Optimized conditions for primary culture of pituitary cells from the Atlantic cod (Gadus morhua). The importance of osmolality, pCO₂, and pH

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A B S T R A C T

Protocols for primary cultures of teleost cells are commonly only moderately adjusted from similar protocols for mammalian cells, the main adjustment often being of temperature. Because aquatic habitats are in general colder than mammalian body temperatures and teleosts have gills in direct contact with water, pH and buffer capacity of blood and extracellular fluid are different in fish and mammals. Plasma osmolality is generally higher in marine teleosts than in mammals. Using Atlantic cod (Gadus morhua) as a model, we have optimized these physiological parameters to maintain primary pituitary cells in culture for an extended period without losing key properties. L-15 medium with adjusted osmolality, adapted to low pCO₂ (3.8 mm Hg) and temperature (12 °C), and with pH 7.85, maintained the cells in a physiologically sounder state than traditional culture medium, significantly improving cell viability compared to the initial protocol. In the optimized culture medium, resting membrane potential and response to releasing hormone were stable for at least two weeks, and the proportion of cells firing action potentials during spawning season was about seven times higher than in the original culture medium. The cells were moderately more viable when the modified medium was supplemented with newborn calf serum or artificial serum substitute. Compared to serum-free L-15 medium, expression of key genes (lhb, fshb, and gnrhr2a) was better maintained in medium containing SSR, whereas NCS tended to decrease the expression level. Although serum-free medium is adequate for many applications, serum supplement may be preferable for experiments dependent on membrane integrity.

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1. Introduction

In many in vitro studies of animal cells in primary culture, it is tacitly assumed that cultured, viable cells have properties similar to those of intact cells in vivo. However, important functional properties of the cells may be lost during dispersion of the cells and their subsequent maintenance in culture, although the cells may stay viable for an extended period. For example, the contractility of dissociated smooth muscle cells [7] and cardiomyocytes [23,43] kept under standard culture conditions may decay within a couple of days, which may be only a fraction of the time the cells stay “healthy” according to common viability tests. However, moderate adjustments of standard culture procedures and media are often sufficient to greatly improve the culture conditions for a specific cell type. Accordingly, a vast literature describes customized culture protocols for a large number of mammalian cell types (for reviews and protocols, see [2,15]). In comparison, specialized methods for culturing cells from exothermic vertebrates, i.e., fish, amphibians and reptiles, are rather few.

When we recently commenced an investigation of the functional properties of pituitary cells from the teleost Atlantic cod (Gadus morhua), we realized that the most commonly used procedures for establishing primary cultures of teleost cells were moderately adjusted protocols for mammalian cells. The main difference between most culture procedures for teleosts and mammals is the temperature, whereas important parameters like osmolality, pCO₂, pH, and serum supplement are usually similar. Still, scientists employing such methods for studying teleost cells in primary culture seem to achieve reasonable results. Therefore, in our initial work on cod pituitary cells [16], we used a slightly adjusted version of an established protocol for dissociation and incubation of pituitary cells from the European eel (Anguilla anguilla) [24]. The main modification of the culture procedure used for Atlantic cod, which is usually adapted to an ambient temperature below about 12 °C, was a reduction in temperature both during dissociation and incubation of the cells.

However, using these primary cultures in electrophysiological experiments, we realized that the culture conditions were suboptimal, and did thus start systematic trials in an attempt to optimize...
the different elements of the culture procedure. We aimed at designing a primary cell culture protocol that fulfilled the following criteria:

- For at least two weeks, the cells should be viable and in addition show all the properties listed below.
- The resting cell membrane potential should stay within the range for pituitary cells reported in other teleost species, for the whole time period.
- At least a subgroup of the cells should be able to generate action potentials, either spontaneously or triggered by current injections.
- The cells should continue to express key genes essential for the endocrine function of the pituitary.

It was an obvious criterion that the cells should stay viable for the required period. Furthermore, a sound negative resting membrane potential and action potential firing require high membrane integrity and consume energy, and do thus indicate healthy cells. Action potentials are important for regulation of secretion in mammalian pituitary cells, for review see [37,38]. Less is known about the role of action potentials in teleost pituitary cells [16,19,42,48], but voltage-activated Ca2+ channels are shown to be involved in regulation of secretion in several species, for reviews see [10,11,20,47]. Finally, genes linked to the functional properties under investigation must of course be expressed under the selected culture conditions. We are currently studying the regulation of sexual maturation and spawning in the Atlantic cod, and are focusing on the gonadotropin releasing hormone (GnRH)-induced production and secretion of gonadotropins, i.e., follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the pituitary gonadotrope cells. Consequently, the last criterion was that cultured cells should continue to express genes for the FSH β-subunit (fshb), the LH β-subunit (lhb), and GnRH receptor 2a (gnrhr2a), and respond to GnRH by a transient elevation of cytosolic free Ca2+ similar to in mammals [38] and goldfish [10].

In our effort to optimize the culture procedures, we focused on the following parameters, which are likely to be rather different in a cod pituitary in vivo compared to the standard mammalian culture conditions: temperature, osmolality, CO2 partial pressure (pCO2) and pH. By optimizing these parameters, we have designed a primary cell culture protocol yielding cod pituitary cells that allow studies of essential, functional properties for at least two weeks.

2. Materials and methods

2.1. Animals

Atlantic cod (1–3 kg) were captured in the Oslo fjord and kept in the aquarium facilities at the University of Oslo for a maximum of 2 weeks before being sacrificed. They were fed shrimps while in captivity. The aquaria were continuously perfused with seawater with salinity of 28% and a temperature of 7–8 °C. The light cycle was adjusted to fit the normal night/day cycle in Oslo. Both male and female fish were used in the study.

2.2. Initial procedure for primary culture of pituitary cells from the Atlantic cod

The initial procedure for preparing primary pituitary cultures from the cod [16] was based on established methods for mammalian cells, and similar to a procedure used successfully for the European eel [24]. Pituitaries were removed immediately after decapitation and placed in M199 medium (Invitrogen, Carlsbad, USA) on ice. The pituitaries were then washed with ice-cold phosphate-buffered saline (PBS; Invitrogen), chopped in approximately 1 mm3 pieces with a scalpel and washed again. The tissue fragments were treated with trypsin (type II-S, 1 mg/ml PBS) for 45 min in a shaker water bath at 18 °C. The trypsin solution was then replaced with PBS containing trypsin inhibitor (type I-S, 1 mg/ml) supplemented with approximately 1 μg/ml DNasel, before the tissue was incubated for another 20 min in the water bath. Subsequently, the tissue fragments were mechanically dissociated in ice-cold PBS by using a plastic pipette. The cell suspension was filtered through a nylon mesh and centrifuged for 10 min at 100 g (4 °C). Cells were then resuspended in M199 medium supplemented with newborn calf serum (NCS; 5%) and seeded at a density of 1.5 × 105 cells/cm2 in 35 mm plastic dishes or 24/96-well plates (Corning, Amsterdam, Netherland) coated with poly-l-lysine. The cells were kept at 12 °C in a humidified atmosphere of 5% CO2 in air (pCO2; 38 mm Hg) until start of experiments. The culture medium M199 contains 26.2 mM HCO3−, which provides a pH of approximately 7.4 in an atmosphere with 5% CO2, and the pH of the PBS used in the first steps of the procedure, was also adjusted to 7.4. The osmolality of the PBS and the M199 medium was 270–280 mOsm. Apart from the culture medium and PBS, all chemicals were purchased from Sigma (St. Louis, MO, USA).

2.3. Modifications of the protocol for primary culture of pituitary cells from the Atlantic cod

In our attempt to develop an improved procedure for making primary culture of pituitary cells from the Atlantic cod, we tested the effects of the following adjustments of the initial protocol:

The culture medium M199, which contains 26.2 mM HCO3−, was replaced with the medium L-15 (Life Technologies (Invitrogen), Paisley, UK) added 1.8 mM (α+)-glucose. This medium does not include a pH buffer, and was supplemented with 10 mM NaHCO3. When using the L-15 medium modified in this manner, the CO2 content of the incubator atmosphere was lowered to 0.5%, corresponding to a pCO2 of 3.8 mm Hg, which resulted in a pH of 7.85 in the medium at 12 °C.

The different L-15-based media tested in the present study were further supplemented with either NCS (5%), the artificial serum substitute SSR (5%) [4], or the medium was completely serum-free. Subsequent to these additions, the osmolality was adjusted to 320 mOsm with NaCl. Finally, the medium was Millipore-filtered (0.2 μm) before added Pen-Strep (20 U/ml, Lonza, Verviers, Belgium). The pH of the PBS was adjusted to 7.85 at 12 °C with NaOH, and the osmolality was increased to 320 mOsm with NaCl. The saline was then Millipore-filtered (0.2 μm) before added Pen-Strep (40 U/ml).

2.4. Solutions

The electrophysiological recordings were performed in the following standard extracellular solution (mM): 150 NaCl, 5 KCl, 2.4 CaCl2, 1.3 MgCl2, 1.8 glucose, 10 HEPES/NaOH, pH 7.85, 320 mOsm. The patch electrodes were filled with the following standard intracellular solution (mM): 120 CH3SO3K, 20 KCl, 10 HEPES/NaOH, 20 sucrose, pH 7.2, 290 mOsm.

2.5. Electrophysiology

The patch-clamp recordings were carried out at 12 °C, using a peltier element for temperature control (TC-10, Dagan Corporation, Minneapolis, MN, USA), as described by Haug et al. [16] and Hodne et al. [17]. All experiments were performed with the perforated patch configuration, which prevents loss of organic molecules from the cytosol. Perforation was achieved using β-escin, following the
procedure described by Sarantopoulos et al. [33]. In brief, β-escin from a stock solution (25 mM) was added to the pipette solution (50 μM), which was then vortexed for 1 min. The stock solution was kept at −20 °C for up to two weeks, whereas the pipette solution was prepared daily. Both the stock solution and the final pipette solution were protected from direct light by aluminum foil. The patch electrodes were made from borosilicate glass with filament, and the electrode resistance was 2–5 MΩ. The electrodes were connected to an EPC-9 patch-clamp amplifier controlled by the software PULSE (HEKA Elektronik, Lambrecht, Germany). The recorded signals were digitized at 4–10 kHz, filtered at 1/3 of the sampling rate, and stored on a computer. Data analysis was performed using PULS-FIT (HEKA) and Origin (OriginLab, Massachusetts, USA).

2.6. Number of surviving cells and cell viability

The cells were seeded at a density of 1.5 × 10⁵ cells/cm² in 96-well plates, corresponding to approximately 50,000 cells per well, and incubated in either serum-free L-15 medium, L-15 medium supplemented with 5% NCS, or L-15 medium with 5% SSR. The absolute number of remaining cells per well at a given time was assessed by counting the cells in a haemocytometer. For each well, the cell suspension for counting was prepared by removing the medium and washing the well with 1 ml PBS, before replacing the PBS with 200 μl 0.25% Trypsin–EDTA (Gibco, Invitrogen). The enzyme solution was then removed after 40 s, followed by 3 min incubation to allow the remaining trypsin to complete detachment of the cells. Finally, 1 ml PBS was added and the cell suspension was collected after stirring with a pipette.

To gain information about the condition of the cells, viability tests using two fluorescent indicator dyes, i.e., AlamarBlue (AB) (Invitrogen) and 5-carboxyfluorescein diacetate–actetoxymethyl ester (CFDA-AM) (Invitrogen), were performed. These tests indicate metabolic activity and membrane integrity, respectively [6]. The AB assay measures the conversion of the non-fluorescent dye resazurin into the fluorescent resorufin by the action of mitochondrial and other enzymes. In living cells able to maintain a cytoplasmic milieu that allows esterase function, non-fluorescent CFDA-AM is converted by non-specific esterases to the fluorescent carboxyfluorescein (CF). The test procedures were based on the original report by Scheer et al. [35] and in accordance with the modifications described by Tollefsen et al. [41]. Cells were seeded in 96-well plates and incubated as described above. At day 7, the culture medium in the wells was replaced with 100 μl Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 μM CFDA-AM (from 4 mM stock in DMSO). The plates were then incubated in the dark on a shaker (85 rpm) for 30 min at room temperature, before the concentration of the fluorescence products was measured simultaneously for both probes with a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., USA). The software Gen5 was used for data collection (Gen5 Data Analysis Software, Bio-Tek Instruments Inc., USA). The employed excitation–emission wavelength pair was 530–590 nm for AB and 485–530 nm for CFDA. The fluorescence signal from each well was corrected for background fluorescence by subtracting the signal from wells without cells, and the adjusted values were expressed as percentage of the values for serum-free medium. As a positive control for cell toxicity, cells were incubated in serum-free medium with 2.6 mM CuSO₄ for 24 h from day 6 in culture. At this concentration, Cu²⁺ is lethal to the cells [41]. All experimental groups were in replicates of five wells and the whole experiment was repeated three times.

2.7. RNA extraction and qPCR

Cod pituitary cells seeded in 24 well plates at a density of approximately 300,000 cells per well were lysed in the wells by removing the culture medium, washing the wells with 300 μl ice-cold PBS, before adding 300 μl of Trizol (Invitrogen). After stirring with a pipette, the cell lysate was instantly frozen on liquid N₂ and stored at −80 °C. In order to extract total RNA, 700 μl Trizol was added to the lysate from each well, and total RNA was extracted following standard procedures and resuspended in 10 μl water (Ambion, Life Technologies, Carlsbad, USA). To remove genomic DNA, the RNA was DNase-treated using TURBO DNase-free (Ambion) according to the manufacturer’s protocol. The quantity of the extracted RNA was determined spectrophotometrically (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the quality was assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies, USA) of the RNA Integrity Number (RIN). Only RNA samples with RIN number above eight were analyzed further.

qPCR analyses were carried out using the LightCycler 480 platform (Roche, Switzerland), as previously described [17,45]. First strand cDNA synthesis was performed on 1 μg total RNA using random hexamers, dNTP mix, and Super Script III reverse transcriptase (all from Invitrogen). The cDNA was stored at −20 °C until qPCR. Each PCR reaction mixture contained Taq DNA polymerase, SYBR green I detection dye, buffer, gene specific primers, and diluted (1:10) cDNA. The qPCR primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Potential primers were further analyzed using Vector NTI (Invitrogen) to test for possible self-annealing and primer dimer formations (see Table 1 for sequence details). In each pair, one primer was targeted to an exon-exon border to avoid amplification of genomic DNA. The primers were synthesized by MWG-Biotech AG (Ebersberg, Germany), diluted to 1 mM with nuclease-free water (Ambion) upon arrival, and stored at −20 °C. From the stock solution, working dilutions of 5 μM were prepared. All samples were run in duplicate, and in every round two non-template negative control (NTC) reactions were conducted for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). In addition, to account for plate-to-plate variation, a standard positive calibrator in duplicate was included on every plate. The qPCR reactions were carried out using an initial step for 10 min at 95 °C to activate Taq polymerase, followed by 45 cycles consisting of 10 s at 95 °C, 10 s at 60 °C, and elongation at 72 °C for 6 s. The fluorescence was measured at the end of each cycle (after each elongation) and used for determining the quantification cycle values (Cq). A melting curve analysis was performed directly following the PCR by continuously reading the fluorescence while slowly heating the reaction mixture from 65 to 98 °C.

The relationship between increasingly diluted cDNA starting material and the corresponding Ct was used for making cDNA dilution curves. After running the qPCR, the Cq was plotted against the logarithm of the relative concentration of the cDNA starting material. The efficiency (E) of the qPCR assay is described by the slope of the regression line E = 10⁻¹/Slope. If the slope of the dilution curve is −3.32, the efficiency equals two, meaning that for each PCR cycle there is a doubling of product. The LC480 software calculates the efficiency directly, which was employed together with the dilution curve and melting curve analyses for optimizing the conditions for the various qPCR assays with regards to primers, elongation time, and annealing temperature.

In the present study, we investigated the expression of three different genes specifically related to pituitary function, i.e., shh (GenBank ID: DQ402373), lhb (GenBank ID: DQ402374), and gnrh2a (GenBank ID: GU332298.1). To allow accurate normalization of the qPCR, we also tested the stability of three reference genes, bactin, arp2/3, and ubiquitin using Bestkeeper Software [29]. The sequences of these genes were obtained by search in the recently sequenced Atlantic cod genome (http://wwwensembl.org/Gadus_morhua/Info/Index). All reference genes were stably expressed with a low standard deviation SD. However, ubiquitin...
proven most stable (SD = 0.35), while the SD of bactin and arp2/3 were 0.53 and 0.42, respectively. Thus, ubiquitin was used for normalizing the qPCR data. The relative expression levels were determined using an efficiency-corrected method [28]:

Relative expression = \frac{E^{C_q(\text{calibrator} – \text{sample})}}{E^{C_q(\text{sample} – \text{calibrator})}} (1)

### 2.8. Ca^{2+} imaging

For Ca^{2+} imaging, the cells were plated in dishes fitted with a central glass bottom and coated with poly-l-lysine. For improved attachment of the cells on the glass bottom, 5% NCS was added to the culture medium. Prior to experiments, the cells were incubated with 5 µM fura-2 AM (Life Technologies, Invitrogen, Carlsbad, CA, USA) in standard extracellular solution for 60 min at 12 °C, followed by washout of the fura-2 ester, and further 30 min incubation. The dish was then mounted on an Olympus IX71 inverted microscope with objectives of high light transmission (Olympus, Tokyo, Japan) for imaging. A Lambda 10-2 filter wheel (Sutter, CA, USA) switched the excitation light from a Lambda LS Xenon Arc Lamp (Sutter). Exposure times at the wavelengths were between 250 and 350 ms for 340 nm, and between 50 and 150 ms for 380 nm, according to the degree of fura-uptake in the cells. The ratio between emissions at these excitation wavelengths (F340/F380) reflects the cytosolic Ca^{2+} concentration ([Ca^{2+}]_i). Emission of fluorescence at 510 nm was recorded using a Hamamatsu ORCA ER camera (Hamamatsu Photonics, Hamamatsu, Japan). The software Imaging Workbench 6 (INDEC Biosystems, Santa Clara, CA, USA) was used for recording and analysis. The digitized data were background-subtracted. In the present study, the relative increase in [Ca^{2+}]_i is used as a measure of response to the GnRH. Therefore, calibration in order to determine the absolute Ca^{2+} concentrations was not performed. Responses were calculated from baseline to peak of the response, and presented as the ratio peak/baseline. In order to stimulate the cells, a mix of 10^{-3} M GnRH 1, 2 and 3 (Bachem, Bubendorf, Switzerland) in extracellular solution was prepared from three individual stock solutions of 10^{-3} M in DMSO. The GnRH solution was applied via a pressure ejection pipette (about 1 kPa) positioned about three cell diameters from the cell. No ejection artifacts were observed from this distance.

### 2.9. Statistics

Numerical data are presented as mean ± SEM if not otherwise stated. The significance of the observed effects was assessed using linear correlation analysis, t-test and one- or two-way ANOVA. A Tukey’s post test was conducted subsequent to one-way ANOVA tests while a Bonferroni multiple comparisons test used following two-way ANOVA. Analysis of the cell count data was performed using Tukey’s test, and the number of remaining cells were tested both as a continuous and a discrete variable with time. The observed values were tested for normality by applying the Shapiro–Wilk test. The differences between the mean fluorescence values obtained in media supplemented with either NCS or SSR and the mean value for serum-free medium were analyzed using the Student’s t-test. In all cases, p-values < 0.05 were regarded as statistically significant. All statistics except for cell viability tests were performed using GraphPad Prism version 5.0d for Mac (GraphPad Software, San Diego, CA, USA). Cell viability data were analyzed using JMP 7 software (SAS Institute Inc, Cary, NC, USA).

### 3. Results

#### 3.1. Condition of cells cultured in traditional medium

The initial protocol for making primary pituitary cell culture from the Atlantic cod was based on the M199 medium and was quite similar to standard procedures for dispersing and maintaining mammalian cells, except for the lower temperature (12 °C) during incubation. As an estimate of the general health of the cells, the resting membrane potential was recorded after 2 and 5 days in culture. The average membrane potential was −37.4 ± 2.2 mV (n = 7) after 2 days and most of the cells were firmly attached to the bottom of the dish. However, after 5 days in culture, the cell density was markedly reduced and the remaining cells were poorly attached to the bottom. The membrane potential of the few cells still possible to record from was −22.4 ± 1.0 mV (n = 5). After 7 days in culture, almost all cells were detached, and inaccessible for electrophysiological experiments.

The osmolarity of the M199 medium is approximately 280 mOsm, whereas the osmolarity of the cod plasma is closer to 320 mOsm. The quality of the cultures was greatly improved by increasing the osmolarity of the incubation medium and all working solutions to 320 mOsm. In the osmolarity-adjusted M199 medium, the resting membrane potential of the cells was recorded at day 1, 2, 4, 5, 7, and 8 in culture (Fig. 1). Compared to the values in the unadjusted medium, the average resting membrane potentials on day 2 and 5 were significantly increased to −43.4 ± 3.1 mV (n = 21) and −41.0 ± 5.1 mV (n = 5), respectively. Furthermore, more cells remained after 5 days in culture. The slope of the regression line showing resting membrane potential as a function of time was significantly different from zero, indicating that resting membrane potential (V_m) still declined with time (X) in culture (F{15.75,4, V_m} = 51.76 + 3.38X, P = 0.0165). In fact, 80% of the variation in resting membrane potential can be explained by the time in culture. These recordings were performed in April, i.e., during the spawning season, and 12% of the recorded cells were able to generate action potentials, either spontaneously or triggered by current injections.

### Table 1

qPCR primers used in the present study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Reference</th>
<th>Primer sequence</th>
<th>Amplicon size (nt)</th>
<th>Efficiency</th>
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<td>lhb</td>
<td>Hodne et al. (2010)</td>
<td>5’-GTGGAGAAGGCGCTGTCCTC-3’</td>
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<tr>
<td>fshb</td>
<td>Hodne et al. (2010)</td>
<td>5’-GAACCGCTCCTCAACAACCC-3’</td>
<td>63</td>
<td>2.02</td>
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<tr>
<td>gnrh2a</td>
<td>Hildahl et al. (2011)</td>
<td>5’-TTCCCCTTCTGGTCTCTC-3’</td>
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</table>
3.2. Condition of cells cultured in modified L15 media

Because the CO₂ and the HCO₃⁻ concentration in blood plasma are very different in mammals and fish, the next obvious step in the attempt to optimize the culture medium was to adjust these parameters to appropriate values for the Atlantic cod. Therefore, the M199 medium, which contains 26.2 mM HCO₃⁻, was replaced with the HCO₃⁻-free medium L-15. Based on the physiological values measured in cod, the L-15 medium was adjusted by adding 10 mM HCO₃⁻ and the pCO₂ in the incubator was reduced to 0.5% (3.80 mm Hg), resulting in a pH of 7.85 in the medium at 12 °C. Finally, 1.8 mM glucose was added and the osmolality adjusted to 320 mOsm. The condition of cells cultured in this serum-free, adjusted L-15 medium was then compared to the properties of cells incubated in the adjusted L-15 medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum substitute (SSR).

3.2.1. Membrane potential

After two days incubation in the serum-free L-15 medium, the average resting membrane potential of the cells was −49.1 ± 1.7 mV (n = 35). The corresponding values for cells incubated in L-15 medium supplemented with NCS or SSR were −44.5 ± 2.4 mV (n = 23) and −48.3 ± 1.9 mV (n = 25), respectively (Fig. 2). The differences between these values are not significant, and none of the values are significantly different from the resting membrane potential of cells incubated for 2 days in the osmolality-adjusted M199 medium. However, whereas the linear correlation analysis shows that the slope of the regression line is significantly different from zero (F_{15.75,4}, P = 0.0165).

3.2.2. Total cell count and cell viability

The total number of cells remaining after 2, 7, and 14 days in culture was used as a coarse estimate of the stability of cultures incubated in the three versions of the L-15 medium (serum-free, 5% NCS, 5% SSR) (Fig. 3A). Double blind counting of the cells was performed to eliminate bias. At day 2 in culture, approximately 50% of the seeded cells were lost, independently of the version of incubation medium. From day 2 until the end of experiment, the total cell number was negatively correlated with time for all media (F_{48.76,4}, P < 0.001). Furthermore, whereas only 12% of the cells cultured in osmolality-adjusted M199 medium during the spawning season in April were excitable, action potentials (either spontaneous or triggered) were recorded from 82% of the cells incubated in the L-15 media during the same season. However, only 12% of the cells cultured in M199 medium had a membrane potential of −51.76 ± 3.317 mV (n = 47) at day 2 and −48.7 ± 1.3 mV (n = 47) at day 12–14. The data are presented as mean ± SEM. There were no statistical differences in membrane potential between days or treatments.

3.2.3. Cell death and cell integrity

An indirect measure of the condition of a primary culture is how long the cells remain sufficiently attached to the dish to allow electrophysiological experiments. Under the initial culture conditions, electrophysiological experiments were possible for 3.8 ± 2.3 days (n = 5). After adjustment of the osmolality of all solutions, the number of experimental days increased significantly to 5.4 ± 2.0 (n = 8), which is an insignificant increase. On the other hand, after changing to L-15 medium and adjusting the HCO₃⁻ concentration, pCO₂, and pH, the average number of experimental days increased significantly to 14.0 ± 4.2 (n = 9) (p < 0.001).

3.2.4. Cell viability

The total number of cells remaining after 2, 7, and 14 days in culture was used as a coarse estimate of the stability of cultures incubated in the three versions of the L-15 medium (serum-free, 5% NCS, 5% SSR) (Fig. 3A). Double blind counting of the cells was performed to eliminate bias. At day 2 in culture, approximately 50% of the seeded cells were lost, independently of the version of incubation medium. From day 2 until the end of experiment, the total cell number was negatively correlated with time for all media (F_{48.76,4}, P < 0.001). Furthermore, whereas only 12% of the cells cultured in osmolality-adjusted M199 medium during the spawning season in April were excitable, action potentials (either spontaneous or triggered) were recorded from 82% of the cells incubated in the L-15 media during the same season. However, only 12% of the cells cultured in M199 medium had a membrane potential of −51.76 ± 3.317 mV (n = 47) at day 2 and −48.7 ± 1.3 mV (n = 47) at day 12–14. The data are presented as mean ± SEM. There were no statistical differences in membrane potential between days or treatments.

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An indirect measure of the condition of a primary culture is how long the cells remain sufficiently attached to the dish to allow electrophysiological experiments. Under the initial culture conditions, electrophysiological experiments were possible for 3.8 ± 2.3 days (n = 5). After adjustment of the osmolality of all solutions, the number of experimental days increased significantly to 5.4 ± 2.0 (n = 8), which is an insignificant increase. On the other hand, after changing to L-15 medium and adjusting the HCO₃⁻ concentration, pCO₂, and pH, the average number of experimental days increased significantly to 14.0 ± 4.2 (n = 9) (p < 0.001).

3.2.8. Cell viability

The total number of cells remaining after 2, 7, and 14 days in culture was used as a coarse estimate of the stability of cultures incubated in the three versions of the L-15 medium (serum-free, 5% NCS, 5% SSR) (Fig. 3A). Double blind counting of the cells was performed to eliminate bias. At day 2 in culture, approximately 50% of the seeded cells were lost, independently of the version of incubation medium. From day 2 until the end of experiment, the total cell number was negatively correlated with time for all media (F_{48.76,4}, P < 0.001). Furthermore, whereas only 12% of the cells cultured in osmolality-adjusted M199 medium during the spawning season in April were excitable, action potentials (either spontaneous or triggered) were recorded from 82% of the cells incubated in the L-15 media during the same season. However, only 12% of the cells cultured in M199 medium had a membrane potential of −51.76 ± 3.317 mV (n = 47) at day 2 and −48.7 ± 1.3 mV (n = 47) at day 12–14. The data are presented as mean ± SEM. There were no statistical differences in membrane potential between days or treatments.
higher metabolic activity of cells incubated in L-15 medium supplemented with either serum (p < 0.0001) or SSR (p < 0.01) compared to cells cultured in serum-free L-15 medium. Membrane integrity measured by the CFDA-AM assay was also significantly higher in L-15 medium supplemented with either 5% NCS or 5% SSR than in serum-free medium (p < 0.01). The CFDA-AM fluorescence signal was also significantly higher in medium containing 5% SSR. Although serum-free medium led to increased expression of both fshb and lhb, which are important in the regulation of cod sexual maturation and spawning (Fig. 4), the mRNA expression of both genes was measured using qPCR and nor-
4. Discussion

4.1. Primary pituitary cultures from teleosts

Prior to our studies on the Atlantic cod, primary pituitary cultures have been made from several teleost species including: carp [31,32]; goldfish [9,48]; rainbow trout [44]; European eel [24]; tilapia [46]; and zebrafish [21]. Trypsin was used for tissue digestion in the cell dispersion step in all these cases except for rainbow trout and zebrafish, where collagenase was used. It is a general feature of the culture protocols that the cells were incubated at a pH between 7.2 and 7.5 in an atmosphere containing 5% CO2. The osmolality of the incubation media varied between 300 and 355 mOsm, and in some protocols it is unclear if all the solutions employed during the culture procedure were adjusted to exactly the same osmolality. Regarding serum supplements, most incubation media contained 1–10% mammalian serum, either horse serum or NCS. Only one protocol comprised serum-free incubation medium [46], whereas an artificial serum substitute was used in one case [44].

Although most of the previous studies on teleost pituitary cells in primary culture have yielded reasonable results, it is uncertain to which degree the cells were in a physiologically normal state. In our initial work on primary pituitary cells from the Atlantic cod, we adopted the established culture protocol for the European eel [24], which we considered to be the most promising. However, the shallow and rapidly decaying resting membrane potential of the dispersed cells strongly indicated that the culture conditions were suboptimal for Atlantic cod.

We did not systematically test different temperature regimes, but simply selected 12 °C, which is moderately above the ambient temperature experienced by the cod before being sacrificed, as the temperature during both incubation and the electrophysiological experiments. The enzymatic digestion of the tissue was performed at 18 °C, as a compromise between the low adaptation temperature of the cod and the much higher temperature required for maximum enzyme activity. For mammals, the most common enzyme for dissociation of pituitary cells is trypsin [15]. For teleost pituitary cultures, mammalian trypsin is also the most commonly used enzyme for tissue digestion (goldfish, Carassius auratus: [9,48]; tilapia, Oreochromis mossambicus: [46]; carp, Cyprinus carpio: [31,32], European eel[24]), but mammalian collagenase has also been used successfully (rainbow trout, Oncorhynchus mykiss: [44]; zebrafish, Danio rerio: [21]). The mammalian isoforms of these enzymes have relative low activity below 20 °C. Trypsin is the most efficient alternative and provides the shortest digestion time, which is a significant benefit when dissociating temperature-sensitive cells from animals acclimated to low temperatures. Therefore, we did not compare the various enzyme treatments, but decided to use trypsin for tissue digestion. In addition to these parameters, the enzymatic treatment for tissue degradation varies considerably between culture protocols.

Fig. 4. Relative gene expression of dispersed Atlantic cod pituitary cells cultured in optimized medium with and without serum supplement. QPCR was used to measure relative expression of the genes fshb, lhb, and gnrhr2a. The cells were maintained in optimized L-15 culture medium (10 mM HCO3−, pCO2 = 3.8 mm Hg, pH = 7.85), and gene expression was compared between cells in serum-free medium and medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum substitute (SSR). The samples were collected from primary cell cultures after 2, 7 and 14 days in culture during the spawning season (March and April). The data are presented as mean ± SEM (n = 6). Lower case letters indicate statistical differences between individual mean values. (A) The fshb mRNA levels were dependent on both time (F2,45 = 67.86, P < 0.0001) and culture medium conditions (F2,45 = 14.96, P < 0.0001). A general decrease in fshb mRNA levels was observed, affected by culture conditions with a statistical significant interaction (F4,45 = 4.01, P = 0.0073). (B) The lhb mRNA expression levels was only time dependent (F2,45 = 193.3, P = 0.0267), with no significant differences between the three culture medium conditions. (C) The gnrhr2a mRNA expression levels were time dependent (F2,45 = 81.47, P < 0.0001), with increased mRNA levels during the first 7 days in culture for all treatments. The expression of gnrhr2a was also affected by culture medium conditions (F2,45 = 55.81, P < 0.0001). Furthermore, there was a statistically significant interaction of the time dependence observed in the three different culture media (F4,45 = 9.59, P = 0.0016).
A rapid change in osmolality is stressful to cells, in particular when being particularly fragile due to the dissociation procedure. It is thus important to measure and adjust the osmolality of all solutions used for establishing and maintaining the cell cultures.

4.2.2. The importance of pCO2 and pH

Because CO2 is easily exchanged over the gills, the pCO2 in fish is only a small fraction of the pCO2 in mammalian blood (40–46 mm Hg or 5.3–6.1 kPa, arterial-venous pCO2) [34]. In teleosts, the arterial pCO2 range from 1.7 to 3.4 mm Hg (0.23–0.45 kPa), while the venous pCO2 range from 3.2 to 5.7 mm Hg (0.42–0.79 kPa), depending on the species [1]. Due to the low pCO2 and the correspondingly low HCO3 concentration, the buffer capacity of the blood and extracellular fluids is lower in fish than in mammals and culture media adapted for mammalian cells. Mammalian culture media usually include buffer systems that result in pH 7.4 when the medium is equilibrated to the standard atmosphere (5% CO2 in humidified air) and temperature (37 °C) in the incubator. Consequently, changing any of these parameters (temperature, pCO2, [HCO3]) in an attempt to adjust to the internal conditions of a teleost will alter the pH of the medium. Furthermore, the value for neutral pH increases with decreasing temperature [8]. Consequently, in cold-water fish, the blood plasma may have a pH about 0.5 units above normal values in mammals, which represents approximately the same alkalinity of the plasma in these animal groups. In teleosts, plasma pH ranges from 7.7 to 7.9, depending on temperature [12]. In the Atlantic cod, the plasma pH is ~7.9 at 12 °C [18]. Thus, it is to be expected that neither the pCO2 nor the pH recommended for incubation of mammalian cells are optimal for teleost cells. Therefore, culture media customized for teleost cells should contain modified buffer systems compared to those used in most media developed for mammalian cells. By adjusting not only the osmolality, but also the interrelated parameters pCO2, [HCO3], and pH of the incubation medium to physiological values for teleosts [12,34], the resting cell membrane potential became stable for at least two weeks in culture. In order to achieve this, the M199 medium, which contains 26.2 mM HCO3, was replaced with the HCO3- free medium L-15 supplemented with 10 mM HCO3. The CO2 content of the incubation atmosphere was set to 0.5% (3.80 mm Hg), resulting in a physiological pH of 7.85 in the medium at 12 °C.

In addition to a deeper and more stable membrane potential, the adjustments of pCO2, [HCO3], and pH significantly increased the electrical excitability of the cells. The cells also stayed attached to the bottom of the dish for about two weeks in the fully adjusted incubation medium, whereas most cells were detached after less than one week when the culture protocol only included osmolality adjustments.

Thus, by simple adjustments of the osmolality of all applied solutions and the pCO2, [HCO3], and pH of the incubation medium, the physiological condition of the cultured cod pituitary cells was dramatically improved compared to the result following a traditional culture protocol adapted for mammalian cells.

4.2.3. The importance of serum supplement

Addition of bovine serum to the culture medium is standard procedure in most protocols for mammalian cell culture. The arguments for adding serum to the culture media range from improved cell adherence to the bottom of the culture vessel, to stronger plasma membranes and a generally improved condition of the cells [15]. However, except for blood cells and the endothelial cells lining the blood vessels, cells are not in direct contact with serum in vivo. Although serum supplement is usually beneficial for cells in culture, serum may also be detrimental for at least some cell types. Several reports have demonstrated that even mammalian primary cell cultures may benefit from serum-free media. For
example, Bowers and Dahm [7] showed that amniotic smooth muscle cells in culture maintained their contractility if serum was omitted from the culture medium. By growing pig liver sinusoidal endothelial cells in a serum-free medium supplemented with an artificial serum substitute, Elvevold et al. [14] managed to maintain functional cells for 20 days, compared to maximum 4 days in traditional serum-containing medium. The serum substitute contained no proteins or peptides that might interfere with the cell function, but did include transferrin, surfactant, adherence promoters, low molecular weight lipids, and replacements for metal ion buffers [4]. In order to keep total control of the experimental conditions, artificial serum supplement has previously also been used in the culture of head kidney macrophages from the Atlantic cod [36]. Thus, it is evident that serum-free media and media supplemented with serum or artificial serum substitutes should be compared when the aim is to develop optimized culture conditions for a specific cell type.

The improvements of the culture conditions discussed in the two previous sections were achieved using serum-free L-15 medium. Therefore, addition of serum (NCS) to the incubation medium is not required in order to maintain seemingly healthy cod pituitary cells in primary culture for at least two weeks. Nevertheless, in order to investigate if supplementing the incubation medium with NCS or the artificial serum substitute SSR is beneficial or not, electrophysiological properties, viability, and gene expression were compared in cells cultivated in serum-free medium and medium supplemented with either NCS or SSR.

Addition of either NCS nor SSR to the incubation medium had any significant effect on the resting cell membrane potential, although it tended to be somewhat shallower when NCS or SSR were present in the medium. The number of surviving cells with time in culture was also unaffected by these supplements. In contrast to this lack of evident effects on electrophysiological properties and survival, both the metabolic activity and the membrane integrity of cells incubated in L-15 medium supplemented with NCS or SSR were significantly higher than in cells cultured in serum-free L-15 medium. The effects of NCS and SSR on these parameters were similar.

The physiological relevance of NCS and SSR supplements was further examined by measuring the expression of selected genes. The expected decrease in the expression of fshb with time in culture, due to absence of GnRH influence, was observed in all medium versions. At day 14 in culture, the expression of fshb was significantly higher in medium supplemented with SSR than in the other media. We did not observe a similar decrease in lhb expression. The differential GnRH-regulation of fshb and lhb will be the topic of further studies.

Transcription of the GnRH receptor gene gorhr2a is essential regarding GnRH responses in pituitary cells. It is common that reduced ligand concentration induces upregulation of the related receptors. Because the tested media were lacking GnRH, it is not surprising that the expression of gorhr2a was higher at day 7 than at day 2 in culture for all medium versions. Also for this receptor gene, the expression level was lowest in medium supplemented with serum, and highest in medium containing SSR. Furthermore, both SSR and NCS supplements caused the elevated gorhr2a expression to remain also at day 14. Thus, for primary culture of cod pituitary cells, the gene expression data indicate that SSR is a preferable medium supplement compared to NCS if the aim is to study the GnRH response.

It is well documented that NCS promotes viability and substrate attachment in primary cultures of many types of teleost cells [5]. Also, we experienced that using NCS was necessary for proper attachment when cells where plated on glass bottom. Fish serum, on the other hand, may be beneficial or toxic, depending on cell type (reviewed in [27]). Barlian et al. [3] showed that it might be favorable to replace the poorly defined serum with calf serum albumin (BSA). However, the success was dependent on the type of BSA. Other similar proteins, like ovalbumin, did not have the same positive effect.

An important concern regarding total removal of serum from incubation media customized for pituitary cells is the fact that gonadotropin releasing hormone (GnRH) receptors, like many G protein-coupled receptors, are dependent on lipid rafts in order to be localized close to their cognate signaling molecules. Navratil et al. [25] showed that the GnRH receptors are localized to the low density membrane fraction together with c-raf kinase, and that depletion of cholesterol disrupts the association between the receptors and the lipid rafts. Cholesterol depletion also reduced the GnRH activation of ERK (extracellular signal-related kinase) and c-fos-regulated gene transcription. There is increasing evidence that also many ion channels are localized to lipid rafts, which can affect channel function [13]. For instance, the channel gating kinetics may be regulated by interactions between channel proteins and both lipids and other associated proteins in the raft.

Tocher et al. [40] showed that the lipid composition of the plasma membrane of fish cells in long-term cultures was affected by the NCS added to the medium, reflecting the lipid components of the serum more than those of the original fish tissue. Therefore, an optimal serum substitute should contain the essential factors for keeping the structure and the constituents of the plasma membrane intact, while omitting unknown growth factors and other signaling molecules that can interfere with the physiology of the cell.

5. Conclusion

By adjusting the osmolality of all solutions included in the culture protocol, and the temperature, pH of the incubation medium, to physiological values for the Atlantic cod, it was possible to maintain viable cod pituitary cells in primary culture for at least two weeks. During this period, the resting membrane potential was completely stable, and the expression pattern of key genes was physiologically reasonable. In addition, the cells responded similarly to GnRH during the whole culture period. In contrast, cells dispersed and maintained following a traditional culture procedure for teleosts displayed rapidly decaying resting membrane potentials and succumbed within one week. The cells were moderately more viable in medium supplemented with either NCS or an artificial serum substitute (SSR). Compared to serum-free medium, the gene expression was better maintained in medium containing SSR, whereas NCS tended to depress the expression level. Although serum-free medium is adequate for many applications, serum supplement may be preferable for experiments dependent on maximizing the membrane integrity and receptor function. Using a well-defined, artificial serum substitute, thus avoiding introduction of undesired signaling molecules, may be the ultimate solution in many experimental situations.

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