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Amplitude-encoded calcium oscillations in fish cells

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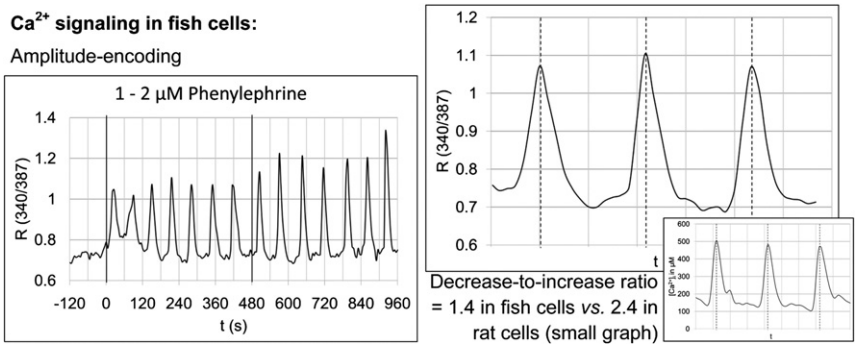
HIGHLIGHTS

► 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.

GRAPHICAL ABSTRACT



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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.

1. Introduction

Calcium plays a fundamental role in many cellular processes in all types of tissues and organisms (for reviews see e.g. [1–4]). 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 [5]. In liver, it regulates many hepatic functions, including glycogenolysis, canalicular contraction, tight junction permeability, and bile secretion [6]. 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. [7–10]).

The versatility of Ca^{2+} signaling is remarkable and is due to the complex mechanisms which help to encode information. Numerous

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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 [11]. 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 [12,13], a great number of experimental and theoretical studies have been carried out [14].

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 [15–18] and purinergic [19] modulation of the intracellular Ca^{2+} level as well as its reaction to angiotensin [20]. 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 [19]. In trout pinealocytes, spontaneous [21,22] as well as Bay K 8644-induced [23] Ca^{2+} oscillations have been observed. Zhang et al. reported catecholamine-induced Ca^{2+} oscillations in eel, but not in trout hepatocytes [18]. 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 [24–26].

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 [27]. 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 [28–31]. 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 [32] 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 [33] and 3,4-dichloroaniline [34] 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 behavior, 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 (*O. 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 libitum* 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 [35] 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 [32] 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 2×10^5cells/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., [18,21,22,36–40]). 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 $CaCl_2\times2\,H_2O$, 1 mM $MgCl_2\times6\,H_2O$, 10 mM D-glucose, 10 mM HEPES) and were loaded with 6 µM Fura-2 AM solution in Ringer for 1 h. 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 s) at 340 nm and 380 nm with a metal halide lamp (Nikon Intensilight). The emitted fluorescence was detected through a 510/30 nm 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 [41]. Sequence alignment was performed using the BLAST implementation therein with default parameters. Predictions of posttranslational modifications were computed using the ELM resource [42]. For the prediction of transmembrane helices we used the TMHMM server [43].

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 [44].

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.

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).

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 (Table 1). 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. 3a), phenylephrine (Fig. 3b), hydrogen peroxide (Fig. 3c, d), and 4-nitrophenol (data not shown). They were also observed in some cells after addition of histamine (Fig. 3e); 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.

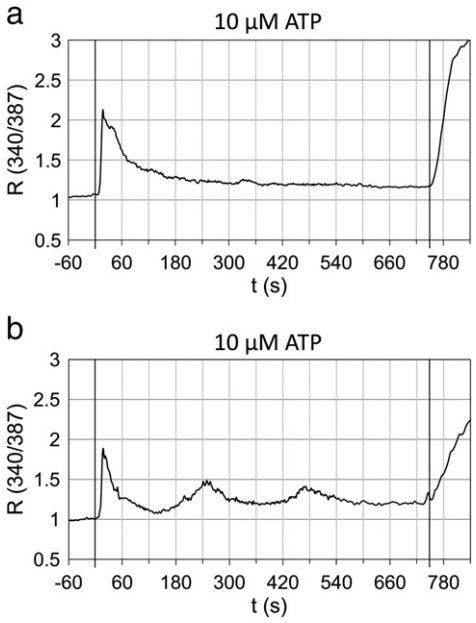

Fig. 1. Alterations of intracellular free Ca^{2+} levels in individual primary rainbow trout hepatocytes after addition of various agonists (first vertical line) exemplified for ATP (10 μM). a. Intracellular free Ca^{2+} rose almost immediately after ATP addition and declined thereafter towards a slightly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

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

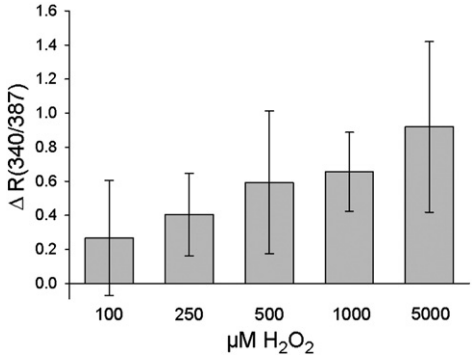

Fig. 2. Difference in the R(340/387) quotient (median ± SD) as a measure for intracellular free Ca^{2+} in primary rainbow trout hepatocytes after addition of different concentrations of hydrogen peroxide. Data from all cells (27–31 per concentration) of one hepatocyte preparation were integrated.

Table 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%).

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. 3d, were observed in some cells for all agonists and seemed to increase in number with higher concentrations.

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; Fig. 3c, d), which sometimes may conceal agonist-specificity.

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 [45], 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 [45], causes simpler oscillations in the fish cells.

In order to find out potential reasons for these different behaviors, 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 [41] 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 Table 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.

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 [46]. 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 Table 3.

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

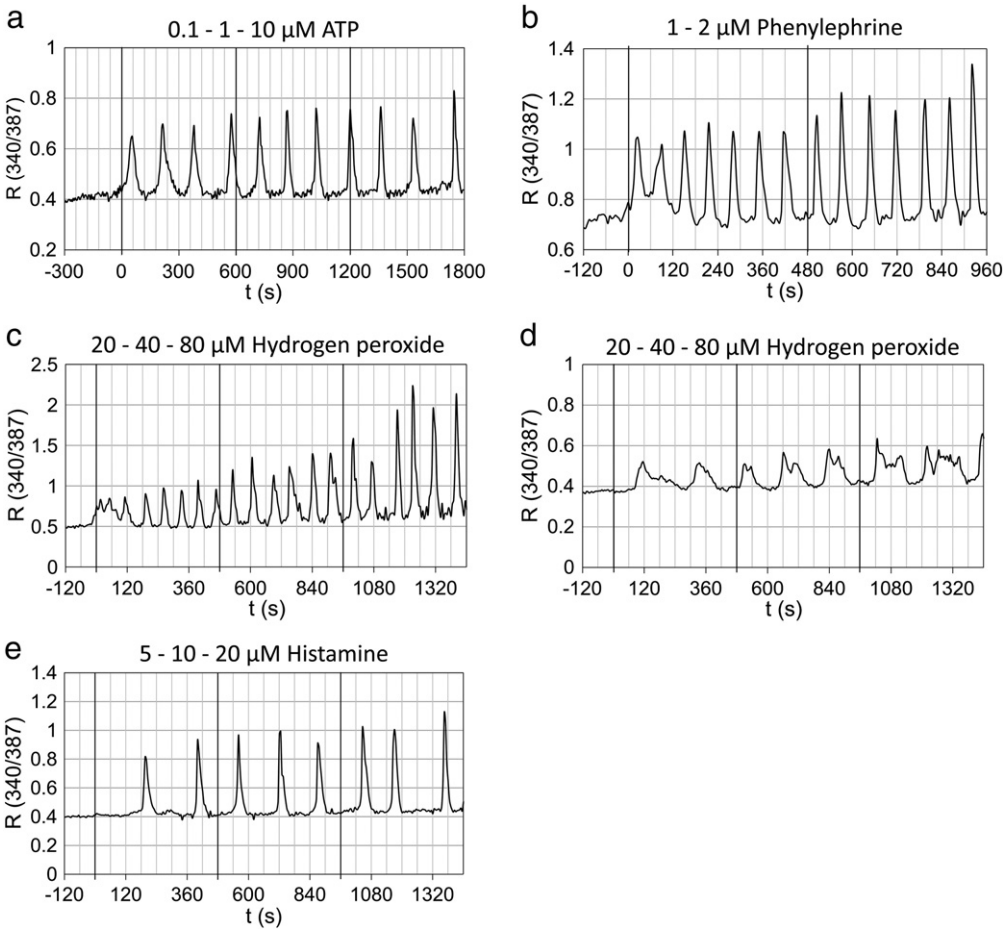

Fig. 3. a–e: Oscillations in the intracellular Ca^{2+} levels in individual RTL-W1 cells after addition of various agonists in different consecutive concentrations (vertical lines). Note the different scaling of the y-axis. a: ATP. b: Phenylephrine. c, d: Hydrogen peroxide: Oscillations mostly displayed 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.

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 [47]. It is given by the following equations:

$$\frac{dG_\alpha}{dt} = k_1 + k_2 * G_\alpha - \frac{k_3 * G_\alpha * PLC}{G_{\alpha\,+}\, K_4} - \frac{K_5 * C\alpha_{cyt} * G_\alpha}{G_\alpha + K_6} \qquad (X.1)$$

Table 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%

Table 3
Prediction of regulatory sides in the cytosolic residues of purinergic receptors. The cytosolic residues were predicted with the TMHMM server [43]. 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	PK, PKA
	221–245	

$$\frac{dPLC}{dt} = k_7 * G\alpha - \frac{k_8 * PLC}{PLC + K_9} \tag{X.2}$$

$$\frac{dC\alpha_{cyt}}{dt} = \left(Ca_{ER} - Ca_{cyt}\right) * \frac{k_{10} * Ca_{cyt} * PLC}{PLC + K_{11}} + k_{12} * PLC + k_{13} * G_a - \frac{k_{14} * Ca_{cyt}}{Ca_{cyt} + K_{15}} - \frac{k_{16} * Ca_{cyt}}{Ca_{cyt} + K_{17}} - \frac{k_{18} * Ca_{cyt}^8}{Ca_{cyt}^8 + K_{19}^8} + \left(Ca_{mit} - Ca_{cyt}\right) * \frac{k_{20} * Ca_{cyt}}{Ca_{cyt} + K_{21}} \tag{X.3}$$

$$\frac{dCa_{ER}}{dt} = -\left(Ca_{ER} - Ca_{cyt}\right) * \frac{k_{10} * Ca_{cyt} * PLC}{PLC + K_{11}} + \frac{k_{16} * Ca_{cyt}}{Ca_{cyt} + K_{17}} \tag{X.4}$$

$$\frac{dCa_{mit}}{dt} = \frac{k^{18} * Ca_{cyt}^8}{Ca_{cyt}^8 + K_{19}^8} - \left(Ca_{mit} - Ca_{cyt}\right) * \frac{k_{20} * Ca_{cyt}}{Ca_{cyt} + K_{21}}. \tag{X.5}$$

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 et al. [47].

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.

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.

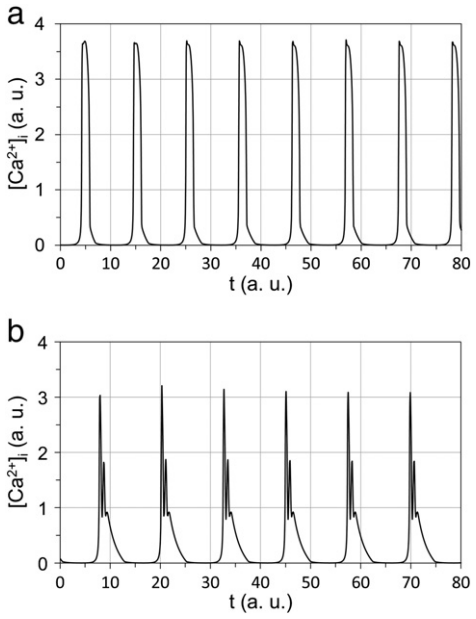

Fig. 4. a, b: Simulations of the model Eqs. (X.1)–(X.5) only differing in the degree of feedback on the receptor. Initial concentrations: a = 0.01, b = 0.01, c = 0.01, d = 10, e = 0.001. Reaction parameters: $k_1 = 0.01$, $k_2 = 2.218$, $k_3 = 0.64$, $K_4 = 0.09$, $K_6 = 1.18$, $k_7 = 2.08$, $k_8 = 32.24$,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

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. [14], 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 [46]), 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; $n = 8$).

Recently, De Pittà et al. [48] investigated a model of Li and Rinzel [49] 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) [50] is a sort of minimalistic model of CICR -induced oscillations and is described by the following equations:

$$\frac{dZ}{dt} = v_0 + v_1 * \beta - v_2 + v_3 + k_f * Y - k * Z \tag{X.6}$$

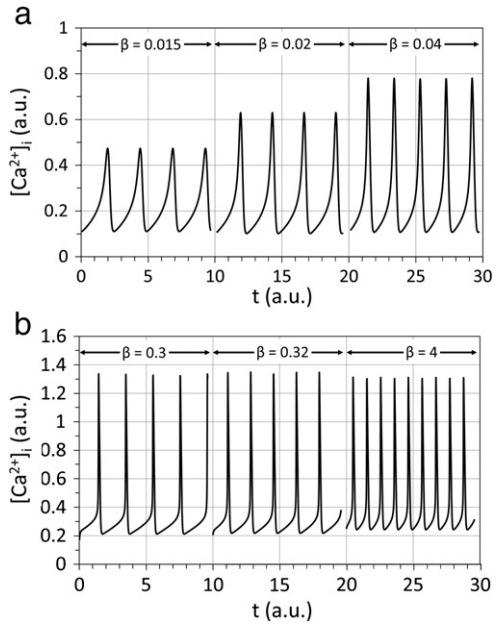

Fig. 5. a, b : Comparison of individual oscillations in rat hepatocytes (taken from [46]) and fish RTL-W1 cells. The oscillations in fish are exhibiting stronger symmetry between uptake and release rates during calcium peaks. One unit on the time scale equates to 10 s in rat or 30 s in RTL-W1, respectively.

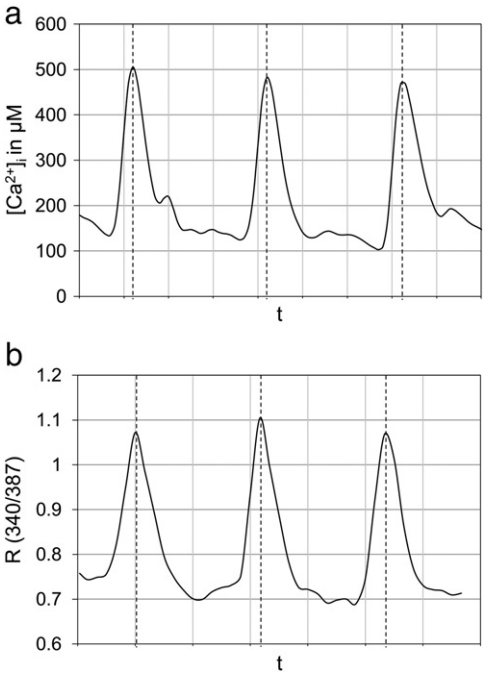

Fig. 6. a, b: Simulation of the model described by Eqs. (X.6)–(X.9). Parameters as in the original publication [50]. In a, following parameters were set to: n = 1.1, m = 1, p = 1.7, k = 5.03, $K_A = 2.2$, $V_{m2} = 34.26$, β as given in the figure.

$$\frac{dY}{dt} = v_2 - v_3 - k_f * Y \tag{X.7}$$

Here, v_0 and k Z represent influx and efflux of Ca^{2+} into and out of the cell and v_1 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:

$$v_2 = V_{M2} * \frac{Z^n}{K_2^n + Z^n} \tag{X.8}$$

$$v_3 = V_{M3} * \frac{Y^m}{K_R^m + Y^m} * \frac{Z^p}{K_A^p + Z^p} \tag{X.9}$$

The model was downloaded from the BioModels Database [51]. 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.

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 and Mariwalla, who found that skate hepatocytes displayed oscillations only when stimulated with lower concentrations of ATP (10–100 nM) [19]. 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 [45].

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 [52] and reflects the poor metabolic response of these cells to phenylephrine found by Moon and Mommsen [53]. Nevertheless, Fabbri et al. 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 [54].

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 [55]. 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. found a dose-dependent epinephrine effect on the maximal amplitude of Ca^{2+} oscillations in eel hepatocytes [18]. 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. [35]). 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 [35]. 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 [56,57]. 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. also found that rainbow trout hepatocytes, contrary to eel and bullhead cells, demonstrated little epinephrine sensitivity, with less than 20% of the cells responding [18]. 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 [56].

The main disadvantage of RTL-W1 is its uncertain origin. The permanent epithelial cell line RTL-W1 was established by Lee et al. by Dispase treatment of liver fragments of a 4-year-old male rainbow trout. Single cells were grown in monolayers and passaged continuously [32]. 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 [32], aromatic hydrocarbons [58,59], various pesticides [60], benzo[a]pyrene [61]) and environmental samples [59,61–63] 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 [58], which makes them useful as a tool for assessing the toxic potency of environmental contaminants.

However, Lee et al. could not definitely identify which among the many cell types in the rainbow trout liver gave rise to the cell line [32]. 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 ([64–66]; 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 [66]. 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.

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