The effects of sex steroids on the formation of gap junctions between folliculo-stellate cells; a study in castrated male rats and ovariectomized female rats

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Summary. We investigated the relationship between gap junction formation and the sex steroids testosterone, progesterone and 17 β -estradiol in the anterior pituitary glands of castrated male rats and ovariectomized female rats. Male and female 30-day-old Wistar-Imamichi strain rats were castrated or ovariectomized, and 30 days later they were subcutaneously injected with the above sex steroids. They were divided into six groups according to the injected materials: sesame oil (control), testosterone, progesterone, 17 β -estradiol, testosterone with 17 β -estradiol, and progesterone with 17 β -estradiol. Five rats from each group were sacrificed 1, 2, 3, 4 and 5 days after the injections, and the anterior pituitary glands were prepared for observation by transmission electron microscopy. We quantified the number of follicles and gap junctions and calculated the rate of occurrence of gap junctions as the ratio of the number of gap junctions existing between folliculo-stellate cells per intersected follicle profile in electron photomicrographs. The administration of testosterone to castrated male rats increased the rate of gap junctions between folliculo-stellate cells; however, progesterone and 17 β -estradiol did not affect the formation of gap junctions. The administration of progesterone to ovariectomized female rats increased the rate of gap junctions between folliculo-stellate cells; this progesterone effect was prevented by the simultaneous administration of 17 β -estra-

diol, which by itself did not affect the rate of gap junctions between folliculo-stellate cells. These observations indicate that the formation of gap junctions within the anterior pituitary gland is regulated differently by sex steroids in castrated male and ovariectomized female rats.

Introduction

Agranular cells with star-like morphological features found within the adenohypophysis of many mammalian species have been named folliculo-stellate cells (Rinehart and Farquhar, 1953; Kagayama, 1965; Vila-Porcile, 1972). Folliculo-stellate cells, which form a cell network extending throughout the whole anterior pituitary gland, are coupled by gap junctions in normal mature rats (Soji *et al.*, 1990; 1991; Yamamoto *et al.*, 1993; Inoue *et al.*, 1999; Sakuma *et al.*, 2001). Gap junctions present between folliculo-stellate cells allow both metabolic and electrical coupling and produce a kind of synchronized syncytium within the anterior pituitary gland (Soji *et al.*, 1990; Guérineau *et al.*, 1998; Inoue *et al.*, 1999; Fauquier *et al.*, 2001). Soji *et al.* (1990, 1991) performed a quantitative electron microscopic study and reported that the gap junctions within the anterior pituitary gland could first be observed after day 20, with this number increasing to the normal adult level by day 40 in both male and female Wistar-Imamichi strain rats.

Both male and female rats undergo a rapid sexual maturation between 20 and 40 days of age that includes changes in the basal level of serum concentration of gonadotropins and sex steroids (Negro-Vilar *et al.*, 1973; Doeblner and Wuttke, 1975). Soji *et al.* (1990, 1991) suggested the existence of specific sex steroids effective for the maturation of gap junctions between folliculo-stellate cells. From results

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of studies on the developmental pattern of gap junctions between folliculo-stellate cells and the synchronous changes of serum sex steroids levels, they speculated that the most applicable sex steroids should differ between male and female rats. They observed a strong correlation between the development of gap junctions in male rats and age-related fluctuation patterns of serum testosterone concentration, while the gap junction development of female rats showed a strong correlation with the temporal pattern of serum progesterone concentration during sexual maturation. On the other hand, gap junctions only appeared in female rats after the fall of serum estradiol levels on day 20.

This study further investigates this topic by documenting the effects of sex steroids over gap junction formation between folliculo-stellate cells within the anterior pituitary glands of Wistar-Imamichi strain rats. It additionally demonstrates that the same sex steroid can promote different effects over the regulation of gap junctions in cases of castrated male rats and ovariectomized female rats.

Materials and Methods

All animal experiments were performed under the NIH Guidelines for the Care and Use of Laboratory Animals. Male and female Wistar-Imamichi strain rats were maintained under conditions of controlled temperature (22–24°C) and illumination (8:00–20:00 daily). All rats were castrated or ovariectomized at 30 days after birth under anesthesia with Nembutal® (pentobarbital). Animals were fed a normal diet and water. At 60 days of age, all animals received subcutaneous injections of the aforementioned sex steroids dissolved in 0.1 ml of sesame oil. Animals of each sex were separated into six groups: Group A received only sesame oil and served as the control, Group B was injected with testosterone, Group C was injected with progesterone, Group D was injected with 17 β -estradiol, Group E was injected with testosterone and 17 β -estradiol, and Group F received progesterone and 17 β -estradiol. Each group consisted of 25 animals. The animals in this study were administered a dose of testosterone, progesterone, and 17 β -estradiol that is known to produce in rats both high physiological levels of the hormones in plasma and sex steroid-inducible biological responses (Shull *et al.*, 1989; Suzuki *et al.*, 1998; Zhang *et al.*, 1999; Cardona-Gomez *et al.*, 2000; Schroeder *et al.*, 2000; Funabashi *et al.*, 2001). We measured and injected 1.5 mg/kg of testosterone, 2.5 mg/kg of progesterone and 1.5 mg/kg of 17 β -estradiol in these experiments. All sex steroids were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Five rats from each group were killed at ages 61, 62, 63, 64, and 65 days, at 1, 2, 3, 4 and 5 days after the injections, respectively. On the scheduled date, the animals were deeply anesthetized with Nembutal® and perfused with a fixative for five minutes through the left ventricle of the heart. The fixative used was 2.5% glutaraldehyde and 2% sucrose in a 0.05 M cacodylate buffer (pH 7.4). After the perfusion, the pituitary gland was removed from each rat, and the anterior lobe of the pituitary gland was dissected into four blocks. The lobes were then refixed by immersion for 1 h in the same solution used for perfusion. The sections were then postfixed for 1 h in 1% osmium tetroxide buffered by 2% sucrose and 0.05 M sodium cacodylate (pH 7.4). After postfixation, they were rinsed in ice-cold water for 5 min and dehydrated in a graded series of ethanol. Following two rinses in 100% ethanol for 10 min each, the specimens were immersed twice in absolute propylene oxide for 15 min each and then embedded in epoxy resin. Two ultrathin sections of 60 nm thickness and separated from each other by at least 100 μ m were prepared from each block. They were placed on copper grids, stained with uranyl acetate and lead citrate, and observed using a Hitachi H-7000 transmission electron microscope. In this way, a total of 8 ultrathin sections were investigated for each rat, amounting to 40 for each group in this study.

The photomicrographs were selected on the basis of their displaying a minimum of one follicle. We confirmed the detail of the trilamellar structure of the gap junction at magnification levels of at least 20,000. Distinction between the types of intercellular junctions was based on the morphological criteria described by Staehelin (1974). It has been suggested that the lattice symmetry of connexons in gap junctions is only exact when the junctions are flat, as gap junctions that are curved often display a disorder of the trilamellar structure under transmission electron microscopy (Peracchia, 1973; Casper *et al.*, 1977; Makowski *et al.*, 1984). Moreover, the cytoplasmic processes of folliculo-stellate cells are usually curved. We therefore, disregarded the interpolated disorder of the trilamellar structure within 20 nm in each gap junction, and counted the whole as one gap junction. We determined the rate of occurrence of gap junctions as the ratio of the number of gap junctions existing between folliculo-stellate cells per intersected follicle profile in electron photomicrographs. The number of follicles and gap junctions was quantified using a total of 400 electron photomicrographs that contained at least one follicle from each animal group, and the mean and standard error for each animal group was calculated. The data were statistically analyzed by an analysis of variance followed by the t-test for determining differences between means.

Fig. 1. Summary of the number of gap junctions present per follicle in the castrated male rat groups at each of the five ages studied.

Results

Observations showed many folliculo-stellate cells encircling the hypertrophied gonadotrophs with their long cytoplasmic processes. Gap junctions with the trilamellar structure of plasma membranes of adjacent cells were found between neighboring folliculo-stellate cells in all samples of the present study. There were no significant morphological differences between the sex steroids treatment groups and control groups.

Castrated male animal group

The rate of gap junctions in the control, sesame oil-injected group rats remained constant (0.17 ± 0.04 to 0.18 ± 0.05)

from day 1 to 5 after injections (Fig.1).

In the groups administered with progesterone, 17β-estradiol, and progesterone with 17β-estradiol, the rate of gap junctions showed no significant difference with control group rats.

In the testosterone injected group rats, an increase in the rate of gap junctions was found on days 2, 3 and 4 after injections. Gap junctions between folliculo-stellate cells were observed at an especially high ratio on day 3 after injections when their numbers reached 0.44 ± 0.07 per follicle (Fig. 1, 2). The rate of gap junctions in this group returned to a level similar to the control group on day 5 after testosterone treatment. The rate of gap junctions for the group which received a simultaneous injection of testosterone with 17β-estradiol showed a pattern similar to the testosterone-injected group from days 1 to 5 (Fig. 1, 3).

Fig. 2 and 3. Legends on the opposite page.

Fig. 4. Summary of the number of gap junctions present per follicle in the ovariectomized female rat groups at each of the five ages studied.

Ovariectomized female animal group

The rate of gap junctions in the control, sesame oil-injected group rats remained constant (0.16 ± 0.04 to 0.17 ± 0.04) from days 1 to 5 after injections (Fig. 4).

Treatment with testosterone, 17β-estradiol, and testosterone with 17β-estradiol caused no significant change in the rate of gap junctions in female rats.

Injection with progesterone resulted in an increase in the rate of gap junctions on days 2, 3 and 4. The rate of gap junctions between folliculo-stellate cells peaked at a maximum of 0.46 ± 0.06 per follicle on day 3 after injections (Fig. 4, 5). The rate of gap junctions returned to a level similar to the control group on day 5 after progesterone treatment.

In contrast with the progesterone-injected group, the rate of gap junctions for the group which received simultaneous

Fig. 2. In a castrated male rat anterior pituitary three days after testosterone treatment, three gap junctions (arrows) between folliculo-stellate cells are seen. The area in the rectangle is shown at a higher magnification in the inset. $\times 10,000$, inset: $\times 40,000$

Fig. 3. A 63-day-old castrated male rat treated with both testosterone and 17β-estradiol. Note one gap junction (arrow) between folliculo-stellate cells forming a follicle. $\times 20,000$, inset: $\times 60,000$

Fig. 5 and 6. Legends on the opposite page.

injection of progesterone with 17β -estradiol showed no significant difference with that of control group rats. The rate of gap junctions remained relatively constant (0.16 ± 0.03 to 0.17 ± 0.05) from days 1 to 5 (Fig. 4, 6).

Discussion

It has been suggested that sex steroids have a strong potential to affect the morphology of folliculo-stellate cells within anterior pituitary glands (Shirasawa *et al.*, 1983; Inoue *et al.*, 1999). Shirasawa *et al.* (1983) reported morphological changes in folliculo-stellate cells such as expanded and ramified cytoplasmic processes in the case of a gonadectomy, but these changes were not seen after the removal of thyroid glands. Inoue *et al.* (1999) confirmed these findings by demonstrating the enlargement of folliculo-stellate cells after a gonadectomy. In the present study, the morphological changes of folliculo-stellate cells closely agreed with these previous investigations. It has also been generally accepted that the formation of hypertrophied gonadotrophs in the anterior pituitary glands may be suppressed by the administration of sex steroids in male and female rats (Low, 1988; Low and Bachmann, 1988). It would seem natural for such changes as a lessening of cell size enlargement in gonadotrophs to be observed in our study; however, we could find no significant morphological changes in folliculo-stellate cells in any experimental group during the investigation period. We believe these phenomena arise from the longer period of time required for folliculo-stellate cells to undergo morphological changes when compared with gonadotrophs.

Gap junctions produce transmembrane channels that allow the free transcellular exchange of small cytoplasmic molecules such as ions, amino acids, sugars, and several second messengers (Loewenstein, 1981; Bennett *et al.*, 1991; Beyer, 1993; Spray, 1994; Wolburg and Rohlmann, 1995; Kumar and Gilula, 1996). It has been suggested that such an intercellular communication via gap junctions between folliculo-stellate cells might have some fundamental importance in maintaining homeostasis within the anterior pituitary gland (Soji *et al.*, 1990; Guerineau *et al.*, 1998; Inoue *et al.*, 1999; Fauquier *et al.*, 2001; Sakuma *et*

al., 2001). The dysfunction of a hypothalamo-pituitary gonadal axis can decrease the rate of gap junction formation between folliculo-stellate cells in male rats (Sakuma *et al.*, 2001; 2002). In addition, we found that the formation of gap junctions within the anterior pituitary gland of male rats lies under the influence of leptin and a ciliary neurotrophic factor that have been thought to share essential functions in the hypothalamo-pituitary gonadal axis in promoting normal sexual maturation (Sakuma *et al.*, 2002). In the present study, the rate of gap junctions was suppressed to a low level in both castrated male rats and ovariectomized female rats. The insufficient development of gap junctions is thought to be a result of the removal of gonads, while the castrated male rats injected with testosterone and ovariectomized female rats injected with progesterone showed a transient pattern of increase of the rate of gap junctions between folliculo-stellate cells on days 2, 3 and 4 after injections.

While the role of gap junctions between folliculo-stellate cells remains largely unknown, some ideas have been proposed for their function. The folliculo-stellate cell network may contribute to the regulation of neighboring granular cells by paracrine effects (Inoue *et al.*, 1999), and the folliculo-stellate cells connected through gap junctions have been shown to communicate by calcium waves (Guerineau *et al.*, 1998; Fauquier *et al.*, 2001). This folliculo-stellate cell network seems to control a variety of Ca^{2+} -dependent cellular processes including the synthesis and release of bioactive substances having a potential for the coactivation of various hormone-producing granular cells. It is possible that the endocrinal insufficiency caused by the removal of gonads can enhance the gap junctional communication between folliculo-stellate cells and lead to changes in the production of bioactive substances.

Many studies have demonstrated that sex steroids, testosterone, progesterone or 17β -estradiol can modulate the development of gap junctions in many kinds of tissues such as uteri, Sertoli cells, cardiac myocytes and motoneurons (Garfield and Hayashi, 1981; Mackenzie *et al.*, 1985; Matsumoto *et al.*, 1988; Kihara *et al.*, 1990; Garfield *et al.*, 1992; Ciray *et al.*, 1995; Hendrix *et al.*, 1995; Risek *et al.*, 1995; Gahr and Garcia-Segura, 1996; Herve *et al.*, 1996; Pluciennik *et al.*, 1996; Verrecchia and Herve, 1997; Lyng

Fig. 5. A 63-day-old ovariectomized female rat treated with progesterone. A gap junction (arrow) is illustrated joining the folliculo-stellate cells adjacent to granular cells. $\times 20,000$, inset: $\times 60,000$

Fig. 6. A 63-day-old ovariectomized female rat that received a simultaneous injection of progesterone with 17β -estradiol. One small gap junction (arrow) is situated between folliculo-stellate cells. The region indicated by the arrow is shown at a higher magnification and illustrates the typical trilamellar structure of the gap junction in more detail (inset). $\times 20,000$, inset: $\times 120,000$

et al., 2000). The findings of these investigations have suggested the existence of tissue specific sex steroids for the rate regulation of gap junctions in many kinds of tissues.

The appearance of gap junctions between myometrial smooth muscle cells is one of the major events associated with the onset of labor in many mammals including humans, and this is thought to contribute to synchronized contractions during delivery (Garfield and Hayashi, 1981; Garfield *et al.*, 1992; Ciray *et al.*, 1995). Treatment with 17β -estradiol induces the expression of connexin 43 in the uterus of rats, and this 17β -estradiol induced connexin 43 expression is almost completely suppressed by progesterone (Mackenzie *et al.*, 1985; Hendrix *et al.*, 1995; Risek *et al.*, 1995).

In the present study, the number of gap junctions between folliculo-stellate cells showed a rising and falling pattern during the relatively brief period of five experimental days. Previous pulse-chase studies of connexin 43 and 45 turnover in cultured neonatal rat myocytes and the liver gap junction proteins connexin 26 and 32 in cultured hepatocytes and mouse liver have demonstrated their half-lives to be only a few hours (Laird *et al.*, 1991; Darrow *et al.*, 1995; Traub *et al.*, 1983; Traub *et al.*, 1989). Other evidence obtained from morphological studies of myometrial gap junctions suggests that these junctions usually complete the formation during the two days before the onset of labor and then rapidly disappear from the plasma membrane within a day after delivery (Garfield *et al.*, 1977; Hendrix *et al.*, 1995). The time course observed in the formation and disappearance of gap junctions in the present study was very similar to that reported for myometrial gap junctions.

Several studies have shown that the developmental regulation of neural gap junctions is dependent on the testosterone level (Matsumoto *et al.*, 1988; Gahr and Garcia-Segura, 1996). In the motoneurons of the spinal nucleus of the bulbocavernosus area in rats, the maturation of gap junctions is regulated by testosterone (Matsumoto *et al.*, 1988). Testosterone-treated female canaries have an increased number of neuronal soma-somatic gap junctions in the caudal nucleus of the ventral neostriatum as compared with untreated females (Gahr and Garcia-Segura, 1996).

On the other hand, gap junctional communication can be interrupted by exposure to testosterone or 17β -estradiol in primary cultures of rat Sertoli cells (Herve *et al.*, 1996; Pluciennik *et al.*, 1996; Lyng *et al.*, 2000), rat cardiac myocytes (Herve *et al.*, 1996; Pluciennik *et al.*, 1996; Verrecchia and Herve, 1997) and human transitional cell carcinoma cell lines (Kihara *et al.*, 1990).

These studies show that the same sex steroid can have

either positive or negative effects over the regulation of gap junctions in different kinds of tissues.

Further research is necessary in order to elucidate the mechanisms by which sex steroids induce changes in the rate regulation of gap junctions; however, it can be stated that testosterone chiefly enhances the rate of gap junctions between folliculo-stellate cells in castrated male rats and progesterone up-regulates them in ovariectomized female rats. Furthermore, the progesterone-induced enhancement of gap junctional coupling between folliculo-stellate cells can be inhibited by the simultaneous administration of 17β -estradiol in ovariectomized female rats. In conclusion, gap junction formation between folliculo-stellate cells in castrated male rats and ovariectomized female rats is regulated differently.

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