Binding of [³H]-muscimol to GABA_A sites in the guinea-pig urinary bladder: biochemical assay and autoradiography

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- 1 The specific binding of [³H]-muscimol, a γ -aminobutyric acid_A receptor (GABA_A) agonist, to whole membranes of the guinea-pig urinary bladder was examined. In addition, the distribution of specific muscimol binding sites within the bladder was visualized by autoradiography.
- 2 It was demonstrated that in a frozen-thawed whole membrane preparation of the organ the specific binding of [³H]-muscimol is reproducible, reversible and saturable.
- 3 Saturable binding was of a single component with an equilibrium dissociation constant (K_d) of 12 nM and a maximal density (B_{max}) of about 80 fmol mg⁻¹ protein.
- 4 In displacement experiments with several model compounds, [³H]-muscimol binding sites showed the characteristics of a GABA_A receptor site.
- 5 Autoradiographic experiments revealed uneven distribution of specifically bound [³H]-muscimol in the bladder. The density of binding sites was high in clusters within the smooth muscle layers of the bladder fundus and of the urethra, while the apex and the neck were not specifically labelled.
- 6 The present findings show that GABA_A type receptor sites in the guinea-pig urinary bladder may be labelled by [³H]-muscimol in a specific and reproducible manner. Moreover, the localization of these binding sites is consistent with the presence of GABA_A receptors in only a subpopulation of vesical ganglia.

Introduction

Pharmacological (Hill & Bowery, 1986), biochemical (Erdö, 1986) and autoradiographic (Amenta, 1986) lines of evidence indicate that specific receptor sites for γ -aminobutyric acid (GABA) are not restricted to the central nervous system, but are present also in various mammalian organs other than the brain.

Ligand binding studies with homogenates revealed the presence of specific, bicuculline-sensitive (GABA_A) receptor sites in several organs, such as female sex organs (Erdö & Lapis, 1982a,b; Erdö *et al.*, 1983; Erdö, 1984; Erdö & Laszlo, 1984), placenta (Erdö *et al.*, 1985), several types of blood vessels

(Krause *et al.*, 1980a,b; Napoleone *et al.*, 1987), hepatocytes (Minuk *et al.*, 1987), and adrenal medulla (Kataoka *et al.*, 1984; Martinez *et al.*, 1987).

On the other hand, binding studies with membrane preparations of the gastro-intestinal tract, and urinary bladder, where GABA is regarded as a neurotransmitter (Jessen *et al.*, 1986, 1987; Taniyama & Tanaka, 1986) have not been published as yet, even though the presence of GABA_A and GABA_B receptors in these organs has been convincingly documented in pharmacological experiments with isolated organs (see Taniyama & Tanaka, 1986; Jessen *et al.*, 1987).

Therefore, we decided to develop a reproducible technique for identification of GABA_A receptor sites in homogenates of the above mentioned organs. Here we show the presence of high-affinity binding

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sites for the GABA_A receptor agonist, muscimol in membranes of the guinea-pig urinary bladder. Moreover, the distribution of binding sites within the bladder has been analysed by autoradiography.

Methods

Animals and membrane preparation

White guinea-pigs, weighing 200–300 g, were decapitated and the urinary bladder as a whole, was dissected and cleaned of external connective tissue and fat. The bladder was opened by a razor blade and repeatedly rinsed by immersion in cold distilled water. The tissue was then placed on dry-ice and stored frozen overnight. On the next day, pooled tissue was homogenized in 10 volumes of ice-cold distilled water by an Ultra-Turrax (Janke & Kunkel, Ika Werk). The crude homogenate was sedimented at 48,000 *g* for 15 min (Hi-Spin, MSE) and the resulting pellet was suspended in water by an ultrasonic disintegrator (Soniprep 150, MSE) for 30 s. This rinsing procedure was repeated once more to reduce endogenous GABA content in the membranes. The resulting pellet was stored at –20°C for at least 16 h, but less than one week. This storage failed to influence the characteristics of [³H]-muscimol binding to the membranes, as controlled in a separate experiment (data not shown). Prior to the assay, membranes were resuspended in ice-cold Tris-citrate buffer (0.05 M, pH 7.1) and the rough tissue fragments, which had been present throughout, were removed by filtration through a fine mesh nylon screen (Eterlon 62 GG).

Binding assay

The binding assay was performed by a minor modification of a previously described centrifugation method (Erdö & Laszlo, 1984), with the following modifications. [³H]-muscimol (specific activity 30.8 Ci mmol^{–1}, New England Nuclear Co.) was used as the ligand instead of labelled GABA and the incubation time was extended to 20 min. The saturability of the specific binding was examined at 8 different [³H]-muscimol concentrations ranging from 2 to 120 nM. Binding parameters were determined by log-probit analysis of the Scatchard data. Non-specific binding was estimated in the presence of 0.1 mM unlabelled muscimol or 0.5 mM GABA. Specific binding was defined as the difference between total binding (in the absence of unlabelled displacers) and non-specific binding.

Protein measurement

Estimates of protein content were made according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Autoradiography

Dissected bladders were divided into the following portions: apex plus corpus (upper and lower bodies), fundus (base), neck, urethra. Terms in parentheses refer to the terminology used by Kusunoki *et al.* (1984). Separated tissue blocks were embedded in a cryoprotectant medium (OCT, Ames, U.S.A.), then rapidly frozen in a mixture of dry-ice and acetone. Serial sections of 7–8 µm thickness were then cut with a –20°C microtome cryostat. Sections were mounted on gelatine-coated microscope slides, air dried, preincubated (20 min) in cold buffer and subsequently incubated (30 min) in Tris-citrate buffer (50 mM, pH 7.1) with 5 nM [³H]-muscimol as described earlier in detail (Napoleone *et al.*, 1987). Control sections were incubated in the same way, but with the addition of 0.1 mM unlabelled muscimol or 0.5 mM GABA. The same non-specific labelling was found with these two compounds.

Slides were postfixed in formaldehyde vapours (60 min, 80°C) and coverslips coated with nuclear emulsion (Ilford L4, 1:1 dilution with distilled water) were attached to the slides (see also Napoleone *et al.*, 1987). This fixation procedure preserved the morphology of the sections, failed to reduce the specifically bound radioactivity, and did not cause any translocation of the ligand, as compared in a pilot experiment with unfixed sections. After an exposure time of 8 to 10 weeks, they were developed in D19 Kodak.

Results

Membranes of the urinary bladder showed the capacity to bind [³H]-muscimol in a specific manner. Non-specific binding was relatively low, representing 20 to 30% of the total binding (data not shown).

The specific binding of 5 nM [³H]-muscimol increased with time up to about 15 min, at 4°C, then equilibrium was achieved (Figure 1). Binding proved to be reversible, as unlabelled GABA could displace equilibrium binding in a few minutes (Figure 1).

Incubations with increasing ligand concentrations revealed the presence of a single class of high-affinity, and saturable binding sites in membrane homogenates of the bladder (Figure 2). The equilibrium

Figure 1 Time-course and reversibility of specific [^3H]-muscimol binding to membranes of the guinea-pig urinary bladder. Membranes were incubated at 4°C in 5 nM radioligand. Note the rapid displacement of specifically bound muscimol (broken line) by the addition of 0.1 mM GABA (arrowhead). Each point is the mean of two triplicate determinations, with less than 7% s.e.mean.

dissociation constant (K_D) and the maximal density of specific binding sites (B_{max}) were calculated from Scatchard plots (Figure 2), and they were found to be 12 ± 3 nM and 78 ± 39 fmol mg^{-1} protein, respectively. Values are mean $\pm$ s.d. of 5 determinations with separate membrane preparations.

Figure 2 Representative Scatchard plot showing a single population of high-affinity binding sites for [^3H]-muscimol on membranes of the guinea-pig urinary bladder. Membranes were incubated at 4°C , for 20 min, in the presence of increasing (2 to 120 nM) muscimol concentrations. Points represent means of triplicate determinations. For binding parameters, see text.

Table 1 Displacement of specific [^3H]-muscimol binding from membranes of the guinea-pig urinary bladder

Compound	IC_{50} value (μM)
Muscimol	0.04
GABA	0.09
THIP	1.2
Bicuculline	44
Baclofen	> 500
L-DABA	> 500

Membranes were incubated for 20 min in the presence of 5 nM radioligand and of at least 5 different concentrations of each model-compound tested. IC_{50} values are given as the mean of 3 independent determinations with less than 10% s.d. THIP = 1,2,3,4-tetrahydro-isoxasolo-pyridinol; L-DABA = L-diaminobutyric acid

In displacement experiments high concentrations of various compounds (see Table 1) known to act competitively at GABA_A sites produced more than 97% reduction of the specific binding, while the uptake-blocker L-diamino-butyric acid (L-DABA) and the GABA_B receptor agonist baclofen were ineffective. The concentrations of the compounds producing 50% displacement of the specific binding, i.e. the IC_{50} values, are presented in Table 1.

In autoradiographic experiments, silver grains representing specific [^3H]-muscimol binding sites were unevenly distributed. No specific binding occurred in the apex and the neck of the bladder. In these parts, only some non-specific accumulation of silver grains was seen in the intercellular spaces or over bundles of the connective tissue dividing bladder smooth muscle (not shown).

Specific binding sites were visualized in the fundus of the bladder, where silver grains were arranged in clusters within the smooth muscle layers (Figure 3). No specific binding was seen in the ureters and in their transitional zone in the bladder wall (not shown).

The highest density of specific binding sites was present in the urethra. Silver grains representing specific binding sites were concentrated in clusters within the smooth muscle (Figure 4). The density of binding sites was lower in the circular than in the longitudinal muscle layer (not shown).

Discussion

It has been demonstrated that GABA and its major biosynthetic enzyme, i.e. glutamate decarboxylase, are present in the guinea-pig urinary bladder, and that their distribution closely correlates with the dis-

Figure 3 Autoradiogram of a section of the guinea-pig urinary bladder (fundus) incubated with 5 nM [^3H]-muscimol. (a) Dark field illumination; (b) bright field picture stained with toluidine blue to verify microanatomical details; (c) dark field picture of an adjacent control section incubated additionally with 0.1 mM unlabelled muscimol. Note the occurrence of silver grains representing specific binding primarily within the smooth muscle, where they appear to be accumulated in clusters ($\times 180$).

Figure 4 Autoradiogram of a section of the guinea-pig urethra incubated with [^3H]-muscimol as above. (a) Picture in dark field illumination; (b) bright field picture showing microanatomical details; (c) dark field picture of an adjacent control section incubated additionally with 0.5 mM unlabelled GABA. Note the high density of specific silver grains in clusters near smooth muscle cells ($\times 160$).

tribution of ganglion cells within this organ (Kusunoki *et al.*, 1984). These authors also reported that electrical stimulation evokes the release of GABA from the bladder and that GABA inhibits the efflux of acetylcholine from strips of the organ in a bicuculline-sensitive manner (Kusunoki *et al.*, 1984). Moreover, through specific GABA_A and GABA_B receptors, GABA induces contractile responses of the isolated bladder (Taniyama *et al.*, 1983; Maggi *et al.*, 1985a,b). These findings, along with electrophysiological observations, have led to the hypothesis of a GABAergic innervation of the urinary bladder (Taniyama & Tanaka, 1986).

In the guinea-pig urinary bladder, GABA_A receptors inhibit the cholinergic component of the contraction to electrical stimulation as well as the nicotine-induced contraction, in a manner consistent with the presence of the receptor sites on postganglionic cholinergic neurones (see Taniyama & Tanaka, 1986).

The occurrence of bicuculline-sensitive GABA receptors in homogenates of several organs other than the urinary bladder has also been demonstrated by ligand binding techniques (see Erdő, 1986). Moreover, autoradiographic evidence has been provided for the occurrence of specific GABA_A binding sites in the guinea-pig ovary (Amenta *et al.*, 1986), in rat cerebral blood vessels (Napoleone *et al.*, 1987), in rat atria (Bowery & Hudson, 1983) and in the pancreas of neonatal rats (Reusens-Billen *et al.*, 1984).

The occurrence of a single population of high-affinity, saturable binding sites for [³H]-muscimol, as well as the capacity of various displacers to reduce specific binding in bladder homogenates are consistent with former findings for peripheral GABA_A sites (see Erdő, 1986). Thus, these binding sites correspond most probably with GABA_A receptors.

It has been proposed that in the guinea-pig urinary bladder, GABA_A receptors are located in vesical ganglia, on postganglionic cholinergic nerves (Kusunoki *et al.*, 1984; Maggi *et al.*, 1985a; Taniyama & Tanaka, 1986) and it has been demonstrated that the number of vesical ganglion cells, and the levels of GABA and glutamate decarboxylase show similar distribution patterns, with the highest densities in the upper body and decreasing levels towards the neck (Kusunoki *et al.*, 1984). By contrast, the present experiments failed to demonstrate any specific binding in the upper portion (apex plus corpus) of the bladder where both ganglion cell density and GABA concentration are high. This lack of correspondence might suggest that the GABA content of the ganglia in this upper region of the bladder may not act as transmitter through GABA_A receptors, but rather through the baclofen-sensitive (GABA_B) receptor subclass which cannot be labelled by muscimol (Bowery *et al.*, 1983). In the fundus and

the urethra, however, specific muscimol binding sites are located in clusters corresponding most probably to vesical ganglia within the musculature. Thus, these specifically labelled sites probably represent ganglionic GABA_A receptors. Consequently, in the fundus of the bladder and in the urethra, GABA may act as transmitter via GABA_A receptors.

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