Korean J Biol Sci 3: 103-113, 1999

Regulation of Prolactin Secretion: Dopamine is the Prolactin-release Inhibiting Factor (PIF), but also Plays a Role as a Releasing Factor (PRF)

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Key Words:
Prolactin
Cyclic AMP
Rough endoplasmic reticulum
Pulsatile secretion
Neurophysin
Ascorbic acid
Dopamine

Many in-depth reviews related to regulations of prolactin secretion are available. We will, therefore, focus on controversial aspects using personal opinion in this review. The neuroendocrine control of prolactin secretion from the anterior pituitary gland involves multiple factors including prolactin-release inhibiting factor (PIF) and prolactin releasing factor (PRF). The PIF exerts a tonic inhibitory control in the physiological conditions. The PIF should be able to effectively inhibit prolactin release for a lifetime, but the inhibitory action of dopamine cannot be sustained for a long period of time. Perifusion of a high concentration of dopamine (1,000 nM) could not sustain inhibitory action on prolactin release but when a small amount of ascorbic acid (0.1 mM) is added in a low concentration of dopamine (3 nM) solution, prolactin release was inhibited for a long period. Ascorbate is essential for dopamine action to inhibit prolactin release. We have, therefore, concluded that the PIF is dopamine plus ascorbate. The major transduction system for dopamine to inhibit prolactin release is the adenylyl cyclase system. Dopamine decreases cyclic AMP concentration by inhibiting adenylyl cyclase, and cyclic AMP stimulates prolactin release. However, the inhibitory mechanism of dopamine on prolactin release is much more complex than simple inhibition of cAMP production. The dopamine not only inhibits cyclic AMP synthesis but also inhibits prolactin release by acting on a link(s) after the cAMP event in a chain reaction for inhibiting prolactin release. Low concentrations of dopamine stimulate prolactin release. Lactotropes are made of several different subtypes of cells and several different dopamine receptors are found in pituitary. The inhibitory and stimulatory actions induced by dopamine can be generated by different subtype of receptors. The GH_4ZR_7 cells express only the short isoform (D_{2s}) of the dopamine receptor, as a result of transfecting the D_{2s} receptors into GH_4C_1 cells which do not express any dopamine receptors. When dopamine stimulates or inhibits prolactin release in GH_4ZR_7 cells, it is clear that the dopamine should act on dopamine D_{2s} receptors since there is no other dopamine receptor in the GH_4ZR_7. Dopamine is able to stimulate prolactin release in a relatively low concentration while it inhibits in a high concentration in GH_4ZR_7. These observations indicate that the dopamine D_2 receptor can activate stimulatory and/or inhibitory transduction system depending upon dopamine concentrations.

Many in-depth reviews related to regulations of prolactin secretion are available (MacLeod 1976; Lambert and MacLeod 1990; Ben-Jonathan 1985). We, therefore, do not intend to cover every aspect of the regulation of prolactin secretion in this review. In order to avoid unnecessary repetition, we will briefly describe the established concepts of regulation, and then focus on less established aspects or "controversial aspects" using personal opinion and experience.

One of the most important endocrine glands is the hypophysis, commonly called the pituitary. The pituitary is comprised of neurohypophysis (posterior pituitary) and adenohypophysis (anterior pituitary). The middle part of pituitary (pars intermedia), which secretes melanocyte stimulating hormone (MSH), is much more active in lower vertebrates than human. The posterior pituitary releases two neurohypophysial hormones; oxytocin and vasopressin, while the anterior pituitary secretes six major protein hormones; growth hormone (GH or somatotropin), prolactin, luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimul-

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ating hormone (TSH) and adrenocorticotrophic hormone (ACTH). Prolactin is one of these six anterior pituitary hormones and mainly promotes milk production. The hormone secreting cells in anterior pituitary are traditionally classified as acidophils (GH and prolactin) and basophils (LH, FSH, TSH and ACTH). These cells can also be identified with immunocytochemical techniques these days, and names of particular pituitary cells can be indicated.

The hormones secreted from basophils are mainly regulated by stimulation of hypothalamic releasing hormones. GH and prolactin are chemically acidic proteins and their actions some times crossover each other. For example, bovine GH (bovine somatoropin, BST) is used to enhance milk production, rather than bovine prolactin. Another common denominator of GH and prolactin is regulation of secretions. Secretions of these two hormones are regulated by dual factors, hypothalamic releasing and inhibiting hormones, in contrast to basophils. GH secretion is mainly stimulated by growth hormone releasing hormone (GRH) and inhibited by somatotropin-release inhibiting hormone (SRIF or somatostatin). GRH plays the major role in most physiological conditions. Prolactin secretion is mainly controlled by the prolactin-release inhibiting factor (PIF), while prolactin releasing factor (PRF) stimulates prolactin secretion in a few limited number of situations, such as sucking breast and stress.

Once chemical structures are known and the physiological functions are well established, they are commonly called releasing or inhibiting hormones while names of releasing or inhibiting factors are used when some uncertainty is still lingering. We will describe this inhibitory agent as prolactin release inhibiting factor (PIF) instead of the prolactin release inhibiting hormone (PIH) because dopamine alone cannot completely satisfy all the criteria to be the inhibiting hormone.

Investigators have been focused on the mechanism of secretion to find means to cure hypersecretion (prolactinemia) rather than to enhance the physiological function of prolactin (milk production). Prolactin secreting tumors are the most common pituitary tumor (usually adenoma), and high plasma concentrations of prolactin disrupt menstrual cycle and suppress libido. Prolactin also antagonizes LH actions. The physical enlargement of pituitary adenoma can press on optic chiasma, which is located just above the pituitary. The physical pressure causes impairment of vision and can result in blindness.

The prolactin-release inhibiting factor (PIF)

The neuroendocrine control of prolactin secretion from the anterior pituitary gland involves multiple factors including PIF and PRF. The PIF usually predominates to exert a tonic inhibitory control in the most physiological conditions (Neill, 1987; Ben-Jonathan et al., 1985; Lamberts and MacLeod, 1990; Shin 1978). When hypothalamic influence is eliminated, prolactin secretion is increased in contrast to other anterior pituitary hormones whose secretions are decreased because hypothalamic releasing hormones regulate their secretions. The concept of inhibitory regulation is supported by evidence that hypothalamic lesions (Bishop et al., 1971) and transplantation of the anterior pituitary to extracranial sites such as the kidney capsule (Chen et al., 1970) resulted in a marked increase in prolactin secretion. The transplantation to the other part of body severs hypothalamic control on pituitary hormone secretions since the hypothalamic releasing or inhibiting hormones are released into hypophysial portal blood in high enough concentrations to generate biological actions. They are then diluted into the general circulation to subthreshold concentrations after passing through capillary bed in anterior pituitary. Therefore, the hypothalamic releasing and inhibiting hormones are not full fledged hormones, but "local hormones".

Convincing evidence has been given that dopamine, secreted by the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus, is the main physiological PIF (MacLeod, 1976; Ben-Jonathan et al., 1985; Lamberts and MacLeod, 1990). The dopamine synthesized in TIDA neurons is released from median eminence and the hypophysial portal system carries the released dopamine to anterior pituitary where dopamine stimulates dopamine D_2 receptors. Stimulation of the dopamine D_2 receptor causes the inhibition of prolactin release.

It would be interesting to point out a historical coincidence. In the early periods (1960s) of *in vitro* prolactin secretion studies, Medium 199 was the most commonly used incubation medium. Medium 199 contains a small amount of ascorbic acid (50 μg/L) in contrast to Dulbecco's modified Eagles medium (DMEM) which does not include ascorbic acid, and has been widely used in later studies. Dopamines inhibitory action on prolactin release from lactotropes is much more sensitive in Medium 199 than in DMEM. Low concentrations of dopamine (2-40 ng/mL) showed some sign of inhibition of prolactin release from rat pituitary halves in Medium 199 during 4 h incubation periods, although they are not statistically significant (Koch et al., 1970). In 1960-70s, rat pituitary halves were used in most studies. Cell dispersion techniques were later introduced to study pituitary hormone secretion (Vale et al., 1973; Hymer et al., 1973). The major advantage of dispersed, cultured cells is elimination of diffusion barrier for stimulation by secretogogues and for hormone release into medium. The dispersed pituitary cells after 2-6 d culture period are much more sensitive to secretagogues than pituitary halves or acutely dispersed cells. The sensitivity declines after 12 d culture and pituitary cells do not synthesize hormones anymore after 2-3 wk culture period though cells appear to be healthy. Therefore, one cannot subculture primary cultured cells. Many investigators believe that the

increased sensitivities are due to recovery of receptors which are damaged during an enzyme (usually trypsin) treatment for cell dispersion. The receptor damage may be one of the reasons, but the entire structure of the cell is altered during the primary culture period. The major change is cell polarity. Each cell in anterior pituitary (about 5 million cells in 10 mg anterior pituitary of a 300 g rat) has direct contact to a capillary blood vessel where hormone secretion occurs. Since only one corner of a cell contacts the capillary, the cell is organized to move secretory granules toward the capillary. However, a part of cultured cells attached to a tissue culture plate. Therefore, hormone granules can be exocytosed through wide area of cell membrane in cultured cells.

Dynamic perifusion systems have been used to analyze rates of hormone secretion using cultured pituitary cells (Shin et al., 1992). The most common perifusion system is composed of a disposable 3 ml plastic syringe packed with inert matrix (either Sephadex or BioRad gel), making a sandwich of the dispersed pituitary cells between two layers of the matrix. Medium is pumped through the perifusion column. In a DMEM perifusion system (containing 0.1% bovine serum albumin) pumped through the matrix containing cells, 46 ng/ml (300 nM) dopamine was able to inhibit prolactin release only for a brief period (15 min) from primary cultured pituitary cells, and a very high concentration of dopamine (153 ng/ml or 1,000 nM) inhibited only for 50-60 min (Shin et al., 1997). Most investigators use DMEM containing bovine serum albumin. The reason is not clear why protein (usually bovine serum albumin) is required for hormone release, but pituitary cells do not respond well to secretagogues without protein in a test medium. If early researchers happen to use DMEM in the *in vitro* study, they would easily miss dopamine as the PIF because the sensitivity of dopamine to inhibit prolactin release would be too high to accept dopamine as the PIF. The physiological concentrations of dopamine in the portal blood are 1-3 ng/ml (Cramer et al., 1979; Gudelsky and Porter, 1979; Plotsky et al., 1978).

It is essential to satisfy a minimum number of criteria for a compound to be acceptable as the releasing or inhibiting factor (or the hormone). The most commonly used criteria are as followings. The releasing or inhibiting factor should be (i) synthesized in the hypothalamus, (ii) released from median eminence, (iii) should change in concentration with the physiological event, (iv) the prolactin release pattern generated by injection of the PIF should be similar to the release pattern seen during the physiological situation, (v) the effect of PIF must be sensitive to both potentiating agents and antagonists, and (vi) the PIF should have ability to directly inhibit prolactin release from lactotropes.

Dopamine satisfies all the criteria to be the PIF as described above except criteria number (iv). The PIF should be able to effectively inhibit prolactin release for a lifetime, but the inhibitory action of dopamine cannot be sustained for a long period of time. That may be the reason why most investigators still use the name PIF instead of the PIH for dopamine. Perifusion of a high concentration of dopamine (1,000 nM) could not sustain inhibitory action on prolactin release during a 2 h perifusion period, but is able to inhibit prolactin release for only 50-60 min in an *in vitro* perifusion system (Shin et al., 1997). However, when a small amount of ascorbic acid (0.1 mM) is added in a low concentration of dopamine (3 nM) solution, prolactin release was inhibited during an entire 2 h experimental period (Shin et al., 1990a). Dopamine (3 nM) in this experiment is substantially lower than the physiological concentrations in the portal blood. Ascorbate (0.1 mM) is at a physiological concentration in plasma. This inability to inhibit prolactin release is not entirely caused by inactivation of dopamine according to following two pieces of evidence: (a) the dopamine half-life is 36 min in DMEM (Shin et al., 1990a) and thus dopamine concentration should not be decreased to less than 1/4 of original concentration during the 60 min perifusion period; 1,000 nM dopamine will be decreased to 250 nM during 72 min period, which is higher than 10 times the physiological concentration. (b) 2 h old dopamine stored in the same condition as perifusion medium, was still able to inhibit prolactin release although 1000 nM dopamine can inhibit prolactin release for less than 60 min during perifusion period (Shin et al., 1990a).

Dopamine is oxidized by oxygen in solution and ascorbate, a reducing agent increases the half-life of dopamine action from 36 to 792 min (Shin et al., 1990a) in DMEM while the half-life of dopamine is much longer in Medium 199 than in DMEM. Dopamine in Medium 199 was lost to about 24% during 3 h incubation period (Marien et al., 1984) which represents approximately the same half-life as dopamine in ascorbate containing DMEM. It used to be believed that the reducing agent enhances dopamine action exclusively by increasing half-life. However, ascorbate pharmacologically potentiates dopamine action and prevents down-regulation of dopamine D_2 receptors in lactotropes (Shin et al., 1997). Ascorbate is essential for dopamine action to inhibit prolactin release. A relatively high concentration of ascorbate (23-85 μM) is found in blood (Scully, 1986) (or 35-130 μM in plasma). The ascorbate concentration in blood is high enough to potentiate dopamine action and prevent rapid down-regulation of D_2 receptors in lactotropes to sustain the receptor sensitivity. From these results, we have concluded that the physiological PIF is dopamine plus ascorbate (Shin et al., 1997).

Another strange twist is that while dopamine is the established inhibiting factor on prolactin release, it also stimulates prolactin release in a low concentration (Shin 1978; Burris et al., 1991; 1992; Denef et al., 1980; Chang et al., 1997a). When dopamine perifusion

is withdrawn, rates of prolactin release rebound to much higher levels of release than previous control values (Hanna and Shin 1992). This matter will be further discussed in the transduction section. The "rebound release" may be related to stimulatory action in a decreased dopamine concentration during the period.

A comprehensive model for the intracellular transport, processing and release of secretory was proposed by Palade (1975; Dannies, 1999). In this model, secretory products undergo synthesis on polysomes, segregation into the lumen of the rough endoplasmic reticulum, followed by concentration and packaging into secretory granules in the Golgi complex, subsequent to post-translational modifications. These granules can then be released by exocytosis or degraded intracellularly through crinophagy (Smith and Farquhar, 1966; Kuriakose et al., 1989; Dannies, 1999). If this orderly process of "synthesis→storage→release" is the sole mechanism, newly synthesized (radioisotope labeled) hormone should be released into medium after all old stored hormone is depleted. However, newly synthesized hormone is preferentially released over stored prolactin (Swearingen 1971; Piercy and Shin, 1981; Stirling and Shin, 1990). These observations have led to the proposal of an alternative pathway for hormone release. Tartakoff and Vassalli (1978) proposed that there are two pathways of hormone release in cells: a constitutive pathway free of short-term stimulation (or under influence of maximum inhibition for prolactin), and a regulated pathway which utilizes the process proposed by Palade (1975). Other models forwarded to explain these observations is the existence of functional heterogeneity among subpopulations of lactotropes (Hopkins and Farquhar 1973; Snyder et al., 1977; Walker and Farquhar, 1980). One subpopulation of lactotropes may rapidly deplete old hormone and secretes new (labeled) hormone while another subpopulation slowly secretes old (non-labeled) hormone. Therefore, the ratios between old and new hormones can be changed in different experimental conditions. TRH stimulates prolactin release on both old and newly synthesized prolactin but in different ratios from the basal release. Specific activities of prolactin released by a treatment with TRH had a lower mean value than control group (Walker and Farquhar, 1980; Morin et al., 1984b; Stirling and Shin, 1990). The newly synthesized prolactin is preferentially released under basal conditions in lactotropes (Swearingen 1971; Walker and Farquhar, 1980; Piercy and Shin, 1981; Morin et al., 1984b). These different ratios between old and newly synthesized prolactin under different experimental condition suggest that prolactin does not secrete in an orderly chronological sequence, and that routes of prolactin secretion are not a single channel of "synthesis→package→storage→secretion", but shunt passage(s) or multiple channels likely exit.

Somatostatin does not inhibit proalctin release in the normal male rat, but can effectively inhibit prolactin release in estrogen-primed male rats. The estrogen induces somatostatin receptors (Cooper and Shin 1981; Kimura et al., 1986). This induction of somatostatin sensitivity may be related to the following observation. Estradiol converts somatotropes which release only GH to mammosomatotropes, cells that release both GH and prolactin (Boockfor et al., 1986). It is possible that somatostatin inhibits the release of prolactin from these mamosomatotropes in the estrogen-primed male rat. The estrogen primed pituitary cells are very sensitive to the inhibitory action of somatostatin (Cooper and Shin, 1981). The PIF plays an important physiological role in both male and female, but somatostatin cannot inhibit prolactin release in male rats. Therefore, the somatostatin cannot be the PIF even though it may contribute to regulation of prolactin release in the female.

Prolactin releasing hormone (PRF)

Many pieces of evidence support the existence of PRF (Boyd et al., 1976; Nicoll et al., 1970; Shin 1980; Frohman 1976; Valverde et al., 1972), though hypothalamic control of prolactin release is mainly achieved by secretion of the PIF.

It is generally believed that stress-induced prolactin release is stimulated by the PRF rather than inhibition of PIF release. We have examined prolactin release induced by stress to demonstrate that the PRF plays a significant role on prolactin release (Shin 1979; 1980). Two possible mechanisms by which hypothalamic factors can enhance prolactin release from lactotropes are (i) stimulation of PRF release and/or (ii) inhibition of PIF release. Under the stimulatory conditions studied, for example, during suckling, the level of dopamine in portal blood is decreased (Mena et al., 1976). Complete blockade of the dopaminergic PIF receptors would eliminate the second possibility since the PIF cannot work on inhibiting prolactin release. Therefore, PRF activity can be demonstrated without interference of the PIF. An excessive amount of pimozide (3 mg/kg), a dopamine receptor blocker, completely blocks dopaminergic receptors (Shin 1979). Plasma prolactin concentration was vastly elevated due to elimination of dopamine-induced tonic inhibition (Piercy and Shin, 1980). In this situation, lacking functional dopaminergic PIF receptors, rats were exposed to ether stress (Shin 1979; 1980). The ether stress elevated the circulating prolactin concentration. The elevation shows that factor(s) other than the PIF stimulates prolactin release, since the PIF influence is completely removed (Shin, 1980). Therefore, the ether-induced prolactin release supports that the PRF is involved in the generation of this prolactin surge. Ether is a well known general anesthetic agent but generates a powerful acute stress on initial exposure, and is commonly used as a stress agent.

However, a possibility that the inhibition of non-dopaminergic PIFs may result in prolactin release is not excluded. Hypothalamic extracts, from which dopamine has been removed by either physicochemical (Enjalbert et al., 1977) or by pharmacological (Quijada et al., 1973) means inhibit prolactin release suggesting existence of other inhibiting factor(s) than dopamine in the hypothalamus. One of the putative, non-dopaminergic PIFs is γ-aminobutyric acid (GABA) (Schally et al., 1977). However, close examination of the effects of GABA showed that it is not a physiological PIF (Shin et al., 1984). GABA and its agonists inhibit prolactin release in a dose-related manner *in vitro* (Enjalbert et al., 1979; Schally et al. 1977; Shin et al., 1984), but a GABA agonist or antagonist does not inhibit or stimulate, respectively, prolactin release in conscious freely moving rats (Shin et al., 1984). GABA cannot be involved in this ether-induced prolactin release since GABA is ineffective on prolactin release in *in vivo* system.

Several peptides have been considered as putative PRFs. The most prominent candidates are thyrotropin releasing hormone (TRH), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) and neurohypophysial hormones (oxytocin and vasopressin). They are synthesized in the hypothalamus, released from median eminence and may stimulate prolactin release. However, none of these peptides satisfies the minimum number of required criteria to be the PRF (Shin et al., 1987). We spent considerable time to resolve whether a neurohypophysial hormone(s) is the PRF since they are synthesized in the hypothalamus (supraoptic nucleus and paraventicular nucleus) and released from median eminence (Zimmerman et al., 1973), and oxytocin has powerful stimulatory action on prolactin release in an *in vitro* system (Samson et al., 1986).

Oxytocin is extremely potent on stimulating prolactin release in an *in vitro* system, 1 nM oxytocin can effectively stimulate prolactin release (Samson et al., 1986). The powerful stimulatory action of oxytocin on prolactin release in *in vitro* system indicates that it acts directly on lactotropes to stimulate prolactin release. If a compound has powerful direct stimulatory action, it should be able to stimulate the hormone secretion in a whole animal since the agent will directly stimulate hormone secretion. However, oxytocin is inactive in free moving rats even in a very high dose. Vasopressin is virtually inactive in an *in vitro* system, but can stimulate proalctin release in an extremely high dose in an *in vivo* system (Shin, 1982). Both *in vitro* and *in vivo* systems normally produce the similar responses when a secretagogue is administered. However, they sometimes react very differently as oxytocin actions have shown. The *in vitro* system is very useful to elucidate mechanisms of action because feedback systems and other interference induced by complex homeostatic mechanisms are eliminated, while the *in vivo* system is closer to the physiological conditions. Therefore, when biological actions are examined, both *in vivo* and *in vitro* system are useful approaches to study effects of a hormone.

We tried to purify the PRF from neurohypophysis using a classical method. Prolactin releasing activities were examined in each fraction after fractionation of neurohypophysial (bovine posterior pituitary) extract using an *in vivo* rat model. The most active fraction was again subfractionated and each subfraction was further assayed. Finally we end up with a pure protein fraction according to gel electrophoresis. This protein has stimulatory action on prolactin release. The active compound turned out to be neurophysin-II (Shin and Obonsawin, 1985). The neurophysin-II is synthesized together with vasopressin in a single protein as a prohormone and processed to produce neurophysin-II and vasopressin. Neurophysin-II is the specific carrier protein for vasopressin and used to believe a biologically inert protein.

All the evidence indicates that the PRF plays an important role on prolactin release during sucking breast as well as stress. However, the chemical nature of PRF is not established. Many known hormones are suggested to be a PRF but not a single compound completely satisfied the criteria to be the PRF. It is now generally agreed that the PRF is combined actions of several stimulatory compounds on prolactin release. Although dopamine is established as the PIF, there is convincing evidence showing that dopamine, at much lower concentrations than those required for inhibition of prolactin secretion, stimulates prolactin release (Shin 1978; Denef et al., 1980; Burris et al., 1992). The low concentrations of dopamine may play a role as another PRF.

Estradiol has a stimulatory effect on prolactin release (Chen and Meites, 1970). The response time of estradiol's stimulatory effect on prolactin release is too slow to be considered a PRF. It takes at least 2 h before any effect on prolactin release can be seen, even if estradiol is directly injected into the right atrium (Shin, 1979b). The stimulatory effect of estradiol on prolactin release is believed to be mostly due to the enhancement of prolactin synthesis (Maura 1979; Seo et al., 1979; Shull and Gorski, 1984; Lee and Shin, 1996; Cho et al., 1993). However, the stimulatory effect of estradiol on prolactin release is far more complex than just an increase in the rate of prolactin synthesis and release. Dopamine inhibits prolactin release by 70-90% from non-estradiol-primed lactotropes (Giguere et al., 1982; Raymond et al., 1978; Shin et al., 1990; West and Danies 1980), while it decreases release by only 50% from estogen-primed lactotropes (Giguere et al., 1982; West and Dannies 1980). Although this phenomenon can partly be explained by a decrease in the number of dopamine receptors (Ali and Peck, Jr., 1985; Heiman and Ben-Jonathan 1982), the complete effect of estradiol on lactotropes is complex.

Progesterone can stimulate prolactin release in estradiol primed experimental animal model (Williams et al., 1985). This stimulation is an indirect stimulation since lactotropes do not have progesterone receptors (Strangers et al., 1989; Fox et al., 1990) and does not stimulate prolactin release in an *in vitro* system. The stimulatory action is blocked by naloxone suggesting that endorphine is involved between the stimulatory action of progesterone and prolactin release. Endorphine acts on TIDA system by inhibiting dopamine secretion (Pecins-Thompson et al., 1996). Therefore, prolactin secretion induced by progesterone should be involved in a link(s) of chain reactions to stimulate prolactin release. In contrast to the stimulation of prolactin release in estradiol primed monkey, progesterone inhibited prolactin synthesis (Cho et al., 1993). These observations suggest that the synthesis and release can be dissociated.

Pulsatile secretion

In endocrine studies, measurements of hormone concentrations in plasma (or serum) are critically important. Hormones, in contrast to other plasma constituents such as albumin and γ-globulin, have relatively short half-lives. The short half-life can cause rapid fluctuations of the plasma hormone concentrations with time if the hormone secretes intermittently. We, therefore, consider that blood sampling technique is important to visualize a true profile of the hormone concentration. Many investigators have been taking relatively large volumes of blood samples every 30 min, or even longer periods, and examine changes of plasma hormone concentrations. An extra amount of blood is taken to get rid of void volume in collection tubing before taking assay samples. The red blood cells in the blood samples are normally recovered by centrifugation and returned them to the experimental animals, usually with suspension in saline. Returning the red blood cells is a good idea to maintain oxygen carrying capacity, but the experimental animals lose plasma proteins and other hormones in plasma, even if the blood cells are returned. We believe that taking smaller amounts of blood would be better than larger amounts even if the red blood cells are returned.

Many pituitary hormones, including prolactin, have short half lives. For example, the half-life of prolactin is 5 min (Chi and Shin, 1978). In principle, 2 or more blood samples should be taken during the half-life period to visualize a reasonable profile of hormone secretion. A commonly used technique for frequent blood sampling in rat is to use an indwelling cannulae implanted in the right atria, and allow one week for recovery from surgery to avoid stress caused by surgery. This indwelling technique is an essentially stress-free blood sampling method. Many hormones are secreted in response to stresses such as handling rats and cardiopuncture. Blood samples are continuously taken by letting blood slowly flow in a rate of 70 μl/2 min. A slow rate of blood flow reduces the "tail" of pulsatile changes. Experimental animals, usually rat in endocrinological studies, have a limited amount of blood (5% of body weight, 15 ml in 300 g rat). We have been taking 60-70 μl blood samples from rats in capillary tubes originally designed for hematocrit measurements. The blood samples in the hematocrit tubes are centrifuged in a hematocrit centrifuge, and cut above the white blood cell line. Plasma in the tube is recovered with a 10 μl Eppendorf pipette and plasma samples are assayed in triplicates. Prolactin in the 10 μl plasma samples is sufficient to quantify the prolactin content by a radioimmunoassay. This type of work generates a massive number of samples to assay, and thus study of pulsatile hormone secretion is only feasible after radioimmunoassay techniques are readily available. One person can handle a few thousand samples in a week using automatic or semiautomatic radioimmunassay systems. Our protocol worked well without serious anemia of the experimental animals. Red blood cells in rats normally have a large reserve capacity to carry oxygen to the organs.

We have established that prolactin is secreting in a pulsatile fashion using the blood sampling technique described above (Chi and Shin, 1978). The best studied pulstile secretion of hormones is GH. GH secretes in a regular interval (3.2 h) in male rats (Tannenbaum and Martin, 1976). The prolactin secretion pattern is not periodic, but rather irregular (Shin 1979b).

Many peptide hormones are secreted in a pulsatile fashion. Pulsatile secretion of a hormone has a biological advantage. According to an old concept, peptide hormone receptors are perceived to be some fixed structures on the membrane. However, they are actually in a dynamic equilibrium state and rates of receptor down-regulation are very rapid when a hormone binds to its receptors. When dopamine D_2 receptors are exposed to dopamine, receptors are down-regulated within a few minutes (Shin et al., 1997). The pulsatile secretion does not persistently stimulate receptors but intermittently activates them. During a trough of hormone concentration, receptors are not stimulated and thus can be recovered from down-regulation. Therefore, the pulsatile secretion of a hormone prevents receptor-down regulation, and thus maintain a physiological level of sensitivities.

Transduction system

The major transduction system for dopamine to inhibit prolactin release is the adenylyl cyclase system. It is well established that dopamine can inhibit adenylyl cyclase activities (Lambert and MacLeod, 1990; Ben-Jonathan 1989; Burris and Freeman, 1994) and prolactin release is decreased due to the inhibition of cyclic AMP (cAMP) production. In other words, dopamine decreases cyclic AMP concentration by inhibiting

adenylyl cyclase, and cAMP stimulates prolactin release. However, the inhibitory mechanism of dopamine on prolactin release is much more complex than simple inhibition of cAMP production. If activation of dopamine D_2 receptors inhibits only cAMP production to decrease prolactin release, cAMP administered in incubation medium should stimulate prolactin secretion without influence of dopamine in the medium. In contrast to the anticipation, dopamine effectively inhibited cAMP-induced prolactin release (Hanna and Shin, 1992). The observation indicates that the dopamine not only inhibits cAMP synthesis but also inhibits prolactin release by acting on a link(s) after the cAMP event in a chain reaction for inhibiting prolactin release. Dopamine generates many different actions by activating different dopamine receptors. For example, the actions of dopamine are mediated through multiple G proteins (Burris et al., 1992; Kineman et al., 1996), activation of G-protein-regulated potassium channels (Lledo et al., 1992; Werner et al., 1996), voltage dependent potassium channels (Login et al., 1990) and changes in cytosolic Ca^{2+} ion concentration (Burris and Freeman, 1993; Malgaroli et al., 1987; Cui et al., 1994; Chang and Shin, 1997b).

Dopamine receptors belong to the G protein-coupled receptor super family. Five distinct dopamine receptor subtypes (D_1-D_5), which are encoded by separate genes, have been identified (Civelli et al., 1993). It is widely believed that the D_2 receptors in the anterior pituitary are responsible for the inhibitory actions on prolactin release (Ben-Jonathan, 1985). The genomic sequence of the dopamine D_2 receptor is alternatively spliced to generate two isoforms (a short isoform [D_{2s}] and a long isoform [D_{2l}]) which differ by a 29 amino acid insert in the third cytoplasmic domain (Dal Toso et al., 1989; Gandelman et al., 1991). D_{2s} and D_{2l} receptors are structurally very similar, and they are pharmacologically indistinguishable (Faladeau 1994).

It is known for some time that low concentrations of dopamine stimulate prolactin release (Shin, 1978; Denef et al., 1980; Burris et al., 1991; Chang et al., 1997a). Lactotropes in pituitary are made of several different subtypes of cells (Luque et al., 1986) and several different dopamine receptors are found in pituitary (Gingrich and Caron, 1993). The inhibitory and stimulatory actions induced by dopamine can be generated by different receptors (inhibitory and stimulatory receptors) (Burris et al., 1991). The presence of inhibitory and stimulatory receptors does not exclude a possibility that dopamine D_2 receptors can both inhibit and stimulate prolactin release. We tried to resolve the question whether D_2 receptors can generate the inhibitory and stimulatory actions. To answer the question, dopamine actions on prolactin release were examined using the GH_4ZR_7 cell line.

The GH_4ZR_7 cells express only the short isoform (D_{2s}) of the dopamine receptor, as a result of transfecting the D_{2s} receptors into GH_4C_1 cells (Albert et al., 1990). The GH_4C_1 cells have many different receptors such as TRH, but do not express any catecholamine receptors, including dopamine receptors (Tashjian, 1979). GH_4ZR_7 cells have a unique advantage over primary cultured cells because GH_4ZR_7 cells express only short form of dopamine D_2 receptor (D_{2s}) while several different dopamine receptors are identified in the primary cultured cells (Gingrich and Caron, 1993). When dopamine stimulates prolactin release in the primary cultured cells, defining which stimulatory dopamine receptor stimulates prolactin release is difficult. However, when dopamine stimulates or inhibits prolactin release in GH_4ZR_7 cells, it is clear that the dopamine should act on the short form of dopamine receptors since there is no other dopamine (or catecholamine) receptor in the GH_4ZR_7. A high concentration of dopamine can stimulate adrenergic β-receptors which transduct stimulatory action on prolactin release (Shin and Barton, 1993). Therefore, if other catecholamine receptors than dopamine are expressed in GH_4ZR_7 cells, there is a possibility that dopamine might activate other catecholamine receptors to stimulate prolactin release. Dopamine is able to stimulate prolactin release in a relatively low concentration while it inhibits in a high concentration in GH_4ZR_7 (Chang et al., 1997a). It sound strange, but is consistent with observations that dopamine D_2 receptors are able to both stimulate and inhibit prolactin release. Pertussis toxin blocks the inhibitory action of dopamine, suggesting that G_i protein is likely involved in prolactin release, but stimulatory actions of dopamine are not blocked by treatment with pertussis toxin (Burris et al., 1992; Chang et al., 1997b).

These observations indicate that the dopamine D_2 receptor can activate stimulatory and/or inhibitory transduction system depending upon dopamine concentrations. The D_2 receptors are 7 transmembrane receptors which reside within the membrane, and G-proteins suspended and mobile in the cytosol. The relationship between D_2 receptors and G-proteins is that they are not physically fixed structures, but flexible. D_2 receptors can activate different G-proteins if conditions are appropriate. The dopamine D_2 receptors may be coupled to both a G_i and a G_s protein, so that a single receptor is linked to both the inhibitory and the stimulatory pathways. More evidence that a single type of receptors can produce different response has been accumulating. For example, adrenergic β_2-receptor not only activates adenylyl cyclase system, but also interact Na^+/H^+ exchanger regulatory factor (Hall et al., 1998).

We propose a hypothesis to explain the dual actions of the dopamine D_2 receptors. Activation of either of the two pathways depends on the rate of dopamine association to the receptors; the stimulatory transduction pathway may be activated when the rates of association between dopamine and D_2 receptors are relatively low, and the inhibitory transduction pathway

may be activated when the rates of association are relatively higher. This hypothesis is based on the rate theory, which was proposed by Paton (1961). The Patons rate theory is less popular these days than Clark's occupation theory, but the dual action of dopamine can be reasonably explained by the rate theory. Initial rates of the association are concentration-dependent, assuming that the number of D_2 receptors in an experimental system is constant. In addition, it is possible that both the stimulatory and the inhibitory actions of dopamine may be activated when the D_2 receptors in GH_4ZR_7 cells are activated by dopamine. However, the stimualtory effect can be masked by the inhibitory effect induced by a high concentration of dopamine, and thus only inhibitory response is observed with high concentrations (Kineman et al., 1994).

Utrastructural changes induced by dopaminergic agents

Ultrastructure in a cell is traditionally believed to be a semi-permanent or permanent feature. However, treatment of lactotropes with dopamine or dopamine agonists caused a massive increase in volume of "rough endoplasmic reticulum" within a minute or two, and this effect did not happen in other pituitary cells (Reifel et al., 1983a; b). The same ultrastructural changes occurred when the lactotropes are treated with somatostatin (Saunders et al., 1982). This "rough endoplasmic reticulum" satisfies all anatomical criteria to be the "rough endoplasmic reticulum", but the physiological significance of the "rough endoplasmic reticulum" is not known. The structure is not the protein synthesis mechanism due to the following reasons: (a) no synthetic apparatus will increase to such a degree within 2 minutes and (b) dopamine treatment inhibits prolactin synthesis (MacLeod, 1976), in contrast to the increased volume of the "rough endoplasmic reticulum".

We have proposed a hypothesis to explain a role of "rough endoplasmic reticulum": the "rough endoplasmic reticulum" is a physical barrier to block migration of prolactin granules toward membrane for secretion. The hypothesis is our tentative proposal and not widely accepted. We originally examined ultrastructure of lactotrophs to see depletion rates of secretory granules in lactotropes after treatment of a dopamine antagonist, pimozide, which causes massive amounts of prolactin release (Shin and Chi, 1979). Lactotropes have a large number of secretory granules which are irregular shapes. Dopamine was used as a reference control group in this study. There was no difference in number of granules between control group and pimozide treated group though a large amount of prolactin is secreted. However, a dopamine-treated group, an experimental control unexpectedly showed a massive ultrastructural changes within 2 min (Reifel et al., 1983a; b), in contrast to conventional wisdom that ultrastructure is a semi-permanent structure in a cell. During dopamine treatment, prolactin granules are increased in numbers as expected since prolactin secretion is inhibited. Secretory granules are localized in a core of the "rough endoplasmic reticulum". Many layers of the "rough endoplasmic reticulum" physically surround a group of prolactin granules. They are likely assembled into a visible structure from an invisible soluble form in cytoplasm since no *de novo* structure can be formed in less than two minutes. We also observed unidentified "intracellular body" in the lactotropes during the dopamine treatment. The prolactin secretory granules are associated with the "intracellular bodies" in projectile shape with granules lined up in an orderly manner (Reifel et al., 1983a). We tentatively postulate that the "intracellular bodies" are a disposal mechanism of surplus prolactin granules.

There is some mystery in secretory granules. As previously pointed out, we could not detect any deference in number of granules even if a huge mount of prolactin is secreted by pimozide treatment. The number of omega (Ω) shaped exocytoses on cell membrane were not increased. Many investigators tend to dismiss these observations by saying that difference cannot be detected if about 5% of granules are secreted. However, according to our calculation, total amounts of released prolactin by pimozide treatment is about 30-50% of total pituitary contents. Another strange fact related to granules is that GH_4 or GH_4ZR_7 cells do not have secretory granules in the cells, but their pattern of secretion is similar to primary cultured pituitary cells which have a large number of granules. The storage granules are supposed to be secreted by exocytotic processes. A question arises; how do GH_4 cells secrete prolactin by exocytosis without storage of granules? Because of these mysteries, some investigators consider the granules as a preparation stage to dispose surplus prolactin which will eventually be removed by lysosomes, or the granule is a "garbage can". I personally feel that there is some doubt about a concept of exocytosis of granules because the number of granules were not decreased when a large amount of prolactin is released and secretion patterns of prolactin from GH_4 and GH_4ZR_7 cells are the same as primary cultured cells.

Acknowledgements

The original works of this review were supported by the Medical Research Council of Canada. Appreciation is expressed to Ms. Kelly Moore for her excellent secretarial help.

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[Received April 28, 1999]