidification observed in this study protects the cells during ATP depletion. Tissue acidification was shown to have a protective effect during ischaemia in several organ systems, including the kidney (Weinberg, 1991; Weinberg et al., 1991; Edelstein et al., 1996). Cellular acidification was found to occur during anoxia in cultured, but not in freshly isolated rabbit proximal tubular cells (Rose et al., 1995). However, the differences in susceptibility to anoxic injury between cultured and freshly isolated proximal tubular cells could not be explained by cellular
acidification in cultured cells because prevention of acidification in cultured proximal tubular cells did not lead to increased cell death (Rose et al., 1995).

The inhibition of oxidative phosphorylation in the presence of glucose did not produce any significant change in cellular ATP content for 60 minutes, despite a substantial increase in cellular NAD(P)H/NAD(P)+ ratio. This result shows that MDCK cells are highly dependent on glycolysis. This is a general characteristic of cultured cells, but may also be related to the properties of the nephron segment of origin. MDCK cells are a model of distal tubular cells, which are known to have a higher glycolytic capacity in vivo as compared to the proximal tubules (Klahr et al., 1992; Gullans & Hebert, 1996; Schoolwerth & Drewrowska, 1997). In medullary segments of the kidney, where the oxygen pressure is quite low (Schoolwerth & Drewrowska, 1997), anaerobic metabolism is more likely to occur. Furthermore, the anaerobic metabolism can support ATP levels in the presence of glucose in collecting tubules (Uchida & Endou, 1988). It has been shown that the ATP content in MDCK cells bathed with normal culture medium was not affected even after 24 hours of hypoxia (Yonehana & Gemba, 1995). This was different from the effect on LLC-PK1 cells, a model of proximal tubular cells, where hypoxia produced a time-dependent reduction in ATP content (Yonehana & Gemba, 1995). This finding supports the idea that the high glycolytic capacity of MDCK cells is partly due to their distal tubules origin. In agreement with the lack of effect on the ATP content, CN− in the presence of glucose did not increase [Mg2+] and [Ca2+] (see Chapter VI). The small acidification observed in this case is likely due to increased lactate production by anaerobic glycolysis.

Mitochondrial functions during chemical hypoxia; mitochondrial ATP synthase operates in the reverse mode in conditions of metabolic inhibition

With inhibition of both electron transport chain and glycolysis, the NAD(P)H/NAD(P)+ ratio rises rapidly and ΔΨ cannot be sustained in the face of leaks. However, mitochondrial inner membrane is not depolarized completely since ulterior application of the mitochondrial uncoupler FCCP produced a further decrease in the emission ratio of JC-1. This suggests that ΔΨ is partially maintained during chemical hypoxia. Furthermore, ΔΨ even recovers partially after the first 25 minutes of the treatment, in the continuous presence of metabolic inhibitors. The inhibition of mitochondrial ATP synthase with oligomycin slowed down significantly the ATP depletion during chemical hypoxia and accelerated the mitochondrial depolarization. In the presence of oligomycin, ΔΨ was significantly lower than in the absence of the inhibitor throughout the application of CN− and DOG (60 minutes). These results suggest that mitochondrial ATP synthase operates in the reverse mode during chemical hypoxia in MDCK cells: it hydrolyses ATP and this process is coupled to the pumping of H+ from the matrix. In this way, ΔΨ is partially preserved in the absence of electron transport. The reversal of mitochondrial ATP synthase in conditions of metabolic inhibition was observed in several types of cells (Duchen & Bischoe, 1992; Di Lisa et al., 1995; Leyssens et al., 1996). It has been suggested that it is useful for the mitochondrion to conserve ΔΨ as this prevents mitochondrial swelling (Duchen, 1999) and maintains the capacity of mitochondria to accumulate Ca2+ via the Ca2+
Energy state and chemical hypoxia in MDCK cells

uniporter (Gunter et al., 1994). At the same time, this process might represent an important source of ATP consumption during metabolic inhibition, so it could be deleterious for the cells.

Our finding that \( \Delta \Psi \) is partially preserved during chemical hypoxia in MDCK cells agrees with the previously published data on renal cells. It has been shown that complete dissipation of \( \Delta \Psi \) occurs after more than 60 minutes of anoxia (in the absence of glucose) in cultured rat proximal tubular cells and cell death follows immediately after mitochondrial depolarization (Chi et al., 1995). Peters et al. (1996) found that mitochondrial potential collapses in anoxic but not hypoxic proximal tubular cells. An increase in \( \Delta \Psi \) in response to hypoxia has been observed in MDCK cells (Díaz et al., 1999). However, in this experiment the cells were bathed with culture medium during exposure to hypoxia. We showed in this study that glycolysis could maintain the ATP content in the absence of oxidative phosphorylation. Consequently, the result reported by Díaz et al. (1999) could be explained by the operation in the reverse mode of mitochondrial ATP synthase which utilizes the ATP produced by glycolysis to pump out \( H^+ \) from the matrix into the cytosol.

Mitochondrial depolarization with FCCP (in the presence of DOG) resulted in an even faster drop in ATP level and \( \text{pH}_i \). This further supports the hypothesis that mitochondrial ATP synthase reverses in metabolically stressed MDCK cells in an attempt to restore \( \Delta \Psi \).

The initial increase in the NAD(P)H/NAD(P)\(^+\) ratio induced by the application of metabolic inhibitors was followed by a slow decline so that after 60 minutes the NAD(P)H/NAD(P)\(^+\) ratio was comparable with the control. A possible explanation for this behaviour consists in a reduction of pyridine nucleotide pool during chemical hypoxia. The restoration of the electron transport on washout (with pyruvate added as substrate for mitochondria) resulted in a rapid drop in NAD(P)H autofluorescence, to a level much lower than the control. This undershoot of NAD(P)H signal could reflect accumulation of ADP during the preceding period; on washout, ADP stimulates the oxidative phosphorylation. However, it is likely that the effect is due to the NAD hydrolysis during chemical hypoxia and to the presence of pyruvate in the recovery solution. Addition of pyruvate decreases the cytosolic NADH/NAD\(^+\) ratio through the lactate dehydrogenase reaction. The significantly smaller drop observed when glucose replaced pyruvate in the reperfusion solution points towards this latter explanation.

Changes in [Mg\(^{2+}\)] are not proportional with the changes in ATP level in MDCK cells

ATP depletion is expected to result in a rise in [Mg\(^{2+}\)], because the affinity of Mg\(^{2+}\) for ATP is more than one order of magnitude greater than for other adenine nucleotides or inorganic phosphate (Veloso et al., 1973). The Mg\(^{2+}\)-sensitive fluorescent indicator MgG was used in this study to monitor the changes in [Mg\(^{2+}\)] during chemical hypoxia. As expected, the application of CN\(^-\) and DOG resulted in a reversible rise of [Mg\(^{2+}\)]. The changes in [Mg\(^{2+}\)] were independent of external Mg\(^{2+}\) and Ca\(^{2+}\). This observation shows first that the increase in [Mg\(^{2+}\)] has an intracellular source, most
likely the release of Mg$^{2+}$ from Mg-ATP$^{2-}$, and second that the signal is not affected by changes in [Ca$^{2+}$], because chemical hypoxia modifies [Ca$^{2+}$], only in the presence of external Ca$^{2+}$ (Chapter VI). The time course of the increase in [Mg$^{2+}$], was similar to that observed for ATP depletion. However, on washout [Mg$^{2+}$], returned to control levels while only a partial recovery in ATP content was observed. This suggests that the changes in [Mg$^{2+}$], and cellular ATP are not proportional, as expected from the 1:1 stoichiometry of the binding. Because we did not calibrate the MgG signal, it is not possible to compare quantitatively the changes in [Mg$^{2+}$], and cellular ATP. Chemical hypoxia produced similar changes in MgG signal in the absence and in the presence of oligomycin, despite significant differences in the level of ATP depletion during the first 15 minutes. The mitochondrial uncoupler FCCP (in the presence of DOG) produced a significantly bigger increase in [Mg$^{2+}$], than did CN$^-$ and DOG. However, similar levels of ATP depletion were reached with either CN$^-$ or FCCP. These findings support the conclusion that in MDCK cells exposed to metabolic inhibitors the rise in [Mg$^{2+}$], is not proportional with ATP depletion. A similar situation was found in opossum kidney cells (Li et al., 1993). Our results imply that part of the Mg$^{2+}$ released from Mg-ATP$^{2-}$ is buffered by other intracellular components. The discrepancies between changes in [Mg$^{2+}$], and [ATP] might also be a consequence of ATP compartmentalization, since we measured ATP in extracts of whole cells while MgG reports the changes in cytoplasmic free [Mg$^{2+}$]. In renal cells, about 20% of the ATP is localized in the mitochondria (Pfaffler et al, 1984). The loss and recovery of ATP in the cytoplasm and in the mitochondrial matrix may not be the same. We cannot exclude the possibility that the increase in [Mg$^{2+}$], is partly due to an impairment of the active extrusion of Mg$^{2+}$ from the cells produced by ATP depletion.

Although most of the ion indicators are somewhat sensitive to pH, it is unlikely that the MgG fluorescence was significantly affected by the changes in pH in these experiments. First, the withdrawal of CN$^-$ and DOG resulted in a return of MgG signal to control levels whereas pH remained low. Second, the rise in the fluorescence intensity of MgG in response to the application of FCCP and DOG was significantly bigger than the effect produced by CN$^-$ and DOG. However, both treatments led to a similar degree of intracellular acidification, although with a different time course.

In summary, this study shows that ATP depletion and intracellular acidification develop in parallel in MDCK cells exposed to chemical hypoxia (CN$^-$ and DOG). An increase in [Mg$^{2+}$], accompanies the drop in cellular ATP content. However, the rise in [Mg$^{2+}$], did not follow exactly the changes in ATP level, suggesting that part of the free Mg$^{2+}$ resulted from ATP hydrolysis is buffered by other intracellular components. Mitochondrial membrane potential is partially preserved during metabolic inhibition by the mitochondrial ATP synthase, which operates in the reverse mode, i.e. it hydrolyses ATP and this process is coupled to the pumping of H$^+$ from the matrix into the cytoplasm.
CHAPTER VI

Changes in \([\text{Ca}^{2+}]_i\) during chemical hypoxia in MDCK cells
ABSTRACT
Fluorescence imaging microscopy was used to monitor the effects of chemical hypoxia (CN⁻ and DOG) on [Ca²⁺], and [Na⁺], in confluent monolayers of MDCK cells. A steady 4-5 fold rise in [Na⁺], occurred during a 60 minutes exposure to chemical hypoxia, followed by a partial recovery on washout. When cyanide was applied in the presence of glucose, [Na⁺], increased to a similar extent at the beginning of the experiment but it stabilized after 20-25 minutes.

[Ca²⁺], increased ≈4 fold during the first 30-35 minutes of chemical hypoxia. This rise was followed by a decrease, in the continuous presence of the metabolic inhibitors, to values close to the resting [Ca²⁺], in about 40 minutes. No effect was produced by cyanide in the presence of glucose. The absence of Ca²⁺ from external solution or the addition of La³⁺ prevented the changes in [Ca²⁺]. The effect of chemical hypoxia on [Ca²⁺], was considerably reduced in the absence of external Na⁺ and partially inhibited by verapamil. Thus, Ca²⁺ increase is due to Ca²⁺ entry, mainly via the Na⁺/Ca²⁺ exchanger which operates in the reverse mode. Inhibition of the plasma membrane Ca²⁺ pumps or of SERCA pumps did not prevent the decay phase of [Ca²⁺]. Application of ionomycin (in Ca²⁺ free solution) during the recovery phase produced a transient in [Ca²⁺], suggesting that Ca²⁺ is accumulated in some internal organelles. Oligomycin, which in combination with cyanide dissipates the mitochondrial membrane potential, prevented the decline phase in [Ca²⁺]. This indicates mitochondria as the organelles likely to buffer cytoplasmic Ca²⁺ in conditions of metabolic stress.

INTRODUCTION
We have shown in the previous chapter that chemical hypoxia (induced by CN⁻ and DOG) produces a rapid ATP depletion in MDCK cells. These cells express many similarities with the mammalian cortical collecting tubules (Valentic, 1981). Impairment of ion homeostasis is one of the earliest events that follow ATP depletion. This is especially important in the renal tubules because these cells are actively involved in ion reabsorption and secretion. One of the main activities of the renal tubular cells is the reabsorption of filtered Na⁺, which provides the driving force for the reabsorption of water and for the coupled transport of organic solutes. Na⁺ reabsorption is an active transport process, mediated by the basolaterally located Na⁺/K⁺ ATPase and driven by cellular ATP. The transmembrane Na⁺ gradient is coupled to the intracellular homeostasis of other ions by co- and anti-transport systems. Consequently, changes in intracellular Na⁺ concentration ([Na⁺]), are expected to have important consequences.

Ca²⁺ is a general signal in both life and death: although elevations in intracellular Ca²⁺ concentration are necessary for it to act as a signal, prolonged increases in [Ca²⁺], can be lethal. Consequently, it is not surprising that the issue of whether elevated [Ca²⁺], plays a major role in the ischemic injury of renal cells has been a matter of debate for some time (Weinberg et al., 1991; Smith et al., 1992; Kribben et al., 1994; Peters et al., 1996; Weinberg et al., 1997). However, the results of several studies regarding [Ca²⁺], in renal cells during ischemia/anoxia differ markedly. Kribben et al. (1994)
demonstrated a rapid increase in \([\text{Ca}^{2+}]_i\) during hypoxic incubation of rat proximal tubules, which preceded and correlated to ensuing cell injury. Weinberg et al. (1991) found that rabbit proximal tubules subjected to chemical anoxia did not develop increases of \([\text{Ca}^{2+}]_i\) until just before loss of viability. Jacobs et al. (1991) showed that \([\text{Ca}^{2+}]_i\) did not increase in rabbit proximal tubules during anoxia or chemical hypoxia. Modest increases in \([\text{Ca}^{2+}]_i\) during anoxia in the absence of substrate were also observed in cultured monkey kidney cells (Snowdowne et al., 1985). Smith et al. (1992) observed a rapid increase in \([\text{Ca}^{2+}]_i\) to more than 1 \(\mu\text{M}\) in rabbit proximal tubular cells exposed to cyanide and iodoacetic acid, but no loss of cell viability. Weinberg et al. (1997) reported that between 30 and 60 minutes after treatment of isolated proximal tubules with antimycin, in the presence of glycine, \([\text{Ca}^{2+}]_i\) increased to more than 100 \(\mu\text{M}\) in 68% of the tubules studied. These different results could be due to distinct sensitivities to hypoxia/ischemia manifested by separate segments of the nephron or to the different sources of the cells. Peters et al. (1996) showed that the level of oxygen deprivation is critical in determining changes in \([\text{Ca}^{2+}]_i\) during hypoxia.

Fluorescence imaging microscopy is used in this study to investigate the time course of \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) during chemical hypoxia in MDCK cells. Preliminary accounts of these results have been reported and published in abstract form (Despa et al., 1999).

**RESULTS**

**Corrections for changes in cellular autofluorescence during chemical hypoxia**

At the wavelengths used with Fura-2 and SBFI, the cellular autofluorescence is attributed to coenzymes and flavins (Schneckenburger et al., 1996). The NAD(P)H and FAD fluorescence depend on the redox state of the cells (Hassinen, 1986) and change during inhibition of the mitochondrial electron transport. These changes can affect the excitation ratio \(F_{340}/F_{380}\) of Fura-2 or SBFI loaded cells, which will indicate false variations in \([\text{Ca}^{2+}]_i\) or \([\text{Na}^+]_i\), respectively. To correct for this, the changes in cellular autofluorescence at 340 and 380 nm during chemical hypoxia were investigated in parallel experiments. Following the inhibition of both oxidative and glycolytic metabolisms (with 2.5 mM CN\(^-\) and 10 mM DOG, respectively), the signal at \(\lambda_{\text{ex}} = 380\) nm \((F_{380})\) increased rapidly to 118 ± 4 % (n=5) as compared with the control level and remained practically constant for 60 minutes (Fig. 1). The signal excited at 340 nm \((F_{340})\) was not significantly affected (Fig. 1). Consequently, the changes in the cellular autofluorescence during metabolic inhibition will result in a decrease in the \(F_{340}/F_{380}\) excitation ratio. On washout (with pyruvate added as a substrate) the signal at 380 nm fell to 110 ± 6 % from control (Fig. 1).

Considering these results, the excitation ratio of Fura-2 and SBFI during chemical hypoxia followed by washout was corrected using the expression:

\[
F_{340} = \frac{F_{340}}{F_{380} - \left( \frac{P}{100} - I \right) A F_{380}}
\]
Figure 1. Effect of chemical hypoxia (2.5 mM CN⁻ and 10 mM DOG) and washout on the cellular autofluorescence at the wavelengths used for Fura-2 and SBF1. The unloaded cells were excited alternately at 340 nm (□) and 380 nm (○) while emission was recorded at 535 nm. The changes in the cellular autofluorescence reflect the effects of chemical hypoxia on the endogenous NAD(P)H and FAD fluorescence. Pyruvate was added to the bath solution during washout as a substrate for mitochondria because DOG irreversibly inhibits glycolysis. Shown are the mean ± S.E.M. values from 5 monolayers.

where p is the percentage increase in the autofluorescence excited at 380 nm (p=118 during chemical hypoxia and p=110 on washout) and AF_{380} is the control autofluorescence at λ_{ex}=380 nm.

Chemical hypoxia produces a transient increase in [Ca²⁺]₁
Inhibition of the oxidative phosphorylation with 2.5 mM CN⁻ in the presence of glucose did not produce any significant change in [Ca²⁺]₁ over a period of 60 minutes (Fig. 2). In the next experiments, both mitochondrial and glycolytic metabolisms were inhibited, using the standard combination of CN⁻ (2.5 mM) and DOG (10 mM). This treatment induced a transient increase in [Ca²⁺]₁ (Fig. 2): the level of free cytoplasmic calcium increased from 68 ± 8 nM to 283 ± 32 nM (n=6) in the first 30 - 35 minutes of chemical hypoxia. During the next 40 minutes, in the continuous presence of CN⁻ and DOG, [Ca²⁺], decreased to 138 ± 21 nM. This decrease in [Ca²⁺]₁ is not an artefact produced by the loss of Fura-2 from the cells because of membrane injury. As Fig. 3 shows, the signal at 340 nm (F₀) decreased and the signal at 380 nm (F₃₈₀) increased during the decay phase of [Ca²⁺]₁. This is the normal behaviour of the indicator with a decrease in [Ca²⁺].

Mechanisms leading to the rise of [Ca²⁺]₁ during chemical hypoxia
To elucidate the sources of the increase in [Ca²⁺]₁, the experiments were repeated in the absence of extracellular Ca²⁺. Resting [Ca²⁺]₁ in Ca²⁺ free conditions was 34±2 nM (n=5), significantly (P<0.05) lower than in the presence of 1.5 mM external [Ca²⁺]. This level was not significantly affected by chemical hypoxia (Fig. 4A), suggesting that the response is due to Ca²⁺ influx across the plasma membrane while Ca²⁺ release
Figure 2. Effect of chemical hypoxia on \([Ca^{2+}]_i\) in MDCK cells. The cells were exposed to the mitochondrial inhibitor CN\(^-\) (2.5 mM) in the presence of either 5.5 mM glucose (○) or 10 mM DOG (●). The arrow indicates the moment of application of the metabolic inhibitors. The data were corrected for the changes in cellular autofluorescence as described in the methods section. The values shown are the mean ± S.E.M. from 6 separate experiments.

Figure 3. Effect of chemical hypoxia on Fura-2 signals in MDCK cells. Representative traces showing the effect of chemical hypoxia (CN\(^-\) and DOG) on the fluorescence intensities of Fura-2 loaded MDCK cells excited at 340 nm (□) and 380 nm (○). Also shown is the excitation ratio \(F_{340}/F_{380}\) (●). The data were corrected for the changes in cellular autofluorescence as described in the methods section. The decline in \(F_{340}/F_{380}\) is accompanied by an increase in the fluorescence intensity excited at 380 nm.

from internal stores has a negligible effect. This conclusion was further substantiated by the finding that La\(^{3+}\) (0.1 mM), a non-specific inhibitor of Ca\(^{2+}\) entry, also abolished the changes in intracellular Ca\(^{2+}\) during metabolic inhibition (Fig. 4B). It has been reported that the L-type Ca\(^{2+}\) channel blocker methoxyverapamil (D600) partially
prevented anoxia-induced increase in [Ca\(^{2+}\)]\(_i\) in rabbit medullary and cortical TAL (Rose et al., 1994a). Consequently, we monitored [Ca\(^{2+}\)]\(_i\) during chemical hypoxia in the presence of verapamil, an inhibitor of Ca\(^{2+}\) channels. Verapamil had no significant effect on resting [Ca\(^{2+}\)]\(_i\). However, the drug reduced the increase in [Ca\(^{2+}\)]\(_i\) during chemical hypoxia (Fig. 4C): the peak value reached in the presence of 20 μM verapamil was 180 ± 13 nM (n=6), significantly (P<0.05) lower than in the absence of the blocker, suggesting that part of Ca\(^{2+}\) influx occurs through Ca\(^{2+}\) channels.

**Figure 4.** Effect of Ca\(^{2+}\) entry on the changes in [Ca\(^{2+}\)]\(_i\) during chemical hypoxia. Filled bars indicates the application of chemical hypoxia (CN\(^-\) and DOG). (A) No change in [Ca\(^{2+}\)]\(_i\) occurred when Ca\(^{2+}\) was excluded from the external solution (with 0.5 mM EGTA). The values shown are the mean±S.E.M. from 3 separate experiments. (B) Inhibition of Ca\(^{2+}\) influx with 0.1 mM La\(^{3+}\), prevented the changes in [Ca\(^{2+}\)]\(_i\) during chemical hypoxia. Shown is the mean ± S.E.M. from 3 separate experiments. (C) The Ca\(^{2+}\) channels inhibitor verapamil (20 μM) reduced the increase in [Ca\(^{2+}\)]\(_i\) produced by chemical hypoxia. The values shown are the mean ± S.E.M. from 6 separate experiments.
In MDCK cells, $[\text{Ca}^{2+}]_i$ is related to the transmembrane $\text{Na}^+$ gradient via the $\text{Na}^+/$Ca$^{2+}$ exchanger (Borde & Bender, 1991). When the antiporter operates in the normal mode, three $\text{Na}^+$ ions enter into the cell in exchange for one $\text{Ca}^{2+}$ ion. Borde & Bender (1991) suggested that in control conditions the $\text{Na}^+/$Ca$^{2+}$ exchanger operates in the reverse mode in MDCK cells: $\text{Na}^+$ is extruded in exchange for Ca$^{2+}$. Depletion of cellular ATP during chemical hypoxia (see Chapter V), will interfere with the activity of the $\text{Na}^+/$K$^+$ pump, consequently $[\text{Na}^+]_i$ is expected to increase. We used fluorescence microscopy and the $\text{Na}^+$-sensitive indicator SBFI to monitor intracellular $\text{Na}^+$ during chemical hypoxia. As illustrated in Fig. 5, application of CN$^-$ and DOG for 60 minutes induced a steady increase in $[\text{Na}^+]_i$ from 15±3 mM to 69±7 mM (n=6). This increase was partially reversible: 60 minutes after the washout, $[\text{Na}^+]_i$ decreased to 42 ± 10 mM. The application of CN$^-$ in the presence of glucose produced a similar effect during the first 20-25 minutes, when $[\text{Na}^+]_i$ increased to 39 ± 8 mM. However, in these conditions $[\text{Na}^+]_i$ did not increase further but it stabilized at this level (Fig. 5).

![Chemical hypoxia and washout](image)

**Figure 5.** Effect of chemical hypoxia on $[\text{Na}^+]_i$ in MDCK cells. Inhibition of both oxidative phosphorylation and glycolysis produced a steady increase in $[\text{Na}^+]_i$ (■). The rise in $[\text{Na}^+]_i$ was reversible on washout (with pyruvate added as a mitochondrial substrate). With an active glycolysis (5.5 mM glucose), CN$^-$ produced a rise in $[\text{Na}^+]_i$ only during the first 20-25 minutes of the treatment (□). The data are the mean ± S.E.M. from 6 separate experiments.

The increase in $[\text{Na}^+]_i$ affects the driving force controlling the activity and the mode of the $\text{Na}^+/$Ca$^{2+}$ antiporter and leads to cellular Ca$^{2+}$ load. To evaluate the contribution of the exchanger to the increase in $[\text{Ca}^{2+}]_i$, the effect of metabolic inhibition was assessed in the absence of external $\text{Na}^+$. The changes in $[\text{Ca}^{2+}]_i$ were markedly reduced (Fig. 6), consistent with the hypothesis that Ca$^{2+}$ entry during chemical hypoxia occurs in exchange for $\text{Na}^+$ via $\text{Na}^+/$Ca$^{2+}$ exchanger.
Mechanisms responsible for the $\text{Ca}^{2+}$ clearance

We focused next on the sources of $\text{Ca}^{2+}$ clearance observed after the first 30 - 35 minutes of chemical hypoxia, in the continuous presence of the metabolic inhibitors. $\text{Ca}^{2+}$ ions can be extruded from cells by the plasma membrane $\text{Ca}^{2+}$-ATPase. This pump is inhibited by o-vanadate, consequently the effect of metabolic inhibition on $[\text{Ca}^{2+}]_i$ was evaluated in the presence of 1 mM o-vanadate. The maximum level reached by intracellular $\text{Ca}^{2+}$ was somewhat higher than in control conditions (435 $\pm$ 100 nM, n=6), but not significantly different (P>0.05). However, o-vanadate did not affect the transient behaviour of $[\text{Ca}^{2+}]_i$ (Fig. 7).

Figure 6. Effect of chemical hypoxia on $[\text{Ca}^{2+}]_i$ in MDCK cells in the absence of external Na$. Omission of Na$ from the bathing solution resulted in a significantly smaller effect of CN$ and DOG on $[\text{Ca}^{2+}]_i$. The presence of the metabolic inhibitors in the external solution is indicated by the solid bar. The data are the mean $\pm$ S.E.M. from 6 separate experiments.

Figure 7. Effect of chemical hypoxia on $[\text{Ca}^{2+}]_i$ in the presence of o-vanadate. Inhibition of the plasma membrane $\text{Ca}^{2+}$ pump with 1 mM o-vanadate did not ablate the decay phase of $[\text{Ca}^{2+}]_i$ following the initial increase during chemical hypoxia (indicated by the solid bar). Shown is the mean $\pm$ S.E.M. from 5 separate experiments.
Besides being extruded from the cells, Ca\(^{2+}\) ions can also be taken up into intracellular Ca\(^{2+}\) stores. Currently, two main intracellular Ca\(^{2+}\) stores are recognized: the ER and the mitochondria. A third store that can be depleted by the Ca\(^{2+}\) ionophores ionomycin or A23187 was found in several mammalian cell lines (Pizzo et al., 1997). Ca\(^{2+}\) accumulation into ER occurs through the SERCA pumps. The involvement of ER in the buffering of [Ca\(^{2+}\)]\(_i\) during chemical hypoxia was investigated by pre-treating the cells with thapsigargin, a specific inhibitor of SERCA pumps. A typical experiment is shown in Fig. 8. Thapsigargin (1 µM) produced a fast increase in [Ca\(^{2+}\)]\(_i\), followed by a decay and a plateau at a higher level than resting [Ca\(^{2+}\)]\(_i\). In the presence of thapsigargin, CN\(^-\) and DOG produced a significantly (P<0.05) bigger increase in [Ca\(^{2+}\)]\(_i\) (mean increase to 660 ± 130 nM, n=5). Nevertheless, the cells were still able to reduce [Ca\(^{2+}\)]\(_i\) after the initial increase, in the continuous presence of CN\(^-\) and DOG. Ionomycin is a Ca\(^{2+}\) ionophore which releases Ca\(^{2+}\) from an internal store not clearly identified (Pizzo et al., 1997). When applied during the recovery phase, in the absence of external Ca\(^{2+}\) (to avoid Ca\(^{2+}\) influx), ionomycin (10 µM) induced a spike of Ca\(^{2+}\) corresponding to Ca\(^{2+}\) release from these unknown stores. Thus, Ca\(^{2+}\) accumulation into ionomycin-releasable stores could contribute to the decrease of [Ca\(^{2+}\)]\(_i\) during metabolic inhibition.

![Figure 8](image_url)

**Figure 8.** Representative trace showing the effect of chemical hypoxia on [Ca\(^{2+}\)]\(_i\) in the presence of thapsigargin. MDCK cells were treated with 1 µM thapsigargin (open bar). After [Ca\(^{2+}\)]\(_i\) reached a plateau, the metabolic inhibitors were applied, also in the presence of thapsigargin (solid bar). During the declining phase of [Ca\(^{2+}\)]\(_i\), the bathing solution was exchanged with a Ca\(^{2+}\) free solution containing 10 µM ionomycin (dashed bar).

The next clearance mechanism investigated was the buffering of cytoplasmic Ca\(^{2+}\) by mitochondria. Ca\(^{2+}\) accumulation into mitochondria occurs through the Ca\(^{2+}\) uniporter and is driven by the mitochondrial membrane potential. We have shown in Chapter V that ΔΨ is partially preserved in MDCK cells subjected to chemical hypoxia by the mitochondrial ATP synthase which operates in the reverse mode. Consequently,
mitochondria could be still able to buffer cytoplasmic Ca\(^{2+}\). Inhibition of mitochondrial ATP synthase with 20 \(\mu\)g ml\(^{-1}\) oligomycin during the application of metabolic inhibitors determined a more profound depolarization of mitochondria while the rate of ATP consumption was reduced. Thus, mitochondrial Ca\(^{2+}\) accumulation in cells exposed to chemical hypoxia is expected to be prevented by oligomycin. When oligomycin (20 \(\mu\)g ml\(^{-1}\)) was used in combination with CN\(^-\) and DOG, [Ca\(^{2+}\)]\(_i\) raised to 211 ± 19 nM (n=5) and this increase was not followed by a recovery phase (Fig. 9). Oligomycin was added to the bath solution 10 minutes before the metabolic inhibitors and did not produce any change in [Ca\(^{2+}\)]\(_i\).

![Graph showing changes in [Ca\(^{2+}\)]\(_i\) during chemical hypoxia in the presence of oligomycin.](image)

**Figure 9.** Changes in [Ca\(^{2+}\)]\(_i\) during chemical hypoxia in the presence of oligomycin. Inhibition of the mitochondrial ATP synthase with 20 \(\mu\)g ml\(^{-1}\) oligomycin prevented the decline in [Ca\(^{2+}\)]\(_i\) after the initial increase during chemical hypoxia. The presence of CN\(^-\) and DOG is indicated by the solid bar. Shown is the mean ± S.E.M. from 5 separate experiments.

**DISCUSSION**

This study investigates the changes in [Ca\(^{2+}\)]\(_i\) during inhibition of both oxidative phosphorylation and glycolysis, as a model of renal ischaemia, in MDCK cells. These cultured cells derived from dog kidney express many similarities with the mammalian cortical collecting tubules (Valentic, 1981) and are known to be particularly resistant to ATP depletion (Sheridan et al, 1993; Wiegele et al, 1998; Feldenberg et al, 1999).

The results show a transient increase in [Ca\(^{2+}\)]\(_i\) in response to chemical hypoxia (2.5 mM CN\(^-\) and 10 mM DOG). [Ca\(^{2+}\)]\(_i\) rises slowly during the first 30 - 35 minutes from 68±8 nM to 283±32 nM, then declines (in the presence of the metabolic inhibitors) to 138±21 nM in about 40 minutes. The fluorescence intensity excited at 380 nm increases during the decline phase of [Ca\(^{2+}\)]\(_i\). This rules out the possibility that the decrease in [Ca\(^{2+}\)]\(_i\) is an artefact produced by the leakage of Fura-2 from cells due to membrane damage. It is also unlikely that the pH-dependence of the dissociation
constant of Fura-2 for Ca\(^{2+}\) affects significantly [Ca\(^{2+}\)]\(_i\) measurements as the time course of changes in pH\(_i\) (see Chapter V) and [Ca\(^{2+}\)]\(_i\) are different.

The published results regarding [Ca\(^{2+}\)]\(_i\) in renal cells during hypoxia/ischemia differ markedly. An increase in [Ca\(^{2+}\)]\(_i\) to more than 100 \(\mu\)M was observed in isolated rabbit proximal tubules treated for 30-60 minutes with antimycin in the presence of glycine (to prevent lytic plasma membrane damage) (Weinberg et al., 1997). Other studies revealed only modest changes in [Ca\(^{2+}\)]\(_i\) (Snowdowne et al., 1985; Rose et al., 1993; Rose et al., 1994a, b; Peters et al., 1996) or even no changes at all (Jacobs et al., 1991). These differences could be due to distinct sensitivities to hypoxia/ischemia manifested by separate segments of the nephron or to the different sources of the cells. Peters et al. (1996) have shown that the level of oxygen deprivation is critical in determining changes in [Ca\(^{2+}\)]\(_i\) during hypoxia. A large increase in [Ca\(^{2+}\)]\(_i\) was generally found immediately before cell death. In surviving cells, the rise in [Ca\(^{2+}\)]\(_i\) was modest, considering the large electrochemical gradient favouring Ca\(^{2+}\) influx. For example, [Ca\(^{2+}\)]\(_i\) increased about 6 times in primary cultures of rabbit TAL in response to substrate free anoxia (Rose et al., 1994a). The maximal increase in [Ca\(^{2+}\)]\(_i\) occurred within 60 minutes and was not accompanied by cell death.

It has been reported (McCoy et al., 1988) that application of 5 mM CN\(^{-}\) and 5 mM DOG to a confluent monolayer of MDCK cells results in a 6 fold increase in [Ca\(^{2+}\)]\(_i\) in 15 minutes (the maximum time interval investigated). The smaller effect observed in this study might be due to the different temperature used for experiments: room temperature in our case and 37°C in the experiments of McCoy et al. (1988).

The only previous study that reported a decline in [Ca\(^{2+}\)]\(_i\) after an initial increase in renal cells exposed to ischemic conditions was performed on rat proximal tubular cells in primary cultures (Chi et al., 1995). Chi et al. (1995) found that in \(\approx\) 25% of cells exposed to glucose free anoxia [Ca\(^{2+}\)]\(_i\) peaked to > 1 \(\mu\)M and then dropped rapidly, stabilising near 500 nM in about 10 minutes. The partial recovery of [Ca\(^{2+}\)]\(_i\) was associated with an extended period of survival. The authors suggested that Ca\(^{2+}\) buffering may be possible because ATP and \(\Delta\Psi\) are maintained at sufficiently high levels.

In the present study, the sources of both the increase and the subsequent decrease of [Ca\(^{2+}\)]\(_i\) were investigated. The increase in [Ca\(^{2+}\)]\(_i\) is due to Ca\(^{2+}\) influx across the plasma membrane, since removal of external Ca\(^{2+}\) or inhibition of Ca\(^{2+}\) influx with La\(^{3+}\) prevented the effect. Similar results were obtained in proximal tubular cells (Smith et al., 1992; Rose et al., 1993; Rose et al., 1994b) but not in TAL, where both Ca\(^{2+}\) influx and Ca\(^{2+}\) release from internal stores seem to contribute to anoxia-induced rise in [Ca\(^{2+}\)]\(_i\) (Rose et al., 1994a). Verapamil partially reduces the increase in [Ca\(^{2+}\)]\(_i\), suggesting a role for Ca\(^{2+}\) influx through Ca\(^{2+}\) channels. This result agrees with the reported finding that in TAL methoxyverapamil was effective in reducing the increase in [Ca\(^{2+}\)]\(_i\) during anoxia. Evidence for the presence of L-type Ca\(^{2+}\) channels in the distal segments of the nephron originates from previous studies in cultures of distal tubular cells (Bacskaï & Friedman, 1990).
In MDCK cells, $[\text{Ca}^{2+}]_i$ is related to the transmembrane $\text{Na}^+$ gradient via the $\text{Na}^+/$$\text{Ca}^{2+}$ exchanger (Borle & Bender, 1991). Generally, the exchanger couples the passive movement of $\text{Na}^+$ to the extrusion of $\text{Ca}^{2+}$ from the cell. However, the $\text{Na}^+/$$\text{Ca}^{2+}$ exchanger was found to operate in the reverse mode (Ca$^{2+}$ enters the cells in exchange for Na$^+$) in conditions of ATP depletion in several cell types, including cardiac myocytes (Haigney et al., 1992), neurons (Schroder et al., 1999) and human epithelial cells (Kiang & Smallridge, 1994). A similar mechanism seems to operate in MDCK cells exposed to chemical hypoxia, because no significant change in $[\text{Ca}^{2+}]_i$ was produced in the absence of extracellular Na$^+$. It is not surprising that Ca$^{2+}$ ions enter the cells via the exchanger after ATP depletion. The direction of Ca$^{2+}$ movement by Na$^+/$Ca$^{2+}$ exchanger is dictated by several variables including membrane potential and intracellular and extracellular concentration of both Na$^+$ and Ca$^{2+}$. Both membrane depolarization and an increase in $[\text{Na}^+]_i$ are expected to occur during ischemia and both these factors favour exchanger mediated cell Ca$^{2+}$ entry rather than exit. We found that $[\text{Na}^+]_i$ steadily increases 4-5 times during 60 minutes of chemical hypoxia (CN$^-$ and DOG). It has been reported (Mason et al., 1981) that $[\text{Na}^+]_i$ is only little affected in distal tubules from kidneys subjected to 20 minutes of ischemia, while a considerable increase in $[\text{Na}^+]_i$ occurred after 60 minutes. This time course was different from that observed in proximal tubules, where $[\text{Na}^+]_i$ raised considerably after 20 minutes of ischemia (Mason et al., 1981). $[\text{Na}^+]_i$ doubled following a 5 minutes exposure of TAL cells to CN$^-$ (Kondo et al., 1993).

The SBFI ratio is somewhat sensitive to pH. It has been reported that a decrease in pH of 0.4 pH units mimicks an apparent decrease in $[\text{Na}^+]_i$ of 3-4 mM in parietal cells (Negulescu & Machen, 1990) and neurons (Rose & Ransom, 1997). We have shown in Chapter V that chemical hypoxia produces an intracellular acidification of 0.6 pH units in MDCK cells. This will result in an underestimation of $[\text{Na}^+]_i$ by about 5-6 mM during metabolic inhibition.

To elucidate the sources of $[\text{Ca}^{2+}]_i$ decay after the initial increase, several buffering mechanisms were investigated: Ca$^{2+}$ extrusion through the plasma membrane Ca$^{2+}$ pump, the active Ca$^{2+}$ uptake into the endoplasmic reticulum, via SERCA pumps, and Ca$^{2+}$ accumulation into mitochondria. Inhibition of the plasma membrane Ca$^{2+}$ pump with o-vanadate and of SERCA pumps with thapsigargin did not prevent the decay phase of $[\text{Ca}^{2+}]_i$. In both cases, the peak value of $[\text{Ca}^{2+}]_i$ was higher than in control conditions. This suggests that these ATPases could be still active, although operating at a lower rate, in the presence of the metabolic inhibitors. Another possible source of the greater increase in $[\text{Ca}^{2+}]_i$ in the presence of thapsigargin consists in the activation of SOC channels in the plasma membrane due to depletion of the endoplasmic reticulum, so that the cells have to deal with a higher Ca$^{2+}$ influx. The presence of SOC channels in MDCK cells has been well documented (Dietl et al., 1996; Gordjani et al., 1997). We found that ionomycin is able to release Ca$^{2+}$ during the decay phase of $[\text{Ca}^{2+}]_i$ in cells exposed to chemical hypoxia in the presence of thapsigargin. The identity of ionomycin-releasable Ca$^{2+}$ stores is not clear. Pizzo et al. (1997) suggested that ionomycin and A23187 release Ca$^{2+}$ from an internal store different from ER and mitochondria. However, the ionomycin - releasable Ca$^{2+}$ pool in Jurkat T cells was
[Ca\textsuperscript{2+}] and chemical hypoxia in MDCK cells

identified as mitochondria (Hoth et al., 1997). Nevertheless, the results with
ionomycin confirm that the decrease in [Ca\textsuperscript{2+}] is due to sequestration of Ca\textsuperscript{2+} into
organelles different from ER.

Mitochondria are generally known to be a safety device: they can accumulate great
amounts of Ca\textsuperscript{2+} and protect cells from a toxic Ca\textsuperscript{2+} overload under pathophysiological
conditions (Gunter et al., 1994). Ca\textsuperscript{2+} accumulation occurs through the Ca\textsuperscript{2+} uniporter
and it is driven by the mitochondrial membrane potential. When oxidative
phosphorylation is inhibited by cyanide, the cells could still maintain ΔΨ at the
expense of ATP reserves. This mechanism involves the reversal of the mitochondrial
ATP synthase that will now act as a proton translocating ATPase (Duchen & Bisceo,
1992; Di Lisa et al., 1995; Leyssens et al., 1996). We showed in Chapter V that such a
mechanism is effective in MDCK cells exposed to chemical hypoxia: ΔΨ decreases
but it is not completely dissipated during a 60 minutes metabolic inhibition with CN-
and DOG. Moreover, ΔΨ even seems to recover after the first =25 minutes in the
continuous presence of the metabolic inhibitors. In these conditions, the capacity of
mitochondria to buffer cytoplasmic Ca\textsuperscript{2+} might be, at least partially, preserved during
chemical hypoxia. ΔΨ was found to collapse if chemical hypoxia is realized in the
presence of oligomycin, an inhibitor of mitochondrial ATP synthase (see Chapter V).
In this study, we found that oligomycin also prevents the decay phase of [Ca\textsuperscript{2+}], thus
suggesting an important role of the mitochondria in buffering [Ca\textsuperscript{2+}] in conditions of
ATP depletion in MDCK cells.

It has been shown that mitochondria have the capacity to sequester Ca\textsuperscript{2+} at
physiological [Ca\textsuperscript{2+}], probably by sensing microdomains of high [Ca\textsuperscript{2+}], close to the
sources such as IP\textsubscript{3} receptors in the ER or Ca\textsuperscript{2+} channels in the plasma membrane
(Rizzuto et al., 1993; Hajnoczky et al., 1995; Lawrie et al., 1996). The spatial
resolution of our imaging system does not allow to detect such possible microdomains
of high [Ca\textsuperscript{2+}].

It has been recently reported (Jan et al., 1999) that mitochondria do not play a
significant role in the decay phase of [Ca\textsuperscript{2+}], in thapsigargin - treated MDCK cells.
Two causes might explain the opposite result found here: first, Jan et al. (1999)
investigated MDCK cells in suspension, while we studied confluent monolayers of
MDCK cells. Second, and more important, in our hands the peak value of the Ca\textsuperscript{2+}
spike produced by 1 μM thapsigargin is much lower than the maximum level of [Ca\textsuperscript{2+}]
during chemical hypoxia. It is known that Ca\textsuperscript{2+} influx into the mitochondria via Ca\textsuperscript{2+}
uniporter depends on [Ca\textsuperscript{2+}] in a non-linear manner, with a Hill coefficient of =2
(Gunter et al., 1994). Also, while other buffering mechanisms (e.g., Ca\textsuperscript{2+} pumps)
depend directly on ATP, Ca\textsuperscript{2+} accumulation into mitochondria is a secondary active
process depending only indirectly, via ΔΨ, on ATP availability. This fact favours the
buffering of cytoplasmic Ca\textsuperscript{2+} by mitochondria in conditions of ATP depletion if ΔΨ
is not dissipated. Ca\textsuperscript{2+} accumulation into mitochondria can be prevented by
mitochondrial uncouplers, such as FCCP, which collapse ΔΨ. However, FCCP was
not used in this study first because it induces itself a profound ATP depletion (see
Chapter V) and second because it absorbs light at the wavelengths used with Fura-2.
We could not use the well known inhibitor of Ca\(^{2+}\) uniporter, Ruthenium Red, because it has to be injected into the cells.

Inhibition of oxidative phosphorylation in the presence of glucose did not produce any effect on [Ca\(^{2+}\)]\(_i\). This result agrees with our observation that glycolysis is able to maintain a normal ATP level in MDCK cells treated with CN\(^-\) (see Chapter V). It was previously reported that anoxia - induced increases in [Ca\(^{2+}\)]\(_i\) in TAL were only apparent in the absence of glucose and acetate (Rose et al., 1994a). Similar behaviour has been noticed in proximal tubule cells in primary culture (Rose et al., 1993). Despite the lack of effect on the cellular ATP content, the application of CN\(^-\) in the presence of glucose induced an increase in [Na\(^+\)]. It has been suggested that mitochondria preferentially provide ATP to Na\(^+\)/K\(^+\) pumps in A6 cells, a renal cell line with distal tubular origin (Guerrero et al., 1997). It is possible that in MDCK cells the ATP produced by mitochondria is also mainly used by the Na\(^+\)/K\(^+\) pumps.

In summary, chemical hypoxia (CN\(^-\) and DOG) produces an increase in [Ca\(^{2+}\)]\(_i\) in MDCK cells during the first 30-35 minutes. This initial increase is followed by a decay phase to levels close to control in the continuous presence of metabolic inhibitors. The rise in [Ca\(^{2+}\)]\(_i\) is due to Ca\(^{2+}\) influx, mainly via the Na\(^+\)/Ca\(^{2+}\) exchanger which operates in the reverse mode. Part of Ca\(^{2+}\) influx occurs through a verapamil sensitive pathway. The decay phase is due to Ca\(^{2+}\) sequestration into internal organelles, most likely the mitochondria. Whether or not these changes in [Ca\(^{2+}\)]\(_i\) are an early event in hypoxia mediated disturbances which lead eventually to cell injury remains an open question.
CHAPTER VII

GENERAL DISCUSSION
AND FUTURE PROSPECTS
Behavior of Na⁺-sensitive indicators in cells

We found (Chapter III) that the frequency domain data of Sodium Green are significantly less sensitive to [Na⁺] in HeLa cells as compared with simple buffer solutions, with and without bovine serum albumin. Nonetheless, it is still possible to perform an in vivo calibration of [Na⁺]. The resting [Na⁺], derived from lifetime measurements of Sodium Green compares well with the value obtained from the steady-state ratio of SBFI and with the values reported by Amorino & Fox (1995) and Zahler et al. (1997). Furthermore, it is possible to follow the time course of the increase in [Na⁺], upon inhibition of the Na⁺/K⁺ pump by monitoring only the phase angle at a single modulation frequency (160 MHz). This suggests that Sodium Green can be used with fluorescence lifetime imaging microscopy (FLIM) and lifetime-based flow cytometry (Pinsky et al., 1993; Yu et al., 1998) to detect physiological changes in [Na⁺]. The use of lifetime measurements, which are independent of the total concentration of the indicator (Szmacinski & Lakowicz, 1994), would offer a real advantage because Sodium Green is a non-ratiometric probe. Steady-state measurements with Sodium Green have therefore all the disadvantages resulting from a signal dependent on the concentration of the indicator in cells: fluorescence intensity is affected by leakage of the dye from cells, photobleaching and changes in the cell volume. Furthermore, in our hands the intensity measurements of Sodium Green in HeLa cells proved to be unreliable (see also Haugland & Johnson, 1999). Although [Na⁺], can be reliably and easily determined using ratiometric measurements with SBFI, there are circumstances where SBFI cannot be used. For example, SBFI cannot be used to investigate the effect of amiloride, an inhibitor of epithelial Na⁺ channels, because amiloride is excited and emits fluorescence at the same wavelengths as SBFI. Sodium Green is also useful in applications where [Na⁺], has to be measured simultaneously with another ion, because it can be used in combination with an indicator excited in UV (e.g., Fura-2).

We show (Chapter IV) that the different behavior of SBFI in HeLa cells as compared to buffer solutions cannot be rationalized in terms of viscosity effects, as initially suggested for Jurkat cells (Harootunian et al., 1989), but rather by the binding of SBFI to intracellular proteins or organelles, as proposed by Baartscheer et al. (1997). In contrast to previous studies, which investigated only the spectral characteristics of SBFI, our conclusion is based on both steady-state and time-resolved data. Mainly the free form of the indicator binds to proteins, similar to the case of Ca²⁺ sensitive probes Quin-2 (Hirshfield et al., 1996) and Indo-1 (Bancel et al., 1992b). Our time-resolved data suggest that in HeLa cells SBFI is bound either to Na⁺ or to intracellular components, so that there is essentially no free dye in the ground-state. However, this species is generated in the excited state, mainly by the dissociation of SBFI-proteins complex. In these conditions, the observed steady-state fluorescence intensities of SBFI in cells merely reflect the fractions of the SBFI bound to intracellular components and of the Na⁺-SBFI complex. Binding to intracellular components affects the intensity decay of SBFI to a greater extent than it affects the Sodium Green decay (investigated in Chapter III). The fluorescence relaxation times and the normalized pre-exponential factors of the decay of SBFI in HeLa cells are essentially
independent of intracellular \([\text{Na}^+]\). This means that SBFI cannot be used, at the excitation (363 nm) and emission (535 nm) wavelength investigated here, for fluorescence lifetime imaging techniques applied to cells. It has been suggested by Srivastava & Krishnamoorthy (1997) that fluorescence relaxation time measurements can be used to discriminate the fraction of the indicator bound to intracellular components. Our results show that this is not possible for SBFI. Although in practice SBFI has to be used under steady-state conditions, time-resolved measurements provide information about the behavior of the dye in cells.

Effect of chemical hypoxia on cellular energetics and intracellular ion homeostasis in MDCK cells

The results obtained in Chapters V and VI suggest the following sequence of events initiated by chemical hypoxia (10 mM DOG and 2.5 mM CN\(^-\)) in MDCK cells: NAD(P)H/NAD(P)\(^+\) ratio increases immediately (in less than three minutes), showing a shift towards a more reduced cellular redox state. Cellular ATP content drops to less than 10% from control in about 20 minutes. There are two direct consequences of ATP hydrolysis: an intracellular acidification (= 0.6 pH units) and an increase in [Mg\(^2+\)]. ATP depletion also reduces the activity of the Na\(^+/K^+\) pump and leads to accumulation of intracellular Na\(^+\). The rise in [Na\(^+\)], results in the reversal of the Na\(^+/Ca^{2+}\) exchanger from the plasma membrane, which now extrudes Na\(^+\) in exchange for Ca\(^{2+}\), consequently [Ca\(^{2+}\)] increases. In the same time, inhibition of the electron transport chain depolarizes the inner membrane of the mitochondria. However, the mitochondrial membrane potential is partially preserved by the mitochondrial ATP synthase, which operates in the reverse mode: it hydrolyses ATP and this process is coupled to the extrusion of H\(^+\) from the matrix. While accelerating ATP depletion, this mechanism enables mitochondria to buffer cytoplasmic Ca\(^{2+}\), so that the initial increase in [Ca\(^{2+}\)] is followed by a recovery to levels close to the resting [Ca\(^{2+}\)]. Many of the mechanisms found here for MDCK cells have also been reported in heart cells (Haigney et al., 1992; Di Lisa et al., 1995; Leyssens et al., 1996). This similarity is rather surprising considering the major differences between these two cellular models: cardiac myocytes are excitable cells and their main ATP source is the oxidative phosphorylation. Also, Ca\(^{2+}\) homeostasis is handled differently in heart cells and MDCK cells.

Metabolic inhibition with CN\(^-\) and DOG has become a standard method to simulate ischemia for \textit{in vitro} models. CN\(^-\) has the advantage of being readily reversible on washout (as shown by the immediate decrease in the NAD(P)H/NAD(P)\(^+\) ratio) but is a rather non-specific inhibitor of the electron transport chain. It has been shown (Yang et al., 1996; Sun et al., 1997) that at very low concentrations (10-100 \(\mu\text{M}\), CN\(^-\) interferes with signal transduction pathways by interacting directly with some receptors. For example, CN\(^-\) interacts with the N-methyl-D-aspartate receptor channel complex in cerebellar granule cells to enhance receptor-mediated responses (Sun et al., 1997). CN\(^-\) was also found to reduce K\(^+\) currents in the type I cells of the carotid body (Peers & O'Donnell, 1990) and this reduction occurred regardless of the presence of
intracellular ATP. However, in our case CN\(^-\) applied in the presence of glucose had no significant effect on the cellular ATP content and neither did it change [Ca\(^{2+}\)], or [Mg\(^{2+}\)]. The only direct (not mediated by the inhibition of oxidative phosphorylation) effect of CN\(^-\) that might affect the changes in the parameters investigated here is its capacity to inhibit the Na\(^+\)-independent Ca\(^{2+}\) efflux from mitochondria (Gunter et al., 1994). As this is the dominant mechanism of Ca\(^{2+}\) extrusion from the mitochondrial matrix in renal cells (Gunter et al., 1994) the inhibition of Na\(^+\)-independent Ca\(^{2+}\) efflux will favor Ca\(^{2+}\) accumulation into mitochondria, thus contributing to the return of cytoplasmic Ca\(^{2+}\) concentration towards its resting level after the initial increase.

Whether or not some of the changes found in this study are early events in hypoxia mediated disturbances, which lead eventually to irreversible cell injury, or whether, on the contrary, they have a role in cell survival remains an open question. It has been reported (Carini et al., 1997) that an uncontrolled increase in [Na\(^+\)] is critical for the development of cell death in hepatocytes exposed to hypoxia or to metabolic inhibition and the protective effect of glycine is associated with the prevention of Na\(^+\) accumulation. However, a 4-5 fold increase in [Na\(^+\)] is not lethal for MDCK cells, suggesting that Na\(^+\) accumulation \textit{per se} does not lead to cell death. Intracellular acidification has been shown to have a protective effect during ATP depletion in several organ systems, including the kidney (Weinberg, 1991; Weinberg et al., 1991; Edelstein et al., 1996). Consequently, one may speculate that the rapid and profound intracellular acidification observed here could, at least partially, explain the high resistance of MDCK cells to energy deprivation (Sheridan et al., 1993; Lieberthal et al., 1997). We think this hypothesis is worth investigating further.

Another problem that deserves further investigation is the role of mitochondria in deciding the survival or death of the injured cells. Our data suggest that mitochondria act as "sinks" for cytoplasmic Ca\(^{2+}\) during chemical hypoxia. On short term, this might protect the cells by preventing a potentially damaging increase in [Ca\(^{2+}\)]. However, a sustained accumulation of Ca\(^{2+}\) into mitochondria induces mitochondrial permeability transition (Lemasters et al., 1997), which is generally associated with irreversible cell injury. Future investigations should be aimed at elucidating (1) the causal relationships between ischemia-induced changes in [Ca\(^{2+}\)], Ca\(^{2+}\) concentration in the mitochondrial matrix, mitochondrial membrane potential and mitochondrial permeability transition and (2) whether any of these events is a primary switch marking a point of no return in cell injury.

A last point that must be discussed here is the significance of the \textit{in vitro} results for the \textit{in vivo} situation. The experiments shown in Chapters V and VI were designed to investigate the cellular effects of energy deprivation as they occur in the non-lethally injured renal cells exposed to hypoxia/ischemia. MDCK cells show a high glycolytic capacity (application of CN\(^-\) in the presence of glucose did not affect the cellular ATP content), which is characteristic for cells in culture. Furthermore, due to long term cultivation, the cells underwent a process of dedifferentiation so that they do not maintain all the structural and functional characteristics of the nephron segment of origin (which is actually unknown!). However, cultured cells offer the advantage of
having a high resistance to energy deprivation. Their long term (several hours) survival in ischemic conditions allows a detailed study of the cellular and subcellular events that occur and enables to identify the causal relationships between them and their possible involvement in cell death or survival. These aspects are more difficult to investigate in primary cultures or freshly isolated cells, because the survival period is confined to a significantly shorter time interval (generally, tens of minutes). Consequently, although the results obtained with MDCK cells have to be tested in conditions closer to the in vivo situation (in primary cultures and freshly isolated cells), they indicate the main mechanisms one should look for in other renal models.
General discussion and future prospects
SUMMARY

The chemical processes that comprise living organisms are the result of precise orchestration, in time and space, of ions, metabolites, macromolecules, the cytoskeleton and organelles. Changes in ion concentrations contribute to the regulation of numerous functions in epithelial cells, including the transepithelial transport, control of metabolism, mediation of signal transduction, cell proliferation and cell death. Various fluorescent techniques are used in this study to investigate ion concentrations in epithelial cells in different conditions.

This study has two main objectives. The first aim is to investigate the behavior of the Na⁺-sensitive fluorescent indicators Sodium Green and sodium binding benzofuran isophthalate (SBFI) in cells, by combining steady-state and time-resolved fluorescence measurements. The fluorescent properties of these probes in the cytoplasm are different from those in buffer solution (Harootunian et al., 1989; Negulescu & Machen, 1990; Borzak et al., 1992; Baartscheer et al., 1997; Haugland & Johnson, 1999), but the causes of these differences are not fully understood. With this study we also explored whether the lifetime of these probes can be used as a sensor for [Na⁺]. As lifetime-based sensing is mostly independent of the total concentration of the indicator (Szymacinski & Lakowicz, 1994), this would offer a real advantage especially in the case of Sodium Green, which is not a ratiometric indicator. The second objective of this thesis is to study the time course of changes in the intracellular ion concentrations in a renal model of ischemia (MDCK cells exposed to chemical hypoxia). Our interest focused on the non-lethal effects, since the regeneration of the kidney after an ischemic insult is based on the capacity of surviving cells to recover and to proliferate.

Chapter I
This chapter contains a general overview of the mechanisms involved in the regulation of intracellular ion concentrations in epithelial cells. The measurement of intracellular ions using fluorescent methods is also briefly reviewed.

Chapter II
The methods used throughout this study are described in detail in Chapter II. Special attention is devoted to fluorescence imaging using steady-state excitation and to time-resolved fluorescence measurements in the frequency domain. Two epithelial cell lines are used in this study: HeLa and MDCK. The conditions of their culturing are also presented in this chapter.

Chapter III
Because in buffer solution Sodium Green proved to be an excellent lifetime-sensitive Na⁺ probe (Szymacinski & Lakowicz, 1997a), Chapter III investigates the applicability of lifetime sensing for this indicator in HeLa cells. The fluorescence decays of Sodium Green in HeLa cells are compared with those recorded in different buffer solutions at various [Na⁺] with and without K⁺ ([Na⁺]+[K⁺]=145 mM) and bovine serum albumin
(BSA). In all cases, the intensity decay is multi-exponential, with decay times independent of [Na\(^+\)]. Three relaxation times are found in the various buffer solutions. For Sodium Green inside HeLa cells, the intensity decay can be approximated by a bi-exponential. The relative contribution of the long decay time (\(\tau_2=2.4 \pm 0.2 \text{ ns}\)) to the fluorescence decay is found to increase with the intracellular Na\(^+\) concentration ([Na\(^+\)]) at the expense of the contribution of the short component (\(\tau_1=0.4 \pm 0.1 \text{ ns}\)). These changes are significantly less pronounced in cells as compared with the corresponding changes observed in buffer solutions. Nevertheless, frequency domain data are still sensitive enough to allow an in vivo calibration. Similar values for the resting [Na\(^+\)], were estimated from lifetime measurements of Sodium Green (12±3 mM, n=5) and ratiometric measurements using SBFI (16±3 mM, n=6).

The analysis of the fluorescence intensity decay of Sodium Green provides useful information about the behavior of the indicator inside the cells. When only the determination of [Na\(^+\)], is important, a more practical approach is to monitor the phase angle at a single modulation frequency. We selected the modulation frequency of 160 MHz and monitored the phase angle of Sodium Green in cells exposed to a K\(^+\) free solution (to inhibit the Na\(^+\)/K\(^+\) pump). These measurements revealed that [Na\(^+\)], increases from 10 to 33 mM during a 40 minutes exposure to K\(^+\) free solution. Reversal of the transmembrane Na\(^+\) gradient (5 mM K\(^+\), Na\(^+\) free solution) results in a decrease of [Na\(^+\)], to values close to the control in about 30 minutes. Similar changes in [Na\(^+\)], were estimated by ratiometric measurements with SBFI. Our data suggest that, although its sensitivity is considerably reduced in cells as compared with buffer solutions, Sodium Green is a good candidate for fluorescence lifetime imaging microscopy (FLIM) in cells.

Chapter IV

Time-resolved fluorescence microscopy is used to investigate the behavior of the Na\(^+\) indicator SBFI in HeLa cells. The fluorescence relaxation of SBFI in HeLa cells can be described by a tri-exponential function for [Na\(^+\)], between 0 and 90 mM. The pre-exponential factor of the shortest decay time is negative. Changes in [Na\(^+\)], affect neither the fluorescence relaxation times (0.21 ± 0.05 ns, 0.60 ± 0.03 ns and 2.7 ± 0.1 ns) nor the average decay time (2.2 ± 0.1 ns). However, the ratio of the fluorescence signal due to excitation at 340 nm to that at 380 nm increases with [Na\(^+\)]. To elucidate the behavior of SBFI in cells experiments are performed on SBFI in buffer at various concentrations of Na\(^+\), K\(^+\) and BSA and at various viscosities. The fluorescence decay is tri-exponential only in the presence of BSA and bi-exponential in all other buffer solutions. The relaxation times are independent of [Na\(^+\)] and [BSA]. The pre-exponential factor of the shortest decay time is negative from a certain [BSA] on which depends on [Na\(^+\)]. The data indicate that interactions with intracellular components rather than microviscosity influence the SBFI behavior in cells. A model is suggested in which the fluorescence intensities are mainly determined by the signals from the Na\(^+\)SBFI and SBFI-proteins complexes.
Chapter V
This chapter investigates the relationship between the energy metabolism and the mitochondrial function in conditions of chemical hypoxia (cyanide and 2-deoxyglucose (DOG), applied for 60 minutes), followed by washout, in confluent monolayers of MDCK cells. This is considered an in vitro model of the reversibly injured cells from the distal tubules of ischemic kidney. The time-course of changes in the intracellular pH (pH_i), Mg^{2+} concentration ([Mg^{2+}]_i), mitochondrial membrane potential (ΔΨ) and cellular redox state was monitored by fluorescence imaging microscopy. Cellular ATP content was determined with a chemiluminescent assay in cell extracts.

Cellular ATP content drops to about 5% as compared with control in 20 minutes. ATP depletion is accompanied by a profound intracellular acidification (0.6 pH units), with a similar time course. A partial recovery in both parameters is noticed on washout. The full or partial recovery observed for the other parameters investigated in this chapter and in Chapter VI ([Mg^{2+}]_i, ΔΨ, cellular redox state, [Ca^{2+}]_i, and [Na^+]_i) suggest that the cells are not lethally injured and so does the fact that no sudden loss of fluorescent indicators from the cells was observed.

Chemical hypoxia results in an increase in [Mg^{2+}]_i, assessed by the increase in the Magnesium Green fluorescence. The rise in Magnesium Green signal is independent of the external Mg^{2+}, implying that the increase in [Mg^{2+}]_i has an intracellular source, most likely the release of Mg^{2+} from Mg-ATP. However, the changes in [Mg^{2+}]_i do not follow exactly the changes in the cellular ATP content, suggesting that part of the free Mg^{2+} resulted from ATP hydrolysis is buffered by other intracellular components.

Chemical hypoxia produces an immediate reduction in the cellular redox state, as assessed by an increase in NAD(P)H autofluorescence and a depolarization of the mitochondrial inner membrane. ΔΨ drops to 26±6 % from the control value during the first 25 minutes of chemical hypoxia. This decrease is followed by a partial recovery of ΔΨ (to 48±5 % from control) during the next 35 minutes, in the continuous presence of the metabolic inhibitors. No significant change in ΔΨ is noticed on washout. Inhibition of the mitochondrial ATP synthase with oligomycin during chemical hypoxia accelerates the mitochondrial depolarization. In the presence of oligomycin, ΔΨ decreases to 18±2 % (n=6) from the control level during the first 6 minutes of chemical hypoxia, compared to 57±10 % in the absence of oligomycin. With oligomycin, ΔΨ was significantly lower throughout the application of the metabolic inhibitors. In the same time, oligomycin slowed down the depletion of cellular ATP. These results suggest that ΔΨ is partially maintained during chemical hypoxia by the mitochondrial ATP synthase, which operates in the reverse mode.

Chapter VI
Fluorescence imaging microscopy was used to monitor the effects of chemical hypoxia on cytoplasmic [Ca^{2+}] and [Na^+] in confluent monolayers of MDCK cells. A steady 4-5 fold rise in [Na^+] occurs during exposure to chemical hypoxia for 60 minutes, from 15±3 mM to 69±7 mM (n=6). On washout, [Na^+] recovers partially (to 42±10 mM).
Summary

When cyanide is applied in the presence of glucose, [Na⁺], increased to a similar extent at the beginning of the experiment but it stabilized after 20-25 minutes to 39±8 mM.

[Ca²⁺]i increases ≈4 fold during the first 30-35 minutes of chemical hypoxia (from 68±8 nM to 283±32 nM, n=6). This rise is followed by a decrease, in the continuous presence of the metabolic inhibitors, to values close to the resting [Ca²⁺]i in about 40 minutes. No effect is produced by cyanide in the presence of glucose. The absence of Ca²⁺ from external solution or the addition of La³⁺ prevents the changes in [Ca²⁺]i. The effect of chemical hypoxia on [Ca²⁺]i is considerably reduced in the absence of external Na⁺ and partially inhibited by verapamil. Thus, Ca²⁺ increase is due to Ca²⁺ entry, mainly via the Na⁺/Ca²⁺ exchanger, which operates in the reverse mode. Part of Ca²⁺ influx occurs through Ca²⁺ channels.

Inhibition of the plasma membrane Ca²⁺ pumps with o-vanadate or of SERCA pumps with thapsigargin did not prevent the decay phase of cytoplasmic Ca²⁺ and [Ca²⁺]i peaked at a higher level. Application of ionomycin (in Ca²⁺ free solution) during the recovery phase produced a transient in [Ca²⁺]i, suggesting that Ca²⁺ is accumulated in some internal organelles. We showed in Chapter V that ΔΨ decreases, but it is not dissipated, during chemical hypoxia, so that Ca²⁺ could be accumulated into mitochondria via a Ca²⁺ uniporter. Oligomycin, which in combination with cyanide dissipates the mitochondrial membrane potential, prevented the decline in [Ca²⁺]i. This indicates mitochondria as the organelles likely to buffer cytoplasmic Ca²⁺ in conditions of metabolic stress.
SAMENVATTING

De chemische processen in levende organismen zijn het resultaat van een nauwkeurig samenspel in ruimte en tijd tussen ionen, metabolieten, macromoleculen, cytoskelet en organellen. Veranderingen in ionenconcentraties dragen bij tot de regeling van talrijke functies in epitheelcellen waaronder epitheliaal transport, controle van het metabolisme, doorgeven en omvormen van signalen, celproliferatie en celdood. Meerdere fluorescentietechieken worden in deze studie aangewend om de ionenconcentraties in epitheelcellen te onderzoeken in verschillende omstandigheden.

Deze studie heeft twee hoofddoelstellingen. De eerste betreft het onderzoek van het gedrag van de fluorescerende Na⁺-gevoelige indicatoren Sodium Green en sodium binding benzofuran isopthalate (SBFI). Hierdie worden stationaire en tijdsgeresolveerde fluorescentiemetingen aangewend. De fluorescentiekarakteristieken van de indicatoren in cytoplasma zijn verschillend van deze in bufferoplossing (Harootunian et al., 1989; Negulescu & Machen, 1990; Borzak et al., 1992; Baartscheer et al., 1997; Haughland & Johnson, 1999). De oorzaken van deze verschillen zijn echter niet volledig gekend. In deze studie wordt ook nagegaan of de meting van de fluorescentieleeftijden van de indicatoren Sodium Green en SBFI kan aangewend worden om de intracellulaire natriumconcentratie, [Na⁺], te bepalen. Het voordeel van deze benadering ligt in het feit dat de fluorescentieleeftijd praktisch onafhankelijk is van de concentratie van de indicator (Szmacinski & Lakowicz, 1994). Dit is belangrijk voor de indicator Sodium Green die niet op een ratiometrische wijze kan worden gebruikt.

De tweede hoofddoelstelling van deze thesis betreft het onderzoek van het tijdverloop van de veranderingen in ionenconcentraties in een model voor niercellen onder ischemie (MDCK cellen onderworpen aan chemisch geïnduceerde hypoxie). Hierbij gaat de aandacht naar subietale effecten omdat het regenereren van de nier na een ischemie berust op het vermogen van de overlevende cellen om te herstellen en zich te vermenigvuldigen.

Hoofdstuk I

In dit hoofdstuk wordt een algemeen overzicht gegeven van de regelmechanismen van intracellulaire ionenconcentraties in epitheelcellen. Tevens worden beknop de fluorimetriscbe methoden ter bepaling van de intracellulaire ionenconcentraties besproken.

Hoofdstuk II

De experimentele methoden die in deze studie zijn gebruikt worden in detail besproken in hoofdstuk II. Hierbij wordt bijzondere aandacht besteed aan de beeldvorming met behulp van fluorescentie onder stationaire excitatie en aan de tijdsopgeloste fluorescentiebepalingen in het frequentiedomein. Twee epitheelcellijnen zijn gebruikt in deze studie: HeLa en MDCK. De condities voor deze cellculturen zijn beschreven in dit hoofdstuk.
Summary

Hoofdstuk III
In bufferoplossingen kan de fluorescentieleeftijd van Sodium Green gebruikt worden als een uitstekende indicator voor $[\text{Na}^+]$ (Smacinski & Lakowicz, 1997a). In hoofdstuk III wordt nagegaan of de fluorescentieleeftijd kan gebruikt worden als $[\text{Na}^+]$ indicator in HeLa cellen. Het fluorescentieverval van Sodium Green in HeLa cellen wordt vergeleken met de vervallen in buffer bij verschillende $[\text{Na}^+]$ met en zonder $\text{K}^+$ (waarbij $[\text{Na}^+] + [\text{K}^+] = 145$ mM) en met en zonder runder serum albumine (BSA). Voor alle onderzochte gevallen kan het fluorescentieverval beschreven worden met een som van exponentieel dalende functies met relaxatietijden die onafhankelijk zijn van $[\text{Na}^+]$. Drie relaxatietijden zijn vereist voor het fluorescentieverval in de verschillende bufferoplossingen. Twee relaxatietijden volstaan om de fluorescentierelaxatie van Sodium Green in HeLa cellen te beschrijven: $\tau_1=0.4\pm0.1$ ns, $\tau_2=2.4\pm0.2$ ns. De relatieve bijdrage van de langere relaxatietijd neemt toe met stijgende $[\text{Na}^+]$. Alhoewel de veranderingen van de relatieve bijdragen significant minder uitgesproken zijn in cellen dan in bufferoplossingen zijn de metingen van de fluorescentierelaxatie in het tijdsdomein voldoende gevoelig om een calibratie in vivo toe te laten. Gelijkwaardige waardes voor de basale $[\text{Na}^+]$ worden bekomen met relaxatietijden van Sodium Green ($12\pm3$ mM, n=5) en de ratiometrische methode met stationaire excitatie van SBFI ($16\pm3$ mM, n=6).

De analyse van het fluorescentieverval van Sodium Green verschafte nuttige informatie over het gedrag van de indicator in cellen. Wanneer echter alleen de bepaling van $[\text{Na}^+]_i$ belangrijk is bestaat een meer praktische benadering erin het faseverschil tussen excitatie en fluorescentie te volgen bij eenzelfde modulatiefrequentie. In deze studie wordt bij een modulatiefrequentie van 160 MHz het faseverschil gevolgd voor Sodium Green in cellen waarvan de $\text{Na}^+/\text{K}^+$ pomp is geïnhibeerd door een $\text{K}^+$ vrije badoplossing te gebruiken. Onder deze voorwaarden neemt $[\text{Na}^+]_i$ toe van 10 tot 33 mM in periode van 40 minuten. Het omkeren van de natriumgradiënt ($5$ mM $\text{K}^+$ vrije oplossing) resulteert in een daling van $[\text{Na}^+]_i$ tot ongeveer de controlewaarde in 30 minuten. Gelijkwaardige veranderingen in $[\text{Na}^+]_i$ worden bekomen met ratiometrische bepalingen met SBFI. Alhoewel de gevoeligheid van de fluorescentierelaxatie in cellen gereduceerd is t.o.v. bufferoplossingen tonen onze resultaten aan dat Sodium Green een goede indicator is voor “fluorescence lifetime imaging microscopy” (FLIM) in cellen.

Hoofdstuk IV
Het gedrag van de $\text{Na}^+$ indicator SBFI in HeLa cellen is bestudeerd met tijdsgeresolideerde fluorescentiemicroscopie. Het fluorescentieverval van SBFI in HeLa cellen kan beschreven worden met drie relaxatietijden voor $[\text{Na}^+]_i$ tussen 0 en 90 mM. De pre-exponentiële factor corresponderend met de kortste relaxatietijd is negatief. Veranderingen in $[\text{Na}^+]_i$ hebben geen effect op de fluorescentierelaxatietijden ($0.21\pm0.05$ ns, $0.60\pm0.03$ ns en $2.7\pm0.1$ ns) noch op de gemiddelde levensduur ($2.2\pm0.1$ ns). Nochtans neemt de verhouding van het fluorescentiesignaal bekomen door excitatie bij 340 nm tot het signaal door excitatie bij 380 nm toe met $[\text{Na}^+]_i$. Om dit gedrag van SBFI in cellen te kunnen verklaren zijn experimenten uitgevoerd op SBFI in buffer bij verschillende concentraties van $\text{Na}^+$, $\text{K}^+$ en BSA en bij verschillende

Hoofdstuk V
Het onderwerp van dit hoofdstuk is de studie van het energiemetabolisme van de cel in condities van chemische hypoxie die geïnduceerd wordt door het toedienen van cyanide en 2-deoxyglucose (DOG) voor een tijdsduur van 60 minuten, en van het gedrag van de cel in de daaropvolgende fase waarin een controle oplossing wordt toegediend. De studie is uitgevoerd op conflente monolagen van MDCK cellen. Deze cellen worden beschouwd als een in vitro model voor cellen van de distale tubulus van de ischemische nier die erin slagen zich te herstellen. Fluorescentiemiicroscopische beeldvorming is gebruikt om het tijdsverloop na te gaan van de veranderingen in intracellulaire pH (pH_i), Mg²⁺ concentratie ([Mg²⁺]), membraanpotentiaal van de mitochondriën (∆Ψ) en de redox-toestand van de cel. De hoeveelheid cellulaire ATP is bepaald met een test gebaseerd op chemiluminescentie in celextracten.

De cellulaire ATP daalt tot ongeveer 5% in vergelijking met de controle in 20 minuten. In deze tijdsbepaling gaat de ATP vermindering gepaard met een belangrijke verzuring van het intracellulair milieu (0,6 pH eenheden). Een gedeeltelijk herstel wordt waargenomen bij het wegnemen van de chemisch geïnduceerde hypoxie. Het volledige of het gedeeltelijke herstel dat waargenomen wordt voor de parameters onderzocht in dit hoofdstuk en in hoofdstuk VI ([Mg²⁺], ∆Ψ, [Ca²⁺], [Na⁺], en de redox-toestand van de cel) geven aan dat de cellen niet dodelijk beschadigd zijn. Ook het feit dat er geen plotselinge toename is in het verlies van de fluorescerende indicator uit de cel wijst in die richting.

Chemisch geïnduceerde hypoxie leidt tot een toename van [Mg²⁺], zoals blijkt uit de toename van de fluorescentie van Magnesium Green. De stijging van het Magnesium Green signaal is onafhankelijk van extern Mg²⁺. Dit impliceert dat de stijging in [Mg²⁺], kan worden toegeschreven aan een intracellulaire bron, heel waarschijnlijk het vrijzetten van Mg²⁺ uit Mg-ATP². De veranderingen in [Mg²⁺], lopen echter niet volledig parallel met de veranderingen in cellulaire ATP inhoud. Deze bevinding suggereert dat een deel van het Mg²⁺ dat vrijkomt door de hydrolyse van ATP gebufferd wordt door intracellulaire componenten.

Chemische hypoxie leidt tot een onmiddellijke verschuiving naar een meer gereduceerde cellulair redox toestand. Dit blijkt uit de toename van de NAD(P)H autofluorescentie en de depolarisatie van het binnenste mitochondriaal membraan. ∆Ψ daalt tot 26 ± 6 % t.o.v. de controlewaarde gedurende de eerste 25 minuten van de chemische hypoxie. Deze daling wordt gevolgd door een gedeeltelijk herstel van ∆Ψ.
Summary

(tot 48 ± 5 % van de controle) in de daaropvolgende 35 minuten in de voortdurende aanwezigheid van de metabole inhibitoren. Bij het gebruik van een controleoplossing werd geen significante verandering in ΔΨ opgemerkt. Inhibitie van de mitochondriale ATP synthase met oligomycine gedurende de chemische hypoxie versnelt de depolarisatie van de mitochondriën. In de aanwezigheid van oligomycine daalt ΔΨ tot 18 ± 2 % (n = 6) van de controlewaarde gedurende de eerste 6 minuten van de chemische hypoxie. Zonder oligomycine daalt ΔΨ tot 57 ± 10%. In de aanwezigheid van oligomycine is ΔΨ significat lager gedurende de chemische hypoxie. Te gelijktijdig vertraagt oligomycine de vermindering van de cellulaire ATP. Deze bevindingen suggereren dat ΔΨ gedurende chemische ischemie gedeeltelijk behouden blijft door het mitochondriale ATP synthase dat in omgekeerde zin werkt.

Hoofdstuk VI
Fluorescentiemicroscopische beeldvorming is gebruikt om de effecten te volgen van chemisch geïnduceerde hypoxie op de [Ca²⁺] en [Na⁺] in het cytoplasma van MDCK cellen in confluente monolagen. Gedurende 60 minuten chemische hypoxie neemt [Na⁺] gelijkmatig toe van 15 ± 3 mM tot 69 ± 7 mM (n = 6). In een controleoplossing herstelt [Na⁺] zich gedeeltelijk tot 42 ± 10 mM. Wanneer cyanide wordt toegevoegd in de aanwezigheid van glucose stijgt [Na⁺], op vergelijkbare wijze bij het begin van het experiment maar stabiliseert na 20 – 25 minuten tot 39 ± 8 mM.

[Ca²⁺] wordt ongeveer 4x groter gedurende de eerste 30 - 35 minuten onder chemische anoxie (van 68 ± 8 nM tot 283 ± 32 nM, n = 6). Gedurende de condities van chemische ischemie wordt deze stijging gevolgd door een daling. In ongeveer 40 minuten bereikt [Ca²⁺], bijna de rustwaarde. Cyanide in de aanwezigheid van glucose heeft geen effect op [Ca²⁺]. De afwezigheid van Ca²⁺ in de externe oplossing of het gebruik van La³⁺ voorkomt veranderingen in [Ca²⁺]. Het effect van chemische hypoxie op [Ca²⁺] wordt merkwaardig gereduceerd door de afwezigheid van extern Na⁺ en gedeeltelijk geïnhibieerd door het toedienen van verapamil. Deze bevindingen geven aan dat de Ca²⁺ toename te wijten is aan een instroom van Ca²⁺ hoofdzakelijk via de Na⁺ / Ca²⁺ uitwisselaar die in omgekeerde richting werkt. Een gedeelte van de Ca²⁺ influx gebeurt via Ca²⁺ kanalen.

Inhibitie van de Ca²⁺ pumps in het plasmamembraan met o-vanadate of van de SERCA pumps met thapsigargin voorkomt de dalende fase van Ca²⁺ in het cytoplasma niet en [Ca²⁺], kan een hogere maximale waarde aannemen. Wanneer ionomycine wordt toegevoegd gedurende de herstelfase (in Ca²⁺ vrije oplossing) wordt een transiënt in [Ca²⁺], waargenomen. Dit suggerereert dat Ca²⁺ wordt opgeslagen in sommige organellen. In hoofdstuk V wordt aangetoond dat ΔΨ afneemt maar niet verdwijnt gedurende chemische hypoxie. Dit houdt de mogelijkheid in dat Ca²⁺ in de mitochondriën wordt opgeslagen via een Ca²⁺ uniporter. Oligomycine doet in combinatie met cyanide de mitochondriale membraanpotentiaal verdwijnen. Bij deze combinatie verdwijnt ook de herstelfase van [Ca²⁺]. Dit geeft aan dat het waarschijnlijk de mitochondriën zijn die de cytoplasmatische Ca²⁺ bufferen onder condities van metabolische stress.
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Acknowledgements

It would be impossible to thank all the people whose help was essential in completing this thesis. This work would not have been possible without the generous help of Prof. Marcel Ameloot. I am extremely grateful to him for the opportunities he has given me and for his excellent supervision. He showed me that the gap between physics and life sciences is less wide than it sometimes appears.

My special thanks go to Prof. Paul Steels whose generous support has been invaluable to realize this thesis. We had many stimulating discussions, which helped me to understand the complexity of cells life. I am also grateful to him for providing a friendly and supportive environment.

I am grateful to the members of the jury, Prof. Van Driessche, Prof Nilius, Prof. De Broe, Prof. Boens, Prof. Lebaqc and Prof. Katona, for their detailed evaluation of this thesis and for their constructive criticism during its preparation. I also want to thank Prof. Van Driessche for facilitating my stay at LUC.

I want to thank Prof. Botana for the wonderful time I had in his lab, where I got introduced in the physiology of mammalian cells. I am also indebted to Prof. V. Topa for his generous support at the beginning of my work.

I want to express my gratitude to the members of the Laboratory of Physiology, Prof. Emmy Van Kerkhove, Agnes Roosen, Bart Laenen, Frank Jans, Georg Klein, Ilse Smets, Ingrid Vandenreyt, Isabelle Vanrooijen, Patrick Pirotte, Robert Bipat, Roland Van Werde and Wilfried Leyssens, for their permanent support and friendship. With Dr. M. Van de Ven I had many interesting discussions on time-resolved fluorescence measurements. I am also indebted to him for the critical review of previous versions of this thesis. I thank Dr. J. Vecer for his generous help with the time-resolved fluorescence measurements at the beginning of my work at LUC. I also want to thank Ilse Smets and Frank Jans for the stimulating discussions and exchange of ideas we had on the cellular effects of ischemia. The technical assistance of Wilfried Leyssens, Patrick Pirotte, Agnes Roosen and Roland Van Werde has been of great help. I also thank Magda leven and Mark Withofs for their help with figures and slides.

My very special thanks go to my husband, Florin. Without his love and support, this thesis would not have been written. I also want to thank my family for their understanding and help. Despite the physical distance of 1600 km between us, I always felt their unconditional support.
CURRICULUM VITAE

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