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D-Serine modulates neurogenic relaxation in rat corpus cavernosum

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ABSTRACT

D-Serine, an endogenous co-agonist for the N-methyl-D-aspartate (NMDA) receptor in mammals, is synthesized from L-serine by serine racemase. Although much attention has been focused on the role of D-serine within the central nervous system, the physiological role of D-serine in peripheral nerves such as corpus cavernosal nerves has not been investigated. The present study was aimed to study the expression, cellular localization and function of serine racemase/D-serine system in isolated rat corpus cavernosum. Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis showed the expression of serine racemase in rat corpus cavernosum. Immunogold electron microscopy demonstrated the cellular localization of serine racemase in the cavernosal nerves' membrane of the tissue. The organ bath studies on isolated rat corpus cavernosum showed that D-serine increases the non-adrenergic non-cholinergic neurogenic relaxation of isolated rat corpus cavernosum *in vitro*. This effect of D-serine was inhibited by a variety of NMDA receptor antagonists (ketamine, MK 801 and ifenprodil), suggesting that NMDA receptors are involved in the effects of D-serine on the neurogenic relaxation of corporal tissue strips. These observations provide the first evidence for the role of D-serine in modulating the neurogenic relaxation of rat corpus cavernosum, and may open new therapeutic avenues for the treatment of impotence.

1. Introduction

The discovery that mammalian brain contains high levels of D-serine overturned the dogma that only L-isomers of amino acids occur in mammalian tissues [1–3]. It is now well accepted that D-serine is a neurotransmitter within the mammalian central nervous system which selectively binds to a co-agonist site at the N-methyl-D-aspartate (NMDA) receptors and, along with glutamate, mediates several important physiological processes [3].

D-Serine is synthesized from L-serine by a pyridoxal-phosphate-dependent enzyme, serine racemase [4], and is rapidly degraded by a variety of enzymes including D-amino acid oxidase (DAAO) [5]. The expression of serine racemase has been demonstrated in astrocytes as well as neurons in various areas of mammalian central nervous system [4,6–8]. Although much attention has been focused on D-serine within the central nervous system, the expression of serine racemase has also been shown in the peripheral tissues including retinal ganglion cells [9], Schwann cells [10], epineural fibroblasts [10] and chondrocytes [11]. However, the physiological role of D-serine in peripheral nervous system remains to be investigated.

Penile tumescence (erection) and de-tumescence are regulated by a complex neurophysiological process of relaxation and contraction, respectively, of the corpus cavernosum [12,13]. There is consensus among scientists that the neural control of erection via cavernosal nerve stimulation involves chemical mediators that are described as non-adrenergic non-cholinergic (NANC) transmitters [14–16]. Nitric oxide is considered to be the main NANC transmitter which mediates the relaxation of corpus cavernosum [15–17]. Apart from nitric oxide, other NANC transmitters are also involved in the relaxation of corpus cavernosum which include purines (e.g., ATP and ADP), eicosanoids, calcitonin-gene-related peptide and anandamide [14,18–20].

It is well known that sexual responses such as penile erection are controlled by neural circuits in the brain and spinal cord that are stimulated by activation of post-synaptic NMDA receptors [21,22]. There is several convincing evidence that central NMDA receptors play a role in modulation of penile erection [23]. Moreover, the pioneering studies by Gonzalez-Cadavid et al. demonstrated that peripheral NMDA receptors may play a role in the relaxation of corpus cavernosum and thereby on erectile function [24,25]. The expression of NMDA receptor subunits has been shown in rat and human corpus cavernosum [24,25].

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Gonzalez-Cadavid et al. also demonstrated that NMDA receptor blockers at sub millimolar concentrations are able to induce relaxation of corpus cavernosum *in vitro* [24]. However, it is yet unclear to what extent these observations with high concentration of these chemicals result from NMDA receptor blockade, or from effects on non-NMDA ion channels in cavernosal smooth muscle [24].

NMDA receptors play a pivotal role as an excitatory neurotransmission which leads to the influx of Na^+ and Ca^{2+} in postsynaptic membrane. Since Ca^{2+} entry through NMDA receptors activates neuronal nitric oxide synthase (NOS) by a Ca^{2+}/calmodulin-dependent mechanism, NMDA receptors are considered as an important modulator of neuronal NOS activity in a variety of model systems [26–27]. Magee et al. showed the co-localization of NMDA receptors with neuronal NOS in the rat and mouse cavernosal nerve [25]. However, the role of such interaction is not well understood in context of corpus cavernosal function.

Since D-serine is involved in modulation of NMDA receptors in a variety of biological systems, we initially wished to confirm that serine racemase is expressed in rat corpus cavernosum and act as a modulator of NANC relaxation *in vitro*. The present study reports our investigations on the expression, cellular localization and function of glutamate/D-serine system in isolated rat corpus cavernosum.

2. Methods and materials

2.1. Reagents

Phenylephrine hydrochloride, guanethidine sulfate, atropine sulfate, ifenprodil tartrate, N^G-L-nitro-arginine methyl ester (L-NAME), dizocilpine (MK 801) and ketamine were purchased from Sigma (Sigma–Aldrich, Bristol, UK). All drugs were freshly dissolved in distilled water. All reagents and enzymes used for PCR and Western blotting were purchased from Promega (Promega, Madison, USA).

2.2. Animals

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from the Comparative Biology Unit at the UCL Medical School (Royal Free Campus, UCL, London, UK). The animals were housed in a light-controlled room with a 12 h day/night cycle and were given free access to food and water. All animal procedures were in accordance with Home Office (UK) recommendations. Each separate experimental group consisted of six animals.

2.3. 2.3. mRNA extraction & reverse transcription polymerase chain reaction (RT-PCR) procedure

Corpus cavernosum, liver and brain were obtained from rats and immediately immersed in liquid nitrogen. Total RNA was extracted from tissue homogenate using RNeasy Fibrous Tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Further elimination of genomic DNA was performed by DNase after RNA extraction. First strand cDNA was then generated by using 1 μg of deoxyribonuclease treated RNA, 1 μl of random hexamer primer (p(dN)6), and ribonuclease free water, heated at 70 °C for 5 min, and then placed on ice. RNasin (ribonuclease inhibitor), 100 unit of Moloney murine leukaemia virus reverse transcriptase, Moloney murine leukaemia virus buffer, and 0.4 mM deoxynucleoside triphosphates were added, and the mix was incubated at 42 °C for 1 h. Oligonucleotide primers used for PCR amplification of rat serine racemase, DAAO and β-actin were as follows:

a. Rat serine racemase (NM 198757), PCR product: 295 bp
   Sense: 5′-ATTGCAAGAAACTGGCCATC
   Anti-sense: 5′-TCAGCAGCGTACACCTTCAC
b. Rat DAAO (NM 053626), PCR product: 460 bp
   Sense: 5′-TTCGAAGAGGTGGTGAGAGGA
   Anti-sense: 5′-AGTGAATCGTGAGCCCGTAAC
c. Rat β-actin (NM 031144), PCR product: 453 bp
   Sense: 5′-AGAGGGAAATCGTGCGTGACA
   Anti-sense: 5′-ACATCTGCTGGAAGGTGGACA

PCR reactions comprised of 1 μl of cDNA template, 100 ng each of sense and anti-sense oligonucleotide primers, 2.5 μl of optimized TaqPCR buffer, 0.4 mM dNTP mixture, and 2 U of Taq polymerase in a total reaction volume of 25 μl. After initial 5 min incubation at 94 °C, PCRs were performed using a 1 min annealing step, followed by a 1 min elongation step at 72 °C and a 45 s denaturation step at 94 °C. Forty PCR cycles were performed for amplification of serine racemase and DAAO cDNAs, 25 cycles for β-actin cDNA, followed by a final elongation for 10 min at 72.0 °C. PCR products were separated by electrophoresis through a 1% agarose gel and detected by ethidium bromide staining.

2.4. Western blotting

Corpus cavernosum and liver (positive control) were obtained from rat and were immediately frozen in liquid nitrogen. Snap frozen tissues were homogenized in ice-cold RIPA buffer containing protease inhibitors (protease inhibitor mixture from Roche, Mannheim, Germany), 50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. Homogenates were then sonicated followed by centrifugation at 10,000 × *g* for 5 min at 4 °C. After determining the protein concentrations of the supernatants (Bradford assay with bovine serum albumin as standard), 10 μg protein of each sample was fractionated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with Tris buffered saline (10 mM Tris, 100 mM NaCl) containing 0.1% Tween-20 for 1 h, the membranes were incubated overnight with rabbit anti-serine racemase antibody (1:500 rabbit polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After rigorous washing, the membranes were incubated with anti-rabbit IgG alkaline phosphatase-linked antibody (1:5000 dilution, Perbio Science Ltd., Northumberland, UK). Alkaline phosphatase was detected using a BCIP/NBT developing kit.

2.5. Immunogold electron microscopy

Freshly isolated corporal tissues from rats were excised and immersed in isotonic fixative (4% paraformaldhyde, 0.5% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4, with 0.1 M sucrose) for electron microscopic immunocytochemistry as has been previously described ($n = 2$). Tissues were then infiltrated and embedded in LR white resin. Thin sections (70–90 nm) were cut and mounted on coated nickel grids. The grids were then blocked (0.1% bovine serum antigen, 0.1 M glycine in PBS) for 30 min and incubated for 2 h with rabbit polyclonal antibody against serine racemase (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following a series of washes, grids were incubated for 1 h with 10 nm Immunogold-linked, EM grade, goat anti-rabbit IgG (1:50 dilution). Following another series of washes, grids were successively stained with uranyl acetate and Reynold's lead citrate before visualization with a transmission electron microscope. Electron micrographs were scanned using a digital imaging system.

2.6. Organ bath studies

Corpora cavernosa strips were excised from rats as previously described [19,20]. They were mounted separately in 25-ml organ chambers with one end tied to an electrode holder and the other to a wire connected to a force transducer (ADInstrument, Australia). The chambers contained Krebs-bicarbonate solution (containing in mM: NaCl, 118.1; KCl, 4.7; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; and glucose, 11.1, pH 7.4) at 37 °C equilibrated with 95% oxygen and 5% carbon dioxide. The corporal strips were allowed to equilibrate under 0.5 g resting tension and after equilibration for 60 min, the contractile response to phenylephrine (7.5 μM) was measured. Electrical field stimulation (EFS) was applied via two parallel platinum electrodes on either side of the corpus strips. In experiments which used electrical field stimulation, atropine (1 μM, to produce cholinergic blockade) and guanethidine (5 μM, to produce adrenergic blockade) were always present in the bathing medium to obtain NANC conditions. In all the experiments, each strip was used only once.

3. Results

3.1. Serine racemase expression and protein in the corporal tissue

To examine the expression of serine racemase and DAAO in corporal tissue, RT-PCR was performed on rat corpus cavernosum and hepatic tissues served as positive control. β-Actin was used as housekeeping gene. As shown in Fig. 1A, serine racemase mRNA was detectable in corporal tissue as well as the liver samples. This was further confirmed by Western blot analysis which showed the expression of a 37 kDa protein in corporal homogenate that reacted with anti-serine racemase antibody (Fig. 1B). We were unable to show the expression of DAAO mRNA in the corporal tissue using RT-PCR (Fig. 1A).

3.2. Electron microscopy

Representative electron micrographs from the corpus cavernosum are shown in Fig. 2. Black electron-dense circles (5 nm gold particles) represent positively stained tissue for serine racemase using anti-serine racemase antibody (Fig. 2.2 and 2.3). An obvious staining of cavernosal nerve membrane was noted within the corporal tissue.

3.3. Organ bath study

Corporal strips, pre-contracted with phenylephrine in the presence of guanethidine and atropine, were relaxed by electrical field stimulation at frequency of 10 Hz (Fig. 3A). The neurogenic relaxations were significantly increased 30 min after incubation with D-serine (10 and 100 μM) but not by 100 μM L-serine (Fig. 3A and B).

To evaluate whether the effect of D-serine is mediated by the NMDA receptor, in separate experimental groups, the non-selective NMDA antagonist ketamine (100 μM) and the specific NMDA antagonists MK 801 (100 μM) and ifenprodil (2 μM) were injected to the organ bath 10 min before administration of D-serine (100 μM). As shown in Fig. 3C, the effect of D-serine was significantly ($P < 0.01$) prevented by NMDA receptor blockade. We also examined the effect of MK 801 30 min after D-serine administration on EFS-induced neurogenic relaxation. The results showed that MK 801 could reverse the effect of D-serine on corporal relaxation *in vitro* (data now shown). Neither of the NMDA antagonists at the mentioned concentrations showed a significant effect on the EFS-induced neurogenic relaxations or on the basal contractile tone. However, higher concentrations of the NMDA receptor antagonists (ketamine, 350–1000 μM; MK 801,

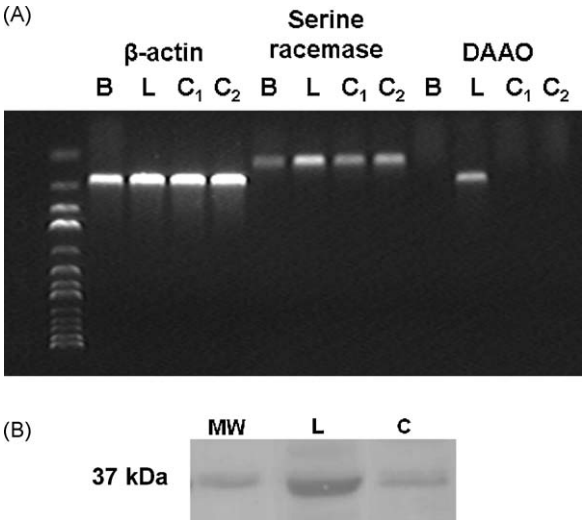

Fig. 1. (A) Expression of β-actin, serine racemase and D-amino acid oxidase (DAAO) in rat corpus cavernosum and in rat forebrain and liver. RNA was isolated from rat corpus cavernosum and analyzed by RT-PCR for β-actin, serine racemase and DAAO. The DNA bands were fractionated on agarose gels and subjected to ethiduim bromide staining. C_1 and C_2 indicate corporal tissues taken from two Sprague–Dawley rats. Liver (L) samples were used as positive control for both serine racemase and DAAO. Forebrain (B) samples were used as positive control for serine racemase and as negative control for DAAO. (B) Western blot of serine racemase protein in a rat corporal tissue strip (C). Proteins extracted from rat liver (L) was used as the positive control for serine racemase immuno-reactivity; MW: molecular weight marker.

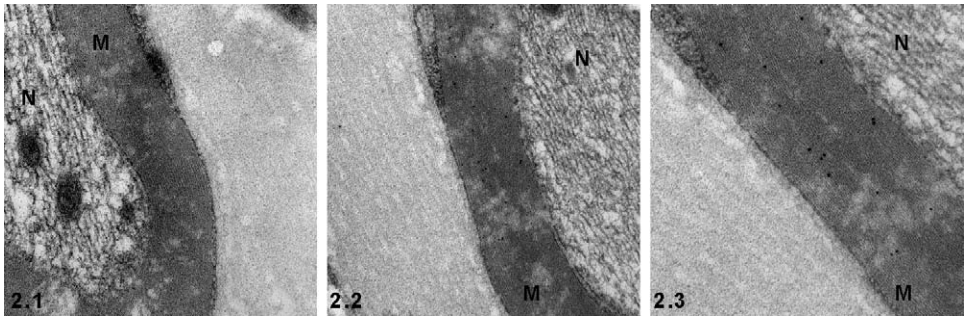

Fig. 2. Immunogold electron microscopy for the serine racemase in the cavernosal nerve from rat. (2.1) Primary antibody omission control. Electron-dense circles in (2.2) and (2.3) indicate positive staining for serine racemase in the cavernosal nerve membrane. Direct magnifications in (2.1), (2.2) and (2.3) are 66,000×, 66,000× and 11,0000×, respectively. N: cavernosal nerve; M: cavernosal nerve membrane.

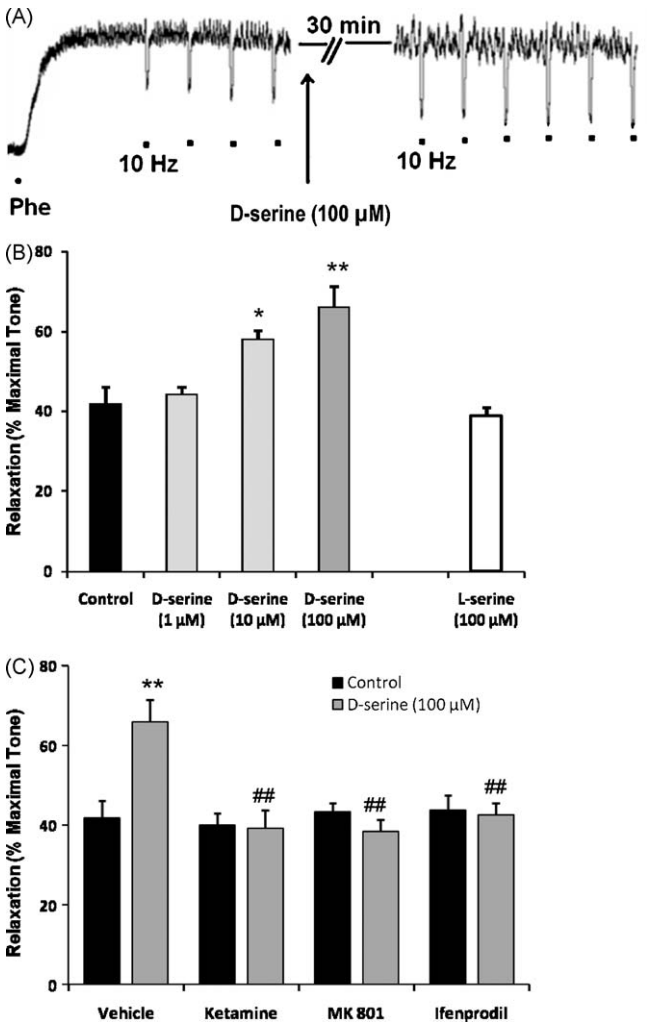

Fig. 3. (A) The NANC relaxation of isolated corpus cavernosum in response to electrical field stimulation (EFS) in cavernosal tissue pre-contracted with phenylephrine (Phe) before and after administration of D-serine (100 μM). (B) Effect of D-serine and L-serine on EFS-induced relaxation of cavernosal tissue pre-contracted with phenylephrine. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with controls. (C) Effect of different NMDA antagonists; ketamine (100 μM), MK 801 (100 μM) and ifenprodil (2 μM) on EFS-induced relaxation of isolated corpus cavernosum in the presence or absence of D-serine (100 μM). $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with D-serine treated group without pre-treatment with the NMDA antagonist.

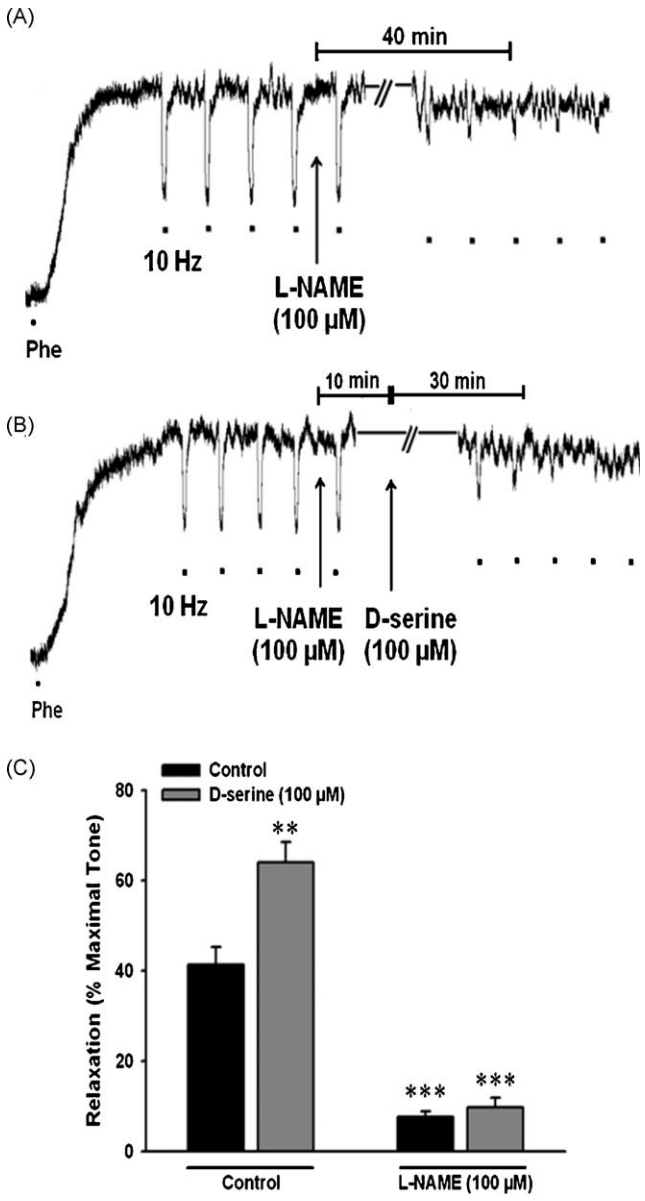

Fig. 4. Effect of the non-selective NOS inhibitor L-NAME (100 μM) on the NANC relaxation of isolated corpus cavernosum in response to electrical field stimulation (EFS, 10 Hz) in cavernosal tissue pre-contracted with phenylephrine (Phe) in the absence (A) or presence (B) of D-serine (100 μM). (C) Effect of the non-selective NOS inhibitor L-NAME (100 μM) on the relaxant responses of isolated rat corpus cavernosum to EFS (10 Hz) in the presence or absence of D-serine (100 μM). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control group; $^{\#}P < 0.05$ compared with corresponding L-NAME group with D-serine.

200–1000 μM and ifenprodil, 30–300 μM) resulted in a significant relaxation of isolated tissue strips in the absence of electrical field stimulation (data not shown).

The NANC relaxation was significantly ($P < 0.001$) inhibited by pre-incubation with a NOS inhibitor L-NAME (100 μM) in the absence or presence of D-serine (100 μM), suggesting that electrically NANC relaxation is mainly mediated by nitric oxide (Fig. 4).

4. Discussion

Neural control of penile erection by cavernosal nerve involves physiological mediators by NANC transmitters [12,13]. Nitric oxide is considered to be the main NANC transmitter which mediates the relaxation of corpus cavernosum through a cGMP-dependent pathway [28]. A variety of transmitter systems are able to modulate nitric oxide-dependent NANC relaxation in smooth muscles. Kohjitani et al. provided the first experimental evidence for the contribution of NMDA receptors in NANC relaxation in rabbit lower esophageal sphincter [29]. They demonstrated that NMDA receptor agonists dose-dependently mediate NANC smooth muscle relaxation which is accompanied by an increase in cGMP production [29,30].

NMDA receptors play a pivotal role as an excitatory neurotransmission which leads to the influx of Na^{+} and Ca^{2+} in post-synaptic membrane. Since Ca^{2+} entry through NMDA receptors activates neuronal NOS by a Ca^{2+}/calmodulin-dependent mechanism, NMDA receptors are considered as an important modulator of neuronal NOS activity within the central nervous system [26,27]. The presence of NMDA receptors have also been shown in some peripheral tissues such as enteric nervous system as well as corpus cavernosum [24,25,29]. Moreover, NMDA receptors are reported to co-localize with neuronal NOS in rat and mouse penile

nerve [25]. These reports suggest that NMDA receptors might be involved in the modulation of NANC relaxation in smooth muscles.

NMDA receptors contain a number of distinct binding sites including a binding site for glycine or D-serine, whose occupancy is required for NMDA receptor activation [27]. Since NMDA receptors are present in the rat corpus cavernosum, we tested whether serine racemase is expressed in this tissue, and confirmed this by RT-PCR and Western blot analysis. These data are the first evidence of the presence of this enzyme in the corpus cavernosum. The potential contribution of D-serine in corpus cavernosal function has not been demonstrated so far. Our data showed that D-serine is able to increases the neurogenic relaxation of rat corpus cavernosum *in vitro*: D-serine, but not L-serine, increased the electrically stimulated NANC relaxation of cavernosal strips at frequency of 10 Hz (Fig. 3). At this frequency, the NANC relaxation was almost completely blocked by the NOS inhibitor (Fig. 4), This phenomenon is a good experimental evidence for neurogenic nitric oxide to be a mediator of corporal tissue relaxation in response to EFS, but even if nitric oxide probably is the most important factor for relaxation of corpus cavernosum, this does not exclude the possibility that other agents released from nerves (e.g., vasoactive intestinal polypeptide, calcitonin-gene-related peptide and ATP [14,20]) may have been involved in the enhanced neurogenic responses to D-serine in the cavernosal tissue.

Interestingly, the effect of D-serine was inhibited by low concentration of selective NMDA receptor antagonists, suggesting that NMDA receptors may be involved in the effect of D-serine on the neurogenic relaxation of corporal tissue strips. These data are in line with the previous study by Magee et al. who showed the co-localization of NMDA receptors with neuronal NOS enzyme in the rat and mouse cavernosal nerve [25]. Using electron microscopy we showed that serine racemase is also present in the cavernosal nerves which play a major role in the corpus cavernosum physiology. We did not use double staining to confirm co-localization of serine racemase with either neuronal NOS or NMDA receptors. This can be further studied in details in future studies.

While low concentrations of NMDA receptor antagonists could inhibit the enhanced neurogenic relaxation induced by D-serine, incubation with higher concentrations was associated with relaxation of pre-contracted cavernosal strips even in absence of electrical field stimulation. Gonzalez-Cadavid et al. demonstrated that a wide variety of NMDA receptor blockers at sub millimolar concentrations are potent *in vitro* relaxants of cavernosal strips under conditions that inhibit other neurotransmission pathways [24]. However, it is yet unclear to what extent these observations with high concentration of these chemicals result from NMDA receptor blockade, or from effects on muscle non-NMDA ion channels [24]. In the present study we used the concentration of NMDA receptor antagonists which did not have any effect on the basal tone of the pre-contracted muscle strips. This enabled us to assess the effect of NMDA antagonists on neurogenic relaxation induced by electrical field stimulation *in vitro*.

It seems paradoxical that moderate concentrations of NMDA receptor blockers did not reduce electrically induced relaxation, although the same concentrations prevented the D-serine effects (Fig. 3C). Conventional NMDA receptors require dual agonists, glutamate and glycine/D-serine, for activation. If D-serine was able to increase electrically induced relaxation, it must have acted on a receptor that it already occupied by the other co-agonist. The failure of NMDA antagonists (ketamine, MK 801 and ifenprodil) to block electrically induced relaxation might be interpreted as indication for a very low endogenous level of D-serine in the corporal tissue. This might be related to rapid degradation of D-serine in the corpus cavernosum. Selective degradation of D-serine is achieved at least in some parts of central nervous system by the enzyme DAAO. Apart from central nervous system, DAAO has also been detected in kidney, liver and to a lesser extent in leukocytes, small intestine, epididymis and adrenal glands [5,31,32]. To assess whether the enzyme is present in the rat corpus cavernosum we used RT-PCR, and this did not show DAAO mRNA expression in rat corporal tissue. Likewise the DAAO levels are almost undetectable in D-serine-rich forebrain [33]. Moreover in DAAO-deficient mice, D-serine levels appear relatively unchanged in this region [33,34]. Thus, other mechanisms probably regulate D-serine elimination. Indeed, serine racemase also catalyzes α,β-elimination of water from D-serine [33]. Besides enzymatic degradation, the clearance of D-serine from the synaptic space is assured by Na^+-dependent transporters on the membrane of glial and neurons in central nervous system [34]. We did not study the clearance of D-serine in the corporal tissue, thus further studies are needed to shed light on the mechanism of D-serine degradation in the corpus cavernosum.

The present study demonstrated that both serine racemase mRNA and protein are present in the rat corpus cavernosum. We also showed that D-serine which is produced from L-serine by serine racemase increases the neurogenic relaxation in isolated rat corpus cavernosum. This effect of D-serine was inhibited by NMDA receptor blockades, suggesting that the effect of D-serine might be mediated via NMDA receptor activation in the rat corpus cavernosum. These observations provide the first evidence for the role of D-serine in modulating the neurogenic relaxation of rat corpus cavernosum, and may open new therapeutic avenues for the treatment of impotence.

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