In Vivo Effects of \(\gamma\)-Aminobutyric Acid on the Urinary Bladder Contraction Accompanying Micturition

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Abstract—We studied the effects of the \(\gamma\)-aminobutyric acid (GABA) receptor agonists, diazepam and muscimol, on the urinary bladder contraction induced by infusion of Tyrode’s solution into the bladder in anesthetized rats. Diazepam (1 mg/kg, i.p.) completely inhibited bladder contraction, causing the bladder pressure to rise until solution leaked from the penis. The inhibitory effects of diazepam were reversed by picrotoxin (1 mg/kg, i.v., twice with an interval of 10 min), and the effects were potentiated and attenuated by pretreatment with aminooxyacetic acid (AA, 10 mg/kg, i.v.) and semicarbazide (200 mg/kg, i.v.), respectively. Only pretreatment with AA inhibited the bladder contraction induced by infusion of Tyrode’s solution into the bladder in six out of eight rats. Diazepam abolished efferent discharges recorded from the left pelvic nerve, but hexamethonium facilitated the generation of efferent discharges after inhibition of bladder contraction. After complete inhibition of bladder contraction by diazepam, electrical stimulation of the left pelvic nerve at 5 Hz for 30 sec was able to induce bladder contraction, and this resulted in micturition. Intracerebroventricular injection or intrathecal injection into the sacral part of the spinal cord of 1 \(\mu\)g muscimol completely inhibited the bladder contraction. It was considered that the inhibitory effects of GABA receptor agonists on bladder contraction were mainly induced through the GABA receptors in the micturition center of the sacral cord, as well as the brain stem.

Materials and Methods

The preparations used and the method of recording bladder contraction were described in our previous paper (6). Male Wistar rats...
(weighing 250–350 g) were anesthetized with urethane (1.0 g/kg, s.c.) and α-chloralose (25 mg/kg, s.c.). The bladder was exposed through a midline incision in the abdomen and the abdominal muscle was transected. The bladder was then prepared for the recording of pressure within the bladder as follows: a needle (1/4) attached to one end of a silicone tube (O.D., 1.0 mm and I.D., 0.5 mm, 30–40 cm in length) was inserted into the bladder through the left ureter. The left ureter was ligated around the needle so that the urine from the left kidney flowed out from the incision. The right ureter was kept intact. The other end of the tube was connected to a syringe and a pressure transducer (Nihon Kohden, MPU-0.5) by means of a T-tube. The whole system was filled with Tyrode’s solution lacking glucose. In order to infuse the Tyrode’s solution into the urinary bladder at a constant rate (0.8–1.0 ml/10 min), the plunger of the syringe was continuously pushed by that of another syringe into which water was infused with a peristaltic pump (Atto, SJ-1211). The pressure signals were delivered by an amplifier (Nihon Kohden, RP-5) and recorded by a D.C. recorder (Watanabe Sokki, SR 6204). Cotton-wool swabs soaked with Tyrode’s solution were laid on the bladder to keep it moist, and the swabs were warmed with a lamp.

When almost the same patterns of bladder contractions were induced at almost constant interval at least for 20 min by the infusion of Tyrode’s solution into the bladder, drugs were administered into the preparations. There were rats, whose proportion was less than 10% of the total rats, in which the infusion of Tyrode’s solution could induce bladder contraction but the contractile force was too weak to produce micturition. In these preparations, the rise of pressure during the bladder contraction gradually decreased and the pressure after bladder contraction gradually rose until the solution leaked from the penis. These preparations were not used for the experiments.

When the efferent activities were recorded from the urinary branches of left pelvic nerve fibers and bladder contraction was induced by electrical stimulation of the nerves, rats with the left pelvic nerve dissected were used. The urinary branch of the left pelvic nerve fibers was isolated from the connective tissue for as long a distance as possible and separated from the fibers innervating the rectum. When the efferent activities were recorded from the urinary branch of the nerves, the central ends of the fibers were placed on a pair of platinum electrodes and covered with paraffin oil. Efferent impulses were amplified with an amplifier (Nihon Kohden, AVB-10) and displayed on an oscilloscope (Nihon Kohden, VC-10). The impulses were counted by means of a tachometer (Sanei Sokki, model 1332). As the amplitudes of these efferent impulses were very small and the spikes could not be completely separated from background noise, the trigger level of the tachometer was adjusted so that it counted only those spikes with a voltage higher than an arbitrary threshold. The output voltage of the tachometer was recorded on a D.C. recorder together with the bladder pressure measured with a pressure transducer (Nihon Kohden, LPU-0.1).

In order to study the effects of diazepam on the pelvic ganglia, bladder contraction was induced by electrical stimulation (1 msec duration, 5 V strength and 5 Hz frequency for 30 sec) generated from a stimulator (Nihon Kohden, S-5039) at the peripheral branch of the left pelvic nerve. Intracerebroventricular (i.c.v.) injection was performed as follows. The animals were anesthetized with pentobarbital (40 mg/kg, s.c.) and placed on a stereotaxic apparatus. A catheter was implanted into the lateral ventricle using the following coordinates with reference to the bregma: 0.3 mm anterior, 1.0 mm lateral to the midline and 5.6 mm deep, from the skull surface. Four or five days after implantation, i.c.v. injection was performed by means of a microsyringe. Each injection volume was 5 μl. The position of the catheter was checked in all animals after termination of the experiments by studying the distribution of injected trypan blue.

When intrathecal (i.t.) injection was performed, the drug was injected into the spinal
subarachnoid space according to the method of Yaksh and Rudy (8). A polyethylene tube (SP-8, Natsume) was inserted to a depth of approximately 8.5 cm through a split between the first and second cerebral vertebrae into the subarachnoid space. At the rat head side, the board onto which the rat was fixed was raised by about 1 cm to flow the injected solution caudally. The injection volume was 20 μl (8). After termination of the experiments, the location of the tube in the subarachnoid space was checked by laminectomy. In order to perform electrical stimulation of the spinal cord at the site of drug injection, a polyethylene tube, through which an insulated copper wire (0.1 mm in diameter) with 1 mm of the tip exposed had been passed, was inserted in the same way as for i.t. injection, but into the space between the vertebrae and the dura mater. The exposed tip of the copper wire was pushed out of the tube once it had been placed in the spinal cord. A silver wire was then placed subcutaneously over the site of the copper wire tip, and the copper and silver wires were connected to a stimulator (Nihon Kohden, SEN-1101). Under artificial respiration and after injection of d-tubocurarine, rectangular pulses of 1 msec duration and 10 mA strength were applied at 5 Hz for 10 sec, and the change in bladder pressure was recorded. During stimulation, the heart rate was measured with a pulse rate tachometer (Nihon Kohden, RT-5) which was triggered by the R wave of the electrocardiogram lead I. In some experiments, the site of the tube tip was checked using a soft X-ray fluoroscope (Softex).

When the influence of aminooxyacetic acid (10 mg/kg, i.v.) and semicarbazide (200 mg/kg, i.v.) on the effects of diazepam were studied, diazepam was injected at least two or four hours after injection of aminooxyacetic acid or semicarbazide, respectively.

For intravenous (i.v.) injection, drugs were dissolved in saline and injected into the femoral vein. For i.c.v. or i.t. injection, drugs were dissolved in artificial cerebrospinal (a.c.s.) fluid (g/l: NaCl, 8.1; KCl, 0.25; CaCl₂, 0.14; MgCl₂, 0.11; and NaHCO₃, 1.0; pH=7.0–7.1). Diazepam was dissolved in propyleneglycol in order to adjust the dose for intraperitoneal (i.p.) injection. After it had been confirmed that the bladder contraction was not influenced by i.c.v. and i.t. injection of a.c.s. fluid for at least 10 min, the solution containing the drug was injected. Picrotoxin was injected 20–25 min after injection of diazepam. If the bladder was quiescent after two injections of picrotoxin, the infusion of solution into the bladder was interrupted to prevent prolonged expansion of the bladder wall and then infusion was resumed 20 min after the interruption of infusion to investigate the recovery of bladder contraction.

Drugs used were: diazepam (Cercine, Takeda Chem. Ind., Ltd.), muscimol (Sigma), GABA, picrotoxin, hexamethonium chloride, semicarbazide HCl and aminoxyacetic acid HCl (Wako Pure Chem.) pentobarbital Na (Nembutal, Daichi Seiyaku Co., Ltd.) and d-tubocurarine (Amerizole, Yoshitomi Pharm. Ind., Ltd.). All drug concentrations are expressed as those of the respective salts.

Results

1) The effects of diazepam on bladder contraction induced by infusion of solution into the bladder: Diazepam (0.1 mg/kg, i.p.) did not interrupt micturition and only caused an increase in the bladder pressure at a point when the bladder pressure rose steeply (micturition threshold) for 19.7±4.6 min (mean±S.E., n=4). However, diazepam (1 mg/kg, i.p.) completely inhibited the bladder contraction and interrupted micturition for at least 40 min in all four experiments (Fig. 1). As the infusion of solution was continued after interruption of micturition, the pressure in the bladder rose to 30–40 cmH₂O until the solution leaked from the penis, and a high pressure was maintained during the infusion of solution into the bladder. The effects of picrotoxin on the bladder contraction were studied in this model. Picrotoxin (1 mg/kg, i.v.) exhibited mild stimulation (e.g., tremble of whisker), but the pattern of the bladder contraction did not change. With picrotoxin (2 mg/kg, i.v.), convulsions repetitively occurred about 5 min after the injection. Though the bladder contractions continued independently of the appearance of convulsions, the interval of
the bladder contractions was irregular, and micturition was not always accompanied by bladder contraction. Picrotoxin (1 mg/kg, i.v.) was injected so as to investigate the interaction between diazepam and picrotoxin with an interval of 10 min between the injections 20–25 min after diazepam injection. The bladder contraction which was completely inhibited by diazepam (1 mg/kg, i.p.) resumed and micturition took place after the second injection of picrotoxin (1 mg/kg, i.v.) without appearance of convulsion (Fig. 1).

2) The influence of pretreatment with semicarbazide or aminooxyacetic acid on the inhibitory effects of diazepam: After pretreatment with semicarbazide (200 mg/kg, i.v.), infusion of solution into the bladder induced bladder contraction; and in two out of four rats, the interruption time of micturition induced by diazepam (1 mg/kg, i.p.) was shortened as compared with that in untreated rats (Table 1). On the other hand, in six out of eight rats pretreated with aminooxyacetic acid (AA, 10 mg/kg, i.v.), infusion of solution into the bladder was unable to induce bladder contraction, and the pressure in the bladder rose until solution leaked from the penis. In three out of four rats pretreated with AA in which the infusion of solution induced no bladder contraction, picrotoxin (1 mg/kg, i.v., once, or twice with an interval of 10 min) induced bladder contraction accompanied by micturition (Fig. 2). In rats in which infusion of solution into the bladder induced bladder contraction after pretreatment with AA, diazepam at a dose (0.1 mg/kg, i.p.) that was

Table 1. The interruption time of micturition induced by diazepam after pretreatment with semicarbazide or aminooxyacetic acid in anesthetized rats

|                | Semicarbazide (200 mg/kg, i.v.) | Aminooxyacetic acid (10 mg/kg, i.v.) |
|----------------|---------------------------------|-------------------------------------|
| Diazepam 0.1 mg/kg, i.p. | 0 min (n=4)                     | 19.2±5.8 min (n=2)                  |
| 1 mg/kg, i.p.            | over 40 min (n=4)               | 10.0±1.0 min (n=2)                  |
                                    | over 40 min (n=2)               |                                     |

Each value indicates the mean with S.E. Semicarbazide or aminooxyacetic acid was injected 4 hr or 2 hr before the injection of diazepam, respectively.
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Fig. 2. Typical tracing of cystometrogram induced by infusion of Tyrode's solution into the bladder in an anesthetized rat preinjected with aminooxyacetic acid and the urinary bladder contraction induced by picrotoxin in the rat. Vertical bar: bladder pressure. Open circle indicates i.v. injection of picrotoxin. Aminooxyacetic acid (10 mg/kg, i.v.) was injected 2 hr before injection of Tyrode's solution into the bladder.

Fig. 3. The effects of diazepam and hexamethonium on the urinary bladder contraction (bottom trace) induced by infusion of Tyrode's solution into the bladder and the efferent discharges (top trace) synchronized with the contraction recorded from the left pelvic nerve in anesthetized rats with the left pelvic nerve resected. Vertical bar: spike frequency of discharges and bladder pressure. Horizontal bar: time after i.p. injection of diazepam and i.v. injection of hexamethonium.

unable to interrupt micturition in untreated rats interrupted micturition for 19.2 min (Table 1).

3) The effects of diazepam and hexamethonium (C6) on the efferent activities recorded from the pelvic nerves: The infusion of solution into the bladder was started at least 60 min after resection of the left pelvic
nerves. In rats with the left pelvic nerve resected, the infusion of solution into the bladder was able to induce bladder contraction in four out of ten or eleven rats, and the efferent discharges synchronized with these could be recorded. Diazepam (1 mg/kg, i.p.) simultaneously abolished both bladder contractions and efferent discharges (Fig. 3). After injection of picrotoxin (1 mg/kg, i.v., twice with an interval of 10 min), efferent discharges and bladder contraction appeared in all four rats. No spontaneous efferent discharges appeared after the first injection of picrotoxin. On the other hand, when C₆ (10 mg/kg, i.v.) inhibited the bladder contraction and caused an increase in bladder pressure after interruption of micturition, the generation of efferent discharges was facilitated (Fig. 3). Afterwards, bladder contraction resumed, the bladder pressure fell due to micturition, and only the efferent discharges which were synchronized with the contraction appeared (Fig. 3).

4) The effects of i.c.v. or i.t. injection of muscimol or GABA on the bladder contraction induced by infusion of solution: GABA at high dose (500 µg, i.c.v. and i.t.) was necessary to suppress the bladder contraction. After i.c.v. injection of GABA, the level of the micturition threshold rose for 9.7±0.7 (mean±S.E., n=4) min (Fig. 4). After i.t. injection of GABA, a rise in the micturition threshold level was observed in two out of four rats and the times were 6 and 10 min. Since i.c.v. or i.t. injection of saccharose (2000 µg), at almost the same osmotic pressure as GABA (500 µg), did not change the bladder motility, the transient inhibition induced by GABA could not have been due to the increase in osmotic pressure of the solution. In the case of muscimol, i.c.v. or i.t. injection of a 1 µg dose completely inhibited bladder contraction (n=4), and the bladder pressure rose until the solution leaked from the penis. The inhibition produced by i.t. injection was reversed by picrotoxin (1 mg/kg, i.v., twice with an interval of 10 min) in three out of four rats, but not by i.c.v. injection in four rats (Figs. 4 and 5). In order to confirm that the i.t. injection site was near the micturition center in the sacral cord, the spinal cord was electrically stimulated. d-Tubocurarine (d-Tc, 0.1 mg/kg, i.v.), which was injected to prevent convulsion, transiently suppressed the contractile force of the bladder, and the frequency of bladder contraction was increased (Fig. 6). After disappearance of the inhibitory effect of d-Tc, electrical stimulation was able to induce bladder contraction.

![Fig. 4. The effects of intracerebroventricular (i.c.v.) injection of GABA and muscimol on the urinary bladder contraction induced by infusion of Tyrode's solution into the bladder in anesthetized rats. Vertical bar: bladder pressure. Horizontal bar: time after artificial cerebrospinal fluid (a.c.s.f.) injection. Solid circles indicate i.c.v. injection of GABA and muscimol, and open circles indicate i.v. injection of picrotoxin.](image-url)
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Fig. 5. The effects of intrathecal (i.t.) injection of muscimol on the urinary bladder contraction induced by infusion of Tyrode's solution into the bladder in anesthetized rats. Vertical bar: bladder pressure. Horizontal bar: time after a.c.s.f. injection. Solid circle indicates i.t. injection of muscimol, and open circles indicate i.v. injection of picrotoxin.

Fig. 6. The response of the urinary bladder to electrical stimulation at the sacral part of the spinal cord after i.v. injection of d-tubocurarine. Urinary bladder contraction induced by infusion of Tyrode's solution in an anesthetized rat. Vertical bar: bladder pressure. Horizontal bar: time after i.v. injection of d-tubocurarine. At the point indicated by the arrow, electrical stimulation at 5 Hz was performed for 10 sec. Top trace shows the heart rate. The artifacts of electrical stimulation were superimposed during stimulation.

5) The effects of diazepam on the bladder response to pelvic nerve stimulation at the peripheral end: In order to study the effects on pelvic ganglia, the response of the bladder to electrical stimulation of the left pelvic nerve was investigated. The nerve stimulation at 5 Hz for 30 sec induced bladder contraction accompanying micturition. After injection of diazepam (1 mg/kg, i.p.), the nerve stimulation induced bladder contraction accompanying micturition, and so the bladder pressure which had been elevated was reduced after stimulation and then increased without any increase in heart rate (Fig. 6).
Fig. 7. The response to electrical stimulation of the left pelvic nerve at the peripheral end before and after i.p. injection of diazepam. Urinary bladder contraction was induced by infusion of Tyrode's solution in anesthetized rats with the left pelvic nerve resected. Vertical bar: bladder pressure. Horizontal bar: time after i.v. injection of diazepam. During (Ⅱ), electrical stimulation at 5 Hz was performed for 30 sec. by infusion of solution (Fig. 7).

Discussion

In the experiments of Sillén et al. (2), bladder motility was induced by administration of L-dopa and carbidopa, and an i.c.v. catheter was implanted into the vicinity of the 4th ventricle. On the other hand, in our present experiments, the bladder contractions were induced in a reflex manner by the infusion of solution into the bladder and an i.c.v. catheter was implanted into the lateral ventricle. However, the dose range of i.p. injection of diazepam or i.c.v. injection of muscimol which was able to exert an inhibitory effect on bladder contraction was very similar in both experiments (Ref. 2 and Figs. 1 and 4). Since GABA receptor agonists were able to inhibit the bladder contraction which was induced without dopaminergic agents, it seems unlikely that GABA receptor agonists exerted their inhibitory effects through presynaptic inhibition of dopaminergic nerves. Our present results support the hypothesis of Sillén et al. (2) that there are GABAergic synapses in the brain stem which are involved in the modulation of urinary bladder function and that GABA receptor agonists exert their inhibitory effects on bladder contraction through the direct activation of the GABA receptors. The inhibitory effect of GABA itself on bladder contraction was very weak in both experiments (Fig. 4) and those of Sillén et al. (2). GABA would not be able to easily penetrate the active sites, and so a high dose of GABA would be necessary to obtain a sufficient concentration around the sites which can exert the inhibitory effects. In addition, the following results were obtained in the present experiments which may confirm the involvement of GABA receptors in the control of bladder function. The inhibitory effect of diazepam was potentiated by the GABA transaminase inhibitor AA and attenuated by the glutamic acid decarboxylase inhibitor semicarbazide (Table 1). In particular, in six out of eight rats pretreated with AA, infusion of solution into the bladder was unable to induce reflex bladder contraction. Since the inhibitory effect of AA was very similar to that of diazepam and was reversed by picrotoxin at the dose which reversed the inhibitory effects of diazepam, this would have resulted from the elevation of GABA concentration.

The main active sites of diazepam which are related to its inhibitory effects on bladder contraction may be in the micturition center of the brain stem (2), but not the pelvic ganglia. This is because presynaptic pelvic nerve stimulation induced bladder contraction after diazepam had completely inhibited the bladder contraction induced by infusion of solution (Fig. 7) and because diazepam simultaneously abolished both bladder contraction and the efferent discharges recorded from pelvic nerves (Fig. 3). In the case of the ganglion blocking agent C6 (10 mg/kg, i.v.), it suppressed bladder contraction but facilitated the generation of efferent discharges (Fig. 3). We (6) previously reported that C6 inhibited bladder contraction and increased the rate of afferent discharges in accordance with bladder filling. Activation of bladder afferents leads to excitation of pelvic nerves via supraspinal pathways originating in the micturition
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There is also an area in the sacral cord which is involved in the control of bladder function (9). In the present experiments, we studied the effects of muscimol on this area through a tube inserted according to the method of Yaksh and Rudy (8). When we observed the rats using a soft X-ray fluoroscope, the tip of the i.t. injection tube was located in the area from the 13th thoracic to the 2nd lumbar vertebra. Since electrical stimulation at the tip of the tube induced bladder contraction (Fig. 6), it was considered that the tip was close to the area in the sacral cord which controlled bladder function. Injection of muscimol (1 µg) i.t. was able to inhibit bladder contraction, and the inhibitory effect was reversed by picrotoxin. These results suggest that there may be GABA receptors in the sacral micturition center and that the inhibitory effects of diazepam on bladder motility are due to activation of GABA receptors in the spinal cord, as well as the supraspinal region. With regard to the opiate receptors which are involved in the inhibitory control of bladder function, these have been reported to be located in the spinal cord and the supraspinal region (10, 11). The inhibitory effect of diazepam on the bladder contraction was not reversed by naloxone at a dose (1 mg/kg, i.v.) which was 10 times higher than that capable of reversing the inhibitory effects of morphine in this model (12). It was thus considered that the inhibitory effects of GABA receptor agonists were unrelated to the opiate mechanisms.

In conclusion, our results suggest that the inhibitory effect of GABA receptor agonists on bladder motility is mainly due to central action and that the active sites are not only in the brain stem but also in the spinal cord. GABA receptor agonists thus directly activate the GABA receptors which are involved in the control of bladder function.

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