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NO and NOS inhibitor affect the generation of retinal spreading depression

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Abstract The effects of nitric oxide (NO) and its synthase (NOS) inhibitor on the generation of spontaneous spreading depression (SD) waves in the frog retina were studied by recording spreading depression potentials (SDPs) as an index. NO-releasing reagents such as sodium nitroprusside and hydroxylamine as well as the substrate of NOS, L-arginine, increased the frequency of the occurrence of spontaneous SDPs. On the other hand, typical NOS inhibitors, Nω-nitro-L-arginine and Nω-nitro-L-arginine methyl ester decreased the frequency. These results imply the participation of NO and its releasing system in the generation of retinal SD. A possible model of SD-generation has been discussed from the viewpoint of potassium accumulation.

Key words: retinal spreading depression; nitric oxide; field potential; extracellular K^+; frog

Introduction

Spreading depression (SD) is now a well-known phenomenon that occurs in the brain cortex (Leão, 1944), and also in the retina (Gouras, 1958). The occurrence of retinal SD can be recognized by a change of light scattering in the retina (Martins-Ferreira and Oliveira Castro, 1966) and of the concomitant extracellular potential (spreading depression potential: SDP) (Mori *et al.*, 1976a). Before the occurrence of retinal SD, some nerve cells are depolarized by an abnormal synaptic activity and release a large amount of glutamate in the inner plexiform layer (IPL)(Van Harreveld and Fifkova, 1970), which further enhances the cell depolarization and results in an accumulation of extracellular K^+ (Mori *et al.*, 1976b). SDP may be generated by the current of increased K^+ which is taken away by Müller cells working as a K^+ siphoning machine (Karwoski *et al.*, 1989; Newman *et al.*, 1984).

SD can be easily evoked by immersing a retina in a conditioning solution which has an increased K^+ or decreased Cl^- concentration. Once the extracellular environment reaches an equilibrium state in such a conditioning solution, SDs tend to appear spontaneously with fairly constant intervals. Therefore measurement of the mean interval serves as a good index of feasibility of SD-occurrence (Fujimoto and Yanase, 1991).

It has been reported, that glutamate in the brain enhances calcium influx into post synaptic neuron from NMDA channels and the calcium/calmodulin complex activates nitric oxide synthase (NOS). Nitric oxide (NO) then diffuses out to act on neighboring cellular elements and stimulates the activity of guanylate cyclase, leading to the generation of cyclic GMP that mediates various kinds of cellular activity (Garthwaite, 1991). It is likely that the same situation might occur in the generation of retinal SD, since there is an accumulation of both K^+ and glutamate in the IPL at SD occurrence. Recently, Ulmer *et al.* (1995) has reported that NO in chicken retina decreases the conduction velocity of retinal SD waves and speeds up their recovery.

The intention of the present study is to examine whether NO and NOS-related reagents affect the intervals of the occurrence of spontaneous retinal SD,

that is, the participation of the NO system in SD generation.

Materials and Methods

Bullfrogs (*Rana catesbeiana*), 150-200 g, were purchased from a local dealer and 20 frogs in total were used. After dark adaptation of more than 1 hr, a frog was rapidly double-pithed and its eye was enucleated. The retina was detached from the pigment epithelium with its receptor side up onto a small piece of filter paper and was set in an experimental chamber. The retina on the filter paper was in contact at the vitreous surface with Ringer solution (about 1.5 ml in volume) through a hole in the chamber (see Fujimoto and Tomita, 1981). The normal Ringer solution in this experiment was a modified Conway's solution (Hanawa *et al.*, 1967) and contained (in mM) NaCl (72.0), KCl (2.5), $MgSO_4$ (1.2), Na_2SO_4 (0.6), $NaHCO_3$ (25.0), Na_2HPO_4 (2.3), NaH_2PO_4 (0.7), Calcium gluconate (0.9), glucose (26.0). The low chloride SD-conditioning solution was made by replacing 95 % of the NaCl and KCl in normal Ringer's with sulfates and an appropriate amount of sucrose was added to maintain isotonicity. The high pH resulted from carbohydrate buffer also made it easy to induce spontaneous SDs.

Reagents used were L-arginine and sodium nitroprusside (Wako, Japan), hydroxylamine (Kanto, Japan), and Nω-nitro-L-arginine and Nω-nitro-L-arginine methyl ester (SIGMA, US). Each reagent was simply dissolved in the SD-conditioning solution.

For recording SDP, a micropipette electrode with a tip diameter of 5-10 μm and filled with the SD-conditioning solution was touched onto the receptor surface. The solution in the experimental chamber was connected by an agar bridge to an Ag/AgCl plate which served as a reference electrode. The transretinal potentials were displayed on an oscilloscope *via* a DC-preamplifier and recorded on a pen recorder. The solutions were changed at the intermission of each continuous recording period of 30 or 40 min. The retina was continuously aerated with humidified oxygen gas and the recordings of the SDPs were made at room temperature 20-25° C.

Results

Generally, within 2 to 3 hrs after the start of conditioning, spontaneous SDs began to occur periodically at almost fixed intervals. The mean was 3-20 min depending on the preparation and chloride content in the conditioning medium. Therefore no attempt was made for statistical analysis because there was little variation in the mean interval for a given preparation.

Figure 1A shows a sample recording on the effect of Nω-nitro-L-arginine (L-NNA), one of the com-

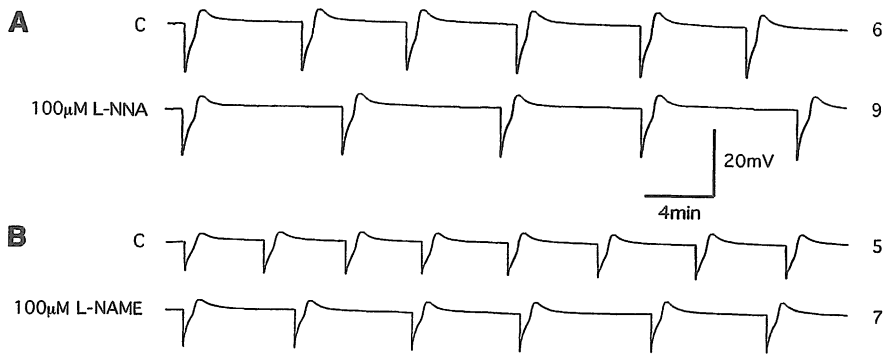

Fig. 1. Typical example of the effect of L-NNA(A) and L-NAME(B) on the occurrence of retinal SDP in the frog. The upper trace in each set shows the control response recorded before the application of the reagent and the lower trace shows the response recorded during the application. The numbers at the right end show mean intervals (min) of SD appearance.

pounds that inhibits NO synthesis. The upper trace is a control response recorded before the application of L-NNA and the lower one is that recorded during the application. The numbers at right show the mean interval (min). In this case, the application of 100 μM L-NNA suppressed SD generation and the mean interval increased from 6 to 9 min showing the decrease in frequency.

To confirm the effect of NOS inhibitor, another NOS inhibitor, Nω -nitro-L-arginine methyl ester (L-NAME), was applied. In the case shown in Fig. 1B, 100 μM L-NAME reduced the frequency of SDP generation. The mean intervals increased from 5 to 7 min.

Sodium nitroprusside (SNP) and hydroxylamine (HA) are known as the reagents which directly liberate NO. To study the effect of these reagents on SD generation, preparations whose frequency of SD generation was rather low, were used, since an increase of the frequency had been expected from the previous experiments. The mean interval in the control in Fig. 2A was 23 min and only one SDP appeared in Fig. 2B. Both substances (10 μM) exhibited a strong positive tendency to promote SD generation.

Finally, the combination experiment was done. Figure 3 shows the effect of 1 mM L-arginine (L-A), the starting material in the cascade of NO-synthes, and 100 μM L-NNA on the occurrence of SDP. The mean interval of control was 9 min in this case. The application of L-A drastically decreased the mean interval to 3 min. L-NNA clearly increased the interval to 11 min, then return to L-A promoted the SD-generation again. Finally in the control solution, the mean interval was almost restored to 9 min. It seems likely that L-A was incorporated into the cascade to generate NO by the action of NOS and then NO affected the occurrence of spontaneous SDs.

The same tendency in the change of the interval was observed in half the preparations. Others showed no appreciable change by the application of the reagents. Some unknown factor other than NO might also be involved in the generation of spontaneous SDs.

Discussion

The present study shows that NO and NOS-related substances can affect the occurrence of spontaneous SDPs. Over the past years, a considerable number of studies have been made on the presence of NO-related systems in retina. NOS has been identified as an enzyme capable of producing a NADPH diaphorase (NADPH-d) reaction (Hope *et al.*, 1991). Therefore it has been suggested that the histochemical demonstration of NADPH-d reflects the presence of NOS. It has been revealed that some kinds of horizontal (Weiler and Kewitz, 1993), amacrine (Darius *et al.*, 1995; Huxlin 1995; Koistinaho *et al.*, 1993; Perez *et al.*, 1995), ganglion (Huxlin, 1995; Weiler and Kewitz, 1993) and Müller cells (Huxlin, 1995; Liepe *et al.*, 1994) are NADPH-d positive. These findings provide strong histological and chemical platforms for supporting the present results since

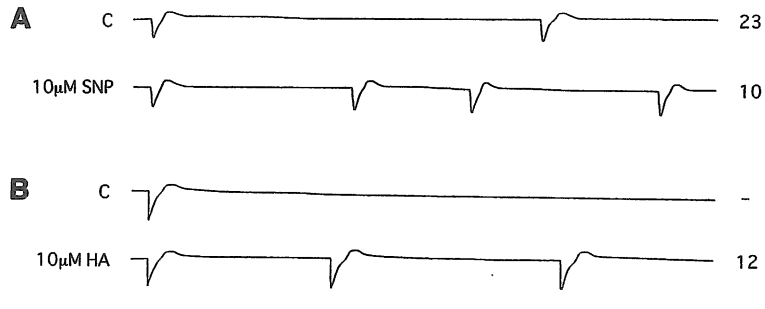

Fig. 2. Examples showing the effect of SNP (A) and HA (B) on the occurrence of retinal SDP. Explanation and calibration are the same as those in Fig. 1.

they showed that one of the main NADPH-d positive sites is the IPL where SD is supposed to originate (Martins-Ferreira and Oliveira Castro, 1966; Mori *et al.*, 1976b).

Taking the current knowledge into consideration, a possible model of the sequence for generation of SDP could be assumed as below. Glutamate released to NOS-containing amacrine cells depolarizes them through the activation of AMPA/Kainate receptors. This depolarization causes a concomitant increase of extracellular K^+. When the postsynaptic membrane is sufficiently depolarized, the magnesium block of the NMDA channel is reduced and calcium enters into the cells. Then the calcium / calmodulin complex activates NOS. The generated NO diffuses out to activate soluble guanylate cyclase in the surrounding cells, leading to the production of c-GMP that modifies the ion channels in the membrane of these cells to depolarize. Therefore, this cascade amplifies the increase of extracellular K^+.

In the normal condition, the increase of K^+ grows only to the extent that the Müller cells give rise to b-wave. In the low Cl^--condition, however, this cascade, beginning with very small fluctuations of the membrane potential, makes up a positive feedback since low-Cl^--condition tends to deprive the retina of inhibitory synaptic processes (Toyoda *et al.*, 1987), that is, the retina is under exclusive conditions for depolarization. Further, accumulated glutamate accelerates the excitability because its uptake by Müllercells might be reversed under high K^+-condition (Szatkowski *et al.*, 1990). This accumulation of excessive K^+ (Grafstein, 1956) and the siphoning process by Müller cells proposed by Newman (1984) might cause periodical occurrence of SDP.

The effects of L-A and L-NNA on ERG waves were also tested (not shown). No modification of the ERG waves was observed at the application of these reagents of the same concentrations as those applied to the SD-experiments. Therefore, NO-related substances seem to affect SD generation simply *via* an activity of neural retina.

Ulmer *et al.* (1995) have reported that the velocity of retinal SD waves decreased at the application of SNP with concentration- and time-dependent manner. It attained 0 velocity at a higher concentration, which resulted in blocking of SD generation. In the present study, the concentration of the reagents was also very critical. In some cases, high doses of SNP tended to block SD generation while low doses exerted no influence. They also pointed out that the recovery of SD waves was speeded up by NO released from SNP. This means that a cycle of the generation of SD accelerates and this acceleration might be reflected in the reduction of the SD interval as shown in the present study.

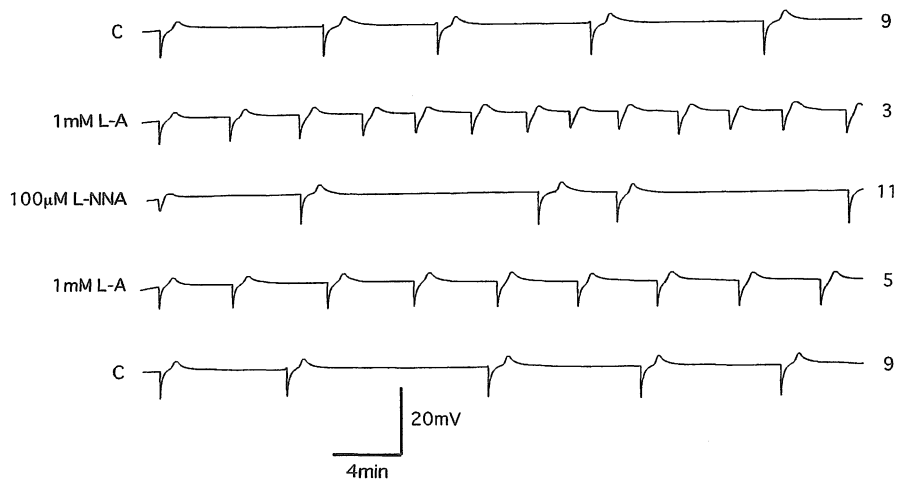

Fig. 3. Enhancement of the appearance of retinal SDPs by L-A and its suppression by L-NNA. Explanation and calibration are the same as those in Fig. 1.

In the theoretical model reported by Wood and Garthwaite (1994), the physiological sphere of influence of a single point source of NO that emits for 1-10 s has a diameter of about 200 μm corresponding to a volume of brain enclosing 2 million synapses. It is very intriguing that an episode of SD, which involves and reflects the activities of a large number of retinal cells, is affected by NO which easily expands into intercellular space and activates its second messenger system.

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