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The role of ion channels and intracellular metal ions in apoptosis of *Xenopus* oocytes

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Cover: Magnified *Xenopus laevis* oocytes injected in the nucleus with red dye (left) and uninjected (right). Photo by Ulrika Englund.

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INFALL (ur Barfotabarn,1933)

”Man dansar däruppe - klarvaket
är huset fast klockan är tolv.
Då slår det mig plötsligt att taket,
mitt tak, är en annans golv.”

-Nils Ferlin

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- II. **Englund UH**, Gertow J, Kågedal K, Elinder F. A voltage dependent non-inactivating Na⁺ channel activated during apoptosis in *Xenopus* oocytes. *PLoS ONE*. 2014 Feb 28;9(2):e88381
- III. **Englund UH**, Brask J, Elinder F. Inhibiting *SCN2A* ortholog upregulation in *Xenopus laevis* oocytes prevents cell death. *Manuscript*

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Abstract

Apoptosis is one type of programmed cell death, important during tissue development and to maintain the tissue homeostasis. Apoptosis comprises a complex network of internal signaling pathways, and an important part of this signaling network is the action of voltage-gated ion channels. The aim of this thesis was to explore the role of ion channels and the role of intracellular metal ions during apoptosis in *Xenopus laevis* oocytes. The reasons for using these oocytes are that they are large, robust, easy to handle, and easy to study electrophysiologically. Apoptosis was induced either chemically by incubation of the oocytes in staurosporine (STS) or mechanically by centrifugation of the oocytes. Ion currents were measured by a two-electrode voltage clamp technique, intracellular ion concentrations were measured either directly by in-house developed K^+ -selective microelectrodes or indirectly by the electrophysiological technique, and apoptosis was measured by caspase-3 activation. Paper I describes that the intracellular K^+ concentration was reduced by about 30 % during STS-induced apoptosis. However, this reduction was prevented by excessive expression of exogenous ion channels. Despite the magnitude of the intracellular K^+ concentration, either normal or reduced level, the oocytes displayed normal signs of apoptosis, suggesting that the intracellular K^+ reduction was not required for the apoptotic process. Because the intracellular K^+ concentration was not critical for apoptosis we searched for other ion fluxes by exploring the electrophysiological properties of *X. laevis* oocytes. Paper II, describes a non-inactivating Na^+ current activated at positive membrane voltages that was upregulated by a factor of five during STS-induced apoptosis. By preventing influx of Na^+ , the apoptotic signaling network involving caspase-3 was prevented. To molecularly identify this voltage-gated Na channel, the *X. tropicalis* genome and conserved regions of the human *SCNA* genes were used as a map. Paper III, shows that the voltage-gated Na channel corresponds to the *SCN2A* gene ortholog and that suppression of this *SCN2A* ortholog using miRNA prevented cell death. In conclusion, this thesis work demonstrated that a voltage-gated Na channel is critical for the apoptotic process in *X. laevis* oocytes by increasing the intracellular Na^+ concentration.

Introduction

Ion channels and intracellular metal-ion concentrations play a vital role in intracellular signaling cascades, and it has been suggested that metal ions were the catalysts before the presence of enzymes for the creation of precursors to nucleic acids, amino acids and lipids in the prebiotic age of our world (Keller et al., 2014). The core of this thesis is on the role of ion channels and metal-ion concentrations in the life and death of cells.

Apoptosis

Apoptosis is a form of programmed cell death which is important during tissue development and to maintain the tissue homeostasis. Disruption of apoptosis can cause diseases where individual cells die prematurely such as neurodegenerative disorders, or cells continue to divide out of control such as in cancer. The apoptotic process involves activation of proapoptotic proteins, mitochondrial membrane permeabilisation, nuclear defragmentation, and loss of cell volume. At the terminal stage of apoptosis the cells budd off apoptotic bodies that are phagocytosed by neighboring cells without initiating an inflammatory response (Kerr et al., 1972; Ziegler and Groscurth, 2004). A more violent way for a cell to end its life is via necrosis, where cell swelling and loss of membrane resistance leads to the intracellular content leaking out, which causes inflammation and damages to the surrounding cells (Festjens et al., 2006; Galluzzi et al., 2011; McCall, 2010).

Apoptosis can be initiated either by an extrinsic pathway or by an intrinsic pathway (Fig. 1). The extrinsic pathway starts when a proapoptotic ligand binds to one of the death receptors on the extracellular side (Dickens et al., 2012). The binding of the ligand leads to the formation of the death-inducible signaling complex on the intracellular side of the membrane which triggers activation of caspase-8 (Muzio et al., 1996; van Raam and Salvesen, 2012). In type I cells, caspase-8 cleaves and activates caspase-3, which in its active form cleaves target proteins leading to apoptosis. In type II cells, the cleavage of caspase-3 is insufficient and therefore amplification of the apoptotic signal is necessary, by activation of Bid, where active Bid translocates and inserts pro-apoptotic protein into the mitochondrial membrane, leading to activation of the intrinsic pathway (Kantari and Walczak, 2011).

The intrinsic pathway starts with the mitochondrial membrane being destabilised, which leads to release of cytochrome c into the cytosol (Garrido et al., 2006). Cytochrome c forms the apoptosome together with dATP and the cytosolic proteins apoptotic protease activating factor 1 and procaspase-9. Activation of caspase-9 occurs when the apoptosome is assembled, which in turn results in activation of caspase-3 by caspase-9 (Li et al., 1997). Caspase-3 has several hundred different substrates and this leads to proteins becoming non-functional, or proapoptotic proteins get activated (Timmer and Salvesen, 2007).

Apoptosis involves a complex network of internal signaling in the cell, and previous studies have revealed that a part of this signaling network involves voltage-gated ion channels. In the next sections, the properties of voltage-gated ion channels will be described followed by what is known about voltage-gated ion channels and apoptosis.

Figure 1. Schematic signaling pathways for initiating apoptosis in mammalian cells. Extrinsic pathway: activation of death receptors by external stimuli leading to formation of death-inducible signaling complex (DISC), which includes recruitment of procaspase-8. Procaspase-8 is cleaved to its active conformation, caspase-8. In type I cells, caspase-8 cleaves procaspase-3 to caspase-3, which starts the proteolytic cascade during apoptosis. In type II cells, caspase-8 also cleaves Bid that translocates and inserts pro-apoptotic protein into the mitochondrial membrane, thereby destabilising the membrane. The intrinsic pathway also starts with destabilisation of the mitochondrial membrane, leading to release of cytochrome c into the cytosol. Cytochrome c forms the apoptosome, leading to cleavage of caspase-3 and apoptosis.

Voltage-gated ion channels

Voltage-gated ion channels are transmembrane proteins that regulate the movement of ions across the hydrophobic cell membrane. This is important during for instance transmission of nerve impulses and contraction of heart and skeletal muscles (Hille, 2001).

Voltage-gated ion channels have a voltage-sensor domain that changes the conformation of the channel between open, closed or inactivated states in response to

changes in the membrane potential. The response is fast (in ms), and a narrow region of the channel pore act as a selectivity filter that recognizes and selects which ions can pass through the channel. Ion channels can conduct ions at an extremely rapid rate (up to 100 million ions per second), and can over time change the intracellular ionic composition of a cell. The voltage-gated ion channel superfamily consist of channels that are selective for either K^+ , Ca^{2+} or Na^+ , but since voltage-gated Na channels turned out to be the main channels discussed in this thesis I will focus here on the structure and function of the Na channels in the following section.

Voltage-gated Na channels

Voltage-gated Na channels play an important role in excitable cells (Hodgkin and Huxley, 1952a). In neurons, action potentials are generated by the opening of voltage-gated Na channels in response to changes in the membrane potential. Na^+ will flow into the cell until the membrane potential almost reaches the equilibrium potential for Na^+ (+60 mV), thereby opening voltage-gated Na channels nearby, leading to propagation of the action potential along the axon of a nerve cell (Hodgkin and Huxley, 1952a). Blockage of voltage-gated Na channels, with e.g. tetrodotoxin (TTX) which is a classical voltage-gated Na channel blocker, prevents influx of Na^+ into the nerve cell and thus prevents action potentials (Narahashi et al., 1964). The crystal structure of a bacterial voltage-gated Na channel has been published (Payandeh et al., 2011), but up to this time point no structure of a mammalian voltage-gated Na channel has yet been published. Voltage-gated Na channels consist of four homologous domains (domain I-IV) and each domain consists of a voltage-sensor domain and a pore domain (Fig. 2). The variability between the different domains in a single channel is larger than between two homologous domains in two different Na channels or even Ca channels (Strong et al., 1993). There are only nine human voltage-gated Na channels ($Na_v1.1-1.9$), and compared to other members of the voltage-gated channel superfamily, these nine Na channels are less diverse. Most Na channels inactivate fast (Hille, 2001); only one of nine human Na channels lacks fast inactivation (Cummins et al., 1999). It is known that there are kinetic differences between TTX-sensitive and TTX-resistant Na channels. TTX-sensitive Na channels open faster and produce larger single-channel currents compared to the TTX-resistant Na channels (Hille, 2001; Weiss and Horn, 1986).

Figure 2. Schematic figure of the α -subunit of a voltage-gated Na channel. The voltage-gated Na channel consists of four homologous domains (Domain I-IV) and each domain consist of six transmembrane segments, where the first four segments are the voltage-sensor domain (orange) and the last two transmembrane segments (blue) form an ion selective pore domain.

Plasma membrane-bound voltage-gated ion channels are important for apoptosis

Voltage-gated ion channels are directly involved in programmed cell death. One of the hallmarks of apoptosis is apoptotic volume decrease where increased efflux of K^+ and Cl^- plays an important role during cell shrinkage (Barbiero et al., 1995; Beauvais et al., 1995; Bortner and Cidlowski, 2007, 2002; Dezaki et al., 2012; Lang and Hoffmann, 2012; Maeno et al., 2000; Wei et al., 2004). Additionally, a dual role of Na^+ influx has been reported, where both absence and enhanced Na^+ influx causes the cell to swell (Bortner and Cidlowski, 2003; Carini et al., 1995; Koike et al., 2000). Inhibiting or activating a specific voltage-gated Na, K, Ca, or anion channel (see Table I), as well as affecting unspecific L-type Ca channels, TTX-sensitive Na channels or volume-sensitive Cl channels, have all been reported to prevent apoptosis (Banasiak et al., 2004; Dargent et al., 1996; Ise et al., 2005; Sribnick et al., 2009; Szabò et al., 1998; Tanaka and Koike, 1997; Wu et al., 2008; Yagami et al., 2004; Zawadzki et al., 2008).

Altered composition of ions has been reported to directly affect activation of proteins involved in apoptosis (cytochrome c, proteases, nucleases etc), as well as formation of the apoptosome, affecting the ratio between pro- and antiapoptotic proteins (Cain et al., 2001; Hampton et al., 1998; Hughes et al., 1997; Karki et al., 2007; Koeberle et al., 2010; Strickland et al., 1991; Thompson et al., 2001; Wondrak et al., 1991).

Reduction of the number of voltage-gated K channels have been shown to reduce the expression of pro- (caspase-3, caspase-9 and Bad) and antiapoptotic (Bcl-xL) genes (Koeberle et al., 2010). It has also been shown that proapoptotic and apoptotic related proteins can activate voltage-gated K, Na, Ca and anion channels directly (Ekhterae et

al., 2001; Koeberle et al., 2010; Platoshyn et al., 2002; Storey et al., 2003; Vander Heiden et al., 2001; Yao et al., 2011).

This thesis aims to investigate the role of ion channels and intracellular metal ion concentrations in *Xenopus laevis* (*X. laevis*) oocytes with the purpose to use oocytes as a model system for exploring the direct role of ion channels and intracellular ionic composition in the apoptotic process.

Table I. Examples of plasma-membrane bound voltage-gated Na, K, Ca and HCN as well as anion channels reported to be directly involved in the intrinsic and extrinsic pathways of apoptosis.

Type of channel	Cell/tissue	Inducer of apoptosis	Reference
Na_v channels			
1.1	Forebrain (rat)	Veratridine	(Dave et al., 2003)
1.4	Skeletal muscle (mice), HEK-293 cells	Absence of dystrophin, expression of Na1.4	(Hirn et al., 2008; Pincin et al., 2005)
1.5	Astrocytoma cells, ventricular myocytes (rat)	Down regulation of 1.5, anemone toxin II,	(Xing et al., 2014; Yao et al., 2011)
1.9	Kidney tissue (rat)	Ischemia-reperfusion	(Dusmez et al., 2014)
K_v channels			
1.1	Cerebellar granule cells (rat), pulmonary artery smooth muscle cells (rat), retinal ganglion cells (rat), hippocampal cells (rat)	Low extracellular serum free-solution, STS, axotomy, glutamate	(Ekhterae et al., 2001; Hu et al., 2008; Koeberle et al., 2010; Shen et al., 2009)
1.3	Retinal ganglion cells (rat), microglia (rat), Jurkat cells, CTLL-2 cells (mouse)	Axotomy, HIV-1 Tat protein, CD95 ligand, STS, actinomycin D	(Bock et al., 2002; Koeberle et al., 2010; J. Liu et al., 2013; Storey et al., 2003; Valencia-Cruz et al., 2009)
1.4	Striatal neuron (rat)	Ischemia	(Deng et al., 2011)
1.5	Pulmonary artery smooth muscle cells (rat), vascular endothelial cells	STS, oxidative stress	(Chen et al., 2012; Ekhterae et al., 2001)
2.1	Cortical cells (rat), cerebellar granule cells (rat), neuroblastoma cells, hippocampal cells (Rat), HEK-293	Serum-deprivation, low extracellular serum free-solution, DTDP, HIV-1 gp120, oxidative stress,	(Dallas et al., 2011; Jiao et al., 2007; H. Liu et al., 2013; Pal et al., 2003; Shepherd et al., 2012; Wu et al., 2013; Yao et

		CXCR4-signaling	al., 2009)
3.4	Hippocampal cells (rat), pheochromocytoma cells (rat)	A β ₁₋₄₂	(Pannaccione et al., 2007)
4.2	Pulmonary artery smooth muscle cells (rat), striatal neuron (rat)	STS, ischemia	(Deng et al., 2011; Ekhterae et al., 2001)
4.3	HEK-293 cells	Inhibition of k _v 4.3	(Li et al., 2012)
11.1	Glioblastoma cells, HEK-293	Inhibition of k _v 11.1	(Obers et al., 2010; Staudacher et al., 2014; Thomas et al., 2008)
BK channels	Pancreatic beta cell (mouse), ovarian cancer cells	H ₂ O ₂ and inhibition of BK channels, BK channel opener	(Düfer et al., 2011; Han et al., 2008)
IK channels	Glioma cells (mouse)	STS	(McFerrin et al., 2012)
HCN channels			
HCN2	Lung carcinoma cells, cerebral cortical neurons (rat)	PKC inhibitors, STS	(Norberg et al., 2010)
Ca_v channels			
1.2	Cortical cells (rat), primary neural cells (rat)	Low intensity static magnetic fields, glutamate excitotoxicity,	(Ben Yakir-Blumkin et al., 2014; Tsuruta et al., 2009)
2.1	Sertoli cells (rat), hippocampal cells, retinal cells (rat+mouse)	Methoxyacetic acid, oxygen-glucose deprivation, ω -conotoxin GVIA	(Barone et al., 2005; Tian et al., 2013; Ueda et al., 2004)
2.3	Hippocampal cells (mice)	Kainic acid-induced excitotoxicity, EFCH1 overexpression	(Suzuki et al., 2004; Weiergräber et al., 2007)
Anion channels			
VDAC	Hippocampal cells (mouse and rat)	STS	(Akanda et al., 2008; Elinder et al., 2005)

The *X. laevis* oocyte as a model system

The *X. laevis* oocyte is a well-established expression system for studying ion channels and is widely used in the field of developmental biology and cell-cycle research (Brown, 2004; Dascal, 1987; Dawid and Sargent, 1988). The oocyte can be up to 1.3 mm in diameter and this makes them easy to handle and to record ion currents from. The size also allows injections to alter ionic composition or regulate the activity of different proteins (Dascal, 1987). Previous studies have shown that the *X. laevis* oocyte displays a normal apoptotic process, which includes activation of caspases, cytochrome c release from the mitochondria, nuclear condensation and ATP depletion (Braun et al., 2003; Johnson et al., 2010; Nutt et al., 2005; Tokmakov et al., 2011). The expression level of endogenous channels is low (Dascal, 1987) and this makes them suitable to explore the importance of ion channels and intracellular ion concentrations in the apoptotic process. Below, I will describe the different endogenous ion channels found in *X. laevis* oocytes followed by how miRNA are processed in *X. laevis* oocytes.

Endogenous ion channels in X. laevis oocytes

Even though the expression level of endogenous ion channels is low, oocytes express a variety of different ion channels and transporters that can change the intracellular composition of ions.

X. laevis oocytes express three different types of K channels. These channels are thoroughly discussed in a number of review articles (Sobczak et al., 2010; Weber, 1999). One type of K channel is blocked by both Tetraethylammonium (TEA) and Ba^{2+} . One type of K channel is blocked by TEA. The third type of K channel can be induced by expressing peptides and other channels. Overall, the function of endogenous K channels, together with the ATP- driven Na^+/K^+ -pump, is to create and to maintain the oocyte membrane potential.

Four different types of Cl channels have been described: a Ca^{2+} -activated Cl channel, a volume sensitive Cl channel activated by hypotonicity, a Cl channel induced by hyperpolarisation, and a Ca^{2+} -inactivated Cl channels. These Cl channels are important to create a functional polarity of the oocyte, prevent polyspermy and to further maturation of the fertilised oocyte (Sobczak et al., 2010; Weber, 1999).

Several voltage-gated Ca channels belonging to the L, N and T-type, have been reported in *X. laevis* oocyte, as well as store-operated Ca channels that activates in response to elevated intracellular Ca^{2+} (Sobczak et al., 2010; Weber, 1999). The increase in intracellular Ca^{2+} through Ca channels and transporters (through the plasma membrane and from internal stores) is reported to be important during fertilisation (Busa and Nuccitelli, 1985; Sobczak et al., 2010; Weber, 1999).

Five different endogenous Na channels have been reported in *X. laevis* oocytes. (i) One Na channel is blocked by amiloride and is only found in every third oocyte (Weber et al., 1995). (ii) One Na channel is activated by high concentrations of extracellular ATP

(Kupitz and Atlas, 1993). (iii) One Na channel is activated by NH_4Cl (Burckhardt and Burckhardt, 1997). (iv) One Na channel is a small transient TTX-sensitive channel that inactivates fast and has only been recorded occasionally in *Xenopus* oocytes (Krafte and Volberg, 1992; Parker and Miledi, 1987). (v) One Na channel reported in *X. laevis* oocytes is the Na_x channel, which is blocked by TTX at high concentrations. Na_x is activated by a long depolarisation to positive voltages, with the activation facilitated by insulin and mobilisation of intracellular Ca^{2+} (Baud et al., 1982; Bossi et al., 1998; Charpentier and Kado, 1999; Vasilyev et al., 2002). Na_x is blocked by extracellular polyvalent cations, intracellular Mg^{2+} and high concentrations of the local anaesthetics lidocaine (Charpentier, 2002; Quinteiro-Blondin and Charpentier, 2001; Vasilyev et al., 2002). This last channel is found in the present thesis work to be upregulated during apoptosis and will be more discussed in the general discussion section.

miRNA expression

In most cell types, cDNA plasmids expressing pre-miRNA can be transfected and transcribed in the cytosol. However, in *X. laevis* oocytes, cDNA plasmids expressing pre-miRNA need to be injected into the nucleus for it to be transcribed (Fig. 3) (Lund et al., 2011). In the nucleus, pre-miRNA is bound to exportin 5 (EXP5) and cofactor Ran that has GTP bound to it. When GTP hydrolyses, pre-miRNA is released into the cytosol (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). The pre-miRNA is then cleaved to mature miRNA by Dicer, a RNase III-like enzyme, together with TAR RNA binding protein. In human cells, the mature miRNA is assembled with one of four Argonaute (AGO) protein creating miRNA-induced silencing complexes (miRISCs) that binds to complementary mRNA leading to mRNA degradation. In the *X. laevis* oocytes, the pre-miRNA is transported out of the nucleus the same way, but the enzyme dicer has 75 % less activity in *X. laevis* oocytes compared to mature *X. laevis* eggs. They also do not express AGO proteins and therefore, no miRISCs are assembled and only inhibition of translation of the complementary mRNA can occur.

Figure 3. Simplified schematic picture describing the maturation and action of miRNA in human cells and *Xenopus laevis* oocytes. In the nucleus, pre-miRNA is bound to exportin 5 (EXP5) and its cofactors and by hydrolysing GTP, pre-miRNA is transported over the nucleus membrane and released in to the cytosol. Pre-miRNA is then bound to a protein complex containing the RNase III-like enzyme dicer. Dicer cleaves pre-miRNA into mature miRNA. The mature miRNA is then assembled with one of four Argonaute (AGO) proteins creating miRNA-induced silencing complexes (miRISCs) that binds to complementary mRNA leading to mRNA degradation. In the *Xenopus laevis* oocyte, the pre-miRNA is transported out of the nucleus the same way, but the enzyme Dicer has 75 % less activity in oocytes compared to mature *Xenopus laevis* eggs. They also do not express AGO proteins and therefore, no miRISCs are assembled and only inhibition of translation of the complementary mRNA can occur.

Aims of the thesis

The **general aim** of this thesis was to study the role of ion-channel activity and the functional role of metal-ion concentrations during apoptosis. To study this, *X. laevis* oocytes were used as a model system, which due to their size and common internal apoptotic-signaling makes it easy to explore this connection.

The specific aims were

1. to develop tools and methods to measure alterations in intracellular K^+ and Na^+ concentrations in *Xenopus* oocytes during apoptosis (papers I and II).
2. to study the role of altered metal-ion fluxes in apoptosis in *Xenopus* oocytes (papers I and II).
3. to measure alterations in endogenous ion channel activity in *Xenopus* oocytes during apoptosis (paper II), and, if alterations in ion channel activity in 3,
4. to molecularly identify and target the channel which specifically alters the apoptotic process (paper III).

Methods

The model system

Oocytes in developmental stage V-VI (as distinguished by morphology) were surgically collected from adult female *X. laevis* frogs were used in this thesis. These oocytes are large (up to 1.3 mm in diameter), they have a distinct darker animal pole (brown) and a lighter vegetal pole (yellow/green) and they are surrounded by a vitelline membrane (Dumont, 1972). Oocytes were stored in Modified Barth's solution at 8 °C after being collected and before each experiment started. Endogenous voltage-gated ion channels were studied in papers II and III. In paper I, cRNA for the non-N-type inactivating voltage-gated Shaker H4 channel (Hoshi et al., 1990; Kamb et al., 1987) was injected into the cytosol of the oocytes to express the channel. All solutions and other details about cell handling are described in papers I-III.

Ethical considerations

All animal experiments were approved by the local Animal Care and Use Committee at Linköping University.

How to measure and analyse currents across a membrane

When voltage is applied across the cell membrane, it activates voltage-gated ion channels that allow ions to pass through the lipid membrane. The ion currents were measured by the two-electrode voltage-clamp technique (Stühmer, 1992). Two electrodes are carefully inserted into the oocyte (Fig. 4), where one of them measure the voltage relative a reference electrode in the extracellular solution. The other electrode injects current into the oocyte to maintain the voltage across the membrane to keep the membrane voltage at a predestined level. The current injected is a direct measure of the ions flowing through the voltage-gated ion channels in the membrane. With this information, the voltage dependence, the reversal potential, the kinetics, and the open probability of voltage-gated ion channels in the membrane can be determined.

The extracellular solution can easily be changed which makes it possible to directly measure the effect of extracellular solution composition on the parameters mentioned above. This also makes it easy to apply different substances to the *Xenopus* oocyte to block the voltage-gated ion channel at interest (papers II and III). In papers I-III, a physiological extracellular solution with high Na^+ and low K^+ concentrations (termed 1K in paper I and 100Na in papers II and III) was used. In paper I and II, Na^+ in the extracellular solution was replaced by K^+ (termed 100K in paper I and 0Na in paper II).

Figure 4. Schematic figure showing setup of the two-electrode voltage-clamp technique with a *Xenopus* oocyte (green and brown). See text for further explanation.

In papers I-III, we measured the reversal potential (V_{rev}), defined as the membrane voltage where the net current of the specific ion is 0. By the use of Nernst's equation and the known extracellular concentration of the monovalent cation X ($[X]_o$), the intracellular concentration of the ion X ($[X]_i$) can be calculated:

$$[X]_i = \frac{[X]_o}{e^{(V_{rev}FR^{-1}T^{-1})}} \quad \text{Eq. 1}$$

F is the Faraday constant, R is the universal gas constant and T is absolute temperature (measured in Kelvin).

In paper II, the conductance (G), which reflects the open probability of the voltage-gated ion channel of interest, was calculated using a modified Ohm's law:

$$G = \frac{I}{(V - V_{rev})} \quad \text{Eq. 2}$$

I is the current, and V is the absolute membrane voltage. To quantify the conductance data we used the Boltzmann equation

$$G(V) = \frac{1}{(1 + e^{(V - V_{50})z_gFR^{-1}T^{-1}})}, \quad \text{Eq. 3}$$

where V_{50} is the voltage at which 50 % of the channels are open, and z_g is the gating charge, that is the number of charges that have to move through the membrane to open the channel.

Construction of a nanorod microelectrode for intracellular K^+ concentration measurements

A ZnO-nanorod microelectrode which measures the intracellular K^+ concentration was developed and tested in collaboration with Magnus Willander's group at the Department of Science and Technology (Linköping University) (Usman Ali et al., 2011). ZnO nanorods were chemically grown on the tip of a borosilicate glass capillary in an aqueous solution of $Zn(NO_3)_2 \cdot 6H_2O$. The ZnO nanorods were similar in length (1.5 μm) and diameter (100-180 nm) which was verified by field emission scanning-electron microscope images. The ZnO-nanorod covered glass tip was coated with a K^+ -selective membrane consisting of a thin polyvinyl-chloride membrane containing valinomycin ionophore (Fig. 5). Valinomycin is a cyclic molecule created by twelve alternating amino acids and esters and it is selective for K^+ ions. When K^+ passes through the ionophore, they interact with the ZnO nanorods, thereby sending an electrical signal to an amplifier. To measure the ion concentration, the selective electrode is inserted into the oocyte. Each microelectrode is calibrated against known concentrations of K^+ before the measurement.

Figure 5. Electron scanning microscope of a ZnO-nanorod microelectrode covered with the ionophore- containing polyvinyl-chloride membrane before intracellular measurements (paper I).

In paper I, the reliability and accuracy of the K^+ -selective microelectrodes was tested by comparing the intracellular K^+ concentration in *X. laevis* oocytes using both the K^+ -selective microelectrode and electrophysiological methods on the same oocyte. Voltage-gated K channels (Shaker channel) was expressed in the oocytes and the intracellular K^+ concentration was also altered by injections of different K^+ and choline $^+$ solutions (figure 1 in paper I).

Using Nernst's equation (Eq. 1), the intracellular K^+ concentration could be determined by the electrophysiological method and be compared with results from the K^+ -selective microelectrodes. The concentration determined with the two methods gave similar results

(Fig. 6). This makes it possible to accurately measure the intracellular K^+ concentration in oocytes not expressing K_v channels using the K^+ -selective microelectrode.

Figure 6. Comparison between electrophysiological recordings and K^+ -selective microelectrodes. Intracellular K^+ concentrations are similar in K_v channel-expressing *X. laevis* oocytes measured with electrophysiological (TEVC) and K^+ -selective microelectrode techniques. Data are expressed as mean values for control oocytes and oocytes injected with 50 nL of indicated test solutions. Error bars show S.E. $n = 3-5$.

Induction and detection of apoptosis

In papers I-III, apoptosis was induced in *X. laevis* oocytes by incubating the cells with different concentrations of staurosporine (STS). STS is a broad-spectrum protein kinase inhibitor that induces apoptosis (Bertrand et al., 1994; Tamaoki et al., 1986). STS-treatment changes the intracellular ionic composition in several different cell types (Arrebola et al., 2005a, 2005b). In paper III, *X. laevis* oocytes were also centrifuged to induce apoptosis mechanically.

X. laevis oocytes display a normal apoptotic process compared to other cells including cytochrome c release and activation of caspase-3 (Braun et al., 2003; Johnson et al., 2010; Nutt et al., 2005; Tokmakov et al., 2011). In papers I-III, caspase-3 activity was measured by the fluorescence of 7-amino-4-methylcoumarin (AMC) resulting from the cleavage of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) by activated caspase-3. The caspase-3 activity level was normalised to total protein content.

Other markers for apoptosis used in paper III was depolarisation of the resting membrane potential (Bhuyan et al., 2001; Bortner et al., 2001) and morphological changes, where white spots on the dark hemisphere and vice versa appeared during apoptosis, and the line separating the animal and vegetable pole became diffuse (Fig. 7). The resting membrane potential was recorded using the two-electrode voltage-clamp technique.

Figure 7. Morphological changes seen in apoptotic (left) compared to non-apoptotic oocyte (right).

Finding a gene in an unsequenced genome

One of the aims of paper III was to find out which gene is coding for the voltage-gated Na channel found in paper II. This was complicated due to the fact that the genome of *X. laevis* is not yet fully sequenced. For this reason, primers were designed using conserved regions in the Na_v channels found in the genome of *X. tropicalis* (see extended data, Table 1 in paper III). The gene sequences for six Na_v channels from *X. tropicalis* are partly known and can be retrieved at www.xenbase.org. (Gilchrist, 2012; Hellsten et al., 2010; Zakon et al., 2011)

To explore the possibility to target specific Na_v channel sequences in *X. laevis* using primers designed for *X. tropicalis*, tissues from *X. laevis* with known expression of different Na_v channel genes (*SCNA* genes) were collected after the frogs were sacrificed (brain, heart, and skeletal muscles). Total mRNA was extracted from the tissues and cDNA created. Because of the lack of sequenced *X. laevis* *SCNA* genes, the PCR protocol had to be designed to allow mismatch of the primers binding to its complementary sequence. With the primers, short PCR products of six different Na_v channels could be obtained from *X. laevis* and it was revealed after sequencing of the PCR products that each one of the six sequences were orthologs to a specific *SCNA* gene in the human and *X. tropicalis* genomes.

Primers for all six Na channels were used with optimised qPCR protocols to measure the mRNA levels in *X. laevis* oocytes. The expression level of *SCN1A*, *SCN3A*, and *SCN4A* mRNA was low or no significant signal was detected ($C_T < 40$). The other three (*SCN2A*, *SCN5A* and *SCN8A*) were selected for further investigation.

Construction, cultivation and purification of pre-miRNA plasmids

Plasmids containing pre-miRNA constructs labeled with emGFP was created using BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit from Life Technologies. Single-stranded oligonucleotide constructs targeting mRNA transcribed from *SCN2A*, *SCN5A* and *SCN8A* in *X. laevis* were created (see extended data table 3 in paper III) using the short sequences obtained from *X. laevis* (see extended data table 2 in paper III). Double-stranded oligonucleotides were generated through annealing and, thereafter cloned into plasmids and transformed to chemically competent *E. coli*. After cultivation, the pre-miRNA plasmids were purified.

Amplification of the pre-miRNA part using the primers included in the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit was followed by sequencing of the PCR amplicon. This was performed to exclude the possibility that mutations had occurred in the pre-miRNA part of the plasmids during cultivation. Also, the plasmids were transfected into CHO cells to confirm that no mutations had occurred in the promoter region of the plasmids. Positive transfected cells expressed miRNA labeled with emGFP.

Suppression of the expression level of the SCN2A, SCN5A and SCN8A gene orthologs in X. laevis oocytes

Exogenous pre-miRNA plasmids need to be injected into the nucleus to be transcribed and processed in *X. laevis* oocytes (Bohnsack et al., 2004; Lund and Dahlberg, 2006). *X. laevis* oocytes were centrifuged to bring the nucleus close to the cell membrane and plasmids containing pre-miRNA targeting mRNA from either *SCN2A*, *SCN5A* or *SCN8A* were injected into the nucleus directly after centrifugation using an air pressure-based microinjector system. A red dye was also injected together with the plasmids to confirm successful injection into the nucleus of the *X. laevis* oocytes. Injected oocytes were incubated for two days before electrophysiological recordings were performed.

Statistics

Data are presented as mean $\pm$ S.E.M, with *n* as the number of oocytes or pools of oocytes investigated. Statistical analysis was performed using unpaired t-test when two variables were compared, whereas one-way ANOVA was used when more than two variables were compared. Two *post hoc* tests were used. The Bonferroni *post hoc* tests were used when all variables were compared against each other and Dunnett's *post hoc* tests were used when all variables were compared to control. All statistical analysis was performed using GraphPad Prism, software 5. Statistical significance was defined as $p < 0.05$ (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

Results & Discussion

The intracellular K^+ concentration during STS-induced apoptosis in *X. laevis* oocytes (Paper I)

Two different methods were used to estimate the intracellular K^+ concentration in *X. laevis* oocytes. The first method was to measure the reversal potential by the two-electrode voltage-clamp technique and to calculate the K^+ concentration by Nernst's equation (Eq. 1). The second method was to measure the concentration directly by a K^+ -selective microelectrode, which we have developed in collaboration with another research group at Linköping University, Sweden (Usman Ali et al., 2011). The two methods gave similar results, suggesting that they could be used interchangeably (grey bars in Fig. 8). Treatment with STS did not alter the intracellular K^+ concentration in oocytes expressing Shaker K_v channels compared to control. This finding was, as above, independent of the method of measurement (four left bars in Fig. 8).

Figure 8. Intracellular K^+ concentrations measured by two different techniques in normal and apoptotic *X. laevis* oocytes with and without expression of Shaker K_v channels. TEVC = the two-electrode voltage-clamp method. STS-labelled bars (red) were obtained from oocytes after six hours incubation in 1 μ M STS. The second bar is obtained by interpolation from the data presented in Paper I. Data expressed as mean $\pm$ SEM. N = 5 for all bars. P values calculated using one-way analysis of variance (ANOVA) with Dunnett's post hoc tests (uninjected and no STS treatment was set as control). * P $\leq$ 0.05

The preservation of the intracellular K^+ concentration during STS treatment was also measured over time (figure 4b in paper I). In contrast to Shaker-expressing oocytes, oocytes not expressing Shaker K_v channels displayed a 27 % decrease in intracellular K^+ (81 ± 8 mM, n

= 5) after six hours in 1 μM STS (two bars to the right in Fig. 8). This reduction was measured by the K^+ -selective microelectrodes and could not be measured by the electrophysiological method due to lack of K channels.

Thus, dense expression of exogenous Shaker channels prevented the STS-induced K^+ loss. Because expression of a non-conducting K channel (Shaker W434F) also prevented the STS-induced K^+ loss, it was concluded that it was the protein expression itself and not the expression of an ion-conducting protein that was critical (Paper I). How can a dense expression of a membrane protein prevent the STS-induced K^+ loss? It is possible that the Shaker-channel protein affects K^+ efflux via direct or indirect interactions with apoptosis-associated channels or pumps (Paper I).

To conclude, treatment by STS reduced the intracellular K^+ concentration by 27 % in normal *X. laevis* oocytes, not expressing Shaker K_v channels. In contrast, expression of Shaker K channels prevented this reduction.

Caspase-3 activity is not dependent on the intracellular K^+ concentration (Paper I)

Reduction in the intracellular K^+ concentration has been suggested to trigger caspase-3 activity, an indicator of apoptosis (Hughes et al., 1997). Therefore, the next step was to explore whether or not the reduced intracellular K^+ concentration was required in the apoptotic process. This was tested by measuring caspase-3 activity in oocytes expressing Shaker K_v channels (normal intracellular K^+ concentration after STS treatment, Fig 8) and in oocytes not expressing K_v channels (reduced intracellular K^+ concentration after STS treatment, Fig. 8) respectively. Caspase-3 activity was measured in oocytes before and after three and six hours exposure to 1 μM STS, and was shown to increase equally much in both groups of oocytes; there was a doubling in activity after three hours and almost a three-fold increase after six hours (Fig. 9). This suggests that the reduction in the intracellular K^+ concentration is not required for the apoptotic process in *X. laevis* oocytes.

Figure 9. Caspase-3 activity measured in oocytes expressing (RNA injected) or not expressing (Not RNA injected) Shaker K_v channels. Oocytes were incubated with 1 μM STS for either three or six hours. The fluorescence after caspase-3 cleavage of Ac-DEVD-AMC was measured with photospectrometry and corrected with total protein level. Data expressed as mean values $\pm$ SE. (n=4. 15 oocytes/n).

The question that follows is whether or not other ions than K^+ are import in the apoptotic process in *X. laevis* oocytes? Therefore, we performed an electrophysiological characterisation of the *X. laevis* oocytes during STS-induced apoptosis to investigate if apoptosis leads to alterations in any endogenous ion currents.

A voltage dependent non-inactivating Na channel is activated during apoptosis in *X. laevis* oocytes (Paper II)

Incubation with 1 μM STS for six hours increased an outward-going current three fold at +100 mV compared to untreated (control) oocytes. (Fig. 10A). This current did (i) not inactivate during a 1-s long pulse, it was (ii) only activated at positive voltages (paper I), and it was (iii) conducted by Na^+ ions; by replacing the extracellular Na^+ with K^+ , that is changing the extracellular solution from 100 mM Na^+ (100Na) to 0 mM Na^+ (0Na) abolished the inward going tail current (Fig. 10B, blue), and reintroduction of Na^+ in the extracellular solution quickly and completely restored the inward tail current (Fig. 10B, grey). Other ions, such as Cl^- or Ca^{2+} ions could not be a part of the current since their concentrations were not altered. K^+ ions are ruled out since the high extracellular K^+ concentration would increase the tail current rather than decrease it.

Figure 10. Electrophysiological recordings in *X. laevis* oocytes. A) Outward current recorded at +100 mV in control (black) and STS-treated oocytes (red) (holding potential -80 mV). The difference between the current in control and apoptotic oocytes is also plotted (dashed line). B) Tail currents recorded at 0 mV after a prepulse to +100 mV. The inward tail current in 100Na solution (black) is abolished in 0Na solution (blue) in oocytes treated with 1 μM STS. The outward current could be reversed to an inward current again, when switching back to 100Na (grey).

This STS-induced Na^+ current is atypical in more than one way compared to human Na^+ currents. Most Na channels inactivate fast (Hille, 2001); only one ($\text{Na}_v1.9$) of nine human Na channels lacks fast inactivation (Dib-Hajj et al., 2002). The STS-induced channel is also insensitive to high concentrations of TTX (known to block the majority of the voltage-gated Na channels) (figure 5a and b in paper II). The non-inactivating $\text{Na}_v1.9$ is in fact TTX-resistant, but in contrast to $\text{Na}_v1.9$ (Dib-Hajj et al., 2002, p. 9), the midpoint of activation for the STS-induced Na channel in *X. laevis* oocytes is +55 mV (figure 3e in paper II) compared to a midpoint of -47 mV for $\text{Na}_v1.9$ (Cummins et al., 1999). Neither is the STS-induced channel sensitive to amiloride (known to block weakly voltage dependent epithelial Na channels). Instead, 200 μM of the Ca channel blocker verapamil, also known to block Na and K channels (Madeja et al., 2000; Roger et al., 2004; Rolf et al., 2000; Yamagishi et al., 1995), almost completely abolished the current (Fig. 11A), leaving only a slowly activating current which most likely is mediated by another type of channel. The response to verapamil was dose-dependent, and 10 μM verapamil blocked 50 % of the channels (figure 5g in paper II). To further explore the verapamil-blocked Na^+ current, the slow current was subtracted from the total current (Fig. 11B). Strikingly, this current is almost identical to the STS-induced current

(dashed curve in Fig. 10A). Furthermore, this also suggests that the three-fold increase in total current in STS-treated oocytes compared to control cells (Fig. 10A) corresponds to a near five-fold increase of the specific Na^+ current (Fig. 11B).

Figure 11. The effects of verapamil on the Na^+ channel at +100 mV. A) 200 μM verapamil blocks the fast activating Na^+ current in oocytes treated with 1 μM STS, leaving a slowly activating current (blue). B) The verapamil-sensitive Na^+ current in STS-treated (red) and control (black) oocytes after subtraction of the remaining, slowly activated verapamil-resistant current.

To conclude, a voltage dependent non-inactivating Na^+ current was found to be upregulated in *X. laevis* oocytes during STS-induced apoptosis. This Na^+ current is blocked by verapamil, but not by the classical Na^+ channel blockers TTX and amiloride. The next step was to investigate if prevention of the Na^+ influx during STS-induced apoptosis in *X. laevis* oocytes could prevent apoptosis.

Low extracellular Na^+ prevents apoptosis in *X. laevis* oocytes (Paper II)

Long incubation with verapamil induced necrosis of the oocytes. Therefore, it was not possible to block the Na^+ influx by verapamil during STS-treatment. Instead, to reduce the Na^+ influx to explore its role in the apoptotic process, extracellular Na^+ was replaced by either choline⁺ (Hodgkin and Huxley, 1952b) or K^+ during STS incubation. Changing the extracellular Na^+ to choline⁺ did not prevent the almost two-fold increase in Na^+ conductance in STS-treated oocytes (Fig. 12A). This suggests that it is not the increased intracellular Na^+ concentration that increases the Na^+ conductance in a positive feed-back loop, but rather that STS directly increases the Na^+ conductance, independent of the intracellular Na^+ concentration.

How does the intracellular Na^+ concentration depend on STS and the extracellular Na^+ concentration? The intracellular Na^+ concentration is doubled during STS-induced apoptosis in normal extracellular solutions (Fig. 12B), and removal of extracellular Na^+ prevented the STS-induced increase in intracellular Na^+ (Fig. 12B). Thus, Na^+ influx is not needed to increase the Na^+ conductance, but Na^+ influx is (not unexpectedly) needed to increase the intracellular Na^+ concentration. A crucial question is whether or not the increase in intracellular Na^+ is needed for STS-induced apoptosis. The caspase-3 activity increased in STS-treated oocytes, but this

increase was prevented if Na^+ was replaced by K^+ (Fig. 12C). The oocytes did not tolerate that Na^+ was replaced by choline $^+$, which resulted in a fragile and swollen cell.

Figure 12. Role of intracellular Na^+ during apoptosis. A) The conductance at +100 mV in STS-treated oocytes (red) compared to control oocytes (black). Replacing extracellular Na^+ with choline $^+$ did not affect the increase in Na^+ conductance (control=grey; STS-treated=blue). Data expressed as mean $\pm$ SEM and P values calculated using unpaired t-test (** $P < 0.01$). B) Intracellular Na^+ concentrations in STS-treated oocytes (red). Replacing extracellular Na^+ by either choline $^+$ (blue) or K^+ (light blue) during STS incubation did not increase the intracellular Na^+ concentration. Intracellular Na^+ concentration for control oocytes (control*, black) were taken from other reports (Asif et al., 2010; Baud et al., 1982; Dascal, 1987). Data expressed as mean $\pm$ SEM and P values calculated using one-way ANOVA test with Bonferroni post hoc tests (* $P < 0.05$, ** $P < 0.01$). C) Low extracellular Na^+ (replaced by K^+) prevented caspase-3 activation (blue). Data expressed as mean $\pm$ SEM and P values calculated using one-way ANOVA test with Bonferroni post hoc tests (* $P < 0.05$, **** $P < 0.0001$).

Previous studies have shown that a high concentration of extracellular K^+ inhibits apoptosis by preventing loss of intracellular K^+ (Bortner and Cidlowski, 2002; Hughes et al., 1997; Singleton et al., 2009; Yu et al., 1997). The results in paper I showed that intracellular K^+ decreases during STS-induced apoptosis in *X. laevis* oocytes. However, paper I also showed that keeping the intracellular concentration of K^+ from decreasing did not prevent apoptosis (Fig. 8 and 9). This suggests that it is the increase in intracellular Na^+ and not the decrease in intracellular K^+ that is needed for the apoptotic process in *X. laevis* oocytes. This is also consistent with other reports on the role of intracellular Na^+ (Banasiak et al., 2004; Hirn et al., 2008; Poulsen et al., 2010).

To conclude, STS-induced apoptosis in *X. laevis* oocytes increased the Na^+ conductance leading to an increased Na^+ influx. This Na^+ influx leads to an increased caspase-3 activity. The

next step was to identify the gene behind the Na channel responsible for the uprelated Na⁺ current during STS-induced apoptosis in *X. laevis* oocytes to find out if specific downregulation of this channel could prevent apoptosis in *X. laevis* oocytes.

The apoptosis-induced Na channel in *X. laevis* oocytes is a *SCN2A* ortholog (Paper III)

Because of the lack of a fully sequenced *X. laevis* genome, the *X. tropicalis* genome was used instead, in which six Na_v channel genes (*SCN* genes) have been identified (Fig. 13) (Hellsten et al., 2010; Zakon et al., 2011). Detailed description of the development of the methods in paper III can be found in the methods section (“Finding a gene in an unsequenced genome” and “Alter the expression level of a *SCN2A* ortholog”). In brief, short sequences of six different Na_v channels were obtained from *X. laevis* and they were all orthologs to a specific *SCN* gene in the human and *X. tropicalis* genomes. Plasmids expressing miRNA against the *SCN2A*, *SCN5A* and *SCN8A* orthologs were constructed. Electrophysiological recordings were performed two days after oocytes had been injected with plasmids containing pre-miRNA. Oocytes that had been injected with miRNA-targeting mRNA related to the *SCN2A* gene (miRNA-*SCN2A*) showed a 58 % reduction of the Na⁺ current at +100 mV compared to control oocytes, whereas miRNA targeting mRNA related to *SCN5A* and *SCN8A* genes had no effect on the Na⁺ current (figure 1c in paper III).

Figure 13. Phylogenetic tree showing the ten human *SCNA* genes (*hSCNXA*), the corresponding Na_v channel proteins and the six orthologs identified in *X. tropicalis* genome (*xtSCNXA*). Modified picture from Zakon et al., 2011.

To conclude, the upregulated Na^+ current in apoptotic *X. laevis* oocytes was found to be conducted through a *SCN2A* channel ortholog. In the following section we investigated whether suppressing the expression of the *SCN2A* channel ortholog could impact the survival of *X. laevis* oocytes.

Inhibiting the *SCN2A* ortholog upregulation in *X. laevis* oocytes prevented cell death (Paper III)

Plasmids must be injected into the nucleus to be transcribed and express miRNA in *X. laevis* oocytes (Bohnsack et al., 2004; Lund and Dahlberg, 2006). To inject plasmids into the nucleus, the oocytes were centrifuged with the purpose to get the nucleus close to the cell membrane. However, the centrifugation also triggered apoptosis as judged by several signs: (i) Morphological changes (white spots in the dark pole and vice versa, and a diffuse line between the poles) were apparent two days after centrifugation for uninjected oocytes, but not for oocytes expressing miRNA-*SCN2A* (Fig. 14A, top). (ii) The resting membrane potential was more positive two days after centrifugation in uninjected oocytes compared to miRNA-*SCN2A* expressing oocytes (Fig. 14A, bottom). Depolarisation of the resting membrane potential is an apoptotic marker in oocytes and other cells (Bhuyan et al., 2001; Bortner et al., 2001). Unpublished data from paper I and II shows that depolarisation of the membrane potential happens within the first six hours after treatment with $1 \mu\text{M}$ STS during apoptosis in *Xenopus* oocytes, Fig. 15).

Figure 14. Rescue of resting membrane potential, sodium current, and viability when oocytes express miRNA against *SCN2A* A) (top) Morphology of *Xenopus* oocytes in miRNA *SCN2A*-expressing oocytes and uninjected oocytes with or without incubation of $20 \mu\text{M}$ STS at day two after centrifugation (bottom). Mean resting membrane potential in the same oocytes. B) Number of cells (%) possible to record from with two-electrode voltage-clamp. Data expressed as mean $\pm$ SEM. P values calculated using one-way analysis of variance (ANOVA). ** $P \leq 0.01$.

(iii) Electrophysiological recordings could only be performed in 40 % of the uninjected oocytes, while it was possible to perform proper electrophysiological recordings on all of the miRNA-SCN2A injected oocytes (Fig. 14B, green column). Thus, miRNA-SCN2A expression clearly rescued the oocytes from the apoptotic signs induced by centrifugation.

Figure 15. Depolarisation of the membrane when *X. laevis* oocytes were treated with 1 μ M staurosporine for 6 hours in room temperature. Data expressed as mean $\pm$ SEM and P values calculated using unpaired t-test (* $P < 0.05$).

Electrophysiological recordings performed two days after centrifugation showed a six-fold increase in Na^+ current in uninjected oocytes compared to control oocytes (see figure 5e (black line) in paper II) (Fig. 16A and B, black trace and column). However, this increase in Na^+ current could be prevented if the oocytes were injected with plasmids expressing miRNA-SCN2A directly after the centrifugation (Fig. 16A and B, green trace and column).

Figure 16. Na^+ current in oocytes when oocytes express miRNA against SCN2A. A) Na^+ current recordings in uninjected oocytes (black), miRNA SCN2A-expressing oocytes (green), and miRNA-expressing oocytes incubated in staurosporine (STS, 20 μ M) (red). Recordings at +100 mV from a holding potential of -80 mV. B) Mean Na^+ current at +100 mV. Data expressed as mean $\pm$ SEM. P values calculated using one-way analysis of variance (ANOVA). * $P \leq 0.05$ and ** $P \leq 0.01$.

Previous results showed that by inducing apoptosis in *Xenopus* oocytes with 1 μ M STS for six hours increased the Na^+ current almost five-fold at +100 mV (Fig. 11B), doubled the intracellular Na^+ concentration, and doubled the caspase-3 activity (Fig 12B and 12C). Injection of miRNA prevented these effects. Despite that the concentration of STS was increased from 1 to 20 μ M, expression of miRNA-SCN2A reduced the current by 50 % compared to oocytes only centrifuged (Fig. 16A and B). Furthermore, expression of miRNA-SCN2A rescued the oocyte from alterations in morphology and resting membrane potential (Fig. 14A and B).

The reversal potential for Na^+ was altered because of a change in the intracellular Na^+ concentration in centrifuged oocytes (Fig. 17A and B). The intracellular Na^+ concentration was five times higher in centrifuged oocytes compared to control oocytes. Expression of miRNA-SCN2A reduced the intracellular Na^+ concentration back to normal (Fig. 17B). In addition, expression of miRNA-SCN2A prevented an increase in intracellular Na^+ concentration provoked by 20 μM STS for six hours (Fig. 17A and B).

Figure 17. Changes in intracellular Na^+ concentration. A) Current versus voltage graphs revealing a change in reversal potential for centrifuged oocytes (black) at day two after centrifugation compared to oocytes that also expresses miRNA *Scn2a* (green) and miRNA-SCN2A expressing oocytes that was incubated with STS (20 μM) for six hours (red). B) Mean intracellular Na^+ concentration that was calculated using Nernst equation. Intracellular Na^+ concentration for control oocytes (control*, black) were taken from other reports (Asif et al., 2010; Baud et al., 1982; Dascal, 1987). Data expressed as mean $\pm$ SEM. P values was calculated using one-way analysis of variance (ANOVA). *** $P \leq 0.001$.

Apoptosis was studied by measuring the caspase-3 activity in centrifuged oocytes. Centrifuged oocytes and centrifuged oocytes injected with miRNA-SCN2A showed similar caspase-3 activity two days after centrifugation (Fig. 18). The reason why no change in caspase-3 activity was detected most likely depends on the time point of the measurement. Caspase-3 increase occurs early after centrifugation (about 6-12 hours) and the caspase-3 activity has already declined after 48 hours. An additional treatment with 20 μM STS for 6 hours did not increase the caspase-3 activity (Fig. 18 black bar). Again, this most likely depends on the late time period; initiation of apoptosis occurred while the oocytes were centrifuged, so STS is unable to reinitiate apoptosis at day two after apoptosis. In contrast, the apoptotic process initiated by centrifugation was prevented in oocytes expressing miRNA and could be initiated, as seen by a two-fold increase of caspase-3 activity, by provocation of 20 μM STS (Fig. 18, red column).

The enzyme dicer that cleaves pre-miRNA into mature miRNA, have only one fourth of the activity in *X. laevis* oocytes compared to matured cells (Lund et al., 2011; Muggenhumer, 2010; Muggenhumer et al., 2014). Therefore, the Na channel activity is only suppressed by 58 % in oocytes expressing miRNA-SCN2A (figure 1c in paper III). This means that the number of active Na channels can be increased to the point where the caspase-signaling pathway can be triggered using enough provocation. That is likely why provocation with 20 μM STS on miRNA-

SCN2A expressing oocytes raises the number of active Na channels and initiate apoptosis in the same way as 1 μ M STS did in paper II.

Figure 18. Mean caspase-3 activity for centrifuged oocytes (black) at day two after centrifugation compared to oocytes that also expresses miRNA *Scn2a* (green) and miRNA-*SCN2A* expressing oocytes that was incubated with staurosporine (STS, 20 μ M) for six hours (red). Also in this figure, centrifuged oocytes that also was incubated with 20 μ M STS for six hours at day two after centrifugation (black column, right). Data expressed as mean $\pm$ SEM (20 oocytes/n) P values was calculated using one-way analysis of variance (ANOVA). * P $\leq$ 0.05.

To conclude, suppressing upregulation of the Na⁺ conducting *SCN2A* channel ortholog prevents mechanically-induced apoptosis in *X. laevis* oocytes.

General discussion

A major role of a voltage-gated Na channel and the intracellular Na⁺ concentration in apoptosis in *X. laevis* oocytes has emerged during the work of this thesis. However, the majority of published studies are about voltage-gated K and Ca channels and their involvement in the apoptotic process. So, why is a voltage-gated Na channel and intracellular Na⁺ important for apoptosis in *X. laevis* oocytes? Below I will discuss the role of intracellular monovalent metal ions in the apoptotic volume decrease, the role of intracellular monovalent metal ions for the apoptotic process, and some properties of the Na channel described in paper III.

The role of ions in apoptotic volume decrease

Decrease in cell volume is caused by a decrease in intracellular K⁺ and Cl⁻ ions, which causes extrusion of water due to osmosis (Dezaki et al., 2012; Ise et al., 2005; Maeno et al., 2000; Wei et al., 2004). There has been contradictory reports on the involvement of Na⁺ during the apoptotic volume-decrease process, where both reduced and increased Na⁺ influx have been suggested to cause the cell to swell (Bortner and Cidlowski, 2003; Carini et al., 1995; Koike et al., 2000). Substituting extracellular Na⁺ with either choline⁺ or tetramethylammonium caused the *X. laevis* oocyte to swell and become fragile, whereas substituting with K⁺ did not swell the oocyte. This is in line with reports on other cell types (Bortner et al., 2001; Bortner and Cidlowski, 2003; Thompson et al., 2001). Even though volume decrease is a hallmark of apoptosis, several studies have shown that it is the increased ionic fluxes across the membrane that are important during apoptosis rather than the change in volume (Bortner and Cidlowski, 2003; Hernández-Enríquez et al., 2010; Maeno et al., 2012).

The role of Na⁺ and K⁺ in apoptosis

Studies have revealed that the decrease of intracellular K⁺ is directly involved in the initiation and regulation of the apoptotic signaling process. The reduced intracellular K⁺ concentration directly affects the cleavage of procaspases (8 and 3) to active caspases, the release of cytochrome c and inhibition of Apaf-1, which in turn affects the formation of the apoptosomes (Cain et al., 2001; Karki et al., 2007; Thompson et al., 2001). Paper I showed that a decrease of intracellular K⁺ occurred in *X. laevis* oocytes, however this decrease was not necessary for the activation of caspase-3.

Several reports that have their main focus on K⁺ and its role in apoptosis also report of the effect of intracellular Na⁺ on the apoptotic signaling cascade (Bortner et al., 2001; Hampton et al., 1998; Hughes et al., 1997; Thompson et al., 2001). These studies indicate that high extracellular K⁺ concentrations suppresses both the extrinsic and intrinsic apoptotic pathways, and that low extracellular Na⁺ suppresses the intrinsic apoptotic pathway (Thompson et al., 2001). One hypothesis is that Na⁺ regulates caspase-2 dependent release of cytochrome c (Robertson et al., 2002). Further evidence for involvement of Na⁺ in apoptosis is reports of

increase in the number of activated voltage-gated Na channels during apoptosis in both neural and cardiac cells, and that blocking or reduction of the number of voltage-gated Na channels prevents cell death (Banasiak et al., 2004; Dave et al., 2003; Hirn et al., 2008; Zhan et al., 2007). This was also seen in the present thesis work during apoptosis in *X. laevis* oocytes, where inhibiting the increase of the number of active voltage-gated Na channel prevents apoptosis (paper III).

Several studies point out that it is not the specific ion *per se* that is involved in the apoptotic signaling, but rather a decrease in intracellular ionic strength (Arrebola et al., 2006, 2005a, 2005b; Hughes et al., 1997; Perez et al., 2000). Furthermore, increase in ionic strength has been shown to block the cytochrome c activated caspase-3 pathway (Hampton et al., 1998). Metal ions, such as Ca^{2+} , Zn^{2+} and Mg^{2+} are well-known to associate with and stabilise different protein structures and are thus important for their function. However, also monovalent ions, such as Na^+ and K^+ have been reported to associate with and affect protein structures. Both Na^+ and K^+ associate with amino acids bearing a hydroxyl group side chain, but Na^+ interacts with a higher affinity than K^+ (Ye et al., 2008). This interaction between monovalent ions with different affinities to amino acids could be one important mechanism regulating proteins involved in the apoptotic process. This is thus one mechanism in which the intracellular Na^+ concentration could be involved in the apoptotic process in *X. laevis* oocytes and other cells.

In either case, regulation of the ionic composition is important during apoptosis. One way to alter the ionic composition is to regulate the activity of plasma-membrane bound ion channels, transporters and pumps (Lang and Hoffmann, 2012). If the intracellular concentrations of ions during apoptosis are not tightly regulated, apoptosis can easily turn over to necrosis (Barros et al., 2001; Koike et al., 2000; Maeno et al., 2012; Okada et al., 2004). *X. laevis* oocytes with their endogenous expression of ion transducing transmembrane proteins and ability to sustain intracellular manipulation can be used as a model system to study the role of ions and ionic signaling during apoptosis (Goldin, 1992; Sobczak et al., 2010).

The results in paper II and III clearly show that the voltage-gated Na channel and intracellular Na^+ concentrations were important for caspase-3 activity, whereas the results in paper I clearly showed that alterations in intracellular K^+ concentration was not necessary for the increased caspase-3 activity. One important aspect to take into consideration is that different apoptotic stimuli can result in different apoptotic signaling responses. Apoptosis can be initiated through several different pathways. Depending on the apoptotic stimuli used, different voltage-gated ion channels and ion concentrations may be most important (Thompson et al., 2001).

The voltage-gated Na channel upregulated during apoptosis in *X. laevis* oocytes is a human voltage-gated Na channel *SCN2A* ortholog

The voltage-gated Na channel described in paper II, and which identity is revealed in paper III, is most likely responsible for causing the Na_x current that has previously been described in *X. laevis* oocytes (Baud et al., 1982; Vasilyev et al., 2002), not to be confused with human *SCN7A*

which also has been called Na_x . The Na_x current activates at positive voltages and does not inactivate. In the present thesis work, this channel was demonstrated to be upregulated during apoptosis, and in previous studies it is upregulated after a prolonged depolarisation. Both our apoptotic-induced Na^+ currents reported here and the Na_x current reported by others are blocked by a high concentration of lidocaine (reported in paper II)(Baud et al., 1982; Charpentier, 2002; Vasilyev et al., 2002). By using miRNA to suppress the Na^+ current upregulated during apoptosis we could identify the channel conducting the Na^+ current as a *SCN2A* ortholog.

We discovered *SCN2A* ortholog was insensitive to TTX, which is a classical Na-channel blocker. TTX binds to the extracellular part of the conducting pore of a sensitive Na channel. However, if a cysteine is introduced to this part of the channel, it reduces the affinity to TTX by a factor of >1000 (Heinemann et al., 1992). All of the TTX-resistant mammalian Na channels ($\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$) have a cysteine in the selectivity filter. However, the voltage-gated Na channel identified in paper III is orthologous to mammalian $\text{Na}_v1.2$. Interestingly, contrary to mammalian voltage-gated Na channels, the genome sequence of the *X. tropicalis* revealed that the $\text{Na}_v1.2$ ortholog has a cysteine in the selectivity filter, while the $\text{Na}_v1.5$ ortholog lacked a cysteine. Thus, it is likely that the *X. laevis* $\text{Na}_v1.2$, reported here in paper II and III, also have a cysteine in its selectivity filter, and this could be why the apoptotic Na^+ current in *X. laevis* oocytes is not affected by TTX.

The $\text{Na}_v1.2$ channel found in this thesis lacked fast inactivation (paper II). For other Na channels, fast inactivation is controlled by a conserved hydrophobic cluster consisting of isoleucine, phenylalanine, and methionine (IFM-cluster), located in the intracellular linker between domain III and domain IV (West et al., 1992). Investigation of the *SCN* orthologs in the *X. tropicalis* genome revealed that all of them have an intact IFM cluster. This suggests that the absence of inactivation of the apoptotic-induced voltage-gated Na channel in *X. laevis* is caused by other parts of the channel.

Conclusions

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The results in this thesis manifests

- that a decrease in intracellular K^+ occurs in *X. laevis* oocytes during STS-induced apoptosis, but this is not necessary for the apoptotic signaling cascade,
- that a Na^+ current is upregulated during STS- and mechanically-induced apoptosis in *X. laevis* oocytes and that the maintaining intracellular Na^+ concentration prevents apoptosis,
- that the identity of the Na channel behind the upregulated Na^+ current during STS-induced apoptosis in *X. laevis* oocytes is an ortholog to the human *SCN2A* gene, and
- that inhibition of the *SCN2A* channel ortholog upregulation during apoptosis, revealed a crucial role for the *SCN2A* channel ortholog during apoptosis in *X. laevis* oocytes.

The *X. laevis* oocyte is relevant as a model system to further study the connection between internal apoptotic-signaling pathways, voltage-gated ion channels and the functional role of metal-ion concentrations during apoptosis.

Future perspectives

The series of three papers presented in this thesis has led to the discovery that the *SCN2A* ortholog codes for an atypical Na channel expressed in and being crucial for the apoptotic process in *X. laevis* oocytes. The electrophysiological properties of the *SCN2A* channel ortholog are a bit different compared to the human *SCN2A*. Future work on this finding would be to amplify and convert the mRNA sequence for the *SCN2A* ortholog to cDNA and sequence it to get the whole-gene sequence. The next step would be to clone the channel and overexpress it in a cell system and further characterise the electrophysiological properties with whole-cell and single-channel recordings.

Three important questions remain to be answered:

1. Why does this *SCN2A* ortholog lack fast inactivation (paper II). All of the *SCN* orthologs in the *X. tropicalis* genome seems to have an intact IFM cluster. This suggests that the absence of inactivation of the apoptotic-induced Na channel in *X. laevis* is caused by other parts of the channel. If the whole-gene sequence could be obtained, the sequence could be investigated to see if the answer lies at the amino acid level. It could also be that the Na channel is built up by an α - and an unknown auxiliary subunit. If this is the case, then expressing the Na channel in other cell system that do not naturally express the auxiliary subunit, could alter the electrophysiological properties of the channel and regain the fast inactivation.

2. Which signal upregulates the channel during apoptosis? There is an increase in Na^+ conductance through this Na channel during apoptosis and both STS and mechanically induced apoptosis triggers upregulation. Is there a release of inhibition through phosphorylation, conformational changes, or is it an increase in the number of Na channels in the membrane, either through vesicle storage or gene transcription? The phosphorylation part of the question can be examined through specific protein kinase inhibitors. STS is an unspecific protein kinase inhibitor and by targeting different kinase signaling pathways, which of them that upregulates the Na channel could be investigated. The second part could be examined if apoptosis could be induced only by injection of mRNA thereby getting an overexpression of the channel. However, this requires that the mRNA sequence is obtained.

3. What happens downstream of the upregulated channel in the apoptotic signaling pathway? Is it the channel-protein itself or the Na^+ ion that have a critical role in the apoptotic pathway or both? A future investigation is to measure caspase-3 activation when *SCN2A* ortholog expression is suppressed by miRNA against its mRNA followed by injections of high Na^+ solution. However, preliminary data has shown that injections of Na^+ and other metal ions do not trigger an upregulation of the voltage-gated Na channel so a positive feedback loop, where an increase in intracellular Na^+ concentration trigger increase in Na channel upregulation, is unlikely. It could be that more membrane proteins are involved in keeping the increase in Na^+ concentration during apoptosis. One possibility is the involvement of Na transporters and exchangers so that increasing the intracellular Na^+ concentration by injections are prohibited by Na^+ being pumped out.

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