Mouse submandibular glands show an androgen-dependent sexual dimorphism, reflected in higher concentrations in males than in females of bioactive peptides, such as epidermal growth factor (EGF), nerve growth factor, and renin in the cells of the granular convoluted tubules (GCT). Biochemical studies have demonstrated androgen receptors in submandibular gland and other androgen-responsive organs in mouse. We have determined the cellular localization of these receptors using steroid autoradiography. Fifteen adult gonadectomized male mice were injected intravenously with 0.13 or 0.26 μg [3H]-dihydrotestosterone (SA 135 Cl/mM); some animals were pre-treated with cycloheximide to stimulate secretion by GCT cells. Animals were killed 15 min, 1, 2, or 3 hr after isotope injection. Steroid autoradiographs were prepared, and some were stained immunocytochemically for EGF. Of the different cell types of submandibular gland, the acinar cells most frequently and intensely concentrated [3H]-DHT; GCT cells also concentrated the hormone, as did a small number of striated duct cells. In the other major salivary glands, the only cells that concentrated the androgen were interlobular striated duct cells in sublingual gland. In prostate, anterior pituitary, and brain a large number of cells concentrated androgen, as has been previously reported. Androgen binding by the GCT cells was a predictable finding, since androgen-induced alterations in composition and form of these cells are well documented. The intense androgen concentration by the acinar cells was an unexpected finding and suggests a hitherto unknown androgen regulation of these cells. An incidental finding was intense concentration of [3H]-DHT in the nuclei of the endothelial cells of the post-capillary venules of the cervical lymph nodes. (J Histochem Cytochem 35:1053–1058, 1987)

KEY WORDS: Steroid autoradiography; Androgen receptor; (Mouse) salivary glands.

Introduction

The submandibular glands of adult mice contain a large variety of polypeptides with striking biological activities, such as epidermal growth factor (EGF), nerve growth factor, kallikrein, and renin in the cells of the granular convoluted tubules (GCT). These peptides are localized in the granular convoluted tubule (GCT) cells (Gresik et al., 1981b; Barka, 1980). These polypeptides may be produced by androgen stimulation of androgen receptors. Recent studies have shown that these peptides are synthesized by these cells.

The submandibular gland exhibits an androgen-dependent sexual dimorphism. The concentration of most, if not all, of these peptides in the GCT cells is androgen-dependent, with adult male mice having higher concentrations of these peptides than adult females. The reduction in peptide content seen in the male after gonadectomy can be completely compensated for by administration of androgens. The sexual dimorphism is dependent on hormone levels and not on genotype. With androgen administration, the submandibular gland of females can be induced to produce high levels of these peptides and their mRNAs (Gubits et al., 1986; Gresik et al., 1985; Mesterovic et al., 1983), indicating that these peptides are synthesized by these cells.

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specificity of the receptor for androgen in mouse submandibular gland is well established (Verhoeven, 1979; Takuma et al., 1977). These receptors have higher affinity for dihydrotestosterone, a somewhat weaker affinity for testosterone, and do not bind estradiol, progesterone, or hydrocortisone to any appreciable extent (Verhoeven, 1979; Takuma et al., 1977). In contrast to these biochemical data showing that progesterone does not importantly compete with androgen for the androgen receptor, there are some data that imply that progenins may exert some of their androgenic activity in mouse submandibular gland through the androgen receptors (Bullock et al., 1975, 1982). In addition to the "typical" androgen receptor, mouse salivary gland has been reported to have a high-capacity 2.7 S cytosolic androgen-binding protein of unknown function (Verhoeven, 1979).

Although testosterone is rapidly metabolized to dihydrotestosterone in most target organs (Chan and O'Malley, 1976), Coffey et al. (1979) have shown that testosterone is largely unmetabolized or is converted to 5-alpha-androstan-3-alpha, 17-beta-diol in mouse submaxillary gland. We chose to use dihydrotestosterone for these experiments in order to consider only androgen binding to the gland; using testosterone would not enable us to rule out binding subsequent to the aromatization of testosterone to estrogen.

Androgen receptors are present in submandibular glands of male and female mice but not in the submandibular gland (or any other tissue) of the testicular-feminized male mouse (Verhoeven, 1979; Takuma et al., 1977; Verhoeven and Wilson, 1976). Androgens can increase the peptide content of the submandibular glands in normal mice (Gresik et al., 1981a, 1981b; Gresik and Barka, 1980), but not the peptide content in the testicular-feminized males. This suggests that the high-affinity, low-capacity, androgen-specific receptors mediate androgen influence on this gland.

Although some data are available on which cells in submandibular glands of rat, guinea pig, and rabbit bind steroid hormones (Herandez et al., 1982; Stumpf and Sar, 1976), we do not know which cells in mouse submandibular gland contain the androgen receptors. The gland is complex and contains five major epithelial cell types: acinar cells, granular convoluted tubule cells, and intercalated, striated, and excretory duct cells, plus the usual complement of stromal cells. The cell types in mouse submandibular gland differ importantly from those of rat, guinea pig, and rabbit. In addition, there is a large literature on the action of androgens on mouse submandibular gland. Steroid autoradiography is a well-known technique used to determine the specific cells that contain steroid hormone receptors (Morrell and Pfaff, 1981; Stumpf and Grant, 1975). We used steroid autoradiography to identify the cells in mouse submandibular gland that concentrate dihydrotestosterone, so that we could understand more precisely how this hormone affects the gland.

Materials and Methods

Fifteen Swiss-Webster adult male mice (body weight 23-36 g) were gonadectomized 6 days before isotope injection. Two were also adrenalectomized 2 days before injection with the isotopically labeled steroid hormone.

We found in preliminary experiments (n = 20 mice; other details similar to those reported here) that the secretory granules in the cytoplasm of GCT cells induced positive chemography. To reduce this, we injected 10 of the animals with cycloheximide, which depletes the GCT cells of their secretory granules (Barka et al., 1978a; Liss et al., 1976). The cycloheximide (1.5 mg per 10 g body weight) was injected intraperitoneally, 3 hours before isotope injection.

Eleven animals (eight cycloheximide pre-treated, three not pre-treated) were injected in the tail vein with 0.13 μg (59 μCi) or 0.26 μg (118 μCi) of [3H]-dihydrotestosterone per 50 g body weight. The dihydrotestosterone (1,2,4,5,6,7-[3H]-5-alpha-androstan-17-beta-ol-3-one) from New England Nuclear (Boston, MA) had a specific activity of 135 Ci/mm. The tritiated dihydrotestosterone ([3H]-DHT) dissolved in ethanol was diluted 1:1 with physiological saline and was injected over a period of several minutes. The tritiated DHT (99% pure) was received from New England Nuclear, which checks the chemical purity of its stock every 3 months; the hormone was used within 1 week of arrival in our laboratory.

The animals were killed by cervical dislocation 0.25, 1, 2, or 3 hours after [3H]-DHT administration. The salivary glands (parotid, submandibular, sublingual) and their associated lymph nodes, as well as the prostate, brain, and pituitary were quickly dissected out and chilled on ice while being blocked, and then half of the tissue was quickly frozen onto cryostat chucks by rapidly stirring freon chilled by liquid nitrogen. The other half of the tissue was fixed by immersion in Zamboni's fixative for 5 hr at room temperature. The Zamboni's fixed tissues were cryoprotected by soaking in 15% sucrose in PBS for 12-15 hr at 4°C, and then frozen as above. The frozen tissues were stored in liquid nitrogen until the steroid autoradiographs were prepared.

The steroid autoradiographs were prepared from sections (6, 12, or 18 μm) cut in a Bright cryostat at 27°C and 30°C. Under safe-light conditions in the darkroom, the sections were picked up on dry slides, previously coated with NTB-3 Kodak emulsion. The autoradiographs were then exposed under light-tight, dry conditions at 20°C for 6-17 months and then developed (Kodak, D-19 developer), fixed (Kodak fixer), counterstained with cresyl violet, dehydrated, and coverslipped. The details of the autoradiographic procedure have been previously described (Morrell and Pfaff, 1981, 1983). The storage temperature for exposure of the autoradiographs was 0°C.
chosen as a compromise between a reduction of chemography and a reasonable exposure time.

After 17 months of exposure, some of the developed autoradiographs were used for a combined steroid autoradiographic-immunocytochemical preparation as previously described (Morrell et al., 1983). These combined procedures were possible only if the tissue was fixed in Zamboni's fixative, since preliminary experiments had demonstrated the absence of immunoreactivity for EGF in unfixed tissue. The primary antibody to EGF was previously characterized (Barka et al., 1978b) and used to localize EGF content in the submandibular gland of mice (Gresik and Barka, 1978).

**Controls.** Two types of controls were prepared: (a) Three of the animals were not injected with cyclocytidine, but were injected with [3H]-DHT. Comparison of these autoradiographs to those from the cyclocytidine-injected animals indicated that the cyclocytidine did not alter the [3H]-DHT binding in any tissue. As anticipated, the drug did decrease the cytoplasmic content of secretory granules of the GCT cells, and hence greatly reduced the positive chemography under the cytoplasm of these cells. (b) As standard controls for chemography (Morrell and Pfaff, 1981; Rogers, 1979), four other animals were not injected with [3H]-DHT; two received cyclocytidine, two did not. The tissues from these animals were prepared exactly as those from isotope-injected animals. The cyclocytidine again greatly reduced the positive chemography under the cytoplasm of the GCT cells. Moreover, chemography was never seen under the GCT cell nuclei nor under any other cells in the other tissues we examined.

**Analysis.** The autoradiographs were systematically analyzed with a Zeiss light microscope (x 25 or x 40 objectives) for the presence of reduced silver grains under the cells' nuclei. A cell was considered to be labeled with [3H]-DHT if it had three times the number of reduced silver grains under its nucleus than were found under immediately adjacent areas of the tissue. This statistically based criterion for a labeled cell results in a very low probability of a false-positive or false-negative cell. The basis for this criterion to establish a cell as labeled has been previously published (Morrell et al., 1986).

**Results**

**Submandibular Gland**

In the glands of mice exposed to [3H]-DHT for 1, 2, or 3 hr, the nuclei of the acinar cells were the most frequently and densely labeled (Figures 1A-1C). Every acinus contained 50-70% labeled nuclei. No labeled cells were seen 15 min after the isotope was administered.

Nuclei of the GCT cells also concentrated [3H]-DHT (Figure 1D). However, these cells were less frequently and less intensely labeled than the acinar cells. By a combined autoradiographic-immunocytochemical procedure we could readily verify that labeled duct cells were GCT cells by the presence of immunoreactive EGF in their cytoplasm (Figure 1E). In GCT cells from animals not treated with cyclocytidine, the cytoplasm was packed with secretory granules immunoreactive for EGF. In GCT cells from animals treated with cyclocytidine, the number of the secretory granules in the cytoplasm was greatly reduced. However, the remaining immunoreactive secretory granules were sufficient to make possible identification of these cells as GCT. The findings on the effect of cyclocytidine on the GCT cells are consistent with previous data (Barka et al., 1978a; Liss et al., 1976).

Occasionally, the nucleus of a striated duct cell was found to be labeled (Figure 1F). The nuclei of intercalated and excretory duct cells did not concentrate the hormone.

**Sublingual and Parotid Glands**

In the sublingual gland, the nuclei of acinar cells were completely unlabeled. However, we occasionally found an interlobular striated duct cell that concentrated DHT in its nucleus. The parotid gland did not concentrate the hormone in any cell type.

The pattern and relative intensity of androgen-concentrating cells were consistent in all experiments, irrespective of experimental manipulations or exposure time of the autoradiographs. Specifically, comparison of the autoradiographs from animals with and without cyclocytidine treatment showed that there was no difference in the pattern or intensity of the nuclear concentration of the hormone in the salivary glands or in the tissues described below.

**Lymph Nodes**

The majority of endothelial cells of the post-capillary venules in the cervical lymph nodes intensely concentrated the hormone in their nuclei (Figure 1G). This labeling was conspicuous throughout these lymph nodes, suggesting that all the endothelial cells of the post-capillary venules concentrated the androgen. No other labeled cells were present in these nodes.

**Prostate, Brain, and Anterior Pituitary**

As a comparison, the other known androgen target organs from the same animals were examined for androgen binding. The majority of cells in the prostate gland and surrounding tissue concentrated the [3H]-DHT in their nuclei intensely. The nuclei of the secretory epithelial cells and ducts (Figure 1H), the surrounding connective tissues, and muscle cells concentrated the hormone. Cells in specific regions of brain concentrated the [3H]-DHT in their nuclei. The vast majority of these cells were found in the medial preoptic area, the bed nucleus of the stria terminalis, the medial nucleus of the amygdala, and in the medial basal hypothalamus, particularly in the arcuate, periventricular, and ventromedial nuclei. Many cells of the anterior pituitary concentrated the hormone.

The results agree with previously published results in mouse (Schleicher et al., 1985; Sheridan, 1978; Hansson et al., 1974). Cyclocytidine pre-treatment had no effect on DHT binding in any target tissue.

**Discussion**

High-affinity, low-capacity androgen receptors are present in submandibular gland, kidney, prostate, seminal vesicles, brain, and pituitary of mouse (Verhoeven, 1979; Wieland et al., 1978; Takuma et al., 1977; Verhoeven and Wilson, 1976). Although previous steroid autoradiographic studies revealed the exact cells in prostate, seminal vesicles, brain, and anterior pituitary of mouse that contain dihydrotestosterone receptors (Schleicher et al., 1985; Sheridan, 1978), to our knowledge this is the first specification of the cells in mouse salivary glands that contain these receptors.

In a review that described autoradiographic identification of the cells that bind various steroid hormones in many different tissues of rat and guinea pig, Stumpf and Sar (1976) briefly mention the concentration of testosterone in the nuclei of acinar and cer-
tained duct cells in the submandibular gland of both species. The only other report on the autoradiographic localization of steroid binding in mammalian salivary gland is of dexamethasone binding in the submandibular gland of the rabbit (Hernandez et al., 1982). Nuclei of the acinar cells as well as the nuclei of the striated and interlobular duct cells concentrate the hormone. The acinar cell nuclei are more heavily labeled than the duct cells.

The GCT cells are under multihormonal control. Androgens, thyroid hormones, and to a lesser extent the adrenal steroid hormones influence these cells (Barka, 1980; Gresik, 1980; Raynaud, 1960). Androgen and thyroid hormones regulate the peptide content of the GCT cells (Gresik et al., 1981b; Gresik and Barka, 1980). Recently, the epidermal growth factor mRNA levels in mouse submandibular gland have been shown to vary with changes in testosterone or thyroid hormone levels (Gubits et al., 1986; Gresik et al., 1985). These experiments suggest that these hormones act at a transcriptional level.

Our finding that GCT cells concentrate androgen was expected, because androgens are known to increase the peptide content of these cells (Gresik et al., 1981b). Since neither the structure nor the composition of acinar cells is known to be affected by androgens, our observation that DHT labeling of acinar cells greatly exceeded DHT labeling of GCT cells was unexpected. The high concentration of androgen receptors in the acinar cells suggests a hitherto unknown androgen regulation of these cells. Furthermore, acinar cells may be responsible for androgen binding in submandibular gland of the prepubescent mouse before the GCT cells differentiate (Takuma et al., 1977). The small number of striated duct cell nuclei that concentrated the DHT may also contribute to the prepubescent binding. Moreover, the striated duct cells that contain androgen receptors may be the direct precursors of GCT cells, which are known to differentiate from this cell type (Chetien, 1977; Harvey, 1952).

In sublingual gland, we found a small number of interlobular striated duct cells that concentrated the dihydrotestosterone. Epidermal growth factor, renin, and protease A immunoreactivity in the striated duct cells in sublingual glands of mice are responsive to testosterone or thyroxin (Gresik and Barka, 1983). Most likely, the DHT-concentrating capacity of these cells serves as the basis for this androgen responsiveness. The sparcity of DHT receptor-containing cells in sublingual gland may explain the fact that androgen binding was not detected in homogenates of the gland (Takuma et al., 1977).

Our finding that the endothelial cells of the post-capillary venules in the cervical lymph nodes intensely concentrated DHT is the first report of steroid binding in this cell type. Steroid autoradiographic studies of the brain, pituitary, and accessory sex organs have never demonstrated steroid binding in any type of endothelial cell nuclei (Morrell et al., 1975; Stumpf and Grant, 1975). The nature of these anatomical localization studies makes it unlikely that the cells were binding steroid hormones and that this binding went unnoticed or unreported. We conclude that this is a specific binding of the hormone in endothelial cell nuclei because (a) the structural preservation was excellent and provided clear visualization of the endothelial cells and their nuclei, (b) no other endothelial cells in the other tissues concentrated the hormone, and (c) the labeled endothelial cells were present in the animals sacrificed 1, 2, or 3 hr after isotope administration, but not in animals sacrificed 15 min after isotope. If the labeling we observed was due mainly to nonspecific diffusion of the isotope from the blood across the endothelial cells, then these observations would not hold. Because of the specialized nature of these endothelial cells and their important functions for the immune system, their ability to concentrate DHT is of considerable interest and deserves further study.

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