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Pregnenolone Sulfate Acutely Enhances NO Production in the Rat Hippocampus: Digital Fluorescence Study Using NO Reactive Dye

Norio Takata[1], Keisuke Shibuya[1], Masashi Okabe[2,], Tetsuo Nagano[3], Hirotatsu Kojima[3], and Suguru Kawato[1,*]**

[1]Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo at Komaba, 3–8–1 Komaba, Meguro, Tokyo 153–0041, Japan, [2]Department of Public Health and Environmental Medicine, Jikei University School of Medicine, 3–25–8 Nishishinbashi, Minato, Tokyo 105–8461, Japan, and [3]Graduate School of Pharmaceutical Sciences, University of Tokyo, 7–3–1 Hongo, Bunkyo, Tokyo 113–0033, Japan.

Summary

Both neurosteroids and nitric oxide (NO) are neuroactive modulators. The effect of pregnenolone sulfate (PREGS), a representative neurosteroid, on the production of NO in hippocampal slices was investigated with digital fluorescence microscopy using a newly synthesized NO-reactive fluorescent dye, diaminofluorescein-FM (DAF-FM). Upon stimulation with N-methyl-D-aspartate (NMDA), DAF-FM fluorescence increased to 126%. Preincubation of slices with 100μM PREGS for 20 min increased NMDA-stimulated DAF-FM fluorescence to 148%. Such fluorescence increase was suppressed by both L-NMMA (NO synthase (NOS) inhibitor) and MK-801 (NMDA receptor antagonist). Preincubation with PREGS also enhanced NMDA-induced Ca^{2+} influx in hippocampal slices, as measured with fura-2. The localization of neuronal NOS protein in pyramidal neurons of the CA1 region was demonstrated using immunohistochemistry combined with *in situ* blotting of unfixed slices. Taken together, these results imply that PREGS acutely enhances NO production in the hippocampal CA1 neurons, due to an increase in Ca^{2+} influx through NMDA receptors. Because NMDA-induced acute synthesis of PREGS in hippocampal pyramidal neurons has been demonstrated, a postsynaptic signal amplification circuit including PREGS and NO may function in pyramidal neurons.

Key words

NO, NOS, hippocampus, neurosteroid, pregnenolone sulfate, DAF

Introduction

Nitric oxide (NO) imaging is very important, because NO imaging could provide dynamic aspects of NO production with spatial resolution which cannot be obtained using conventional biochemical or NO-electrode assay.

The hippocampus, which is involved essentially in learning and memory processes, is a target of the neuromodulatory action of steroid hormones (McEwen et al., 1999; Schumacher et al., 1997). In addition to classical genomic effects of peripheral steroids via intracellular steroid receptors, neurosteroids in the brain have been shown to rapidly alter neuronal excitability via a nongenomic pathway by modulating cell surface receptors (Baulieu, 1997; Paul et al., 1992). Electrophysiological studies have shown that pregnenolone sulfate (PREGS), a representative neurosteroid, enhanced the opening probability of NMDA receptors but suppressed that of GABAA receptors in cultured hippocampal neurons (Bowlby, 1993; Irwin et al., 1992; Rupprecht et al., 1999; Wu et al., 1991). Using single-cell Ca^{2+} imaging, PREGS was demonstrated to potentiate Ca^{2+} influx through genetically expressed NMDA receptors in CHO cells, indicating direct binding of PREGS to NMDA receptors (Mukai et al., 2000). The synthesis of neurosteroids in the brain has been suggested, due to the presence of significant amount of

*To whom all correspondence should be addressed.
E-mail: kawato@phys.c.u-tokyo.ac.jp
**Current Address
Environmental Bio-Science Lab., Experimental Research Section, Bldg. No. 12, Hosei University, 4342 Aihara-cho, Machida, Tokyo 194–0298, Japan.

Abbroviations:
DAF-FM; diaminofluorescein-FM, eNOS; endothelial NOS, LTP; long-term potentiation, NC membrane; nitrocellulose membrane, NMDA; N-methyl-D-aspartate, nNOS; neuronal NO synthase, NO; nitric oxide, PREGS; pregnenolone sulfate.

PREG(S) (Baulieu, 1998). We recently demonstrated the localization of a complete set of steroidogenic proteins, including cytochrome P450scc and hydroxysteroid sulfotransferase, that conducts PREGS synthesis in pyramidal neurons and granule cells in the hippocampus (Kawato et al., 1999; Kawato et al., 2001; Kimoto et al., 2001). This neuronal machinery of PREGS synthesis was shown to be driven acutely by an NMDA receptor-mediated Ca^{2+} influx (Kimoto et al., 2001). Local acute synthesis and acute action of PREGS in hippocampal neurons imply that PREGS is a paracrine neuromessenger (Kawato et al., 2001).

Nitric oxide (NO), a free radical gas, acts as an intracellular and intercellular messenger (Hawkins et al., 1998; Yun et al., 1996). NO production by neuronal NO synthase (nNOS) may be regulated by Ca^{2+} influx through NMDA receptors, because nNOS is a Ca^{2+}/calmodulin-dependent enzyme linked to NMDA receptors by an intermediary adapter protein, PSD95, through the PDZ-PDZ interaction (Christopherson et al., 1999). NO is involved in long-term potentiation (LTP) in the hippocampus. The induction of LTP was reduced in hippocampal slices from mice that were doubly knocked-out in nNOS and endothelial NOS (eNOS) (Son et al., 1996). Digital fluorescence imaging using NO-reactive fluorescent dyes is a powerful way to investigate real-time, 2-dimensional NO production (Kojima et al., 1998; Kojima et al., 1999), which is not possible with such conventional procedures as NO electrodes, citrulline assay, or electron spin resonance analysis.

NO production in the hippocampus upon NMDA stimulation was measured previously using a NO-sensitive dye, DAF-2 (Kojima et al., 1998). However, the results were not very reliable, because DAF-2 fluorescence was not always increased but occasionally decreased to 82~93% upon NMDA stimulation when we tried to replicate the result. We concluded that the fluorescence decrease of DAF-2 was due to pH decrease of hippocampal neurons, because intensity of DAF-2 fluorescence decreased when pH fell below pH7.0 (Kojima et al., 1999), and because intracellular pH of hippocampal neurons decreased to nearly pH6.0 from 7.2 upon NMDA stimulation (Yamamoto et al., 1998; Zhan et al., 1998). We therefore used DAF-FM (an improved DAF-2 dye), whose fluorescence is pH-independent between pH5.8 and pH12. This has the advantage of obtaining an accurate assay of NO production in the hippocampus, because intracellular pH is acidified in hippocampal neurons upon NMDA stimulation (Yamamoto et al., 1998; Zhan et al., 1998).

Although PREGS was shown to increase Ca^{2+} influx through NMDA receptors, it is not known whether the augmentation of Ca^{2+} influx is high enough to potentiate NO production (Reddy et al., 1998). In this study, we tried to measure NO production in hippocampal slices using DAF-FM and studied the effect of PREGS on NO production.

Materials and Methods

Chemicals

The diacetate derivative of DAF-FM (DAF-FM/DA) was synthesized as detailed elsewhere (Kojima et al., 1999). Fura-2 AM was purchased from Dojindo (Japan), PREGS and MK-801 from Sigma (USA), NMDA from RBI (USA), and L-NMMA from Wako Pure Chemicals (Japan). All other chemicals were of the highest purity commercially available.

Preparation of hippocampal slices

Male Wistar rats aged 8 weeks and purchased from Saitama Experimental Animal Supply Co. (Japan) were deeply anesthetized with ethyl ether and decapitated. The brains were quickly removed and placed in O_2 equilibrated artificial cerebrospinal fluid (ACSF) at 2-4 °C. The hippocampus was dissected and 400μm transverse slices were prepared with a vibratome (D.S.K. ZERO 1; Dosaka, Japan) at 0 °C. Slices were then incubated in ACSF at room temperature for 1 hr. ACSF consisted of (in mM): 127 NaCl, 1.6 KCl, 1.24 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose and was equilibrated with 95% O_2/5% CO_2.

Slice loading with fluorescence indicators

Hippocampal slices were incubated for 60 min in a plastic tube containing 6 ml of 10μM DAF-FM/DA or fura-2 AM (taken from a stock solution in dimethyl sulfoxide) filled with 95% O_2 and 5% CO_2 at room temperature. After dye loading, slices were postincubated in ACSF for at least 30 min. After incorporation into cells, DAF-FM/DA and fura-2 AM were hydrolyzed to yield DAF-FM and fura-2 by intracellular nonspecific esterase.

Fluorescence imaging and analysis

Prior to fluorescence observation, hippocampal slices were incubated for 20 min in modified ACSF (mACSF) containing 100μM Mg^{2+} instead of 1.3 mM Mg^{2+}. For DAF-FM measurement, 10 mM pyruvate was substituted for glucose in mACSF. Each slice was then transferred to a fluorescence-recording chamber perfused with 95% O_2/5% CO_2 equilibrated mACSF. Perfusion was at 1 ml/min, and perfusates were maintained at 30 °C throughout the experiments. Fluorescence imaging was conducted using fluorescence microscopy consisting of an inverted microscope (Nikon TMD 300, Japan) with an intensified CCD

camera (Hamamatsu Photonics C2400-77, Japan). We used a 20 ×, 0.75 numerical aperture for the fluorescence objective. The microscope had a chamber that maintained air at 30 °C around the sample using a warm air supply. Fluorescence was measured in the CA1 region of hippocampal slices.

To measure NO production, DAF-FM was excited at 480 nm. Fluorescence above 520 nm was measured with an IF excitation filter, a DM510 dichroic mirror, and a BA520 emission filter. To reduce photobleaching of DAF-FM during long-time measurement, fluorescence images were recorded for 1 s each 20 s, using a rotary excitation wheel and the following time sequence: 480 nm exposure for 1 s, closure for 19 s; 480 nm exposure for 1 s; closure for 19 s; etc. The time resolution of measurement was thus 20 s. Images were analyzed using an ARGUS-50 (Hamamatsu Photonics, Japan). Acquired images were stored on a hard disk, using 512 × 482 pixels resolution at an 8-bit depth. Time-dependent fluorescence intensity f(t) was calculated for a square region (100 × 100 pixels, Fig. 1 (B)) by subtracting background fluorescence, which showed a time-dependent gradual decrease due to photobleaching. Normalized fluorescence intensity f(t)/f(0) was used to demonstrate net NO production.

To determine the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), we measured the fluorescence of fura-2 above 520 nm with a DM510 dichroic mirror and a BA520 emission filter. The excitation wavelength was cycled between 340 nm and 380 nm with a rotary excitation wheel using the following time sequence: 340 nm exposure for 1 s, 380 nm exposure for 1 s; closure for 4 s; 340 nm exposure for 1 s; 380 nm exposure for 1 s; etc. The intracellular concentration of Ca^{2+} is expressed as F340/F380, the ratio of fura-2 fluorescence intensity at 340 nm excitation (F340) to that at 380 nm excitation (F380). The time resolution of measurement was 6 s.

In situ blotting and immunostaining for nitrocellulose (NC) membrane-transferred proteins of hippocampal slices without chemical fixation

In situ blotting was conducted as described elsewhere (Okabe et al., 1993). Male Wistar rats, aged 8 weeks, were transcardially perfused with Tris buffer solution (TBS; pH7.4), without perfusion of a fixative solution, following an overdose of pentobarbital anesthesia. Hippocampi were frozen-sliced coronally at 20μm thickness with a cryostat (Leica 2800E, Germany) at −20 °C. Sections were mounted on dry NC membranes in a cryostat box and thawed at room temperature. Membranes were then incubated on filter paper prewetted with TBS. Tissue was removed from the blotting membrane by a high-pressure TBS jet spray; then blots were soaked in a 2% Blocking Reagent (Boehringer Manheim, Germany) for 1 hr. Membranes

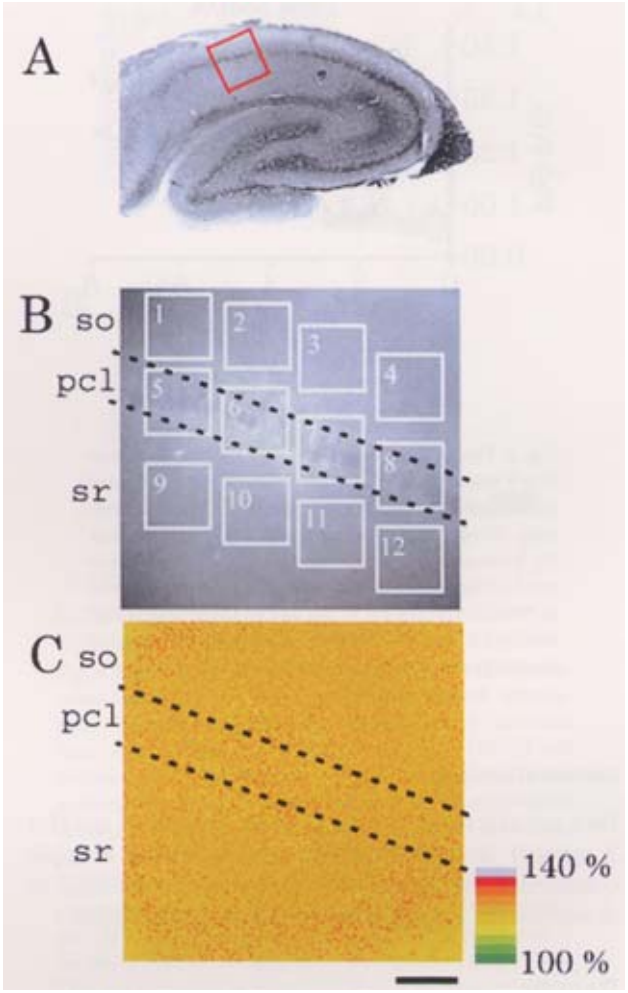

Fig. 1. Representative image of a DAF-FM-loaded hippocampal slice: (A) Immunohistochemical staining with anti-neurofilament IgG after DAF-FM fluorescence imaging (immunohistochemistry was performed essentially as described in Kimoto et al. (2001)). Red square shows region used for fluorescence imaging of (B) and (C). (B) Representative fluorescence image of the CA1 region of a DAF-FM-loaded hippocampal slice before NMDA stimulation. This fluorescence demonstrates an almost uniform distribution of DAF-FM. Squares 1-4, 5-8, and 9-12 are placed at the stratum oriens (so), pyramidal cell layer (pcl; indicated by broken lines), and stratum radiatum (sr). (C) 5 min after NMDA stimulation without PREGS. The DAF-FM fluorescence increase is pseudo-color-coded. Scale bar = 400μm for (A), 100μm for (B) and (C).

were next incubated overnight with primary monoclonal anti-nNOS IgG (1:500) (Sigma, USA). Sections were then washed with TBS and reacted with biotin-tagged antimouse IgG secondary antibody (1:500) for 1 hr, after which membranes were incubated in Immunogold conjugate solution (1:50, British BioCell Int., U.K.) for 1 hr. After membranes were washed, they were incubated in Silver enhancement reagent (Boehringer Manheim, Germany) for 3 hr. To preabsorb the anti-nNOS antibody with antigens, we used 32μg/ml of purified nNOS (generously provided by Dr. S. Kominami).

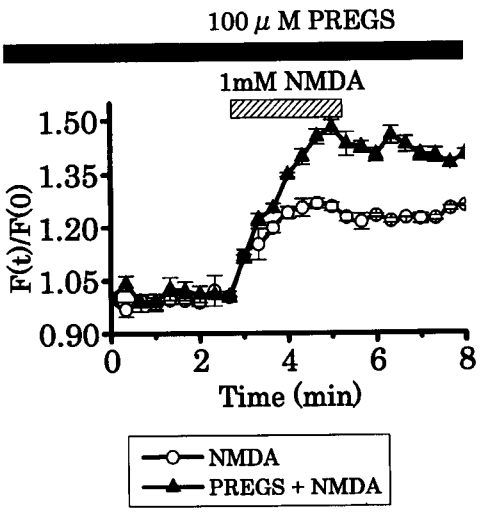

Fig. 2. Time courses of an average DAF-FM fluorescence intensity over squares 1-12, F(t)/F(0), induced upon application of 1 mM NMDA in PREGS presence (closed triangles) and absence (open circles). To measure the effect of PREGS, hippocampal slices were perfused with 100μM PREGS from 20 min prior to NMDA stimulation to the end of the measurement, indicated by the closed bar above the graph. The hatched bar designates the period of 1 mM NMDA perfusion.

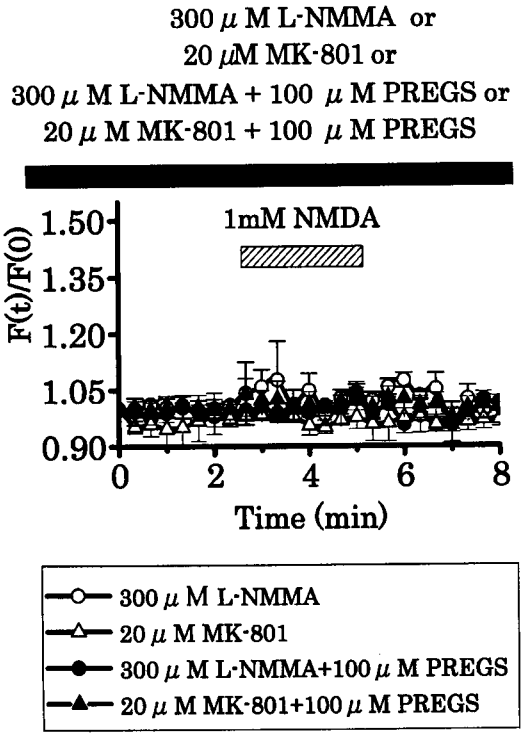

Fig. 3. Effect of inhibitors on average DAF-FM fluorescence intensity over squares 1-12, F(t)/F(0), upon NMDA stimulation in hippocampal slices. The time courses of DAF-FM fluorescence induced upon NMDA stimulation, in the presence of: 300μM L-NMMA (open circles), 20μM MK-801 (open triangles), 300μM L-NMMA + 100μM PREGS (closed circles), and 20μM MK-801 + 100μM PREGS (closed triangles). In each case, hippocampal slices were perfused with mACSF containing inhibitors from 20 min prior to NMDA stimulation to the end of measurement, indicated by the closed bar above the graph. The hatched bar designates the period of 1 mM NMDA perfusion.

Statistical analysis.

Data are expressed as mean ± SEM. Student's t test (for 2 groups) and ANOVA followed by Tukey multiple comparison test (for 3 or more groups) were used to determine the statistical significance of differences.

Results

Measurement of NO production in hippocampal slices with DAF-FM

The fluorescence intensity of DAF-FM-loaded hippocampal slices was measured to determine NO production. Fluorescence intensity in the CA1 region (Fig. 1 (A)) was examined using a set of square regions, numbered 1 to 12 (Fig. 1 (B)). In comparison with a bright field image, squares 1-4, 5-8, 9-12 were placed at the stratum oriens, pyramidal cell layer, and stratum radiatum, respectively. Upon NMDA stimulation, no significant difference was observed among squares 1-12 during the time course of the fluorescence intensity, f(t)/f(0) (see Fig. 1 (C)). We then used the average fluorescence intensity over squares 1-12, F(t)/F(0), to improve the signal-to-noise ratio of measurement. Upon 1 mM NMDA application, F(t)/F(0) increased to 126 ± 3% (4 slices) during the first 1.3 min, then remained unchanged within the observation time (Fig. 2). Time course F(t)/F(0) demonstrates that NO production continued for the first 1.3 min and no further NO production occurred thereafter, i.e., DAF-FM fluorescence remains constant when there is no NO production. No significant difference occurred in the fluorescence increase between stratum oriens (average of squares 1-4), the pyramidal cell layer (average of squares 5-8), and the stratum radiatum (average of squares 9-12) (Fig. 1 (C)). When the NMDA concentration was reduced to 100μM, NMDA-induced increase of F(t)/F(0) was also reduced to 112 ± 4% (3 slices). When hippocampal slices were incubated in mACSF containing 300μM L-NMMA (a competitive NOS inhibitor) or 20μM MK-801 (a noncompetitive NMDA receptor antagonist) for 20 min prior to NMDA stimulation, the increase of average fluorescence intensity over squares 1-12, F(t)/F(0), upon 1 mM NMDA stimulation was markedly suppressed (4 slices each; Fig. 3).

Effect of PREGS on NO production in the hippocampus

To study the effect of PREGS on NO production, DAF-FM-loaded hippocampal slices were incubated with 1～100μM PREGS for 20 min prior to NMDA stimulation. The production of NO showed a dose-dependent increase on the concentration of PREG (1～100μM) (Fig. 4 (A)). The PREGS dosage yielding the half maximum (EC_{50}) was estimated to be 19.5μM by calculation using a logistic equation. Treatment with 100μM PREGS, which should be the saturation concentration for modulating NO production, enhanced the normalized DAF-FM fluorescence increase in the CA1 region upon 1 mM NMDA stimulation to 148 ± 5% (4 slices), nearly twice the net fluorescence increase obtained without PREGS (Fig. 2). Fluorescence intensity increased during the first 2 min, then remained unchanged. Fluorescence intensity increases observed in the stratum oriens, pyramidal cell layer, and stratum radiatum were essentially identical. Treatment with L-NMMA or MK-801 suppressed fluorescence increases accompanying NMDA stimulation in the presence of PREGS (Fig. 3). Such increases, observed under different conditions of PREGS and inhibitors, are summarized in Fig. 4 (B).

Effect of PREGS on Ca^{2+} elevation in hippocampal neurons induced by NMDA

In the absence of PREGS, the CA1 region showed a transient rapid increase in F340/F380 of fura-2 during the first 0.5 min upon stimulation with 1 mM NMDA. F340/F380 reached a maximum of 0.83 ± 0.01 (6 slices; Fig. 5), then decreased to a plateau of 0.63 ± 0.03. Preincubation with 100μM of PREGS for 20 min augmented the transient rapid increase of F340/F380 induced by NMDA stimulation. In the presence of PREGS, F340/F380 attained a maximum of 1.02 ± 0.01 (5 slices) within 0.25 min - roughly 1.2 times that obtained in the absence of PREGS (Fig. 5). After peaking, F340/F380 then gradually decreased to the plateau of 0.64 ± 0.03.

Immunohistochemical detection of nNOS in hippocampus

To examine the presence of nNOS in the hippocampal CA1 region, we conducted immunohistochemical staining, combined with *in situ* blotting of proteins from hippocampal slices onto nitrocellulose membranes without using fixative reagents. We observed nNOS immunoreactivity in pyramidal neurons of the CA1 region (Fig. 6 (B)). Preabsorption of the antibody with an excess amount of purified nNOS antigen (32μg/ml) resulted in a complete absence of nNOS immunoreactivity in the hippocampus (Fig. 6 (C)). Nonimmunized serum did not cause any positive staining of the hippocampus.

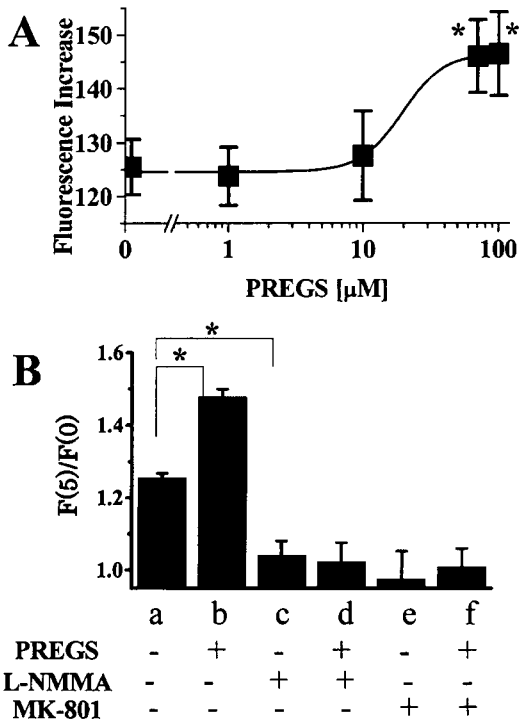

Fig. 4. Panel A: Dose dependency on PREGS of fluorescence increase of DAF-FM upon 1 mM NMDA stimulation. The solid line indicates a sigmoidal curve calculated from logistic equations. Panel B: Effect of PREGS and inhibitors on the fluorescence increases upon 1 mM NMDA application. (a) NMDA application only; (b) in the presence of 100μM PREGS; (c) in the presence of 300μM L-NMMA; (d) in the presence of 100μM PREGS plus 300μM L-NMMA; (e) in the presence of 20μM MK-801; (f) in the presence of 100μM PREGS plus 20μM MK-801. Comparison of DAF-FM fluorescence F(5)/F(0) was performed at the end of NMDA perfusion (t = 5 min) in hippocampal slices. Data are expressed as mean ± SEM of 4 independent determinations. $^*P < 0.01$, compared to NMDA-only stimulation.

Discussion

PREGS enhanced NO production in the CA1 region upon NMDA stimulation. Because NO production was suppressed by MK-801, this NO production was triggered by the Ca^{2+} influx through NMDA receptors. Many studies have dealt with the modulating effect of PREGS on cellular and receptor Ca^{2+} signaling. PREGS has been shown to enhance the Ca^{2+} influx through NMDA receptors, but to suppress the Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mukai et al., 2000; Rupprecht et al., 1999). Enhancement of the increase observed in intracellular Ca^{2+} concentration cannot be due to PREGS modulation in voltage-dependent Ca^{2+} channels.

Because of its linkage to NMDA receptors, nNOS should be very sensitive to NMDA-receptor-mediated

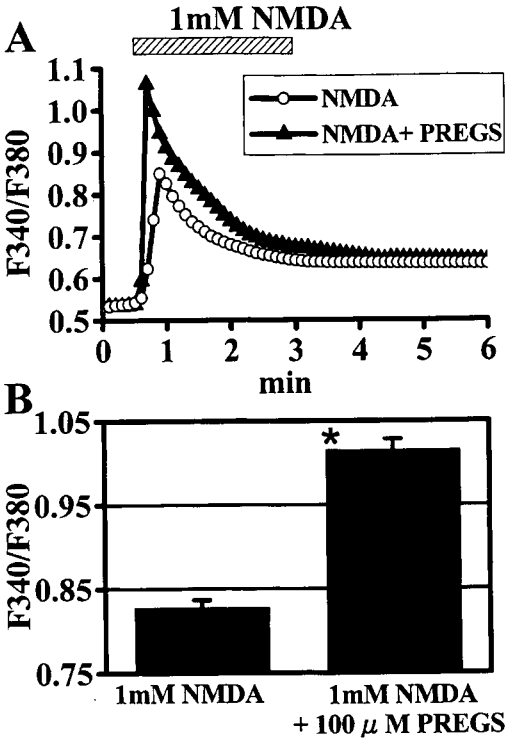

Fig. 5. Effect of PREGS on NMDA-induced $[Ca^{2+}]_i$ elevation in the hippocampal CA1 region: (A) Time course of F340/F380 of fura-2 in the CA1 region of hippocampal slices in 100μM PREGS absence (open circles) or presence (closed triangles). To measure the PREGS effect, hippocampal slices were incubated with 100μM PREGS for 20 min prior to NMDA stimulation, and during all measurement. The hatched bar designates the period of 1 mM NMDA perfusion. The horizontal axis is time in min. (B) Comparison of peak magnitudes of F340/F380 elevation induced upon 1 mM NMDA in 100μM PREGS absence and presence in the CA1 region of hippocampal slices. Each column represents mean ± SEM of 5 or 6 independent determinations. *$P < 0.01$ compared to NMDA-only stimulation.

Ca^{2+} influx. The time course of $[Ca^{2+}]_i$ showed an increase during either the first 0.5 min (NMDA-only) or 0.25 min (NMDA + PREGS) just after NMDA application. This increase was followed by a decrease toward the plateau (Fig. 5). The initial rate of increase of $[Ca^{2+}]_i$ was greater for NMDA + PREGS stimulation than for NMDA-only stimulation. The initial increase in F(t)/F(0) of DAF-FM occurred just after NMDA application, and followed almost the same time course for both NMDA + PREGS and NMDA-only stimulation. F(t)/F(0) plateaued, however, after 1.3 min for NMDA-only stimulation and after 2 min for NMDA + PREGS stimulation (Fig. 2). The longer time duration of NO production observed for NMDA + PREGS stimulation, related to NMDA-only stimulation, may correlate with the longer time of high $[Ca^{2+}]_i$ (e.g. F340/F380 above 0.75), also observed for NMDA + PREGS stimulation, related to NMDA-only stimulation.

The duration of NO production should depend on high local $[Ca^{2+}]_i$ around nNOS, assuming that EC_{50} of the activity of nNOS on Ca^{2+} concentration was 1μM (Lee et al., 1998). Because F340/F380 represents average intracellular $[Ca^{2+}]_i$ rather than local $[Ca^{2+}]_i$ around nNOS, a direct comparison of the time duration of NO production and local $[Ca^{2+}]_i$ around nNOS could not be made.

In addition to nNOS, eNOS may exist in the hippocampus. The relative contribution of nNOS and eNOS to NO production was not clarified in our study. Ca^{2+}-dependent NOS activity in homogenates of hippocampal tissue from nNOS knock-out mice and measured by citrulline production was only 1.7% of that from wild mice (Son et al., 1996). This implies that the contribution of eNOS is very small, compared to total NO production in the hippocampus, and indicates that observed NMDA-induced NO production is mainly due to nNOS activity.

The distribution of nNOS was observed along the pyramidal cell layer in the CA1 region with *in situ* immunoblotting (Fig. 6). Although nNOS reactivity was restricted to cell bodies of pyramidal neurons and little immunoreactivity was observed in dendrites in Fig. 6, the presence of nNOS in dendrites has been reported in Wendland et al. (1994). Immunostaining of nNOS in dendrites in Fig. 6 was probably too weak to be observed because the amount of nNOS in dendrites would be much smaller than that in cell bodies, due to small volume of intracellular contents of dendrites.

NO production in the hippocampus had been measured previously using a NO-sensitive dye, DAF-2 (Kojima et al., 1998). Resulting fluorescence increased to 127~143% upon 1 mM NMDA stimulation of the CA1 pyramidal cell layer. This increase was, however, often not reproducible; in fact, DAF-2 fluorescence was occasionally observed to decrease to 82~93% upon NMDA stimulation. This fluorescence decrease may be because: (i) DAF-2 fluorescence intensity is pH-dependent for pH < 7.0, but pH-independent for pH > 7.0. When pH decreased from 7.0 to 6.0, DAF-2 fluorescence intensity decreased to about 60% (Kojima et al., 1998). (ii) Neuronal pH of hippocampal slices decreased from pH6.9 to 6.7 upon 1 mM NMDA stimulation (Zhan et al., 1998) and pH of cultured hippocampal neurons decreased from pH7.1 to 6.1, upon 1 mM glutamate stimulation (Yamamoto et al., 1998). To avoid the influence of pH on fluorescence intensity, we used DAF-FM instead of DAF-2. DAF-FM fluorescence is pH-independent for pH > 5.8 (Kojima et al., 1999). In DAF-FM experiments, we substituted pyruvate for glucose in mACSF in order to completely avoid the influence of pH drop on DAF-FM fluorescence. This substitution significantly attenuates the pH decrease in hippocampal neurons accompanying NMDA

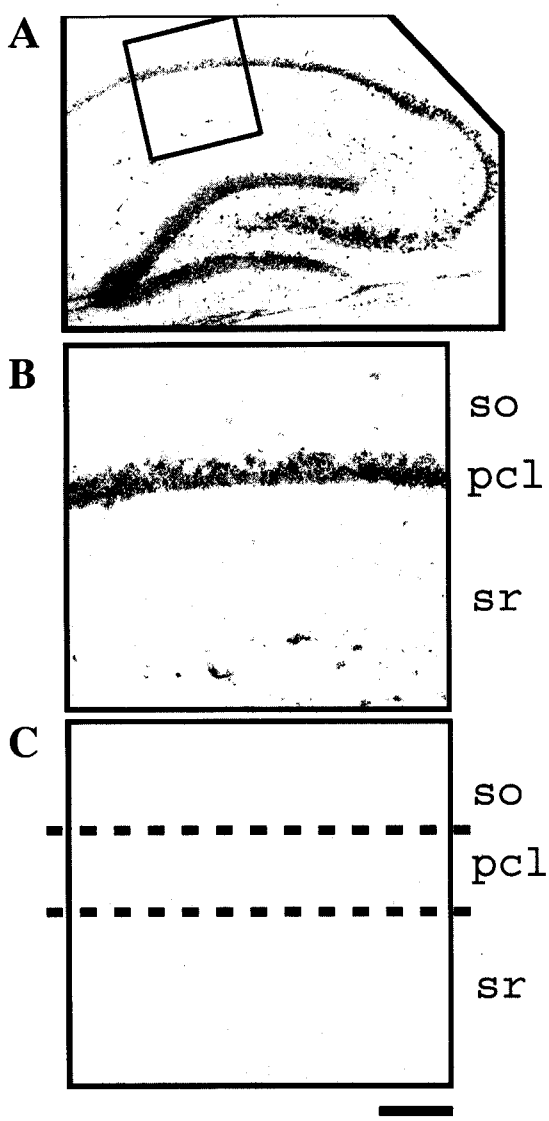

Fig. 6. Immunohistochemistry of nNOS in hippocampal slices from an adult male rat: (A) Immunohistochemical staining of *in situ* blotted proteins on the nitrocellulose membrane using anti-nNOS IgG. Immunoreactivity was found in all pyramidal neurons of the CA1-3 region and in granule cells of dentate gyrus. (B) Higher magnification image of square region indicated in (A). Immunoreactivity was found in all pyramidal neurons of the CA1 region. (C) Staining with anti-nNOS IgG preincubated with a saturation concentration of purified nNOS in the CA1 region. Pyramidal cell layer is indicated by broken lines. Scale bar = 600μm for (A), 250μm for (B) and (C). so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum.

stimulation. Zhan et al. (1998) reported that, upon 20μM NMDA stimulation, hippocampal pH decreased by about 0.15 units in normal ACSF, while pH decreased by only 0.04 units in pyruvate-substituted ACSF. Note that DAF-FM fluorescence also sometimes decreased upon 1 mM NMDA stimulation when we used conventional ACSF. When pyruvate-substituted mACSF was used, however, no fluorescence decrease was observed upon NMDA stimulation, even in the presence of L-NMMA. It is known that pyruvate can be substituted for glucose as an energy source (Izumi et al., 1997; Schurr et al., 1988). Due to these considerations, we observed the reproducible production of NO in the hippocampus using DAF-FM. In other cells such as bovine aortic endothelial cells where no pH decrease was observed, even DAF-2 was successfully used to measure NO production without these improvements (Kojima et al., 1999).

This study demonstrates the possibility of the additive effect of neurosteroids and NO on neuronal excitability in the hippocampus, by overcoming the disturbance of pH decrease upon NMDA receptor-mediated Ca^{2+} influx.

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