Recent advances in the hormonal regulation of gonadal maturation and spawning in fish

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Seasonal reproductive cycle in fish is precisely regulated by different hormones, which lead them to breed at a specific time of the year. Environmental signals are presumably received by the brain, which releases a decapeptide hormone, gonadotropin releasing hormone (GnRH). GnRH is the central regulator of hormonal cascades; it stimulates pituitary to release gonadotropic hormone (GTH), the primary mediator of germ cell growth and maturation. GTH induces the synthesis and release of estradiol-17β from ovarian follicular cells or 11-ketotestosterone from testicular Leydig cell at the initial stage. At a later stage, i.e. during the maturation of germ cell, GTH induces 17α,20β-dihydroxy-4-pregnene-3-one or maturation inducing hormone (MIH) from ovarian follicular cells, and testicular somatic and germ cells. Although MIH is a steroid, it has nongenomic receptors. MIH binds to the membrane receptor and induces the formation of protein factor, i.e. maturation promoting factor (MPF), a complex between cdc 2 kinase and cyclin B protein. During premature condition of the gonad, MPF remains inactive. For the maturation of germ cells, MIH activates MPF by amino-phosphorylation. The hormonal cascade of events is so perfectly coordinated that male and female fish release their germ cells exactly at the same time to the external aquatic environment to ensure fertilization.

HORMONAL regulation of reproductive control mechanism in fish is grossly similar to their vertebrate counterpart but differs remarkably in details. Although hypothalamo-hypophyseal-gonadal axis is the main stream of regulation, hormones released from each of these organs are not very similar to those of other vertebrates, and tuning of control mechanism has certain distinguishing features. Fish brain (hypothalamic portion) secretes gonadotropin releasing hormone (GnRH): the key regulator of reproductive events in all vertebrates. It is a decapeptide and four different forms of it, so far isolated from various piscine sources, vary from mammalian GnRH (ref. 1). GnRH reaches pituitary via portal circulation and binds to its receptor in the pituitary gonadotroph cell membrane. This causes release of gonadotropic hormone (GTH) from gonadotroph cells. GTH then travels through circulation and finds its target organ, ovary or testis, where it occupies specific receptor in the cell membrane of theca and granulosa cell of ovary or Leydig cell of testis. GTH induces the synthesis and release of sex steroids which are, in turn, responsible for growth and maturation of female and male germ cells. Although this profile looks the same as in other vertebrates, GTH in fish is designated as GTH I and GTH II (ref. 2) instead of LH and FSH as termed in other vertebrates. In certain teleosts only one form of GTH is found[3,4]. Both GnRH and GTH on occupation of target cell membrane receptor transduct specific signals for accomplishing their function. In contrast to other vertebrates, steroidogenesis in fish, although induced by GTH, directed in two predominant forms; 17β-estradiol and 17α,20β-dihydroxy-pregnene-3one (17α,20β-DP). 17β-estradiol has a peak during vitellogenic stage (phase of yolk protein, i.e. vitellogenin formation and deposition in the oocytes), while 17α,20β-DP is the major steroid in post-vitellogenic phase or phase of final maturation. Such a clear shift of steroidogenesis is indeed a remarkable phenomenon in teleostean fish[5]. Target site of 17β-estradiol is liver where it binds to the nuclear receptor of hepatocyte and expresses vitellogenin gene. In contrast to this, 17α,20β-DP binds to oocyte membrane and produces signals for final maturational event. In mammal, steroid receptors are always genomic, i.e. they bind to the nuclear receptor which in turn binds to a specific site of DNA and induces a specific gene expression. While 17β-estradiol in fish follows the same mode of action, 17α,20β-DP in fish is a striking exception as it binds to the oocyte membrane receptor followed by signal transduction mechanism which results in final maturation and ovulation or spermiation.

All the above-mentioned intricate hormonal interactions will be discussed briefly in this review. In present aquacultural practice, some of the above mentioned knowledge has been utilized for successful breeding of economically important fish, and better management of fry and fingerling growth. Reproduction in majority of fish is seasonal. Hormonal regulation for gonadal recrudescence, vitellogenic event and final maturation occur at certain specific times of the year depending on the geographical location and environment of a particular place. However, regulatory mechanism of piscine gonadal recrudescence, maturational process and ovula-

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tion or spermiation will be dealt in the light of current information.

GnRH regulation of GTH release

GnRH is the central regulator of reproductive hormonal cascade. Till date, eleven GnRH structures have been elucidated from different vertebrates and protochordates (Figure 1) including four structures from fishes, i.e. seabream, catfish, salmon and dogfish[1]. This ten amino acid structure in fish varies considerably from other vertebrates, although there is striking conservation in peptide length and in NH_2 terminus and COOH terminus, suggesting terminal part to be functionally critically important. Position 8 is the most variable one followed by positions 7 and 5. GnRH receptor is a G-protein-coupled receptor with extracellular amino terminal, transmembrane and intra-cellular carboxy terminal domains. In fish, GnRH receptors on pitutary cell membrane have been detected to be of different types: (i) high affinity/low capacity[6], (ii) low affinity/high capacity[6], (iii) only one high affinity[7] or (iv) two high affinity receptors[8]. Such a variation in GnRH-receptor has not been observed in other vertebrates. Occupation of GnRH receptors on fish pitutary gonadotroph cell membrane by GnRH follows several intra-cellular signal cascades, ultimately leading to the release of GTH. Unfortunately, major part of these cascades is still unknown. However, there are evidences of three possible pathways; (i) Ca^{2+}-calmodulin-protein kinase C (PKC), (ii) adenylate cyclase-cAMP-protein kinase A (PKA) and (iii) combination of both these pathways where Ca^{2+} and/or cAMP behaves as second messenger, independently or in a dependent manner. The presence of voltage sensitive Na^+, K^+ and Ca^{2+} channels has been shown in goldfish pituitary cells[9]. Involvement of voltage-sensitive calcium channel (VSCC) in GnRH-augmented GTH release has been implicated in a number of teleostean fish, e.g. the African catfish[10], the goldfish[11,12], hybrid tilapia[13] and Indian murrel[14–16]. The presence of VSCC indicates the requirement of extracellular calcium influx in GnRH action. Our studies have shown that GnRH-augmented GTH release from Indian carp and murrel is dependent on the influx of extracellular Ca^{2+} through VSCC[14,17]. In GnRH-stimulated GTH release from fish pitutary cell, Ca^{2+} appears to play an obligatory role. Removal of calcium source from the murrel pitutary cell culture medium almost completely waived GnRH stimulatory effect on GTH release[14,16]. A correlation between medium calcium and calcium uptake by the pituitary cell is noticed between 5 and 15 min of GnRH addition. Medium calcium decreases with concomitant increase in cells, the peak was observed at 15 min. This influx of calcium into the cell coincided with the GTH release. Addition of verapamil, a Ca^{2+}-channel blocker, blocks Ca^{2+} uptake and also stops GTH release at the same time[16]. Surprisingly, calmodulin (CaM), a calcium binding protein, clearly augmented GnRH + Ca^{2+} effect on GTH release from fish pituitary cells[15]. It is rather puzzling to find that CaM, whose molecular weight is 17 kDa, is stimulating GTH release when added in the presence of GnRH + Ca^{2+}. Calmidazolium, a specific inhibitor of CaM could effectively inhibit the CaM stimulatory effect. Detection of a CaM binding protein in murrel pitutary cell plasma membrane indicates the rationality of CaM effect[15]. However, Ca^{2+}–*CaM–GnRH interaction in GTH release is still unclear* and requires further investigations.

The second part of the signal transduction by GnRH in fish appears to be adenylate cyclase–cAMP–PKA pathway[15,18]. GnRH clearly stimulates PKA activity in murrel pitutary cell at 30 min, which reaches peak at 60 min and is maintained at this level till 120 min. This profile corresponds to cellular cAMP level. During the same time, GTH synthesis in the cell increases[16]. Recently, cAMP binding to pituitary cell DNA has been detected in an Indian major carp *Catla catla*, indicating a possible role of cAMP on GTH gene expression[19]. A study on time kinetics of GnRH action suggests a meaningful sequence of signal transduction pathway. GnRH binding to the pituitary cell receptor immediately causes influx of extracellular calcium followed by elevation of cellular calmodulin (CaM), which then stimulates phosphodiesterase (PDE) activity (CaM is a known stimulator of PDE activity)[15]. Increase of Ca^{2+} is closely related to increase of PKC activity[11,16]. Inhibitors of VSCC, removal of extracellular Ca^{2+}, inhibitor of PKC and down regulation of cellular PKC content, inhibited GnRH-induced GTH release[14,17,20–22]. Activator of PKC, on the other hand, increased GTH release[23]. In the second phase of GnRH-stimulated GTH release, there is an increase in cAMP and PKA[16,18]. With the evidences so far obtained in GnRH signal transduction cascade, a model on GnRH action can be

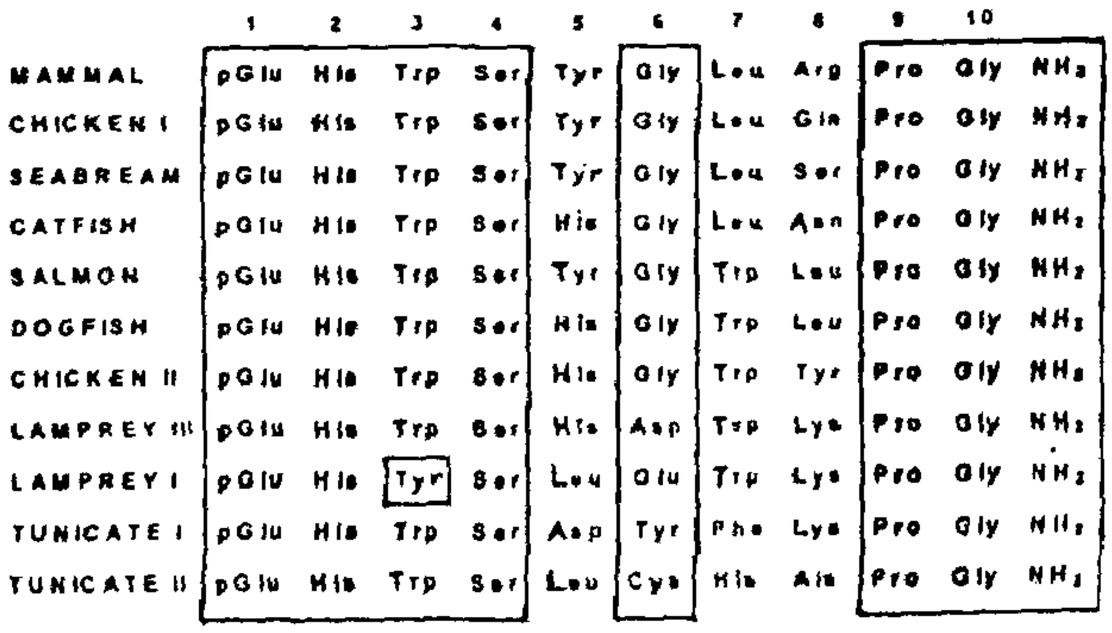

Figure 1. Structures of GnRH peptides isolated from different vertebrate brains. Blocked areas show conserved regions.

constructed. In the first phase, GnRH causes a rapid influx of extracellular Ca^{2+} with concurrent rise in cellular PKC activity resulting in an acute release of stored GTH, and during the second phase a sustained release of GTH occurs concurrently with its continued synthesis involving cAMP and PKA. The cessation of the signal is affected by an increase in PDE activity due to higher amount of CaM. All these GnRH-induced cellular signals have been described in Figure 2.

GTH regulation of gonadal function

In majority of fish, pituitary glands secrete two types of gonadotropins, GTH I and GTH II. In salmonids, GTH I is secreted during the period of vitellogenesis and most probably accelarate ovarian growth and synthesis of specific steroid hormone required at this stage. On the other hand, GTH II acts during the period of gonadal maturation[24,25]. However, there may be one type of GTH in certain teleosts which regulates biphasic gonadal growth and maturation[26]. In this review, the term GTH refers to all types of GTH.

Control of ovarian function

Teleost ovary consists of oogonia, oocytes and surrounding follicle cells, inner granulosa cell layer and outer thecal cell layer. Enormous growth of fish oocytes occurs during vitellogenesis, which involves selective sequestration and packaging of hepatically derived plasmatic vitellogenin into yolk protein[27–29]. It is now well known that vitellogenesis in fish is promoted by a two-step mechanism: (i) GTH binds to theca and granulosa cell receptor in the ovarian follicle and thereby increases estradiol-17β secretion, which in turn, induces synthesis and secretion of vitellogenin, a highly complex phospholipo-glycoprotein[27,30–32]. Moreover, GTH most probably stimulates incorporation of vitellogenin into the oocytes from the circulating blood[33,34]. Besides GTH, insulin and thyroxine also influence vitellogenin uptake by the oocyte[35]. In the vitellogenic phase, estradiol appears to be the major regulator and is synthesized in a coordinated manner, in which both theca and granulosa cell layers participate. GTH influences the secretion of androgen substrate (testosterone) from thecal cells which diffuses into the granulosa cell layer, where the aromatase is exclusively localized and converts testosterone to estradiol-17β (ref. 36). A diagrammatic model on hormonal regulation of oocyte growth during vitellogenic stage is shown in Figure 3.

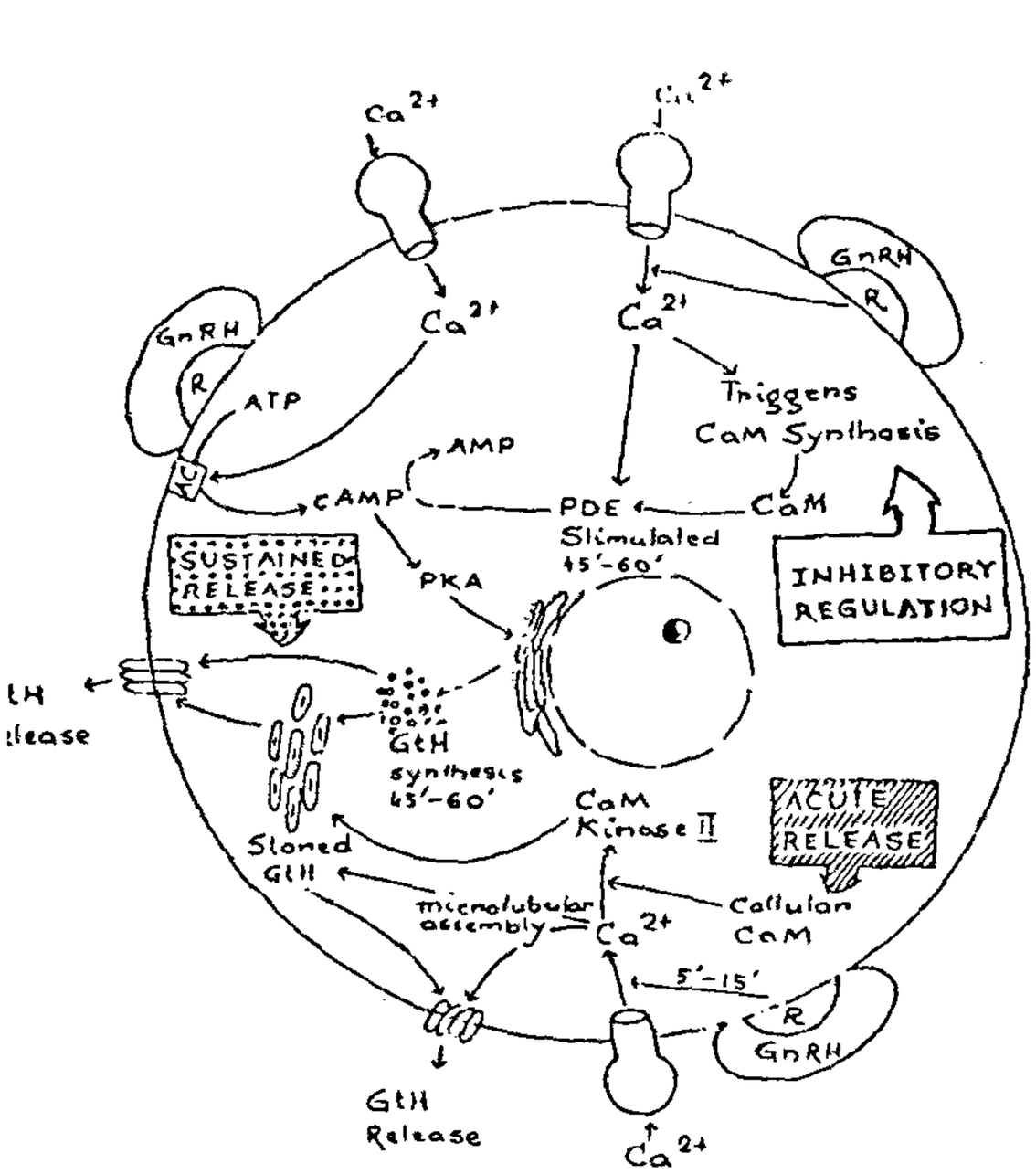

Figure 2. Proposed model of GnRH mechanism of action on the pituitary gonadotroph cell. Acute release of GTH is followed by sustained release. Dissociation of GnRH–receptor complex (GnRH–R) initiates inhibitory regulation. AMP – adenosine monophosphate, cAMP – cyclic AMP, CaM – calmodulin, PDE – phosphodiesterase.

Maturation of oocyte

After the growth phase, oocytes enter into the stage of maturation. Full-grown post-vitellogenic oocytes in the ovary of teleostean fish are physiologically arrested at the late G_2 in the first meiotic prophase and awaits final maturation, which is hormonally controlled. This involves germinal vesicle breakdown (GVBD), chromosome condensation, assembly of the first meiotic spindle and extrusion of the first polar body. In some fish, the first part of oocyte maturation can be visible under the microscope. The events are as follows: The germinal vesicle migrates toward the micropyle of animal pole, the membrane of the germinal vescicle then breaks down and its contents mix with the surrounding cytoplasm. At this time, chromosomes condense, align on the first metaphase spindle, complete meiosis I, and then realign on the second metaphase spindle, when they are considered matured egg and again remain arrested until fertilization[36]. Over and above these nuclear changes, there are certain remarkable alterations in cytoplasm during oocyte maturation, which include the coalescence of lipid droplets and yolk globules, rapid increase of oocyte size due to hydration and a visible increase in oocyte translucency.

Maturation-inducing hormone

In contrast to the growth of oocyte which is regulated mainly by estradiol-17β, maturation of oocytes is regu-

lated by another steroid, i.e. 17α,20β-dihydroxy-4-pregnene-3-one [17α,20β-DP], also known as maturation-inducing hormone (MIH). GTH acts on ovarian follicle cells of post-vitellogenic phase and causes MIH synthesis and release[37]. Production of this steroid is effected via the interaction of two follicular cell layers, the thecal and granulosa layers[38]. It is interesting to find that atleast in salmonid fish, a distinct steroidogenic shift takes place, i.e. from estradiol-17β during vitellogenic phase (this steroid induces vitellogenin synthesis in liver) to 17α,20β-DP (MIH) during post-vitellogenic phase immediately prior to oocyte maturation, a pre-requisite for the growing oocytes to enter maturation stage[36,39]. In a number of teleostean species, MIH has been shown to be a potent maturation-inducing factor *in vitro* and present at high levels in plasma, when fish is undergoing final maturation[40–43]. Interestingly, microinjection of MIH into full-grown immature goldfish oocytes fails to induce oocyte maturation[39], this indicates that MIH possibly acts via membrane-bound receptor. Membrane receptors for MIH have been shown in salmonids and flounder oocytes[44–46]. The amount of MIH receptor considerably increases in flounder oocytes undergoing final maturation[47]. *In vitro* treatment of fish ovarian follicles with GTH greatly stimulated MIH receptor concentrations[47] which suggests that GTH not only induces the synthesis and release of MIH but also stimulates its receptor concentration required for final maturation to occur. MIH receptor is a G-protein coupled receptor with both Gs and Gi components; these are associated with the regulation of adenylate cyclase activity, indicating involvement of cAMP in MIH signal transduction[48]. Binding of MIH to membrane receptors produces protein factor (MPF) which causes final maturation. These possible pathways have been summarized in Figure 4.

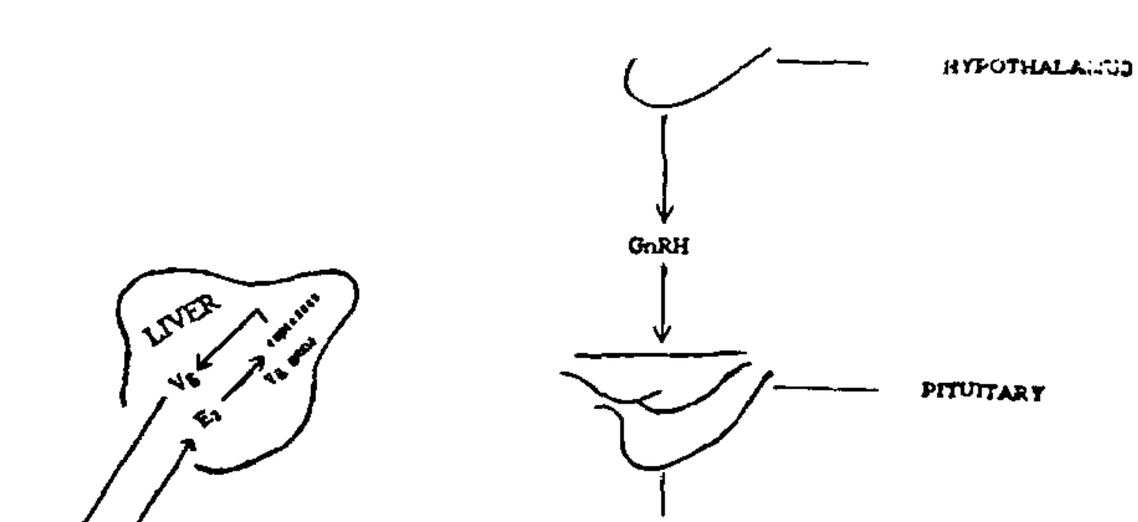

Figure 3. Regulation of oocyte growth during pre-vitellogenic stage. TC – thecal cell, GC – granulosa cell, GTH-R – GTH receptor complex, Chol – cholesterol, Preg – pregnenolone, P_4 – progesterone, T – testosterone, Aro – aromatase, E2 – estradiol-17β, V_g – vitellogenin, GV – germinal vesicle, YG – yolk granules.

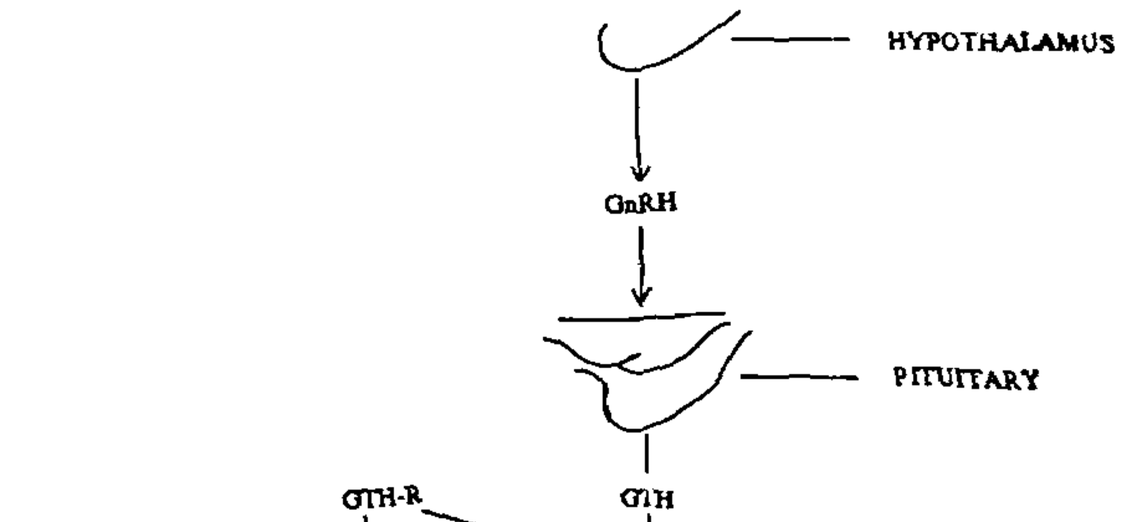

Figure 4. Hormonal regulation of final maturation of oocytes during post-vitellogenic stage. TC – thecal cell, GC – granulosa cell, Chol – cholesterol, Preg – pregnenolone, P_4 – progesterone, 17a,OHP – 17a-hydroxy progesterone, MIH – maturation inducing hormone, MPF – maturation promoting factor, GVBD – germinal vesicle breakdown, GV – germinal vesicle.

Maturation promoting factor

The presence of MIH receptors on oocyte surface of fish indicates the existence of a cytoplasmic factor that mediates MIH action. This factor is termed maturation-promoting factor (MPF). MPF contains two predominant proteins with relative molecular mass of 34- and 46–48 kDa[49]. 34 kDa protein is catalytic cdc2 kinase and 46–48 kDa is cyclin B regulatory protein.

In immature goldfish oocytes, 35-kDa cdc 2 kinase exists in inactive form, while cyclin B is absent. Cyclin B appears at the time when oocytes undergo GVBD and is related to the activation of cdc 2 kinase[50,51]. Immature goldfish oocytes contain mRNA for cyclin B but this is not further processed to synthesize the protein. In maturing oocytes the pre-existing 35 kDa inactive cdc 2 kinase binds to newly synthesized cyclin B, which in turn, makes its rapid conversion to 34 kDa active form. Activation of cdc 2 kinase is not only due to the reduced molecular weight but also due to the phosphorylation of threonine residue at 161 position. This active cdc 2 kinase and cyclin B complex is known as MPF. MPF triggers all changes observed during oocyte maturation, i.e. GVBD, chromosome condensation and spindle formation[52]. Just after fertilization, MPF activity remarkably decreases, coinciding with the degradation of cyclin B protein[48]. Figure 5 is a diagrammatic representation of MIH-regulated MPF formation and activation.

Regulation of testicular spermatogenesis

As compared to the regulation of oocyte growth and maturation, very little is known on the regulation of spermatogenesis and spermiation. Spermatogenesis in teleostean fish is also under GTH control. The line of regulation is grossly similar in male fish, i.e. GnRH stimulates synthesis and release of GTH, which in turn acts on testicular Leydig cells to produce 11-keto-testosterone (11-KT), and this event triggers spermatogenesis. Sperm maturation, on the other hand, is affected by 17α,20β-DP (MIH) but the site of MIH production is different. Injection of GTH to male Japanese eel markedly activates Leydig cells and Sertoli cells, subsequently followed by proliferation of spermatogonia and beginning of meiosis in them, which leads to spermatids and spermatozoa[53]. Addition of GTH into the eel testicular fragments *in vitro* induced entire process of spermatogenesis from pre-meiotic spermatozoa, which coincides with the rapid increase in 11-KT production from Leydig cells[54]. In fact, 11-KT could induce all

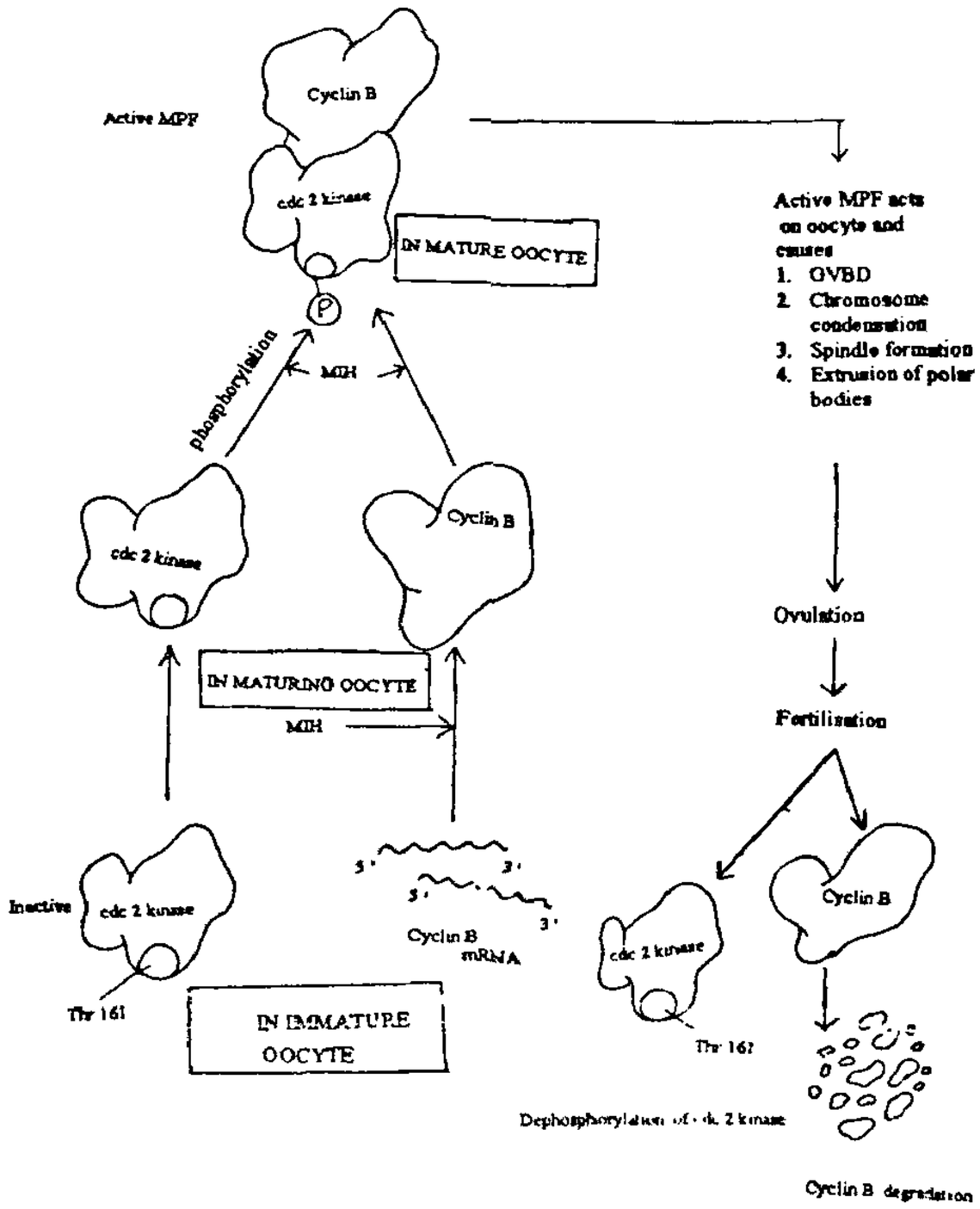

Figure 5. Diagrammatic representation of MPF activation in fish oocytes by MIH.

stages of spermatogenesis, including proliferation of spermatogonia, and meiotic division[55]. 11-KT also effected a marked cytological activation of Sertoli cells. Probably, under 11-KT influence, Sertoli cell releases meiosis-initiating substance, which in turn, stimulates spermatogenesis[56].

Testicular maturation

MIH is the principal hormone responsible for final maturation of testicular germ cells. MIH concentration is greatly increased during spermiation of salmonids[57,58]. MIH is low during spermatogenesis but sharply increases at the time of spermiation[59]. For the production of MIH, GTH does not act on endocrine cell but on somatic and germ cells. MIH is produced from salmonid sperm and is necessary during spermiation[60], but this may not be the case in other teleosts[61].

Regulation of sperm motility

Very little is known about the hormonal regulation of sperm motility in fish. In masu salmon, sperm in the testis and in the sperm duct are not motile. If sperms in the duct are diluted with freshwater, they gain motility but this does not happen in the case of testicular sperm[62]. This may be due to the change of pH; at higher pH range (8.0–9.0) spermatozoa acquire motility with almost two-fold increase in cAMP level. Immotile testicular spermatozoa can attain motility when treated with dbcAMP (ref. 56). 17α,20β-DP alone can raise the percentage of motile sperms and the duration of sperm motility, while 11-KT or testosterone is ineffective in causing sperm motility[56,63]. These findings may be summarized as follows: GTH stimulates the production of 17α,20β-DP from testis which possibly effects increase of pH in the sperm duct; this in turn, elevates cAMP in sperm-permitting the acquisition of sperm motility.

Spawning

Most fish select a specific spawning ground for their natural spawning behaviour. The factors involved for selecting a specific spawning ground have not been identified properly. But sufficient oxygen supply and protection from predators are probably the primary factors. Involvement of certain steroids, which are synthesized in higher level during sexual behaviour, has also been indicated[64,65]. Spawning essentially means successful ovulation and spermiation from female and male partners respectively. In a suitable environment GnRH is released from brain, acts on pituitary gonadotroph cells causing GTH release, which in turn stimulates 17α,20β-DP (MIH) secretion from ovarian theca and granulosa cells, and testicular somatic cell. MIH produces MPF, and MPF causes final maturation and release of fertilizable germ cells. The details of the cascade of hormonal regulation at the downstream level for setting the spawning are yet unknown.

In present day aquaculture practice, GnRH or its chemical analogues are used for induced spawning of culturable fish. This is not only cheaper but also extremely dependable as failure rarely occurs. Being a decapeptide, GnRH is comparatively more stable than GTH or pituitary extract and can be stored for a long time in an ordinary refrigerator. From the above description of the hormonal events which produces various signal cascades causing ovulation or spermiation in fish, it is clear that GnRH-stimulated GTH release is the primary regulatory mechanism. Since GTH acts on the gonads for their developement, growth, maturation and release of germ cells by producing other regulatory factors, this hormone at one time was used as pituitary extract for induced spawning. To spawn (i.e. to release the finally matured germ cells, oocytes and sperms), a sudden rise of endogenous GTH level is essential which may be three- to five-fold in excess of the basal level[66]. Problem with the commercially important culturable fish is that they do not breed in confined waters and require induction of hormone for spawning. In our country, the Indian major carps are richest source of food fish and their induced breeding is still practised by injecting pituitary extract[67]. But there are many problems in this process. As the demand for carp seed production is increasing, the pituitary glands are becoming more expensive and their availability is decreasing. Often, batches of these pituitaries are bad as they are collected from the market where dead fishes are purchased under ice. Decrease in their potency is leading to failure in spawning. To overcome these problems, induced spawning of carps and other important fishes in many countries is now carried out with GnRH or its analogue which releases endogenous GTH and effects spawning in a much more surer manner than the crude pituitary extract. Pituitary extract very often also contains pathogenic microorganisms causing infection in fishes. Moreover, pituitary GTH is a glycoprotein hormone and is extremely sensitive to temperature denaturation. Hence, GnRH is no doubt a better alternative for induced spawning. But there is one problem with GnRH use, its activity is inhibited by endogenous dopamine[68,69]. Dopamine occupies GnRH-receptor and thus blocks its action on pituitary gonadotroph cells. Use of domperidone (or pimozide), a dopamine antagonist, increases GnRH-receptor capacity, thus enhancing GnRH responsiveness[68]. Therefore, for induced ovulation, together with salmon GnRH-analogue, pimozide (anti-dopamine) has been used[70]. On the basis of this information, Ovaprim, a commercial product has been prepared by the Syndel Laboratories, Canada, which

is now marketed by Glaxo Laboratories Ltd for induced breeding of fish.

The first successful report in using synthetic GnRH analogue for induction of ovulation and spawning of cultured carps is available from a research group in China[71–73]. This successful application of GnRH analogue has been later followed for induced breeding of various fishes including common carp[74], rainbow trout[75], Atlantic salmon[76], milk fish[77] and grey mullet[78]. Addition of dopamine with GnRH produces better results in spawning[79–83]. Spawning of two Indian major carps by using murrel GnRH, with pimozide and Ca^{2+} has been sucessfully conducted earlier[66]. This has been modified later by introducing two forms of highly biologically active GnRH, GnRH I and GnRH II, which have been isolated from Indian murrel brain, and in combination with other chemicals it is producing excellent results in the spawning of Indian major carps[84]. However, attempts are in progress to use the downstream regulators, i.e. MIH or its precursor for induction of spawning but it has not met with success yet. Spawning is a complex behaviour, where not only MIH but other steroids and/or peptide factors are possibly involved, all of them are triggered by GnRH-mediated GTH release and its action on the gonads.

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