Molecular characterization and gene expression analysis of calcium-activated potassium channels in Atlantic cod

(Gadus morhua)

Master thesis by Stine Berg Vaule

The Physiology Programme

Department of Molecular Biosciences

Faculty of Mathematics and Natural Sciences

University Of Oslo

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Stine Berg Vaule

Oslo, juni 2011
Abstract

Calcium-activated potassium ($K_{Ca}$) channels are found in virtually all multicellular organisms. The channels play important roles in several physiological processes. One of these processes is hormone secretion, where the $K_{Ca}$ channels in mammals are shown to be important components in the signaling pathway regulating hormone secretion from endocrine cells in the pituitary, e.g. gonadotropes. The $K_{Ca}$ channels are also believed to be important in the regulation of hormone secretion in teleost fishes. Unpublished results (Hodne et al.) indicate that $K_{Ca}$ channels are present in gonadotropes in the teleost Atlantic cod (*Gadus morhua*). I have cloned and sequenced the BK, SK1 and IK channels, and investigated their gene expression in pituitaries from female Atlantic cod during their reproductive season (November-April) using qPCR. The BK channel was significantly up-regulated in April, compared to November and December, while both the SK1 and the IK channel shows a decreasing trend from November until April. In addition, qualitative analyses have been performed, to investigate which tissues outside the central nervous system that express the three cloned $K_{Ca}$ channels. Two months were compared, one prior to spawning and one during spawning. The BK and SK1 channels were expressed in all tissues except the liver in both months, whereas the IK channel was expressed in all tissues in both months. The results correspond to the gene expression pattern of lhβ, fshβ and gpα, indicating that $K_{Ca}$ channels may regulate hormone secretion in Atlantic cod.
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### Abbreviations

<table>
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<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin related protein 2/3</td>
</tr>
<tr>
<td>BK</td>
<td>big conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BPG</td>
<td>brain-pituitary-gonad</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_i</td>
<td>cytosolic calcium concentration</td>
</tr>
<tr>
<td>CaMBD</td>
<td>calmodulin binding domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxy ribonucleic acid</td>
</tr>
<tr>
<td>CDS-primer</td>
<td>cDNA synthesis primer</td>
</tr>
<tr>
<td>cGnRH-II</td>
<td>chicken GnRH-II</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cq</td>
<td>quantification cycle</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy ribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy ribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EF1α</td>
<td>elongation factor 1 alpha</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>ΔG</td>
<td>change in Gibbs free energy</td>
</tr>
<tr>
<td>GAP</td>
<td>GnRH associated peptide</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<tr>
<td>gmGnRH</td>
<td>gadus morhua GnRH</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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<tr>
<td>GnRH-R</td>
<td>GnRH receptor</td>
</tr>
<tr>
<td>gpa</td>
<td>glycoprotein alpha</td>
</tr>
<tr>
<td>GSI</td>
<td>gonadosomatic index</td>
</tr>
<tr>
<td>IK</td>
<td>intermediate conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K$_{Ca}$ channel</td>
<td>calcium-activated potassium channel</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth (bacterial growth medium)</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>L-type</td>
<td>long lasting-type</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NTC</td>
<td>non template control</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>pars intermedia</td>
</tr>
<tr>
<td>PN</td>
<td>pars nervosa</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>PPD</td>
<td>proximal pars distalis</td>
</tr>
<tr>
<td>ppt</td>
<td>parts per thousand</td>
</tr>
<tr>
<td>PRL</td>
<td>prolactin</td>
</tr>
<tr>
<td>pS</td>
<td>picosiemens</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RCK domain</td>
<td>regulating conductance of K⁺ (potassium) domain</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPD</td>
<td>rostral pars distalis</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>sGnRH</td>
<td>salmon GnRH</td>
</tr>
<tr>
<td>SK</td>
<td>small conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>Tₘ</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TN</td>
<td>terminal nerve</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside</td>
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1. Introduction

Puberty can be defined as the period in life when sexual activity commences. In vertebrates, sexual maturation is characterized by the activation of the brain-pituitary-gonad (BPG) axis, and in teleosts like in other vertebrates, it seems that an activation of the gonadotropin-releasing hormone (GnRH) system is a key event in the onset of puberty. What leads to this activation is not fully explained in any vertebrate (Schulz and Goos, 1999) (for review, see Bliss et al., 2010).

Our research group studies the physiological control mechanisms involved in puberty, focusing on the gonadotropin-producing (gonadotrope) cells and how they function. I have in this project performed a molecular characterization of calcium-activated potassium (K\text{Ca}) channels and studied the gene expression of these channels in the Atlantic cod (Gadus morhua). The K\text{Ca} channels are believed to be important components in the signaling pathway regulating hormone secretion from the gonadotropes.

1.1 The brain-pituitary-gonad (BPG) axis

The BPG axis consists of three physiologically connected components. Neuroendocrine neurons in the brain represent the link between the central nervous system (CNS) and the endocrine system. Some of these neurons produce an integrated output in the form of GnRH. GnRH stimulates gonadotrope cells in the pituitary to produce and release the two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In some species, notably in several teleost fish, dopamine opposes the effect of GnRH by inhibiting production and release of gonadotropins. The gonadotropins are heterodimeric glycoproteins, consisting of a common \(\alpha\) subunit linked to a specific \(\beta\) subunit, with distinct genes encoding the FSH\(\beta\), LH\(\beta\) and the common \(\alpha\) subunit (Norris, 1997). It is the \(\beta\) subunit that determines the biological activity and the specificity of the hormone. FSH and LH bind to their specific receptors in the gonads, which subsequently initiate gametogenesis and steroidogenesis (Figure 1). In fish, the most important androgen and estrogen are 11-ketotestosterone and 17\(\beta\)-estradiol, respectively (for review, see Borg, 1994). The sex steroids control the different
stages of gametogenesis together with FSH, and they can have a positive or negative feedback on the pituitary and the brain depending on the maturational stage (Schulz and Goos, 1999).

In addition to these feedback mechanisms within the axis itself, the BPG axis is influenced by a variety of external and internal factors, ensuring that reproduction takes place when offspring survival is optimal. Examples of external factors that have been shown to influence the BPG axis include temperature, photoperiod, lunar phase, and pheromones, whereas

Figure 1. The brain-pituitary-gonad (BPG) axis. A simplified version of the BPG axis in teleosts. Stimulating and inhibiting signals produce an integrated output in the brain, resulting in the release of the stimulatory gonadotropin-releasing hormone (GnRH) and the inhibitory dopamine. GnRH binds to receptors in the pituitary, and activation of these receptors leads to synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Dopamine can either bind to receptors on the gonadotropes or on the cells producing GnRH, directly or indirectly leading to inhibition of FSH and/or LH release. FSH and LH act on target cells in the gonads, and initiate the production of sperm and eggs (gametogenesis), in addition to synthesis and secretion of steroid hormones (steroidogenesis). The sex steroids can have a positive or negative feedback on the pituitary and the brain, depending on the maturational stage.
internal factors include those related to nutritional status, such as leptin, ghrelin and neuropeptide Y (Schulz and Goos, 1999, Bromage et al., 2001).

The basic organization of the BPG axis is highly conserved and the axis is vital to reproductive maturation in all vertebrates (Al-Kindi et al., 2001, Lake et al., 2008) (for reviews, see Francis et al., 1993, Sisk and Foster, 2004). During the transformation from a sexually immature juvenile to a mature adult, the BPG axis achieves its full hormonal and gametogenetic capacity (Norris, 1997).

1.1.1 The brain and GnRH

GnRH and dopamine from hypophysiotropic neurons originating in the hypothalamus are the two main factors that can influence the axis either stimulatory or inhibitory. GnRH has a stimulatory effect in all vertebrates, while dopamine in some species can have an inhibitory effect on the synthesis and secretion of FSH and LH (Peter et al. 1986, cited in Dufour et al., 2010). Dopamine inhibits both basal and GnRH-stimulated LH secretion in many teleost species, but not many studies have focused on what role dopamine plays in the inhibition of puberty. In the pre-pubertal eel (Anguilla anguilla), dopamine inhibition has to be removed before GnRH-stimulated LH synthesis and release can be triggered (Dufour et al., 1988, Vidal et al., 2004). Vidal et al. (2004) showed that in eel, dopamine acts directly at the pituitary level to counteract both GnRH-stimulated LH synthesis and release and that these inhibitory actions represents a true block of puberty.

GnRH is a well conserved decapeptide. The hormone is synthesized as an inactive pre-pro-hormone, consisting of a signal sequence, GnRH, a proteolytic cleavage site and a GnRH associated peptide (GAP) (White and Fernald, 1998). In all vertebrate GnRH sequences, the amino acids in position 1, 4, 9 and 10 are identical. To date, 14 variants of GnRH have been described in vertebrates. Most vertebrate species including teleosts (Chow et al., 1998, Okubo et al., 2000), express two or three forms of GnRH encoded by distinct genes (for review, see Dubois et al., 2002, Lethimonier et al., 2004). Three pre-pro-GnRH genes have recently been identified in Atlantic cod (gadus morhua, gmGnRH) (Hildahl et al., 2011a).
In most vertebrates, GnRH1 is the hypophysiotropic form of GnRH, and GnRH1-expressing neurons have their cell bodies located in the preoptic area (POA) and the caudal hypothalamus. This GnRH form varies between species and is the form that induces the release of gonadotropins from the adenohypophysis, which in turn promotes gonad development (Krieger et al., 1982, Khakoo et al., 1994, Whitlock et al., 2006). In Atlantic cod, GnRH1 is suggested to be a pseudogene (Hildahl et al., 2011a). Pseudogenes are residues of genes that have lost their protein-coding ability, or for other reasons are no longer expressed in cells (for review, see D’Errico et al., 2004). In all jawed vertebrates, GnRH2, also called chicken GnRH-II, is expressed in the midbrain tegmentum, and is considered to have neuromodulatory functions related to reproductive behavior (Yamamoto et al., 1997, Temple et al., 2003, Barnett et al., 2006). GnRH3, also called salmon GnRH, is a teleost-specific form. It is located in the terminal nerve (TN) ganglion, but the function of TN GnRH3 is not clear (White and Fernald, 1998, Ogawa et al., 2006). In species where GnRH1 has been lost or inactivated, GnRH3-expressing neurons are located both in the TN and in several nuclei of the hypothalamus, including the POA. Depending on if the fish has two or three forms of GnRH, the hypothalamic GnRH1 or GnRH3 is the hypophysiotropic isoform regulating FSH and LH (Whitlock et al., 2003). In Atlantic cod, both GnRH2 and GnRH3 are expressed in the ovary, pituitary, and brain, and sequence and expression analysis suggests that GnRH3 is likely to be the hypophysiotropic form of GnRH in this teleost species (Hildahl et al., 2011a).

As many as five GnRH-receptor (GnRH-R) genes have been identified in two teleost species; the European sea bass (Dicentrarchus labrax) (Moncaut et al., 2005) and spotted green puffer fish (Tetraodon nigroviridis) (Ikemoto and Park, 2005). Hildahl et al. (2011b) have recently identified four GnRH-Rs in Atlantic cod (gmGnRH-R), and have shown that three of these, gmGnRH-R1b, gmGnRH-R2a and gmGnRH-R2c are expressed in the pituitary. Of these three, only gmGnRH-R2a gene expression varies significantly in accordance with reproductive stage, showing increased expression in spawning females. This indicates that gmGnRH-R2a is the most likely candidate to mediate the hypophysiotropic function of GnRH in Atlantic cod (Hildahl et al., 2011b).

Although the basic organization is similar, there are important differences between mammals and teleosts concerning the BPG axis. In mammals, the activation of the BPG axis results in a pulsatile secretion of GnRH from the hypothalamus. GnRH is transported to the pituitary via
the hypophyseal portal circulation, where GnRH binds to its cognate receptors located in the membrane of the gonadotropes. Binding of GnRH to its receptors induces synthesis and secretion of the two gonadotropins; FSH and LH. The pulsatory release of GnRH is required for appropriate production and release of gonadotropins in mammals (Belchetz et al., 1978) (for reviews, see Tsutsumi and Webster, 2009, Bliss et al., 2010). In teleosts, on the other hand, the GnRH neurons directly innervate the pituitary. Fibers either end in close vicinity to the gonadotropes, or synapse directly on them (Peter et al., 1990, Anglade et al., 1993, Mousa and Mousa, 2003). A pulsatory release of GnRH does not seem to be required in teleosts. However, the picture is still not clear as medaka (*Oryzias latipes*) GnRH1 neurons show an action potential profile indicative of a pulsatory release of GnRH (Wayne et al., 2005), similar to mammals.

### 1.1.2 The pituitary and the gonadotropes

In both teleosts and mammals, the pituitary constitutes two main parts: the neurohypophysis, which originates from a down-growth from the floor of the diencephalon and represents the neural compartment of the pituitary, and the adenohypophysis, which is the non-neural part of the gland, and originates as an ectodermal up-growth (Rathke’s pouch) from the anterior roof of the embryonic oral cavity (Wingstrand 1966, cited in Weltzien et al., 2004). The teleost adenohypophysis is divided into three zones; *rostral pars distalis* (RPD), *proximal pars distalis* (PPD) and *pars intermedia* (PI, Figure 2).
Figure 2. A schematic diagram of the Atlantic halibut pituitary. The adenohypophysis of the Atlantic halibut has a general morphology similar to other evolutionary advanced teleost fish. The different hormone-producing cell types have a specific localization in the pituitary. Both FSH- and LH-producing gonadotropes can be localized in the PPD, indicated by orange and purple squares. Only LH-producing gonadotropes can be found in the periphery of the PPD and the PI. Abbreviations used in the figure are; P = lactotropes, C = corticotropes, T = thyrotropes, S = somatotropes, GF = FSH-producing gonadotropes, GL = LH-producing gonadotropes, SL = somatolactotropes, M = melanotropes, RPD = rostral pars distalis, PPD = proximal pars distalis, PI = pars intermedia and PN = pars nervosa. From Weltzien et al. (2004).

The hormone-producing cell types in the pituitary of adult tetrapods are randomly arranged in a mosaic pattern (for reviews, see Doerr-Schott, 1976, Voss and Rosenfeld, 1992). Teleosts on the other hand, have preserved the embryonic compartmental organization, where hormone-producing cells of the same type are localized to a specific area in the pituitary (Ball and Baker, 1969, Schreibman et al., 1973). Most teleosts have separate gonadotrope cell types secreting FSH or LH (Nozaki et al., 1990, Naito et al., 1991, Naito et al., 1993, Kagawa et al., 1998, Weltzien et al., 2003), in contrast to mammals that only have one gonadotrope cell type producing both hormones. This makes teleosts good model organisms for separately studying the regulation of FSH and LH production and secretion. Teleosts are useful complementary model organisms to human medicine, since knowledge about the regulation of FSH and LH are transferable to the mammalian GnRH system.

In teleosts, the gonadotropes are located in the PPD of the adenohypophysis. Expression of FSHβ and LHβ mRNA has been found throughout the PPD in several species, e.g. Atlantic
halibut (*Hippoglossus hippoglossus*), and the gonadotropes seem not to be in close contact with the PN (Weltzien et al., 2003). LHβ immunoreactivity was also found throughout the PPD, and in addition, along the periphery of the PI (Figure 2). Similar results have been observed in other teleosts, like Atlantic croaker (*Micropogonias undulatus*), spotted sea trout (*Cynoscion nebulosus*), red drum (*Sciaenops ocellatus*), Mediterranean yellowtail (*Seriola dumerilii*), and white sea bream (*Diplodus sargus*) (Yan and Thomas, 1991, García-Hernández et al., 1997, Segura-Noguera et al., 2000).

1.1.2.1 Electrophysiological signaling in gonadotropes

The neurohormones released from the hypothalamus bind to membrane receptors on pituitary cells. This typically triggers a rise in the cytosolic calcium concentration ([Ca$^{2+}$]$_i$), which may in turn stimulate hormone release, first by exocytosis of stored hormone vesicles and later following gene transcription. In this way, Ca$^{2+}$ is an important second messenger in all cells. The increase in [Ca$^{2+}$]$_i$ can be caused by different mechanisms in different pituitary cell types but typically involves release from intracellular Ca$^{2+}$ stores, e.g. the smooth endoplasmic reticulum (ER). The Ca$^{2+}$ source can also be extracellular, and the increase in [Ca$^{2+}$]$_i$ may be caused by influx through voltage-gated Ca$^{2+}$ channels or other Ca$^{2+}$-conducting channels. Action potentials can promote extracellular Ca$^{2+}$ influx in cells expressing voltage-gated Ca$^{2+}$ channels. This Ca$^{2+}$ signal is encoded by both the frequency and the shape of the action potential (Hille, 2001). Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels is the major link between electrical phenomena at the cell membrane and intracellular processes.

In mammals, pituitary endocrine cells exhibit spontaneous action potentials that are partially Ca$^{2+}$-dependent (Sims et al., 1991, Tse and Hille, 1993, Sankaranarayanan and Simasko, 1996, Kwiecien et al., 1998, Bonnefont et al., 2000, Beltran-Parrazal and Charles, 2003). In somatotropes and lactotropes, action potentials occur in bursts linked to depolarizing plateaus, and generate Ca$^{2+}$ signals that can trigger hormone release. In these cells, the membrane potential oscillates. When these oscillations reach a threshold level, an action potential can be generated. If the oscillations are large enough, several action potentials can be generated on top of the depolarized plateau. This phenomenon is called plateau-bursting action potentials. Resting gonadotropes in mammals fire single high-amplitude action potentials, which have
limited ability to promote Ca\(^{2+}\) influx and hormone secretion. However, GnRH has the ability to transform single action potentials into plateau-bursting-type electrical activity and hence to trigger hormone secretion (for reviews, see Freeman et al., 2000, Ben-Jonathan and Hnasko, 2001, McArdle et al., 2002, Sun et al., 2003). Still, for mammalian gonadotropes the major Ca\(^{2+}\) source for control of hormone secretion appears to be intracellular stores.

The prolactin-producing GH\(_{4}\) cell line from the rat anterior pituitary is a particularly well described model system for pituitary secretion control. These cells can be stimulated with thyrotropin-releasing hormone (TRH), which induces biphasic changes in the electrical activity and [Ca\(^{2+}\)]\(_i\) (Figure 3) (for review, see Ozawa and Sand, 1986). The first phase of the TRH response is caused by an activation of phospholipase C and formation of inositol triphosphate (IP\(_3\)), causing Ca\(^{2+}\) release from intracellular stores. The second phase of the response is caused by enhanced Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels. The release of prolactin (PRL) from the GH\(_{4}\) cells follows the pattern of the Ca\(^{2+}\) response seen in Figure 3B (Iijima et al., 1990).

![Figure 3. Effects of thyrotropin-releasing hormone (TRH) on a clonal rat lactotrope (GH\(_{4}\) cell). (A) Application of TRH (indicated by arrow) induced an initial hyperpolarization followed by a delayed depolarization and increased frequency of action potentials. (B) Concomitant with the first phase hyperpolarization, [Ca\(^{2+}\)]\(_i\) transiently increased, and subsequently stayed at a moderately raised level during the second phase depolarization. Hyperpolarizing current was injected during the period indicated by the horizontal bar. The second phase of the Ca\(^{2+}\) response was then reversed. From Iijima et al. (1990).](image-url)
Much less is known about the electrophysiological signaling and control of \([\text{Ca}^{2+}]_i\) in teleost pituitary cells. The best studied species in this respect is goldfish (Carassius auratus) (for reviews, see Chang et al., 2000, Chang et al., 2009). Goldfish gonadotropes have been identified based on their unique morphological characteristics (Van Goor et al., 1994). This is a method that does not separate the FSH- from the LH-producing cells. In morphologically identified goldfish gonadotropes, the application of GnRH increases \([\text{Ca}^{2+}]_i\), mainly through intracellular stores. The GnRH action on LH release also requires extracellular \(\text{Ca}^{2+}\) entry through L (long-lasting)-type voltage-gated \(\text{Ca}^{2+}\) channels as well as activation of calmodulin kinase and protein kinase C. The presence of L-type voltage-gated \(\text{Ca}^{2+}\) channels (Van Goor et al., 1996) and the ability of both GnRH2 and GnRH3 to increase \([\text{Ca}^{2+}]_i\) have been described in goldfish gonadotropes (Johnson et al., 1999). Most goldfish gonadotropes display spontaneous action potentials. Interestingly, GnRH does not increase the action potential frequency in these identified gonadotropes (Van Goor et al., 1996).

Levavi-Sivan et al. (2005) showed that electrical activity in anterior pituitary cells from a teleost, tilapia (Oreochromis sp.), are synchronized by coupling through gap junctions, and regulation of this coupling may play an important role in determining complex patterns of pituitary hormone secretion. Haug et al. (2007) showed that spontaneous action potentials were observed in about 30% of the largest pituitary cells from Atlantic cod. The largest cells are believed to be gonadotropes (Montero et al., 1996, Van Goor et al., 1996). The shape of these spontaneous action potentials suggests that the action current is carried by both \(\text{Na}^+\) and \(\text{Ca}^{2+}\). Thus, action potentials may be important for \(\text{Ca}^{2+}\) influx in these cells.

Increased \([\text{Ca}^{2+}]_i\), may lead to activation of \(K_{\text{Ca}}\) channels located in the plasma membrane, leading to membrane hyperpolarization and reduced excitability. When present, these channels thus play an important role in modulating the electrophysiological response to hypothalamic releasing hormones (for reviews, see Israel and Vincent, 1990, Naor et al., 1998, Pawson and McNeilly, 2005).
1.2 Calcium-activated potassium (K\(_{\text{Ca}}\)) channels

Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) channels are a link between changes in [Ca\(^{2+}\)]\(_i\) and membrane potential (for reviews, see Petersen and Maruyama, 1984, Vergara et al., 1998, Fettiplace and Fuchs, 1999). They are activated by an increase in [Ca\(^{2+}\)]\(_i\), and they can shape the amplitude and duration of Ca\(^{2+}\) transients and in this way influence the downstream signaling pathways that are triggered by changes in [Ca\(^{2+}\)]\(_i\) (for review, see Fakler and Adelman, 2008).

K\(_{\text{Ca}}\) channels are found in virtually all multicellular organisms in both neuronal and non-neuronal tissues, the latter including epithelial cells and smooth muscle cells. The activity of K\(_{\text{Ca}}\) channels is implicated in several physiological processes, including neurosecretion, regulation of action potential shape and frequency (for review, see Vergara et al., 1998), cell volume regulation, epithelial transport, and cell proliferation and migration (Ouadid-Ahidouch et al., 2004, Fioretti et al., 2005) (for review, see Schwab, 2001).

The K\(_{\text{Ca}}\) channels can be divided into three subfamilies: BK (big conductance K\(_{\text{Ca}}\)), IK (intermediate conductance K\(_{\text{Ca}}\)) and SK (small conductance K\(_{\text{Ca}}\)) channels, based on the primary amino acid sequences, their single-channel conductance and pharmacological properties (for review, see Vergara et al., 1998).

The K\(_{\text{Ca}}\) channels have only been cloned from a few teleosts, e.g. zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss) (For GenBank accession numbers, see Appendix V), and their function has not yet been characterized in fish.

I have studied the K\(_{\text{Ca}}\) channels because of their assumed role in the signaling pathway regulating hormone secretion in gonadotropes in Atlantic cod.

1.2.1 Big conductance K\(_{\text{Ca}}\) (BK) channels

Big conductance K\(_{\text{Ca}}\) (BK) channels are activated by both elevated [Ca\(^{2+}\)], and membrane depolarization. BK channels have a single channel conductance of around 250 pS in symmetrical KCl (for reviews, see Kaczorowski et al., 1996, Calderone, 2002, Orio et al.,
These channels are abundant in e.g. smooth muscle, neuronal membranes and pancreas. BK channels have several physiological roles, including control of action potential frequencies in neurons, modulation of smooth muscular tone in blood vessels, and tuning of the electrical resonance of inner ear hair cells (for reviews, see Sah, 1996, Toro et al., 1998, Vergara et al., 1998, Fettiplace and Fuchs, 1999). Cell swelling caused by various factors, like hormones, paracrine agents and hypotonic stress, may elevate $[\text{Ca}^{2+}]_i$ and hence activate BK channels if present (for review, see Pasantes-Morales and Mulia, 2000). The subsequent efflux of $\text{K}^+$ (and associated anions) may then contribute to the regulatory volume decrease (Hafting et al., 2006).

BK channels often appear as complexes of two different subunits: pore-forming $\alpha$-subunits and regulatory $\beta$-subunits. The BK channel pore is a tetrameric assembly of $\alpha$-subunits encoded by a single gene KCNMA1 (previously named Slo1) in tetrapods, and duplicate genes in teleosts (Rohmann et al., 2009). Different splice variants of this gene can generate functional diversity (Adams et al., 1982, Elkins et al., 1986, Tseng-Crank et al., 1994, Saito et al., 1997, Yan et al., 2008) (for reviews, see Adelman et al., 1992, Vergara et al., 1998). The primary sequence of the BK channel $\alpha$-subunit is highly conserved between mammals and teleosts, and BK channels are identified in all vertebrate classes.

The $\alpha$-subunit of the BK channel consists of seven transmembrane domains (S0-S6), where the short NH$_2$ terminus is situated at the extracellular side of the membrane and the large COOH terminus is situated at the intracellular side (Figure 4) (Meera et al., 1997). The intracellular domain of the BK channel contains four hydrophobic segments (S7-S10), two domains regulating the conductance of $\text{K}^+$ (RCK domains) and a stretch of aspartate residues known as the Ca$^{2+}$ bowl. The tertiary folding of these domains create binding site(s) for Ca$^{2+}$. The binding of Ca$^{2+}$ provides one source of energy for opening of the channel (Schreiber and Salkoff, 1997, Jiang et al., 2001, Xia et al., 2002, Bao and Cox, 2005, Sweet and Cox, 2008) (for review, see Magleby, 2003). The other source of energy is the membrane depolarization, which induces movement of the voltage-sensing S4 segments (Horrigan and Aldrich, 1999, Horrigan et al., 1999, Ma et al., 2006, Pantazis et al., 2010).
The β-subunits (β1-β4) can modify the Ca$^{2+}$ sensitivity of the channels, in addition to regulating channel activity and its pharmacological properties. They are encoded by four distinct genes (KCNMB1-4) (Knaus et al., 1994a, Xia et al., 1999, Brenner et al., 2000, Meera et al., 2000, Uebele et al., 2000). The β-subunit of the BK channel has two transmembrane segments flanking a glycosylated extracellular loop and short intracellular NH$_2$ and COOH termini (Knaus et al., 1994a) (for reviews, see Orio et al., 2002, Torres et al., 2007).

Tetramers of the BK α-subunits are functional, but in the plasma membrane of vertebrate cells, the large majority of BK channels are complexes of α- and β-subunits. The interaction of α- and β-subunits involves multiple contact sites. The transmembrane segment 0 (S0) of the α-subunit is in contact with the extracellular extension of the second transmembrane domain of the β-subunit, and S1 and S2 of the α-subunit touches the first transmembrane domain of the β-subunit (Liu et al., 2008, Wu et al., 2009). Knaus et al. (1994b) proposed that the α-subunit tetramer associates with up to four β-subunits, but this is still discussed. Functional studies suggest that the α-subunit tetramer is associated with at least one of the β-subunits (Tanaka et al., 1997, Toro et al., 2006).
1.2.2 Small conductance $K_{Ca}$ (SK) channels

Small conductance $K_{Ca}$ (SK) channels are divided into three subtypes called SK1, SK2 and SK3. The pore-forming $\alpha$-subunits of the SK1, SK2 and SK3 channels are encoded by three homologous genes (KCNN1-3) (Köhler et al., 1996). KCNN1-3 are differentially expressed in neuronal and non-neuronal tissues (for reviews, see Stocker, 2004, Bond et al., 2005, Pedarzani and Stocker, 2008, Lujan et al., 2009). SK channels have been identified in mammals, birds, amphibians and teleosts (NCBI). In symmetrical KCl, the SK channels display a single channel conductance of approximately 10 pS (Köhler et al., 1996, Hirschberg et al., 1998).

The SK channels play a fundamental role in most excitable cells. SK1 and SK2 are predominantly found in CNS neurons, while SK3 is expressed both in neuronal and glial cells, as well as in diverse endothelial and smooth muscle cells and in secretory cells (Köhler et al., 1996, Ishii et al., 1997, Joiner et al., 1997, Logsdon et al., 1997, Stocker and Pedarzani, 2000, Hosseini et al., 2001, Tacconi et al., 2001, Boettger et al., 2002, Arnold et al., 2003). The SK channels have long been known to be present in gonadotropes in sheep (Heyward et al., 1995) and rat (Kukuljan et al., 1992, Tse and Hille, 1992).

SK channels are activated by an increase in $[Ca^{2+}]_i$, such as occurs during an action potential. The activation of SK channels causes membrane hyperpolarization, which typically reduces action potential frequency (for review, see Vergara et al., 1998).

The SK channel shares the tetrameric six-transmembrane domain architecture of voltage-gated cation channels, but it lacks the typical features of voltage-sensing S4 segments (Figure 5). As a consequence, the gating of SK channels is independent of the transmembrane voltage, in contrast to BK channels. The opening and closing of SK channels is solely driven by changes in the $[Ca^{2+}]_i$ (Köhler et al., 1996, Hirschberg et al., 1998). SK channels constitutively bind calmodulin, which mediates the $Ca^{2+}$ gating of the channel (for review, see Stocker, 2004).
Figure 5. Small/intermediate conductance calcium-activated potassium (K_{Ca}) channel. SK and IK channels share the same overall structure, with six transmembrane domains. Both NH\textsubscript{2} and COOH termini are located on the intracellular side of the membrane, and a calmodulin binding domain (CaMBD) is located on the COOH terminal end. From Berkefeld et al. (2010).

1.2.3 Intermediate conductance K_{Ca} (IK) channels

Intermediate conductance K_{Ca} (IK) channels are encoded by the gene KCNN4, and were previously named SK4 due to the great structural similarity to the three SK channels (Figure 5). The IK channel is now considered a distinct type of K_{Ca} channels, based on its single channel conductance of 20-80 pS in symmetrical KCl (for review, see Latorre et al., 1989) and pharmacological differences from the SK channels. IK channels are activated in the same way as SK channels, by an increase in [Ca\textsuperscript{2+}]\textsubscript{i}. Calmodulin also mediates Ca\textsuperscript{2+} gating of the IK channel. The IK channels are mostly found in non-neuronal tissues such as muscle, epithelia and blood cells (Köhler et al., 1996, Ishii et al., 1997, Joiner et al., 1997, Stocker and Pedarzani, 2000).

IK channels are involved in diverse tasks, including volume regulation of erythrocytes (for review, see Brugnara, 1995) and K\textsuperscript{+} homeostasis in brain capillary endothelial cells (Van Renterghem et al., 1995). IK channels also participate in membrane potential regulation in clonal epithelial cells (T84 cells) (Devor et al., 1996).

1.2.4 K_{Ca} channels in pituitary cells

The activation of voltage-gated Ca\textsuperscript{2+} channels in the plasma membrane and intracellular Ca\textsuperscript{2+} release channels, and the subsequent rise in [Ca\textsuperscript{2+}]\textsubscript{i}, are components mediating pituitary
hormone release in mammals. In gonadotropes, intracellular Ca\(^{2+}\) stores are the major Ca\(^{2+}\) source for control of hormone secretion. Ca\(^{2+}\) released from intracellular stores can activate K\(_{Ca}\) channels and thus activation of these channels influence the gonadotropin response to GnRH (for reviews, see Naor, 1990, Chang and Jobin, 1994, Stojilkovic et al., 1994). The predominant Ca\(^{2+}\)-activated ion channels in rat and mouse gonadotropes are K\(_{Ca}\) channels (Ritchie, 1987, Kehl and Wong, 1996, Shipston et al., 1996, Waring and Turgeon, 2006). Membrane hyperpolarization due to activation of these K\(^+\) channels removes voltage-dependent channel inactivation and on subsequent depolarization leads to entry of extracellular Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels (Van Goor et al., 2001a).

Electrophysiological analysis have identified K\(_{Ca}\) channels in multiple pituitary cells, for example immortalized anterior pituitary cells (AtT20 mouse pituitary corticotrope tumor cells) and native intermediate lobe pituitary cells from rats (Ritchie, 1987, Lang and Ritchie, 1990, Kehl and Wong, 1996, Shipston et al., 1996). BK channels (but not SK and IK channels) are expressed in rat somatotropes and lactotropes. In these cell types, the activation of the BK channel is associated with voltage-gated Ca\(^{2+}\) influx (Van Goor et al., 2001b). In rat gonadotropes, SK channels (but not BK and IK channels) are expressed and co-localized with intracellular Ca\(^{2+}\) release sites (Kukuljan et al., 1992).

All three types of K\(_{Ca}\) channels (BK, SK and IK) are activated during the first phase of the previously described TRH response in the GH\(_4\) cells (section 1.1.2.1). The initial peak in [Ca\(^{2+}\)], activates the K\(_{Ca}\) channels, resulting in an outward hyperpolarizing K\(^+\) current (Ozawa and Kimura, 1979). Mørk et al. (2005) showed that, contrary to previous assumptions, the IK channels were the most important contributor of the three types of K\(_{Ca}\) channels when it comes to the TRH induced outward current and hyperpolarization. This was quite unexpected, because the IK channels had not previously been demonstrated to play a major role compared to BK and SK channels in excitable cells (Mørk et al., 2005). Our group has also shown that TRH through an unidentified signaling pathway leads to reduced activity of BK channels during the second phase of the response (Haug et al., 2004). This reduced channel activity may contribute to the depolarization and increased action potential frequency seen during the second phase, directly increasing Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Thus, K\(_{Ca}\) channels may be involved in the response to hypothalamic releasing hormones in several ways.
K\textsubscript{Ca} channels, which are essential in the regulation of membrane excitability in mammalian pituitary cells, seem to be lacking in goldfish gonadotropes (Van Goor et al., 1996). While Van Goor et al. concluded that K\textsubscript{Ca} channels are probably lacking in goldfish gonadotropes, Xu and Cooke (2007) demonstrated the presence of K\textsubscript{Ca} current in tilapia pituitary cells. The K\textsubscript{Ca} current was not investigated any further, and it is therefore not known which of the channels that contributes to this current. Romano et al. (1996) performed a detailed electrophysiological analysis of K\textsuperscript{+} currents in lactotropes of goby (*Gillichthys mirabilis*), showing the presence of big conductance K\textsubscript{Ca} channels similar to mammalian BK channels.

As mentioned above, approximately 30% of unidentified pituitary cells (presumed to be gonadotropes due to their size) from Atlantic cod fired occasional action potentials spontaneously during current clamp recordings (Haug et al., 2007). It was further demonstrated that cod pituitary cells exposed to extracellular Co\textsuperscript{2+}, which blocks inward Ca\textsuperscript{2+} current, also significantly reduced the outward current. This strongly suggests the presence of K\textsubscript{Ca} channels in cod pituitary cells. Unpublished results (Figure 6) show that the response to a mix of GnRH1, 2 and 3 in identified cod gonadotropes includes a hyperpolarization, which further indicates activation of K\textsubscript{Ca} channels by the increase in the free [Ca\textsuperscript{2+}]. This response is similar to the response seen in GH cells from mammals. (Hodne et al., unpublished results).

Figure 6. Response to a GnRH-mix in an identified LH-producing gonadotrope from Atlantic cod. The current clamp recording shows reduced excitability and a tendency to hyperpolarize in response to a mix of GnRH1, 2 and 3. When the cell starts to fire action potentials again, it is with a higher frequency. This response is similar to the TRH response in GH cells presented in figure 3 and indicates that K\textsubscript{Ca} channels are present in the cell membrane. From unpublished results by Hodne et al.
In teleosts, knowledge about the role of $K_{Ca}$ channels in pituitary cells is very scarce. The details of how hormone secretion from gonadotropes is regulated are still unknown, but the $K_{Ca}$ channels may be important, as we know they are in mammals. This project, with its characterization of the $K_{Ca}$ channels in Atlantic cod, is a contribution to the ongoing research in this field, trying to reveal the mechanisms behind the regulation of hormone secretion in teleosts.

1.3 Atlantic cod as model organism

Our research group is investigating the basic physiological mechanisms behind pubertal development in fish, and for this purpose we are using the Atlantic cod as one of our model organisms. The Atlantic cod is a teleost belonging to the order Gadiformes and the family Gadidae.

This group of teleosts is yet little studied. Other teleost models like medaka, zebrafish, and goldfish, have a short juvenile period. In contrast, cod has a relatively long juvenile period, which makes it a good model organism to study pubertal development. Growth and age of puberty varies between cod populations. Wild Norwegian costal cod attains sexual maturity at the age of 3 and older (Berg and Albert, 2003). The cod is a multiple batch spawner, and generally spawns from January until April (for review, see Taranger et al., 2010), depending on the stock and water temperature.

The Atlantic cod is an important commercial species, but successful farming has proven to be difficult. Precocious sexual maturation and an unpredictable timing of brood-stock ovulation constitute two main obstacles for further development of commercial fish farming, and this is also the case for cod. Precocious maturation and spawning in the net-pens can also lead to genetic pollution. Knowledge on the regulatory mechanisms controlling pubertal development, especially the neuroendocrine control of the pituitary, is limited. It is thus important to gain more information about this topic. This study is investigating some of the factors believed to play a part in the signaling pathway regulating hormone secretion from the gonadotropes.
1.4 Aims of this study

The aims of this study were to:

1. Perform a molecular characterization of the putative five different genes for $K_{Ca}$ channels in Atlantic cod
2. Study the gene expression pattern of these channels in Atlantic cod
   a) in whole pituitaries during the reproductive season
   b) in single FSH- and LH-producing cells
3. Perform a tissue screen to investigate where these channels are expressed outside the CNS
2. Materials and methods

![Diagram](image)

Figure 7. Overview of the methodological approach used in this study.

2.1 Animal handling and tissue sampling

Atlantic cod were captured in the Oslo fjord and at Austevoll, south of Bergen, between January 2010 and May 2011, and kept in indoor aquarium facilities at the Department of Molecular Biosciences, University of Oslo, for no longer than 2 weeks before tissue sampling. The holding tanks were continuously supplied with seawater with a salinity of 32 ppt and a temperature of 8-12 °C. The cod were fed shrimps while in captivity. The light regime was adjusted every two weeks to mimic the natural photoperiod in Oslo.

The cod were sacrificed by quickly severing the spinal cord and the dorsal aorta. Following decapitation, the skull was cut open, enabling sampling of the pituitary and the brain in addition to samples from the retina, gills, intestine, liver, muscle, skin and gonads.
Tissue samples of approximately 5 x 5 x 1 mm were immediately placed in 1 ml RNAlater (Ambion, Austin, TX, USA) in eppendorf tubes on ice before being stored at -20 °C until further processing.

Before tissue sampling, total body and gonad weights were measured, whereby the gonadosomatic index (GSI) was calculated for each fish according to the formula: GSI = (gonad weight / body weight) * 100. The sex of each cod was registered. For cloning and sequencing, both female and male cod were used, but for the gene expression analysis, only female cod were used. The GSI for the female cod used in gene expression analysis, can be found in Appendix I.

Figure 7 shows an overview of the methodological approach used in this study.

### 2.2 Isolation of total RNA and DNase treatment of tissue samples

The different tissue samples were homogenized in 1 ml trizol reagent (Invitrogen, Carlsbad, CA, USA) using the FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Solon, OH, USA) at a setting of 6.0 m/s for 45 s. The tube was incubated on ice for minimum 2 min, and then homogenized once more at 6.0 m/s for 45 s. The tube was centrifuged at 4 °C for 10 min at 12 000 g using a Kubota 3500 centrifuge (Kubota, Japan). The supernatant, about 700 µl, was transferred to an RNase free 1.5 ml eppendorf tube and incubated at room temperature for 10 min to gain full dissociation of the nucleoprotein complexes. For each 1 µl homogenized sample, 0.2 µl chloroform was added prior to vortexing for 30 s. The sample was incubated at room temperature for 5 min and then centrifuged at 4 °C for 15 min at 12 000 g. After centrifugation, 300 µl of the upper phase was transferred to an RNase free 1.5 ml eppendorf tube, and isopropanol was added in 1:1 proportion. The content was mixed by hand and the mixture was incubated at room temperature for 10 min. The tube was centrifuged at 4 °C for 10 min at 12 000 g. The supernatant was removed, and the pellet was washed twice with 500
µl cold 75 % ethanol. The tube was centrifuged at 4 °C for 5 min at 7500 g following the two washings. After removing the liquid the last time, the samples were centrifuged briefly using a tabletop microcentrifuge (Galaxy MiniStar centrifuge, VWR, Radnor, PA, USA), to remove the last traces of ethanol. The pellet was set to dry for 5-10 min at room temperature before being eluted in 20 µl RNA Storage Solution (Ambion). The eluted pellet was incubated at 50 °C for 10 min before DNase treatment using the TURBO DNA-free kit (Ambion) to remove any contaminating DNA from the sample. The DNase treatment was performed according to the manufacturers’ protocol, adding 0.1 volume 10x TURBO DNase buffer and 1 µl TURBO DNase to the RNA and mixing the content gently. The tube was incubated at 37 °C for 20-30 min. Then, 0.1 volume of resuspended DNase inactivation reagent was added, and everything was mixed well. The sample was incubated for 5 min at room temperature with occasional mixing. The tube was centrifuged at 10 000 g for 1.5 min, and after the centrifugation, the supernatant, which contains the RNA, was carefully transferred to an eppendorf tube and stored at -80 °C.

For quantity control, the RNA was tested using the NanoDrop spectrophotometer (NanoDrop, Thermo Fisher scientific, USA). Only RNA with optical density (OD) 260/280 between 1.8 and 2 was used for synthesis of cDNA. The RNA was also tested using the Agilent 2100 bioanalyzer and a RNA labchip kit (Agilent Technologies, USA) to determine the RNA integrity. When using this method, only RNA with an RNA integrity number (RIN) higher than 8 was used.
2.3 cDNA synthesis

First-strand cDNA was prepared using Superscript III reverse transcriptase (RT) (Invitrogen), according to the protocol supplied by Invitrogen. To a 0.5 ml microcentrifuge tube, 1 µl of random hexamer primers (50 ng/µl) (Invitrogen), 1 µl 10 mM dNTP mix, and 1 µg total RNA were added. Nuclease-free water (Ambion) was added to a total volume of 13 µl. The mixture was heated at 65 °C for 5 min and incubated on ice for at least 1 min. By a brief centrifugation using a tabletop microcentrifuge, the contents of the tube were collected, and 4 µl 5x first-strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT recombinant RNase inhibitor (Invitrogen, 40 U/µl) and 1 µl of Superscript III RT (Invitrogen, 200 U/µl) were added. The content was mixed by pipetting gently up and down before incubating at 25 °C for 5 min. The tube was then incubated at 50 °C for 60 min followed by heat inactivation at 70 °C for 15 min. The cDNA was stored at -20 °C until use.

2.3.1 RACE cDNA synthesis

RACE cDNA was made using the protocol for the SMARTer RACE cDNA amplification kit (Clontech, CA, USA). For 5’ and 3’ RACE cDNA, 1 µg RNA and 1 µl 5’-3’-CDS primer A were added to separate microcentrifuge tubes. Nuclease-free water (Ambion) was added to each tube to a total volume of 3.75 µl for 5’ RACE and 4.75 µl for 3’ RACE before a brief spin in a microcentrifuge. The tubes were incubated at 72 °C for 3 min, then at 42 °C for 2 min. After the cooling step, the tubes were centrifuged for 10 s at 14 000 g. To the 5’ RACE cDNA synthesis reaction, 1 µl of SMARTer IIA oligo was added.

To the RNA, 2.0 µl 5x first-strand buffer, 1.0 µl DTT (20 mM), 1.0 µl dNTP mix (10 mM), 0.25 µl RNase inhibitor (40 U/µl), and 1.0 µl SMARTScribe reverse transcriptase (100 U) (all from Clontech) were added to a total volume of 10 µl. The contents of the tubes were mixed by gently pipetting, and then incubated at 42 °C for 90 min followed by incubation at 70 °C for 10 min. The first-strand reaction product was then diluted with tricine-EDTA buffer by adding 20 µl if starting with ≤ 200 ng of total RNA or by adding 100 µl if starting with ≥ 200 ng of total RNA. The RACE cDNA was stored at -20 °C until use.
2.4 Cloning and sequencing

None of the target genes had been sequenced previously in the Atlantic cod. However, the $K_{Ca}$ channels have been sequenced in other fish species, and these sequences were used to make a consensus sequence for each of the five genes. The consensus sequences were used when performing a BLAST (Basic Local Alignment Search Tool) search on the University of Oslo Bioportal (www.bioportal.uio.no), searching in a database containing the newly sequenced cod genome. The cod genome was not published when the work started, but after some time we had access to it through the University of Oslo Bioportal (www.bioportal.uio.no). The results from these BLAST searches were transferred to the software Vector NTI (Invitrogen) and used as templates for primer design for rapid amplification of cDNA ends (RACE) PCR and reverse transcriptase (RT) PCR. Through this approach, fragments of three $K_{Ca}$ channel genes were isolated: BK, SK1 and IK, whereas the two remaining channels, SK2 and SK3, could not be identified in the genome database. Based on the large degree of similarity between the SK channels, gene specific primers for SK2 and SK3 were also designed based on conserved sequences from other fish species. However, despite numerous attempts using both RACE- and RT PCR, I was not able to isolate SK2 or SK3.

2.4.1 Primer design

Primers were designed using Vector NTI (Invitrogen). Primers for 5’ RACE and 3’ RACE were designed for the genes encoding the BK, SK1 and IK channel, based on partial sequences obtained from BLAST search in the cod genome database (see above). Based on the consensus sequences from different fish species, primers for 5’ RACE and 3’ RACE were designed for the genes encoding the SK2 and SK3 channel. The RACE primers were designed using the following criteria: $T_m$ (melting temperature) between 70.0 °C and 75.0 °C, GC content between 50.0 % and 70.0 %, and primer length between 23 and 50 bp.

RACE primers were first designed for all of the five genes. For the genes that gave sequencing results using RACE primers, these sequencing results made the basis for RT-PCR primer design. For two of the genes, SK2 and SK3, RACE primers did not give any results. For these genes, RT-PCR primers were designed based on the consensus sequences from
different fish species. After running RACE-PCR and obtaining sequencing results, new RT-PCR primers were designed based on the sequencing results. These primers were designed to confirm overlapping sequences from 5'-RACE and 3'-RACE, and to fill in gaps between sequences from 5'-RACE and 3'-RACE. The criteria used for RT-PCR primers were: T_m between 50 °C and 60 °C, GC content between 40.0 % and 60.0 % and primer length between 18 and 25 bp. All primers were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany) and diluted to 5 µM before use.

2.4.2 RACE-PCR

RACE-PCR was performed to obtain the cDNA ends of the sequences for the five genes; BK, SK1, SK2, SK3, and IK. The RACE-PCR was carried out using the SMARTer RACE cDNA amplification kit (Clontech). The SMART technology allows first-strand cDNA to be used directly in 5'- and 3' RACE-PCR reactions, after reverse transcription. The cDNA used for RACE-PCR has universal primer binding sites incorporated, which eliminates the need for second-strand synthesis and adaptor ligation (SMARTer RACE cDNA Amplification Kit, www.clontech.com).

RACE was performed on RACE cDNA from both female and male Atlantic cod brains, using 10x advantage 2 PCR buffer, dNTP mix (10 mM), and 50x advantage 2 polymerase mix (all from Clontech). For 5' RACE and 3' RACE, 10x universal primer A mix (Clontech, 10 µM) or nested universal primer A (Clontech, 10 µM) was used as sense and antisense primer, respectively. Gene specific primers designed in Vector NTI (Invitrogen) were used as antisense primers for 5' RACE and sense primers for 3' RACE. RACE-PCR was performed by touchdown PCR, where the first 7 cycles had an initial denaturation step for 30 s at 94 °C, followed by an annealing step for 30 s where the temperature dropped one degree for each cycle from 72–65 °C, and an elongation step for 3 min at 72 °C. After these 7 cycles, an additional 30 cycles consisting of 30 s at 94 °C, 30 s at 65 °C, and 3 min at 72 °C was performed. The PCR ended with a final extension step for 5 min at 72 °C.
2.4.3 RT-PCR

RT-PCR was used to confirm overlapping sequences from 5’- and 3’- RACE, and to fill in gaps. RT-PCR was performed on brain first-strand cDNA from Atlantic cod, using 10x AccuPrime PCR buffer II and AccuPrime Taq DNA polymerase (both from Invitrogen). The RT-PCR was carried out using an initial denaturation step for 2 min at 94 °C, followed by 35 cycles consisting of 15 s at 94 °C, 15 s at 48-50 °C (depending on the primers), and 2 min at 68 °C. After the 35 cycles, there was a final step for 5 min at 68 °C.

2.4.4 Cloning protocol

After running RACE-PCR and RT-PCR, the PCR products were analyzed by agarose gel electrophoresis on a 1 % agarose gel stained with SYBR Safe (Invitrogen). Electrophoresis was conducted using an electrical field of 4 V/cm for 40-60 min. DNA was extracted from the gel using a modified version of the protocol for QIAGEN QIAquick gel extraction kit (Qiagen, Hilden, Germany). The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was placed in a 1.5 ml eppendorf tube, and 1 ml of buffer QG (solubilization buffer) was added. The tube was incubated at room temperature until the gel slice had completely dissolved. To bind DNA, the sample was transferred to a QIAquick spin column placed in a 2 ml collection tube. The QIAquick spin column was centrifuged at room temperature for 1 min using a microcentrifuge. Flow-through was discarded and the QIAquick column was placed back in the same tube. An additional 500 ml of solubilization buffer was added, the tube was centrifuged once more and the flow-through discarded. For washing, 500 ml buffer PE (wash buffer) (with ethanol, 96-100 %, added) was added to the QIAquick column and it was centrifuged for 1 min in a microcentrifuge. The flow-through was discarded and the column was placed back in the same tube. The tube was then centrifuged at 20 600 g at room temperature for an additional 1 min (Kubota 3500). After this, the QIAquick column was placed in a 1.5 ml eppendorf tube, and remaining drops of liquid were removed from the inside of the QIAquick spin column. To elute DNA, 20 µl of elution buffer (preheated to 50 °C) was added to the center of the QIAquick membrane. It was
incubated at room temperature for 5 min, and then centrifuged at 20,600 g for 1 min at room temperature. The sample was stored at -20 °C until use.

For ligation, the pGEM-T Easy vector system (Promega, Madison, WI, USA), was used. The DNA fragment was ligated into the pGEM-T Easy vector (50 µg/µl) with the use of 5 µl 2x rapid ligation buffer and 1 µl T4 DNA Ligase (3 U/µl). Three µl of ligation mix was transformed into 100 µl JM109 competent cells (>10⁸ cfu/µg) (Promega), according to the manufacturer’s protocol. Transformed cells were cultured on LB plates containing 30 µl ampicillin (100 mg/ml), 20 µl X-Gal (50 mg/ml) (Promega) and 100 µl IPTG (0.1 M) at 37 °C over night. Five positive colonies and 1 negative colony were picked from each plate and grown in separate 50 ml falcon tubes, each containing 10 ml LB medium and 10 µl ampicillin (100 mg/ml). The tubes were incubated at 37 °C with shaking for 8-16 hr.

To check if the transformation had been successful, the sample was run on a 1 % agarose gel stained with SYBR Safe (Invitrogen). One hundred µl of the liquid culture was mixed with 50 µl phenol-chloroform and 3 µl 6x blue/orange loading dye (Promega). The samples were vortexed prior to a centrifugation at 20,600 g for 5 min at room temperature (Kubota 3500). Eighteen µl of the upper phase was loaded in each well in the gel before starting the gel electrophoresis.

From competent cells transformed with the vector containing the insert, DNA was isolated using a modified version of the protocol for QIAGEN plasmid mini kit (Qiagen). The 50 ml falcon tubes containing the liquid culture were centrifuged using a Beckman Coulter Allegra X-22R centrifuge at 3901 g for 15 min at 4 °C. After centrifugation, the liquid was removed, and the bacterial pellet was resuspended in 600 µl of resuspension buffer (P1). The mixture was vortexed, and everything had to be dissolved before adding 600 µl of lysis buffer (P2). The content was mixed by inverting the tube 2-3 times. It is important not to vortex at this step, as this will result in shearing of genomic DNA. The tube was incubated at room temperature for 4-5 min. Six hundred µl of neutralization buffer (P3, stored on ice) was added and immediately mixed with the other content by inverting the tube 2-3 times, before the tube was incubated on ice for 5 min. After incubation, the sample was transferred to a 2 ml eppendorf tube, and centrifuged at 20,600 g for 5 min at 4 °C (Kubota 3500). To equilibrate the QIAGEN-tip 20, 1 ml of equilibration buffer (QBT) was applied to the column. The column was allowed to empty by gravity into a waste tray. The liquid from the centrifuged
tube (containing buffer P1, P2 and P3) was then transferred to the equilibrated column. The liquid entered the resin by gravity flow. The QIAGEN-tip 20 was then washed 2 times with 2 ml of washing buffer. When the washing buffer had moved through the QIAGEN-tip 20 by gravity flow, the column was placed in a 1.5 ml eppendorf tube, and DNA was eluted by adding 800 µl elution buffer to the column. The flow-through was collected in the eppendorf tube, and 540 µl isopropanol was added to the flow-through. The content was mixed well, and the tube was centrifuged at 20 600 g for 30 min at 4 °C. After centrifugation, the liquid was removed, and the pellet was washed by adding 1 ml 70-75 % ethanol to the tube. The tube was centrifuged at 20 600 g for 5-10 min at 4 °C. After centrifugation, the ethanol was removed and the tubes were left with the lid open until the ethanol had evaporated and the pellet was almost transparent. The DNA was dissolved in 20 µl nuclease-free water (Ambion), and the samples were stored at -20 °C until use.

2.4.5 Sequencing

The plasmid DNA used for sequencing had a concentration of between 250 and 500 ng/µl. For sequencing, the forward primer pUC/M13 (Promega, 10 µg/ml) and the reverse SP6 promoter primer (Promega, 10 µg/ml) were used. To a 0.5 ml tube, 3.5 µl primer, 1 µl plasmid DNA and 5.5 µl nuclease-free water (Ambion) were added. The samples were delivered for sequencing at the ABI-lab at the University of Oslo.
2.5 Sequence analyses

Fish K<sub>Ca</sub> channel sequences were obtained by searching at NCBI (http://www.ncbi.nlm.nih.gov/). Additionally, similar sequences were obtained from at least one representative species from the other vertebrate classes whenever possible; *Homo sapiens*, *Macaca mulatta*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Sus scrofa*, *Bos taurus*, *Oryctolagus cuniculus* (mammals); *Gallus gallus*, *Taeniopygia guttata* (birds); *Trachemys scripta* (reptile); *Xenopus laevis* and *Xenopus tropicalis* (amphibians). For GenBank accession numbers used in the analysis, see Table 1, 2 and 3.

The Atlantic cod genome, available on the University of Oslo Bioportal (www.bioportal.uio.no), was used to analyze the sequencing results. Multiple sequence alignments and pairwise comparisons of the collected amino acid sequences were performed using the CLC DNA Workbench Version 5.7.1.

Table 1. Latin name, common name and GenBank accession numbers for the BK channel sequences used in this study.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Gene</th>
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<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK</td>
<td>KCNMA1</td>
<td>Anguilla anguilla</td>
<td>European eel</td>
<td>EU267177.1</td>
</tr>
<tr>
<td>BK</td>
<td>KCNMA1</td>
<td>Anguilla rostrata</td>
<td>American eel</td>
<td>FJ265725.1</td>
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<td>BK</td>
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<td>Cattle</td>
<td>NM_174680.2</td>
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<td>Chicken</td>
<td>NM_204224.1</td>
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<td>BK</td>
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<td>Three-spined stickleback</td>
<td>FJ269030.1</td>
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<td>Human</td>
<td>NM_002247.3</td>
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<td>Rhesus monkey</td>
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<td>House mouse</td>
<td>NM_010610.2</td>
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<td>BK</td>
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<td>Rainbow trout</td>
<td>FJ269023.1</td>
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<td>Pan troglodytes</td>
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<tr>
<td>BK</td>
<td>KCNMA1</td>
<td>Porichthys notatus</td>
<td>Plainfin midshipman</td>
<td>FJ269026.2</td>
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<tr>
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<td>KCNMA1</td>
<td>Rattus norvegicus</td>
<td>Norway rat</td>
<td>NM_031828.1</td>
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<td>BK</td>
<td>KCNMA1</td>
<td>Sus scrofa</td>
<td>Pig</td>
<td>NM_214219.1</td>
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<td>BK</td>
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<td>Trachemys scripta</td>
<td>Red-eared slider turtle</td>
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<td>BK</td>
<td>KCNMA1</td>
<td>Xenopus laevis</td>
<td>African clawed frog</td>
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Table 2. Latin name, common name and GenBank accession numbers for the SK1 channel sequences used in this study.

<table>
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<th>Channel</th>
<th>Gene</th>
<th>Latin name</th>
<th>Common name</th>
<th>GenBank Accession number</th>
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<tbody>
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<td>SK1</td>
<td>KCNN1</td>
<td><em>Bos taurus</em></td>
<td>Cattle</td>
<td>XM_002688571.1</td>
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<td>SK1</td>
<td>KCNN1</td>
<td><em>Danio rerio</em></td>
<td>Zebrafish</td>
<td>BC153898.1</td>
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<td><em>Homo sapiens</em></td>
<td>Human</td>
<td>NM_002248.3</td>
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<td>Rhesus monkey</td>
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<td>SK1</td>
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<td><em>Musculus</em></td>
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<td><em>Oryctolagus cuniculus</em></td>
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<td>XM_002723873.1</td>
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<td>KCNN1</td>
<td><em>Pan troglodytes</em></td>
<td>Chimpanzee</td>
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<td><em>Petromyzon marinus</em></td>
<td>Sea lamprey</td>
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<td>SK1</td>
<td>KCNN1</td>
<td><em>Rattus norvegicus</em></td>
<td>Norway rat</td>
<td>NM_019313.1</td>
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<tr>
<td>SK1</td>
<td>KCNN1</td>
<td><em>Scophthalmus maximus</em></td>
<td>Turbot</td>
<td>EF495177.1</td>
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<td>SK1</td>
<td>KCNN1</td>
<td><em>Sus scrofa</em></td>
<td>Pig</td>
<td>XM_003123502.1</td>
</tr>
<tr>
<td>SK1</td>
<td>KCNN1</td>
<td><em>Xenopus (Silurana) tropicalis</em></td>
<td>Western clawed frog</td>
<td>NM_001079016.1</td>
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</table>

Table 3. Latin name, common name and GenBank accession numbers for the IK channel sequences used in this study.

<table>
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<tr>
<th>Channel</th>
<th>Gene</th>
<th>Latin name</th>
<th>Common name</th>
<th>GenBank Accession number</th>
</tr>
</thead>
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<td>IK</td>
<td>KCNN4</td>
<td><em>Danio rerio</em></td>
<td>Zebrafish</td>
<td>XP_696782.5</td>
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<tr>
<td>IK</td>
<td>KCNN4</td>
<td><em>Homo sapiens</em></td>
<td>Human</td>
<td>NM_002250.2</td>
</tr>
<tr>
<td>IK</td>
<td>KCNN4</td>
<td><em>Mus musculus</em></td>
<td>House mouse</td>
<td>NM_008433.4</td>
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<td>IK</td>
<td>KCNN4</td>
<td><em>Rattus norvegicus</em></td>
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<tr>
<td>IK</td>
<td>KCNN4</td>
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<td><em>Tetraodon nigroviridis</em></td>
<td>Spotted green puffer fish</td>
<td>CAF94198.1</td>
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2.6 Quantification of mRNA expression using qPCR assay

The qPCR assay for quantification of mRNA expression was developed and validated using the LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany), with SYBR green I non-specific detection. Since SYBR green I binds to any double-stranded DNA, the qPCR assays had to be carefully evaluated by performing a melting curve analysis and PCR amplicon sequencing. Melting curve analysis is performed immediately following the PCR, within the same LightCycler 480 machine, without breaking the seal over the samples. This removes the risk of contaminations and pipetting errors.

2.6.1 qPCR primer design

Primers for qPCR, based on the cloned and sequenced $K_{Ca}$ channels, were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were designed to bind different exons, or if possible, bind to exon-exon borders. The settings for the primer design were: Primer length between 18 and 22 bp, primer $T_m$ between 58 °C and 62 °C, and primer GC content between 40 % and 60 %. Maximum $T_m$ difference was set to 2 °C, maximum PolyX was set to 3, and the PCR product size should be between 60 and 90 bp. Potential primers were analyzed for hairpins, primer dimers and $\Delta G$ value using Vector NTI (Invitrogen). Only primers that theoretically did not make hairpins or primer dimers, and that had a $\Delta G$ value $>-4$ kcal/mol were tested in the qPCR. Because of difficulties designing primers for the KCNN1 gene (SK1 channel), the size of the PCR product had to be increased to 117 bp. Primers for the reference genes EF1α, β-actin, Arp2/3 and ubiquitin were designed and validated by other members of my group.

The primers were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany) and diluted to 5 µM before use. For qPCR primer sequences, see Table 4.
Table 4. List of primers used for qPCR experiments on cod. KCNMA1 (BK channel), KCNN1 (SK1 channel), and KCNN4 (IK channel). Primer sequences for the reference genes EF1α, β-actin, Arp2/3, and ubiquitin are also listed in the table. The primers are listed in the 5’ to 3’ direction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→ 3’)</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNMA1 (BK channel)</td>
<td>Forward: GGCCTGAGGTTGATACAGT</td>
<td>87 bp</td>
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<tr>
<td></td>
<td>Reverse: CCATCAAGCTAGTGAACCTGTG</td>
<td></td>
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<tr>
<td>KCNN1 (SK1 channel)</td>
<td>Forward: CGACACCACCAGAGAAGTT</td>
<td>117 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACATCGCAAAGACCCAGAAC</td>
<td></td>
</tr>
<tr>
<td>KCNN4 (IK channel)</td>
<td>Forward: CTCCGAAGATGCAGATGAT</td>
<td>60 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACTGGAACAACTCGTACCAGG</td>
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<tr>
<td>EF1α</td>
<td>Forward: CTTCAACGCCCAGGTGTCAT</td>
<td>100 bp</td>
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<tr>
<td></td>
<td>Reverse: AACTTGCGAGGCGATGAGG</td>
<td></td>
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<tr>
<td>β-actin</td>
<td>Forward: TTCTACAACGAGCTAGAGTG</td>
<td>102 bp</td>
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<tr>
<td></td>
<td>Reverse: CATGATCTGGGTGTCATTCTTCCC</td>
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<tr>
<td>Arp2/3</td>
<td>Forward: GGAGGTTAGAAGTAGCAAGGAGC</td>
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<td></td>
<td>Reverse: TGCTGACTCTCACGGAGGTTG</td>
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<tr>
<td>Ubiquitin</td>
<td>Forward: TTGAGCGCTTCCTCAGAATG</td>
<td>120 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAAGAAGAAGTGCGGACACA</td>
<td></td>
</tr>
</tbody>
</table>

2.6.2 qPCR assay

qPCR was performed on cDNA from female cod using LightCycler 480 SYBR Green I Master kit (Roche). For testing and validation of primers, a total reaction volume of 10 µl was used; containing 5 µl of SYBR Green I master mix (Roche), 1 µl (5 µM) of forward primer, 1 µl (5 µM) of reverse primer, and 3 µl of diluted cDNA. A non-template control (NTC) was included for every primer pair on every qPCR plate, and contained nuclease-free water (Ambion) instead of cDNA. For testing of primer pairs specific for the KCa channels, all samples were run in triplicate, and each run included a NTC, a RNA (minus RT) control, and a genomic DNA control.
After the initial Taq activation at 95 °C for 10 min, LightCycler PCR was performed using 42 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. Directly after the PCR, a melting curve analysis was performed by continuously reading the fluorescence while slowly increasing the temperature from 65 to 98 °C.

For making dilution curves, the relationship between increasingly diluted cDNA and the corresponding Cq (quantification cycle) value was used. After running a qPCR with the dilution series, the Cq values were plotted against the logarithm of the relative cDNA concentration. The qPCR efficiency (E) was calculated using the slope of the regression line (Equation 1). A slope of -3.32 gives an efficiency of the qPCR equal to 2 (100 %), which means that there is a doubling of product for each PCR cycle.

\[
\text{Efficiency} = 10^{-\frac{1}{\text{slope}}} \quad \text{(Equation 1)}
\]

For the gene expression analysis, each assay included a calibrator, to adjust for assay to assay variations, and this was run in duplicate for both target and reference genes. The reference genes tested for use on pituitary cDNA was EF1α, β-actin, Arp2/3, and ubiquitin. See Table 4. cDNA used for gene expression analysis was in a 1:10 dilution. An efficiency-correlated relative expression method (Equation 2) was used as quantification of relative expression levels because the efficiency was higher than 2 for some of the genes (Weltzien et al., 2005):

\[
\text{Relative expression} = E_{\text{target}}^{\Delta Cq(\text{calibrator-sample})} \times E_{\text{reference}}^{\Delta Cq(\text{sample-calibrator})} \quad \text{(Equation 2)}
\]
2.6.3 Confirming qPCR-product identity

As part of the development of the qPCR assays, the identity of the qPCR products were confirmed by agarose gel electrophoresis and sequencing.

2.6.3.1 Agarose gel electrophoresis

The qPCR products were analyzed by electrophoresis using a 2 % agarose gel stained with SYBRsafe DNA gel stain (Invitrogen) in order to visualize the nucleic acids. Before loading the qPCR products on the gel, 2 µl of 6x blue/orange loading dye (Promega) were added to 8 µl of qPCR product. Electrophoresis was conducted using an electrical field of 3 V/cm for approximately 60 min.

2.6.3.2 Sequencing

The qPCR products were purified using the Wizard SV gel and PCR clean-up system from Promega. Two µl forward or reverse primers (5 µM) were added to 8 µl 10x diluted purified qPCR sample before the samples were sequenced at the ABI lab at the University of Oslo.
2.7 Single-cell qPCR analysis of $K_{Ca}$ channels

Our group has recently developed a protocol allowing gene expression analysis from single cells in culture (Hodne et al., 2010). This single-cell qPCR method was used for analyzing the presence of $K_{Ca}$ channels in the two gonadotrope cell types.

2.7.1 Tissue sampling for single-cell qPCR analysis

The cod were treated and sacrificed as described in section 2.1. After decapitation, pituitaries destined for primary cell culture were transferred to ice-cold L-15 growth medium (Invitrogen) containing penicillin (25 U/ml) and streptomycin (25 µg/ml; both from Invitrogen), 1.8 mM glucose, osmolarity adjusted to 325 mOsm and pH adjusted to 7.80.

2.7.2 Primary culture of pituitary cells from Atlantic cod

The procedure for preparing primary pituitary cultures was adapted from a similar procedure used successfully for the European eel (Montero et al., 1996) and goldfish (Chang et al., 1990), see Hodne et al. (2010) for details. The pituitaries were transferred from the modified L-15 medium and washed in ice cold PBS (Invitrogen) with penicillin (25 U/ml) and streptomycin (25 µg/ml; both from Invitrogen), before chopped in approximately 1 mm$^3$ pieces. Subsequently, the tissue fragments were subjected to a combination of chemical and mechanical dissociation before the dispersed cells were plated in 35 mm plastic petri dishes coated with poly-L-lysine (Sigma, MO, USA). The cell densities in each dish were between 150 000 and 200 000 cells/cm$^2$. The dishes were kept at 12 °C in a humidified atmosphere, with 0.5 % CO$_2$ and 99.5 % air.
2.7.3 Harvesting of cytosol

From the primary pituitary cultures, cytosol from single cells was harvested using a patch pipette made with a P-97 horizontal puller (P-97 Sutter Instrument CO, CA, USA). The patch pipette was silanized in Sigmacote (Sigma), before fire polishing. The patch pipette was filled with 3-4 µl β-escin-containing RNase-free intracellular solution. The intracellular solution consisted of (mM): 120 CH₃O₃SK, 20 KCl, 20 sucrose, 10 HEPES/NaOH, pH 7.2, adjusted to 300 mOsm. All the chemicals in the intracellular solution were purchased from Sigma and certified ultrapure or RNase-free.

Prior to electrophysiological recording and harvesting of cytosol, the growth medium in the petri dish containing the dispersed pituitary cells, was replaced with extracellular solution. The extracellular solution consisted of (mM): 150 NaCl, 5 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 1.8 glucose, 0.1 % BSA, 10 HEPES/NaOH, pH 7.8, adjusted to 320 mOsm. All the chemicals in the extracellular solution were, similar to the intracellular solution, purchased from Sigma and certified ultrapure or RNase-free. The petri dish was placed on a microscope stage cooled to 12 °C by a peltier element (TC-10, Dagan Corporation, MN, USA). The cells were observed through an inverted phase-contrast microscope (Nikon, Tokyo, Japan).

As described in Hodne et al. (2010), a giga seal was made between the patch pipette and the cell membrane. Instantly after a giga seal was made, β-escin started to perforate the membrane, thus creating electrical contact and making electrophysiological recordings possible.

Following these recordings, which will not be reported in this thesis, a complete hole was made in order to harvest the cytosol. The cytosol was harvested according to the protocol by Hodne et al. (2010), and after the harvesting was stopped, the content in the pipette was transferred to an RNase-free 0.5 ml tube (Ambion) containing 7 µl of a sodium citrate EDTA- and EGTA-free solution (Ambion). One µl (40 U/µl) Rnasin Plus RNase Inhibitor (Promega) was added to the tube, to inactivate RNA-degrading RNases. The outermost part of the content in the pipette was transferred to the tube by applying positive pressure. The positive pressure was maintained, and the tip of the pipette was crushed gently against the wall of the
tube. In this way, the remaining cytosol content in the pipette was emptied into the tube. The tube was snap frozen in liquid nitrogen before being stored at -80 °C until further processing.

2.7.4 Synthesis of cDNA for single-cell qPCR

cDNA for single-cell qPCR was prepared directly from the harvested cytosol according to the protocol in Hodne et al. (2010). After the cDNA synthesis, the cDNA was precipitated by adding 2.5 µl 2 M sodium-acetate (pH 4.0), 1 µl glycogen (1 µg), 1 µl poly C RNA (250 ng), 1 µl poly cD DNA (250 ng) and 85 µl 96 % ethanol. The mixture was then stored at -20 °C until the next day. Then, the mixture was centrifuged at 4 °C for 60 min at 20 600 g. After centrifugation, the supernatant was removed and the pellet was washed by adding 100 µl 75 % ethanol to the tube. The tube was then centrifuged at 4 °C for 15 min at 20 600 g. The pellet was dried at a 40 °C heating block, before it was eluted in 10 µl nuclease-free water and heated at 45 °C for 180 min. The tube was centrifuged every 60 min, and placed at -20 °C after the precipitation was finished.

2.7.5 Single-cell qPCR assays

qPCR assays were developed and validated using SYBR green I detection dye on a LC480 platform (both from Roche), as described by Hodne et al. (2010). The qPCR was performed as described in section 2.6.2. Primers for FSHβ and LHβ (Table 5) were used in the qPCR analyses, to investigate if the cells were FSH-producing or LH-producing gonadotropes. These qPCR primers were designed, developed and tested by Hodne et al. (2010).

Table 5. qPCR primers used for identifying gonadotropes in single-cell qPCR analyses in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' → 3')</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>fshβ</td>
<td><strong>Forward</strong> GAACCGAGTCCATCAACACC</td>
<td>63 bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> GGTCATCGGGGTCTCCT</td>
<td></td>
</tr>
<tr>
<td>lhβ</td>
<td><strong>Forward</strong> GTGGAGAAGAAGGGCTGTCC</td>
<td>81 bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> GGACGGGTCCATGGTG</td>
<td></td>
</tr>
</tbody>
</table>
2.8 Statistical analyses

Statistical calculations were performed using one-way ANOVA with Tukey’s post test in GraphPad Prism version 4.00 for Mac (GraphPad Software, San Diego, California, USA). All data are expressed as mean + standard error of mean (SEM) unless otherwise stated. For the tissue distribution analyses, the statistical calculations were performed using student t-test for comparing two values (here; the data from two months). The level of significance was set to $P < 0.05$. 
3. Results

3.1 Cloning and sequence analysis

3.1.1 Amino acid sequence comparisons of the $K_{Ca}$ channels in Atlantic cod

Sequences for the BK, SK1 and IK channel from Atlantic cod were obtained by performing RACE- and RT-PCR on brain cDNA from female and male Atlantic cod. Despite numerous attempts by RACE- and RT-PCR, in addition to data mining in the cod genome, I could not identify any other SK channels (SK2 and SK3) that potentially might be expressed in cod.

The three sequences for the BK, SK1 and IK channels cloned in this study include the consensus amino acid “signature sequence” for $K^{+}$ channels; GYGD, located in the pore region (Figure 8). This sequence made the basis for multiple sequence alignments identifying conserved regions within the genes, and made it possible to evaluate the outcome of the cloning.
Figure 8. Schematic representation of the general structure of vertebrate $K_{Ca}$ channels. Green/pink lines indicate the obtained sequences of the three cloned Atlantic cod $K_{Ca}$ channels. A) Sequence analysis of the Atlantic cod BK channel sequence identified the S1-S6 transmembrane segments, S7 and S8 intracellular segments, and the pore region. B) Sequence analysis of the Atlantic cod SK1 channel sequence identified the S3-S6 transmembrane segments, the pore region and the two calmodulin binding domains CaMBDα1 and CaMBDα2. C) Sequence analysis of the Atlantic cod IK channel sequence only identified the part of the pore containing the GYGD signature sequence for $K^+$ channels and a small part of the sequence downstream of this.

3.1.1.1 BK

Based on the alignment of the (KCNMA1) BK channel, I identified putative S2, S3, S4, S5, and S6 transmembrane segments, intracellular S7 and S8 segments, and the pore region (Figure 8). However, the S0 transmembrane segment and the intracellular S9 and S10 segments were not identified in the Atlantic cod sequence, indicating that a small part of the 5’ end and 3’ end of the sequence has not been obtained in my work. The putative S2, S3, S4, S5 and S6 transmembrane segments, intracellular S7 and S8 segments and the pore region were all a part of the manually sequenced Atlantic cod BK channel. The sequence was
obtained by RACE- and RT-PCR. When analyzing the sequence results, several C’s were identified in both ends of the sequence. This can influence the RACE primers, and prevent obtaining the 5’ and 3’ ends of the sequence. Analysis using the University of Oslo Bioportal and BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) did not identify more of the Atlantic cod BK channel sequence. The manually sequenced part of the BK channel can be found in Appendix II.

The alignment of the KCNMA1 sequences was performed using both full-length sequences and partial sequences from different vertebrates, but only a part of the alignment is presented (Figure 9), due to the variation in size of the sequences. The alignment displays a large degree of conservation among different vertebrate species, including the teleosts. The pore region and the S6 transmembrane segment are considered to be the most conserved regions within the KCNMA1 gene. The first amino acid in the pore region differs between the species. The teleosts and the amphibian species in this alignment have the amino acid serine in this position, while the other species (mammals, birds and reptiles) have the amino acid asparagine in this position. Both serine and asparagine are amino acids with uncharged polar side chains. The amino acid in the third position in the pore region varies between several of the species in the alignment, and also between species within the same vertebrate classes. The fifth amino acid in the pore region varies in the same way as amino acid number one. Teleosts and amphibian species have the amino acid serine in this position, while other species have the amino acid threonine in this position. Also threonine has an uncharged polar side chain. The S6 transmembrane segment is 100 % conserved among the species in the alignment of the KCNMA1 gene.
3.1.1.2 SK1

In the alignment of the SK1 channel (KCNN1), the putative S3, S4, S5 and S6 transmembrane segments, the pore region, and the intracellular calmodulin binding domains (CaMBD) α1 and CaMBDα2 were identified (Figure 8). By performing RACE- and RT-PCR, the pore region, the S6 transmembrane segment and the two CaMBDs were identified. Data mining analyses also identified the S3, S4 and S5 transmembrane segments. The manually sequenced SK1 channel, indicated in Figure 8, lacked a part of the 5’ end and the 3’ end of the sequence. Analysis using the University of Oslo Bioportal and BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/), resulted in more of the 5’ end of the sequence. However, in this work, the S1 and S2 transmembrane segments were not identified in the Atlantic cod sequence. As for the BK channel sequence, it was also identified several C’s in the 5’ and 3’ end of the sequence, interfering with the RACE primers. This is probably the reason why I was not able to identify both ends of the gene. The manually sequenced part of the SK1 channel can be found in Appendix III.

The alignment shows that also the amino acid sequences for the SK1 channel is conserved, but not to the same degree as the BK channel sequence. When analyzing the amino acids in the pore region and the S6 transmembrane segment for the SK1 channel, the conservation is well defined. The pore region, comprising 25 amino acids, shows variation at position 21. In some species we find histidine, while other species has an asparagine in this position. It is no clear pattern between the vertebrate classes. The Atlantic cod has the amino acid histidine at this position, and the same has two of the three other teleost species in the alignment. The S6 transmembrane segment is 100 % conserved in the alignment of the SK1 channel (Figure 10).
Figure 10. Conserved residues among vertebrate species for the SK1 channel. Alignment of amino acid sequences of the SK1 channel from Atlantic cod and other vertebrate species. Alignments were performed using CLC DNA Workbench software. Percent conservation between amino acid sequences is indicated at the bottom of the alignment in pink. The signature sequence for K⁺ channels, GYGD, is marked with a red box, the S1-S6 segments are highlighted in pink (only a part of S5 and the whole S6 segment are included in the figure), the pore region is highlighted in yellow, and the calmodulin-binding domain (CaMBD) α1 and α2 are highlighted in blue. Gadus morhua = Atlantic cod, Homo sapiens = human, Mus musculus = mouse, Rattus norvegicus = rat, Sus scrofa = pig, Oryctolagus cuniculus = rabbit, Bos taurus = cattle, Macaca mulatta = rhesus monkey, Pan troglodytes = chimpanzee, Xenopus tropicalis = Western clawed frog, Danio rerio = zebrafish, Scophthalmus maximus = turbot and Petromyzon marinus = sea lamprey.
The part of the IK channel (KCNN4) sequence obtained is considerably shorter than both the BK and the SK1 channel sequences. RACE- and RT-PCR was performed to obtain this sequence. Even though the RACE-PCR gave PCR products, it was not successful. A large part of the 3’ end of the sequence does not code for any protein. The obtained sequence can be found in Appendix IV. Sequence analyses have been performed using the University of Oslo Bioportal and BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/) to try to reveal more of the Atlantic cod IK channel. However, at this point I have not been able to identify additional parts of the sequence.

The GYGD part of the pore region in the IK channel is identified (Figure 11). The alignment further shows that there are more similarities between the Atlantic cod sequence and the sequences from the two fish species than there are between the Atlantic cod and the mammalian species. The degree of conservation is not as high in the alignment of the IK channel as was shown for the BK and SK1 channel (Figure 9 and 10).
Figure 11 Conserved residues among vertebrate species for the IK channel. Alignment of amino acid sequences of the IK from Atlantic cod and other vertebrate species. Alignments were performed using CLC DNA Workbench software. Percent conservation between amino acid sequences is indicated at the bottom of the alignment in pink. The signature sequence for K⁺ channels, GYGD, is highlighted in yellow and marked with a red box. *Gadus morhua* = Atlantic cod, *Homo sapiens* = human, *Mus musculus* = mouse, *Rattus norvegicus* = rat, *Sus scrofa* = pig, *Tetraodon nigroviridis* = spotted green puffer fish, *Danio rerio* = zebrafish.

3.1.2 Sequence analysis

An amino acid alignment of the three K<sub>Ca</sub> channels isolated from Atlantic cod and other vertebrates was performed. The alignment was performed in the area where sequences from all the three K<sub>Ca</sub> channels were obtained. The sequence of the Atlantic cod IK channel was
thus the restraint for this alignment. The deduced amino acid sequences reveal that the three genes group together in three distinct groups, where there are more similarities between the SK1 and IK channel sequences, than it is between the BK channel sequence and the two other sequences (see Figure 12 and Table 6). The alignment also shows that the teleost species within each channel type group together, and that there are some amino acid differences between teleosts and the other vertebrate species investigated, as described in section 3.1.1.1, 3.1.1.2 and 3.1.1.3.

The figure continues on the next page.
Figure 12 Conserved residues among vertebrate species for the three sequenced $K_{Ca}$ channels. Alignment of amino acid sequences for the BK, SK1 and IK channel. The alignment includes a part of the pore region containing the GYGD signature sequence, and approximately 200 amino acid residues downstream of this region. Glycine = G, Alanine = A, Valine = V, Leucine = L, Isoleucine = I, Methionine = M, Proline = P, Phenylalanine = F, Tryptophan = W, Serine = S, Threonine = T, Asparagine = N, Glutamine = Q, Tyrosine = Y, Cysteine = C, Lysine = K, Arginine = R, Histidine = H, Aspartic acid = D, and Glutamic acid = E.
Based on the alignment (Figure 12), the three Atlantic cod sequences have been pairwise compared with the other species in the alignment. Some of the species, and the sequence identity between them and the Atlantic cod BK, SK1 and IK channels, are presented in Table 6. The sequence identity of the same gene in different fish species are all above 83 %. The Atlantic cod BK channel shows the largest sequence identity with BK channel sequences from other species, with sequence identities above 96 %. The SK1 channel sequences have sequence identities above 77 %, and the IK channel shows sequence identity between species above 52 %.

Table 6. Percent identity between the Atlantic cod BK, SK1 and IK channel sequences and those of representatives of other teleost and mammalian species. Values in bold identify comparisons between the same gene.

<table>
<thead>
<tr>
<th></th>
<th>Atlantic cod BK channel (KCNMA1)</th>
<th>Atlantic cod SK1 channel (KCNN1)</th>
<th>Atlantic cod IK channel (KCNN4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK-H. sapiens</td>
<td>98.96</td>
<td>17.00</td>
<td>15.50</td>
</tr>
<tr>
<td>BK-M. musculus</td>
<td>98.96</td>
<td>17.00</td>
<td>15.50</td>
</tr>
<tr>
<td>BK-D. rerio</td>
<td>98.96</td>
<td>17.99</td>
<td>15.50</td>
</tr>
<tr>
<td>BK-O. mykiss</td>
<td>98.45</td>
<td>17.00</td>
<td>15.50</td>
</tr>
<tr>
<td>BK-A. anguilla</td>
<td>96.89</td>
<td>16.50</td>
<td>15.50</td>
</tr>
<tr>
<td>BK-G. morhua</td>
<td></td>
<td>17.00</td>
<td>15.50</td>
</tr>
<tr>
<td>SK1-H. sapiens</td>
<td>16.50</td>
<td></td>
<td>48.86</td>
</tr>
<tr>
<td>SK1-M. musculus</td>
<td>15.50</td>
<td>77.33</td>
<td>46.02</td>
</tr>
<tr>
<td>SK1-D. rerio</td>
<td>17.00</td>
<td>86.98</td>
<td>50.87</td>
</tr>
<tr>
<td>SK1-X. tropicalis</td>
<td>17.00</td>
<td>84.62</td>
<td>51.45</td>
</tr>
<tr>
<td>SK1-G. morhua</td>
<td>17.00</td>
<td></td>
<td>47.40</td>
</tr>
<tr>
<td>IK-H. sapiens</td>
<td>15.00</td>
<td>52.66</td>
<td>52.60</td>
</tr>
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<td>IK-D. rerio</td>
<td>15.50</td>
<td>48.82</td>
<td>83.72</td>
</tr>
<tr>
<td>IK-T. nigroviidis</td>
<td>16.50</td>
<td>47.65</td>
<td>85.47</td>
</tr>
<tr>
<td>IK-G. morhua</td>
<td>15.50</td>
<td>47.40</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Quantitative expression of K\textsubscript{Ca} channel genes in Atlantic cod

3.2.1 Development and optimization of qPCR assay for K\textsubscript{Ca} channel genes in Atlantic cod

qPCR assays for the three genes, KCNMA1, KCNN1, and KCNN4 (BK, SK1 and IK channel, respectively), were developed and validated by first performing melting curve analysis, checking the specificity of the different primer pairs for the different genes. Melting curves that showed one specific peak indicated that the PCR only had amplified one product. Second, the efficiency of the SYBR green I assay for the three genes was evaluated using a cDNA template dilution curve. Cod pituitary cDNA was prepared with two-fold dilutions from 1:10 to 1:5120 for BK and IK, and from 1:2 to 1:128 for SK1. Efficiencies between 1.9 and 2.1 were considered acceptable. Selected primer pairs were also tested on genomic DNA to assure no interference.

The primer pairs for BK, SK1, and IK all had an optimal annealing temperature of 60 °C, resulting in specific melting curves (Figure 13A, B and C). The qPCR products were analyzed by agarose gel electrophoresis. The agarose gel electrophoresis showed only one distinct band for BK, SK1 and IK, with approximate sizes of 87, 117, and 60 bp, respectively (Figure 13D). The identity of each product was also confirmed by sequencing (for details see section 2.6.3).
Figure 13. Specificity of the SYBR green I assays using specific primers for the BK channel, SK1 channel and IK channel. A) BK channel (KCNMA1) B) SK1 channel (KCNN1) and C) IK channel (KCNN4). The melting curve analysis is plotted as the negative change in fluorescence per time as a function of temperature. D) The qPCR products are visualized on a 2% agarose gel stained with SyberSafe (Invitrogen). Lane 1: molecular size ladder (100 bp molecular weight ladder, Promega). Lane 2: qPCR sample with specific primers for the BK channel containing a 87 bp product. Lane 3: qPCR sample with specific primers for the SK1 channel containing a 117 Bp product. Lane 4: qPCR sample with specific primers for the IK channel containing a 60 bp product. Lane 5: non template control (NTC) for BK. Lane 6: NTC for SK1. Lane 7: NTC for IK.

The PCR efficiencies were calculated as described in section 2.6.2. The chosen primer pairs had the efficiencies 2.085, 2.052 and 2.058 for BK, SK1 and IK, respectively. See Figure 14.
Figure 14. Efficiency of the SYBR green I assays using specific primers for BK, SK1 and IK. Primer pairs that showed a specific peak in the melting curve analysis and that did not amplify any genomic DNA were tested using a dilution curve with cDNA dilutions between A) (BK) 1:10 and 1:5120, B) (SK1) 1:2 and 1:128, and C) (IK) 1:10 and 1:5120. The Cq (quantification cycle) values are plotted against the logarithmic concentration of the cDNA. The slope of the regression line is used to calculate the efficiency of each assay. The efficiencies were 2.085, 2.052, and 2.058 for the primer pairs for the BK, SK1 and IK channel, respectively.

3.2.2 Reference genes

To investigate the relative gene expression of the $K_{Ca}$ channels in the pituitary, different reference genes were tested to see which one was best suited. The reference genes tested were EF1α, ubiquitin, β-actin, and Arp2/3. All primer pairs had specific peaks in the melting curve analysis (Figure 15), and an efficiency between 1.9 and 2.1 (Figure 16).
Figure 15. Specificity of the SYBR green I assays using specific primers for A) EF1α, B) ubiquitin, C) β-actin, and D) Arp2/3. The melting curve analysis is plotted as the negative change in fluorescent per time as a function of temperature. The non-template control (NTC) for A) EF1α and D) Arp2/3 shows a small peak to the left of the gene-specific peak, indicating a primer dimer.
Figure 16. Efficiency of the SYBR green I assays using specific primers for A) EF1α, B) ubiquitin, C) β-actin, and D) Arp2/3. Primer pairs that showed a specific peak in the melting curve analysis and that did not amplify any genomic DNA were tested using a dilution curve with cDNA dilutions between 1:25 and 1:390625. The Cq (quantification cycle) values are plotted against the logarithmic concentration of the cDNA. The slope of the regression line is used to calculate the efficiency of each assay. The efficiencies were 2.010, 2.030, 1.957, and 1.979 for EF1α, ubiquitin, β-actin, and Arp2/3, respectively.

None of the reference genes had significant differences in the expression between the months we conducted the qPCR study. However, we chose β-actin for normalizing the qPCR data, as this reference gene had the lowest variance from month to month (Figure 17).
Figure 17. Cq values ± SEM for the four reference genes tested on cDNA from Nov (November), Dec (December), Mar (March) and Apr (April). A) EF1α, B) ubiquitin, C) β-actin, and D) Arp2/3.
3.2.3 Pituitary gene expression

Pituitaries from female cod, sampled in November, December, March, and April (n=4 at each sampling) were analyzed for potential seasonal variation in gene expression of K\textsubscript{Ca} channels using the developed qPCR assays. The genes for the BK (encoded by the KCNMA1 gene), SK1 (encoded by the KCNN1 gene), and IK (encoded by the KCNN4 gene) channels were all expressed in the four months investigated. Regarding the KCNMA1 gene (BK channel) (Figure 18 A), the expression is significantly higher in April compared to November and December, and the expression is increasing between all samplings from November until April. Although not significant, the expression of the KCNN1 gene (SK1 channel) and KCNN4 (IK channel) (Figure 18 B and C) show a decreasing trend from November until April. In addition, there is a large variation in the relative expression of KCNN1 and KCNN4 in November, compared with the other months investigated (Figure 18 B and C).
Figure 18. Gene expression of $K_{Ca}$ channels in the pituitary of female cod. Quantitative gene expression of A) the BK channel, B) the SK1 channel and C) the IK channel in female cod pituitaries sampled in November, December, March and April. Data are presented as mean ± SEM. There is a significant difference in gene expression of the BK channel (A) between November and April, and December and April (p < 0.05), and the expression is increasing from November until April. Different letters indicate significant difference. The SK1 channel (B) and the IK channel (C) show a decreasing trend from November until April. qPCR data are normalized to β-actin expression. Change in gonadosomatic index (GSI) is identified by a grey, solid line, and values are given on the right y-axis. GSI data are presented as mean ± SEM.
3.2.4 Tissue distribution of the KCa channel genes

In addition to gene expression of the KCa channels in the brain and pituitary, these genes are known to be expressed in several tissues outside the CNS in other species. The gene expression in December (before spawning) was compared to the expression in the same tissue in March (during spawning) using the same female cod as were used in the pituitary analyses.

3.2.4.1 Tissue distribution of KCNMA1

In Atlantic cod, tissue expression analyses of the KCNMA1 gene (BK channel) revealed that the KCNMA1 gene is expressed in all the investigated tissues, except for liver (Figure 19). The KCNMA1 gene was expressed in 3 out of 3 muscle samples from December, and in 4 out of 4 samples in all of the other tissues. Based only on the Cq values, relatively high gene expression levels (low Cq values) of KCNMA1 were measured in muscle, brain and pituitary, and relatively low gene expression levels (high Cq values) were measured in gills. There were no significant differences in expression levels between December and March.
Figure 19. Relative quantification of gene expression of the BK α subunit in various tissues from female Atlantic cod. Gene expression of the KCNMA1 gene (BK channel) in female Atlantic cod. N = 4 for all tissues, except muscle (December) where n = 3. Tissues in which the gene was not detected is labeled nd (not detectable). The tissues investigated were gills, gonad, eye (retina), liver, intestine, muscle, skin, brain, and pituitary. Data are presented as mean + SEM. The abbreviations are D = December and M = March.

3.2.4.2 Tissue distribution of KCNN1

The KCNN1 gene (SK1 channel) was expressed in all tissues analyzed, except in liver (Figure 20). The KCNN1 gene was expressed in 3 out of 4 gill samples from December, in 2 out of 3 muscle samples from December, and in 4 out of 4 samples in all of the other tissues. For the KCNN1 gene, relatively low expression was measured in all of the tissues, except in the brain. Interestingly, there is a significant difference in gene expression between the measurements done in December and March in the gills and the intestine. Because the KCNN1 gene is expressed only in 2 out of 3 muscle samples from December, standard error
of mean (SEM) could not be calculated for these data, and it is not possible to say anything about the significance of the differences between the measurements done in those two months.

**Figure 20. Relative quantification of gene expression of the SK1 channel in various tissues from female Atlantic cod.** Gene expression of the KCNN1 gene (SK1 channel) in female Atlantic cod. N = 4 for all tissues, except for muscle (December) where n = 3. Tissue in which the gene was not detected is labeled nd (not detectable). Significant differences at the 0.05 level in gene expression in December and March were detected in the gills and intestine. These differences are marked with a star. The tissues investigated were gills, gonad, eye (retina), liver, intestine, muscle, skin, brain, and pituitary. Data are presented as mean + SEM. The abbreviations are D = December and M = March.
3.2.4.3 Tissue distribution of KCNN4

Expression analysis of the KCNN4 gene (IK channel) in Atlantic cod reveals that the gene is expressed in all tissues, in both December and March (Figure 21). The KCNN4 gene was expressed in 3 out of 4 liver samples from December, in 3 out of 4 muscle samples from December, and in 4 out of 4 samples in all of the other tissues. There is a significant difference in gene expression between measurements done in December and March in the liver samples.

Figure 21. Relative quantification of gene expression of the IK channel in various tissues from female Atlantic cod. Gene expression of the KCNN4 gene (IK channel) in female Atlantic cod. N = 4 for all tissues, except liver (December) and muscle (December) where n = 3. Significant differences in gene expression in December and March were detected in the liver. This difference is marked with a star. The tissues investigated were gills, gonad, eye (retina), liver, intestine, muscle, skin, brain, and pituitary. Data are presented as mean + SEM. The abbreviations are D = December and M = March.
3.3 Single-cell qPCR

When performing these experiments, specific primers for FSHβ and LHβ were used to identify gonadotropes. In a total of 32 cells analyzed, 9 were FSH-positive and 5 were LH-positive. The FSH- and LH-positive cells were further analyzed for gene expression of the K_{Ca} channels, but there were no detection of these genes. K_{Ca} channels are expressed at low levels, and the expression level was most likely under the detection limit for the qPCR assays.
4. Discussion

This study reports cloning, sequencing and identification of the BK, IK and SK1 \(K_{\text{Ca}}\) channels in Atlantic cod, and also development and validation of qPCR assays for the three genes. Gene expression of the \(K_{\text{Ca}}\) channels in female cod pituitaries has been investigated during the reproductive season. In addition, a tissue screening has been performed, analyzing the gene expression in various tissues one month prior to spawning (December) and during spawning (March). Despite numerous attempts, the isolation and cloning of the putative SK2 and SK3 (KCNN2/KCNN3) channels were not successful.

4.1 Sequence analysis

4.1.1 The \(K_{\text{Ca}}\) channels

The gene for the BK channel, KCNMA1, is highly conserved, particularly the pore region and the S6 transmembrane segment. The same is true also for the SK1 channel (KCNN1 gene). With only minor differences in the amino acid sequences, all conserved parts of the Atlantic cod BK channel sequence, except the S0 and S1 transmembrane segments, and the S9 and S10 intracellular segments, are identified in this project. In the Atlantic cod SK1 channel sequence, it is possible to identify all conserved regions, also with minor amino acid differences, except the S1 and S2 transmembrane segments. Concerning the IK channel (KCNN4 gene), only a short part of the sequence was obtained, and there are not many published sequences to compare with. The GYGD part of the pore region is identified, but limited information regarding the IK channel sequence has made further identification difficult.

In all the \(K_{\text{Ca}}\) channels, several exons are identified. The KCNMA1 gene, for instance, is highly conserved, and as many as 35 exons have been identified in tetrapods (Beisel et al., 2007). Many exons give several alternative splicing possibilities. The cloned and sequenced
products may lack exons containing e.g. transmembrane segments, making identification of the complete gene difficult. In addition, mostly partial sequences are available in the GenBank from different fish species. The degree of conservation is known to be large in the pore region and the adjacent areas. If the degree of conservation is not as large in the unidentified regions, this could also influence the outcome of the sequence analyses.

The alignment of the three isolated $K_{Ca}$ channels shows that when there are differences in the conserved regions between teleosts and the other species investigated, the Atlantic cod sequences are similar to the other teleosts. In the alignment, the SK1 channel shows more similarities with the IK channel than with the BK channel. This is as expected, considering the known similarity in structure of the SK channels and the IK channel. The similarities between these channels are reflected in the fact that the IK channel was previously named SK4. Thus, the conservation in the alignment, and the high percent identity between the Atlantic cod sequences and sequences from other vertebrates strongly indicates that the obtained sequences truly are the Atlantic cod $K_{Ca}$ channels.

Concerning the SK2 and SK3 channels, the cloning and sequencing were as mentioned above, not successful. I was not able to identify the SK2 or SK3 channel sequences in the Atlantic cod genome database at the University of Oslo Bioportal. One reason why the sequences were not identified could be due to the large sequence similarities between the three SK channels, or it could also be that the genes encoding the SK2 and SK3 channels are not present in the Atlantic cod genome. In the human, mouse, and rat genomes, the genes encoding the five $K_{Ca}$ channels have been identified (http://www.ncbi.nlm.nih.gov/, for GenBank accession numbers, see Appendix V). To my knowledge, the genes encoding the BK, SK1, SK2 and IK channel, but not the SK3 channel, have been identified in zebrafish (for GenBank accession numbers, see Appendix V), while in rainbow trout, all genes except the genes encoding the SK1 channel and the IK channel have been identified (for GenBank accession numbers, see Appendix V).
4.2 Gene expression studies

4.2.1 Choice of reference gene

Normalization with reference genes is important in a reliable qPCR assay. Reference genes are known to vary among different types of tissues, and normalization should therefore be performed against multiple reference genes (for review, see Bustin et al., 2010). Based on this, 4 different reference genes were tested for the pituitary analyses, and β-actin was chosen because it showed the lowest variance and was the most stable reference gene. For the qualitative tissue distribution analysis, no reference gene was used, and the data are presented as quantification cycle (Cq) to give a qualitative measure of gene expression.

4.2.2 Gene expression in the pituitary

The gene encoding the BK channel is significantly up-regulated in April compared to November and December. A trend indicates that the gene encoding the SK1 channel is down-regulated at the same time as the BK channel is up-regulated. The cod matures in the period between November and March, following increased activity in the BPG axis, e.g. increased synthesis and release of gonadal steroids. Interestingly, when mouse gonadotropes pretreated with the steroid 17β-estradiol were stimulated with GnRH, the BK current was increased, while the SK current was reduced, compared to in control cells. This up-regulation of the BK channel and down-regulation of the SK channel leads to an increase in membrane excitability and an increase in secretion (Waring and Turgeon, 2009). The gene expression analyses in the current project were performed on whole pituitaries from Atlantic cod, which make it difficult to assess whether eventual up- or down-regulation occurs in particular cell types. Other hormone-producing cell types, e.g. somatotropes, also express Ke4 channels and are likely to have a seasonal variation in the gene expression of the channels. In many fish species, somatic growth decreases prior to and during the spawning period, and this is often followed by a period of compensatory growth, e.g. in Atlantic cod (for review, see Taranger et al., 2010). In largemouth bass (Micropterus salmoides), it has also been shown that growth hormone (GH) mRNA varies significantly over the year in females, and follows closely gonadotropin
expression in the pituitary (Martyniuk et al., 2009). Thus, regulation of $K_{Ca}$ channels in somatotropes could potentially confound my results.

The gene expression data shows large sample variability in November for both SK1 and IK. A possible biological explanation for this variation is that in November, the fish prepares for the spawning season, meaning that some of the fish could have reached further in the development compared to other fish captured at the same time. From December and until April, the fish are at a more similar developmental stage, which could explain why the expression data for these months vary less than they do in November. The large variation was only found in the data for SK1 and IK channels, and not in the data for gene expression of the BK channel in Atlantic cod pituitaries. In rat anterior pituitary cells, there have been shown to be differences in the ionic channels expressed in three hormone secreting cell types; somatotropes, lactotropes and gonadotropes (Van Goor et al., 2001b). The same can be true for anterior pituitary cells from Atlantic cod, and since these analyses have been performed on whole pituitaries containing 8 hormone-producing cell types, variation can be expected.

One weakness of the analyses is the low number of individuals for each sampling point. Unfortunately, the qPCR results are limited to few fish samples. This is one disadvantage of using wild Atlantic cod, and not other model organisms, e.g. medaka or mice and rats. The sampling was hampered due to an unusually long and cold winter, making the inner area of the Oslo fjord freeze. Because of this, it was not possible to get any cod in January and February. The ideal situation would have been to have more individuals from each month during one year.

Relative expression for each month and each gene was compared to the GSI of each fish. No direct correlation between the GSI and the gene expression of the $K_{Ca}$ channels was found. However, the GSI varies a lot for the fish from the same sampling date, and there were only 4 cod from each sampling. If more cod had been available for the analyses, the variation in GSI might have been less. Relative expression and GSI for each fish used in the experiment can be found in Appendix I.
4.2.3 Tissue distribution

The results from the tissue distribution analyses are presented as Cq values. As mentioned in section 4.2.1, the data were not normalized to any reference gene. This was because the analyses were qualitative, confirming if the genes were expressed or not in a specific tissue. The gene for the BK channel is expressed in all tissues investigated, except in the liver. These results correspond to what is known about BK channels, that they can be found in most tissues (Marty, 1981, Adams et al., 1982, Elkins et al., 1986, Smith and Thompson, 1987, Tseng-Crank et al., 1994, Saito et al., 1997, Yan et al., 2008) (for reviews, see Adelman et al., 1992, Vergara et al., 1998). In the pituitary, the tissue distribution analysis did not show any difference in the gene expression of the BK channel between December and March. These data are presented as Cq values. This is probably the reason why we do not see a difference between the expression in December and March, even though we see a difference in the gene expression analyses of the pituitary using β-actin as reference gene.

The gene for the SK1 channel is also expressed in all tissues investigated, except in the liver. These results also correspond to previous results concerning the expression of SK channels (Köhler et al., 1996, Ishii et al., 1997, Joiner et al., 1997, Logsdon et al., 1997, Stocker and Pedarzani, 2000, Hosseini et al., 2001, Tacconi et al., 2001, Boettger et al., 2002, Arnold et al., 2003). The gene for the IK channel is the only gene expressed in all tissues investigated. When interpreting these results, it is important to consider that the IK channel is highly expressed in red blood cells, and hence tissues containing blood (Köhler et al., 1996, Ishii et al., 1997, Joiner et al., 1997, Stocker and Pedarzani, 2000). All tissues contain some traces of blood, and this influences the expression data. The gene expression is relatively low in the liver samples from December. This is a tissue that normally contains a lot of blood, so it could be that the IK channel is even less expressed in these samples than what the Cq values indicate, or not expressed at all.
4.3 **K\textsubscript{Ca} channels and their possible role in regulation of hormone secretion**

Not much is known about the role of K\textsubscript{Ca} channels in the regulation of hormone secretion in teleosts. In mammals, the channels have been shown to take part in the regulation of hormone secretion. However, differences are observed between mammalian species. Rat gonadotropes express mainly SK channels, while the BK channel is expressed in somatotropes and lactotropes (Van Goor et al., 2001b). However, mouse gonadotropes express both BK and SK channels (Waring and Turgeon, 2009).

In mouse gonadotropes, both BK and SK channels are involved in regulation of LH secretion. One outcome of increased excitability when SK channel activity is reduced is enhancement of GnRH-stimulated LH secretion. The situation concerning the BK channel is more complex, but activation of BK channels may lead to an increase in the LH secretion stimulated by GnRH (Waring and Turgeon, 2009). Gene expression analyses of female Atlantic cod lh\beta, fsh\beta, and gpα (glycoprotein α) have been performed on individual pituitaries during a complete reproductive cycle (Mittelholzer et al., 2009). The expression of lh\beta closely followed the GSI, increasing during gonadal growth with a peak during spawning in February-March. In contrast, fsh\beta and gpα expression showed two expression peaks, one in December and one at spawning. The increase in gene expression of the BK channel and the decrease in gene expression of the SK1 and IK channels during spawning in Atlantic cod thus correlate well with the gene expression pattern of lh\beta, fsh\beta, and gpα, since we would expect increased membrane excitability in periods with high hormone production and secretion. We believe that the electrophysiological properties of Atlantic cod and mammalian pituitary cells are similar (Haug et al., 2007). In mammals, the K\textsubscript{Ca} channels play an important role in modulating the electrophysiological response to hypothalamic releasing hormones. The results presented in this thesis are in agreement with the notion that K\textsubscript{Ca} channels may contribute to the regulation of hormone secretion also in Atlantic cod.
4.4 Future work

The results obtained in this study make it very interesting to follow up with other analyses and experiments. The search for the genes encoding the SK2 and SK3 channels in Atlantic cod should continue, and one possible strategy is in silico synteny search in the cod genome database, followed by cloning. By performing synteny search, it is possible to confirm if the genes are present or not in the genome.

Single-cell qPCR is another type of experiment that would be interesting to follow up with. All the qPCR experiments are performed on whole pituitaries, which in teleosts consists of eight distinct hormone-producing cell types; lactotropes, corticotropes, thyrotropes, somatotropes, FSH-producing gonadotropes, LH-producing gonadotropes, somatolactotropes and melanotropes. The ideal situation would have been to do the gene expression analyses of the \(K_{Ca}\) channels on identified gonadotropes.

Another method to investigate the expression of specific \(K_{Ca}\) channels in gonadotropes is double-labeling in situ hybridization. Using this method, it would be very interesting to see if fsh\(\beta\) and lh\(\beta\) is co-localized with any of the three cloned and sequenced \(K_{Ca}\) channels.

When performing electrophysiological experiments, it is possible to isolate the \(K_{Ca}\) current by recording the outward current at the holding potential following a long depolarizing voltage pulse inducing maximum inward Ca\(^{2+}\) current (tail current). The voltage-activated channels will be closed during the tail current, while the Ca\(^{2+}\)-sensitive channels will be open as long as the \([Ca^{2+}]_i\) is increased. It would be interesting to see if the size of the tail current is correlated with the gene expression of the different \(K_{Ca}\) channels in identified gonadotropes or pituitary cell cultures.
In conclusion, partial sequences of the tree genes encoding the BK channel, SK1 channel and IK channel were successfully cloned and sequenced from Atlantic cod. The genes encoding the BK, SK1 and IK channels were expressed in all tissues investigated, except that the BK and SK1 channels were not expressed in the liver. Quantitative expression analyses of the genes in the pituitary revealed different expression levels in different months. The expression patterns correlate well with results concerning the expression pattern of lhβ, fshβ and gpα in Atlantic cod, in addition to functional studies of the role of \( K_{Ca} \) channels in mice and rats, as described in section 4.2.2 and 4.3. This indicates that \( K_{Ca} \) channels may be involved in the regulation of hormone secretion in Atlantic cod.
References

Bao L, Cox DH (2005) Gating and ionic currents reveal how the BK$_{Ca}$ channel's Ca$^{2+}$ sensitivity is enhanced by its beta 1 subunit. Journal of General Physiology 126:393-412.


Montero M, Le Belle N, Vidal B, Dufour S (1996) Primary cultures of dispersed pituitary cells from estradiol-pretreated female silver eels (\textit{Anguilla anguilla} L):


Tse A, Hille B (1993) Role of voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels in gonadotropin-releasing hormone-induced membrane potential changes in identified rat gonadotropes. Endocrinology 132:1475-1481.


Appendices

Appendix I: GSI for fish used in gene expression analysis
Appendix II: Atlantic cod BK sequence
Appendix III: Atlantic cod SK1 sequence
Appendix IV: Atlantic cod IK sequence
Appendix V: GenBank accession numbers
## Appendix I  GSI for fish used in gene expression analysis

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Table presenting the GSI (gonadosomatic index) for each fish used in the gene analysis, and the relative expression of each of the three K\textsubscript{Ca} channel genes investigated.
Appendix II  Atlantic cod BK channel sequence

The nucleotide sequence and the deduced amino acid sequence for the manually sequenced part of the Atlantic cod BK channel. Exon-exon boundaries identified in silico are highlighted in grey, qPCR primer sequences are boxed, the conserved residues in the pore are marked in yellow, the transmembrane S1-S6 segment are marked in green, and the intracellular S7 and S8 segments are marked in pink. The signature sequence for K+ channels, GYGD, are in bold and underlined.

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Appendix III  Atlantic cod SK1 channel sequence

The nucleotide sequence and the deduced amino acid sequence for the manually sequenced part of the Atlantic cod SK1 channel. Exon-exon boundaries identified in silico are highlighted in grey, qPCR primer sequences are boxed, the conserved residues in the pore are marked in yellow, and the S6 segment is marked in green, the CaMBD (calmodulin binding domain) α1 is marked in turquoise, and the CaMBD α2 is marked in purple. The signature sequence for K⁺ channels, GYGD, are in bold and underlined.

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T C I G M C A G C T A L Y V A V Y Y A R K S
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Appendix IV

Atlantic cod IK channel sequence

The nucleotide sequence and the deduced amino acid sequence for the manually sequenced part of the Atlantic cod IK channel. Exon-exon boundaries identified in silico are highlighted in grey, and qPCR primer sequences are boxed. The signature sequence for K+ channels, GYGD, are in bold and underlined.

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## Appendix V  GenBank accession numbers

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