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Light-evoked NMDA receptor-mediated currents are reduced by blocking D-serine synthesis in the salamander retina

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Abstract

Experiments were carried out in the retina of the tiger salamander (*Ambystoma tigrinum*) to evaluate the importance of D-serine synthesis on light-evoked N-methyl D-aspartate (NMDA) receptor-mediated components of ganglion cells and contributions to the proximal negative field potential. We blocked the synthesis of D-serine through brief exposures of the retina to phenazine ethosulfate and validated the changes in the tissue levels of D-serine using capillary electrophoresis methods to separate and measure the amino acid enantiomers. Ten minute exposures to phenazine ethosulfate decreased D-serine levels in the retina by about 50% and significantly reduced the NMDA receptor contribution to light responses of the inner retina. This is the first report of a linkage between D-serine synthesis and NMDA receptor activity in the vertebrate retina.

Keywords

D-serine; ganglion cells; glia; NMDA receptors; retina; serine racemase

Introduction

D-serine plays a major role as an N-methyl D-aspartate (NMDA) receptor coagonist in the vertebrate retina [1,2], where it is synthesized from L-serine by the enzyme serine racemase (SR) [3]. In the retina, SR is primarily localized to glial cells, including Müller cells and astrocytes [1], though it appears to be transiently expressed during development in mouse retinal ganglion cells [4] and appears early during synaptogenesis of the retina [5]. In this study, we pursued the relationship between D-serine synthesis and its effect on NMDA receptor activation using electrophysiological recordings of synaptic currents in the inner retina combined with measurements of D-serine using capillary electrophoresis techniques. For these experiments, we evaluated the SR blocking agent phenazine ethosulfate (Phz-ES, Sigma Aldrich, St. Louis, Missouri, USA), which, during brief applications, reduced D-serine and concomitantly reduced light-evoked NMDA receptor currents in the inner retina. These results further establish a tight relationship between the synthesis of D-serine and its impact on NMDA receptors by serving as the primary coagonist of the retina.

Methods

Electrophysiology

All recordings were carried out using a superfused retina-eyecup preparation of the tiger salamander (*Ambystoma tigrinum*) described earlier [2]. Light stimulation was provided by a computer-controlled LCD projector system described previously [6].

All chemicals and other agents used in these experiments were purchased from Sigma Chemical (St. Louis, Missouri, USA) with the exception of hyaluronidase and collagenase (Worthington Chemical, Lakewood, New Jersey, USA); tetrodotoxin (Alomone labs, Jerusalem, Israel); picrotoxin (Fluka, Neu-Ulm, Switzerland); KCH_3SO_4 (Pfaltz & Bauer, Waterbury, Connecticut, USA); D, L-2-amino-phosphonoheptanoate (AP7); 5,7-dichlorokynurenic acid (5,7-DCK) and 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxalinedione (DNQX) from Tocris (Ellisville, Missouri, USA).

Tiger salamanders were purchased from a dealer (Charles D. Sullivan Co., Nashville, Tennessee, USA) and maintained in circulated cold-water tanks (4°C) with a 12 h room light/dark cycle. Animal maintenance and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Animals were killed by decapitation followed by double pithing.

Antibody preparation

A polyclonal antibody was generated in rabbits (Sigma Genosys) against the N-terminal peptide (NH_2 -AQYCIS-FADVE-COOH) of mouse SR (Swiss-prot accession number Q9AZX7). We affinity-purified the serum over CNBr-sepharose beads (Sigma) conjugated to the same peptide. Two eluent fractions containing the highest protein content were pH neutralized, pooled, and used for these experiments.

Immunostaining

Retinal tissue was harvested from the neonous tiger salamander, *A. tigrinum*, and processed for immunostaining using the methods described earlier [1]. Confocal images were processed and rendered using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, California, USA) with three-dimensional reconstructions generated using Imaris software (Bitplane Inc., Saint Paul, Minnesota, USA).

D-serine measurements

D-serine and L-serine measurements were made through the use of capillary electrophoresis methods that have been described earlier [7]. Limited animal availability restricted our chemical measurements to 12 animals for two separate experiments.

Retinal protein determination

All amino acid measurements were normalized to the total retinal protein content. The protein pellets from each sample were suspended in 120 μl of 2 M NaOH and diluted 1 : 20 in distilled water. The protein concentration in the diluted sample was quantified using a Pierce (Rockford, Illinois, USA) bicinchoninic acid assay kit; absorption was measured at 562 nm.

Results

Experiments were done under voltage-clamp conditions while continuously superfusing the preparation with a normal Ringer. Following these initial observations, the bathing solution was changed to a nominally Mg^{2+} -free Ringer to which tetrodotoxin (0.5 μM) and

strychnine (10 μ M) were added (the control Ringer). When steady-state conditions with the toxin control Ringer were established, voltage-clamp studies were initiated and the holding potential was adjusted to give zero holding current. The range of holding potentials, which were used with these criteria ranged from -53 mV to -72 mV (average -64.9 ± 4.8 mV).

Serine racemase is located in retinal Müller cells

Figure 1 (top left) shows a single section of the salamander retina immunostained with the SR antibody developed for this report. Prominent labeling is evident in the Müller cells and the photoreceptor layer, including some photoreceptor terminals. A western blot using this antibody against different concentrations of a protein homogenate of the salamander retina revealed a single band near the 37 kDa (lower right panel) size of SR [3]. The single image on the upper left was one of a stack of images from different depths that were used to generate a three-dimensional reconstruction of the staining pattern (top right). Cross-sections of the retina were also exposed to the SR N-terminal peptide antibody after it had been pretreated with the peptide (lower left), indicating that the staining pattern observed could be attributed to the distribution of SR.

Blocking serine racemase decreases NMDA currents in retinal ganglion cells

Phz-ES inhibits the enzyme SR and reduces the synthesis of D-serine [8]. We compared the effects of Phz-ES with the actions of its parent compound Phz. Figure 2a illustrates the transient On and Off light-evoked inward currents (stimulus duration indicated by bar under first trace) recorded while the On-Off ganglion cell was bathed in the control Ringer. Addition of D-serine (100 μ M) substantially enhanced both the On and Off responses to the light stimulus, indicating a contribution from NMDA receptors [2]. When the selective and potent NMDA receptor coagonist site competitive antagonist, 5,7-DCK (30 μ M) was introduced, the On and Off responses were diminished compared with control values. After returning to a control Ringer (not illustrated), the cell was exposed to Phz (100 μ M), which had no significant effect on the response amplitude. After the wash out of Phz, the addition of Phz-ES (10 μ M) to the control bathing medium rapidly diminished the light-evoked responses. In the presence of continuous Phz-ES, the addition of exogenous D-serine resulted in a response enhancement similar to that observed when D-serine was added to the control Ringer. The last trace in the sequence illustrates the responses observed when the bathing medium consisted of 5,7-DCK added to the Phz-ES Ringer. In this case, the decline in response amplitude was slightly greater but similar to that observed when 5, 7-DCK was added to the control Ringer.

Figure 2b summarizes the actions of Phz, Phz-ES, and the actions of D-serine added to the Phz-ES bathing medium (Phz-ES + DS) and contrasts that with D-serine added to the control bathing environment (DS). The comparisons are expressed as a percentage of the control response for light-evoked charge entering the cell during the On and Off responses. No significant change in response amplitude was observed when Phz was added to the bathing medium, but the application of Phz-ES resulted in a significant decrease in the light evoked currents ($P < 0.05$). When D-serine was added to either the control Ringer or the Phz-ES bathing solution, a significant and equivalent enhancement of the responses was evident.

Figure 2c shows the actions of Phz-ES on light responses and interactions with the NMDA receptor antagonist 5,7-DCK. Bath applied Phz-ES significantly decreased both On and Off responses. In the presence of Phz-ES, the addition of 5,7-DCK (30 μ M) decreased the response slightly, similar to that observed when 5,7-DCK was added to the control Ringer.

Figure 2d illustrates the time course of the effects of Phz-ES on whole cell recordings (■) and the proximal negative field potential (PNFP, ●). Through trial and error, we determined

that the curve relating the actions of Phz-ES on the light responses was better fit by a Boltzman relationship rather than one or more exponential functions, suggesting that a mechanism other than simple diffusion of Phz-ES is required to describe its mode of action.

Actions of phenazine on the proximal negative field potential

Figure 3a illustrates a recording of the PNFP from the salamander retina, evoked by a 120 μm spot of light. A long light exposure was used to evoke both On and Off responses, but only the On response is illustrated. When phenazine (100 μM) was added to the bathing medium for 10 min, the response to light was superimposed on the control response (not illustrated). After returning to the control environment for 10 min, the introduction of Phz-ES (10 μM) decreased the response amplitude. In the presence of Phz-ES, the addition of D-serine (100 μM) increased the light response (Phz-ES + DS). Figure 3b shows the results of seven different experiments and illustrates a consistent decline in PNFP amplitude resulting from Phz-ES, and an increase in PNFP amplitude when D-serine was added to the Phz-ES bathing medium. When Phz-ES exposures were longer than 10 min, we did not see a return of the responses to control values and for that reason, we carried out our chemical determinations using a 10 min exposure time line for Phz-ES.

Phenazine ethosulfate decreases D-serine in the retina

We analyzed the effects of Phz-ES on D-serine and L-serine levels in the salamander retina. As the D-serine tissue levels are low, we pooled 12 retinas for each of two experiments, with one retina from each animal serving in either the control or the Phz-ES bathing solutions. Figure 3c shows the D-serine changes that resulted from two repetitions of this procedure. During a 10 min exposure the D-serine levels decreased by approximately 50%. We also measured L-serine levels (3d) in these experiments, which were not significantly changed.

In summary, findings with whole-cell recordings from retinal ganglion cells, the PNFP and chemical determinations converge to support the idea that Phz-ES decreased tissue levels of D-serine, which, in turn, decreased the light response of ganglion cells without compromising the sensitivity of ganglion cell NMDA receptors to exogenous D-serine. The time course of changes in D-serine and measured changes in NMDA receptor-mediated synaptic currents suggests a fairly tight coupling between synthesis, release and availability of D-serine as a coagonist for NMDA receptors.

Discussion

Although SR has been localized to the retina with immunostaining techniques, this is the first report of a direct relationship between D-serine synthesis and the light-evoked NMDA receptor-mediated responses of the inner retina. Measurable changes in tissue levels of D-serine, generated by brief exposures to Phz-ES, were correlated with a decrease in the light-evoked synaptic activity of ganglion cells that could be attributed to NMDA receptors. There is now compelling evidence that D-serine plays a major role as an NMDA receptor coagonist in the retina [1,2,9], as it does in the brain [10]. The present experiments bear on this interpretation, since they revealed a relatively tight coupling between D-serine synthesis and light-evoked currents generated by NMDA receptors. Indeed, the depressive actions of Phz-ES on NMDA receptor light-evoked currents were comparable to those observed when the light response was attenuated by blocking the NMDA receptor with 5,7-DCK. This is also consistent with other experiments [2] in the salamander, which showed that enzymatically degrading D-serine, using D-serine deaminase or D-amino acid oxidase reduced NMDA receptor currents to an amplitude similar to that observed when NMDA receptors were blocked with selective antagonists. Kalbaugh *et al.* [11] have recently suggested that glycine serves a dynamic role as an NMDA receptor coagonist. In the present experiments,

we cannot eliminate a small contribution of glycine as a coagonist, but it is clear that glycine does not rapidly substitute for D-serine, since the measured decrease in D-serine levels was not compensated for by a return of the NMDA receptor-mediated component of the light response, at least over the time course of our experiments.

Source of D-serine in the retina

We developed an antibody against SR, using a peptide segment unique to the enzyme. This antibody provided a single band of staining in the western blot with a molecular weight of 37 kDa, the same as SR [3,12]. When this antibody was used for immunostaining in the salamander retina, the most obvious labeling was apparent in the Müller cells, the prominent radial glia in the retina. This result confirms earlier studies of the retina using antibodies for D-serine and SR obtained from other sources [1]. In addition to the prominent staining found in Müller cells, it appeared that the inner segments of photoreceptors, particularly those of cones, were labeled, although they appeared more lightly stained than Müller cells. But, like the Müller cells, photoreceptor staining was eliminated with preadsorption of the antibody with the peptide fragment. It is worth noting that D-amino acid oxidase has been reported in amphibian cones [13], so the possibility that D-serine is a functional constituent of photoreceptors requires further study.

While we generally focus on NMDA receptors as an inner retinal glutamate receptor of ganglion and amacrine cells [14-16], evidence for NMDA receptors in horizontal cells of the fish retina has been known for many years [17]. In a recent study in the carp retina Shen *et al.* [18] have shown that when glycine was added to the bathing medium NMDA receptor contributions to horizontal cell responses were observed [18]. The possibility that photoreceptors might use D-serine does not diminish the more obvious presence of SR in the inner retina, where it appears that Müller cells are the major cellular elements containing SR. For that reason, it seems reasonable to conclude that in the inner retina, where NMDA receptor function is prominent, the source of D-serine is likely to come from Müller cells. The immunostaining carried out in this study supports the idea that D-serine subserves one component of a glial-neuronal control pathway.

Conclusion

A rapid reduction in tissue levels of D-serine was observed following the introduction of Phz-ES to inhibit D-serine synthesis by the enzyme SR, using the isolated tiger salamander retina. The decrease in D-serine was correlated with reductions in light-evoked currents mediated by NMDA receptors, characterized through whole-cell recordings of retinal ganglion cells and extracellular recordings of the proximal negative field potential. These observations provide the first direct link between tissue levels of D-serine, D-serine synthesis and the critical role that D-serine plays as a coagonist for NMDA receptors in the inner retina.

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Fig 1.

Serine racemase (SR) distribution in the tiger salamander retina shows a prominent staining pattern in retinal Müller cells. An antibody to SR (green) labeled Müller cells in this cross-section of salamander retina (upper left). SR label can also be appreciated in the photoreceptor layer. A three-dimensional reconstruction of the staining from the SR antibody (red) from a z-series in this same section of retina (upper right). This label is severely curtailed in an adjacent retinal section when the antibody was preadsorbed with peptide (lower left). A western blot of protein extracted from salamander at different concentrations labeled with the SR antibody displayed a single band at approximately 37 kDa, the anticipated weight of the enzyme.

Fig 2.

Blocking serine racemase (SR) decreased *N*-methyl-D-aspartate currents in retinal ganglion cells. (a) A whole-cell voltage-clamp recording ($V_{\text{Hold}} = -67$ mV) from a retinal ganglion cell in an eyecup preparation showed the response to a 2 s light stimulus in control Ringer, which was enhanced by the addition of D-serine (100 μ M), and blocked by 5,7-dichlorokynurenic acid (5,7-DCK, 30 μ M). When the SR antagonist, phenazine ethosulfate (Phz-ES; 10 μ M, thick bar) was added, the response was diminished in amplitude similar to that observed in 5,7-DCK. This reduced response could still be potentiated by the addition of D-serine (DS). Phz (100 μ M) did not alter the response to a significant degree. (b) Summary data from seven cells showing reduced On and Off responses when Phz-ES was added to the bathing medium and an unaltered potentiation by DS and the lack of change in the presence of Phz. (c) Summary data from seven cells showing a small, nonsignificant additional reduction in inward current when 5,7-DCK was added to Phz-ES. (d) Time course of the block by the Phz-ES fit with a Boltzman. Whole cell recording (WCR, ■) and proximal negative field potential (PNFP, ●) measurements were averaged from responses at 20 s intervals (error bars not shown). Transit time of enzyme from reservoir subtracted before plotting. LS: L-serine; L-E PSP: light-evoked post-synaptic potential (* $P < 0.01$ compared with control).

Fig 3.

(a) Extracellular recording of the proximal negative field potential (PNFP). Application of phenazine-ethosulfate (Phz-ES) decreased the amplitude of the PNFP. An example set of traces shows a decrease in PNFP amplitude after bath application of Phz-ES (light gray trace) when compared with the control cocktail response (black trace). The addition of exogenous D-serine (DS) to the Phz-ES bathing media (gray trace) increased the response beyond that of the original control. (b) Cumulative results showed a significant decrease of $26.8 \pm 2.6\%$ in the PNFP in the presence of Phz-ES and a significant increase of $9.1 \pm 1.1\%$ after addition of DS (both compared with control, $n = 6$). (c) Shows the change in measured levels of DS for the intact retina exposed for 10 min to Phz-ES, which produced an approximate 50% decline in DS levels. (d) Shows that L-serine levels measured from the same retinas were not significantly changed.