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Calcium signaling in fish cells

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Für meine Kinder

The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

Albert Einstein

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Contents

Summary

One basic cellular response towards a multitude of physical and chemical factors is the modulation of intracellular Ca^{2+} levels. There is also considerable evidence that a number of toxicants have an impact on Ca^{2+} signaling processes, alter them, and may induce cell death. Given the immense versatility of Ca^{2+} modulation due to the complex mechanisms which help to encode information, recording of the intracellular Ca^{2+} signal might eventually be a useful tool for the detection and identification of environmental stressors. Notwithstanding the universal character of Ca^{2+} signaling and the highly conserved pathways, research on Ca^{2+} as a second messenger has mainly been restricted to mammalian models. Much less is known about its function and mode of action in fish and only a handful of papers deal with the question whether there is a Ca^{2+} response to environmental toxicants or not.

The present thesis aims at closing some of the remaining gaps. Therefore, after adapting the cell culture and Ca^{2+} imaging protocols for the needs of this study, the reaction of intracellular Ca^{2+} to different "classical" agonists such as phenylephrine and ATP was investigated systematically in order to find out the basic principles of Ca^{2+} dynamics in teleost fish cells. Two cell types were used and compared to one another: primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*), and the permanent fish cell line RTL-W1 derived from rainbow trout liver, both established model systems in aquatic ecotoxicology. From an ecotoxicological point of view, we tried to answer the question whether Ca^{2+} imaging can be applied for the early detection of environmental stress with cell death as a last consequence. Therefore, selected model environmental toxicants and stressors such as 4-nitrophenol, 3,4-dichloroaniline, and hydrogen peroxide were used to elucidate possible interactions between contaminants and Ca^{2+} signaling in RTL-W1 cells.

Ca^{2+} oscillations in response to several stimuli were recorded in RTL-W1 cells and to a lesser extent in primary hepatocytes. Interestingly, these Ca^{2+} oscillations are amplitude-encoded in contrast to their mammalian counterpart. Moreover, Ca^{2+} release in rat cells during oscillations is markedly faster than the uptake, whereas this relation is more symmetric in the fish cells. Bioinformatics and computational analysis were employed to identify key players of Ca^{2+} signaling in fish and to determine likely causes for the experimentally observed differences between the Ca^{2+} dynamics in fish cells compared to those in mammalian liver cells. Different binding characteristics of the IP_3R, e.g. responsible for the Ca^{2+}-induced Ca^{2+} release, could be the origin of the observations.

The present thesis also indicates that the fish cell line RTL-W1 is a suitable tool for the investigation of Ca^{2+} signals in consequence of toxicant exposure. Evidence is provided that ecotoxicologically relevant substances take influence on intracellular Ca^{2+}. Namely hydrogen peroxide and 4-nitrophenol showed a clear response and produced marked Ca^{2+} oscillations at sublethal concentrations. Effect intensity and threshold varied from cell to cell; however, general effects were reproducible and dose-dependent. At concentrations below those inducing elevated cytotoxicity and apoptosis rates (assessed by the neutral red assay and the apoptosis assay with Hoechst 33342), there is a lasting, unspecific increase in the intracellular Ca^{2+} level which might be interpreted as a precursor of apoptotic or necrotic processes in the cell.

Generally, Ca^{2+} signals seem to be dependent on the pathway activated or non-specifically interfered by the respective substance. The question whether specific types of Ca^{2+} responses are specific of and may be used to characterize different types of stressors still cannot be answered at present. However, there is evidence that Ca^{2+} imaging might provide a highly sensitive, yet non-specific indicator of toxic impact, since, as a second messenger, intracellular Ca^{2+} integrates toxic effects of multiple other sublethal parameters.

Zusammenfassung

Die Modellierung des intrazellulären Ca^{2+}-Spiegels ist eine grundlegende zelluläre Reaktion auf eine Vielzahl physikalischer und chemischer Faktoren. Es deutet vieles darauf hin, dass toxische Substanzen einen Einfluss auf Ca^{2+}-Signalwege ausüben, sie modifizieren und den Zelltod einleiten können. Die Ca^{2+}-Modulierung zeichnet sich durch große Variabilität aus, welche durch die komplexen Mechanismen ermöglicht wird, die zur Codierung der Information beitragen. Möglicherweise könnte sie daher als Werkzeug zur Identifizierung von Umweltstressoren dienen. Ungeachtet der Universalität von Ca^{2+} als sekundärem Botenstoff und der stark konservierten Signalwege beschränkt sich die Ca^{2+}-Forschung bisher weitgehend auf Säugermodelle. Über die Funktionen und Wirkmechanismen in Fischzellen ist wenig bekannt, und die Frage nach einer Ca^{2+}-Antwort auf Umweltchemikalien wird nur in wenigen Studien behandelt.

Die vorliegende Arbeit soll einige dieser Lücken schließen. Nach der Anpassung und Optimierung der Zellkulturbedingungen und Ca^{2+}-Imaging-Protokolle wurde daher die Reaktion des intrazellulären Ca^{2+}-Spiegels auf verschiedene „klassische“ Agonisten wie Phenylephrin und ATP untersucht, um die grundlegenden Prinzipien der Ca^{2+}-Modellierung in Fischzellen zu erkennen. Zwei Zelltypen und etablierte Modellsysteme der aquatischen Ökotoxikologie wurden miteinander verglichen: Primärhepatocyten der Regenbogenforelle (*Oncorhynchus mykiss*) sowie die permanente Fischzelllinie RTL-W1 aus der Leber der Regenbogenforelle. Aus ökotoxikologischer Sicht sollte ferner die Frage beantwortet werden, ob Ca^{2+}-Messungen zur frühzeitigen Erkennung von Umweltstress – mit dem Zelltod als letzter Konsequenz – eingesetzt werden können. In RTL-W1-Zellen wurden daher ausgewählte Umweltchemikalien wie 4-Nitrophenol, 3,4-DCA und Wasserstoffperoxid untersucht, um mögliche Interaktionen zwischen Schadstoffen und Ca^{2+}-Signal aufzudecken.

Es konnten Oszillationen des Ca^{2+}-Spiegels als Reaktion auf verschiedene Stimuli sowohl in RTL-W1-Zellen als auch (in geringerem Maße) in Primärhepatocyten nachgewiesen werden. Bemerkenswerterweise sind diese im Gegensatz zu Ca^{2+}-Oszillationen in Säugerzellen amplitudencodiert. Darüber hinaus ist das Verhältnis Anstieg/Abfall des Ca^{2+}-Gehalts während der Oszillation in Fischzellen symmetrischer als in Säugerzellen. Mit Hilfe von Bioinformatik und Computeranalysen sollten die Hauptkomponenten der Ca^{2+}-Signalwege in Fischzellen identifiziert und mögliche Ursachen für die experimentell beobachteten Unterschiede zwischen Fisch- und Säugerzellen erkannt werden. Unterschiedliche Bindungseigenschaften des IP_3-Rezeptors, verantwortlich beispielsweise für den CICR (Ca^{2+}-induced Ca^{2+} release, Ca^{2+}-induzierte Ca^{2+}-Freisetzung), könnten demnach die Ursache der Beobachtungen sein.

Die vorliegende Studie zeigt auch, dass die Fischzelllinie RTL-W1 ein gutes Mittel zur Untersuchung von Veränderungen des Ca^{2+}-Spiegels in Folge toxischer Exposition ist. Es konnte nachgewiesen werden, dass ökotoxikologisch relevante Substanzen intrazelluläre Ca^{2+}-Konzentrationen beeinflussen. Insbesondere Wasserstoffperoxid und 4-Nitrophenol zeigten eine klare Wirkung und riefen deutliche Oszillationen in subletalen Konzentrationen hervor. Effektintensität und Wirkschwelle variierten von Zelle zu Zelle; die allgemeinen Effekte waren jedoch reproduzierbar und konzentrationsabhängig. Konzentrationen, welche noch keine erhöhte Cytotoxizität und Apoptoseraten hervorriefen, bewirkten einen anhaltenden unspezifischen Anstieg des intrazellulären Ca^{2+}-Gehalts, der als Vorbote apoptotischer oder nekrotischer Prozesse in der Zelle gedeutet werden kann.

Generell scheinen Ca^{2+}-Signale von dem Signalweg abzuhängen, welcher durch die jeweilige Substanz aktiviert oder in welchen unspezifisch eingegriffen wird. Die Frage, ob bestimmte Ca^{2+}-Signaltypen spezifisch für bestimmte Stressortypen sind und zu deren Charakterisierung genutzt werden können, kann zum gegenwärtigen Stand der Forschung nicht beantwortet werden. Dennoch könnten Untersuchungen des zellulären Ca^{2+}-Spiegels als sehr sensitiver, wenn auch unspezifischer, Indikator toxischer Belastung dienen, da dieser als sekundärer Botenstoff die Effekte vieler weiterer subletaler Parameter integriert.

Chapter 1

Introduction

Die Natur
Sie hat keine Sprache noch Rede, aber sie schafft Zungen und Herzen, durch die sie fühlt und spricht.

Johann Wolfgang von Goethe (1749 - 1832)

1 Motivation and structure of the present study

1.1 Motivation

Notwithstanding the universal character of Ca^{2+}-signaling and the highly conserved pathways, research on Ca^{2+} as a second messenger has mainly been restricted to mammalian models. Much less is known about its function and mode of action in fish. There are several publications on the adrenergic (Krumschnabel et al. 2001, Moon et al. 1993, Zhang et al. 1992a, Zhang et al. 1992b) and purinergic (Nathanson and Mariwalla 1996) modulation of the intracellular Ca^{2+} level as well as its reaction to angiotensin (Russell et al. 2001). However, most of these studies are very specific and do not analyze the modulation of the Ca^{2+} signal systematically and in a dose-dependent manner. Moreover, regular agonist-induced Ca^{2+} oscillations have been reported only for few cell types and substances. Finally, only a handful of papers deal with the question whether there is a Ca^{2+}-response to environmental toxicants or not, mostly related to oxidative stress (Betoulle et al. 2000, Burlando and Viarengo 2005, Nawaz et al. 2006). Due to their taxonomic and ecological positions, however, fish are important model organisms for both vertebrates and aquatic systems.

The present study aims at closing some of the remaining gaps. Therefore, the reaction of intracellular Ca^{2+} to different agonist stimuli in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*), an established model system in aquatic ecotoxicology, as well as in the permanent fish cell line RTL-W1 originally derived from primary cultures of rainbow trout liver (Lee et al. 1993) was investigated systematically in order to find out the basic principles of Ca^{2+} dynamics in teleost fish cells. Since liver is a key organ in detoxification, primary hepatocytes as well as the liver-derived cell line seemed appropriate experimental systems.

In concrete terms, the present study was designed to answer the following questions:

(1) Are there Ca^{2+} alterations/oscillations in isolated fish hepatocytes derived from rainbow trout and RTL-W1 cells as models for fish cells?

(2) Are there differences between intracellular Ca^{2+} signaling in fish cells compared to mammalian cells? Which pathways are playing a role in fish? Is it possible to create a general model for the behavior of intracellular Ca^{2+} in fish cells?

(3) Is it possible to transfer the *in vitro* results to the conditions in fish *in vivo*? Which experimental system – the primary hepatocytes or the cell line RTL-W1 – is more appropriate to serve as a model for the behavior of intracellular Ca^{2+}?

(4) Which parameters take an influence on intracellular Ca^{2+} alterations in fish cells?

(5) May Ca^{2+} imaging be applied for the early detection of cell death?

(6) Does Ca^{2+} imaging provide new chances in environmental toxicology? Can intracellular Ca^{2+} alterations/oscillations be used as biomarkers of contamination at a long term?

1.2 Structure

The present thesis consists of five parts (chapters): Chapter 1 (this) gives a general introduction to the mechanisms of intracellular Ca^{2+} signaling and the experimental systems and assays applied in the study. The chapters 2 to 4 are individual sections consisting of their own abstract, introduction, material & methods, results, discussion and conclusions, as a whole reflecting the development of this thesis.

Chapter 2 describes the adaption of an isolation and culture protocol of primary hepatocytes for the routine application of primary hepatocytes in laboratory in order to get viable and robust cells and reproducible results. Several factors were investigated to find out the most adequate culture conditions, especially with respect to the application of primary hepatocytes in Ca^{2+} imaging.

The two following chapters 3 and 4 have been submitted for publication in international journals. In chapter 3 (Schweizer et al. 2011b), the reaction of intracellular Ca^{2+} to different agonist stimuli in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*) as well as the permanent fish liver cell line RTL-W1 was investigated systematically. Bioinformatics and computational analysis were employed to identify key players of Ca^{2+} signaling in fish and to determine likely causes for the differences observed experimentally between the Ca^{2+} dynamics in fish liver cells compared to that in mammalian liver cells.

In chapter 4 (Schweizer et al. 2011a), selected model environmental toxicants and stressors such as 4-nitrophenol, 3,4-dichloroaniline, and hydrogen peroxide were used to elucidate possible interactions between contaminants and Ca^{2+} signaling. For better differentiation between agonist-specific effects and general cytotoxic or apoptotic effects on intracellular Ca^{2+}, the neutral red assay according to (Borenfreund and Puerner 1984) and apoptosis assay with Hoechst 33342 were performed.

Chapter 5 provides an overall discussion and conclusions aiming at answering the questions raised in part 5.1 of the introduction.

2 Calcium in intracellular signaling

Calcium (Ca^{2+}) plays a fundamental role in many cellular processes in all types of tissues and organisms (for reviews see e.g. Berridge et al. 2000a, Berridge et al. 2000b, Petersen et al. 2005, Putney Jr 1998). One important function is its role as a second messenger in cellular signaling transmitting information from the cell surface to specific targets within the cell and controlling a wide range of cellular reactions. In non-excitable cells, Ca^{2+} modulates diverse processes such as cell proliferation, egg activation and early development, contraction, secretion, gene regulation, the control of various enzymes, and apoptosis (Parekh and Penner 1997). In liver, it regulates many hepatic functions, including glycogenolysis, canalicular contraction, tight junction permeability, and bile secretion (Dupont et al. 2000). Ca^{2+}-signaling also seems to play a role in the response to environmental stress including the induction of cell death as a consequence of the loss of intracellular Ca^{2+} homeostasis (see e.g. Duchen 2000, Kass and Orrenius 1999, Orrenius 2004, Orrenius et al. 2003).

The versatility of Ca^{2+} signaling is remarkable and is due to the complex mechanisms which help to encode information. Numerous Ca^{2+}-providing and -detracting pools and reactions allow the temporal and spatial modulation of the Ca^{2+} signal: Ca^{2+} can operate locally or throughout the entire cell. The signal can last over different periods of time, as short transient or as sustained signal (Berridge et al. 1999). Special emphasis has been put on oscillations of the cytosolic Ca^{2+} concentration, as they permit a large number of possibilities for the encoding of information by the modulation of amplitude, wavelength and waveform of the transients. Since the 1980s, when Ca^{2+} oscillations were found experimentally (Cuthbertson and Cobbold 1985, Woods et al. 1986), a great number of experimental and theoretical studies have been carried out (Schuster et al. 2002).

2.1 Ca^{2+} pools, receptors, and signaling pathways

The extracellular Ca^{2+} concentration in the organism is > 1 mM, whereas the cytosolic Ca^{2+} concentration is approximately 100 nM at the resting state. Ca^{2+} concentrations in the mitochondrial and nuclear matrices are similar. In contrast, the endoplasmic/sarcoplasmic reticulum (ER/SR) features high Ca^{2+} concentration (100 - 500 µM) as compared to Ca^{2+} in the cytosol (Roy and Hajnoczky 2008). Various channels, transport ATPases, uniporters, and antiporters in the plasma membrane, endoplasmic and sarcoplasmic reticulum, and mitochondria are responsible for the transport of Ca^{2+} across membranes and Ca^{2+} mobilization from the intracellular pools (for review see e.g. Saris and Carafoli 2005).

2.1.1 Cytosolic Ca^{2+}

The gradients between Ca^{2+} in the cytosol on the one side and the internal storage sites and the extracellular compartment on the other side are maintained by the action of various

receptors and pumps. The activity of Ca^{2+}/Mg^{2+} ATPases, the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) and the plasma membrane Ca^{2+} ATPases (PMCAs), ensure primarily the maintenance of the large Ca^{2+} gradients (Misquitta et al. 1999, Roy and Hajnoczky 2008). They are necessary to reduce cytosolic Ca^{2+} to the low resting levels by energy consumption. In addition there are exchangers that utilize gradients of other ions to provide the energy to transport Ca^{2+} out of the cell, e.g. Na^{+}/Ca^{2+} exchange (Bootman et al. 2001). Depending on the concentrations of these cations in the cytosol and external medium and on the direction of the transmembrane potential, Ca^{2+} can be also taken up by exchange against relatively high internal Na^{+} (Saris and Carafoli 2005).

External Ca^{2+} enters the cell through several different types of plasma membrane Ca^{2+} influx channels, which can be grouped on the basis of their activation mechanisms (Berridge et al. 2000b, Bootman et al. 2001):

Voltage-operated channels (VOCCs) are activated by plasma membrane depolarisation and are employed largely by excitable cell types such as muscle and neuronal cells.

Receptor-operated channels (ROCCs) open in response to binding of an extracellular ligand (e.g. ATP, serotonin, glutamate and acetylcholine) and are particularly prevalent on secretory cells and at nerve terminals.

Store-operated channels (SOCCs) open in response to depletion of the internal Ca^{2+} stores, either by physiological Ca^{2+}-mobilizing messengers or pharmacological agents. Enhanced Ca^{2+} entry following Ca^{2+} pool depletion has been shown in many different cell types. Presumably SOCCs are homologues of a protein named transient receptor potential (TRP) that functions in *Drosophila* photoreception.

Mechanically activated Ca^{2+} channels are present on many cell types and respond to cell deformation. Such channels convey information into the cell concerning the stress/shape changes that a cell is experiencing.

In addition to these established Ca^{2+} influx mechanisms, evidence is accumulating in favor of Ca^{2+} channels that are activated by intracellular lipid messengers, such as diacylgylcerol and arachidonic acid in the absence of Ca^{2+} store release (Bootman et al. 2001).

2.1.2 Sarco/endoplasmic reticulum Ca^{2+}

The endoplasmic reticulum (sarcoplasmic reticulum in muscle cells; ER/SR) is the most important internal Ca^{2+} store. As already mentioned in section 1.1.1., several isoforms of an ATP-dependent Ca^{2+}/Mg^{2}-ATPase termed sarco/endoplasmic reticulum Ca^{2}-ATPase (SERCA) play an important role for the accumulation of Ca^{2+} within the ER which is achieved by using the energy of ATP. Besides, SERCAs play a role in the generation of Ca^{2+} waves in various cell types. They are specifically and potently inhibited by thapsigargin (Landgraf et al. 2004, Misquitta et al. 1999).

Ca^{2+} storage in the ER and release of Ca^{2+} to the cytosol is regulated by two types of ligand gated Ca^{2+} release channels: the IP_3 (myoinositol-1,4,5-triphosphate) and ryanodine receptors. Opening of the ER Ca^{2+} release channels provokes increase in cytosolic and decrease in ER Ca^{2+} (Roy and Hajnoczky 2008). It is modulated by numerous factors, including phosphorylation, adenine nucleotides, thiol-reactive compounds, pH and the Ca^{2+} load of the ER/SR (Berridge et al. 2000b). The principal activator of the IP_3 and ryanodine receptors is Ca^{2+} itself. Due to the sensitivity of IP_3 and ryanodine receptors to cytosolic Ca^{2+}, the ER functions as an excitable system to spread signals through the process of the Ca^{2+} induced Ca^{2+} release (CICR) amplifying the smaller trigger event. Ca^{2+}-mobilizing second messengers that are generated when stimuli bind to cell surface receptors determine whether Ca^{2+} can activate these channels (Bootman et al. 2001).

IP_3 receptors

One important second messenger is myoinositol-1,4,5-triphosphate (IP_3), which controls many cellular processes by generating internal calcium signals through binding to the IP_3 receptors (myoinositol-1,4,5-triphosphate receptors; IP_3Rs; Berridge 1993). The IP_3R is almost ubiquitously expressed in mammalian tissues. It consists of four subunits, each with six transmembrane domains (total molecular mass ~ 1200 kDa; Bootman et al. 2001). Three paralogs exist in fish, mammals and amphibians, with IP_3R-1 being the most widely expressed (Hercht 2011).

The binding of many hormones and growth factors to specific receptors on the plasma membrane leads to the activation of phospholipase C (PLC) which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the intracellular messengers myoinositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is highly mobile in the cytoplasm and diffuses into the cell interior. On the ER/SR, IP_3 binds specifically to IP_3Rs effecting conformation change of the receptor such that an integral channel is opened leading to Ca^{2+} entry from the ER/SR into the cytosol along the concentration gradient. IP_3Rs absolutely require IP_3 to open; however, modest increase in Ca^{2+} (0.5 - 1 µM) enhance IP_3 opening, whereas higher Ca^{2+} concentrations (> 1 µM) inhibit their opening. This dependence of IP_3R activity on cytosolic Ca^{2+} allows the complex modulation of intracellular Ca^{2+} signals (Bootman et al. 2001).

Ryanodine receptors

The molecular and physiological properties of the ryanodine receptors (RyRs) are structurally and functionally analogous to IP_3Rs, although they have approximately twice the conductance and molecular mass (Bootman et al. 2001). RyRs are complex molecules, with unusually large cytoplasmic domains containing numerous binding sites for agents that control the state of activity of the channel-forming domain of the molecule (Coronado et al.

1994, Franzini-Armstrong and Protasi 1997). There are four different versions of the ryanodine receptor; RyR-4 is expressed exclusively in fish (Hercht 2011). In contrast to IP_3Rs, RyRs are principally expressed in excitable cells and exhibit affinity to ryanodine, a plant alkaloid, which is an antagonist to the receptor family (Bennett et al. 1996). Interestingly, low ryanodine concentrations (1 - 10 µM) lock the RyRs into a long-lived subconductance state, whilst higher concentrations (~ 100 µM) irreversibly inhibit channel opening (Bootman et al. 2001).

Like IP_3Rs, RyRs are sensitive to cytosolic Ca^{2+} concentrations, although they are generally > 10 µM; Bootman et al. 2001). Caffeine at lower concentrations enhances the sensitivity of the RyRs to Ca^{2+} promoting the CICR such that basal Ca^{2+} concentrations become activating (Wakui et al. 1990).

2.1.3 Mitochondrial Ca^{2+}

The mitochondria are the second cellular storage site. Regulation of intracellular Ca^{2+} by the mitochondria plays a role in many cellular functions (Gunter et al. 2004, Putney and Thomas 2006): During the process of energy production, Ca^{2+} stimulates and controls the rate of oxidative phosphorylation in ATP generation. In addition, Ca^{2+} uptake by mitochondria helps to modulate and "shape" cytosolic Ca^{2+} signals by important feedback mechanisms. Last but not least, Ca^{2+} uptake into mitochondria may be an important step in the initiation of cellular apoptosis (see section 1.2.).

Several Ca^{2+} entry and release mechanisms between the mitochondria and the cytosol are known; however, the precise physiological role and the molecular structure still need to be determined (Hoppe 2010). Close apposition between mitochondria and endoplasmic reticulum (ER) Ca^{2+} release sites facilitates Ca^{2+} uptake into the mitochondria (Putney and Thomas 2006).

The mitochondrial matrix is separated from the cytoplasm by two membranes. The inner mitochondrial membrane has a very limited permeability to ions. The mitochondrial Ca^{2+} uniporter (MCU), at least one non-MCU Ca^{2+} channel and the mitochondrial ryanodine receptor (mRyR) promote Ca^{2+} entry to the mitochondria when cytosolic Ca^{2+} increases, however with relatively low affinity and depending on the inner mitochondrial membrane potential. Therefore, only long-lasting global cytosolic Ca^{2+} signals yield rapid and high elevations in mitochondrial Ca^{2+} from 100 nM to at least micromolar and in some cases > 100 µM (Hoppe 2010, Roy and Hajnoczky 2008).

The role of the mitochondrial influx mechanisms is to remove the Ca^{2+} sequestered during the pulse from the cytosol during the period between pulses in order to maintain intramitochondrial Ca^{2+} homeostasis for repetitive physiological processes (Gunter et al. 2004). There exist two different Ca^{2+} uptake mechanisms: the Na^+ dependent (Na^+/Ca^{2+} cation exchangers) and the Na^+ independent (H^+/Ca^{2+} cation exchangers; Hoppe 2010, Ryu et

al. 2010). The Na^+-dependent mechanism is dominant in heart, brain, skeletal muscle, parotid gland, adrenal cortex, brown fat, and many other tissues, and is inhibited e.g. by tetraphenyl phosphonium, trifluroperazine, diltiazem, verapamil, clonazepam, and amiloride, whilst the Na^+-independent mechanism dominates in liver, kidney, lung, and smooth muscle. There are only a few known inhibitors such as tetraphenyl phosphonium, cyanide, and low levels of uncouplers (Gunter et al. 2000, Gunter and Pfeiffer 1990).

2.2 Ca^{2+} homeostasis and cell death

Ca^{2+} plays an important role as a second messenger in fundamental cellular processes. This makes the regulation of Ca^{2+} an essential prerequisite for the survival of cells and organisms. Deregulation of its homeostasis provokes that Ca^{2+} acts as an intrinsic stressor, producing (further) cell damage. The cell responds to the insult with repair and death mechanisms leading to cell survival or apoptosis, respectively – both mediated by further Ca^2-requiring signaling pathways (Cerella et al. 2010). On one hand, dysregulation of Ca^{2+} signaling may cause cell death; on the other hand, Ca^{2+} signaling can trigger apoptosis (Roy and Hajnoczky 2008). Apoptosis is defined as a specific mode of cell death characterized by morphological changes such as chromatin condensation, fragmentation of the nucleus, contraction of the cytoplasmic volume, and emission of “apoptotic bodies” containing seemingly intact organelles (Belloc et al. 1994). The multiple and in part overlapping mechanisms involving local or global cytosolic Ca^{2+} transients make it difficult to differentiate which of the two fundamental routes – survival or death – has been selected by the cell, and raise the question how the cellular effectors distinguish the respective Ca^{2+} signals determining future of the cell.

In a number of tissues and cell types, toxic insults by different agents and conditions have been found to cause an increase of intracellular Ca^{2+} levels. Studies indicate that the perturbation of Ca^{2+} homeostasis can be interrelated to alterations in mitochondrial functions and the development of cellular injury. Beside their function in the energy metabolism of the cell, it could be demonstrated that mitochondria play an important role in the regulation of cell death (Bellomo et al. 1991, Boehning et al. 2004, Dong et al. 2006, Duchen 2000, Ermak and Davies 2001, Hajnoczky et al. 2003, Ichas and Mazat 1998, Kass and Orrenius 1999, Krumschnabel et al. 2005, Orrenius 2004, Orrenius et al. 2007, Orrenius et al. 2003, Poern-Ares et al. 1998, Putney and Thomas 2006, Rizzuto et al. 2003).

Under normal physiological conditions, Ca^{2+} signal propagation to the mitochondria results in stimulation of ATP production through activation of the Ca^{2+}-sensitive dehydrogenases. However, Ca^{2+} overloading of the mitochondria triggers the mitochondria permeability transition (MPT) leading to uncoupling of the mitochondria membrane culminating in mitochondrial swelling and disruption of the outer mitochondrial membrane (Costantini et al. 1995, Roy and Hajnoczky 2008).

The MPT was proposed in 1979 (Haworth and Hunter 1979). It is a result of the opening of mitochondrial permeability transition pores (MPTP) for molecules of less than 1500 Da. This protein pore is a regulated, voltage-dependent channel of the inner mitochondrial membrane. The MPT strongly compromises the bioenergetic functions in mitochondria. Membrane energization appears to be a prerequisite to the induction of this phenomenon (Toninello et al. 2000). The MPT leads to collapse of the membrane potential and the onset of a bidirectional trafficking of solutes through the inner membrane, which, as a first consequence, induces swelling of the matrix and rupture of the outer membrane. Another consequence of MPT induction is the release of cytochrome c and other proteins from the intermembrane space of the mitochondria into the cytosol, where a protein complex is formed activating several downstream actions ultimately causing apoptosis (Gerasimenko et al. 2002).

The MPT seems to be an important step in the signaling cascade leading to apoptosis. There is evidence for the MPT as a final common point at which the pathways of many toxic agents converge (Toninello et al. 2000). The herbicide paraquat, e.g., induces a Ca^{2+}-dependent permeability increase of the inner mitochondrial membrane leading to membrane depolarization, uncoupling and matrix swelling due to inappropriate opening of the permeability transition pore through a shift of the gating potential to more negative values, allowing pore opening at physiological membrane potential (Costantini et al. 1995). In a subpopulation of rainbow trout hepatocytes, copper induced disruption of Ca^{2+} homeostasis and onset of the MPT as a prerequisite for the stimulation of apoptosis, whereas necrotic cell death in other cells was independent from the MPT (Krumschnabel et al. 2005).

So far, it is difficult to clearly relate a definite Ca^{2+} signal and its cellular response and even more to differentiate between apoptosis and necrosis only considering Ca^{2+} signaling. Criddle et al. (2007) found evidence suggesting that different patterns of cytosolic Ca^{2+} rises influence both apoptotic and necrotic cell death pathways. Physiological agonists evoke cytosolic Ca^{2+} spikes but do not depolarise mitochondria and fail to induce apoptosis. There is increasing evidence that most (if not all) pathologies involve alterations of oxidative metabolism leading to oxidative stress. Oxidation and redox imbalance, however, cause ER and plasma membrane Ca^{2+} channels malfunctions; this increases cytosolic Ca^{2+} and depletes Ca^{2+} in the ER. Moreover, oxidative stress impairs the buffering capacity of mitochondria, lowering the internal Ca^{2+} threshold level of MPTP opening (Cerella et al. 2010). Gerasimenko et al. (2002) suggest that both intracellular release of Ca^{2+} and induction of the MPTP are required for initiation of apoptosis. More severe insults or second and/or continued low stress cause sustained pathological Ca^{2+} elevations which lead to irreversible inhibition of mitochondrial function, cellular ATP production and necrosis.

2.3 Ca^{2+} in environmental toxicology

Environmental toxicology is an interdisciplinary science dealing with toxicants in the environment. The toxicants usually are contaminants or ecotoxicants, which are a diverse group of substances. Ecotoxicants can be defined as substances discharged into the environment through human actions and having the potential to impact on ecosystems at relatively low concentrations. Environmental toxicology can be divided in two subdisciplines with different focus levels: Toxicology studies the effects of toxicants on individual organisms (usually man), whereas ecotoxicology studies the impact of ecotoxicants on ecosystems (Bols et al. 2005).

The main objective of ecotoxicology is to study structural and functional disturbances induced over short-, medium-, and long-term periods by contamination factors in ecological systems. These factors, including all physical, chemical, and sometimes biological agents, result essentially from the direct and indirect effects of anthropogenic activities (Boudou and Ribeyre 1997). Adverse effects can be analyzed at different levels of biological organization, from the molecular, cellular, tissue, organ and organism level, up to populations and ecosystems (Fent 2001). Responses at higher biological organization levels (e.g. population and community responses) are directly indicative of ecosystem health and hence, much more relevant to environmental management. However, they are more difficult to determine, less specific and only manifest at a late stage, when environmental damage has already occurred. On the other hand, responses at lower biological organization levels (e.g., molecular and biochemical responses) are more specific, sensitive, reproducible, and easier to determine, but more difficult to relate to ecological changes (Au 2004).

Interactions of ecotoxicants with biota first take place at the cellular level. During the past decades, new methods in the field of molecular and cellular ecotoxicology have been developed in order to improve our understanding of the effects manifesting at higher biological levels. Cellular toxicology provides a suitable tool for the early and sensitive detection of chemical exposure and helps us to understand ecotoxicological processes and predict toxicological effects at higher biological levels (Fent 2001).

One basic response in cell physiology towards a multitude of physical and chemical factors is the modulation of the intracellular Ca^{2+} level. Ca^{2+} signaling also seems to play a role in the response to environmental stress including cell death (see e.g. Duchen 2000, Kass and Orrenius 1999, Orrenius 2004, Orrenius et al. 2003). Cells possess a complex system to maintain intracellular Ca^{2+} homeostasis. The loss of Ca^{2+} compartmentalization can be reversible or irreversible and often arises in the form of cytoplasmic increases. Irreversible perturbation of the intracellular Ca^{2+} balance activates multiple destructive processes that target subcellular structures including the plasma membrane, the cytoskeleton, mitochondria, and the nucleus, and may lead to cell injury and tissue damage (Dong et al. 2006).

There is considerable evidence that a number of toxic environmental chemicals have an impact on Ca^{2+} signaling processes, alter them, and induce cell death by apoptosis. Toxic agents such as the environmental pollutant tributyltin or the natural plant product thapsigargin, which deplete the ER Ca^{2+} stores, will induce – as a direct result of this effect – the opening of plasma membrane Ca^{2+} channels and a stress response in the endoplasmic reticulum (Kass and Orrenius 1999). This might provide an additional application for Ca^{2+} imaging in ecotoxicology. Given the immense versatility of Ca^{2+} modulation due to the complex mechanisms which help to encode information, the recording of the intracellular Ca^{2+} signal might eventually be a useful tool for the detection and identification of environmental stressors.

3 Agonists

In the present study, the reaction of intracellular Ca^{2+} to different agonist stimuli was investigated systematically in order to find out the basic principles of Ca^{2+} dynamics in teleost fish cells. In addition to "classical" agonists such as phenylephrine and ATP, model environmental toxicants like 4-nitrophenol and 3,4-dichloroaniline were used to elucidate possible interactions between toxin effects and Ca^{2+} signaling.

3.1 "Classical" Ca^{2+} agonists

3.1.1 Adenosine 5-triphosphate (ATP)

ATP (Fig. 1) is a nucleotide with a multitude of functions in the cell, and the main energy source for the majority of cellular functions. It is generated by energy-consuming processes (cellular respiration and photosynthesis) and is consumed by energy-releasing processes (biosynthesis of proteins and nucleic acids, cell division and motility, cytoskeleton, muscle contraction, transport of macromolecules across cell membranes), this way providing energy transfer between spatially separated metabolic reactions.

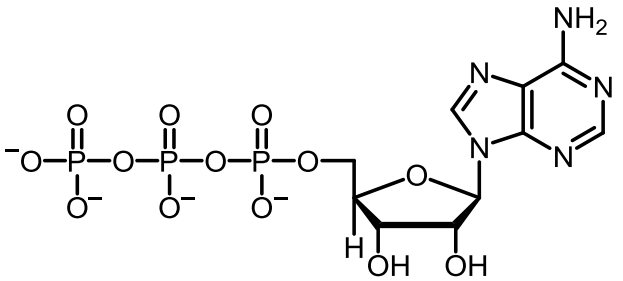

Fig. 1: Adenosine 5-triphosphate (ATP).

Besides, ATP plays a fundamental role in signal transduction pathways: In intracellular signaling, ATP is used as a substrate by kinases which phosphorylate proteins and lipids. Phosphorylation of a protein by a kinase can initiate a signal transduction cascade. The adenylate cyclase transforms ATP to the second messenger cyclic AMP, which is involved in triggering Ca^{2+} signals by the release of Ca^{2+} from intracellular stores. During extracellular signaling, purinergic receptors in the cell membrane recognize ATP, adenosine and other nucleosides as a substrate. These receptors modulate intracellular Ca^{2+} and cyclic AMP levels transducing information from outside into the cell.

Since the lifetime of ATP is short, it is not normally a hormone. Extracellular ATP, however, at micromolar concentrations, induces significant functional changes in a wide variety of cells and tissues (for reviews see (Burnstock 1993, 1997, 2007, Conigrave and Jiang 1995, Dubyak and el-Moatassim 1993, Gordon 1986, Putney and Bird 1993). ATP acts as a neurotransmitter in the autonomic and central nervous system and as a paracrine and, possibly, autocrine modulator (Conigrave and Jiang 1995). Purinergic signaling is involved in exocrine and endocrine secretion, immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation. Cell proliferation, differentiation and

death that occur in development and regeneration are also mediated by purinergic signaling (Burnstock 2007).

ATP and extracellular nucleotides in general are potent Ca^{2+} mobilizing agents. The mechanisms by which ATP increases Ca^{2+} in hepatocytes have been examined in detail in rodents. The diverse biological responses to ATP are mediated by a variety of cell surface receptors, the functionally identified nucleotide or P_2-purinergic receptors (P_1 purinoceptors are activated by adenosine and AMP increasing cAMP; Charest et al. 1985), which are activated when ATP or other nucleotides are bound (for reviews see (Abbracchio and Burnstock 1994, Burnstock 2007, Carew et al. 1994, Conigrave and Jiang 1995, Fredholm et al. 1994, Gordon 1986, Ralevic and Burnstock 1998). These receptors have a fundamental role to play in cell physiology. They include (1) ATP receptors that stimulate G protein-coupled effector enzymes and signaling cascades, including inositol phospholipid hydrolysis and the mobilization of intracellular Ca^{2+} stores; (2) ATP receptors that directly activate ligand-gated cation channels in the plasma membranes of many excitable cell types; (3) ATP receptors that, *via* the rapid induction of surface membrane channels and/or pores permeable to ions and endogenous metabolites, produce cytotoxic or activation responses in macrophages and other immune effector cells; and (4) ADP receptors that trigger rapid ion fluxes and aggregation responses in platelets (Dubyak and el-Moatassim 1993). Several P_2 receptors exist that are classified on the basis of molecular structure and transduction mechanisms, as ligand-gated cationic channel receptors or P_{2X} receptors and G protein-coupled P_{2Y} receptors (Abbracchio and Burnstock 1994, Dubyak and el-Moatassim 1993, Ralevic and Burnstock 1998). This nomenclature has been widely adopted, and currently seven P_{2X} subtypes and eight P_{2Y} receptor subtypes are recognized (Burnstock 2007).

Many reports exist on how extracellular ATP and related compounds act on P_2 purinoceptors coupled to the phosphatidylinositol pathway immediately increasing cytosolic free Ca^{2+} concentration in hepatocytes from several species (Capiod 1998, Charest et al. 1985, Dixon et al. 1990, Green et al. 1995, Green et al. 1999, Nathanson and Mariwalla 1996, Nathanson et al. 1999, Sistare et al. 1985; for reviews see Conigrave and Jiang 1995, Putney and Bird 1993). Stimulation of P_2 receptors leads to the hydrolysis of phosphoinositides (PI) and the production of myoinositol-1,4,5-triphosphate (IP_3; Charest et al. 1985, Dixon et al. 1990, Dubyak and el-Moatassim 1993). The increase in IP_3 is dose-dependent and directly releases intracellular Ca^{2+} stores into the cytosol. This results in a rapid, transient increase in Ca^{2+} independently of extracellular Ca^{2+} depletion. Depletion of IP_3-sensitive Ca^{2+} stores then stimulates influx of extracellular Ca^{2+} (Putney Jr and Pedrosa Ribeiro 2000), which results in a late, sustained Ca^{2+} increase. In contrast to the first transient increase in intracellular Ca^{2+}, the sustained plateau phase requires influx of external Ca^{2+}. Ca^{2+} influx occurs most likely through a capacitative Ca^{2+} entry mechanism, which was shown to exist by experiments performed with thapsigargin (Schöfl et al. 1999). It has also been demonstrated in a variety of other non-excitable cells that ATP induces a biphasic increase in intracellular Ca^{2+} (Ikeda et

al. 1995, Nathanson and Mariwalla 1996, Paredes-Gamero et al. 2006, Popper and Batra 1993). Raising agonist concentration increases the frequency of Ca^{2+} oscillations; the duration of individual transients is unchanged (Green et al. 1999).

In hepatocytes, the research focus of this study, namely the G protein-coupled P_{2Y} receptor family plays a fundamental role in many physiological processes. An increase in Ca^{2+} is one of the key signals for the regulation of hepatic enzymes, such as glycogen phosphorylase, the rate-limiting enzyme for glycogenolysis. Extracellular nucleotides have been reported to activate glycogen phosphorylase and to stimulate glycogenolysis, most likely by binding to specific P_2 receptors in both rat and human hepatocytes (Keppens and De Wulf 1986, Keppens et al. 1989). In addition, activation of the PI-Ca^{2+} signaling cascade and an increase in intracellular Ca^{2+} may be involved in the regulation of other hepatic functions such as protein synthesis or gene expression (Gardemann et al. 1992). Furthermore, extracellular ATP has been suggested to be involved in canalicular contraction of hepatocyte doublets (Kitamura et al. 1991) and in intercellular communication between neighboring hepatocytes and between hepatocytes and bile duct cells in the rat liver (Schlosser et al. 1996). This signaling between non-contacting cells seems to be mediated by release of ATP or other nucleotides into the extracellular space. The evidence provided by Schlosser et al. (1996) suggests a paracrine signaling pathway for epithelia, which previously were thought to communicate exclusively via gap junctions.

There are only few reports about Ca^{2+} signaling in fish. In a study of Frojdo et al. (2002) on primary astrocytes from juvenile rainbow trout, 44 % of the cells responded to 100 µM ATP with an increase in Ca^{2+}. Nathanson and Mariwalla (1996) found a biphasic pattern of increased Ca^{2+} in skate primary hepatocytes (0.1 - 1 µM ATP) similar to the pattern induced by ATP in rodent hepatocytes. Since the initial Ca^{2+} peak was observed even in Ca^{2+}-free medium, it is due to Ca^{2+} released from intracellular stores. The Ca^{2+}-ATPase inhibitor thapsigargin was used to indirectly identify the IP_3-sensitive Ca^{2+} pool. Also as in rodent hepatocytes, the late sustained Ca^{2+} increase in skate hepatocytes was abolished in Ca^{2+}-free medium, suggesting that in skate hepatocytes this portion of the Ca^{2+} signal also is due to activation of plasma membrane Ca^{2+} channels. At low concentrations (10 - 100 nM), ATP induced Ca^{2+} oscillations. A maximal concentration of ATP (100 µM) caused a marked, transient increase in bile flow in the isolated perfused skate liver. These findings suggest that, similar to mammal hepatocytes, skate hepatocytes possess P_2 purinoceptors that link to intracellular plus extracellular Ca^{2+} mobilization pathways, which in turn regulates bile secretion (Nathanson and Mariwalla 1996). Indeed, a primitive P_{2Y} ATP receptor with broad pharmacologic specificity of the response to ATP and similar compounds and related to the evolutionary forerunner of P_{2Y1} receptors of higher organisms has been identified in skate hepatocytes (Dranoff et al. 2000).

3.1.2 Caffeine

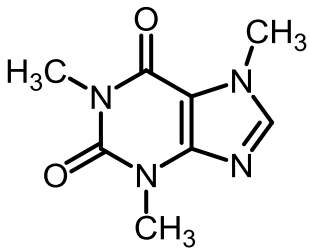

Fig. 2: Caffeine = 1,3,7-trimethylxanthine.

Caffeine is the most widely consumed behaviorally active substance in the world (for reviews see e.g. Anaya et al. 2006, Arnaud 1987, Ashihara et al. 2008, Bryan 2008, Daly 2000, Eteng et al. 1997, Ferré 2008, Ferré et al. 2008, Fisone et al. 2004, Fredholm et al. 1999, Ruxton 2008).

Chemically it is a methylxanthine alkaloid (like theobromine and theophylline), based on the purine structure. Purine alkaloids are secondary metabolites derived from purine nucleotides. They have been found in nearly 100 species in 13 orders of plant kingdom (Ashihara et al. 2008). Caffeine is found e.g. in *Comellia thea*, *Coffea arabica* and *C. sinensis*, *Theobroma cacao* and *Ilex* sp.

Xanthines affect many tissues, producing bronchodilatory, cardiotonic and diuretic effects, some of which have therapeutic importance (Snyder and Sklar 1984). In the liver, caffeine is metabolized to form dimethyl and monomethylxanthines, dimethyl and monomethyl uric acids, trimethyl- and dimethylallantoin, and uracil derivatives (Arnaud 1985, 1987).

There is information on various well-established pharmacological actions of caffeine; however different concentrations are required (for review see Fredholm et al. 1999). Blood and brain concentrations of theophylline and caffeine at doses commonly employed are 10 - 50 µM (Parker and Ivorra 1991). Among the diverse actions of caffeine, two consist in affecting important receptors which function as Ca^{2+} release channels and mobilize Ca^{2+} from intracellular, non-mitochondrial storage sites, however not at doses commonly applied in humans: (1) Caffeine has an inhibitory action on the IP_3 receptor (Berridge 1993) and/or reduces/inhibits IP_3 production in various tissues (e.g., rat cerebellum, Brown et al. 1992; rat hepatocytes, Nathanson et al. 1994, Sanchez-Bueno et al. 1994; *Xenopus* oocytes, Parker and Ivorra 1991; mouse pancreatic acinar cells, Toescu et al. 1992). (2) Caffeine at lower concentrations enhances the sensitivity of the Ca^{2+} sensitive Ca^{2+} release channel (Wakui et al. 1990) which releases Ca^{2+} in the presence of Ca^{2+} (Ca^{2+} induced Ca^{2+} release, CICR; Endo 1977) and is blocked by ryanodine (Coronado et al. 1994, Goldbeter et al. 1990). Ryanodine receptors seem to play a major role in electrically excitable cells, however, are also present in other tissues like liver (Feng et al. 1992, Somogyi and Stucki 1991).

In addition, caffeine blockades $GABA_A$ (γ-aminobutyric acid) receptors (Ferré et al. 1996) and acts as an inhibitor of adenosine receptors (Conlay et al. 1997, Ferré et al. 2008, Fisone et al. 2004, Fredholm 1985, 1995, Fredholm et al. 1999, Kuzmin et al. 2006, Ongini and Fredholm 1996, Snyder and Sklar 1984). Caffeine is able to significantly block adenosine effects on A2A (most potent) and A1 receptors already at the low concentrations achieved after a single cup of coffee. At concentrations in the micromolar range, comparable to blood and brain levels

of these drugs after ingestion of a few cups of coffee or treatment with therapeutic doses of theophylline in asthma, xanthines occupy 50 % of adenosine receptors (Fredholm et al. 1999, Snyder and Sklar 1984). Therefore, the stimulant actions of caffeine are thought to arise principally through binding to adenosine receptors.

Even though the primary action of caffeine may be to block adenosine receptors this leads to very important secondary effects on many classes of neurotransmitters, including noradrenaline, dopamine, serotonin, acetylcholine, glutamate, and GABA. This in turn will influence a large number of different physiological functions (Fredholm et al. 1999). Whereas the behavioral effects of caffeine at pharmacologically relevant levels appear linked to antagonism of adenosine receptors, the toxicological effects are likely mediated by other sites, such as calcium-release channels and $GABA_A$ receptors (Shi et al. 2003). Here, we focus on the effects of caffeine on the intracellular Ca^{2+} level.

There is controversy whether there is a caffeine-mediated increase in intracellular Ca^{2+} in epithelial cells or not: In some studies, caffeine up to 20 mM concentration did not induce any significant change in single rat hepatocytes (Sanchez-Bueno et al. 1994, Somogyi and Stucki 1991). Nathanson et al. 1994, however, found that caffeine increases Ca^{2+}, even in Ca^{2+}-free medium, suggesting that caffeine induces discharge of endogenous CICR stores (Nathanson et al. 1994). This effect of caffeine is consistent with observations of Schmid et al. (1990) who reported an increase of the open-state probability of intracellular Ca^{2+} channels independent of the free Ca^{2+} concentration in endoplasmic reticulum vesicle preparations from rat exocrine pancreas (Schmid et al. 1990).

Data indicate that higher concentrations of caffeine are toxic to cells. Several working groups found apoptotic effects of caffeine for concentrations of approximately ≥ 10 µM (e.g., Chinese hamster ovary cell line CHO-K1, Fernandez et al. 2003; mouse epidermal cell line JB6-Cl41, He et al. 2003; human neuroblastoma cell line SK-N-MC, Jang et al. 2002).

3.1.3 Histamine

Histamine (Fig. 3) is a monoamine derived from the decarboxylation of the amino acid histidine. It is one of the neurotransmitters that act *via* G-protein-coupled receptors. In the organism, histamine has diverse functions in the immune and inflammatory response, as a neuro-transmitter and in the regulation of physiological functions. In various peripheral tissues, histamine is stored in mast cells, basophils, enterochromaffin cells and specific neurons. Histamine stored in mast cells plays a very important role in the pathogenesis of various allergic conditions. After mast cell degranulation, release of histamine will lead to many of the symptoms of allergic conditions in skin and airway preparations (Leurs et al. 1995). In the

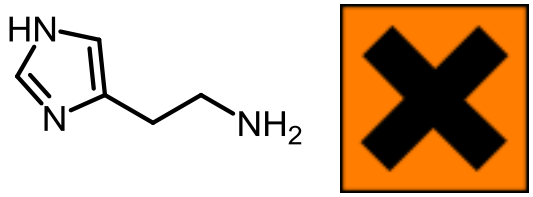

Fig. 3: Histamine.

CNS, histamine is synthesized in a restricted population of neurons located in the tuberomammillary nucleus of the posterior hypothalamus. They project in most cerebral areas and have been implicated in various functions of the brain of mammalian species (e.g. sleep/wakefulness, hormonal secretion, cardiovascular control; Leurs et al. 1995).

Histamine exerts its actions by binding with specific cellular receptors, the histamine receptors H_1, H_2, H_3, and H_4. Relevant with respect to the modulation of intracellular Ca^{2+} is especially the H_1 receptor. H_1 receptors have been detected in most smooth muscle, endothelial cells, adrenal medulla, heart and the CNS. They are coupled to phospholipase C and the IP_3 signaling pathway through pertussis toxin-insensitive G-proteins (Hill 1992). Several working groups have reported histamine evoked repetitive Ca^{2+} spikes (e.g. HeLa cells (Bootman and Berridge 1996), HLEC cells (Riach et al. 1995), smooth muscle cells from guinea pig urinary bladder (Rueda et al. 2002).

3.1.4 Phenylephrine

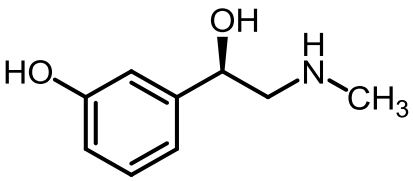

Fig. 4: Phenylephrine.

The catecholamine (Fig. 4) is an α_1-adrenergic receptor agonist used primarily as a vasoconstrictor in decongestants. In mammalian cells, the signal transduction pathways of the catecholamines are well defined (Exton 1985): They exert their actions through adrenergic receptors by altering the concentrations of Ca^{2+} or cAMP in the cytosol. An increase in cytosolic Ca^{2+} is mediated specifically by the α_1-subtype of adrenergic receptor, whereas effects attributable to an increase or decrease in cAMP are regulated by α_2- and β-adrenergic receptors. In the liver, the regulation of glucose metabolism is in part under the control of catecholamines and it has been shown that in mammals both α_1- and β-adrenergic receptors are involved, however with significant differences in the relative abundance of the two receptor types (Fabbri et al. 1995). α_1-adrenergic receptors may activate glycogenolysis, although this response is more potently elicited by activation of β-adrenergic receptors in most species (Exton 1985). α-adrenergic regulation of cytosolic free Ca^{2+} concentrations occurs, at least in part, through the intermediary IP_3, which causes mobilization of Ca^{2+} ions from intracellular stores (Zhang et al. 1992b). In rat hepatocytes and other tissues, this mobilization results in oscillations of intracellular free Ca^{2+} levels (Cobbold et al 1991), (Fewtrell 1993, Rooney et al. 1989). Glycogenolysis is stimulated by Ca^{2+} activation of glycogen phosphorylase and inhibition of glycogen synthase (Exton 1985).

For more primitive vertebrates, however, the players involved in the catecholamine transduction are discussed controversially and seem to vary among different species. The presence of β-adrenergic receptors on the membrane of higher fish liver cells and their role

in the catecholamine-modulated carbohydrate metabolism are established (Fabbri et al. 1994). β-adrenergic receptors have been characterized on rainbow trout hepatocytes (Reid et al. 1992) and catfish liver membrane (Fabbri et al. 1992) showing the coupling to the adenylyl cyclase system. Brighenti et al. (1987a,b) showed that in catfish hepatocytes, epinephrine, norepinephrine, isoproterenol, and phenylephrine increase the cytosolic cAMP level (Brighenti et al. 1987a) and stimulate phosphorylase activity accompanied by a decrease of glycogen content in cells and by an increase in glucose output into the medium (Brighenti et al. 1987b). In trout liver, epinephrine binds to β-adrenoceptors and increases ACase activity and cAMP biosynthesis (Fabbri et al. 1995).

There is also indirect evidence for α_1-adrenergic receptors in the liver of eel and bullhead, since Zhang et al. (1992) detected changes in intracellular Ca^{2+} levels induced by catecholamines (epinephrine) and blocked by α_1-antagonists (Zhang et al. 1992b). The epinephrine-induced Ca^{2+} fluctuations in eel cells have features qualitatively similar to those observed in rat hepatocytes exposed to α-agonists (Cobbold et al. 1991), i.e., spontaneous generation of Ca^{2+} transients with usually stable amplitudes and latency periods, marked heterogeneity among individual cells, and the ability of epinephrine to induce these oscillations in the absence of external Ca^{2+} relying therefore primarily on intracellular stores. Despite the species differences, the similarity of the responses with those reported for mammalian hepatocytes suggests that an α-adrenergic/Ca^{2+} transduction system is involved in eel and bullhead hepatocytes (Zhang et al. 1992b). Fabbri et al. (1994) provided evidence that α_1-adrenergic receptors as well are present in catfish liver and are involved in the regulation of carbohydrate metabolism in addition to the β-adrenergic receptor population formerly characterized.

3.2 Ecotoxicants

3.2.1 3,4-Dichloroaniline (3,4-DCA)

3,4-Dichloroaniline (3,4-DCA, Fig. 5) was selected in this study as a model environmental contaminant and positive control in the fish egg assay with *Danio rerio* (DIN 38415-6 in German waste-water testing and submitted to standardization to ISO; Braunbeck et al. 2005, Nagel 2002). It is an intermediate in the production of diuron, linuron, lindane and other herbicides, azo dyes and pharmaceuticals and a degradation product of several organochlorines. It is known that 3,4-DCA and analogues endanger growth, development, and propagation of aquatic organisms. Data suggest that 3,4-DCA induces free radical generation and antioxidant depletion, and causes

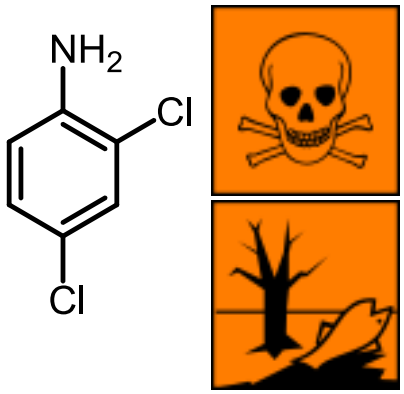

Fig. 5: 3,4-Dichloroaniline.

oxidative stress and lipid peroxidation in liver of crucian carp (Li et al. 2003). In fish, prolonged exposure to 3,4-DCA causes a progressive increase in levels of methaemoglobin leading to methaemoglobinaemia. These effects may be reversible when the fish is transferred to uncontaminated water (Crossland 1990). In addition, linuron generates tumors in rat and evidence exists that linuron, 3,4-DCA and other degradation products of diuron behave as antiandrogens (Cook et al. 1993). 3,4-DCA displaces testosterone from the androgen receptor with an IC_{50} of 110 µM (concentration which causes 50 % of inhibition) in ventral prostate cytosol from rat. Research indicates that 3,4-DCA acts as a toxicant on different levels within the cell and the whole organism arising the question whether it also influences on intracellular Ca^{2+} in a specific or non-specific way.

3.2.2 Hydrogen peroxide

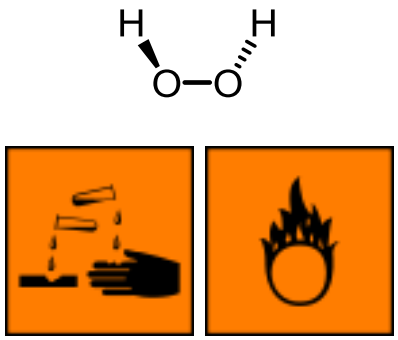

Fig. 6: Hydrogen peroxide.

It is known that high concentrations of oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, and lipid hydroperoxides (i.e., reactive oxygen species) can lead to damage to cellular components and ultimately be cytotoxic. Notwithstanding its toxic potential, hydrogen peroxide (Fig. 6) is a relevant component of living cells. It plays important roles in host defense and oxidative biosynthetic reactions. All aerobic organisms appear to regulate their intracellular hydrogen peroxide concentrations at relatively similar levels. In addition, in animals and plants, regulated enzymatic systems for generating hydrogen peroxide have been described (Stone and Yang 2006).

Exposure of cells to sublethal oxidative stress initiates a signaling response which results in a variety of downstream effects including increased expression of protective and repair enzymes (Winterbourn and Hampton 2008). It has been proposed that changes in the redox potential stimulate signal transduction components such as phospholipase and protein kinases and inhibit protein phosphatases effecting tyrosine and serine/threonine phosphorylation (Suzuki et al. 1997). This can lead to the activation of oxidative stress-responsive transcription factors.

The observations that ligand-receptor interactions produce reactive oxygen species and that antioxidants block receptor-mediated signal transduction led to a proposal that reactive oxygen species themselves may be second messengers for transcription factor activation, apoptosis, bone resorption, cell growth, and chemotaxis (Suzuki et al. 1997). Winterbourn and Hampton (2008) found evidence that redox signaling is fundamental to a wide range of receptor-mediated pathways. However, weather H_2O_2 really acts as a second messenger in non-stressed cells still needs to be elucidated.

3.2.3 4-Nitrophenol (p-nitrophenol)

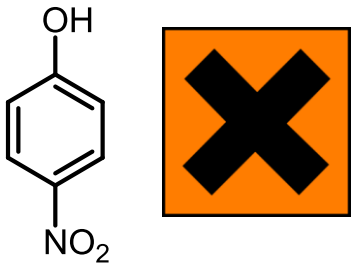

Fig. 7: 4-Nitrophenol.

Literature data about the mode of action of 4-nitrophenol (Fig. 7) are scarce; however, it is important to know the cellular targets of a substance to understand how it influences in Ca^{2+} signaling pathways. As the name implies, some properties of 4-nitrophenol can be explained considering the chemical characteristics of phenolic compounds. Besides being lipophilic, nitrophenols are H^+-ionophores; i.e. they eliminate proton gradients in mitochondria by transporting H^+ cations across the inner mitochondrial membrane leading to the break-down of proton gradient and transmembrane potential. A collapse of the mitochondrial membrane potential results in a reduced ATP production, and finally leads to ATP depletion and disruption of ion homeostasis (Bellomo et al. 1991). The decrease in cellular ATP has been proposed as one of the critical events in the development of irreversible injury caused by a variety of ionophores and chemical toxins (Bellomo et al. 1991).

The increasing amount of protons in the mitochondria induced by an uncoupling agent leads to a release of Ca^{2+} from mitochondria into the cytosol via reversal of the uniport Ca^{2+} carrier (Bernardi et al. 1984). This initial increase in intracellular Ca^{2+} can trigger Ca^{2+}-induced Ca^{2+} release (CICR), when ion channels in the plasma membrane are secondarily activated, thus effecting an influx of Ca^{2+} from the extracellular space.

4 Experimental systems applied in the present study

For the present study, two different test systems were selected in order to approach the main principles of Ca^{2+} in fish: (1) primary hepatocytes from the rainbow trout (*Oncorhynchus mykiss*), and (2) the permanent rainbow trout cell line RTL-W1 (rainbow trout liver - Waterloo 1; Lee et al. 1993). Both present various characteristics as well as conveniences and disadvantages making them more or less adequate for different research objectives. Differences among the two cell types as well as between the *in vitro* systems in general and other test systems are discussed in the following.

4.1 The rainbow trout (*Oncorhynchus mykiss*)

Fig. 8: Adult rainbow trout (*Oncorhynchus mykiss*; taken from www.karnbaum.de).

Rainbow trout (Fig. 8) is an established model organism in research on teleosts featuring various advantages: Fish are easy available from local suppliers (trout farms) and can be kept in laboratory for adaption without complications. Physiology of trout has been studied in detail in the past and is well understood. Last, but not least, due to its role in toxicity testing during decades, namely in the acute fish test, there already exist comprehensive data permitting the comparison and evaluation of new results.

4.2 Reduction of animal testing

Animal testing is performed in the fields of biomedical research, in toxicity testing to assess the potential hazards and risks posed by products and substances, and education. In 2001, about 300,000 fishes per year were tested in Germany, two to three times as much as in the years 1995 - 2000. This can in particular be attributed to basic research, since the number of fish applied in (eco)toxicological testing is regressive due to the rising application of alternative methods in this area (Tierschutzbericht 2003). For example, the *Danio rerio* embryo test has become a valuable model organism in ecotoxicology for effect assessment of environmental samples (Braunbeck et al. 2005, Nagel 2002). This embryo assay has already replaced the former mandatory acute fish test in German waste-water testing (DIN 38415-6) and has been submitted to standardization to ISO. Recent efforts are targeted to use the zebrafish- and other fish embryo tests for the effect analysis of environmental samples and chemicals, too (Kuster and Altenburger 2007).

There is still a high demand to reduce, replace and refine animal testing (3R-concept; Russel and Burch 1959). Both national and European legislation requires that non-animal, alternative methods should be used in place of animal procedures where possible (Jenkins & Langley 2002). The reduction of animal experimentation is also of high interest for the development, screening and registration of chemicals within REACH (Kuster and Altenburger 2007). The Second High Level Meeting of the Chemicals Group of the OECD adopted the following statement: Animal testing "cannot be eliminated at present, but every effort should be made to discover, develop and validate alternative testing systems" (see OECD website; http://www.oecd.org/document/48/0,3746,en_2649_34377_40695856_1_1_1_1,00.html).

Alternative possibilities could be the application of cell culture (primary culture and cell line), tissue slices in culture, organ cultures or embryos. The method should be simple, cost-effective, sensitive, fast and reproducible (Ahne 1985, Lange et al. 1995). New imaging technology (e.g. ultrasound, nuclear magnetic resonance) provides a non-invasive means to examine research animals, thus providing a significant refinement opportunity. Organisms such as invertebrates, early-stage vertebrate embryos, and microorganisms can be regarded as either replacements or refinements when replacing vertebrates. State-of-the art mathematical and computer modeling approaches to biomedical research and testing are likewise replacing and reducing the need for animal testing. Toxicologists use mathematical models known as Quantitative Structure Activity Relationships (QSARs) to predict biological (toxic) activity associated with chemical structure (Hakkinen and Green 2002). Increasingly, new molecular methods (so-called "omics" technologies) are becoming available that hold the promise to detect tissue-specific changes with increasing sensitivity. These methods permit the simultaneous analysis of thousands of genes, proteins or metabolites and, thus, the global detection of treatment-related changes on the transcriptional and translational expression levels in animal experiments becomes possible (e.g. toxicogenomics, toxicoproteomics and metabolomics). By using these technologies, the chance arises to gain valuable information on the underlying toxicity mechanisms and potentially to fill the 'black hole' that exists between treatment and the classical morphological or clinical outcome (Kroeger 2006).

4.3 Fish in ecotoxicology

From the perspective of ecotoxicants, fish are especially important. With approximately 24,000 different species occupying all aquatic niches, fish are the most diverse group of vertebrates. Thus, understanding the actions of ecotoxicants on fish assists in evaluating the health of the aquatic environment. By a variety of routes, ecotoxicants are often released first into or end up in aquatic environments (Bols et al. 2005).

Because of their taxonomic position, fish are important model organism in research in general and in ecotoxicology in particular: Animal welfare legislation and ethics demand the

use of more primitive vertebrates in animal experimentation. The universality of many basic biological processes among eukaryotic organisms means that lower animals can serve as useful research models for problems in toxicology (Baksi and Frazier 1990). Because of their taxonomic relation, results from fish tests should be at least partially transferrable to other vertebrate groups including humans. As a result, fish, by sharing with mammals a large number of important characteristics (e.g. similar organ systems, developmental organization and physiologyical/biochemical mechanisms) and by presenting various technical advantages (Berghmans et al. 2005, McGonnell and Fowkes 2006, Patton and Zon 2001), have become a promising vertebrate model for most biological studies and a suitable alternative to mammalian systems (Braga et al. 2006, Hightower and Renfro 1988, Kelly et al. 1998, Marques et al. 2007).

For the regulation of chemicals and waste-water testing, the acute fish test according to OECD Guideline 203 is specified by German legislation. The new European chemical regulation "Registration, Evaluation and Authorization of Chemicals (REACH)" also requires a short-term fish test for chemicals, when the level of production exceeds 10 tons per year. The increasing demand of testing for hazard and risk assessment in health and environment, exemplified by REACH, subsequently triggers laboratory animal testing. This holds especially true, as a limited number of non-animal methods have been developed and received regulatory acceptance (Garthoff 2005, Jenkins and Langley 2002).

A problem for the implementation of alternative test methods is the fact that animal tests are often used as the "gold standard" the *in vitro* test is compared to. Even when the *in vitro* test is clearly better than the animal test, it takes a long time to implement owing to the problem of more rigorous evaluation, which the *in vivo* tests never had to undergo (Kroeger 2006). Besides, in comparison to the costs involved, animal experiments are not expensive and they may even offer a perspective for less severe safety requirements, if a lower hazard classification class can be proven (Hofer et al. 2004).

However, single species LC_{50} data may be highly questionable, since there are differences in orders of magnitude not only between species, but also for the same species between laboratories (Kuster and Altenburger 2007). Furthermore, the significance for environmental risk assessment of the death of individuals after short-term exposure to high toxicant concentrations is low – except in cases of accidental spills (Nagel 2002), since an extrapolation from acute to chronic effects is critical and since sublethal effects are not included.

In addition to such scientific considerations, this approach is also in conflict with the principles of animal experimentation as it uses typically welfare-sensitive parameters, relatively high (stressful) concentrations and disregards potentially valuable information, which is clearly not compatible with current animal welfare legislation (Wester et al. 2002). There is little doubt that fish subjected to acutely toxic concentrations of chemicals suffer severe distress and pain.

Although toxicity testing represents only a relatively minor part of laboratory animal use, it has attracted a great deal of attention from the animal welfare movement. According to (Balls and Fentem 1999), this is for three main reasons: (1) Many toxicity tests inevitably result in animal suffering, because the causation of effects is integral to the design of the test itself. (2) Many toxicity tests are required by laws, guidelines and regulations, before chemicals and products of various kinds can be manufactured, transported or marketed. (3) The performance of many toxicity tests is routine, without case-by-case scientific justification, or even without any scientific justification at all. For these reasons, toxicity testing in animals is unpopular in the general public, and high efforts are being made in seeking ways of replacing animal tests.

4.4 Role of the liver

The liver is of key importance for the maintenance of internal homeostasis in vertebrates. It adapts to fluctuating environmental conditions by continuously readjusting hepatocellular structures and functions, such as: metabolism of nutrients, storage of energy (glycogen, lipid), synthesis and secretion of proteins (e.g. albumin, vitellogenin, lipoproteins), maintenance of plasma glucose levels, elimination of nitrogen components after urea or ammonia formation, metabolism of hormones, metabolism of xenobiotics, and bile formation (Segner 1998).

In vertebrates, the liver plays a fundamental role in the metabolism and the excretion of xenobiotics through enzymatic and non-enzymatic mechanisms. Therefore, the liver is the most appropriate organ for evaluating responses to most environmental pollutants. Generally, biotransformation occurs in hepatocytes through the activity of enzymes of the endoplasmic reticulum. Due to their respective catalase and superoxide dismutase (SOD) activity, peroxisomes and mitochondria are also involved at some stage of biotransformation. Furthermore, hepatocytes are protected against chemical injury by agents such as glutathione (GSH), which acts as a reducing agent and participates in the detoxification of xenobiotics and in the metabolism of numerous cellular compounds (Fenoglio et al. 2005).

Ultrastructural studies demonstrate that livers of fish are remarkably heterogenous organs. In trout liver, hepatocytes are the most numerous cells, occupying approximately 80 % of the liver volume. Other cell types include biliary epithelial cells; sinusoidal endothelial cells; perisinusoidal, fat-storing, stellate cells of Ito; perisiunusoidal and melanomacrophages (Blair et al. 1990, Hampton et al. 1989).

4.5 Primary hepatocytes

In 1969, (Berry and Friend 1969) first reported the isolation of hepatocytes in mammals using a combination of hyaluronidase and collagenase for the enzymatic digestion of liver tissue. Since then, hepatocytes have been used as a model cell system. The techniques, however, have subsequently undergone a number of refinements. (Seglen 1976) published the modified collagenase method used by most investigators today (Moon 2004).

For teleosts, the first report on the use of isolated liver cells was the work by Birnbaum et al. (1976) on hormone-stimulated glycogenesis in goldfish hepatocytes. During the 80s and 90s, the application of freshly isolated fish hepatocytes greatly expanded (Babich and Borenfreund 1991, Baksi and Frazier 1990, Blair et al. 1990, Blair et al. 1995, Borenfreund and Shopsis 1985, Braunbeck and Storch 1992, Maitre et al. 1986, Segner 1998). Fig. 9 shows primary hepatocytes isolated for the present study by collagenase digestion of the liver after (Braunbeck and Storch 1992).

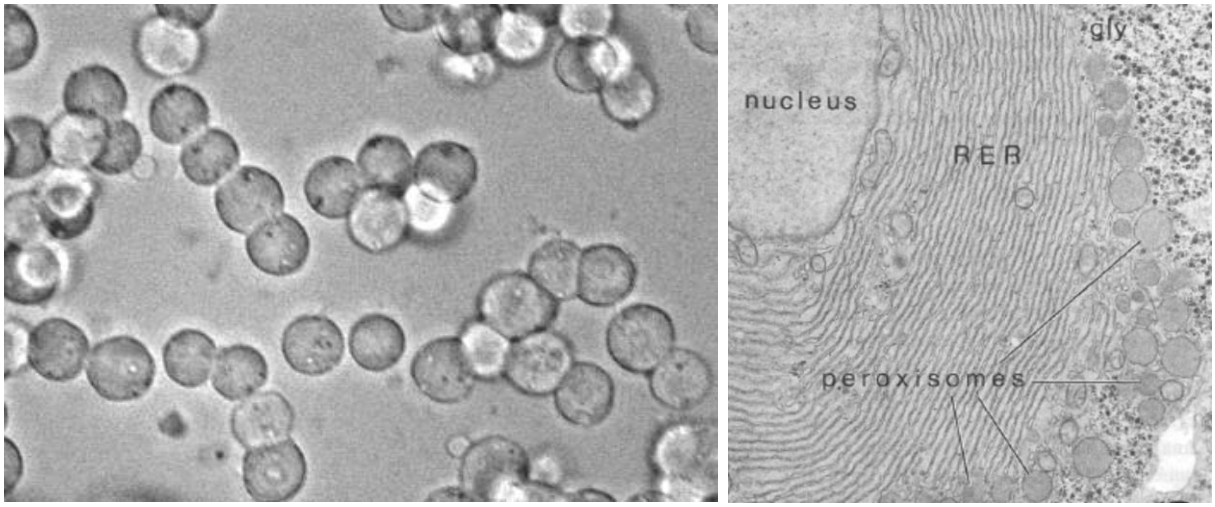

Fig. 9: left Primary hepatocytes of the rainbow trout under the light microscope after 24 h in culture. Diameter of the individual cell is approx. 10 µM (15 - 25 µM *in vivo*). **right** Detail of an individual cell under transmission electron microscope (taken from Braunbeck and Storch 1992).

Cells isolated from intact organs can be used directly after isolation as short-term (2 - 4 h) suspension cultures, or they can be plated on culture dishes for longer-term experiments. Under appropriate culture conditions, the attached primary hepatocytes remain viable for at least one week. With mammalian hepatocytes, techniques to maintain the cells after isolation for several days or weeks in primary culture have been introduced during the early seventies (Bissell et al. 1973). Primary hepatocytes, either freshly isolated or cultured, generally maintain many of their differentiated *in vivo* characteristics, which facilitates extrapolation of the results to the *in vivo* situation. Compared to fresh isolates, cultures have

several advantages: they allow the investigation of longer-term, time-dependent processes, they permit studies of structures and functions which require intercellular contact and organization and the cells have time to recover from damage sustained during the isolation procedure (Moon 2004, Pesonen and Andersson 1997, Segner 1998).

Compared to mammalian hepatocytes, there is evidence that the loss of differentiated liver function is less rapid in teleost primary cultures, since the available data indicate a greater stability of biotransformation enzymes in piscine liver cells than in mammalian hepatocytes. Activity of xenobiotic enzymes of both phase I (7-ethoxyresorufin-*O*-deethylase, EROD) and phase II (e.g. glutathione-*S*-transferases, GST, and UDP-glucuronyltransferases, UDPGT) seem to be conserved during monolayer culture (Segner 1998, Segner and Cravedi 2001).

The use of isolated cells in general and in environmental risk assessment in particular, provides numerous advantages (for review see Segner 1998):

(1) The *in vitro* system gives nearly all the benefits of an intact cell (functional organelles, enzyme interactions, physiological cofactor and metabolite concentrations, etc.), but

(2) it is less complex than the intact animal and could therefore greatly advance the understanding of basic liver properties and environmental adaptive responses. It eliminates interactive systemic effects which can confound the experimental situation.

(3) Technically, cell cultures are easier to handle and allow more rapid and less expensive testing than *in vivo* systems. Defined experimental conditions can be readily set and maintained. Known numbers of isolated cells may be exposed to controlled doses of individual xenobiotics. This facilitates quantitative and reproducible dose-response studies on toxicity, such as drug uptake kinetics, enzyme induction, biotransformation and excretion (Baksi and Frazier 1990, Mothersill and Austin 2003, Segner et al. 2001).

(4) Multiple types of experimental conditions can be initiated with populations of cells obtained from a single animal, and analyzed individually or in combination.

(5) Morphological changes in hepatocytes as a result of toxicity are easily visible in isolated cells (Pesonen and Andersson 1997).

(6) Only small quantities of xenobiotics are needed. This is particularly important when studying the effects of micropollutants extracted from environmental samples, the quantities of which are usually small (Pesonen and Andersson 1997). Accordingly, quantities of toxic waste are smaller.

(7) From an ethical point of view, the use of isolated cells is preferable, since fewer animals have to be sacrificed. *In vitro* testing provides a lot more information but acute toxicity without animals suffering from acute exposure.

In contrast to the many benefits of primary hepatocytes, there are also limitations:

(1) Generally, *in vitro* cultures represent complex organs by using only one or a limited number of cell types. This fact makes them easier to handle, but at the same time they do not reflect organ integrity and thus cannot show the same treatment response to chemicals and drugs compared with the in vivo model (Kroeger 2006).

(2) Primary hepatocytes, both freshly isolated and in culture, are only viable for a relatively short period of time. Braunbeck and Storch (1992) found that with prolonged culture, the number of viable cells declined almost linearly to < 80 % after eight days in culture at 14 °C.

(3) In fact, not all cells classified as “alive” by light microscopic observation, are ultrastructurally intact. As shown by electron microscopy by Braunbeck and Storch (1992), the number of ultrastructurally intact cells was consistently about 15 % lower than the number of viable hepatocytes determined by trypan blue exclusion.

(4) Further limitations include loss of membrane specialization, possible down regulation of cytosolic enzymes and loss of the ability to form bile. These effects mean that direct extrapolation of results from isolated hepatocytes to intact liver is often problematic (Braunbeck and Storch 1992).

(5) Cells from different preparations may vary as a result of differences in the health and reproduction status of the fish or in the isolation procedure (e.g., time to clear liver from blood, extent of collagenase digestion).

(6) Even though the use of primary cells reduces the number of organisms suffering from acute exposure, animals still have to be kept in laboratory and sacrificed for each cell preparation.

Primary cultures of teleost hepatocytes have been used to study various physiological and biochemical functions of hepatocytes, e.g. energy metabolism (e.g. Bains and Kennedy 2004, Pannevis and Houlihan 1992), protein synthesis (e.g. Koban et al. 1987, Pannevis and Houlihan 1992), gluconeogenesis and glycolysis (e.g. Birnbaum et al. 1976, Mommsen and Lazier 1986), lipid metabolism (e.g. Hazel 1983, Voss and Jankowsky 1986), vitellogenin synthesis (e.g. Maitre et al. 1986), membrane transport, intracellular pH (e.g. Walsh 1986), temperature acclimatization (e.g. Koban 1986), cell senescence (e.g. Braunbeck and Storch 1992) and endocrine regulation (for review, see Moon 2004). During the last decades, cultured hepatocytes have also been applied in the fields of pharmacology and (eco)toxicology (e.g. Braunbeck 1993, Christianson-Heiska and Isomaa 2008, Filipak Neto et al. 2007, Finne et al. 2007, Laville et al. 2004, Nakari and Pessala 2005, Navas and Segner 2006, Strmac and Braunbeck 2000). The knowledge of how xenobiotics impair normal functions at the cellular level can facilitate risk assessment (Pesonen and Andersson 1997).

4.6 Fish cell lines

Ross Harrison was the first to culture cells *in vitro*. In 1907, he published his findings on the growth of nerve fibers (Harrison 1907). Since then, the methods of isolation and culture of cell lines have evolved, and the cell lines used today have their origin in various species and tissues. The fields of application of cell lines are expanding as well.

Cell lines are a useful tool in many areas of research like medicine, basic research and (eco)toxicology. To overcome points (12) and (13) of the disadvantages of primary cultures listed above, continuous or permanent cell lines seem to be the optimal cell culture: Like primary cells, they reduce animal testing and allow the observation of potential toxic effects at the lowest living level, the cell. Additionally, they are easy to obtain and handle, and provide genetically identical material over a long period without the necessity to revert on intact animals. The higher homogeneity of permanent cell cultures therefore good reproducibility of cell tests makes them preferable in routine laboratory use. Test data from cell lines show good reproducibility, what makes cell lines particularly suitable as tool in screening studies and environmental diagnostics (Segner 1998).

In order to analyze toxicological impacts on aquatic organisms, it is useful to culture cells derived from fish; even more since they provide various advantages: In contrast to mammalian cells, fish cell lines grow well at room temperature without the need of thermoregulated and CO_2 buffered incubators. Because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time (Wagg and Lee 2005). Originally, fish cell lines were developed for the studies of fish virus diseases (Wolf and Quimby 1962); however, today they have grown in number covering a wide variety of species and tissues of origin. Fish cell lines have contributed to various research areas, e.g. fish immunology, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture (Wagg and Lee 2005). Nevertheless, immortalized cell lines are not normal cells and serious questions arise as to whether responses of cell lines to chemical toxins are in fact representative of the response of differentiated cells in vivo (Baksi and Frazier 1990). This limits their use in physiological or metabolic studies.

The potential roles of fish cell lines in toxicology and ecotoxicology are only beginning to be exploited (Bols et al. 2005). However, cell lines are a promising tool in environmental toxicology since, studies with cultured cells permit the determination of molecular and cellular mechanisms by which contaminants lead to toxic effects in organisms at sublethal and chronic levels. Furthermore, cell lines are more amenable to toxicogenomic technologies and up or down regulation of genes or proteins. The homogeneity of cell lines makes these responses to toxicants easier to detect and with less variability than whole organisms (Castano et al. 2003; Schirmer 2006).

4.7 The RTL-W1 cell line

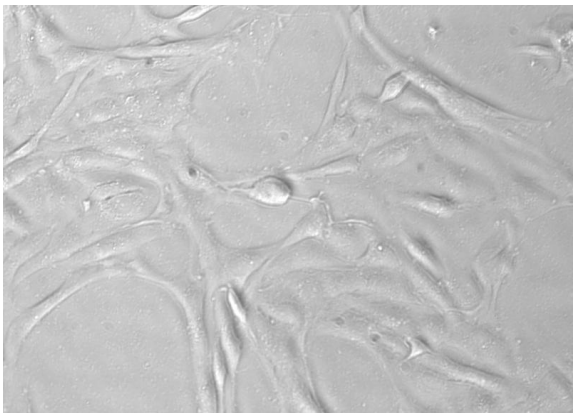

Fig. 10: The fibroplastic RTL-W1 cells after 24 h in culture under the light microscope.

Development of the permanent fish cell line RTL-W1 (rainbow trout liver-Waterloo 1) was initiated by Lee et al. (1993) by Dispase treatment of liver fragments of a 4-year-old male rainbow trout. Attached single cells were grown and passaged continuously. For confluence, supplementation with 5 to 10 % FBS is necessary (Lee et al. 1993). When maintained as a confluent culture, shape of RTL-W1 cells is predominantly bipolar or fibroblast-like (Fig. 10).

The cell line was selected to overcome some limitations presented by primary hepatocytes and complete our investigations on intracellular calcium signaling in fish. There are different permanent cell cultures derived from rainbow trout primary cultures such as R1, RTG-2 (gonads), RTgill-W1 and RTL-W1 (liver, Lee et al. 1993). Among these, the liver cell line was selected for this study to complete data from primary trout hepatocytes.

In contrast to other fish cell lines, RTL-W1 express cytochrome P450 enzymes and induce EROD activity in response to several chemicals (e.g. TCDD, Lee et al. 1993; aromatic hydrocarbons, Behrens et al. 2001, Billiard et al. 2004, Brack et al. 2002; various pesticides, Babin and Tarazona 2005; benzo[a]pyrene, Schirmer et al. 2000; and environmental samples, Brack et al. 2002, Keiter et al. 2006, Rastall et al. 2004, Schirmer et al. 2004). Results are similar to those from primary rainbow trout hepatocytes. This leads to the conclusion that during transformation into an immortal cell line, the liver-derived RTL-W1 cells have conserved the essential elements of the aryl hydrocarbon receptor-mediated CYP1A induction pathway (Behrens et al. 2001) and makes them useful as a tool for assessing the toxic potency of environmental contaminants.

However, the main disadvantage of RTL-W1 is its unclear origin, since Lee et al. (1993) could not determine unequivocally from which cell type in rainbow trout liver the cell line arose (Lee et al. 1993), making it more difficult to transfer results to the conditions in trout liver *in vivo*. Nevertheless, since Ca^{2+} signaling is a common phenomenon in cells, results should be transferrable at least to a certain extent to trout cells and more generally to other fish. Therefore, data from the cell line are supposed to supplement data from primary cells and to compensate their lacks. This holds especially true, since one motivation of this study was the application of Ca^{2+} imaging in ecotoxicology. Because of the named reasons, in particular the good reproducibility of the results and the expression of biotransformation enzymes such as cytochrome P450, and since their exist already certain data on the toxicity of environmental contaminants for reference, RTL-W1 is especially suited for toxicity testing.

5 Assays

5.1 Ca^{2+} imaging

Ca^{2+} imaging techniques allow the quantitative measurement of cytosolic free Ca^{2+} concentrations by the means of fluorescent Ca^{2+} dyes that change their light absorption and/or emission characteristics upon Ca^{2+} binding. A large number of fluorescent Ca^{2+} indicators are available for studying Ca^{2+} in cells, most of them based on the Ca^{2+} chelators EGTA or BAPTA modified to incorporate fluorescent reporter groups. At the same time, development of confocal and fluorescence microscopy allowed to visualize rapid Ca^{2+} fluctuations in subcellular volumes. Researchers take advantage of the fact that the Ca^{2+} stains are membrane impermeant in their Ca^{2+}-sensitive form; however, acetoxymethyl (AM)-ester linkage allows introducing indicators into cells. AM-ester groups are split off by endogenous esterases thus releasing the membrane-impermeant Ca^{2+} dye intracellularly and allowing imaging of Ca^{2+} signals by means of fluorescence microscopy (Thomas et al. 2000).

Early Ca^{2+} indicators such as quin-2 signaled Ca^{2+} by increasing its fluorescence intensity without much shift in either excitation or emission wavelengths. Unfortunately, fluorescence intensity is also dependent on many other poorly quantified or variable factors such as illumination intensity, emission collection efficiency, dye concentration, and effective cell thickness in the optical beam. For this reason, so-called ratiometric Ca^{2+} indicators have been developed that – among other advantages – respond to Ca^{2+} by shifting wavelengths while maintaining strong fluorescence. The ratio of the fluorescences at two suitably chosen wavelengths then signals Ca^{2+} (Grynkiewicz et al. 1985).

In the present study, the Ca^{2+} indicator of choice was fura-2 (Fig. 11). Fura indicators arose from the salicylaldehyde derivative XXV, itself synthesized from *p*-hydroquinone. Emission of fura-2 is maximal at a wavelength of 510 nm (Grynkiewicz et al. 1985). Fura-2 is a ratiometric Ca^{2+} indicator with two wavelengths with maximum excitation (340 and 380 nm, respectively). When the concentration of Ca^{2+} bound to Fura-2 increases, the fluorescence at 340 nm increases, whereas it decreases at 380 nm. This shift in the excitation maxima allows the estimation of Ca^{2+} concentrations by forming the ratio of the fluorescences at the two wavelengths independent of possible variability.

Fig. 12 shows the principle mechanism of ratio imaging with Fura-2 as Ca^{2+}-indicator: At each time point two pictures at the respective excitation wavelengths – (a) 340 and (b) 380 nm – are taken depicting the fluorescence intensities (colored in green and red, respectively). Image (c) shows the overlapping fluorescence (yellowish), and in images (e) and (f) false colours represent the ratio of the fluorescence signals at 340 nm/380 nm after subtraction of the background (white ellipse) at different time points. Ratio serves as a measure for the intracellular Ca^{2+} concentration in individual cells.

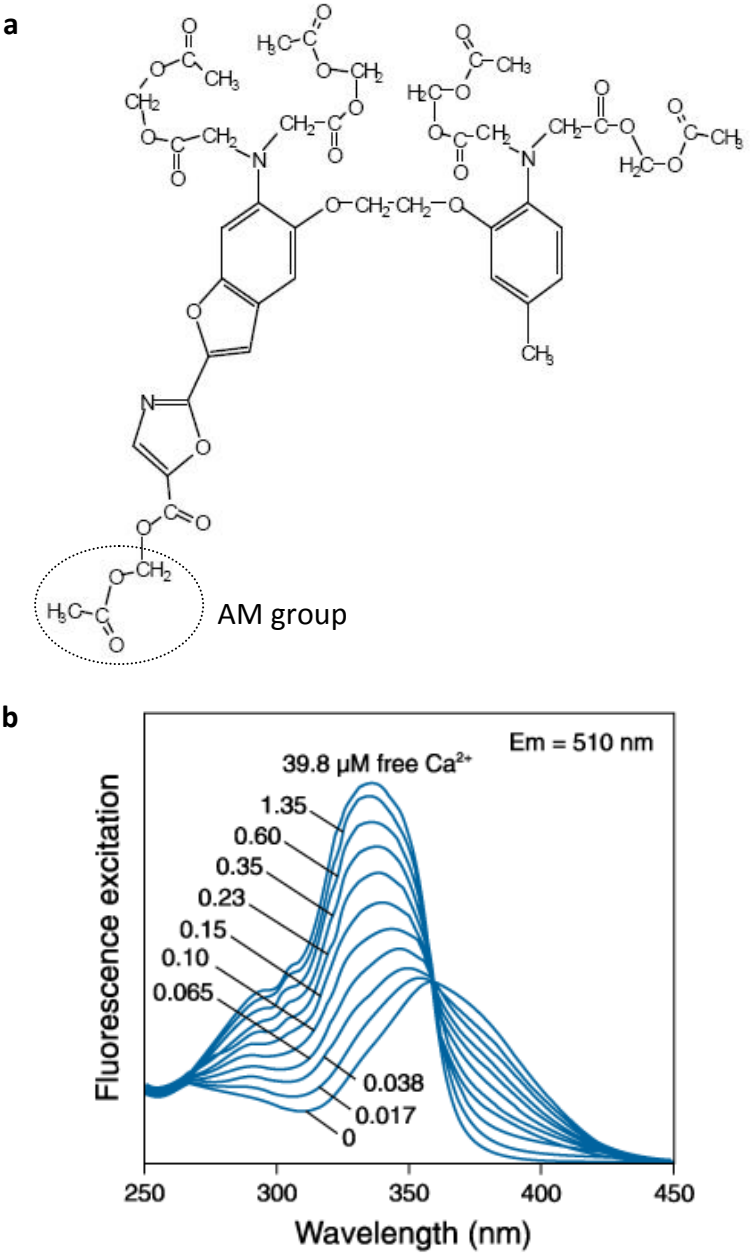

Fig. 11: (a) Fura-2 in the Ca^{2+}-insensitive acetoxymethyl (AM) ester form. After cleavage of the AM group by intracellular esterases the Ca^{2+} sensitive indicator remains. **(b)** Increase of bound Ca^{2+} produces a shift in the excitation maxima of Fura-2; the fluorescence at 340 nm increases whereas it decreases at 380 nm.

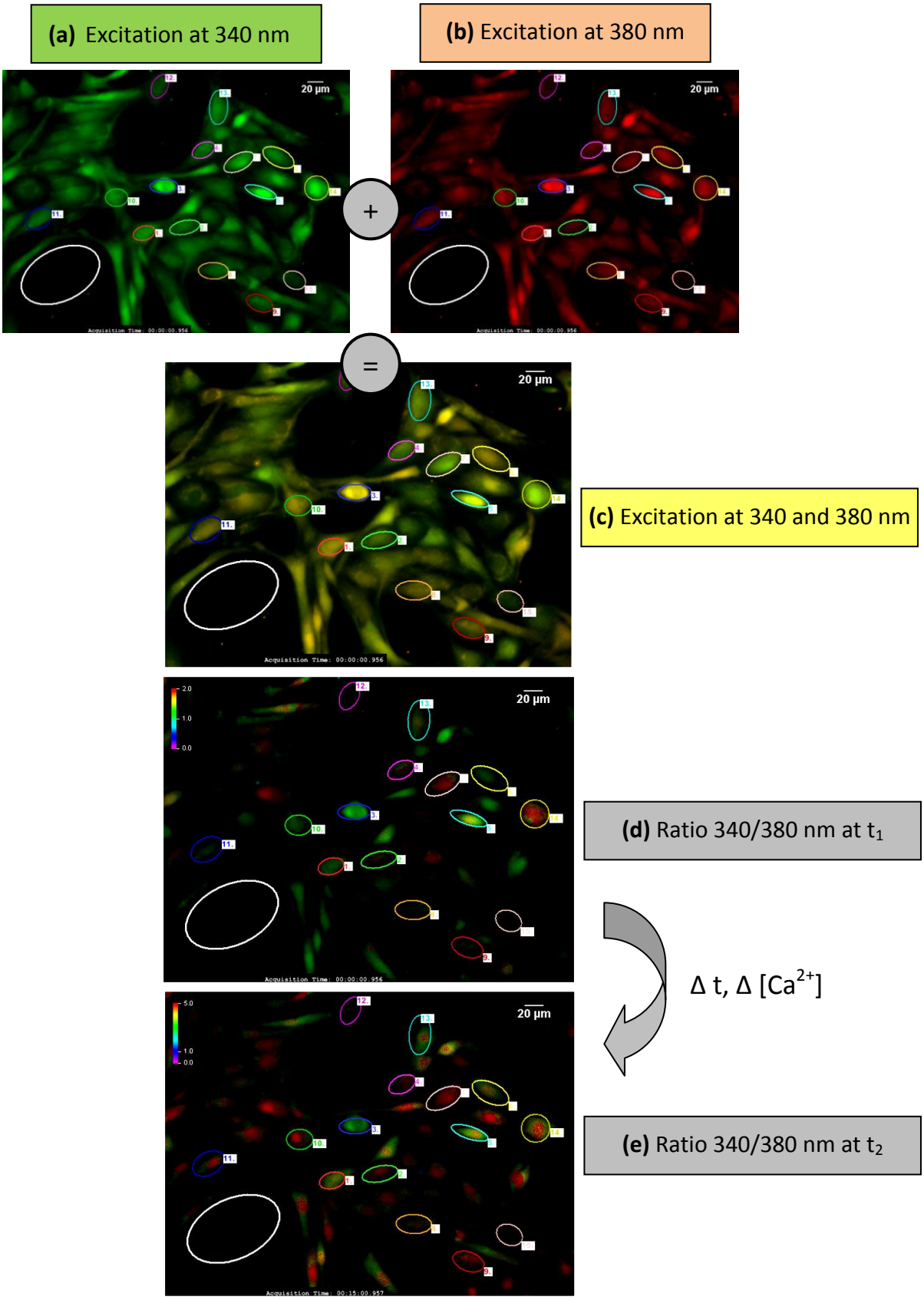

Fig. 12: Principle mechanism of Fura-2-ratio imaging with RTL-W1 cells (see text). Note that false colours in images **(d)** and **(e)** represent different ratio scales.

Fluorescence staining and microscopy were carried out according to many other established protocols (for fish cells, see e.g. Furimsky et al. 1996, Green et al. 1997, Harwood et al. 2000, Johnson et al. 2000, Zhang et al. 1992b), but adapted to the conditions in rainbow trout hepatocytes. Fig. 13 gives a schematic overview of the final protocol.

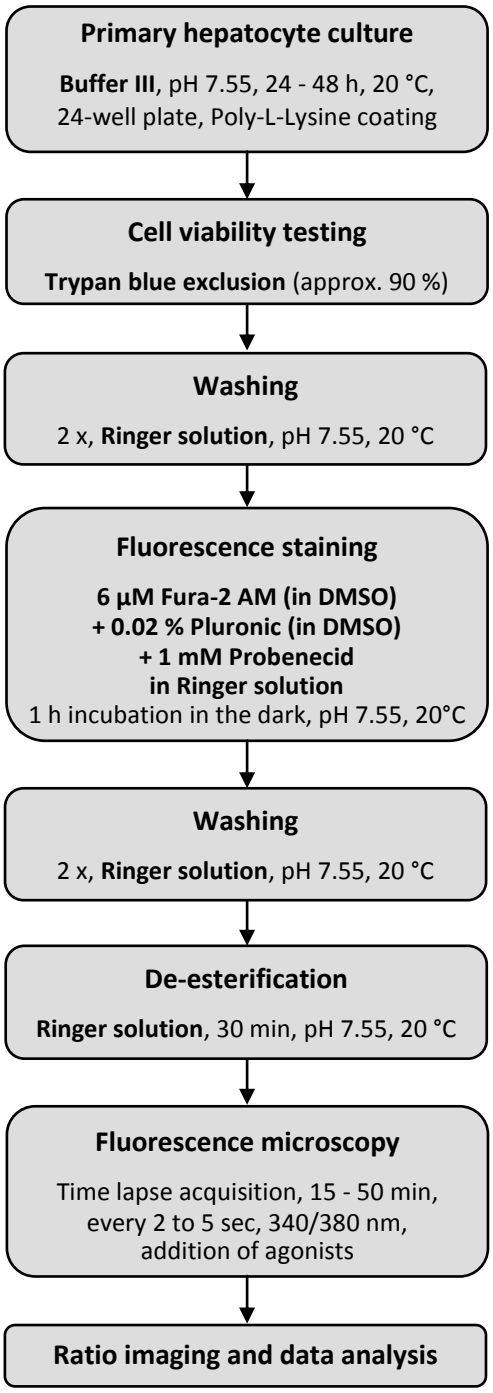

Fig. 13: Ca^{2+} imaging protocol for primary rainbow trout hepatocytes as established in the present study. For RTL-W1 cells, the procedure was basically the same starting with the first washing step after attachment of the cells to the culture plate.

5.2 Cytotoxicity and apoptosis assays

In the present thesis, selected model environmental toxicants were used to elucidate possible interactions between contaminants and Ca^{2+} signaling. For better differentiation between agonist-specific effects and general cytotoxic or apoptotic effects on intracellular Ca^{2+}, two range finding tests were carried out with the tested substances in RTL-W1 cells prior to Ca^{2+} imaging.

5.2.1 Neutral red assay

Acute cytotoxicity was determined using the neutral red assay according to (Borenfreund and Puerner 1984). In this assay, the ability of neutral red (2-Methyl-3-amino-7-dimethylaminophenazin) retention of the cells is tested serving as a measure of lysosomal integrity and therefore cell viability. Correlation with the acute fish test is high, notwithstanding the lower sensibility of the cytotoxicity assay due to the differences in bioavailability of cells and fish (Braunbeck et al. 1997). At the same time, animal testing can be reduced, making cytotoxicity tests useful in ecotoxicology.

5.2.2 Apoptosis assay with Hoechst 33342

The nucleic acids stain Hoechst 33342 was performed to detect apoptotic cells and determine agonist concentrations with a relevant apoptotic potential. Previously the optimal staining conditions for RTL-W1 cells had to be determined.

Hoechst 33342 is a an easily applicable fluorescent synthetic dye which consists of two adjacent benzimidazole rings with *N*-methyl-piperazine and phenolic groups at the ends (Garner 2009). One advantage of Hoechst 33342 is that it is membrane permeant and, thus, can stain live cells (Chazotte 2011). After penetrating the cell, it binds selectively to A-T base pairs exposed in the minor-groove of double stranded DNA. On binding to DNA, the fluorescence greatly increases. Hoechst 33342 is a known mutagen, since binding the DNA can disrupt DNA replication during cell division. Care should be taken in handling.

Hoechst 33342 has multiple applications, including detection of DNA, determination of cell number and chromosome sorting. It is the fluorophore used routinely to measure DNA in X- and Y-chromosome-bearing mammalian sperm so they can be separated by flow sorting. A difference of <3% in DNA mass can be detected (Garner 2009). The fluorescence of Hoechst 33342 is very sensitive to DNA conformation and chromatin state allowing the detection of gradations of nuclear damage (e.g., (Belloc et al. 1994, Ellwart and Dormer 1990, Reid et al. 1996).

Chapter 2

Isolation and culture of rainbow trout primary hepatocytes

The best way to observe a fish is to become a fish.

Jacques-Yves Cousteau (1910 - 1997)

Abstract

The use of primary hepatocytes from rainbow trout in research provides various advantages. However, for the routine application of primary hepatocytes in the laboratory, a highly standardized and reliable isolation and culture protocol is necessary in order to get viable and robust cells and reproducible results. Therefore, based on an established collagenase isolation protocol, we investigated several factors in varying combinations to find out the most adequate culture conditions: (1) fetal calf serum (FCS) concentration in the medium, (2) cell density, (3) coating substrate, and, (4) culture time.

Immediately after isolation, the mean number of liver cells was $7.5 \pm 1.9 \times 10^8$ cells/fish and $2.5 \pm 0.2 \times 10^6$ cells/g body weight with generally > 90 % of viable cells (average of 94.5 ± 3.7 %). Attachment rate, visibility of individual cells and cell viability served as criteria for the selection of the following culture conditions as most appropriate: Hepatocytes in solution containing 2 % FCS were seeded in 24-well plates with a cell density of approx. 4×10^5 cells/well or 2.3×10^5 cells/cm^2 and were allowed to attach for 24 or 48 h. The findings indicated that FCS concentration, cell density and culture time had only minor influence on the viability and attachment rate of primary hepatocytes (within the tested ranges) compared to the substrate used for coating the culture plate. Among the substrates tested in this study, poly-L-lysine proved to be the most effective in promoting firm attachment of the cells and in allowing them to maintain a differentiated “hepatocyte-like” phenotype.

1 Introduction

For decades, hepatocytes have been used as a model cell system for many purposes. The first isolation was reported in mammals (Berry and Friend 1969), and ten years later the first successful isolation in teleosts was published (Birnbaum et al. 1976). During the 80s and 90s, the application of freshly isolated fish hepatocytes expanded (Babich and Borenfreund 1991, Baksi and Frazier 1990, Blair et al. 1990, Blair et al. 1995, Borenfreund and Shopsis 1985, Braunbeck and Storch 1992, Maitre et al. 1986, Segner 1998).

The use of primary hepatocytes from rainbow trout (*Oncorhynchus* mykiss) in research provides various advantages: In vertebrates, liver is a key organ in the detoxification and excretion of xenobiotics. This makes liver and hepatocytes especially suitable models for ecotoxicology, as is the rainbow trout among fish. Given their taxonomic and ecological positions, fish are important model organisms for both vertebrates and aquatic systems. Fish, by sharing with mammals a large number of important characteristics and by presenting various technical advantages, have become a promising vertebrate model for most biological studies and a suitable alternative to mammalian systems (Braga et al. 2006, Hightower and Renfro 1988, Kelly et al. 1998, Marques et al. 2007). The motivation of using primary cultures instead of continuous cell lines is their supposed similarity to the conditions *in vivo*.

Nevertheless, it cannot be ignored that primary hepatocytes also bear some limitations: It has to be considered that cells from different preparations may vary as a result of differences in the health and reproductive status of the donor fish or specific conditions during the isolation procedure (e.g., time to clear liver from blood, extent of collagenase digestion, temperature of fish maintenance prior to cell isolation). Therefore, results may be supposed to be less reproducible, if compared to the use of the more homogeneous cell lines which provide genetically identical material over a long period without the necessity to revert on intact animals. Preparation and handling of primary hepatocytes may also produce difficulties: One disadvantage is the limited viability of primary hepatocytes. An additional problem is that not all cells classified as “alive” by light microscopic observation are ultrastructurally intact. Further limitations include loss of membrane specialization, possible down-regulation of cytosolic enzymes and loss of the ability to form bile. These effects mean that direct extrapolation of results from isolated hepatocytes to intact liver may be problematic (Braunbeck and Storch 1992).

The primary cells used in this study were given time to attach to the culture plate and were only 24 to 48 h old in order to limit degeneration processes. Compared to mammalian hepatocytes, there is evidence that the loss of differentiated liver function is less rapid in teleost primary cultures, since available data indicate a greater stability of biotransformation enzymes in piscine liver cells than in mammalian hepatocytes. Activities of xenobiotic enzymes of both phase I (e.g. 7-ethoxyresorufin-*O*-deethylase, EROD) and phase II (e.g. glutathione-*S*-transferases, GST, and UDP-glucuronyltransferases, UDPGT) seem to be

conserved during monolayer culture (Segner 1998, Segner and Cravedi 2001). Nevertheless, loss of some cellular functions during isolation or the first days in culture is a risk which cannot be ruled out completely. Therefore, for the routine application of primary hepatocytes in laboratory, a highly standardized and reliable isolation and culture protocol is indispensible in order to get robust cell populations and reproducible results.

So far, several laboratories have investigated liver cell preparations isolated from rainbow trout, in fresh suspension and in culture. *In vivo*, rainbow trout liver cells have a diameter of 15 - 25 µm; they are typical epithelial cells. *Ex vivo*, their diameter is approx. 10 µM (Braunbeck and Storch 1992). Unlike hepatocytes from rat, attachment of rainbow trout liver cells in culture is difficult and has not been generally adopted in many laboratories (Blair et al. 1990). Nevertheless, attachment is important not only for the observation of individual cells, but also for the maintenance of differentiated liver functions in the isolated cells. Hepatocellular function *in vivo* depends on the presence of various factors, including nutritional and hormonal components, extracellular matrix elements, cell-cell interactions with both hepatocytes and non-hepatocytes, and three-dimensional tissue organization.

Since an *in vitro* environment lacks many of these factors, special emphasis has to be paid to improve the prerequisites for isolation and culture of primary hepatocytes to impede loss of liver-specific structures and functions. Several working groups have studied different conditions, and existing protocols, especially with respect to the isolation procedure, do not vary very much and seem to be reliable. The protocol for collagenase isolation of liver cells applied in this study was established by Braunbeck and Storch (1992). Concerning the culture conditions of primary (trout) hepatocytes, results are more heterogeneous. Therefore, we investigated several factors in varying combinations to find out the most adequate conditions:

(1) Fetal calf serum (FCS) concentration in the medium

(2) Cell density

(3) Coating substrate

(4) Culture time

According to literature data, among the named factors the application of an adequate culture substrate seems to be especially important. Following the conditions *in vivo*, elements of the extracellular matrix are supposed to fulfill this requirement. The extracellular matrix is composed of components which are important for adhesion, i.e., fibronectin, vitronectin, laminin and tenascin, as well as structural components such as collagen and elastin. In primary cultures of rat hepatocytes it has been shown that interactions between cells and the extracellular matrix activate regulate many fundamental processes during development. These include regulation of growth and death, migration of the cells and differentiation programs during organ formation. Studies in rat indicate that

the extracellular matrix modulates hepatic development, regeneration and maintenance of the liver architecture in the normal differentiated state (Sanchez et al. 2000).

Results from different working groups with respect to the most adequate surfaces for the attachment of rainbow trout hepatocytes are heterogeneous. Therefore we tested several components of the extracellular matrix (fibronectin, collagen I and collagen IV; Tab. 1) as well as poly-L-lysine in order to find out if they promoted attachment and *in vivo*-like aspect of primary hepatocytes in culture. The coating should

(1) allow attachment of primary hepatocytes to the culture plate and permit certain manipulations (pipetting, media change etc.),

(2) not interact negatively with the hepatocytes reducing viability,

(3) promote *in-vivo* like cell morphology,

(4) be easily applicable, reproducible and economic to allow large scale use on a daily routine basis.

Tab. 1: Coating agents for the primary culture of rainbow trout hepatocytes applied in the present study. All tested substances were purchased at Sigma, Germany and applied according to the listed references (adapted) and in the named concentration.

Agent	Substance class	Function	Source	c (µg/cm^2)	References
Collagen I	Protein	Extracellular matrix component	Rat tail	5.6	Rabergh et al. (1995) Sanchez et al. (2000) Scholz et al. (1998) Sigma product information
Collagen IV	Protein	Extracellular matrix component, basement membrane	Human placenta	5.6	Sigma product information
Fibronectin	Glyco-protein	Extracellular matrix component	Bovine plasma	2.8	Sanchez et al. (2000)
Poly-L-lysine	Small polypeptide	Natural preservative, cationic surfactant	Synthetic	20	Rabergh et al. (1995) Sigma product information

2 Materials and methods

2.1 Animals

Two year old rainbow trout (*Oncorhynchus mykiss*) of both sexes, 20 - 25 cm long and with a weight of 200 - 400 g, were purchased at a local supplier in Ittlingen, Germany. Fish were kept in lots of 10 - 20 individuals in 600 L plastic tanks supplied with permanent intensive aeration for at least two month prior to experiments. In a flow-through system, non-chlorinated water adjusted to 16 - 17 °C with a total hardness of 325 mg/L $CaCO_3$ (pH 7.6 ± 0.1, NH3 < 0.01 mg/L) was continuously replaced at an exchange rate of 50 L/h. Fish were fed once daily with a commercially available rainbow trout food at a daily rate of 3 - 4 % body weight. Light and dark phases were 12 h each.

2.2 Isolation of primary hepatocytes

Primary hepatocytes were isolated according to (Braunbeck and Storch 1992) with some minor revisions (Fig. 1, 2; for media composition see Tab. 2). In concrete terms, rainbow trout were anaesthetized by immersion in a saturated solution of ethyl-4-aminobenzoate (benzocaine), disinfected superficially with 70 % ethanol, and placed ventral side up on a metal tray. The following steps were carried out under sterile conditions at a laminar flow bench. The abdominal and heart cavities were opened by a midline incision from the anus to the pectoral fins. A second incision along the lateral line was made from the urogenital pore to the pectoral girdle. A blunt 21 gauge butterfly canula (o.d. 0.8 mm, i.d. 0.6 mm) was inserted into the hepatic portal vein and loosely fixed by means of an arterial clamp. The liver was cleared of blood by *in situ*-pre-perfusion with well-aerated Ca^{2+}- and Mg^{2+}-free Dulbecco's medium (buffer I) adjusted to pH 7.55 and supplemented with 2 mM EDTA for 10 - 15 min. Blood and perfusion medium were released by ventrally severing the *sinus venosus*. Perfusion was carried out at an initial flow-rate of 1.5 ml/min x g liver, which was raised to approx. 2.5 ml/min x g liver, at 16 °C. During perfusion, the liver was separated from adhering adipose tissue. The major bile duct and the gall bladder were removed, and the liver was transferred to a perfusion apparatus after cutting the portal vein, the hepatic artery and vein, as well as strands of connective tissue.

After 15 min, perfusion was switched to MEM-Hanks medium containing 25 mM HEPES and 0.25 mg/ml collagenase H from *Clostridium histolyticum* (Clostridiopeptidase A, 150 mU/mg; buffer II) to allow connective tissue digestion. Collagenase perfusion was continued for 30 - 40 min at 20 °C with recirculation of the isolation medium, until the liver tissue became soft and malleable.

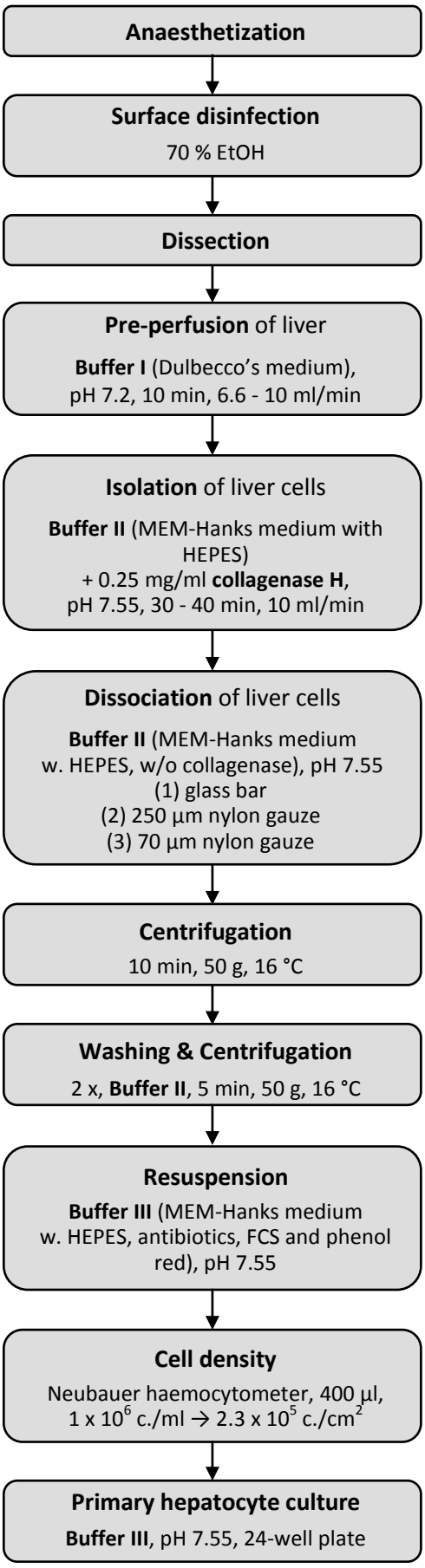

Fig. 1: Scheme for the isolation of rainbow trout primary hepatocytes.

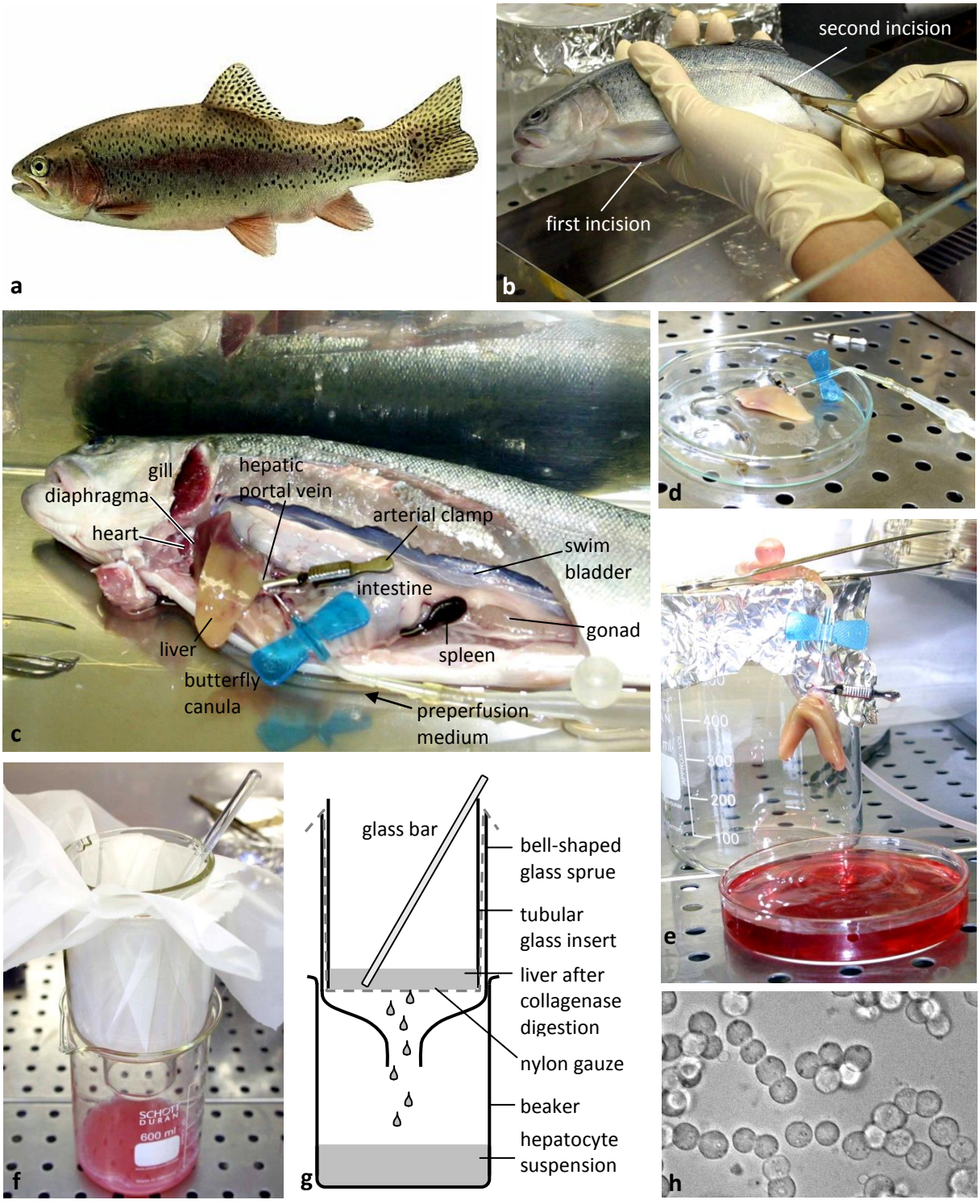

Fig. 2: Primary hepatocyte isolation. (a) Adult rainbow trout (*Oncorhynchus mykiss*; www.karnbaum.de), **(b)** Opening of the abdominal and heart cavities **c** Abdominal and heart cavities after starting pre-perfusion with Dulbecco's medium. Liver is partially cleared of blood. **(d)** *In situ*-pre-perfusion with Dulbecco's medium. **(e)** Perfusion with MEM-Hanks medium containing collagenase. **(f) and (g)** Dissociation of liver cells by filtration through 250 and 70 µm nylon gauze. **(h)** Primary hepatocytes in culture forming monolayer.

Tab. 2: Composition of primary hepatocyte isolation and culture media. All media and other substances were purchased from Sigma, Germany

	Buffer I Pre-perfusion	**Buffer II Isolation**	**Buffer III Culture**
NaCl (mM)	136.89	136.89	136.89
KCl (mM)	2.68	5.37	5.37
$CaCl_2$ (mM)		1.26	1.26
KH_2PO_4 (mM)	1.47	0.44	0.44
$MgSO_4$ x 7 H_2O (mM)		0.81	0.81
NaH_2PO_4 x 2 H_2O (mM)	6.06	0.41	0.41
Na_2HPO_4 (mM)	1.30		
$NaHCO_3$ (mM)		4.17	4.17
EDTA (mM)	2		
HEPES (mM)		25	25
Glucose (mM)		5.55	5.55
Collagenase H (mg/ml)		0.25	
Fetal bovine serum (%)			0 - 10
MEM amino acid mixture		*	*
Cholin chloride (µg/ml)		1	1
Folic acid (µg/ml)		1	1
i-Inositol (µg/ml)		2	2
Nicotinamide (µg/ml)		1	1
Pantothenic acid (µg/ml)		1	1
Pyridoxal-HCl (µg/ml)		1	1
Riboflavine (ng/ml)		100	100
Thiamine-HCl (µg/ml)		1	1
Penicillin G (10,000 IU; µg/ml)			60
Streptomycin sulphate (µg/ml)			126
Amphotericin B (ng/ml)			250
Phenol red (µg/ml)			10
pH	7.2	7.55	7.55
Temperature (°C)	20	20	20
Perfusion time (min)	10 - 15	30 - 40	
Perfusion rate (ml/min)	6.6 - 10	10	
Incubation time (h)			24/48

* including L-forms of arginine-HCl, cystine, glutamine, histidine-HCl, isoleucine, leucine, lysine-HCl, methionine, phenylalanine, threonine, tryptophane, tyrosine, valine

Following digestion, the liver was placed in a sterile glass petri dish with collagenase-free MEM-Hanks medium, and liver cells were dissociated from the hepatic stroma by means of steel tweezers and a glass bar. To remove cell aggregates and connective tissue remnants, the crude cell suspension was passed through 250 and 73 µm nylon gauze into 50 ml sterile centrifuge tubes and span for 10 min at 50 g and 16 °C to collect cells. After resuspension of the pellet in collagenase-free MEM-Hanks buffer, the cells were washed and span twice and taken up in MEM-Hanks medium supplemented with 25 mM HEPES, 5.55 mM glucose, antibiotics, 20 ml MEM amino acid mixture, 10 ml BME vitamin mixture, 0 - 10 % fetal calf serum (FCS) and 10 µG/ml phenol red (buffer III).

2.3 Cell culture and coating

All steps were carried out at 20 °C if no differing temperature is indicated. After taking up the cells in culture medium, cell density was determined in a Neubauer haemocytometer and adjusted. Coating agents (Tab. 1) were applied to the 24-well plates as described in the following:

Collagen I: A 0.05 % collagen solution in 0.02 M acetic acid was stirred at room temperature for 1 - 3 hours until dissolved. The collagen solution was transferred to a glass bottle with a screw cap and carefully layered chloroform at the bottom (10 % of the volume of collagen solution). The glass bottle was allowed to stand overnight at 2 °C without shaking or stirring. The top layer containing the collagen solution was removed aseptically and diluted 10-fold with sterile PBS. 24-well plates were coated overnight at 4 °C with 200 µl solution per well, resulting in a final concentration of 5.6 µg collagen I/cm^2.

Collagen IV: 50 µg/ml were dissolved in 0.05 M HCl for several hours at 4 °C, occasionally swirling. 24-well plates were coated overnight at 4 °C with 200 µl solution per well, resulting in a final concentration of 5.6 µg collagen IV/cm^2.

Fibronectin: 50 µg/ml were dissolved in sterile PBS. 24-well plates were coated for 4 hours at room temperature with 100 µl solution per well, resulting in a final concentration of 2.8 µg fibronectin/cm^2.

Poly-L-lysine: 24-well plates were coated for 4 hours at room temperature with 35 µl of a 100 µg/ml solution per well, resulting in a final concentration of 20 µg poly-L-lysine/cm^2.

Prior to use, excess fluid was removed from the coated surface. Wells were washed twice with sterile PBS and allowed to dry for 3 h in a laminar flow culture hood. Dried plates were sterilized by exposure to UV light for 30 min.

To find out the most adequate culture conditions, most appropriate FCS concentration in the culture medium, cell density and culture time were tested besides the adequate culture substrate to improve cell vitality. All variable factors were tested in different combinations (5 independent replicates; Tab. 3). For each combination, 400 µl of hepatocyte suspension

were incubated in four cavities of a 24-well plate. After 24 or 48 h, cells were photographed under a light microscope. The medium was taken up and released carefully three times with a pipette to simulate several washing steps (mixing the cells of the four wells). After "washing" the cells, the percentage of non-detached cells was determined and used to provide an indicator for cell attachment. Moreover, viability of attached cells was estimated via trypan blue exclusion (0.4 % in 0.81 % sodium chloride and 0.06 % potassium phosphate; Sigma, Germany).

Tab. 3: Culture conditions of rainbow trout primary hepatocytes. All factors were experimented in different combinations. Conditions finally applied are shaded in grey.

Variable	**Tested**		
FCS concentration in culture medium (%)	0		
	1		
	2		
	5		
	10		
Cell density	0.25	1	0.6
(x 10^6 cells/ml - x 10^5 cells/well - x 10^5 cells/cm^2)	0.5	2	1.1
	1	4	2.3
	2	8	4.5
Culture time	24 h		
	48 h		
Coating agent	None		
	Collagen I		
	Collagen IV		
	Fibronectin		
	Poly-L-lysine		

3 Results and discussion

3.1 Hepatocyte attachment and viability

The mean number of liver cells isolated in the present study was 7.5 ± 1.9 x 10^8 cells/fish and 2.5 ± 0.2 x 10^6 cells/g body weight (n = 24; Fig. 3). Immediately after isolation, cell viability was estimated *via* trypan blue exclusion and was 94.5 ± 3.7 %. Only preparations with a viability > 90 % (88 % of all preparations) were used.

In literature, only little information about cell yield and viability can be found. The mean number of viable liver cells isolated from rainbow trout obtained by Klaunig et al. (1985) was 2.81 x 10^6/g body weight. The viability of 96.3 % was also similar to that observed in this study. Braunbeck and Storch (1992) found a viability > 90 %, (Lipsky et al. 1986) of 96 %.

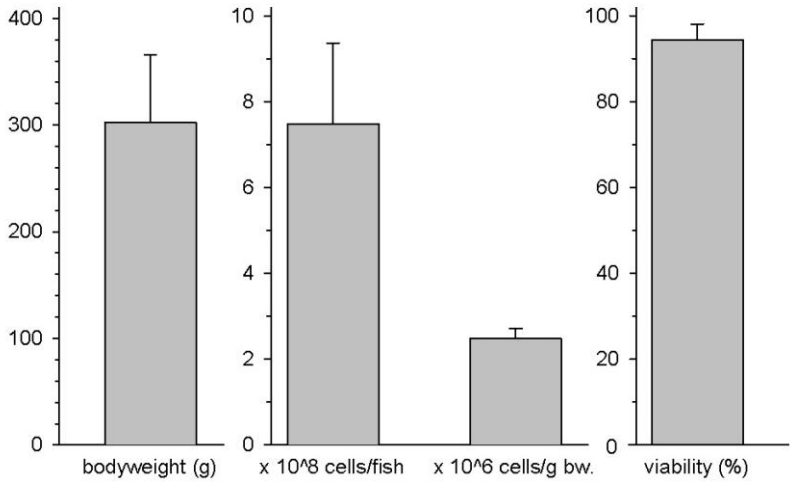

Fig. 3: Average body weight (left), cell yield (middle) per fish and per g bodyweight and viability (right) of liver cells immediately after isolation. All data are given as mean ± SD (n = 24).

To find out the most adequate culture conditions for the further application of primary hepatocytes, several variables were experimented in different combinations. Attachment rate, visibility of individual cells and cell viability served as criteria. Generally, a great variability between different preparations could be observed.

3.2 Fetal calf serum (FCS) concentration

The necessity of serum or hormone addition to the culture media is controversial. In this study, the addition of 2 - 10 % FCS in the medium was found to increase cell attachment (Fig. 4 a), but lowered cell viability (Fig. 4 b). These results are in accordance with those found by Braunbeck and Storch (1992), who also observed a negative influence of FCS. Klaunig et al. (1985), however, found a positive effect of serum addition on survival of cultured rainbow trout hepatocytes and concluded that 5 % FBS was optimal (greatest

survival at the lowest serum concentration) for maintenance of rainbow trout hepatocytes. A possible reason for this discrepancy might be the different culture conditions: Whereas (Klaunig 1984) kept hepatocytes in suspension in L15 medium, (Braunbeck and Storch 1992) plated cells on culture dishes and used MEM-Hanks medium as in the present study.

Since the use of 2 % FCS enhanced the attachment of hepatocytes on 24-well plates coated with poly-L-lysine, but did not reduce viability significantly, this concentration was selected for subsequent experiments.

Kocal et al. (1988) reported the use of fish serum to obtain rainbow trout hepatocytes in culture on collagen-coated plates; whereas fetal bovine serum had little influence on the attachment. Blair et al. (1990), however, found no effect of serum.

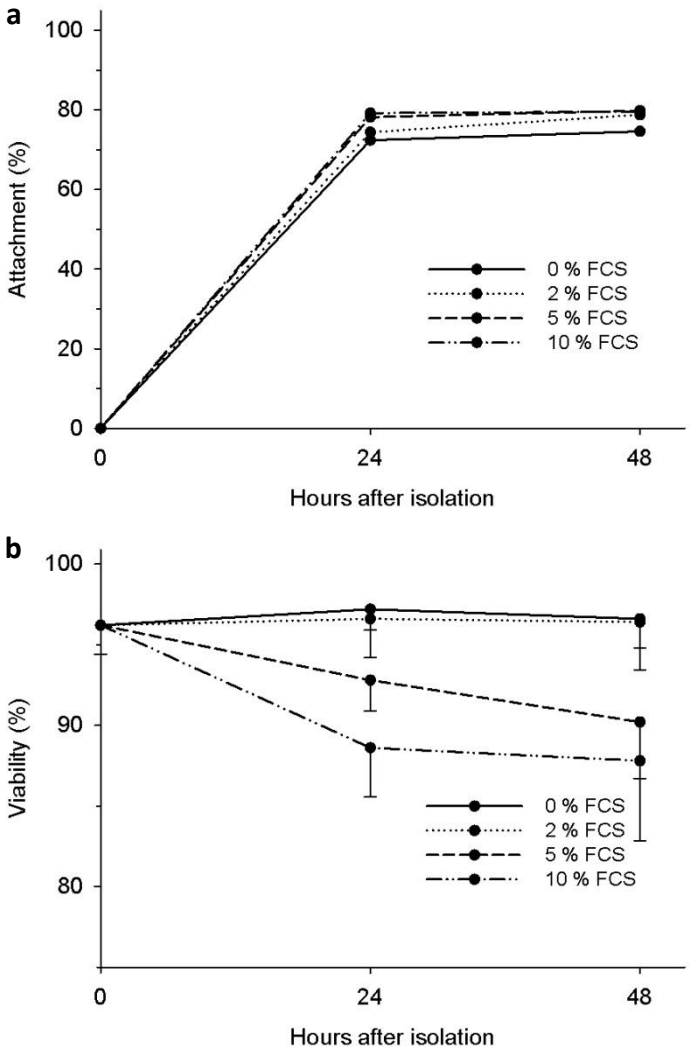

Fig. 4: (a) Attachment and **(b)** viability (assessed by trypan blue exclusion) of isolated rainbow trout hepatocytes to 24-well plates coated with poly-L-lysine, incubated with different FCS concentrations at a density of 2.3 x 10^5 cells/cm^2 (n = 5). Viability after 24 and 48 h cannot be compared directly to that immediately after isolation, since detached and potentially unviable cells are discarded during washing steps.

3.3 Cell density

In order to optimize culture conditions for primary rainbow trout hepatocytes, different cell densities were tested with respect to cell attachment and cell viability on different culture substrates. Fig. 5 shows only data for poly-L-lysine, since attachment on other substrates was poor and cells tended to form aggregates disturbing the determination of cell viability (cf. Fig. 7).

For all substrates, percentage of attached cells did not vary very much when different cell densities were compared (cf. Fig. 6). Cell attachment and viability of attached cells were maximum for intermediate densities (1.1 and 2.3 x 10^5 cells/cm^2). At 1.1 x 10^5 cells/cm^2, however, hepatocytes did not cover the whole surface of the well. At higher densities (> 2.3 x 10^5 cells/cm^2), cells tended to form aggregates instead of a monolayer, which impeded the identification of single cells (see light microscopic photos in Fig. 7). 2.3 x 10^5 cells/cm^2 proved to be the most adequate cell density, taken into regard all factors, providing an average cell attachment of 76.8 ± 9.6 % after 24 h and 72.8 ± 3.7 % after 48 h and a cell viability of 97.6 ± 3.2 % after 24 h and 96.2 ± 3.1 % after 48 h for poly-L-lysine.

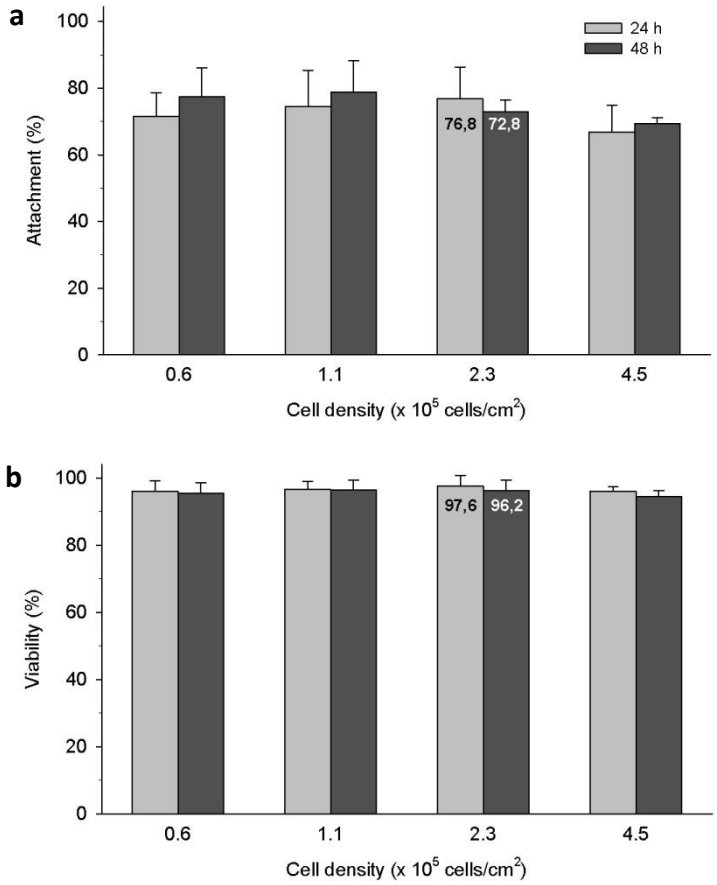

Fig. 5: (a) Attachment and **(b)** viability (assessed by trypan blue exclusion) of isolated rainbow trout hepatocytes on poly-L-lysine at different cell densities after 24 and 48 h (n = 5). FCS concentration in the medium was 2 %.

3.4 Coating substrate

Several coated surfaces were tested and compared with respect to their suitability in the culture of rainbow trout hepatocytes. The culture substrate should not only improve the attachment, but also support good viability and *in vivo*-like appearance of the hepatocytes. Figs. 6 and 7 show the attachment of primary hepatocytes on different coating substrates and on untreated plastic-well plates after 24 and 48 h of culture.

Freshly isolated cell suspensions contained predominantly single cells. Once in culture, cells sank to the bottom quickly, establishing cell-to-cell interactions. As can easily be seen, only the polypeptide poly-L-lysine provided good attachment of primary rainbow trout hepatocytes at an average of > 70 %. In contrast to mammalian hepatocytes, which flatten and spread out on the surface of the plate, cells mainly formed a monolayer of easily observable individual hepatocytes and had a round, only slightly flattened appearance.

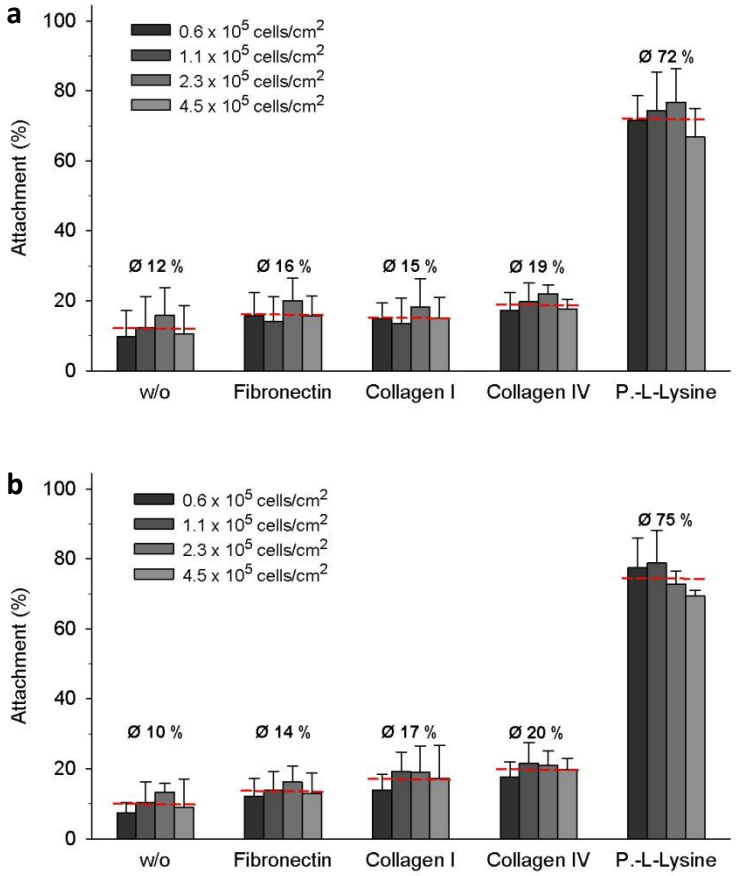

Fig. 6: Attachment of isolated rainbow trout hepatocytes to untreated 24-well plates and various physical surfaces at different cell densities after **(a)** 24 h and **(b)** 48 h, respectively (n = 5). FCS concentration in the medium was 2 %.

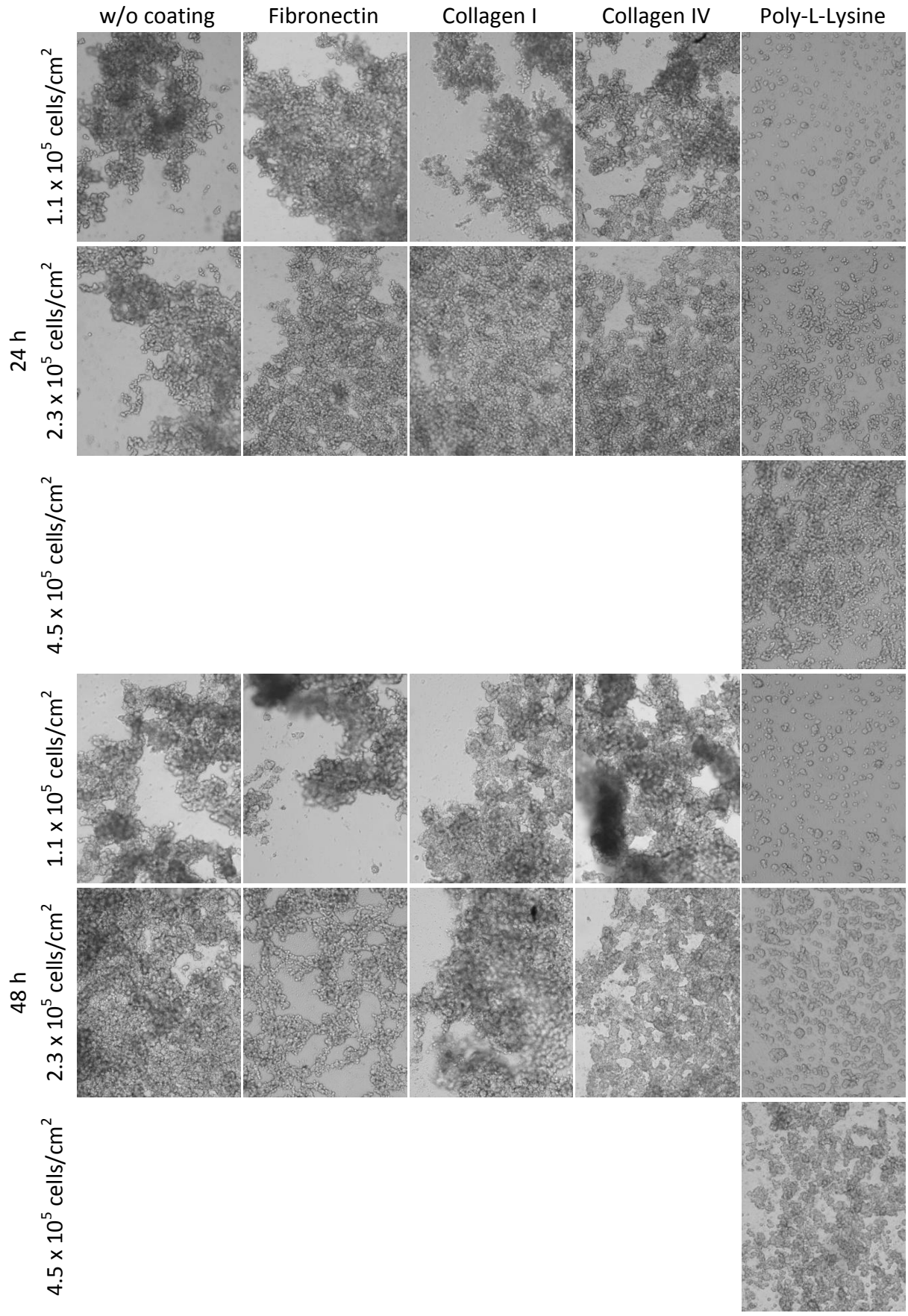

Fig. 7: Attachment of isolated rainbow trout hepatocytes on different substrates and at different cell densities after 24 h and 48 h, respectively.

On untreated plates as well as on all extracellular matrix components applied in this study, attachment was poor and was generally < 20 %. Cells tended to form three-dimensional aggregates, growing larger with time, which detached easily from the bottom of the well plate and floated in the medium. Apparently, rainbow trout hepatocytes have strong affinity to each other and show a tendency to aggregate. Trypan blue exclusion tests suggest that detached cells were still viable in their majority; however it turned out to be impossible to determine exact viability rates of the aggregated cells.

In the present experiments, **polylysine** was by far the most promising substrate for the attachment of primary hepatocytes. Attachment efficiency exceeded 70 % and was optimal at a cell density of 2.3 x 10^5 cells/cm^2 (76.8 ± 9.6 % after 24 h and 72.8 ± 3.7 % after 48 h). Viability of attached cells was almost 100 % (97.6 ± 3.2 % after 24 h and 96.2 ± 3.1 % after 48 h; Fig. 7, 8). Interestingly, polylysine is no extracellular matrix component, but a cationic compound which apparently promotes the attachment of the hepatocytes with a negative surface charge (Sherbet et al. 1972) by increasing the net-positive charge of the culture dish.

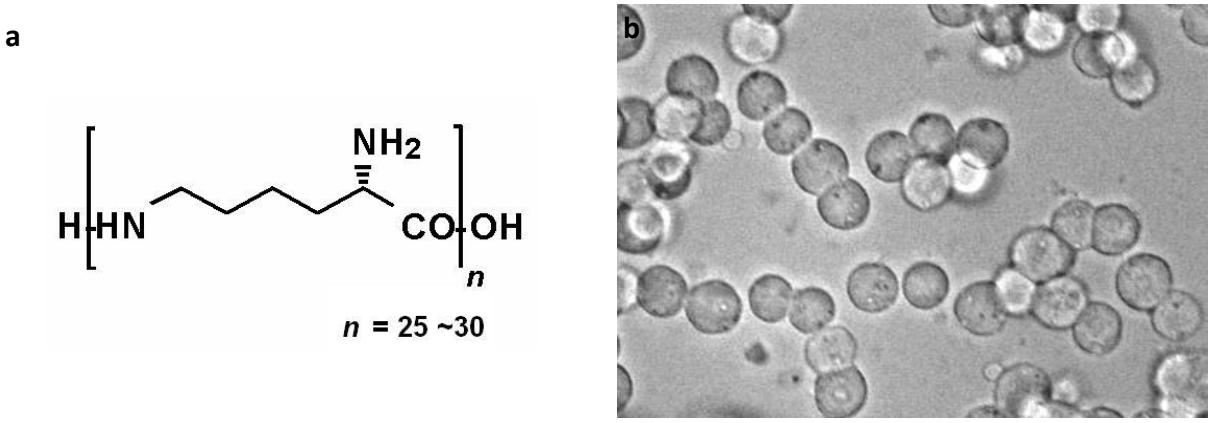

Fig. 8: (a) Poly-L-lysine, a homo-polypeptide of approx. 25 - 30 L-lysine residues. **(b)** Primary hepatocytes grown on poly-L-lysine for 24 h.

Uncoated well-plates proved to be unsuitable for the culture of primary rainbow trout hepatocytes. These results are in concordance with those from other working groups: Generally, untreated plastic cell culture plates from various manufacturers proved ineffective in several studies reporting attachment efficiency of only 15 - 20 % and easy dislodging of the cells (Blair et al. 1990, Braunbeck 1993, Klaunig et al. 1985, Lipsky et al. 1986, Maitre et al. 1986, Rabergh et al. 1995). Conflicting reports exist on the attachment of Falcon Primaria dishes. Pesonen and Andersson (1997) reported good attachment for 5 - 6 days. Blair et al. (1990) and Rabergh et al. (1995) observed attachment of fresh preparations; however, the cells did not remain attached for more than 24 h, where after the cells started to float and were easily detached by media change.

Poor attachment of rainbow trout hepatocytes on extracellular matrix components like **collagen** and **fibronectin** is in clear contrast to the results reported for mammalian hepatocytes. Sanchez et al. (2000) found that all the extracellular matrix proteins promoted attachment of fetal rat hepatocytes in a similar way. Only fibronectin, however, was shown to facilitate the formation of elongated structures reminiscent of liver plate organization. In the present study, however, no positive effects on the attachment or morphology of rainbow trout hepatocytes could be observed when fibronectin-coated substrates were used. (Blair et al. 1990) and (Rabergh et al. 1995) also reported fibronectin that did not promote attachment of rainbow trout hepatocytes.

Culture plates coated with collagen were found to be unsuitable for the culture of rainbow trout hepatocytes by many working groups (Blair et al. 1990, Klaunig 1984, Kocal et al. 1988, Lipsky et al. 1986, Maitre et al. 1986, Rabergh et al. 1995). Scholz et al. (1998) found that seeding of hepatocytes on collagen-coated dishes did not alter cell attachment or detachment from the culture substrate, but had a small, but not significant effect on cell viability and metabolic parameters. Similar data are available for mammalian hepatocytes, indicating improved viability and metabolism of cells maintained on collagen substrate (Guguen-Guillouzo and Corlu 1993, Sawada et al. 1987).

Type and origin of the collagen might have an influence on the attachment of rainbow trout hepatocytes. Most authors used collagen of mammalian origin. In our experiments, commercial collagen preparations from rat and human tissues were used. However, (Kaneko et al. 1995) demonstrated that for fish cell culture, the use of extracellular matrix proteins of piscine origin might be more advantageous.

In literature, several other purchasable or self-made substrates were tested with respect to the attachment of rainbow trout hepatocytes:

Haschemeyer and Mathews (1983) reported a rainbow trout **skin extract** served well as a matrix for attaching primary hepatocytes from fish. According to (Blair et al. 1990), cells quantitatively attach to culture plates coated with rainbow trout skin extract and remain attached for 4 - 6 days. The hepatocytes initially form a monolayer over the first few hours in culture, at a time when they are attaching to the culture plate. In culture, the cells rapidly establish cell-to-cell interactions and move together forming aggregated mounds. After 4 to 5 days in culture, these aggregates grow larger and individual cells retain only minimal contact points with the culture plate, and may detach. Notwithstanding the positive results of these working groups, we excluded rainbow trout skin extract from our study, trying to find an easily applicable and reproducible substrate.

(Lipsky et al. 1986) demonstrated that rainbow trout hepatocytes readily and firmly attached to dishes coated with a commercially available **extracellular matrix** (ECM; attachment efficiency 93 %). Differentiated liver function was maintained in cells cultured on ECM. Supporting these results, Rabergh et al. (1995) reported that cells flattened and formed a confluent monolayer on day 2 in culture. **Matrigel** is also proven to provide good attachment

of liver cells (Rabergh et al. 1995). However, we did not test ECM and matrigel due to their high costs impeding large scale use on a routine basis.

Laminin was reported by Rabergh et al. (1995) to be the only extracellular matrix component in promoting firm attachment of fish hepatocytes to the culture dish. Cells grown on laminin showed an even higher attachment efficiency of > 90 % than polylysine and inducibility of TAT (tyrosine aminotransferase) in response to corticosteroids as an indicator of differentiated liver function through 19 days. The authors were not able to detect any differences in viability or differentiation in rainbow trout hepatocytes grown on laminin or polylysine. Polylysine is, however, more economical than laminin.

It seems that attention has to be paid not only to effects of medium composition and different substrates on the attachment, but also on the physiology of liver cells. Blair et al. (1990) reported for rainbow trout skin extract, rapid establishment of junctional complexes with neighboring hepatocytes and re-creation *in vitro* of cell-to-cell attachments and tissue structures (tubules, ductules), similar to those observed in sections of intact rainbow trout liver. Transmission electron microscopy findings suggest that processes from adjacent biliary epithelial cells form junctions and seem to be overlapping to form *in vitro* equivalents of bile ductules. A similar morphologic appearance was reported by Rabergh et al. (1995) with cells cultured on matrigel, laminin and polylysine substrates. Cells had an oval to rounded appearance with establishment of cell-to-cell contacts and tubular formation after the second day in culture. The authors concluded that the “liver-like” appearance of the cells might be of importance in maintaining a more differentiated phenotype as indicated with rat hepatocytes grown on floating collagen membranes (Michalopoulos and Pitot 1975) or in aggregate cultures (Yuasa et al. 1993).

We could also observe that cells grown on poly-L-lysine attached with relatively high efficiency, but had quite different morphologic features compared to cells cultured on other substrates: Cells attached to uncoated plates, collagen and fibronectin not only in minor degree, the attached cells were also flat and very closed one to another, leaving huge vacancies without cells. On poly-L-lysine, the hepatocytes spread out more or less regularly all over the surface (cf. Fig. 7). Single cells as well as smaller groups and strands of 2 - 10 cells could be observed (Fig. 8), probably allowing neighboring hepatocytes to build out cell junctions and cell-to-cell interactions like those observed by Blair et al. (1990) for rainbow trout hepatocytes grown on rainbow trout skin extract. Cells were rounded in appearance and did not flatten. From day 3 or 4 on (not shown), cells moved closer together forming a network of aggregates and tubular structures. Blair et al. (1990) reported a similar morphologic appearance already after the second day in culture, postulating the formation of tissue-like structures *in vitro*. This morphology, however, made it difficult to observe individual cells. Therefore we decided to use 24 or 48 h old hepatocytes for further applications.

4 Conclusions

In the present study, several factors were tested in different combinations in order to find out the most adequate culture conditions for primary hepatocytes. Attachment rate, visibility of individual cells and cell viability served as criteria for the selection of the following culture conditions which proofed to be most adequate for the application of rainbow trout hepatocytes for further investigations (grey shaded in Tab. 3): 400 µl of hepatocyte suspension with a cells density of 1 x 10^6 cells/ml and containing 2 % FCS were seeded into each cavity of 24-well plates, resulting in a cell density of approx. 4 x 10^5 cells/well or 2.3 x 10^5 cells/cm^2. Since no significant differences were found between the culture times 24 and 48 h, hepatocyte preparations from one fish could be used on two consecutive days.

The findings indicate that FCS concentration in the medium, cell density and culture time had only a relatively small influence on the viability and attachment rate of primary hepatocytes (within the tested ranges) compared to the substrate used for coating the culture plate. Among the substrates tested in this study, poly-L-lysine proved to be the most effective promoting firm attachment of the cells and allowing them to maintain a differentiated “liver-like” phenotype.

In general, primary rainbow trout hepatocytes are an excellent model cell system providing several advantages. However, isolation and culture must strictly follow the protocole to guarantee maximum quality of cells and reproducibility of results.

Chapter 3

Amplitude-encoded calcium oscillations in fish cells

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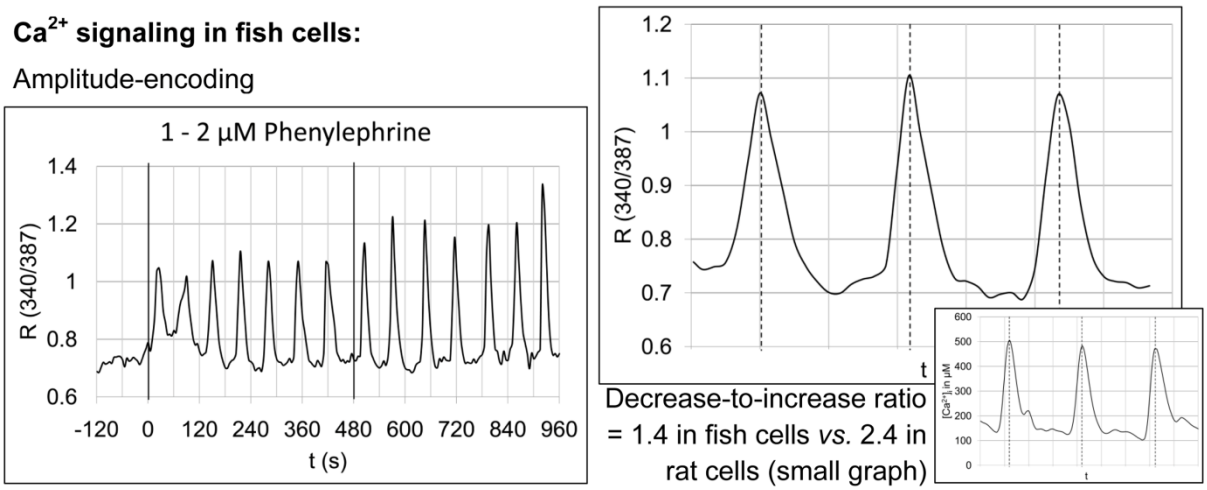
L’universo non potrà essere letto finché non avremo imparato il linguaggio e avremo familiarizzato con i caratteri con cui è scritto. E’ scritto in linguaggio matematico, e le lettere sono triangoli, cerchi e altre figure geometriche.

Galileo Galilei (1564 - 1642)

Graphical abstract



Highlights

- The reaction of intracellular Ca^{2+} was investigated in two cell types from fish.
- Bioinformatics and computational analysis were employed to identify key players.
- We report Ca^{2+} oscillations in RTL-W1 cells and in primary hepatocytes.
- In contrast to mammalian cells, these Ca^{2+} oscillations are amplitude encoded.

Abstract

The reaction of intracellular Ca^{2+} to different agonist stimuli in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*) as well as the permanent fish cell line RTL-W1 was investigated systematically. In addition to "classical" agonists such as phenylephrine and ATP, model environmental toxicants like 4-nitrophenol and 3,4-dichloroaniline were used to elucidate possible interactions between toxic effects and Ca^{2+} signaling. We report Ca^{2+} oscillations in response to several stimuli in RTL-W1 cells and to a lesser extent in primary hepatocytes. Moreover, these Ca^{2+} oscillations are amplitude-encoded in contrast to their mammalian counterpart. Bioinformatics and computational analysis were employed to identify key players of Ca^{2+} signaling in fish and to determine likely causes for the experimentally observed differences between the Ca^{2+} dynamics in fish cells compared to those in mammalian liver cells.

Keywords

Calcium signaling – oscillations – rainbow trout – amplitude encoding – teleost fish – cell line RTL-W1

1 Introduction

Calcium plays a fundamental role in many cellular processes in all types of tissues and organisms (for reviews see e.g. Berridge et al. 2000a, Berridge et al. 2000b, Petersen et al. 2005, Putney Jr 1998). One important function is its role as a second messenger in cellular signaling transmitting information from the cell surface to specific targets within the cell and controlling a wide range of cellular reactions. In non-excitable cells, Ca^{2+} modulates diverse processes such as cell proliferation, egg activation and early development, contraction, secretion, gene regulation, the control of various enzymes, and apoptosis (Parekh and Penner 1997). In liver, it regulates many hepatic functions, including glycogenolysis, canalicular contraction, tight junction permeability, and bile secretion (Dupont et al. 2000). A number of receptors connected to Ca^{2+} signaling pathways have been identified, e.g. adrenergic and purinergic, as well as vasopressin and angiotensin receptors. Ca^{2+}-signaling also seems to play a role in the response to environmental stress including the induction of cell death as a consequence of the loss of intracellular Ca^{2+} homeostasis (see e.g. Duchen 2000, Kass and Orrenius 1999, Orrenius 2004, Orrenius et al. 2003).

The versatility of Ca^{2+} signaling is remarkable and is due to the complex mechanisms which help to encode information. Numerous Ca^{2+}-providing and -detracting pools and reactions allow the temporal and spatial modulation of the Ca^{2+} signal: Ca^{2+} can operate locally or throughout the entire cell. The signal can last over different periods of time, as short transient or as sustained signal (Berridge et al. 1999). Special emphasis has been put on oscillations of the cytosolic Ca^{2+} concentration, as they permit a large number of possibilities for the encoding of information. Since the 1980s, when Ca^{2+} oscillations were found experimentally (Cuthbertson and Cobbold 1985, Woods et al. 1986), a great number of experimental and theoretical studies have been carried out (Schuster et al. 2002).

Notwithstanding the universal character of Ca^{2+}-signaling and the highly conserved pathways, research on Ca^{2+} as a second messenger has mainly been restricted to mammalian models. Much less is known about its function and mode of action in fish. There are several publications on the adrenergic (Krumschnabel et al. 2001, Moon et al. 1993, Zhang et al. 1992a, Zhang et al. 1992b) and purinergic (Nathanson and Mariwalla 1996) modulation of the intracellular Ca^{2+} level as well as its reaction to angiotensin (Russell et al. 2001). However, most of these studies are very specific and do not analyze the modulation of the Ca^{2+} signal systematically and in a dose-dependent manner. Moreover, regular agonist-induced Ca^{2+} oscillations have been reported only for few cell types and substances. In isolated skate hepatocytes, 10 to 100 nM ATP induced Ca^{2+} oscillations (Nathanson and Mariwalla 1996). In trout pinealocytes, spontaneous (Kroeber et al. 1997, Meissl et al. 1996) as well as Bay K 8644-induced (Kroeber et al. 2000) Ca^{2+} oscillations have been observed. Zhang et al. (1992) reported catecholamine-induced Ca^{2+} oscillations in eel, but not in trout hepatocytes (Zhang et al. 1992b). Finally, only a handful of papers deal with the question

whether there is a Ca^{2+}-response to environmental toxicants or not, mostly related to oxidative stress (Betoulle et al. 2000, Burlando and Viarengo 2005, Nawaz et al. 2006).

Because of their systematical position, however, fish are important model organisms. On the one hand, results are at least partially transferrable to other vertebrate groups including mammals and humans; on the other hand, investigations in fish systems give credit to animal welfare legislation and ethics requiring the use of less developed vertebrates in animal experimentation wherever possible. Given the universality of many basic biological processes among eukaryotic organisms, lower animals can serve as useful research models (Baksi and Frazier 1990). Fish, by sharing with mammals a large number of important characteristics and by presenting various technical advantages, have become a promising vertebrate model for most biological studies and a suitable alternative to mammalian systems (Braga et al. 2006, Hightower and Renfro 1988, Kelly et al. 1998, Marques et al. 2007). Finally, differences in biochemical mechanisms between fish and mammalian cells can give insight into evolutionary processes.

In this study, the reaction of intracellular Ca^{2+} to different agonist stimuli in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*), an established model system in aquatic ecotoxicology, as well as in the permanent fish cell line RTL-W1 originally derived from primary cultures of rainbow trout liver (Lee et al. 1993) was investigated systematically in order to find out the basic principles of Ca^{2+} dynamics in teleost fish cells. In addition to "classical" agonists such as phenylephrine and ATP, model environmental toxicants like 4-nitrophenol (Fent and Hunn 1996) and 3,4-dichloroaniline (Monteiro et al. 2006) were used to elucidate possible interactions between toxin effects and Ca^{2+} signaling.

Since liver is a key organ in detoxification, primary hepatocytes as well as the liver-derived cell line seemed appropriate experimental systems for this study. We report Ca^{2+}-oscillations in response to several stimuli in RTL-W1 cells and to a lesser extent in primary hepatocytes. These Ca^{2+} oscillations are amplitude encoded in contrast to their mammalian counterpart. In order to investigate likely mechanisms for this behaviour, we used a combined bioinformatical and computational approach to identify the key players of Ca^{2+} signaling in fish. For this reason, focus was put on the classic agonists of the Ca^{2+} signaling pathway.

2 Materials and methods

2.1 Chemicals

Fura-4 acetoxymethylester (AM) and Pluronic F-127 (20 % solution in DMSO) were obtained from Invitrogen/Molecular Probes (Darmstadt, Germany). All other chemicals were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany).

2.2 Animals

Two year old rainbow trout (*Oncorhynchus mykiss*) of both sexes, 20 - 25 cm long and with a weight of 200 - 400 g, were purchased from a local supplier at Ittlingen, Germany. Fish were kept in lots of 10 - 20 individuals in a flow-through system in 600 L plastic tanks with an exchange rate of 50 L/h and supplied with permanent intensive aeration for at least two months prior to experiments. Fish were fed *ad libidum* once daily with a commercially available trout chow. Light and dark phases were 12 h each.

2.3 Isolation and culture of primary hepatocytes

Primary hepatocytes were isolated *via* collagenase perfusion of the liver according to the protocol described in (Braunbeck and Storch 1992) with minor modifications. Briefly, after digestion of the liver, cells were taken up in MEM-Hanks medium supplemented with 25 mM HEPES, 5.55 mM glucose, antibiotics, 20 ml MEM amino acid mixture, 10 ml BME vitamin mixture, 2 % fetal calf serum and 10 µg/L phenol red. Hepatocytes were seeded into the poly-L-lysine-coated cavities of 24-well plates (Greiner, Frickenhausen, Germany) at a cell density of approx. 4×10^5 cells/well or 2.3×10^5 cells/cm^2, respectively. Since no significant differences were found between 24 and 48 h of culture time, hepatocyte preparations from one fish could be used on two consecutive days.

2.4 Cell line RTL-W1

RTL-W1 cells (Lee et al. 1993) were cultured in Leibovitz-L15 media with 10 % fetal calf serum at 20 °C. For Ca^{2+} imaging, RTL-W1 cells were seeded into the cavities of 24-well plates at a density of 2×10^5 cells/well (1.2×10^5 cells/cm^2) and were allowed to attach for 24 h before the onset of exposure.

2.5 Ca^{2+}-Imaging in rainbow trout hepatocytes

Prior to use, cell viability was estimated *via* 0.18 % trypan blue exclusion and was generally around 90 %. Only test approaches with a viability > 85 % were used for Ca^{2+} measurement.

Fluorescence staining and microscopy were carried out according to many other established protocols on Ca^{2+} imaging in fish cells using 2 - 10 µM Fura-2 AM and incubation times between 10 and 90 min (see e.g., Furimsky et al. 1996, Green et al. 1997, Harwood et al. 2000, Johnson et al. 2000, Kroeber et al. 1997, Marchi et al. 2005, Meissl et al. 1996, Zhang et al. 1992b). In the present study, the following conditions were convenient for Ca^{2+}-imaging in rainbow trout primary hepatocytes: Cells were carefully washed twice with Ringer solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ x 2 H_2O, 1 mM $MgCl_2$ x 6 H_2O, 10 mM D-glucose, 10 mM HEPES) and were loaded with 6 µM Fura-2 AM solution in Ringer for one hour. The Fura-2 solution also contained 0.02 % Pluronic to facilitate the solubilisation of the water-insoluble dye and 1 mM Probenecid to inhibit organic anion transporters and block the efflux of intracellular dyes. Fura-2 AM and Pluronic were solubilised in DMSO at a final concentration < 0.2 %. After dye incubation, cells were washed twice with Ringer solution to remove extracellular Ca^{2+} and left for de-esterification for 30 min.

Ca^{2+} measurements were carried out at the Nikon Imaging Centre at the University of Heidelberg, Germany. For time lapse acquisition (15 - 50 min), 24-well plates were transferred to the stage of a Nikon Ti-E inverted epifluorescence microscope (Nikon, Japan) and cells were illuminated sequentially (every 2 - 5 sec) at 340 nm and 380 nm with a metal halide lamp (Nikon Intensilight). The emitted fluorescence was detected through a 510/30nm bandpass filter with a Hamamatsu ORCA-AG high-sensitive black and white camera (Hamamatsu, Japan). After subtraction of the background, the ratio of fluorescence signals at 340 nm/380 nm served as a measure for the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in individual cells (n = 15 to 30 cells/run). After 5 - 10 min equilibration, cells were stimulated by agonist addition. Depending on the specific experimental objectives, further agonist solution was added to increase effective concentrations after certain periods of time. The Ca^{2+} ionophore ionomycin (5 µM) was used as a positive control.

2.6 Ca^{2+} imaging in RTL-W1 cells

The Ca^{2+} imaging procedure was basically the same as for the primary hepatocytes. All steps were carried with Ringer solution at 20°C in the dark as described above.

2.7 Bioinformatics

Protein sequences were obtained from UniProt (UniProt 2010). Sequence alignment was performed using the BLAST implementation therein with default parameters. Predictions of posttranslational modifications were computed using the ELM resource (Gould et al. 2010). For the prediction of transmembrane helices we used the TMHMM server (Sonnhammer et al. 1998).

2.8 Computational modeling

Computational modeling was done using sets of ordinary differential equations (ODEs). Simulation was performed using the LSODA algorithm as implemented in COPASI (Hoops et al. 2006).

3 Results

3.1 Primary rainbow trout hepatocytes

In rainbow trout primary hepatocytes, for all tested substances (ATP, caffeine, 2,4-dichloroaniline, 3,4-dichloroaniline, histamine, hydrogen peroxide, 4-nitrophenol, phenylephrine) clear effects on the intracellular Ca^{2+} level could be observed in most cells (Fig. 1), but varied in extent between individual cells. Oscillations, however, were evident only in rare occasions, were mostly irregular and showed small amplitudes. Only ATP provoked a characteristic pattern in almost all cells: Intracellular Ca^{2+} rose almost immediately after ATP addition and then declined again to slightly elevated levels. In some cells, this increase could be observed repetitively.

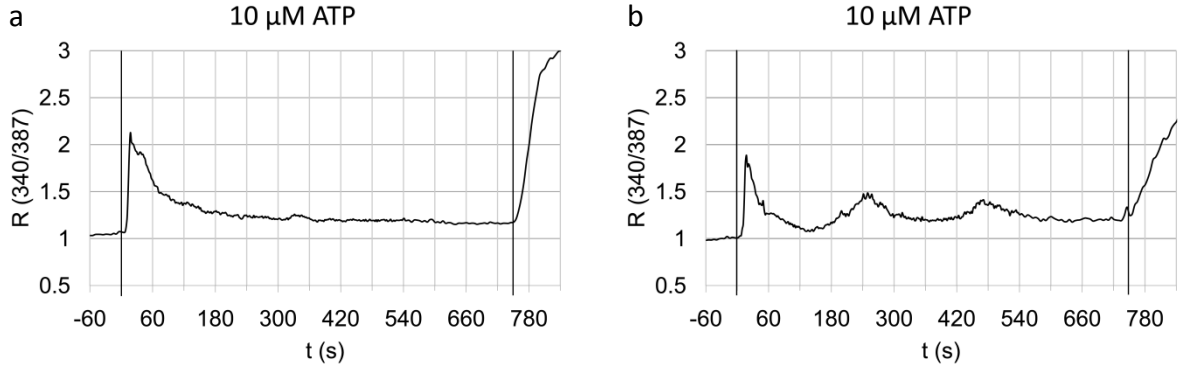


Fig. 1: Alterations of intracellular free Ca^{2+} levels in individual primary rainbow trout hepatocytes after addition of various agonists (first vertical line) exemplified for ATP (10 µM). **(a)** Intracellular free Ca^{2+} rose almost immediately after ATP addition and declined thereafter towards a slightly elevated level. **(b)** In some cells, Ca^{2+} increased repeatedly. Ionomycin addition (5 µM; last vertical line) provoked maximum influx of Ca^{2+}.

For the remaining agonists, the majority of hepatocytes showed a more or less rapid and pronounced elevation of intracellular Ca^{2+} (data not shown). This effect was most pronounced for hydrogen peroxide, the dichloroanilines and 4-nitrophenol. In some hepatocyte preparations, the height of the increase seemed to be concentration-dependent (e.g. hydrogen peroxide; Fig. 2). By addition of ionomycin (control), further Ca^{2+} could be recruited *via* extracellular influx.

Another noticeable response in a fraction of the hepatocyte populations was an abrupt and mostly sustained rise in intracellular Ca^{2+} levels after addition of the agonist. In some preparations, the number of hepatocytes reacting in this way increased in a concentration-dependent manner (details not shown).

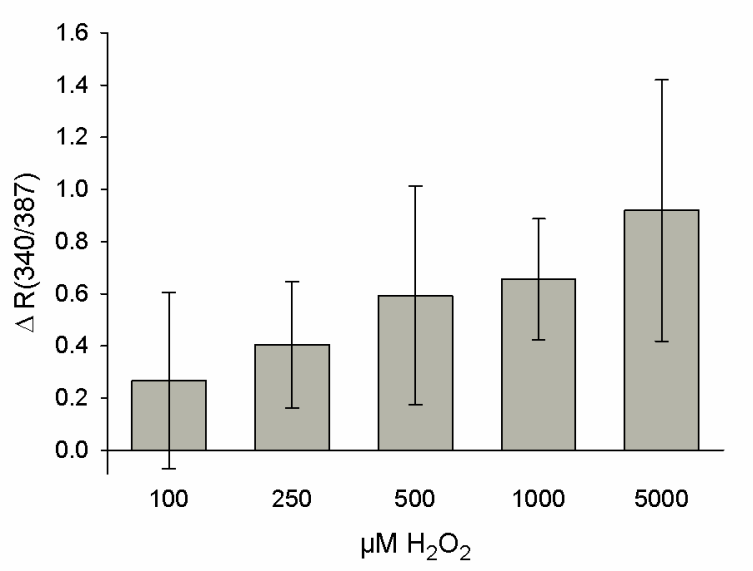


Fig. 2: Difference in the R(340/387) quotient (median ± SD) as a measure for intracellular free Ca^{2+} in primary rainbow trout hepatocytes after addition of different concentrations of hydrogen peroxide. Data from all cells (27 - 31 per concentration) of one hepatocyte preparation were integrated.

3.2 Agonist-specific reactions in the cell line RTL-W1

Results for the permanent fish cell line RTL-W1 differed considerably from those for primary rainbow trout hepatocytes. Oscillations in the intracellular Ca^{2+} level were consistently observed for all agonists tested (ATP, caffeine, 3,4-dichloroaniline, histamine, hydrogen peroxide, 4-nitrophenol, phenylephrine) in the majority of cells. The signals, however, displayed some variation in shape, height and frequency (Fig. 3). Regular oscillations were the predominant reaction for ATP (about half of the cells; Fig. 3 a), phenylephrine (Fig. 3 b), hydrogen peroxide (Figs. 3 c, d), and 4-nitrophenol (data not shown). They were also observed in some cells after addition of histamine (Fig. 3 e); however, this agonist evoked irregular transients in most cells. Hydrogen peroxide and 4-nitrophenol mainly provoked oscillations with pronounced high peaks, whereas 3,4-dichloroaniline gave rise to wide oscillations of small amplitude, which seemed to need time to establish and become regular (data not shown). It has also to be mentioned that only a small number of cells showed a clear response to 3,4-dichloroaniline.

Like the mode of Ca^{2+} alteration itself, individual agonist concentrations required to elicit any reaction of intracellular Ca^{2+} varied from cell to cell, making it difficult to define exact threshold values; however, clear trends were evident. For phenylephrine, e.g., approx.. 0.5 µM were necessary to stimulate regular oscillations. In contrast, for 4-nitrophenol and 3,4-dichloroaniline there was an upper limit for an oscillatory Ca^{2+} response: At concentrations ≥ 5 and 25 µM (4-nitrophenol and 3,4-dichloroaniline, respectively), the agonists rarely had any effect on Ca^{2+}. In other cells, with increasing concentrations of 4-

nitrophenol and hydrogen peroxide, oscillations disappeared, and concentration of free intracellular Ca^{2+} seemed to increase in an uncoordinated manner and remained at elevated levels. Bursts, as illustrated for hydrogen peroxide in Fig. 3 d, were observed in some cells for all agonists and seemed to increase in number with higher concentrations.

Tab. 1: Overview on the alterations in the intracellular Ca^{2+} level observed in RTL-W1 cells after the addition of different agonists. For each agonist, data from 2 - 4 runs with 15 - 30 individual cells each were integrated.

Agonist	Concentration range (µM)	Modulation of intracellular Ca^{2+}		Concentration-dependency
		Rate	Characteristics	
ATP	0.1 - 100	++	Oscillation, singular rise and decrease or irregular transients	Probably amplitude increase at rising concentrations
Caffeine	50 - 10000	+	Rarely regular oscillation	Not perceptible
3,4-Dichloro-aniline	0.1 - 50	+	Oscillation with small amplitude and frequency; baseline decrease ≥ 25 µM	Probably amplitude increase at rising concentrations
Histamine	5 - 400	++	Rarely regular oscillation, mostly irregular transients	Not perceptible
Hydrogen peroxide	5 - 800	++	Oscillation with high amplitude; bursts in higher concentrations	Clear amplitude increase at rising concentrations
4-Nitrophenol	0.5 - 20	++ (≤ 4 µM)	Oscillation with high amplitude; baseline decrease after first addition and subsequent gradual rise; bursts at higher concentrations	Clear amplitude increase at rising concentrations
Phenylephrine	0.1 - 25	++ (≥ 0.8 µM)	Mostly oscillation	Probably amplitude increase at rising concentrations

+ Alterations in the intracellular Ca^{2+} level observed in few cells

++ Alterations in the intracellular Ca^{2+} level observed in most cells (≥ 80 %)

Agonists may also have an impact on free Ca^{2+} baseline concentrations. When 4-nitrophenol was added for the first time, Ca^{2+} baseline levels declined before oscillations started after several minutes. This effect could not be seen when further 4-nitrophenol was added; however, baseline levels increased slowly, but constantly with time.

Finally, agonists not only provoked typical Ca^{2+} reactions in RTL-W1 cells, but individual cells also showed individual patterns with respect to extent and frequency of transients (see, e.g., hydrogen peroxide; Figs. 3 c, d), which sometimes may conceal agonist-specificity.

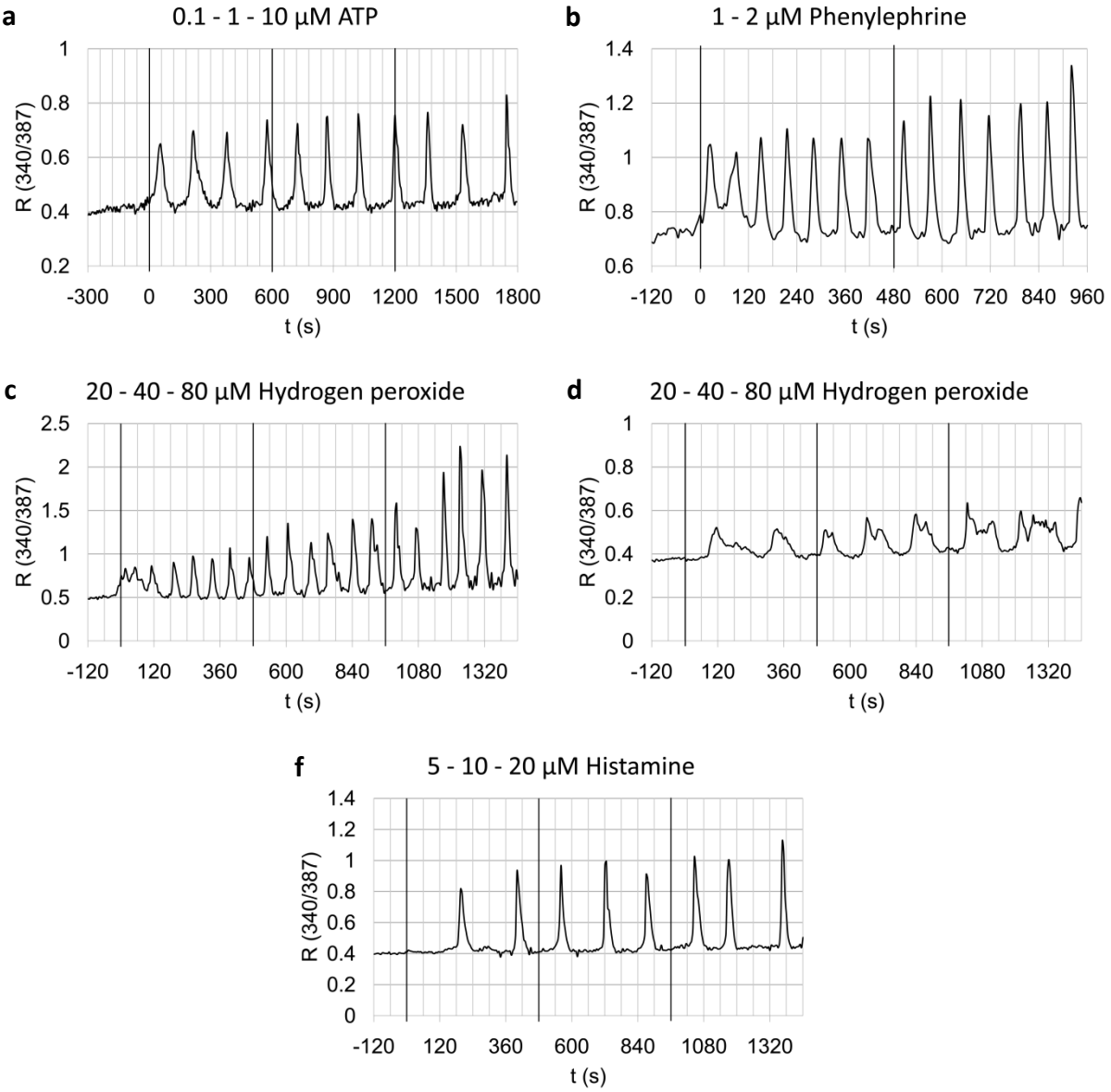


Fig. 3: Oscillations in the intracellular Ca^{2+} levels in individual RTL-W1 cells after addition of various agonists in different consecutive concentrations (vertical lines). Note the different scaling of the y-axis. **(a) ATP. (b) Phenylephrine**. **(c) and (d) Hydrogen peroxide:** Oscillations mostly displayed a high amplitude and relatively small wavelength. In some cells, bursts were observed, here exemplified for hydrogen peroxide. **(e) Histamine**: Regular oscillations were only observed in few cells.

3.3 Amplitude encoding of concentration

Since this study was also motivated from an ecotoxicological point of view, special emphasis was given to a potential concentration-dependency of Ca^{2+} reactions. Hence, different agonist concentrations were tested for all substances. Surprisingly, for almost any agonist, increasing concentrations induced an increase in the amplitude of the Ca^{2+} oscillations. Amplitude-encoding of Ca^{2+} concentrations, e.g., was evident for hydrogen peroxide, 4-nitrophenol and phenylephrine. For ATP, however, a dose-response relationship was less obvious. Thus, an increase in ATP concentrations of a factor 10 was needed to raise the amplitude of the Ca^{2+} oscillation moderately. In contrast to the ATP-induced oscillations in rat liver cells, which exhibit so-called bursting oscillations (Green et al. 1993), the oscillations in RTL-W1 are simple and rather sinusoidal.

3.4 Bioinformatics and modeling of Ca^{2+} oscillations

As described above, there are two major differences in Ca^{2+} oscillations in fish liver cells, if compared to mammalian cells. Most strikingly, there seems to be amplitude- rather than frequency-encoding with respect to the strength of the stimulus. Additionally, ATP, which is known to trigger bursting oscillations in rat hepatocytes (Green et al. 1993), causes simpler oscillations in the fish cells.

In order to find out potential reasons for these different behaviours, we employ computational modeling of Ca^{2+} oscillations.

So far, in contrast to mammalian cells, e.g. hepatocytes, there is no computational model for Ca^{2+} signaling in fish, nor is there comprehensive, detailed information about the biochemical mechanism of Ca^{2+} signaling in fish. Therefore, we first studied literature and analyzed the genetic information available to identify likely key players of Ca^{2+} signaling.

Basically, all key players responsible for classical Ca^{2+} signaling in mammalian cells are also present in fish liver cells. Thus, UniProt (UniProt 2010) entries as well as the literature report the presence of phospholipase C, IP_3 receptors, ryanodine receptors, SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) and different agonist receptors. In order to roughly assess similarities between these and the mammalian counterparts, we performed sequence alignments. As a representative for mammals we used *Rattus norvegicus*. This organism has been used in most experiments on Ca^{2+} signaling in mammalian hepatocytes. Since not all sequences are yet known for the rainbow trout, we used salmon (*Salmo salar*) or zebra fish (*Danio rerio*) as general representatives of fish. The results are summarized in Tab. 2. Obviously, due to the partial lack of species-specific information and in a couple of cases the lack of complete sequences, this only gives a crude insight into the similarities between fish and mammals. One quite eye-catching observation is that there are stronger discrepancies between the agonist-specific receptors of the respective species than in the more general machinery employed in Ca^{2+} signaling.

Tab. 2: Sequence comparison of fish proteins central to Ca^{2+} signaling and mammalian counterparts. Fish sequences from phylogenetic related species were taken, if the sequence for rainbow trout was not available. Mammalian sequences were taken from rat (*Rattus norvegicus*). Sequence alignments were performed using BLAST and default parameters. Only short fragments for the IP_3 receptor, type 1 and some isoforms of the ryanodine receptor exist so far.

Protein	Identity	Homology
P2RY1 purinergic receptor (*Salmo salar*: B5XFC0; *Rattus norvergicus*: P49651)	38 %	63 %
P2RY2 purinergic receptor (*Oncorhynchus mykiss*: C1BHK5; *Rattus norvergicus*: P41232)	31 %	49 %
β_2-adrenergic receptor (*Oncorhynchus mykiss*: QBUUYB; *Rattus norvegicus*: P10608)	63 %	76 %
Phospholipase C (Isoform D) (*Salmo salar*: C0HACB; *Rattus norvegicus*: Q62711)	52 %	71 %
SERCA (*Danio rerio*: Q642Z0; *Rattus norvegicus*: Q64578)	85 %	93 %
IP_3 receptor, type 2 (*Danio rerio*: Q1LV14, long fragment; *Rattus norvegicus*: P29995)	75 %	85 %
IP_3 receptor, type 3 (*Danio rerio*: ABWG42; *Rattus norvegicus*: C7E1V1)	82 %	90 %
Ryanodine receptor (*Oncorhynchus mykiss*: A4KUJ3, 274 aa fragment; *Rattus norvegicus*: BOLPN4)	68 %	80 %

Despite biochemical and genetic information on the presence of the respective key players, there is no kinetic information available for the individual reactions. Since it is impossible to infer these parameters reliably from the Ca^{2+} time-series measured here, our analysis is restricted to qualitative behavior.

Addressing the qualitative difference in ATP-induced Ca^{2+} signaling, it is important that earlier studies on rat hepatocytes have indicated differences in feedbacks on the receptor level to be the cause for qualitative differences (spiking *vs.* bursting) in Ca^{2+} oscillations (Kummer et al. 2000). Interestingly, the purinergic receptors in fish responsible for ATP binding, shows indeed a low homology with its mammalian counterpart. Moreover, analyzing potential motifs that could be responsible for a modulation of the receptor activity using the ELM server, both receptor subtypes in mammals show binding sites for a SH3 domain, whereas this is absent in fish for type 1. There are also fewer amino acids in the fish sequence allowing phosphorylation, if compared to the mammalian sequence. In order to

analyze the potential phosphorylation sites, we used the TMHMM server for the prediction of the transmembrane domains and afterwards investigated those sequences that are likely exposed to the cytosol. The results are summarized in Tab. 3.

Tab. 3: Prediction of regulatory sides in the cytosolic residues of purinergic receptors. The cytosolic residues were predicted with the TMHMM server (Sonnhammer et al. 1998). The potential regulation sites within the cytosolic residues were predicted with the ELM server.

Protein investigated	**Cytosolic residues**	**Predicted regulatory sites**
P2RY1 *Salmo salar* (B5XFC0)	43 - 62	GSK3
	128 - 139	
	217 - 139	
P2RY1 *Rattus norvergicus* (P49651)	38 - 55	
	111 - 130	
	191 - 221	CK2, GSK3
	279 - 373	2*CK1, CK2, GSK3, 2*PKA, PLK, ProDKin
P2RY2 *Oncorhynchus mykiss* (C1BHK5)	72 - 83	CK1, GSK3
	144 - 163	
	226 - 258	
	319 - 346	GSK3, PKA
P2RY2 *Rattus norvergicus* (P41232)	60 - 70	GSK3
	131 - 155	
	221 - 245	PK, PKA

Differences in sequence could also account for differences in interaction with Ca^{2+} channels in the plasma membrane leading to different influxes of Ca^{2+} upon stimulation. Altogether, even though we cannot provide an exact and mechanistic explanation at this point, we conclude, that differences in the regulation of the purinergic receptor can be the origin of the experimentally observed differences in the dynamics of ATP induced Ca^{2+} oscillations in fish liver cells compared to mammalian cells.

To underline this point, we present simulated time-series of a previously published simple model of Ca^{2+} signaling including feedbacks on the receptor level published in (Larsen and Kummer 2003). It is given by the following equations:

(X.1)

$$\frac{dG_\alpha}{dt} = k_1 + k_2 * G_\alpha - \frac{k_3 * G_\alpha * PLC}{G_{\alpha+}K_4} - \frac{k_5 * Ca_{cyt} * G_\alpha}{G_\alpha + K_6} \quad \text{(X.2)}$$

$$\frac{dPLC}{dt} = k_7 * G_\alpha - \frac{k_8 * PLC}{PLC + K_9} \quad \text{(X.3)}$$

$$\frac{dCa_{cyt}}{dt} = \left(Ca_{ER} - Ca_{cyt}\right) * \frac{k_{10} * Ca_{cyt} * PLC}{PLC + K_{11}} + k_{12} * PLC + k_{13} * G_\alpha - \frac{k_{14} * Ca_{cyt}}{Ca_{cyt} + K_{15}} - \frac{k_{16} * Ca_{cyt}}{Ca_{cyt} + K_{17}} - \frac{k_{18} * Ca_{cyt}^8}{Ca_{cyt}^8 + K_{19}^8} + \left(Ca_{mit} - Ca_{cyt}\right) * \frac{k_{20} * Ca_{cyt}}{Ca_{cyt} + K_{21}} \quad \text{(X.4)}$$

$$\frac{dCa_{ER}}{dt} = -\left(Ca_{ER} - Ca_{cyt}\right) * \frac{k_{10} * Ca_{cyt} * PLC}{PLC + K_{11}} + \frac{k_{16} * Ca_{cyt}}{Ca_{cyt} + K_{17}} \quad \text{(X.5)}$$

$$\frac{dCa_{mit}}{dt} = \frac{k^{18} * Ca_{cyt}^8}{Ca_{cyt}^8 + K_{19}^8} - (Ca_{mit} - Ca_{cyt}) * \frac{k_{20} * Ca_{cyt}}{Ca_{cyt} + K_{21}}$$

Here, G_α represents the G_α-subunit activated by the receptor, PLC the active phospholipase C, Ca_{cyt}, Ca_{ER} and Ca_{mit} the Ca^{2+} concentrations in the cytosol, ER and mitochondria respectively. For the exact explanation of the individual kinetics, we would like to refer to Larsen and Kummer (2003).

Simulating this model, qualitatively different behavior for different feedbacks and feedback strength at the receptor level can be observed. Thus, Fig. 4 illustrates two different time-series of Ca^{2+} oscillations, which only differ in the strength (rate of Ca^{2+} (via PKC) induced inactivation) of one of the feedbacks on the receptor binding ATP. The first one representing oscillations similar to the ones observed in fish cells in the present study, the second one representing bursting oscillations in mammalian cells. We conclude that the differences in receptor sequence and structure can indeed be the basis for the differences in dynamics observed.

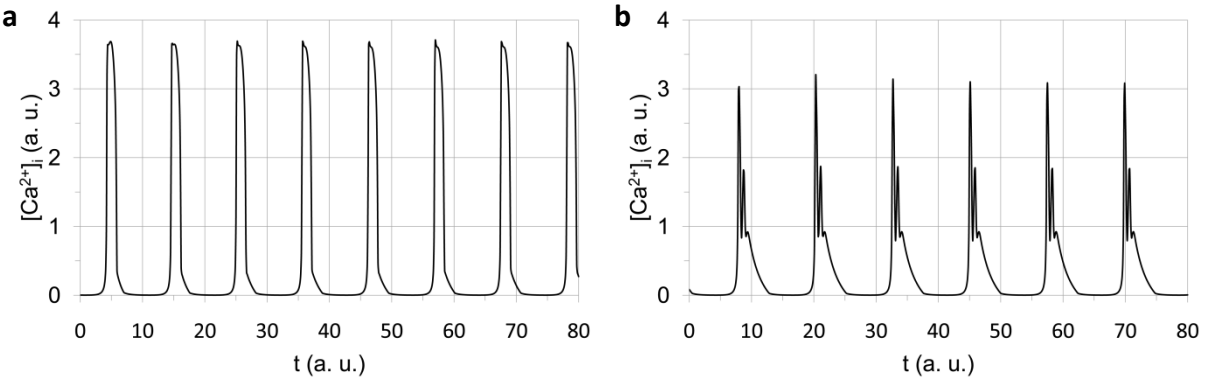


Fig. 4 a, b: Simulations of the model equations X.1 - X.5 only differing in the degree of feedback on the receptor. Initial concentrations: a=0.01, b=0.01, c=0.01, d=10, e=0.001. Reaction parameters: k_1=0.01, k_2=2.218, k_3=0.64, K_4=0.09, K_6=1.18, k_7=2.08, k_8=32.24, K_9=29.09, k_{10}=1, K_{11}=1, k_{12}=2.8, k_{13}=13.4, k_{14}=153, K_{15}=0.16, k_{16}=7, K_{17}=0.05, k_{18}=79, K_{19}=3.5, k_{20}=0.81, K_{21}=4.5. Feedback parameter k_5=3.96988 in **(a)** and 6.81736 in **(b)**.

Another purpose of the modeling is to derive hypotheses which mechanisms are responsible for the amplitude-encoding observed, if compared to the frequency-encoding seen in mammalian hepatocytes. The origin of this phenomenon must mainly lie in processes downstream of the actual receptor, since we observe amplitude-encoding also with agonists unspecifically causing Ca^{2+} oscillations without actual receptor-binding. This is especially true for hydrogen peroxide which operates *via* depolarization of membranes followed by Ca^{2+} entry. This of course does not mean that the actual dynamics is not also highly influenced by the feedbacks on the receptor level, as seen above, but at least one major cause for the amplitude encoding must be further downstream.

Models focusing solely on the processes downstream of the receptor, especially on the processes of Ca^{2+} release from and uptake into the endoplasmic reticulum (ER) usually reproduce frequency encoding behavior. The origin of the oscillations lies in the CICR (Ca^{2+} induced Ca^{2+} release) which represents an autocatalytic system displaying relaxation oscillations. As pointed out also in the review by Schuster et al. (Schuster et al. 2002), these oscillations arise due to the existence of a sudden release of Ca^{2+} from the ER and the comparatively slow pumping back into this store. Relaxation oscillations are the simplest way to maintain fairly constant amplitudes even close to the bifurcation point, if they arise from a subcritical Hopf-bifurcation. More sinusoidal oscillations often arise from supercritical Hopf-bifurcations and here, one expects an increase in amplitude at least in the vicinity of the bifurcation point. Thus, the relative time-scales of the uptake and release properties at the Ca^{2+} channel are likely to be important for frequency- or amplitude-encoding. Investigating the exact shape of individual peaks in Ca^{2+} oscillations (Fig. 5) and comparing the oscillations in fish as seen in this study with those in rat (taken from Kummer et al. 2000), it is evident that the Ca^{2+} release in rat cells during oscillations is markedly faster than the uptake with a ratio “decrease time to increase time” of 2.4 ± 0.8 (average of eight

consecutive peaks ± S.D.), whereas this relation is more symmetric in the fish cells (decrease-to-increase ratio 1.4 ± 0.4; n = 8).

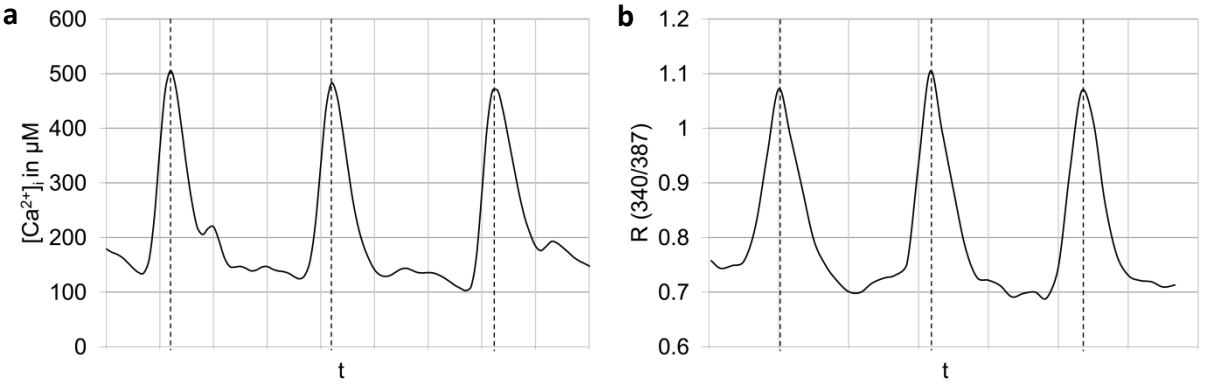


Fig. 5 a, b: Comparison of individual oscillations in rat hepatocytes (taken from Kummer et al. 2000) and fish RTL-W1 cells. The oscillations in fish are exhibiting stronger symmetry between uptake and release rates during calcium peaks. One unit on the time scale equates to 10 sec in rat or 30 sec in RTL-W1, respectively.

Recently, De Pittà et al. (2009) investigated a model of Li and Rinzel (1994) in the context of information processing in astrocytes. They showed that some parameters can change the model from exhibiting amplitude-encoding to frequency-encoding as well as mixed forms. These findings also hold in a more general context and are very much in accordance to the above said. We investigated this further by employing another simple prototypic Ca^{2+} model for non-excitable cells which focuses on the processes around the IP_3 receptor. This model by Goldbeter et al. (1990) is a sort of minimalistic model of CICR-induced oscillations and is described by the following equations:

(X.6)

$$\frac{dZ}{dt} = v_0 + v_1 * \beta - v_2 + v_3 + k_f * Y - k * Z$$

(X.7)

$$\frac{dY}{dt} = v_2 - v_3 - k_f * Y$$

Here, v_0 and k*Z represent influx and efflux of Ca^{2+} into and out of the cell and v1*β describes a saturation function triggered by agonist signal. The rest of the terms describe Ca^{2+} release and uptake from the ER, given by the following equations:

(X.8)

$$v_2 = V_{M2} * \frac{Z^n}{K_2^n + Z^n}$$

(X.9)

$$v_3 = V_{M3} * \frac{Y^m}{K_R^m + Y^m} * \frac{Z^p}{K_A^p + Z^p}$$

The model was downloaded from the BioModels Database (Le Novère et al. 2006). In contrast to de Pitta et al. (2009), we specifically tried to modulate the speed of the release and uptake rates of Ca^{2+} in the ER such that they become more similar to each other to investigate if this can also mutate the Ca^{2+} oscillations from frequency-encoding into amplitude-encoding. This is indeed the case. Thus, as shown in Fig. 6, changes in the rates of the SERCA and the CICR which lead to a more balanced uptake and release result in a model which shows amplitude-encoding with almost constant frequencies. Therefore, similar changes in the characteristics of the fish Ca^{2+} channel or SERCA pump could be very well the origin of the observed differences. Given the fact that the SERCA is very highly conserved between species, we conclude that it is more likely that the origin actually lies in differences of binding characteristics of the IP_3 receptor.

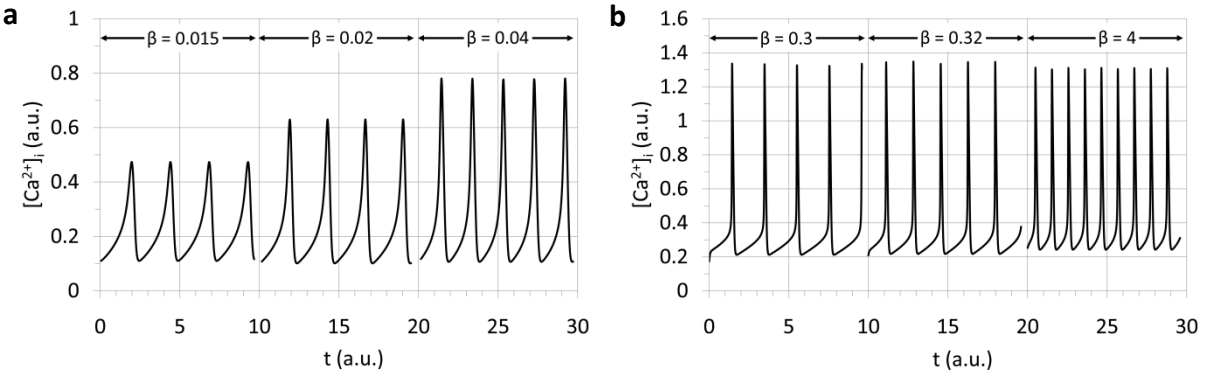


Fig. 6 a, b: Simulation of the model described by equations X.6 - X.9. Parameters as in the original publication (Goldbeter et al. 1990). In **(a)**, following parameters were set to: n=1.1, m=1, p=1.7, k=5.03, K_A=2.2, V_{m2}=34.26, β as given in the figure.

4 Discussion

The present study was initiated to characterize the possible regulation of intracellular Ca^{2+} levels in primary hepatocytes isolated from rainbow trout and the permanent fish cell line RTL-W1. Results document that (1) all agonists tested can modulate Ca^{2+} in both primary hepatocytes and the permanent cell line. Evidence is provided that there are purinergic and adrenergic modulations of intracellular Ca^{2+} and that Ca^{2+} plays a role in the response to toxicants in the examined fish cells. (2) At least in RTL-W1 cells, several agonists can induce Ca^{2+} oscillations, and (3) increasing concentrations provoked an increase in the amplitude of Ca^{2+} oscillations in the cell line. This was evident for hydrogen peroxide, 4-nitrophenol and phenylephrine, but was less obvious for ATP. To the best of our knowledge, these are the first data indicating clear amplitude-encoding of concentrations in non-excitable cells.

In RTL-W1 cells, ATP induced repetitive Ca^{2+} increases. Oscillations were observed in approximately 40 % of the cells with no clear tendency with regard to the ATP concentration (0.1 - 100 µM). This was in contrast to the findings of Nathanson & Mariwalla (1996), who found that skate hepatocytes displayed oscillations only when stimulated with lower concentrations of ATP (10 - 100 nM; Nathanson and Mariwalla 1996). In a small number of RTL-W1 cells, 0.1 µM ATP induced a single biphasic increase in Ca^{2+} which passed into regular oscillations when higher ATP concentrations were added. Generally, peaks were more pronounced with increasing concentration. The oscillations were of simple sinusoidal nature in contrast to the bursting oscillations found in rat hepatocytes (Green et al. 1993).

In the present study, not only purinergic, but also adrenergic responses of intracellular Ca^{2+} after exposure of the cells to phenylephrine could be observed at least in the cell line. Trout hepatocytes showed only little epinephrine sensitivity, with less than 20 % of the cells responding and relatively weak changes in intracellular Ca^{2+}, indicating that a certain population of the cells may respond to epinephrine. This fact might be a consequence of hepatocyte heterogeneity in rainbow trout (Mommsen et al. 1991) and reflects the poor metabolic response of these cells to phenylephrine found by Moon & Mommsen (1990). Nevertheless, Fabbri et al. (1995) could provide direct evidence for the presence of α_1-adrenergic and IP_3 receptors in the liver of the rainbow trout, however, with a minor role of this transduction system in the modulation by epinephrine of hepatic metabolism compared to the β-adrenoceptor pathway (Fabbri et al. 1995).

In the present study, we found that phenylephrine modulated intracellular Ca^{2+} in RTL-W1 cells at concentrations ≥ 800 nm. Around 50 % of the reacting cells displayed oscillations with a period of 0.7 - 0.8 min. Frequency did not change, when higher concentrations were applied; however, the amplitude of the oscillations rose. This is in clear contrast to the findings obtained for mammals: In rat hepatocytes, phenylephrine induced cytosolic dose-dependent Ca^{2+} oscillations, i.e., with increasing agonist concentration (0.5 - 10 µM phenylephrine), the frequency of oscillations increased (Rooney et al. 1989). In fish hepatocytes, in addition to the pronounced inter-species variability with respect to the

players involved in catecholamine response, the main focus of most working groups was not on dose-dependency. Interestingly, Zhang et al. (1992) found a dose-dependent epinephrine effect on the maximal amplitude of Ca^{2+} oscillations in eel hepatocytes (Zhang et al. 1992b). These findings suggest that amplitude-encoding could be a phenomenon typical for fish cells, thus making the Ca^{2+} response different between classes of vertebrates.

We used a combination of bioinformatics and modeling approaches to investigate the likely mechanisms behind the differences observed between fish and mammalian liver cells. According to our analysis, differences in purinergic receptor-sequence including less potential feedbacks and interactions with Ca^{2+} channels in fish cells should be responsible for the simpler ATP induced oscillations, if compared to the more complex oscillations observed in mammalian cells.

In addition, we investigated mechanisms causing amplitude-encoding in contrast to frequency-encoding in mammalian cells. The required differences in bifurcation scenarios can be caused by similar uptake and release rates of Ca^{2+} from the ER. The proteins mainly responsible for these two processes are the SERCA and the IP_3R, the latter being less conserved between fish and mammal whereas the SERCA shows remarkable homology. Therefore, we conclude that different binding characteristics, e.g. responsible for the Ca^{2+} induced Ca^{2+} release, can be the origin of the observations.

One important question that came up during this study was how the differences between the two used cell types – the primary hepatocytes and the RTL-W1 cell line – could be explained and which results could serve as a model for Ca^{2+} modulation in fish *in vivo*. Findings for the cell line are promising and seem to be reliable, since Ca^{2+} answers are a lot more differentiated than in the primary hepatocytes and are highly reproducible. Nevertheless, the motivation of using primary cultures instead of continuous cell lines is their supposed similarity to the conditions *in vivo*. Primary hepatocytes, however, are only viable for a relatively short period of time. An additional problem is that not all cells classified as “alive” by light microscopic observation, are ultrastructurally intact (cf. Braunbeck and Storch 1992). Further limitations include loss of membrane specialization, possible down-regulation of cytosolic enzymes and loss of the ability to form bile. These effects mean that direct extrapolation of results from isolated hepatocytes to intact liver may also be problematic (Braunbeck and Storch 1992). Moreover, with respect to the reproducibility of the results, it has to be considered that cells from different preparations may vary as a result of differences in the health and reproduction status of the fish or specific conditions during the isolation procedure (e.g., time to clear liver from blood, extent of collagenase digestion).

The primary cells used in this study were given time to attach to the culture plate and were only 24 to 48 h old when studied using Ca^{2+} imaging. Compared to mammalian hepatocytes, there is evidence that the loss of differentiated liver function is less rapid in teleost primary cultures, since available data indicate a greater stability of biotransformation enzymes in

piscine liver cells than in mammalian hepatocytes. Activities of xenobiotic enzymes of both phase I (e.g. 7-ethoxyresorufin-*O*-deethylase, EROD) and phase II (e.g. glutathione-*S*-transferases, GST, and UDP-glucuronyltransferases, UDPGT) seem to be conserved during monolayer culture (Segner 1998, Segner and Cravedi 2001). Nevertheless, it cannot be ruled out that loss of some cellular functions, in particular with respect to the Ca^{2+} signaling pathway, already occurred during isolation or the first days in culture. Zhang et al. (1992) also found that rainbow trout hepatocytes, contrary to eel and bullhead cells, demonstrated little epinephrine sensitivity, with less than 20 % of the cells responding (Zhang et al. 1992b). It might be possible that the meager response of trout primary hepatocytes towards agonists of the Ca^{2+} pathway is not (only) due to species differences, but also to the specific isolation procedures or culture conditions.

To overcome this problem, the permanent cell line RTL-W1 was selected. Continuous or permanent cell lines are easy to obtain and handle, and provide genetically identical material over a long period without the necessity to revert on intact animals. The higher homogeneity of permanent cell cultures and, therefore, good reproducibility of cell tests makes them preferable in every-day laboratory routine and particularly in screening studies and environmental diagnostics (Segner 1998).

The main disadvantage of RTL-W1 is its uncertain origin. The permanent epithelial cell line RTL-W1 was established by Lee et al. (1993) by Dispase treatment of liver fragments of a 4-year-old male rainbow trout. Single cells were grown in monolayers and passaged continuously (Lee et al. 1993). When maintained as a confluent culture, the shape of RTL-W1 cells is predominantly bipolar or fibroblast-like. In contrast to other fish cell lines, RTL-W1 express cytochrome P450 enzymes and induce EROD activity in response to several chemicals (e.g. TCDD, Lee et al. 1993; aromatic hydrocarbons, Behrens et al. 2001, Brack et al. 2002; various pesticides, Babin and Tarazona 2005; benzo[a]pyrene, Schirmer et al. 2004; and environmental samples, Brack et al. 2002, Keiter et al. 2006, Rastall et al. 2004, Schirmer et al. 2004) in a manner similar to primary rainbow trout hepatocytes. This leads to the conclusion that during transformation into an immortal cell line, RTL-W1 cells have conserved the essential elements of the aryl hydrocarbon receptor-mediated CYP1A induction pathway (Behrens et al. 2001), which makes them useful as a tool for assessing the toxic potency of environmental contaminants.

However, Lee et al. (1993) could not definitely identify which among the many cell types in the rainbow trout liver gave rise to the cell line (Lee et al. 1993). The most probable potential source, which is suggested by work with rat liver epithelial cell lines, is a compartment of stem cells located in bile ductular structures. Another possible, but less probable origin is the spontaneous immortalization of hepatocytes. However, the original primary culture likely had few hepatocytes, because rainbow trout hepatocytes attach poorly to plastic tissue culture dishes (Blair et al. 1990, Klaunig et al. 1985, Lipsky et al. 1986; own research). Other possible origins that are unlikely, but cannot be ruled out are

connective tissue and endothelial cells, which, relative to hepatocytes and biliary epithelial cells, are much less abundant in the rainbow trout liver (Blair et al. 1990). Finally, there is a possibility that RTL-W1 contains multiple cell lineages.

The unclear origin of the RTL-W1 cell line makes it more difficult to transfer results to the conditions in trout liver *in vivo*. Nevertheless, since Ca^{2+} signaling is a common phenomenon in different cell types, results should be transferrable at least to a certain extent to trout cells and, more generally, to other fish. So far, it seems more promising to revert to the established and highly standardized cell line to get a picture of how Ca^{2+} signaling is principally organized in fish cells. In the long term, however, it would be preferable to establish a Ca^{2+} imaging procedure applicable to primary hepatocytes in order to get better transferability of the data to the conditions *in vivo*.

5 Acknowledgment

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Chapter 4

Calcium imaging in the permanent fish cell line RTL-W1 - a novel tool in ecotoxicology

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The least movement is of importance to all nature;
all the sea changes when a stone is thrown.

Blaise Pascal (1623 – 1662)

Abstract

One basic response towards a multitude of physical and chemical factors is the modulation of intracellular Ca^{2+} levels. There is also considerable evidence that a number of toxic environmental chemicals have an impact on Ca^{2+} signaling processes, alter them, and induce cell death by apoptosis. Given the versatility of Ca^{2+} modulation due to the complex mechanisms mediating the coding of information, intracellular Ca^{2+} signal might be a useful tool for the detection of environmental stressors. Therefore, effects of the model environmental toxicants 4-nitrophenol, 3,4-dichloroaniline, and hydrogen peroxide on Ca^{2+} signaling were studied in the permanent fish cell line RTL-W1. Cytotoxicity tests were carried out to differentiate agonist-specific effects from general cytotoxic or apoptotic effects.

The permanent fish cell line RTL-W1 proved to be a suitable tool for the investigation of Ca^{2+} signals: Effects on intracellular Ca^{2+} were observed for all agonists, and hydrogen peroxide and 4-nitrophenol induced marked Ca^{2+} oscillations at sublethal concentrations. Despite considerable variability in the threshold and extent of cellular effects between individual cells, effects were reproducible and dose-dependent. At levels below concentrations inducing cytotoxicity and apoptosis, there is a lasting, unspecific increase in the intracellular Ca^{2+} level which might be interpreted as a precursor of apoptotic or necrotic events in the cell.

Ca^{2+} signals seemed to depend on either specific activated pathways or on unspecific interference by the respective substance. The question whether certain types of Ca^{2+} responses are specific and may be used to characterize different types of stressors cannot yet be answered. However, there is evidence that Ca^{2+} imaging might provide a highly sensitive, yet non-specific indicator of toxic impact, since, as a second messenger, intracellular Ca^{2+} also integrates toxic effects on multiple other sublethal parameters.

Keywords: calcium oscillation − apoptosis – fish − hydrogen peroxide –4-nitrophenol – 3,4-dichloroaniline – RTL-W1 cell line

1 Introduction

Interactions of anthropogenic chemicals with biota first take place at the cellular level. Over the past decades, numerous new methods have been developed in the field of molecular and cellular ecotoxicology in order to improve our understanding of the mechanisms underlying chemical effects, which manifest at higher levels of biological organization. Cellular toxicology may provide a valuable tool for the early and sensitive detection of chemical exposure. Based on an improved understanding of fundamental toxicological processes, prediction of (eco-)toxicological effects at higher biological levels become possible (Fent 2001, Hinton et al. 2001, Segner and Braunbeck 1998, 2003).

In general cell physiology, one basic response towards a multitude of physical and chemical factors is the modulation of intracellular Ca^{2+} levels. Ca^{2+} plays a fundamental role in all types of cells, tissues and organisms (for reviews, see e.g. Berridge et al. 2000a, Berridge et al. 2000b, Petersen et al. 2005, Putney Jr 1998). One important Ca^{2+} function is its role as a second messenger in cellular signaling controlling a wide range of cellular processes. After decades of intensive Ca^{2+} research, there are well-established concepts of intracellular Ca^{2+} homeostasis and signaling at least for mammals. Despite the immense versatility of Ca^{2+} as a second messenger in signal transduction, the number of proteins controlling Ca^{2+} signaling is relatively limited, and the players and mechanisms of Ca^{2+} transport between cells and extracellular space and within cells have been elucidated: It is the combination of different Ca^{2+} pools, pathways and feedback mechanisms which allows the encoding of information by amplitude, frequency and shape of the Ca^{2+} signal. A further component of Ca^{2+} signaling is the spatial encoding caused by compartmentalization (Petersen et al. 2005).

Ca^{2+} signaling also seems to play a role in the response to environmental stress including cell death (see e.g. Duchen 2000, Kass and Orrenius 1999, Orrenius 2004, Orrenius et al. 2003). Cells possess a complex system to maintain intracellular Ca^{2+} homeostasis. The loss of Ca^{2+} compartmentalization can be reversible or irreversible and often becomes apparent in the form of an increase of cytoplasmic Ca^{2+} concentrations or even Ca^{2+} oscillations. Irreversible perturbation of intracellular Ca^{2+} balance activates multiple destructive processes that target subcellular structures including the plasma membrane, the cytoskeleton, mitochondria, and the nucleus, and may lead to cell injury and tissue damage (Dong et al. 2006).

There is considerable evidence that a number of toxic environmental chemicals have an impact on Ca^{2+} signaling processes, alter them, and induce cell death by apoptosis, e.g. *via* the induction of oxidative stress. Apoptosis is a specific mode of cell death characterized by morphological changes such as chromatin condensation, fragmentation of the nucleus, contraction of the cytoplasmic volume (cell shrinkage), and expulsion of “apoptotic bodies” containing apparently intact organelles (Belloc et al. 1994). Toxic agents such as the environmental pollutant tributyltin or the natural plant product thapsigargin, which deplete the endoplasmic reticulum (ER) Ca^{2+} stores, will induce − as a direct result − opening of plasma membrane Ca^{2+} channels and a stress response in the endoplasmic reticulum (Kass

and Orrenius 1999). This example illustrates further application of Ca^{2+} imaging in ecotoxicology: Given the immense versatility of Ca^{2+} modulation due to the complex mechanisms which help to encode information, recording of the intracellular Ca^{2+} signal might eventually be a useful tool for the detection and identification of environmental stressors.

In mammals, the role of Ca^{2+} in intracellular signal transduction has been investigated in detail. Much less is known about Ca^{2+} modulation in fish. Given their taxonomic and ecological positions, however, fish are important model organisms for both vertebrates and aquatic systems. Therefore, in a prior study (Schweizer et al. 2011b), Ca^{2+} responses towards "classic" agonists such as ATP, phenylephrine, histamine and caffeine were investigated in rainbow trout primary hepatocytes and the permanent cell line RTL-W1 (Rainbow Trout Liver-Waterloo 1; Lee et al. 1993). Ca^{2+} oscillations were found in response to several stimuli in RTL-W1 cells and to a lesser extent in primary hepatocytes. Bioinformatics and analysis of literature and databases provided information about signal transduction pathways in fish compared to mammals: In general, the essential players of signal transduction do not differ very much between piscine and mammalian cells; Ca^{2+} pathways seem to be conserved well during vertebrate evolution.

Nevertheless, one fundamental difference in the modulation of intracellular Ca^{2+} in fish could be observed compared to mammals (Schweizer et al. 2011b): In contrast to the mammalian counterpart, Ca^{2+} oscillations in fish systems seem to be amplitude-encoded, i.e. increasing agonist concentrations provoked an increase in the amplitude of the Ca^{2+} oscillations. Different binding characteristics responsible for, e.g., the Ca^{2+} induced Ca^{2+} release could be the origin of the observations. (Zhang et al. 1992b) also found a dose-dependent epinephrine effect on the maximal amplitude of Ca^{2+} oscillations in eel hepatocytes; however, they did not investigate this phenomenon systematically.

Based on the general model, typical environmental toxicants and stressors such as 4-nitrophenol, 3,4-dichloroaniline, and hydrogen peroxide were used in the present study to elucidate possible interactions between contaminants and Ca^{2+} signaling. For a better differentiation between agonist-specific effects and general cytotoxic or apoptotic effects on intracellular Ca^{2+}, the neutral red assay according to (Borenfreund and Puerner 1984) was used as a range-finding test to elucidate cytotoxicity. The nucleic acids stain Hoechst 33342 was performed to detect apoptotic cells and determine agonist concentrations with an apoptotic potential (Kiechle and Zhang 2002, Moore et al. 1998).

Since the liver is a key organ in detoxification and rainbow trout is an established model organism in aquatic toxicology, the permanent fish cell line RTL-W1 derived from rainbow trout liver (Lee et al. 1993) seemed to be a promising experimental system to investigate intracellular Ca^{2+} responses to environmental pollutants in fish cells, and to quantify to which extent Ca^{2+} signaling in fish cells can be used for the detection of environmental stress. Moreover, the question should be discussed whether specific types of Ca^{2+} responses are specific of and may be used to characterize different types of stressors.

2 Material and methods

2.1 Chemicals

Fura-4 acetoxymethylester (AM) and Pluronic F-127 (20 % solution in DMSO) were obtained from Invitrogen/Molecular Probes (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) at the highest purity available.

2.2 RTL-W1 cell line

The permanent rainbow trout cell line RTL-W1 was established in the early 1990s (Lee et al. 1993), and, given the fact that it strongly expresses biotransformation enzymes such as cytochrome P450, it soon developed into a favorite tool to assess the toxicity of environmental contaminants. RTL-W1 cells were cultured in Leibovitz-L15 media with 10 % fetal calf serum at 20 °C. For Ca^{2+} imaging, RTL-W1 cells were seeded into the cavities of 24-well plates (TTP, Renner, Darmstadt, Germany) at a density of 2×10^5 cells/well (1.2×10^5 cells/cm^2) and were allowed to attach for 24 h.

2.3 Neutral red assay

The neutral red assay with RTL-W1 cells was performed according to (Borenfreund and Puerner 1984) with slight modifications. In this assay, the ability of neutral red (2-methyl-3-amino-7-dimethylaminophenazin) retention of the cells is tested as a measure of lysosomal integrity and, therefore, cell viability. For each agonist, seven sequential dilutions in medium were tested as well as a negative and positive control (medium and 40 mg/L 3,5-dichlorophenol, respectively) with six replicates each. Acute cytotoxicity was determined photometrically as the retention of neutral red by living cells as described by Borenfreund & Puerner (1984). The viability was expressed as a percentage of the negative controls, and data were plotted as concentration-response curves. Non-linear regression analysis was performed using SigmaPlot 11.0 (Systat, Erkrath, FRG), and the extract concentrations inducing 20 % mortality (NR_{80}) were calculated accordingly. In order to get information about the kinetics of cytotoxicity, cells were exposed over different periods of time (0.5, 1, 2, 6, 24 or 48 h), and NR_{80} values were compared.

2.4 Apoptosis assay with Hoechst 33342

Hoechst 33342 was used to detect apoptosis in RTL-W1 cells and determine agonist concentrations with a statistically significant apoptotic potential. RTL-W1 cells were allowed to attach in 24-well plates for 24 hours and were incubated with different agonist dilutions in medium for 0.5, 1, 2 or 6 h. For each exposure time, a negative and a positive control (medium and 40 mg/L 3,5-dichlorophenol, respectively) were conducted. After exposure,

cells were incubated for 20 min with 2 µg/ml Hoechst 33342 in medium and placed on the stage of a Nikon Ti-E inverted epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a DAPI-filter and a Hamamatsu ORCA-AG high-sensitive black and white camera (Hamamatsu, Japan) at the Nikon Imaging Centre, University of Heidelberg, Germany. For each concentration and exposure time, three regions of the well with approximately 100 cells were randomly selected and photographed. Then, a total of 100 cells were analyzed per concentration and exposure time (with an average of 33 - 34 cells per micrograph) in order to determine morphological changes (chromatin condensation and fragmentation; Fig. 1). The apoptosis rate was determined as percentage of affected cells as compared to the negative control. Non-linear regression analysis of the medians of three to four independent replicates was performed using SigmaPlot 11.0, and the extract concentrations inducing 20 % mortality (EC_{20}) were calculated accordingly. For statistical interpretation, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 3.5; Systat, Erkrath, Germany). In case of significant differences, a *post hoc* test according to Dunnett ($p < 0.05$) was employed to identify concentrations that differed significantly from the negative control.

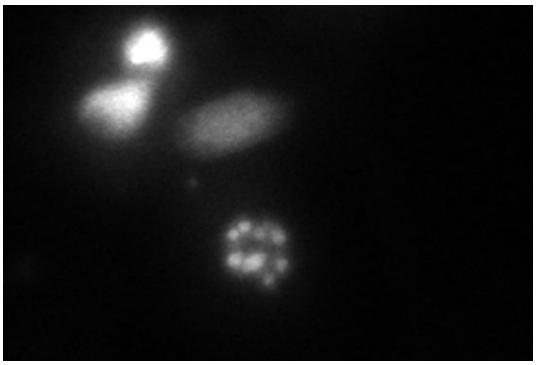

Fig. 1: RTL-W1 cells stained with the fluorescent dye Hoechst 33342 showing morphological changes: chromatin condensation (two leftmost cells) and fragmentation (lower cell). Cells were exposed to 40 mg/L 3,5-dichlorophenol for 2 h.

2.5 Ca^{2+}-Imaging

Ca^{2+}-imaging was carried out as described previously (Schweizer et al. 2011b). All steps were carried out with Ringer solution at 20 °C in the dark. In brief, cells were carefully washed twice with Ringer solution and loaded with 6 µM Fura-2 AM solution in Ringer containing 0.02 % pluronic F-127 and 1 mM probenecid. After dye incubation, cells were washed twice with Ringer solution to remove extracellular Ca^{2+} and left for de-esterification.

Ca^{2+} measurements and time lapse acquisitions were carried out with a Nikon Ti-E inverted epifluorescence microscope (Nikon, Tokyo, Japan) and a Hamamatsu ORCA-AG high-sensitive black and white camera (Hamamatsu, Japan) at the Nikon Imaging Centre at the University of Heidelberg, Germany. The Ca^{2+} ionophore ionomycin (5 µM) was used as a positive control.

3 Results and discussion

3.1 Acute cytotoxicity (neutral red retention)

Acute toxicity data are given in Tab. 1 as NR_{80} values (80 % neutral red retention (NR), i.e. 20 % effect concentrations). Cytotoxicity was determined at six time points between 0.5 and 48 h of exposure; however, only NR_{80} values after 6 and 48 h are presented in Tab. 1, since these exposure times proved to be most significant. In Fig. 2, the entire NR_{80} time-response curves are exemplified for 4-nitrophenol and hydrogen peroxide.

Tab. 1 shows cytotoxic effects within the tested concentration ranges only for four agonists. For ATP, histamine, and phenylephrine, no cytotoxicity could be determined within the range of test concentrations. Since these concentrations are far above those usually applied in Ca^{2+} imaging, no further cytotoxicity or apoptosis tests were performed. For caffeine, 3,4-dichloroaniline, and 4-nitrophenol, NR_{50} data for cytotoxicity were within the same range as literature data for acute fish toxicity; for hydrogen peroxide, no reference data were found. Considering the design of the neutral red assay in the present study as a range finding test, the determination of a lower effect level (i.e. EC_{20} or NR_{80}) seemed more appropriate than the NR_{50}. For all substances, NR_{80} values were 3 to 4 times higher after 6 h than after 48 h, except for 3,4-dichloroaniline where NR_{80} after 6 h is only based on one replicate because of problems of solubility in higher concentrations; therefore, reliability of this value is not guaranteed (Tab. 1).

In most cases, cytotoxicity was negligible; it did not increase for exposure times < 6 h and did not rise very much until between 24 and 48 h of exposure (e.g., 4-nitrophenol, Fig. 2). One exception was hydrogen peroxide with a clear increase in cytotoxicity within the first hours of exposure indicating a rapid entry and action of hydrogen peroxide (Fig. 2). Given the high oxidative potential of hydrogen peroxide, immediate disturbance and/or destructive activity at all cellular levels, including the lysosomal membrane and enzymes, have to be expected.

For Ca^{2+} imaging, concentrations well below those with cytotoxic effects had to be chosen (see last column in Tab. 1). Besides, exposure times in the neutral red assay (0.5 - 24 h) were as high as or above those chosen for measurements of the intracellular Ca^{2+} levels (approx. 30 min). Nevertheless, notwithstanding the good correlation of the neutral red assay with the acute fish test, the sensitivity of the cytotoxicity test is lower than LC values in the acute fish test (Braunbeck et al. 1997). Since the neutral red assay first of all reflects integrity and functionality of the lysosomes, and since we aimed at excluding all general cytotoxic effects in Ca^{2+} signaling which might interfere with specific effects of the agonists on intracellular Ca^{2+}, the apoptosis assay with Hoechst 33342 was performed as a second pre-test.

Tab. 1: Acute cytotoxicity and apoptosis rates in RTL-W1 cells as revealed by the neutral red cytotoxicity assay and the apoptosis assay with Hoechst 33342, respectively, after different exposure times. For all assays, at least 3 replicates were tested. For better comparison of the toxic potential, reference data for acute toxicity in fish (LC_{50}) are given. The last column gives the maximum agonist concentration tested in Ca^{2+} imaging. References (ref.) in brackets.

Agonist	Cytotoxicity assay (mM)			LC_{50} fish (µM; ref.)	Apoptosis assay (mM)		Ca^{2+} imaging (mM)
	NR_{80} - 48h	NR_{80} - 6h	NR_{50} (ref.)		LOEC -6h	EC_{20} -6h	
ATP	> 5*	> 5*					0.1
Caffeine	4 ± 1.16	16	5.12 (1)	0.5 (6)	50	22.14	10
3,4-DCA	0.61 ± 0.05	0.72**	> 0.15 (2)	~25/52 (7)	0.2	> 0.8*	0.05
				78/12 (8)			
				25/15 (9)			
				~50 (10)			
Histamine	> 5*	> 5*					0.4
H_2O_2	1.28 ± 0.71	2.23 ± 1.57			2	1.21	0.8
4-Nitro-phenol	5.6 ± 1.73	9.94 ± 0.1***	3.5 (3)	100 (10)	1	0.96	0.02
			1.81 (4)	57 (11)			
			2.5 (5)	60 (12)			
Phenyl-ephrine	> 10*	> 10*					0.03

*NR_{80} or EC_{20} respectively could not be calculated, since it exceeded the highest concentration applied in the assay.
**Only one replicate could be used, since the NR_{80} exceeded the highest concentration applied in the assay (1,000 µM) in two further test runs.
***Only two replicates could be used, since the NR_{80} exceeded the highest concentration applied in the assay (10,000 µM) in one further test run.
(1) Fish cell line (Castano and Gomez-Lechon 2005)
(2) RTG-2, 72 h (Lange et al. 1995)
(3) RTG-2, 72 h (Lange et al. 1995)
(4) RTG-2, 48 h (Castano et al. 1996)
(5) PLHC-1, 24 h (Babich and Borenfreund 1987)
(6) Fathead minnow, 48 h
(7) Rainbow trout/zebrafish (Nagel 1988)
(8) Zebrafish/rainbow trout, 96 h (Ensenbach and Nagel 1995)
(9) Guppy/common goby, 48 h (Adema and Vink 1981)
(10) Rainbow trout, 96 h (Lange et al. 1995)
(11) Rainbow trout, 96 h (Castano et al. 1996)
(12) Trout, 96 h (Babich and Borenfreund 1987)

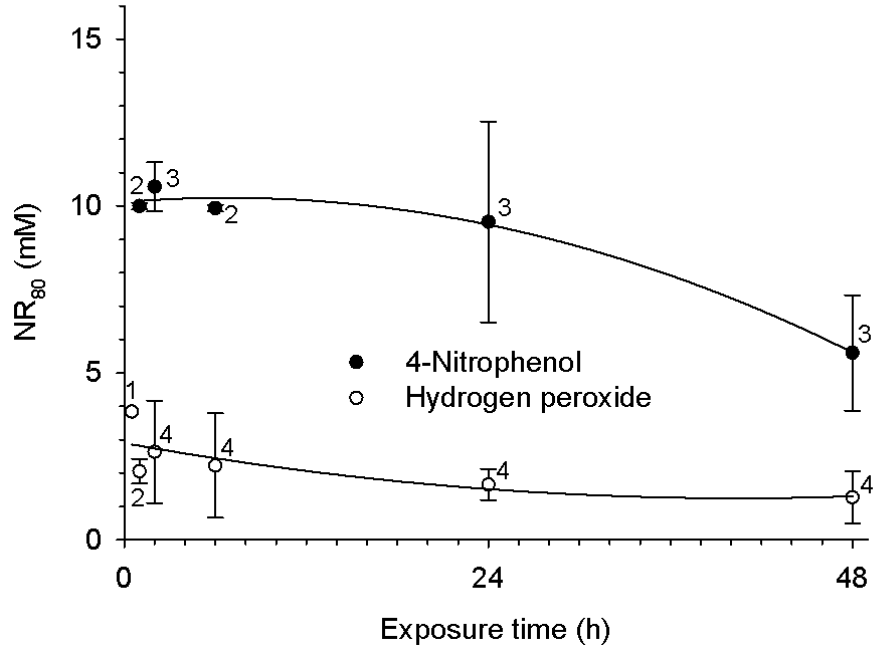

Fig. 2: Kinetics of cytotoxicity of 4-nitrophenol and hydrogen peroxide given in RTL-W1 cells as NR_{80} values (median ± standard deviation in mM) as assessed by the neutral red assay. The number of replicates is given for each data point. Fewer replicates were used in cases when the NR_{80} in further test runs could not be calculated, because concentrations applied were too low (≤ 10 mM for 4-nitrophenol and 5 mM for hydrogen peroxide).

3.2 Apoptosis assay

The apoptosis assay with Hoechst 33342 was performed with all substances with a cytotoxic potential in concentration ranges relevant for Ca^{2+} imaging (Tab. 1, Fig. 3). Fig. 3 shows apoptosis rates for different agonist concentrations and exposure times. For all agonists, apoptosis rates were only significantly elevated at higher concentrations (LOECs = lowest observed effect concentrations) and increased with exposure time. For a better comparison with cytotoxicity data from the neutral red assay, EC_{20} values for each exposure time were determined where possible (see Tab. 1 for 6 h). The apoptosis assay proved to be more sensitive than the cytotoxicity assay after shorter time spans (6 h) for three of the four substances tested. For 3,4-dichloroaniline and hydrogen peroxide, viability assessed with the apoptosis assay after 6 h (EC_{20}) was in the same range as in the neutral red assay after 48 h. For 4-nitrophenol, effect concentrations were even lower in the apoptosis assay. Only for caffeine, the apoptosis assay proved less sensitive.

Results of both toxicity tests as well as literature data of acute toxicity in fish (LC_{50}; see Tab. 1) were taken into consideration for the selection of agonist concentrations for Ca^{2+} imaging. Likewise, literature data about Ca^{2+} modeling (mostly for mammals) were taken into account.

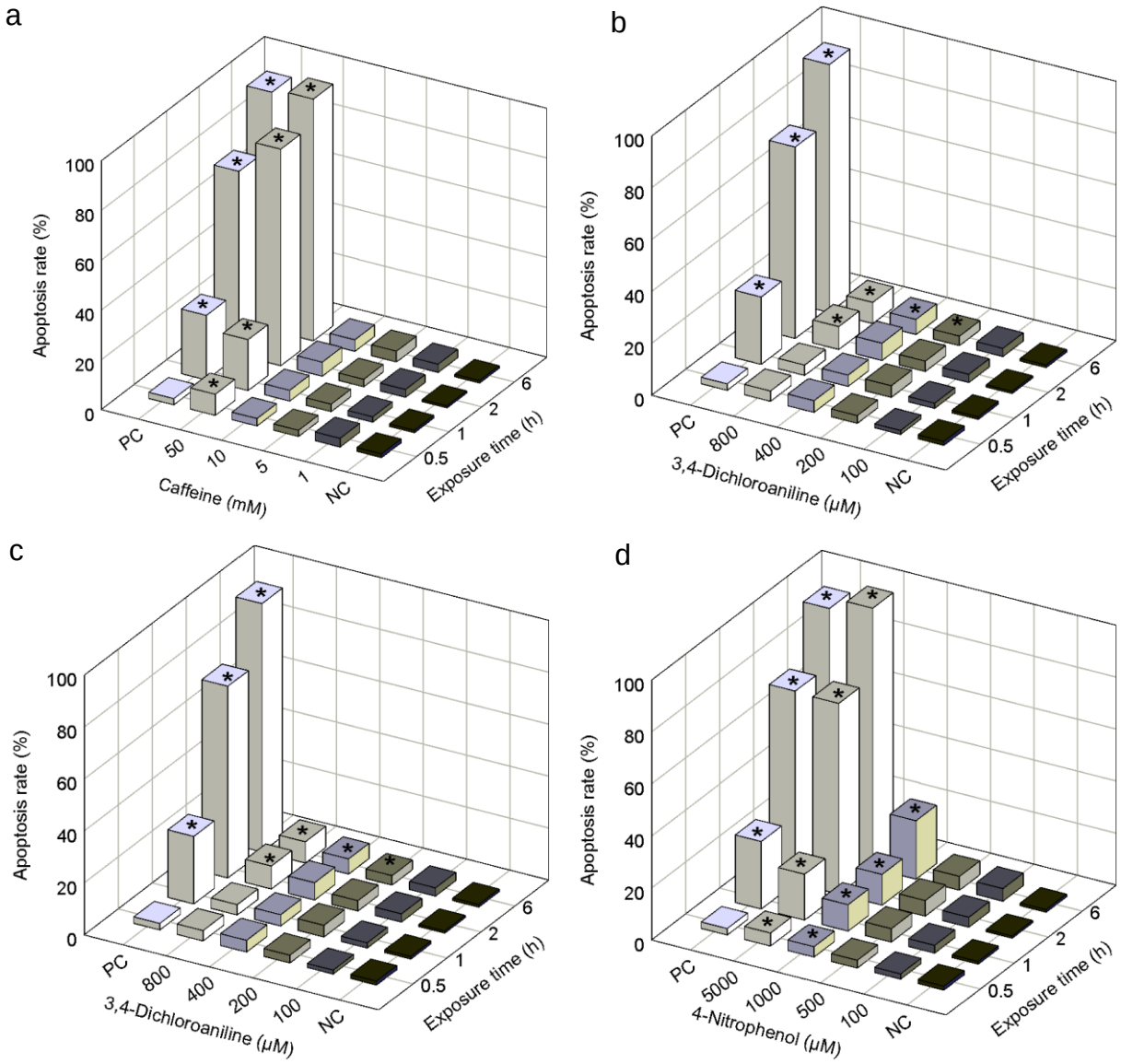

Fig. 3 a-d: Apoptosis in RTL-W1 cells for different agonist concentrations and exposure times assessed with the apoptosis assay with Hoechst 33342 (median from 3 - 4 independent runs). * Apoptosis rates significantly increased over negative controls (one-way ANOVA; *post-hoc* test according to Dunnet; $p < 0.05$).

3.3 Ca^{2+} imaging in fish cells

Hydrogen peroxide

At non-toxic doses, hydrogen peroxide (H_2O_2) provoked mainly oscillations with pronounced high peaks in RTL-W1 cells (Fig. 4a). The characteristic Ca^{2+} pattern of H_2O_2 might indicate that this agent initiates its own signal transduction pathway or that the disturbance of the intracellular redox homeostasis influences other cell processes, which in turn trigger the

activation of signaling procedures. With increasing H_2O_2 concentration, the amplitude of Ca^{2+} oscillations increased. Concentrations of H_2O_2 between 1 and 2 mM were found to be cytotoxic and to induce apoptosis in RTL-W1 cells (Tab. 1, Fig. 3c). High concentrations of oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, and lipid hydroperoxides (i.e., reactive oxygen species, ROS) are known to be able to lead to damage to cellular components *via* the oxidation of lipids, proteins, and nucleic acids, and ultimately be cytotoxic (Avery 2011, Ermak and Davies 2001, Valko et al. 2007).

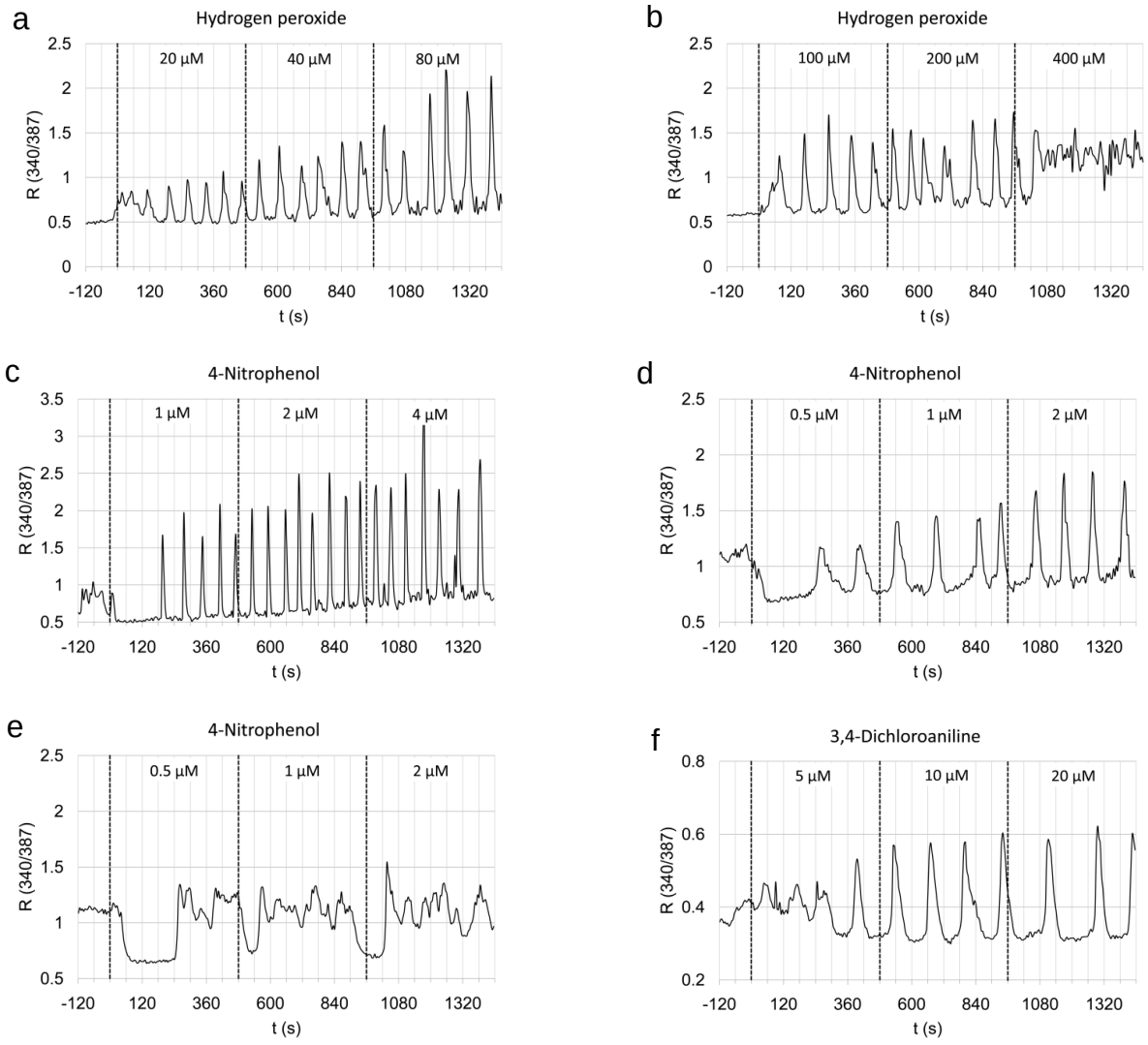

Fig. 4: Oscillations in the R(340/387) quotient as a measure for intracellular free Ca^{2+} levels in individual RTL-W1 cells after addition of various agonists in different consecutive concentrations (vertical lines). Please note differences in ordinate scaling. **(a) and (b) Hydrogen peroxide:** Oscillations displayed a high amplitude and relatively small wavelength. **(c) - (e) 4-Nitrophenol:** Most cells answered with marked Ca^{2+} oscillations. Note the baseline decline after first addition of 4-nitrophenol and its subsequent gradual rise. In some cells, bursts were observed (also for other agonists). **(f) 3,4-Dichloroaniline:** Note the small amplitude of the oscillations for this agonist. It also has to be mentioned that oscillations were observed only in few cells.

In rat hepatocytes, 400 µM H_2O_2 induced a sustained Ca^{2+} elevation (Sato et al. 2009). This was explained by impairment of mitochondrial functions *via* oxidative stress leading to reduced intracellular ATP production. In turn, ATP-sensitive, non-specific cation channels were opened, provoking Ca^{2+} influx. A high concentration (1 mM H_2O_2) induced an additional transient elevation of Ca^{2+} which seemed to be mediated *via* activation of the phospholipase C (PLC) signaling pathway and subsequently, by mobilization of Ca^{2+} from intracellular Ca^{2+} stores.

In contrast, in mouse pancreatic acinar cells (Granados et al. 2006), micromolar concentrations of H_2O_2 induced an oscillatory pattern, whereas 1 mM H_2O_2 caused a slow and sustained Ca^{2+} increase also in the absence from extracellular Ca^{2+}, i.e. recruiting Ca^{2+} from intracellular pools. The authors concluded that, at micromolar concentrations, H_2O_2 induced Ca^{2+} release from agonist-sensitive stores, and that, at millimolar concentrations, H_2O_2 could also evoke Ca^{2+} release from the mitochondria. The action of H_2O_2 seems to be mediated by oxidation of sulphydryl groups of Ca^{2+} ATPases and to be independent of IP_3 generation, since neither inhibition of the phospholipase C nor of the IP_3 receptor could block Ca^{2+} release.

Due to their origin from fish liver, RTL-W1 cells might be expected to react to H_2O_2 in a way similar to the Ca^{2+} response in rat hepatocytes. However, after addition of micromolar H_2O_2 concentrations, Ca^{2+} signaling observed in RTL-W1 cells rather resembled the oscillatory pattern observed in mouse pancreatic acinar cells. In addition, a dose-dependence in the Ca^{2+} amplitude was seen, which was not observed by Granados et al. (2006). Whether the phospholipase C pathway and the IP_3 receptor are involved, remains to be investigated. So far, the oxidation of sulphydryl groups and membrane depolarization followed by Ca^{2+} influx might be a logical explanation for the observed Ca^{2+} response in RTL-W1 cells. In contrast, the sustained Ca^{2+} elevation observed in individual cells for higher H_2O_2 concentrations might be a consequence of the Ca^{2+} release from mitochondria (see below).

Notwithstanding its toxic potential, H_2O_2 is an important component of living cells; e.g., it plays important roles in host defense and oxidative biosynthetic reactions. All aerobic organisms apparently regulate their intracellular H_2O_2 concentrations at relatively similar levels. In addition, both in animals and plants, regulatory enzyme systems for the generation of H_2O_2 have been described (Stone and Yang 2006). Exposure of cells to sublethal oxidative stress initiates a signaling response which results in a variety of downstream effects including increased expression of protective and repair enzymes (Winterbourn and Hampton 2008). It has been proposed that changes in the redox potential stimulate signal transduction components such as phospholipase and protein kinases and inhibit protein phosphatases effecting tyrosine and serine/threonine phosphorylations. This can lead to the activation of oxidative stress-responsive transcription factors (Suzuki et al. 1997).

The observations that ligand-receptor interactions produce reactive oxygen species and that antioxidants block receptor-mediated signal transduction led to the proposal that reactive

oxygen species themselves may function as second messengers for transcription factor activation, apoptosis, bone resorption, cell growth, and chemotaxis (Suzuki et al. 1997). Winterbourn and Hampton (2008) found evidence that redox signaling is fundamental to a wide range of receptor-mediated pathways. However, weather H_2O_2 acts indeed as a second messenger in non-stressed cells still needs to be elucidated.

4-Nitrophenol

4-Nitrophenol concentrations far below cytotoxic levels (1 - 10 mM) turned out to have a strong influence on intracellular Ca^{2+}. Concentrations in the lower micromolar range provoked mostly marked oscillations in intracellular free Ca^{2+} (Figs. 4c-e). Frequency was around one per minute, however with individual differences between cells. Prior to the oscillations, a decrease of the baseline followed by a typical lag period of several minutes could be observed. This might be due to the time required by 4-nitrophenol to reach its cellular targets.

Literature on the mode of action of 4-nitrophenol is scarce; however, it is important to know the cellular targets of a substance to understand its influences on Ca^{2+} signaling pathways. Part of the properties of 4-nitrophenol can be explained by the chemical characteristics of phenolic compounds: In addition to being lipophilic, nitrophenols are H^+-ionophores; i.e., they destroy mitochondrial proton gradients by transporting H^+ cations across the inner mitochondrial membrane, thus leading to the break-down of transmembrane potentials. A collapse of the mitochondrial membrane potential results in reduced ATP production and finally leads to ATP depletion and disruption of ion homeostasis (Bellomo et al. 1991). The increasing amount of protons inside the mitochondrial matrix induced by such uncoupling agents leads to a release of Ca^{2+} from mitochondria into the cytosol *via* reversal of the uniport Ca^{2+} carrier (Bernardi et al. 1984). This initial increase in intracellular Ca^{2+} may trigger Ca^{2+}-induced Ca^{2+}-release (CICR), when ion channels in the plasma membrane are secondarily activated effecting an influx of Ca^{2+} from the extracellular space.

The decrease in cellular ATP has been proposed as one of the critical events in the development of irreversible injury caused by a variety of ionophores and chemical toxins (Bellomo et al. 1991) and might explain cytotoxicity of 4-nitrophenol at higher concentrations. However, these effects of uncoupling ATP synthesis and disturbance of intracellular Ca^{2+} homeostasis do not seem sufficient to explain the regular Ca^{2+} oscillations observed in RTL-W1 cells after application of Ca^{2+} concentrations around 1,000-fold below those inducing cytotoxicity.

3,4-Dichloroaniline

In the present study, only a minority of RTL-W1 cells (approximately 10 %) responded to the addition of sublethal concentrations of 3,4-dichloroaniline (3,4-DCA; 0.1 - 20 µM) with a

regular modulation of the intracellular Ca^{2+} level (Fig. 4f). It took several minutes for the cells to establish regular oscillations after first addition of 3,4-DCA. Once established, oscillations displayed relatively small amplitude and high wavelength (2 - 3 min), if compared to other agonists. Albeit conspicuous, amplitude-encoding of concentration was less pronounced for 3,4-DCA than for the other agonists.

Although effects by 3,4-DCA on intracellular Ca^{2+} were minor, research showed that 3,4-DCA acts as a toxicant at different levels: It is an intermediate in the production of diuron, linuron, lindane and other herbicides, azo dyes and pharmaceuticals and a degradation product of several organochlorines. 3,4-DCA and its analogues are known to compromise growth, development, and reproduction of aquatic organisms. Data suggest that 3,4-DCA induces the generation of free radical and depletion in the levels of antioxidants. In crucian carp (*Carassius carassius*), e.g., 3,4-DCA caused oxidative stress and lipid peroxidation in liver (Li et al. 2003). In fish, prolonged exposure to 3,4-DCA caused a progressive increase in levels of methaemoglobin leading to methaemoglobinaemia. These effects may be reversible when the fish is transferred to uncontaminated water (Crossland 1990). In addition, linuron generates tumors in rat and evidence exists that linuron, 3,4-DCA and other degradation products of diuron behave as antiandrogens (Cook et al. 1993).

The relatively small proportion of cells responding to 3,4-DCA and the moderate oscillations in case of response might be explained by the lack of a specific Ca^{2+}-implying pathway induced by 3,4-DCA. It might be possible that 3,4-DCA induces other non-specific pathways, e.g. depending on the osmotic or redox state of cellular components. One explanation might be the formation of oxidative radicals and the resulting oxidative stress. In crucian carp liver, e.g., lipid oxidation was observed after exposure of fish to 3,4-DCA (Li et al. 2003). At higher concentrations, 3,4-DCA could provoke similar effects on intracellular Ca^{2+} like H_2O_2; however, it was not possible to increase the test concentrations due to a beginning overlap with acute toxicity (cf. Tab. 1).

3.4 Ca^{2+} signaling as a predictor of cell death?

The maintenance of homeostasis and coordinated modulation of intracellular Ca^{2+} is a prerequisite for the organization of many cellular processes. In many cases, breakdown of intracellular Ca^{2+} homeostasis seems to trigger reactions ultimately leading to carcinogenesis and cell death, including perturbation of physiological ion homeostasis, protein and lipid destruction, DNA fragmentation and condensation, swelling of ER and mitochondria, formation of cell surface alterations (blebbing), and loss of plasma membrane integrity, (Orrenius et al. 2003, Trump and Berezesky 1995). Since Ca^{2+} homeostasis is controlled by so many diverse proteins, it is quite likely that multiple insults or physico-chemical alterations end up in dysregulation. As a consequence, multiple pathways are initiated in an uncoordinated manner, overlapping and superimposing one another, eventually leading to

cell collapse. The Ca^{2+} stress answer may consist of either depletion of ER Ca^{2+}, increase of cytosolic/mitochondrial/nuclear Ca^{2+} or both (Cerella et al. 2010).

For all toxicants tested, one apparently unspecific phenomenon in intracellular Ca^{2+} signaling could be observed in individual RTL-W1 cells when exposed to higher concentrations and for longer times: Regular oscillations ceased and intracellular Ca^{2+} seemed to increase in an uncoordinated manner and remained at elevated levels (Fig. 5). For hydrogen peroxide and 4-nitrophenol, this was observed when concentrations reached or exceeded 400 or 1 µM, respectively. The induction rate for this phenomenon was around 5 - 10 %, i.e. only a small sub-population of RTL-W1 cells responded in this way. These concentrations did not show any significant effect neither in the neutral red assay nor in the apoptosis assay for the exposure times selected for Ca^{2+} imaging (approx. 30 min).

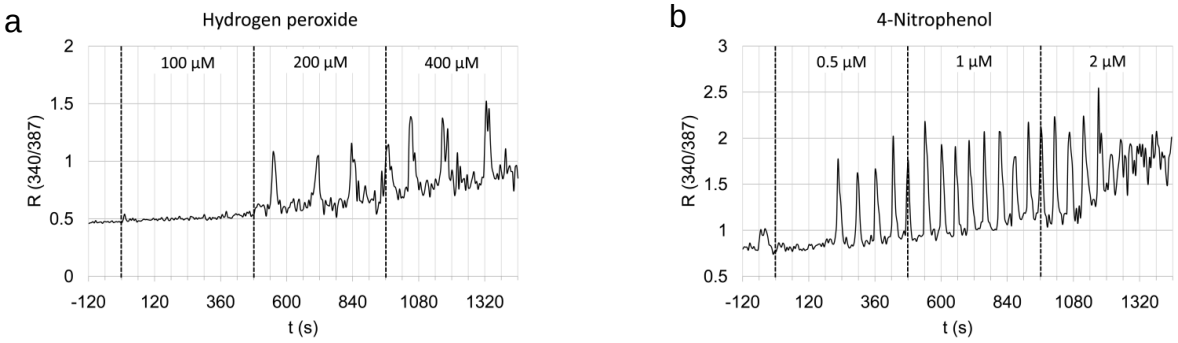

Fig. 5 a, b: At higher concentrations (varying from cell to cell) of hydrogen peroxide (≥ 400 µM) and 4-nitrophenol (≥ 2 µM), free intracellular Ca^{2+} often seemed to increase in an uncoordinated manner and remained at an elevated level.

In literature, however, such an abrupt rise of intracellular Ca^{2+} has often been related to events preceding controlled cell death. In a number of tissues and cell types, toxic insults by different agents and conditions have been found to cause an increase of intracellular Ca^{2+} levels. Furthermore, studies on hepatocytes indicated that the preservation of the mitochondrial membrane potential can be critical for the maintenance of cell viability and that alterations in mitochondrial functions can be related to the perturbation of Ca^{2+} homeostasis and the development of irreversible hepatocellular injury (Bellomo et al. 1991). A shortage of mitochondrial ATP will always lead to the down-regulation of energy requiring processes in the cell, including DNA repair and ATP-dependent Ca^{2+} transport processes. In consequence, it would be more difficult for the cells to counteract the negative effects of Ca^{2+} elevation (Bellomo et al. 1991).

Several studies with aquatic animals attempted to demonstrate mitochondrial damage as a proof of irreversible cell injury after exposure to toxicants by means of microscopy (Benedeczky and Nemcsok 1997, Lemaire et al. 1992). Benedeczky and Nemcsok (1997)

proposed the appearance of so-called “giant mitochondria” as a possible, however non-specific bioindicator for the damage by environmental xenobiotics in fish liver. Toninello et al. (2000) provided evidence that these events are linked to induction of the mitochondrial permeability transition (MPT) in liver cells of the green goby (*Zosterisessor ophiocephalus*).

The concept of MPT was introduced in 1979 by Haworth and Hunter (1979). It is a result of the opening of mitochondrial permeability transition pores (MPTP) for molecules of less than 1500 Da. This protein pore is a regulated, voltage-dependent channel of the inner mitochondrial membrane. In mammalian mitochondria, the MPT strongly compromises the bioenergetic functions. (Toninello et al. 2000) demonstrated that the induction of the permeability transition in fish liver mitochondria has characteristics similar to those described in rat liver mitochondria. The authors found membrane energization to be a prerequisite to the induction of this phenomenon. The MPT leads to a collapse of the membrane potential and the onset of a bidirectional trafficking of solutes through the inner membrane, which, as a first consequence, induces swelling of the matrix and rupture of the outer membrane. Another consequence of MPT induction is the release of cytochrome c and other proteins from the intermembrane space of the mitochondria into the cytosol, where a protein complex is formed activating several downstream actions ultimately causing apoptosis (Gerasimenko et al. 2002).

The MPT seems to be an important step in the signaling cascade leading to apoptosis. Evidence exists for the MPT as a final common point at which the pathways of many toxic agents converge (Toninello et al. 2000). The herbicide paraquat, e.g., induces a Ca^{2+}-dependent permeability increase of the inner mitochondrial membrane leading to membrane depolarization, uncoupling and matrix swelling due to inappropriate opening of the permeability transition pore through a shift of the gating potential to more negative values, allowing pore opening at physiological membrane potentials (Costantini et al. 1995). In a subpopulation of rainbow trout hepatocytes, copper induced disruption of Ca^{2+} homeostasis and onset of the MPT as a prerequisite for the stimulation of apoptosis, whereas necrotic cell death in other cells was independent of the MPT (Krumschnabel et al. 2005). There is increasing evidence that most (if not all) pathologies involve alterations of oxidative metabolism leading to oxidative stress. Oxidation and redox imbalance, however, cause ER and plasma membrane Ca^{2+} channels malfunctions; this increases cytosolic Ca^{2+} and depletes Ca^{2+} in the ER. Moreover, oxidative stress impairs the buffering capacity of mitochondria, lowering the internal Ca^{2+} threshold level of MPTP opening (Cerella et al. 2010).

So far, it is difficult to establish a clear relationship between a definite Ca^{2+} signal and its cellular response, and even more to differentiate between apoptosis and necrosis only considering Ca^{2+} signaling. Criddle et al. (2007) found evidence that different patterns of cytosolic Ca^{2+} rises influence both apoptotic and necrotic cell death pathways in pancreatic acinar cells. Mild stimuli such as oxidant stress (e.g., by the oxidant menadione;

Gerasimenko et al. 2002) evoked transient oscillatory Ca^{2+} rises which promoted partial mitochondrial depolarization, cytochrome c release and apoptosis, when an additional factor, e.g. generation of reactive oxygen species was present. The increase of intracellular Ca^{2+} could be inhibited by the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). ATP production was not negatively influenced. Physiological agonists also evoked cytosolic Ca^{2+} spikes but did not depolarize mitochondria and failed to induce apoptosis (Gerasimenko et al. 2002). The authors suggested that both intracellular release of Ca^{2+} and induction of the MPTP are required for the initiation of apoptosis. More severe insults or secondary and/or continued low stress caused sustained pathological Ca^{2+} elevations, which lead to irreversible inhibition of mitochondrial function, cellular ATP production and necrosis.

If this held true also for RTL-W1 cells, the observed Ca^{2+} oscillations after addition of the agonists would indicate moderate stress and partial mitochondrial depolarization. In contrast, the non-transient elevation in intracellular Ca^{2+} observed after addition of higher agonist concentrations would indicate necrosis rather than apoptosis in the affected cells. On the other hand, regular Ca^{2+} oscillations might also be interpreted as the onset of a (specific) pathway by the respective agonist, whereas the uncoordinated increase of intracellular Ca^{2+} indicates the perturbation of Ca^{2+} homeostasis which could lead to apoptosis or necrosis depending on severity and duration of the insult. Presumably, the exact generation site of the Ca^{2+} signal also influences cell fate.

The present study shows that disturbance of intracellular Ca^{2+} levels can be detected long before effects can be seen at the level of DNA or membrane integrity. The ecological relevance of these results for higher levels of biological organization, however, is probably minor, as holds true for many lower tier endpoints. Yet, Ca^{2+} imaging provides striking evidence that apparently untoxic concentrations of chemicals or chemical mixtures might influence cell metabolism and integrity, even more when different stressors act in an additive or synergistic manner. Moreover, Ca^{2+} imaging might be a useful tool in ecotoxicology to provide information about the mode of action of a certain substance.

4 Conclusions

The present study indicates that the fish liver cell line RTL-W1 is a suitable tool for the investigation of Ca^{2+} signals in consequence of toxicant exposure. Evidence is provided that ecotoxicologically relevant substances take influence on intracellular Ca^{2+}. Namely hydrogen peroxide and 4-nitrophenol induced a clear response and produced marked Ca^{2+} oscillations at sublethal concentrations. Effect threshold and intensity showed considerable variability between individual cells; however, general effects were reproducible and dose-dependent. At concentrations below those inducing elevated cytotoxicity and apoptosis rates, there is a lasting, unspecific increase of intracellular Ca^{2+} levels which might be interpreted as a precursor of apoptotic or necrotic processes.

Despite the sensitivity, specificity, reproducibility and dose-dependency, Ca^{2+} imaging is far from being a routine method in ecotoxicology testing and requires further standardization. In addition, the question arises how the cellular effector systems identify relevant signals, since the possibility to interpret Ca^{2+} signals and to decode the information encoded previously by the cell is a fundamental prerequisite for the application of Ca^{2+} imaging in ecotoxicology. The basic mechanisms of intracellular Ca^{2+} signal encoding are fairly well understood for mammals and are supposed to be at least partially transferrable to other vertebrate groups. Much less is known, however, about the cellular targets and their interpretation of the Ca^{2+} signal, i.e. its specificity and decoding.

The problem of signal encoding/decoding is particularly important for understanding the mechanisms by which Ca^{2+} signals control gene expression. The link between nuclear Ca^{2+} changes and the process of transcription is clearly established; yet, how Ca^{2+} entering the nucleus carries specific information about which types of genes should be expressed, is at present unknown (Petersen et al. 2005). To answer the question of how the Ca^{2+} signal is read out by the cell and can be interpreted by the scientist, further research at the subcellular level is necessary. There are indications that even the precise location and the way of Ca^{2+} signal generation are of importance; however, existing experimental data are controversial (Petersen et al. 2005).

Overall, there is evidence that Ca^{2+} imaging might provide a highly sensitive, yet non-specific indicator of toxic impact, since, as a second messenger, intracellular Ca^{2+} also integrates toxic effects on numerous other sublethal parameters.

5 Acknowledgment

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Chapter 5

Final discussion and conclusions

Kein einzelner Teil konnte entstehen als in diesem Ganzen,
und dieses Ganze selbst besteht nur in der Wechselwirkung seiner Teile.

Friedrich Schelling (1775 - 1854)

1 Ca^{2+} imaging in fish cells

- Finally applied culture conditions for primary hepatocytes yielded good attachment (approx. 75 %) and viability (approx. 97 %).
- An adequate culture substrate (poly-L-lysine) is especially useful.
- Attention has to be paid to the physiology and "liver-like" morphology of cells.
- The established Ca^{2+} imaging protocol allowed detection of Ca^{2+} signals and time lapse acquisition in both cell types.
- Ca^{2+} imaging results showed differences between primary and RTL-W1 cells.
- At least for RTL-W1 cells, the imaging conditions proofed to be optimal.
- Possible reasons for the meager response of trout primary hepatocytes might be: species differences; problems with the isolation procedure or culture conditions and, therefore, reduced viability or functionality of the isolated cells; hepatocyte heterogeneity in trout; too high signal-to-noise ratio during Ca^{2+} imaging; and overstimulation of the cells due to excessive agonist concentrations.
- Both test systems provide various advantages as model systems in aquatic ecotoxicology.
- The Ca^{2+} answer in RTL-W1 cells is more differentiated, and results show better reproducibility than in hepatocytes.
- Primary hepatocytes are supposed to be closer to *in vivo* conditions; however: are they ultrastructurally and physiologically intact?
- The cell line RTL-W1 features several advantages, if compared to primary cells, namely cost efficiency, good handling, ease of standardization, reproducibility, cytochrome P450 expression, and EROD induction.
- The main disadvantage of the cell line is its unclear origin.
- At the moment, results from RTL-W1 seem more reliable, especially considering their application in ecotoxicology.
- In the long term, however, it would be preferable to establish a Ca^{2+} imaging procedure applicable to primary hepatocytes in order to get better transferability of the data to the conditions *in vivo*.

1.1 Isolation and culture of primary hepatocytes

Primary hepatocytes from rainbow trout provide a multitude of advantages when used as a test system (cf. section 3.5 of chapter 1 and introduction of chapter 2). The principal motivation of using primary cultures in the present study was their supposed similarity to the conditions *in vivo*. For this purpose, existing isolation and culture protocols had to be adapted to the given conditions and objectives.

Despite the many advantages of primary hepatocytes from rainbow trout, it cannot be ignored that these cells also present some limitations in comparison to cell lines, such as: (1) the necessity to go back intact animals for each cell preparation; (2) more difficult preparation and handling of cells; (3) variability between different preparations due to differences in the health and reproductive status of the fish or specific conditions during the isolation procedure; (4) limited viability and adhesion of primary hepatocytes.

Bullets (1) and (2) are supposed to be tolerable considering the advantages of primary hepatocytes in context of the aims of the present study – taken for granted the accurate and ethically correct handling of donor animals and cells. Points (3) and (4), however, were problems to face before starting with Ca^{2+} imaging in primary hepatocytes. It is obvious that the routine application of primary hepatocytes in the laboratory and especially the observation of biochemical processes at the level of individual cells require highly standardized and reliable isolation and culture protocols in order to get viable and robust cells and reproducible results. Furthermore, the Ca^{2+} imaging procedure applied requires several washing steps and media changes making optimal adherence indispensable. Attachment is important not only for the observation of individual cells, but also for the maintenance of differentiated liver functions in the isolated cells.

Isolation and culture of primary hepatocytes from rainbow trout require several elaborateness and routine. Then, however, the technique yields good results. Immediately after isolation, the mean number of liver cells was $7.5 \pm 1.9 \times 10^8$ cells/fish and $2.5 \pm 0.2 \times 10^6$ cells/g body weight with generally > 90 % of viable cells (average of 94.5 ± 3.7 %). The following culture conditions proofed to be most adequate for the application of trout hepatocytes for further investigations: Hepatocyte suspension containing 2 % FCS was seeded into each cavity of poly-L-lysine coated 24-well plates with cell density of approx. 4×10^5 cells/well or 2.3×10^5 cells/cm^2, providing an average cell attachment of 76.8 ± 9.6 % after 24 h and 72.8 ± 3.7 % after 48 h and a cell viability of 97.6 ± 3.2 % after 24 h and 96.2 ± 3.1 % after 48 h for poly-L-lysine.

The findings – in accordance with literature data (cf. references in chapter 2) – indicate that the fetal calf serum (FCS) concentration in the medium, cell density and culture time had only a minor influence on the viability and attachment rate of primary hepatocytes (within the ranges tested) compared to the substrate used for coating the culture plate. Among the substrates tested in this study, poly-L-lysine proved to be the most effective promoting firm attachment of the cells at an average of > 70 %. Under the here-used test conditions, on

untreated plates as well as on all extracellular matrix components applied in this study, attachment was poor and was generally < 20 % after washing and media changes. Results are in accordance with many literature reports. Furthermore, poly-L-lysine provoked a "hepatocyte-like" appearance of the cells, which might be of importance in maintaining a more differentiated phenotype as well as physiological integrity of the cells.

In general, it seems that primary trout hepatocytes are an excellent model cell system providing several advantages. However, isolation and culture must strictly follow the protocol to guarantee maximum quality of cells and reproducibility of results.

1.2 Development of a Ca^{2+} imaging protocol

In the present study, fluorescence staining and microscopy were carried out with the ratiometric Ca^{2+} indicator Fura-2 according to many other established protocols, but adapted to the conditions in rainbow trout hepatocytes and RTL-W1 cells (cf. section 4.1 of chapter 1). The established Ca^{2+} imaging protocol allowed detection of Ca^{2+} signals and time lapse acquisition in both cell types. At least for RTL-W1 cells, the imaging conditions proofed to be optimal: Signal-to-noise ratio was tolerable, signal intensity remained already constant during acquisition time (up to two hours), and results were clear and reproducible.

In contrast, in primary hepatocytes, clear effects on the intracellular Ca^{2+} level could be observed for all tested agonists in most cells; however, Ca^{2+} signals varied in extent between individual cells. Oscillations were not only rare, but were mostly irregular and showed small amplitudes. Little Ca^{2+} response of trout hepatocytes to various agonists was also found by other authors (Moon and Mommsen 1990, Zhang et al. 1992b), despite the fact that the principal components of Ca^{2+} signaling pathways are supposed to be present in rainbow trout hepatocytes, as they are in mammals and other fish species. It might be possible that the meager response of trout primary hepatocytes towards agonists of the Ca^{2+} pathway is not (only) a consequence of species differences (Zhang et al. 1992b) or hepatocyte heterogeneity in rainbow trout (Mommsen et al. 1991), but also due to problems in the isolation procedure or culture conditions and therefore reduced viability or functionality of the isolated cells. "Black boxes" in the culture medium like, e.g., FCS, and poly-L-lysine used as a culture substrate, both applied with the objective to optimize adherence and viability of the cells, as well as antibiotics, might modify cell physiology and alter Ca^{2+} signaling in an unpredictable manner. It would be preferable to optimize the culture conditions for primary cells in order to get robust cells without the necessity to come back these substances.

Furthermore, it cannot be ruled out that the imaging protocol established in this study was not totally adequate for the – maybe especially sensitive – primary hepatocytes, in spite of its good suitability for the permanent cell line and the broad application of similar protocols for many other cell types. For this reason, an adaptation of the protocol should be

attempted in order to improve the imaging conditions for primary hepatocytes to obtain more differentiated results if possible.

One possible explication for the little differentiated Ca^{2+} signal in the primary cells might be an excessive signal-to-noise ratio implying that smaller Ca^{2+} signals could not be detected as a consequence of an elevated background signal.

Last, but not least, in some cases over-stimulation of the cells and Ca^{2+} pathways because of too high agonist concentrations might be a reason of the poor Ca^{2+} response of primary hepatocytes. However, this is less probable, since Ca^{2+} imaging also provided only few results when minor agonist concentrations comparable to those applied in literature and with RTL-W1 cells in the present study were applied.

1.3 Primary hepatocytes *versus* RTL-W1 cells

One important question that came up during this study was how the differences between the two used cell types – the primary hepatocytes and the RTL-W1 cell line – could be explained and which system could better serve as a model for Ca^{2+} modulation in fish *in vivo* (see also discussion of chapter 3). Both cell types fulfill the requirements as a model for both vertebrates and aquatic systems and an alternative to mammalian systems. Considering the role of the liver in the detoxification and excretion of xenobiotics, hepatocytes seem especially suitable models for ecotoxicology.

With respect to the Ca^{2+} imaging results of the present study, findings for the cell line look more promising and seem to be more reliable, since the Ca^{2+} response appears a lot more differentiated than in the primary hepatocytes, and results in RTL-W1 cells are highly reproducible. Generally, cell lines present various advantages, if compared to freshly isolated cells, namely cost efficiency, good handling, and a higher potential for standardization and reproducibility of the results making them preferable in every day laboratory routine and particularly in ecotoxicology. This application is favored by the strong expression of biotransformation enzymes such as cytochromes P450.

In contrast, the primary cells from different preparations may vary as a result of differences in the health and reproduction status of the fish or in the isolation procedure. Besides, primary hepatocytes are only viable for a relatively short period of time, and the question arises whether all cells classified as “vital” by light microscopic observation, are also ultrastructurally and physiologically intact. Independently of the routine and accurateness of the applier, it cannot be ruled out that loss of some cellular functions, in particular with respect to specific Ca^{2+} signaling pathways, already occurred during isolation or the first days in culture.

To overcome the limitations presented by primary hepatocytes and to complete our investigations on intracellular Ca^{2+} signaling in fish, the cell line RTL-W1 was selected for further experiments. The main disadvantage of the cell line is its uncertain origin (for an

extensive discussion, see chapter 3), making it more difficult to extrapolate results to the conditions in trout liver cells *in vivo*. Nevertheless, since Ca^{2+} signaling is a common phenomenon in different cell types, results should be transferrable at least to a certain extent to trout (cells) and, more generally, to other fish. Therefore, data from the cell line are supposed to supplement data from primary cells and to compensate for their shortcomings. This holds especially true since one motivation of this study was the application of Ca^{2+} imaging in ecotoxicology. In particular due to the good reproducibility of the results and the EROD expression, RTL-W1 is especially suited for toxicity testing and there is already information on the toxicity of environmental contaminants to refer to.

So far, it seems more promising to revert to the established and highly standardized cell line to get an impression of how Ca^{2+} signaling is principally organized in fish cells. In the long term, however, it would be preferable to establish a Ca^{2+} imaging procedure applicable to primary hepatocytes (cf. section 1.1) in order to get better transferability of the data to the conditions *in vivo*.

2 Ca^{2+} signaling in fish cells

- All agonists tested can modulate Ca^{2+} in both primary hepatocytes and the cell line RTL-W1.
- Evidence is provided that there are purinergic and adrenergic modulations of intracellular Ca^{2+} and that Ca^{2+} plays a role in the response to toxicants in the examined fish cells.
- At least in RTL-W1 cells, several agonists can induce Ca^{2+} oscillations.
- The modulation of intracellular Ca^{2+} in fish cells displayed some fundamental differences compared to mammals.
- Ca^{2+} oscillations seem to be amplitude-encoded in fish cells.
- The relation between Ca^{2+} release (decrease) and uptake (increase) is more symmetric in fish cells compared to rat hepatocytes.
- Different binding characteristics of the IP_3R, e.g. responsible for the Ca^{2+}-induced Ca^{2+} release, could be the origin of the observations in RTL-W1 cells.
- The simpler oscillations induced by ATP in RTL-W1 cells compared to mammals might be due to differences in purinergic receptor sequence including less potential feedbacks and interactions with Ca^{2+} channels.

The present study was initiated to characterize the possible regulation of intracellular Ca^{2+} levels in primary rainbow trout hepatocytes and the permanent fish cell line RTL-W1. Results document that (1) all agonists tested can modulate Ca^{2+} in both primary hepatocytes and the permanent cell line. Evidence is provided that there are purinergic and adrenergic modulations of intracellular Ca^{2+} and that Ca^{2+} plays a role in the response to toxicants in the examined fish cells. (2) At least in RTL-W1 cells, several agonists can induce Ca^{2+} oscillations, and (3) increasing concentrations provoked an increase in the amplitude of Ca^{2+} oscillations in the cell line. This was evident for hydrogen peroxide, 4-nitrophenol and phenylephrine, but was less obvious for ATP. To the best of our knowledge, these are the first data indicating clear amplitude-encoding of concentrations in non-excitable cells.

As already mentioned in section 1 of this chapter, the Ca^{2+} response towards the selected substances was little differentiated in primary hepatocytes. Possible reasons for this phenomenon are discussed in the named section; results for both cell types are described and discussed extensively in chapter 3. In the following, we concentrate on a short resume of the results as well as a comparison to mammals, and attempt to give a first model.

2.1 Primary hepatocytes

In rainbow trout primary hepatocytes, for all tested substances (ATP, caffeine, 2,4-dichloroaniline, 3,4-dichloroaniline, histamine, hydrogen peroxide, 4-nitrophenol, phenylephrine), clear effects on the intracellular Ca^{2+} level could be observed in most cells, but varied in extent between individual cells. Oscillations, however, were evident only in rare occasions, were mostly irregular and displayed small amplitudes. Only ATP provoked a characteristic pattern in almost all cells: a biphasic Ca^{2+} increase. For the remaining agonists, the majority of hepatocytes showed a more or less rapid and pronounced elevation of intracellular Ca^{2+}. In some cells, Ca^{2+} raised abruptly within seconds several times after addition of the agonist (Fig. 1, insert). This effect was also observed by Bauer (1994) and was most pronounced for hydrogen peroxide, 2,4- and 3,4-dichloroanilines and 4-nitrophenol. These substances were chosen not because they are physiological inducers of Ca^{2+} signaling pathways, but because of their known ecotoxicological relevance – at least at higher concentrations. Therefore, the observed increase in intracellular Ca^{2+} might simply be an unspecific response of the cell towards a toxic stressor and potentially indicate (upcoming) apoptosis (cf. section 3 of this chapter). In some preparations, the number of hepatocytes reacting in this way increased in a concentration-dependent manner (Fig. 1) providing further evidence for this phenomenon initiating apoptosis in cellular stress response.

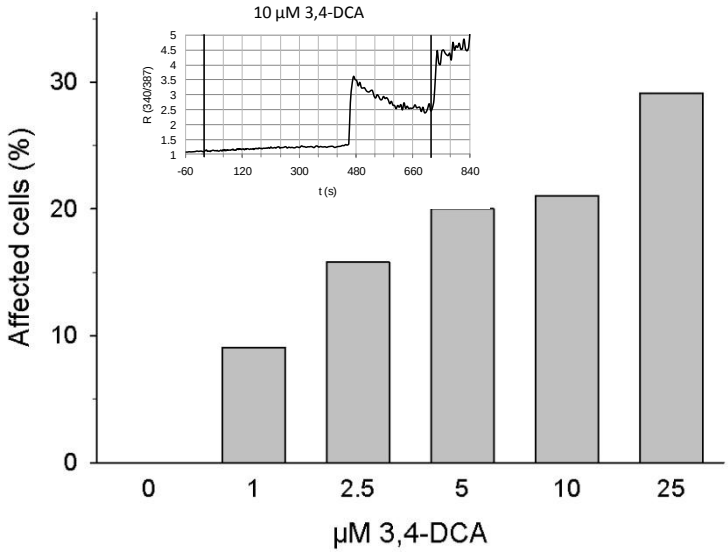

Fig. 1: Abrupt rise in intracellular Ca^{2+} several time after agonist addition in one primary hepatocyte (insert) and relative proportion of affected cells (in %), here exemplified for 3,4-dichloroaniline. Data from all cells (15 - 30 per concentration) of one hepatocyte preparation were integrated.

2.2 RTL-W1 cells

Results for the permanent fish cell line RTL-W1 differed considerably from those for primary rainbow trout hepatocytes. In the vast majority of cells, oscillations in the intracellular Ca^{2+} level were consistently observed for all agonists tested (ATP, caffeine, 3,4-dichloroaniline, histamine, hydrogen peroxide, 4-nitrophenol, phenylephrine). The signals, however, displayed some variation in shape, height and frequency.

Bioinformatics and analysis of literature and databases provide information about signal transduction pathways in fish compared to mammals: In general, the essential players of signal transduction do not differ much between piscine and mammalian cells, and Ca^{2+} pathways seem to be well-conserved during vertebrate evolution. Nevertheless, three fundamental differences in the modulation of intracellular Ca^{2+} in fish can be observed compared to mammals, as illustrated in Fig. 2 and summarized in the following: (1) in contrast to the mammalian counterpart, Ca^{2+} oscillations in fish systems seem to be amplitude-encoded, i.e. increasing agonist concentrations provoked an increase in the amplitude of the Ca^{2+} oscillations. This was evident for hydrogen peroxide, 4-nitrophenol and phenylephrine, but was less obvious for ATP. (2) Ca^{2+} release in rat cells during oscillations is markedly faster than the uptake, whereas this relation is more symmetric in the fish cells. (3) ATP provoked Ca^{2+} oscillation in fish cells. However, the oscillations are of simple sinusoidal nature in contrast to the bursting oscillations found in rat hepatocytes.

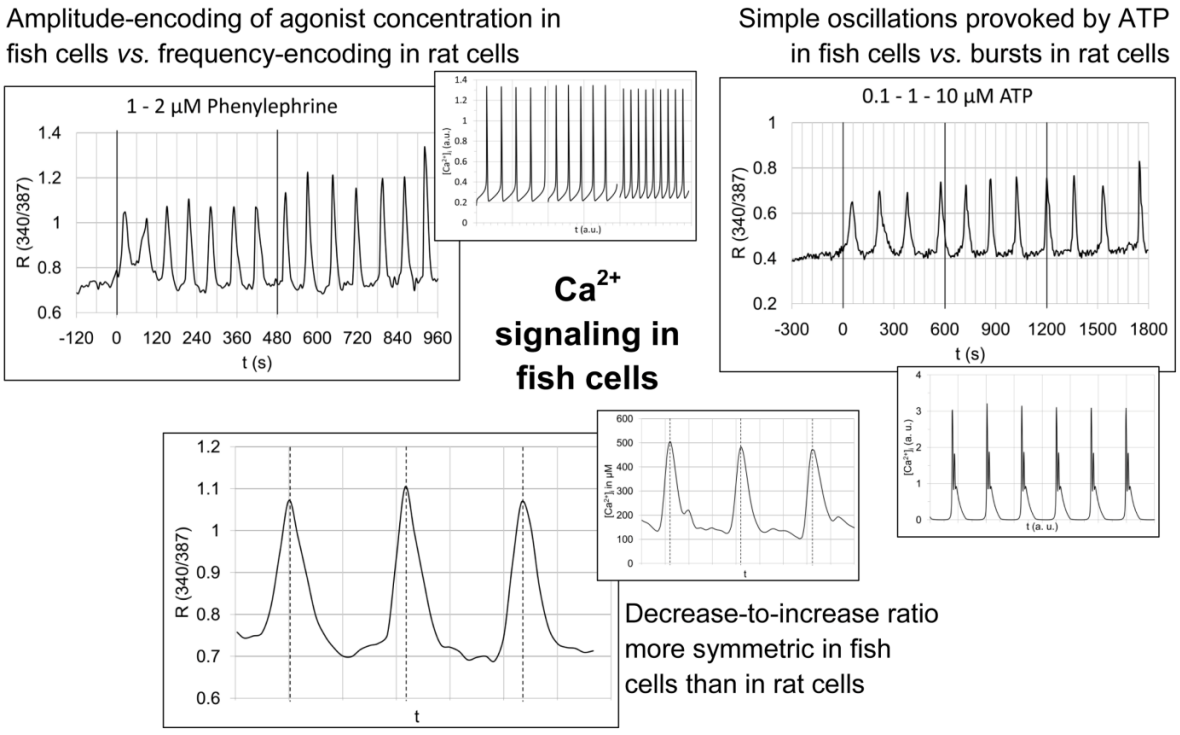

Fig. 2: Fundamental differences in Ca^{2+} signaling in RTL-W1 cells compared to mammalian cells (small graphs). All graphs are taken from chapter 3 and references named therein.

2.2.1 Amplitude-encoding of concentration

In the present study, we found that phenylephrine modulated intracellular Ca^{2+} in RTL-W1 cells at concentrations ≥ 800 nm. Around 50 % of the reacting cells displayed oscillations with a period of 0.7 - 0.8 min. Frequency did not change, when higher concentrations were applied; however, the amplitude of the oscillations increased. This is in clear contrast to the findings obtained for mammals: In rat hepatocytes, phenylephrine induced cytosolic dose-dependent Ca^{2+} oscillations, i.e., with increasing agonist concentration (0.5 - 10 µM phenylephrine), the frequency of oscillations increased (Rooney et al. 1989). In fish hepatocytes, in addition to the pronounced inter-species variability with respect to the players involved in catecholamine response, the main focus of most working groups was not on dose-dependency. Interestingly, Zhang et al. (1992b) found a dose-dependent epinephrine effect on the maximal amplitude of Ca^{2+} oscillations in eel hepatocytes; however, they did not follow up this issue. These findings suggest that amplitude-encoding could be a phenomenon typical for fish cells and distinguishing the Ca^{2+} response in different classes of vertebrates.

At least one major cause for this phenomenon must mainly lie in processes downstream of the actual receptor, since we observe amplitude-encoding also with agonists unspecifically causing Ca^{2+} oscillations without actual receptor-binding. This is especially true for hydrogen peroxide, which operates *via* depolarization of membranes followed by Ca^{2+} entry.

2.2.2 Decrease-to-increase ratio

Investigating the shape of individual peaks in Ca^{2+} oscillations and comparing the oscillations in fish with those in rat, it is evident that the relation between Ca^{2+} release (decrease) and uptake (increase) is more symmetric in fish cells (decrease-to-increase ratio 1.4 ± 0.4; n = 8), whereas in rat cells the release is markedly faster than the uptake (decrease-to-increase ratio 2.4 ± 0.8; n = 8).

The relation of the uptake and release properties at the Ca^{2+} channel is likely to be important both for frequency- and amplitude-encoding. Relaxation oscillations as observed in rat cells arise from a subcritical Hopf-bifurcation maintaining fairly constant amplitudes even close to the bifurcation point. More sinusoidal oscillations often arise from supercritical Hopf-bifurcations leading to an increase in amplitude at least in the vicinity of the bifurcation point.

The origin of the relaxation oscillations lies in the CICR which represents an autocatalytic system provoking a sudden release of Ca^{2+} from the ER and the comparatively slow pumping back into this store (Schuster et al. 2002). The proteins mainly responsible for uptake and release of Ca^{2+} from the ER are the SERCA and the IP_3R. In chapter 3, using a model downloaded from the BioModels Database (Le Novère et al. 2006), we showed that changes in the rates of the SERCA and the CICR which lead to a more balanced uptake and release

result in a model which shows amplitude-encoding with almost constant frequencies. Therefore, similar changes in the characteristics of the fish SERCA pump or Ca^{2+} channel could be the origin of the observed differences. Since the IP_3R is less conserved between fish and mammal whereas the SERCA shows remarkable homology, we conclude that different binding characteristics of the IP_3R, e.g. responsible for the Ca^{2+} induced Ca^{2+} release, could be the origin of the observations.

2.2.3 Simple oscillations

We observed that ATP provoked oscillations of simple sinusoidal nature in RTL-W1 cells, in contrast to the bursting oscillations found in rat hepatocytes. According to our analysis, there are stronger discrepancies between the agonist-specific receptors of fish and mammals than in the more general machinery employed in Ca^{2+} signaling. Differences in purinergic receptor sequence including less potential feedbacks and interactions with Ca^{2+} channels in fish cells should be responsible for the simpler ATP-induced oscillations.

3 Ca^{2+} imaging in environmental toxicology

- Ecotoxicologically relevant substances take influence on intracellular Ca^{2+}.
- Hydrogen peroxide and 4-nitrophenol produced marked Ca^{2+} oscillations at sublethal concentrations.
- Higher, however sublethal concentrations produced a Ca^{2+} increase which might precede cell death.
- It is difficult to clearly relate a definite Ca^{2+} signal and its cellular response and to differentiate between apoptosis and necrosis only considering Ca^{2+} signaling.
- For application in ecotoxicology, further standardization and research on the subcellular level is required.
- We suggest longer exposure times and the combination of Ca^{2+} measurements and toxicity tests in the same test run.
- Nevertheless, Ca^{2+} imaging might be a useful additional tool in ecotoxicology.

Based on the general model for Ca^{2+} signaling in fish cells established in chapter 3, in chapter 4 of this thesis, selected model environmental toxicants and stressors were used to elucidate possible interactions between contaminants and Ca^{2+} signaling and to quantify to which extent Ca^{2+} signaling in fish cells can be used for the detection of environmental stress with cell death as a last consequence. As a long-term target, the question should be addressed whether specific types of Ca^{2+} responses are specific of and may be used to characterize different types of stressors. Prior to Ca^{2+} imaging, two range-finding tests were carried out with the test substances to enable a better differentiation between agonist-specific effects and general cytotoxic or apoptotic effects on intracellular Ca^{2+}: the neutral red assay and the apoptosis assay with Hoechst 33342.

Evidence could be provided that ecotoxicologically relevant substances take influence on intracellular Ca^{2+}. Namely hydrogen peroxide and 4-nitrophenol showed a clear response and produced marked Ca^{2+} oscillations at sublethal concentrations. Effect intensity and threshold varied from cell to cell; however, general effects were reproducible and dose-dependent.

For all test toxicants, one apparently unspecific phenomenon in intracellular Ca^{2+} signaling could be observed in individual RTL-W1 cells when exposed to higher concentrations and for longer times: Regular oscillations ceased, and intracellular Ca^{2+} seemed to increase in an uncoordinated manner and stayed at an elevated level. The respective concentrations showed no significant effect neither in the neutral red nor the apoptosis assay for the exposure times selected for Ca^{2+} imaging (approx. 30 min).

In literature, however, such an abrupt rise of intracellular Ca^{2+} has often been related to events preceding controlled cell death. Breakdown of intracellular Ca^{2+} homeostasis seems to trigger reactions ultimately leading to cell death, including perturbation of physiological ion homeostasis, protein and lipid destruction, DNA fragmentation and loss of plasma membrane integrity as evident from the formation of cell surface alterations (blebbing). Especially the disturbance of the mitochondrial membrane potential as a consequence of many toxic substances (e.g., 4-nitrophenol) might compromise cell viability. So far, it is difficult to relate clearly a definite Ca^{2+} signal and its cellular response and even more to differentiate between apoptosis and necrosis only considering Ca^{2+} signaling (cf. discussion of chapter 4). The present study shows that apoptosis as a result of disturbance of intracellular Ca^{2+} can be detected very early before effects can be observed at the level of DNA or membrane integrity.

Notwithstanding the good sensitivity, specificity, reproducibility and dose-dependency, Ca^{2+} imaging is far from being a standard test in ecotoxicity testing and requires further standardization. In addition, research is necessary especially in the mechanistic field: the possibility of an interpretation of the Ca^{2+} signal and decoding of the information encoded previously by the cell is a basic prerequisite for the application of Ca^{2+} imaging in ecotoxicology. However, in contrast to the mechanisms of intracellular Ca^{2+} signal-encoding, which are fairly well understood, much less is known about the cellular targets and the interpretation of the Ca^{2+} signal, i.e. its specificity and decoding. To answer the question of how the Ca^{2+} signal is readout by the cell and can be interpreted by the scientist, further research at the subcellular level is necessary, e.g. into the precise site and manner of Ca^{2+} signal generation. We suggest to test longer exposure times and to combine Ca^{2+} measurements and toxicity tests in the same test run in order to clearly relate the Ca^{2+} signal detected in one individual cell and the long-term impacts on cell viability. So far, we can only relate Ca^{2+} signaling and viability tests statistically, since the assays are performed in two separate test approaches using different cells. Further toxicity testing at the subcellular level (e.g., EROD induction) performed in parallel to Ca^{2+} measurements could provide information about biochemical pathways in the cell induced by Ca^{2+} signaling

Another difficulty for the application of Ca^{2+} signaling in ecotoxicology results from its high sensitivity: The present thesis shows that apoptosis as a result of disturbance of intracellular Ca^{2+} can be detected long before effects are observable at the level of DNA or membrane integrity. The ecological relevance of these results for higher levels, however, must supposed to be little, as is usually the case for a lower tier endpoint. Nevertheless, Ca^{2+} imaging provides striking evidence that apparently untoxic concentrations of a chemical or a mixture of various substances, e.g. in an environmental sample, might influence cell metabolism and stability, even more when different stressors act in an additive or synergistic manner. Additionally, Ca^{2+} imaging might be a useful additional tool in ecotoxicology providing information about the signaling pathway of a certain substance.

4 Conclusions

In the present thesis, the reaction of intracellular Ca^{2+} to different agonist stimuli in primary hepatocytes from rainbow trout and in the permanent fish cell line RTL-W1 was investigated systematically in order to find out the basic principles of Ca^{2+} dynamics in teleost fish cells.

Culture conditions for primary hepatocytes could be optimized and yielded good viability of the cells. Nevertheless, the question arises whether “black boxes” in the culture medium like, e.g., FCS, antibiotics and poly-L-lysine used as a culture substrate, might modify cell physiology and alter Ca^{2+} signaling in an unpredictable manner. It would be preferable to further optimize the culture conditions for primary cells in order to get more robust cells without the necessity to use these substances.

The established Ca^{2+} imaging protocol allowed detection of Ca^{2+} signals and time lapse acquisition in both cell types. At least for RTL-W1 cells, the imaging conditions proofed to be optimal: The signal-to-noise ratio was tolerable, signal intensity remained already constant during acquisition time (up to two hours), and results were clear and reproducible. In contrast, it cannot be ruled out that the imaging protocol established in this study was not totally adequate for the – maybe especially sensitive – primary hepatocytes, in spite of its good suitability for the cell line and the broad application of similar protocols for many other cell types. For this reason, an adaptation of the protocol should be attempted in order to improve the imaging conditions for primary hepatocytes to obtain more differentiated results if possible.

To overcome the limitations presented by primary hepatocytes and complete our investigations on intracellular Ca^{2+} signaling in fish, the cell line RTL-W1 was selected. Despite its uncertain origin data from the cell line are supposed to supplement data from primary cells and to compensate their lacks. In the long term, however, it would be preferable to establish a Ca^{2+} imaging procedure applicable to primary hepatocytes in order to get better transferability of the data to the conditions *in vivo*.

Experimental and bioinformatical results substituted by literature and database analyses provide information about signal transduction pathways in fish compared to mammals: In general, the essential players of signal transduction do not differ very much between piscine and mammalian cells and Ca^{2+} pathways seem to be conserved well during vertebrate evolution. All agonists tested can modulate Ca^{2+} in both primary hepatocytes and the permanent cell line. Evidence is provided that there are purinergic and adrenergic modulations of intracellular Ca^{2+} and that Ca^{2+} plays a role in the response to toxicants in the examined fish cells. At least in RTL-W1 cells, several agonists can induce Ca^{2+} oscillations. Interestingly, increasing concentrations provoked an increase in the amplitude of Ca^{2+} oscillations in the cell line. Findings suggest that amplitude-encoding could be a phenomenon typical for fish cells, thus making the Ca^{2+} response different between classes of vertebrates.

In the present study, in addition to “classical” agonists, model environmental toxicants were used to elucidate possible interactions between toxic effects and Ca^{2+} signaling. Striking evidence is provided that concentrations far in the sublethal range might influence cell metabolism and stability, even more when different stressors act in an additive or synergistic manner. Therefore, Ca^{2+} imaging might be a useful additional tool in ecotoxicology providing information about the signaling pathway of a certain substance. Nevertheless, notwithstanding the good sensitivity, specificity, reproducibility and dose-dependency, Ca^{2+} imaging requires further standardization before its application in ecotoxicology testing becomes realistic. Furthermore, it is difficult to clearly relate a definite Ca^{2+} signal and its cellular response and even more to differentiate between apoptosis and necrosis only considering Ca^{2+} signaling.

Based on the results of this study, new research perspectives emerge: We suggest to test longer exposure times and to combine Ca^{2+} measurements and toxicity tests in order to clearly relate the Ca^{2+} signal detected in one individual cell and the long-term impacts on cell viability. Further toxicity testing at the subcellular level (e.g., EROD induction) could provide information about biochemical pathways induced by Ca^{2+} signaling.

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Presentations

Seitz, N. et al. (2009). Calcium-Imaging in Fischhepatocyten. Annual meeting SETAC GLB in Weihenstephan, Germany.

Hollert, H. et al. (2008): Assessing sediments and fish health using a weight-of-evidence approach – in search for the causes of fish decline in the Danube river. Invited oral presentation, Bundesanstalt für Gewässerkunde.

Seitz, N. (2007): Der ökologische Zustand der oberen Donau – Eine integrierte Bewertung auf der Grundlage des Makrozoobenthos und Sedimentkontakttests mit *Danio rerio*. Invited oral presentation, Annual meeting SETAC GLB in Leipzig (Nachwuchsförderpreis für die beste Diplomarbeit 2006).

Grund, S. et al. (2007): Integrative Sedimentbewertung der Donau mit Hilfe von EROD-Assay, Histopathologie und Makrozoobenthos-Arterhebung. Invited oral presentation, Annual meeting SETAC GLB in Leipzig, Germany.

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Poster proceedings

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Eidesstattliche Erklärung

Hiermit erkläre ich, Nadja Schweizer, geb. Seitz, geboren am 20.12.1978 in Bad Schwalbach, Deutschland, an Eides statt, dass ich die vorliegende Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Ich, Nadja Schweizer, geb. Seitz, geboren am 20.12.1978 in Bad Schwalbach, Deutschland, erkläre zudem an Eides statt, dass ich an keiner anderen Stelle ein Prüfungsverfahren beantragt habe, dass ich die Dissertation nicht in dieser oder anderer Form bereits anderweitig als Prüfungsarbeit verwendet habe und dass ich sie an keiner anderen Fakultät als Dissertation vorgelegt habe.

Heidelberg, den 10.11.2011

Nadja Schweizer