Dopamine Release and Presynaptic Dopaminergic Regulation in Guinea Pig Spinal Cord†

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Abstract—Using pharmacological approaches, we obtained evidence for the release of dopamine (DA) and its dopaminergic regulation in the guinea pig spinal cord. Electrical stimulation of the cord pieces increased the endogenous DA release and the efflux of [^3H]dopamine ([^3H]DA) from tissues preloaded with [^3H]DA. The evoked release of [^3H]DA was current- and frequency-dependent and was prevented by tetrodotoxin (10^{-6} M) or Ca^{2+} -free medium containing EGTA (10^{-4} M), while bntropine allowed a recovery of a more extensive amount of [^3H]DA in the superfusing medium by inhibiting the reuptake process. The stimulated [^3H]DA release was reduced by LY-171555, but not by SKF-38393 and 8-Br-cAMP. (–)Sulpiride enhanced the stimulated endogenous DA and [^3H]DA release. (–)Sulpiride reversed the inhibition of evoked [^3H]DA release induced by LY-171555, while SKF-38393 and 8-Br-cAMP did not affect LY-171555-induced inhibition of evoked [^3H]DA release. These findings provide additional evidence for the neurotransmitter role of DA in the spinal cord of the guinea pig, and they strongly suggest the presence of presynaptic regulation of DA release via the dopamine receptor which is the D_2 type.

Several studies have suggested the possibility that dopamine (DA) in the spinal cord is not only a precursor of norepinephrine but can also act as an independent neurotransmitter in the separate DA neuron system (1, 2). The dopaminergic pathways were considered to descend from the nigral (A9) (1, 3), diencephalic (A11) (4–6) and hypothalamic (A13) (7) cell groups to the spinal cord. Recently, the descending spinal DA system has been shown to originate primarily in the diencephalic A11 cell group, forming a diencephal-spinal DA pathway (8). We obtained autoradiographical evidence for the localization of dopaminergic terminals in the spinal cord (9). However, the criteria for a neurotransmitter in case of the spinal cord

has not been fully satisfied. In particular, there has been no evidence of a stimulus dependent release of DA from the cord.

The presynaptic dopamine receptors appear to modulate the rate of DA biosynthesis and impulse-induced DA release by a local negative feedback mechanism in the nigrostriatal (10, 11) and mesolimbic systems (10–12), while the mesocortical (13) and tuberoinfundibular DA systems (11) appear to lack DA autoreceptor. There is, however, no information concerning autoreceptors in the spinal DA system. Here, we report the first data obtained about the release of DA from the spinal cord and its presynaptic regulation via the DA receptor.

Materials and Methods

Guinea pigs of both sexes, weighing 350–450 g, were decapitated, and tracheotomy was performed to identify the segments. The segments C_1 – C_5 were quickly removed by a complete laminectomy and divided into left

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and right halves by the cutting at the posterior median sulcus. Using small scissors under a dissecting microscope, the dorsal half of the gray matter was carefully removed from the white matter.

Endogenous DA release: The tissue preparations (approximately 55 mg each) were set in the superfusion chamber. The tissue was then superfused at a constant rate of 0.2 ml/min with Krebs-Ringer bicarbonate solution oxygenated with 95% O₂ and 5% CO₂ and maintained at 37°C. Krebs-Ringer bicarbonate solution had the following composition (mM): NaCl, 118; KCl, 3; MgCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 23; glucose, 11; pargyline, 0.1; and ascorbic acid, 0.5.

The superfusate was continuously collected into a test tube containing 0.1 ml of 0.4 M HClO₄ and sodium metabisulphite (2 mg) every 5 min. Each superfusate was gathered respectively from 4 cord pieces of two animals. The superfusate was adjusted to pH 8.3 with 2 M Tris-HCl buffer, pH 8.3, and 0.5 M NaOH solution. Endogenous DA in the superfusate was absorbed with 5 mg of activated aluminum oxide and eluted with 20 µl of 0.1 M HCl. DA in the eluates was determined using HPLC (Hitachi 655 liquid chromatograph) with an electrochemical detector (IRCA E-502) as described in our previous reports (14, 15).

[³H]DA release: The preliminary uptake experiment was performed to determine the best conditions for the [³H]DA loading to the tissues. The dorsal half of the gray matter (approximately 5 mg each) was preincubated for 10 min at 37°C in 2 ml of Krebs-Ringer bicarbonate solution containing benztropine, DMI or ouabain equilibrated with 95% O₂ and 5% CO₂ and then incubated with [³H]-DA (10⁻⁷ M) for 15 min at 37°C in the same medium. After incubation, the tissues were rinsed in 10 ml of fresh medium 3 times every 10 min and then dissolved by sonication in 0.5 ml of sodium laurylsulfate (10%). The radioactivity in the tissue was measured in a liquid scintillation spectrometer.

In the release experiments, the spinal cord pieces (approximately 5 mg) were loaded with the medium containing [³H]DA (10⁻⁷ M) and DMI (5×10⁻⁶ M) for 45 min at 37°C. After rinsing with fresh medium, the pre-

paration was mounted in the superfusion chamber, then superfused with the medium as described above. The superfusion chamber was saturated with 95% O₂ and 5% CO₂ throughout the experiment. The period of equilibration before the start of the experiment was 30 min. The superfusate was continuously collected every 5 min, and the radioactivity determined in a liquid scintillation spectrometer. The unchanged [³H]-DA in the superfusate was estimated using HPLC (14, 15). A half-volume (0.5 ml) of the samples was used to separate unchanged [³H]DA, and the remaining parts of the samples were prepared for estimation of the total radioactivity. To the sample was added 10 ng of labeled DA as the carrier, and DA in the samples was extracted by the alumina absorption method and identified by HPLC, as described above. The fractions containing DA were collected from the outflow of HPLC, and radioactivity in these fractions was determined by liquid scintillation spectrometer. The amounts of unchanged [³H]DA in the sample obtained before and during the stimulation period were 75±4.85 and 76±7% of the total radioactivity, respectively. Accordingly, the total radioactivity in the superfusate was considered to represent the approximate amount of [³H]DA and, therefore, will further be denoted as [³H]DA release.

Electrical stimulation: The preparation was impaled with a pair of parallel platinum electrodes (0.1 mm in diameter; distance, 1.5 mm). Stimulation composed of monophasic pulses (1 msec duration) were applied for 2 min at various intensities of current (0.5–3.0 mA) and different frequencies (1–20 Hz).

Drug and chemicals: The following drugs were gifts: LY-171555 (Eli Lilly), (–)-sulpiride (Fujisawa), SKF-38393 (Smith Kline), benztropine (Merck). The following drugs were from commercial sources: [³H]-dopamine (45.4 Ci/mmol, New England Nuclear), dopamine HCl and pargyline HCl (Nakarai), desmethylimipramine (Fujisawa), tetrodotoxin (Sankyo), ouabain (Takeda) and 8-Br-cAMP (Sigma). All other chemicals were of reagent grade.

Statistical analyses: Data were analyzed by Student's *t*-test, and differences were

accepted as significant at the 0.05 level of probability.

Results

Release of endogenous DA: The contents of NA and DA of the cervical spinal cord were 218.4 ± 6.2 ng/g ($n=6$) and 22.53 ± 0.75 ng/g ($n=6$), respectively.

When the pieces of cervical spinal cord were superfused with Krebs-Ringer bicarbonate solution, the spontaneous release of endogenous DA reached a steady state level after 10 to 15 min. The amount of spontaneous release of DA was 61.5 ± 17 pg/g tissue/5 min. Electrical stimulation (1.5 mA current, 10 Hz frequency, 1 msec pulse duration) for 2 min produced a release of endogenous DA of 242 ± 44 pg/g tissue/5 min (Table 1). The fractional rate constant of spontaneous and stimulated DA release was approximately 0.003 and 0.012 per min, respectively.

Release of labeled DA: When the dorsal half pieces of cervical cord gray matter were incubated with 10^{-7} M of [3 H]DA at 37°C , the accumulation of [3 H]DA into tissue were 0.132 ± 0.004 pmoles/mg tissue/10 min. Ouabain (10^{-4} M) reduced the accumulation of [3 H]DA by about 22% of the control. This result indicates that the uptake system of DA is an active transport through an energy-dependent process. The accumulation of [3 H]DA was suppressed to 68% of control values by benztropine (10^{-6} M), to 53% by DMI (5×10^{-6} M) and to 26% by benztropine plus DMI. These findings indicate

that 74% of DA uptake in the spinal cord is composed of the benztropine sensitive dopaminergic and the DMI sensitive noradrenergic components. Therefore, in the following release experiments, the tissues were incubated with 10^{-7} M [3 H]DA in the presence of DMI (5×10^{-6} M) to prevent the accumulation of [3 H]DA into noradrenergic nerve terminals.

The spontaneous tritium efflux assumed a fairly constant level in each experiment after 60 min superfusion. The fractional rate constant was about 0.002 per minute. Electrical stimulation produced an increase in the tritium efflux above the level before stimulation. The stimulated [3 H]DA release is dependent on the applied current (0.5–3.0 mA) or frequency (1–20 Hz) (Fig. 1). The [3 H]DA release induced by stimulation of a current less than 1.5 mA was completely suppressed by TTX (10^{-6} M) or Ca^{2+} -deprivation, indicating the requirement of a rapid Na^+ influx and the Ca^{2+} influx (Fig. 1A). Significant increases in the spontaneous and stimulated [3 H]DA release were produced by adding 10^{-5} M benztropine to the superfusion medium (Fig. 2A), by which recapture of the released [3 H]DA from the dopaminergic nerve terminals seems to be prevented. In the tissues preloaded with [3 H]DA in the medium containing both benztropine and DMI, the electrical stimulation did not produce any alteration in [3 H]DA release (Fig. 2B).

Presynaptic dopaminergic regulation of DA release: When the spinal cord pieces were superfused with the medium containing 10^{-5}

Table 1. Effect of (–)sulpiride on endogenous dopamine release evoked by electrical stimulation from guinea pig spinal cord pieces

	Before	(pg/g/5 min) During stimulation	After	Rst/Rsp	%
Control	62 ± 17	242 ± 44	55 ± 18	3.56 ± 0.21	100
(–)Sulpiride (10^{-6} M)	70 ± 11	332 ± 59	49 ± 13	$4.74 \pm 0.34^*$	146

Tissues were superfused with the medium containing (–)sulpiride 40 min before the electrical stimulation (1.5 mA, 10 Hz, 1 msec) for 2 min. Each value represents the mean $\pm$ S.E. of 4 experiments. Rst/Rsp indicates the ratio of the stimulated release (Rst) to the spontaneous release (Rsp) of endogenous DA. The percent was calculated from the formula: $\frac{(A-1)}{(B-1)} \times 100$, where A indicates (Rst/Rsp) of experiments involving (–)sulpiride and B corresponds to (Rst/Rsp) of control experiments. * $P < 0.01$ (Student's *t*-test, significant difference from Rst/Rsp of the control).

Fig. 1. The electrically stimulated release of [^3H]DA from the spinal cord pieces preloaded with [^3H]DA (10^{-7} M). The tissues were incubated in the medium containing [^3H]DA (10^{-7} M) in the presence of DMI (5×10^{-6} M) for 45 min and then superfused with the fresh medium. Each point represents the mean $\pm$ S.E. of 6 experiments. **A:** Relationship between the currents of stimulation (0.5–3.0 mA) and the ratio of the stimulated release (Rst) to the spontaneous release (Rsp) of [^3H]DA (●). Other stimulus parameters were 10 Hz, 1 msec for 2 min. Effect of tetrodotoxin (□) and calcium-free medium (○) on the current dependent-stimulated [^3H]DA release from the spinal cord pieces preloaded with [^3H]DA. **B:** Relationship between the frequencies of stimulation (1–20 Hz) and the ratio of the stimulated release (Rst) to spontaneous release (Rsp) of [^3H]DA. Other stimulus parameters were 1 mA, 1 msec for 2 min.

M LY-171555, a D_2 receptor agonist, (16) the stimulated [^3H]DA release was markedly suppressed without any changes in the spontaneous release. The stimulated [^3H]DA release was reduced to 63% of control values by 10^{-6} M LY-171555. Whereas, SKF-38393 (10^{-5} M, a D_1 agonist) and 8-Br-cAMP (3×10^{-3} M) did not affect the evoked [^3H]DA release (Table 2). (–)sulpiride (10^{-6} M) enhanced the stimulated [^3H]DA release to 150% of the control. Inhibition of stimulated [^3H]DA release induced by LY-171555 (10^{-6} M) was also reversed by 10^{-6} M (–)sulpiride, but not by 10^{-5} M SKF-38393 or 3×10^{-3} M 8-Br-cAMP. When the preparation was superfused with the medium containing 10^{-6} M (–)sulpiride, endogenous DA release induced by electrical stimulation (1.5 mA, 10 Hz, 1 msec) for 2 min was increased to 154% of control values (Table 1). From these findings, DA release in the spinal cord seems to be regulated via dopamine receptors.

Discussion

The present results revealed that endogenous DA can be released from tissue stores in the isolated guinea pig spinal cord in response to electrical stimulation. Since the DA contents in the spinal cord is lower than in the dopaminergic brain area, it was difficult to detect endogenous DA released from the cord into the superfusion medium. In the present study, the electrically stimulated DA release was measured using HPLC with a sensitive electrochemical detector. Electrical stimulation (1.5 mA, 10 Hz, 1 msec) for 2 min produced a release of endogenous DA of approximate 242 pg/g/5 min, which was 4-fold spontaneous DA efflux. The properties of the stimulated DA release were analyzed using labeled DA. Labeled DA is known to be taken up by nerve terminals other than dopaminergic terminals such as noradrenergic and serotonergic terminals (17, 18). The

Fig. 2. A: Effect of benztropine on the electrically stimulated [³H]DA release from guinea pig spinal cord pieces preloaded with [³H]DA in the presence of DMI (5×10^{-6} M). The tissues were incubated for 15 min in the medium containing DMI (5×10^{-6} M) before and during loading with [³H]DA and then superfused with the same medium. The horizontal bar indicates the addition of benztropine (10^{-5} M) to the superfusing medium. B: Effect of the electrical stimulation on [³H]DA release from the guinea pig spinal cord pieces preloaded with [³H]DA in the presence of benztropine and DMI. The tissues were incubated for 15 min in the medium containing benztropine (10^{-6} M) and DMI (5×10^{-6} M) before and during loading with [³H]DA and then superfused with the same medium. Est indicates electrical stimulation (1.5 mA, 10 Hz, 1 msec) for 2 min. Each point represents the mean $\pm$ S.E. of 6 experiments.

Table 2. Effect of various drugs on the electrically evoked release of [³H]DA from guinea pig spinal cord pieces preloaded with [³H]DA

Drugs	Rst/Rsp	(n)	%
None (Control)	2.68 $\pm$ 0.04	(13)	100
LY-171555 (10^{-6} M)	2.05 $\pm$ 0.08*	(4)	63
SKF-38393 (10^{-5} M)	2.69 $\pm$ 0.05	(3)	101
8-Br-cAMP (3×10^{-3} M)	2.54 $\pm$ 0.09	(3)	92
(-)-Sulpiride (10^{-6} M)	3.52 $\pm$ 0.10*	(4)	150
(-)-Sulpiride (10^{-6} M)+LY-171555 (10^{-6} M)	2.57 $\pm$ 0.04	(4)	94
SKF-38393 (10^{-5} M)+LY-171555 (10^{-6} M)	2.10 $\pm$ 0.09*	(3)	66
8-Br-cAMP (3×10^{-3} M)+LY-171555 (10^{-6} M)	2.13 $\pm$ 0.06*	(3)	67

Tissues were superfused with the medium containing various drugs 40 min before the electrical stimulation (1.5 mA, 10 Hz, 1 msec) for 2 min. Rst/Rsp indicates the ratio of the stimulated release (Rst) to the spontaneous release (Rsp) of [³H]DA. *P<0.05 (Student's *t*-test, significant difference from control values). The number of experiments is given in parenthesis. The percent was expressed as indicated in Table 1.

dopamine contents in the cervical cord is less than one-tenth of the NA and 5-HT contents (19, 20). Based on histochemical evidence that the spinal dopaminergic innervation is mainly confined to the dorsal horn (8), while the serotonergic and noradrenergic terminals are supplied more densely in the ventral horn rather than in the dorsal horn at the cervical level (21). [^3H]DA release in response to electrical stimulation was determined in the dorsal half pieces of the cervical cord gray matter preloaded with [^3H]DA and in the presence of DMI, the objective being to prevent [^3H]DA uptake into the noradrenergic nerve terminals. The fractional rates of spontaneous and stimulated release of [^3H]DA were in similar range to those of endogenous release, respectively. The [^3H]DA release induced by stimulation of a current less than 1.5 mA was completely suppressed by TTX or Ca^{2+} -deprivation, indicating the requirement of a rapid Na^+ influx and the Ca^{2+} influx. The [^3H]DA release induced by low current stimulation indicates a characteristic of neurotransmitter release from presynaptic axon terminals (22, 23).

Benztropine produced increases in the spontaneous and stimulated tritium efflux. The presence of benztropine seems to prevent reuptake of the released [^3H]DA by the dopaminergic terminals. Furthermore, the electrical stimulation did not induce [^3H]DA release from the tissues preincubated with [^3H]DA in the presence of benztropine and DMI. These findings together with the data that the unchanged [^3H]DA accounted for 76% of the tritium suggest that the total tritium efflux by stimulus of low current reflects well the unchanged [^3H]DA released from the dopaminergic nerve terminals. The DA receptor mediated modulation of the release of DA from striatal slices under in vitro conditions is by now well established (24, 25). To analyze whether the spinal DA system is regulated via presynaptic DA receptors, effects of the DA receptor agonist and antagonist on the stimulus induced [^3H]DA release were examined. Two types of DA receptor whose stimulation affects cellular cAMP have recently been identified. In the mammalian neostriatum, stimulation of the

DA receptor called D_1 increases formation of cAMP, whereas stimulation of the second type of DA receptor called D_2 reduces cAMP formation induced by stimulation with D_1 agonist (26, 27). The stimulated [^3H]DA release in the spinal cord was reduced by LY-171555, a selective D_2 receptor agonist. This inhibitory effect was antagonized by (-)-sulpiride, a selective D_2 antagonist. It thus appears that DA receptors involved in the inhibition of DA release in the spinal cord have pharmacological properties similar to presynaptic dopamine receptors of the D_2 type found in the striatum (24, 25, 28). On the other hand, SKF-38393, a selective D_1 agonist, and 8-Br-cAMP did not have an effect on the stimulus evoked [^3H]DA release, suggesting that the D_1 receptor linked to adenylate cyclase is not involved in the modulation of transmitter release from the spinal dopaminergic nerve terminals. It has been reported that drugs stimulating cAMP efflux did not affect ACh release or D_2 agonist induced inhibition of ACh release from rat neostriatum, although the pharmacological characteristics of the DA receptors mediating inhibition of both ACh release and cAMP efflux appeared to be similar (29).

By analogy with the situation of ACh (27) and glutamate (29) release from the striatum, the D_2 receptor mediated inhibition of DA release was not quite affected by the D_1 agonist and 8-Br-cAMP, suggesting that the D_2 receptor mediating inhibition in the spinal DA release is independent of the inhibition of cAMP generation.

Molecular characteristics of the D_2 receptor inhibiting the stimulus-induced release of DA from the spinal cord still remain to be clarified.

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