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[SHORT COMMUNICATION]

Immunohistochemical Analysis of Androgen Receptor in the Abdominal Glands of the Cloaca of Male Red-Bellied Newts, *Cynops pyrrhogaster*

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ABSTRACT—Androgen receptor immunoreactivity was examined in the abdominal glands of the cloaca in adult male red-bellied newts, *Cynops pyrrhogaster*, using a polyclonal anti-androgen receptor antibody, PG21. In castrated males treated with saline, prolactin, testosterone propionate or both prolactin and testosterone propionate, all displayed androgen receptor immunostaining of nuclei in the epithelial cells of the glands. Androgen receptor-immunoreactive signals were distributed uniformly in the nuclei in the castrates treated with saline or prolactin. On the other hand, the signals were distributed reticulately in the nuclei in the castrates treated with testosterone propionate or both prolactin and testosterone propionate. Following treatment with testosterone propionate or both prolactin and testosterone propionate, shape of the androgen receptor-immunoreactive nuclei was altered from nonspherical profiles toward spherical profiles and its size became increased. There were no differences in these changes between the testosterone propionate- and both prolactin and testosterone propionate-treated castrates. These findings suggest that androgen participates in regulating size and shape of the nuclei and distribution of androgen receptor in the nuclei of the epithelial cells of the abdominal glands of male newts and that the structural reorganization is necessary for gene expression under the influence of androgen.

INTRODUCTION

Chemical signaling is thought to play a crucial role in courtship behavior in urodeles (Twitty, 1955; Dawley, 1984; Malacarne and Vellano, 1987). It has been suggested that male newts emit olfactory attractant(s) for luring females (Cedrini and Fasolo, 1971; Malacarne and Vellano, 1982; Malacarne *et al.*,1984). Removal of the abdominal glands of the cloaca in male newts induces a decline in female receptivity (Malacarne *et al.*, 1984) and attenuates the female-attracting activity of the water in which the males have been kept (Toyoda *et al.*, 1994). The evidence indicates the possibility that the abdominal glands of male newts are main source of female-attracting substance(s). Recently, a decapeptide, sodefrin that has a potent female-attracting activity was isolated from the abdominal glands of male red-bellied newts (Kikuyama *et al.*, 1995). Immunoelectron microscopic study using antiserum against synthetic sodefrin revealed the presence of immunoreactivity within the secretory granules in the epithelial cells of the abdominal glands (Toyoda *et al.*, 1995)

According to Benson (1965), androgen enhances development of the abdominal glands in male newts. On the other hand, development of the abdominal glands is facilitated by treatment with a combination of prolactin and androgen (Kikuyama *et al.*, 1975; Norris *et al.*, 1989). Based on their behavioral studies, Toyoda *et al.* (1994) stated that a combination of prolactin and androgen is effective for enhancing secretion of female-attracting substance(s) from the abdominal glands of males. Blood levels of prolactin and androgen are higher in males in the breeding season than those in the non-breeding season (Lofts, 1974; Tanaka and Takikawa, 1983; Matsuda *et al.*, 1990). In the breeding season, a marked development of the abdominal glands occurs in male newts (Aron, 1924; Tanaka and Iwasawa, 1979). The evidence supports the hypothesis that prolactin and androgen could participate in facilitating development of the abdominal glands

and secretion of female-attracting substance(s) from the glands. Prolactin and androgen are known to act on target cells by binding to a specific receptor. In the present study, as one step to clarify mode of action of these hormones on the abdominal glands, we examined expression of androgen receptor (AR) in the glands in male red-bellied newts by immunohistochemical analysis, and whether androgen and/or prolactin regulate expression of AR in the abdominal glands in the male newts.

MATERIALS AND METHODS

Tissue preparation

Adult male red-bellied newts, *Cynops pyrrhogaster*, weighing 8-9 g, were obtained from a commercial dealer. They were kept in the laboratory under conditioned photoperiod (12 hr light/12 hr dark) and temperature (23 ± 1°C) , and fed daily with *Tubifex* worms.

Twenty male newts were used, 5 per group. They were castrated under anesthesia with 0.01% MS222 (Sigma) three weeks prior to experiments. Castrated animals were treated with physiological saline, sheep prolactin (PRL, 1 IU, Sigma), testosterone propionate (TP, 5 μg, Sigma) or both PRL (1 IU) and TP (5 μg) every day for 10 days. Dosage of hormones and duration of treatment were determined on the basis of our previous experiments (Kikuyama *et al.*, 1975; Toyoda *et al.*, 1993, 1994). The day following the last injection, the abdominal glands were removed rapidly under 0.01% MS222 anesthesia and were frozen in liquid N_2. Frozen sections of 10 μm thickness were cut in a cryostat at -10°C and mounted on glass coated with bovine serum albumin (BSA). The sections were air-dried immediately after mounting. Then, the sections were immersed in a mixture of 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB)(pH 7.4) for 5 min at 4°C. After rinsing with 0.01 M phosphate buffered saline (PBS)(pH 7.4), the sections were stored in deep-freezer (-80°C) until they were processed for immunohistochemical analysis.

Immunohistochemistry

The sections were washed three times with PBS for 5 min each time. To reduce endogenous peroxidase activity, the sections were incubated with 0.3% H_2O_2 in 0.01M PBS for 20 min at room temperature. After rinsing in PBS, the sections were incubated with

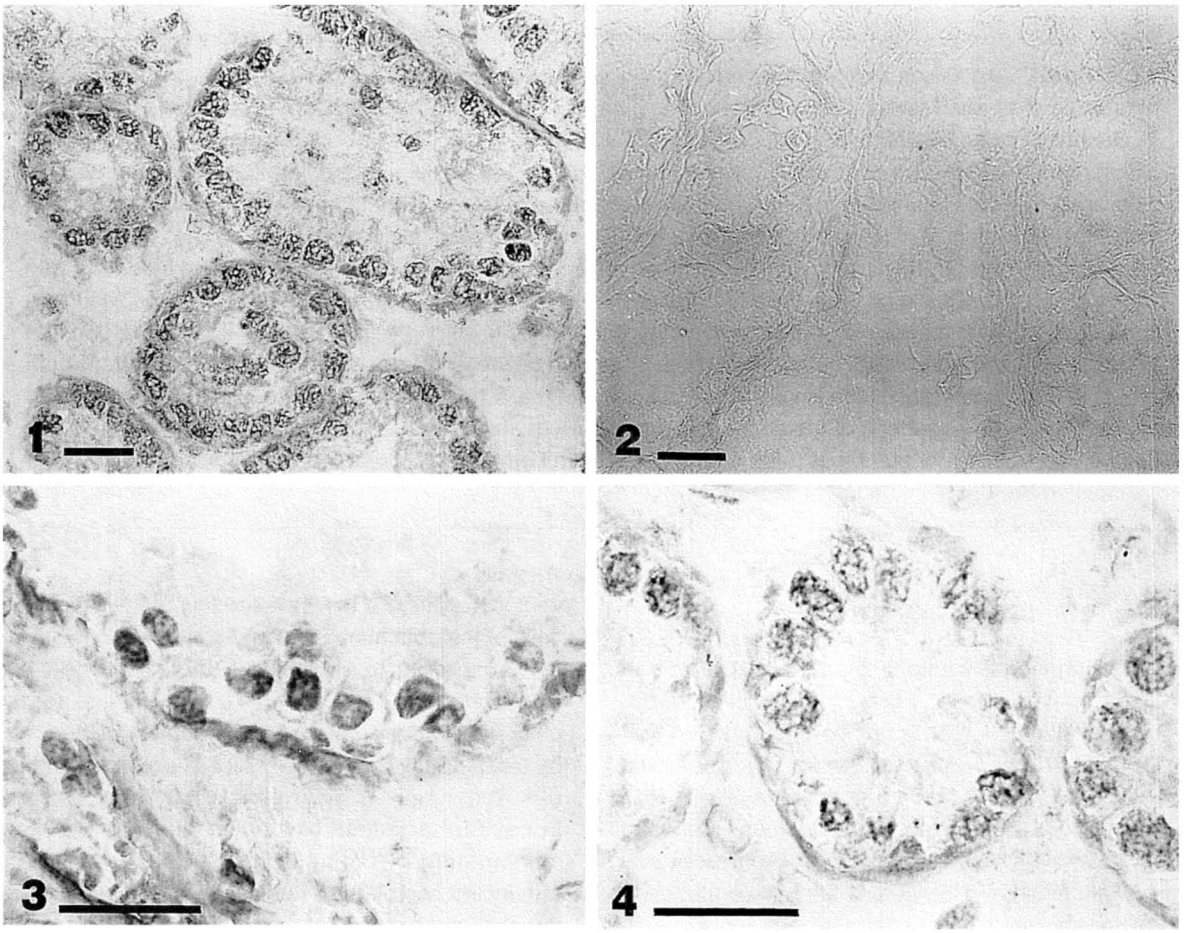

Fig. 1. Immunohistochemical distribution of AR in the abdominal glands of a castrated newt treated with PRL and TP. The AR-immunoreactive signals are localized in the nuclei of the epithelial cells of the glands. Bar: 50 μm.

Fig. 2. Preincubation of the primary antibody with an excess amount of the peptide antigen eliminates AR-immunostaining of the abdominal glands. Bar: 50 μm.

Fig. 3. Distribution of AR-immunoreactive signals in the nuclei of the epithelial cells of the abdominal glands in a castrated newt treated with saline. The signals are distributed uniformly in the nuclei of which shape is nonspherical. Bar: 50 μm.

Fig. 4. Distribution of AR-immunoreactive signals in the nuclei of the epithelial cells of the abdominal glands of a castrated newts treated with both PRL and TP. The signals are distributed reticulately in the nuclei of which shape is spherical. Bar: 50 μm.

0.05% saponin in distilled H_2O for 20 min at room temperature. They were then immunostained using the streptavidin-biotin-peroxidase complex method (Histofine SAB-PO Kit, Nichirei, Tokyo). The sections were blocked with 10% normal goat serum for 20 min at room temperature and incubated for 48 hr at 4°C with PG21, a rabbit polyclonal anti-AR antibody raised against a synthetic peptide corresponding to the first 21 amino acids of the rat AR (Prins *et al.*, 1991), which was diluted with 0.01M PBS containing 1% BSA and 0.1% sodium azide (dilution 1:1000). After rinsing in PBS, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G for 10 min, followed by an incubation with streptavidin-biotinylated peroxidase complex for 10 min at room temperature. The sections were finally treated with 0.0002% 3, 3'-diaminobenzidine (Sigma) and 0.005% H_2O_2 in 0.05M Tris-HCl buffer (Sigma)(pH 7.6). The sections were dehydrated in graded ethanols, cleaned with xylene and covereslipped with Permount. Specificity of the PG21 was confirmed by preabsorption of the antibody with a 20-fold molar excess of the antigenic peptide. We randomly selected 50 AR-immunopositive nuclei of the epithelial cells of the abdominal glands in each animal. Each nucleus was photographed and its cross-sectional area was measured at a final magnification of x420 using a digitizing tablet and microcomputer.

Statistical analysis

Statistical analysis was made by ANOVA and Duncan's multiple range test.

RESULTS

Abdominal glands of the cloaca in male newts were tubular glands of which parenchyma consisted of a single layer of epithelial cells. In castrated male newts treated with saline, PRL, TP or both PRL and TP, all displayed AR immunostaining of nuclei in the epithelial cells of the glands (Figs. 1, 3 and 4) indicating that PG21 could react with both androgen-occupied and androgen-unoccupied AR in the nuclei. When the PG21 was preabsorbed with an excess amount of the antigenic peptide, immunostaining of the nuclei was eliminated in the abdominal glands (Fig. 2). These results confirm the specificity of the PG21. Shape of the AR-immunoreactive nuclei in the castrates treated with saline or PRL was nonspherical, polygonal profiles (Fig. 3), whereas the shape was spherical, smooth profiles in the castrates treated with TP or both PRL and TP (Fig. 4). Distribution pattern of AR-immunoreactive signals in the nuclei was found to be different among the experimental groups. The AR-immunoreactive signals were observed to be distributed uniformly in nuclei in the castrates treated with saline or PRL (Fig. 3). On the other hand, the signals were found to be distributed reticulately in the nuclei in the castrates treated with TP or both PRL and TP (Fig. 4).

There was significant difference in mean cross-sectional area of AR-immunoreactive nuclei of the epithelial cells between the saline- or PRL-treated groups and TP- or both PRL and TP-treated ones ($F=38.10$, $p<0.01$, Fig. 5). The mean cross-sectional area of AR-immunoreactive nuclei of the castrates treated with TP or both PRL and TP was significantly greater than that of the castrates treated with saline or PRL ($p<0.01$). There was no significant difference in the mean cross-sectional area of AR-immunoreactive nuclei between the TP- and both PRL and TP-treated groups. The value in the castrates treated with PRL was not significantly different from that of the castrates treated with saline.

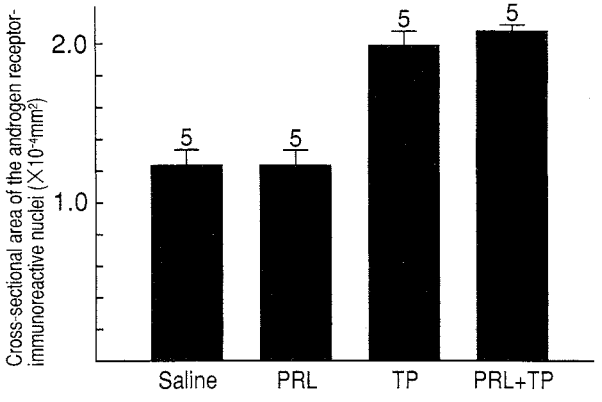

Fig. 5. The mean cross-sectional area of AR-immunoreactive nuclei of the epithelial cells of the abdominal glands in castrates treated with saline, PRL, TP or both PRL and TP. Vertical lines indicate S.E.M. Numbers at the top of columns refer to the number of newts examined.

DISCUSSION

In the present study, striking changes were detected in size and shape of AR-immunoreactive nuclei and in distribution pattern of AR-immunoreactive signals in the nuclei of the epithelial cells of the abdominal glands in the castrates treated with androgen alone or both prolactin and androgen. There were no difference in the nuclear size and shape and AR-immunoreactivity of the nuclei between the androgen- and both prolactin and androgen-treated animals. It seems likely, therefore, that androgen is substantially involved in these changes. It has been well documented that the nucleus is a dynamic structure influencing the physiological state of the cell (Berezney, 1979). Increase in nuclear size has been found to be associated with corresponding increase in the level of transcriptional activity (Merriam, 1969; Busch and Smetana, 1970). Since sex steroids have been shown to regulate gene expression first by binding to their specific receptors and then by interaction of the hormone-receptor complex with specific steroid hormone responsive elements on the DNA which act as trascriptional enhancers (Yamamoto, 1985; Evans, 1988), it is possible that sex steroids are implicated in changes in nuclear size and shape of target cells. In fact, sex steroids have been reported to induce transformation of nuclear shape, from nonspherical to spherical profiles and increment in its size in their target cells (Price and William-Ashman, 1961). Evidence is also accumulating that nuclear matrix is involved in the activational effects of sex steroids on target cells (Ciejek *et al.*, 1983; Luke and Coffey, 1994). Androgen-induced decondensation of AR-immunoreactive signals scattered throughout the nucleoplasm that was observed in the present study implies conformational changes in the nuclear matrix. Actively transcribed messenger ribonucleic acids appear to contribute to increase in elements which make a space

between androgen-AR complex masses. Thus, it is reasonable to speculate that the enlarged AR-immunoreactive nuclei and decondensation of AR-immunoreactive signals observed in the present study reflect expansion of the nuclear matrix as it undergoes structural reorganization necessary for gene expression.

It is well known that both prolactin and androgen are involved in reproductive events in male urodeles, especially in *Cynops* and *Triturus*. Administration of a combination of prolactin and androgen to the male has been demonstrated to elicit tail vibration in front of the female (Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982; Toyoda *et al.*, 1993) and to induce enlargement of somatic size of Mauthner cells (Matsumoto *et al.*, 1995) which may be responsible for the tail movement. These hormones also induce development of peripheral organs such as tail fin (Vellano *et al.*, 1970; Kikuyama *et al.*, 1986) and abdominal glands (Kikuyama *et al.*, 1975). In the present study, differences could not be detected in size and shape of the AR-immunopositive nuclei and distribution pattern of the AR-immunoreactive signals in the nuclei of the epithelial cells of the abdominal glands between the treatment with androgen alone and the treatment with both prolactin and androgen. Treatment of prolactin alone was not effective. The immunohistochemical evidence appears to be consistent with previous findings indicating that there were not remarkable histological differences in the abdominal glands between the two groups (Kikuyama *et al.*,1975). It is plausible, therefore, that prolactin does not participate in inducing enlargement of AR-immunoreactive nuclei and changes in distribution pattern of AR-immunoreactive signals in the nuclei. However, the abdominal gland weight (Kikuyama *et al.*, 1975) and female-attracting activity of the abdominal gland extract (Toyoda *et al.*, 1994) in castrates treated with both prolactin and androgen were much more increased than in castrates treated with androgen alone. The occurrence of a synergism between prolactin and androgen in sustaining the functional development of the abdominal glands is suggested. Prolactin has been reported to be synergistic with androgens on prostate growth in male rats (Grayhack *et al.*, 1955; Assimos *et al.*, 1984). In addition, prolactin alters androgen uptake and metabolism in the tissue (Lloyd *et al.*, 1973; Manandhar and Thomas, 1976). Prolactin receptors have been identified in prostatic tissue (Aragona and Friesen, 1975; Witorsch and Smith, 1977). In male newts, however, cellular mechanisms by which prolactin exerts a stimulatory influence on the abdominal glands remain to be clarified.

The present immunohistochemical studies using PG21 show that the signals for AR-immunoreactivity are localized on the nuclei of epithelial cells of the abdominal glands in male newts. The signals rarely occurred in the cytoplasm of the epithelial cells. Preabsorption of the PG21 with an excess amount of antigenic peptide resulted in elimination of the signals. These findings suggest AR in the abdominal glands of male newts specifically reacts with antibody for rat AR, PG21. The antigenic peptide used for the production of PG21 corresponds to a part of the AR (amino acids 1-21, Prins *et al.*, 1991) where there is sequence identity between rats and humans (Lubahn *et al.*, 1988), and where there is a low degree of homology with other steroid hormone receptors (Evans, 1988). Dorlöchter *et al.* (1994) recently pointed out that the PG21 reacts with nuclei of frog muscular cells. Together with their observation, it is suggested that evolutionary conservation of this portion of AR may extend to amphibia.

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