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IDENTIFICATION AND DISTRIBUTION OF PERIPHERAL BENZODIAZEPINE BINDING SITES IN MALE RAT GENITAL TRACT

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Abstract—In the present study we identified and characterized the distribution of high-affinity peripheral benzodiazepine binding sites (PBzS) in male rat vas deferens (whole, and prostatic and epididymal portions), prostate, seminal vesicles, and Cowper's glands. [^{3}H]PK 11195, an isoquinoline carboxamide derivative, was used as a radioligand specific for PBzS. Scatchard analysis of saturation curves of [^{3}H]PK 11195 binding in the whole vas deferens, the prostatic and epididymal portions of the vas deferens, the prostate, the seminal vesicles, and Cowper's glands yielded mean maximal numbers of binding sites of 1211 ± 158, 1012 ± 311, 1451 ± 156, 1805 ± 86, 865 ± 51, and 2251 ± 135 fmol/mg protein, respectively. The equilibrium dissociation constant values ranged between 1 and 3 mM in all the above tissues. The ability of various drugs to displace the specific binding of [^{3}H]PK 11195 from PBzS in Cowper's gland membranes was also tested. The inhibition constants for Ro 5-4864, diazepam, and PK 11195 were 28, 330, and 4 nM, respectively, whereas clonazepam, Ro 15-1788, and testosterone were inefficient in displacing [^{3}H]PK 11195. The presence of high densities of PBzS in the male genital tract suggests a functional role in these hormone-dependent organs.

The benzodiazepinergic system consists of two distinct types of binding sites, central-type benzodiazepine (BZ) receptors (CBR) and peripheral-type BZ binding sites (PBzS). CBR are located in the central nervous system and mediate the pharmacological effects of BZs as anxiolytics, anticonvulsants, and muscle relaxants [1, 2]. They are a component of a macromolecular complex containing sites for γ-aminobutyric acid (GABA) receptor and a chloride ion channel [3, 4] and bind with high affinity the BZ clonazepam. PBzS are located in various peripheral tissues as well as in low density in the brain [5] and are different from CBR in their subcellular localization [6] and lack of coupling to GABA receptors and to chloride channels [7]. Both Ro 5-4864 (4'-chlorodiazepam) and PK 11195 (an isoquinoline carboxamide derivative) bind with high affinity to PBzS but not to CBR, whereas clonazepam binds with high affinity to CBR but not to PBzS [8]. PBzS in the female genital tract have been thoroughly investigated. The highest densities have been found in the ovary and uterus, which were more than three-fold higher than those in the oviduct [9]. In contrast, except for the testis [10, 11], PBzS have not yet been examined in male sex organs.

The purpose of the present study was to identify and characterize the distribution of PBzS in various regions of the male rat genital system, namely, the vas deferens, prostate, seminal vesicles, and Cowper's glands. Regional distribution of PBzS in the genitalia may shed light on their function in these hormone-dependent organs.

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MATERIALS AND METHODS

Materials. [^{3}H]PK 11195 (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Unlabeled PK 11195 was donated by Dr A. Bouvier (Rhône-Poulenc Santé, Vitry-sur-Seine, France). Ro 5-4864 was purchased from Fluka (Buchs, Switzerland). Clonazepam was a gift from Drs H. Gutman and E. Kyburz (Hoffmann-La Roche, Basel, Switzerland). Testosterone acetate was purchased from the Sigma Chemical Co. (St Louis, MO). Lumax was purchased from Lumac (Schaesberg, The Netherlands). Other chemicals were obtained from commercial sources.

Animals. Five male Sprague–Dawley rats (2 months, 200–220 g) were housed in air-conditioned quarters at 25° with a 12-hr light/dark cycle, and standard food and water were available *ad lib*. The rats were killed by decapitation, and the vas deferens, prostate, seminal vesicles, and Cowper's glands were removed and carefully cleaned of fat and connective tissues. The vas deferens was bisected so that two portions were obtained, the prostatic region and the epididymal region. The capsule of the Cowper's glands was dissected out, and only their glandular portion was collected. All tissues were stored at −20° until used for binding studies.

Membrane preparation. After thawing, tissues were weighed, homogenized separately in 20 mL of ice-cold 50 mM Tris–HCl buffer, pH 7.4, by a tissue homogenizer (Ystral GmbH, Dottingen, F.R.G.) (setting 10) for 20 sec, and centrifuged at 49,000 *g*

Table 1. Regional distribution of PBzS binding characteristics in rat vas deferens, seminal vesicle, prostate, and Cowper's glands

Tissue	K_d (nM)	B_{max} (fmol/mg protein)
Vas deferens (whole)	1.3 ± 0.3	1211 ± 158
Vas deferens (prostatic)	1.1 ± 0.2	1012 ± 311
Vas deferens (epididymal)	1.5 ± 0.2	1451 ± 156
Prostate	2.1 ± 0.2	1805 ± 86
Seminal vesicles	3.0 ± 1.4	865 ± 51
Cowper's glands	2.1 ± 0.2	2251 ± 135

[^{3}H]PK 11195 binding characteristics were determined by Scatchard analysis of saturation curves in the concentration range 0.3 to 40 nM in the absence (total binding) or presence (non-specific binding) of 10 μM PK 11195. Results are the means ± SE of three separate experiments.

for 15 min at 4°. The supernatant was discarded, and the precipitate was resuspended in 200 vol. of the same buffer and filtered through two layers of medical gauze to remove coarse particles (mostly connective tissues). The final concentrations achieved were 0.25–0.43 mg protein/mL.

Binding assay. Binding assay contained 400 μL membranes and 25 μL [^{3}H]PK 11195 (0.3–40 nM final concentration) in the absence (total binding) or presence (non-specific binding) of 10 μM unlabeled PK 11195. Seven concentrations of the tritiated ligand were used for Scatchard analysis. After incubation for 60 min at 4°, samples were filtered under vaccum over Whatman GF/B filters and washed three times with 4 mL of ice-cold Tris buffer. Filters were placed in vials containing 5 mL of a 1:3 mixture of Lumax–xylene and counted for radioactivity.

Displacement studies. Displacement studies were conducted on Cowper's gland membranes prepared as described above. [^{3}H]PK 11195 (2 nM) was used as a radioligand, and binding was conducted in the presence of various concentrations (10^{-9}–10^{-5} M, final concentration) of unlabeled Ro 5-4864, diazepam, PK 11195, Ro 15-1788, clonazepam, and testosterone acetate. The BZs, PK 11195, and testosterone were dissolved in ethanol, and the assay contained 1% final concentration of the ethanol.

Statistical analysis. Statistical analysis of differences in PBzS characteristics between various regions of the genital tract was evaluated by two-tailed Student's *t*-test.

RESULTS

Regional [^{3}H]*PK* 11195 *binding in male genital tract*

The mean maximal binding capacity (B_{max}) and equilibrium dissociation constant (K_d) values of [^{3}H]PK 11195 binding to membranes from the whole vas deferens, its prostatic and epididymal portions, the prostate, seminal vesicles, and Cowper's glands are summarized in Table 1. The K_d values were in the nanomolar range (1–3 nM) in all the above-tested tissues. The B_{max} values in the various regions of the vas deferens did not significantly vary from each other. The B_{max} values in the prostate gland were about 20–80% higher than in two regions of the vas deferens and 110% higher than in the seminal

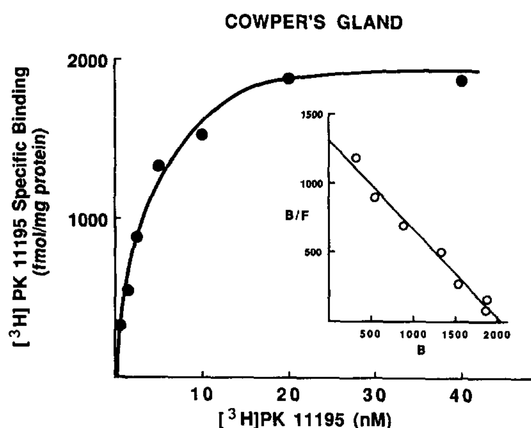

Fig. 1. Representative saturation curve of specific [^{3}H]PK 11195 binding to membranes of rat Cowper's glands. Membranes (100 μg protein) were incubated with seven adequately spaced concentrations of [^{3}H]PK 11195 (final concentration 0.3–40 nM) for 60 min at 4°. Inset shows Scatchard plot of [^{3}H]PK 11195 binding to membranes of rat Cowper's glands ($r = 0.99$).

vesicles, but 20% lower than in Cowper's glands. Seven adequately spaced concentrations of the tritiated ligand, used for Scatchard analysis of saturation curves of [^{3}H]PK 11195 binding to the various tissues, yielded linear plots ($r = 0.95$–0.99). These results indicate the presence of a single population of binding sites. Representative saturation curve and Scatchard analysis of [^{3}H]PK 11195 binding to rat Cowper's glands are depicted in Fig. 1. Specific binding reached a plateau at 15 nM, with a K_d value of 1.7 nM and B_{max} value of 2046 fmol/mg protein. Non-specific binding at 2 nM [^{3}H]PK 11195 was 15% of total binding.

In vitro *inhibitory effects of BZs, PK* 11195 *and testosterone acetate on* [^{3}H]*PK* 11195 *binding to Cowper's glands*

In order to examine the *in vitro* pharmacologic activity of various drugs at the PBzS, we tested their ability to displace [^{3}H]PK 11195 binding in the rat Cowper's glands. Of all drugs tested, PK 11195 was

Table 2. Potencies of BZs, PK 11195, and testosterone acetate to displace [^{3}H]PK 11195 binding from PBzS in rat Cowper's gland membranes

Displacer compound	K_i (nM)
Ro 5-4864	28
Diazepam	330
PK 11195	4
Clonazepam	>10,000
Ro 15-1788	>10,000
Testosterone acetate	>10,000

Binding of [^{3}H]PK 11195 (final concentration 2 nM) to rat Cowper's gland membranes was determined in the presence of 10–12 concentrations (10^{-9}–10^{-5} M) of the above compounds to estimate IC_{50} values (concentrations causing 50% inhibition of [^{3}H]PK 11195 specific binding). K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_d)$, where L = [^{3}H]PK 11195 concentration. Results are the means of three separate experiments with less than 15% variability.

the most potent displacer: one order of magnitude more potent than Ro 5-4864 and two orders of magnitude more potent than diazepam. Clonazepam, Ro 15-1788 and testosterone acetate were inefficient in displacing [^{3}H]PK 11195 ($K_i > 10$ μM). The K_i values for these drugs are listed in Table 2.

DISCUSSION

Apart from the testis, PBzS have not yet been identified and characterized in other regions of the male genital tract. The present study, using [^{3}H]PK 11195 as a radioligand, demonstrated the presence of typical PBzS in the rat vas deferens, prostate, seminal vesicles and Cowper's glands. The vas deferens consists of two regions, the prostatic and the epididymal regions. Differences in neuronal distribution were demonstrated between the prostatic and the epididymal portions of the rat vas deferens. For example, the sympathetic adrenergic nerve supply is denser in the prostatic portion than in the epididymal one [12], and the endogenous concentration of norepinephrine is higher in the prostatic than in the epididymal zone [13]. In addition, voltage- and norepinephrine-induced contractions exhibit different sensitivities to calcium channel blockers between the various parts of rat bisected vas deferens [14]. It has been proposed that calcium channels are coupled to PBzS, as has been shown by the ability of peripheral BZ ligands to inhibit [^{3}H]nitrendipine binding to mouse astrocytes [15]. Therefore it was expected that PBzS densities would correspond to calcium channel distribution in the two portions of the vas deferens. However, our results do not suggest major differences in PBzS densities in both vas deferens regions, which might be present if the PBzS were connected, for example, to sympathetic innervation or to calcium channels. Nevertheless, Holck and Osterrieder [16] propose that PBzS and calcium channels in the heart are not coupled.

In the prostate and Cowper's glands, PBzS densities were significantly higher than in the whole vas deferens and in the seminal vesicles. The function of the accessory sex glands is to produce and store the various components of the semen. In common, they exhibit intensive metabolism compared to the vas deferens, which acts as a duct for the spermatozoa. PBzS have been shown to be a part of the outer mitochondrial membrane and to be involved in cellular metabolism [6]. It is not clear whether an uneven metabolism within the various regions of the male genital system reflects PBzS density differences observed in these tissues. However, although the seminal vesicle is active in production of semen, it exhibits low PBzS density similar to that of the vas deferens (a ductal tissue), a fact which does not support this speculation.

PBzS are extensively involved in the endocrine system and are regulated by various hormones [9–11]. In the rat testis PBzS are dependent on the trophic influence of pituitary hormones. Hypophysectomy causes a decrease in PBzS density in the testis [15], and 10 days of thyroxine treatment causes an up-regulation of PBzS in various peripheral tissues, including the testis [10]. Autoradiographic studies reveal a high density of PBzS in the testicular interstitial tissue, where Leydig cells, which produce testosterone, are present. Lower concentrations of PBzS are found in the epithelium of the seminiferous tubules, where sperm cells and Sertoli cells predominate [17]. In addition, chronic diazepam administration to male patients has been shown to increase plasma testosterone levels [18], and an *in vitro* stimulatory effect of diazepam on testicular testosterone production has also been observed [19].

Testosterone is essential to the normal development and function of male sex organs. After castration, there is a striking reduction in size of the prostate, as well as in the other accessory sex glands, although not all cells in the prostate are equally affected [20]. On the other hand, administration of testosterone to castrated rats elicited an increase in prostatic epithelial mitotic activity [21]. However, the present study showed that, at least at the *in vitro* level, testosterone did not affect [^{3}H]PK 11195 binding to Cowper's gland membranes, even at a concentration as high as 10 μM. The PBzS-specific ligands demonstrated a typical peripheral pattern, with PK 11195 and Ro 5-4864 demonstrating low K_i values, diazepam showing intermediate values, and clonazepam being actually an ineffective displacer. The relatively high K_i values obtained for Ro 5-4864 and diazepam (Table 2) are consistent with a previous study presenting similar values in rat cardiac membrane [22]. The presence of high densities of PBzS in the male genital organs may indicate a functional role of PBzS in these hormone-dependent organs.

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