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NMDA receptors and nitric oxide regulate prostaglandin D_2 synthesis in the rabbit hippocampus *in vivo*

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Abstract. The aim of this *in vivo* microdialysis study was to characterise the regulation of prostaglandin D_2 (PgD_2) synthesis by NMDA receptors in the rabbit hippocampus in relation to changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) and nitric oxide (NO) levels. Samples of dialysate were analysed for changes in PgD_2 concentration, in $[Ca^{2+}]_e$ and in the level of NO. The results demonstrated that a 20-min pulse application of 0.1 - 2.5 mM NMDA *via* a microdialysis probe induced a prolonged stimulation of PgD_2 release that was sensitive to competitive NMDA receptor antagonists. An inhibitor of voltage-sensitive Na^+ channels, tetrodotoxin, did not influence this effect but significantly suppressed basal PgD_2 production, whereas a NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), prevented NMDA-evoked NO release and inhibited NMDA-induced PgD_2 release in an L-arginine-sensitive manner. NO donors, *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside, stimulated PgD_2 release. NMDA-evoked decrease in $[Ca^{2+}]_e$ was insensitive to TTX and L-NAME. These results demonstrate an *in vivo* NMDA receptor-mediated modulation of PgD_2 synthesis in the brain, in which NO participates.

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INTRODUCTION

Prostaglandin D_2 (PgD_2) is the main prostanoid in the mammalian brain (Shimazu and Wolfe 1990). It is the product of prostaglandin D synthase (Hoffmann et al. 1993, Urade et al. 1993). Its precursor, prostaglandin H_2, is produced by the arachidonic acid oxidation cascade through a pathway mediated by cyclooxygenase (COX) and peroxidase (Maccarrone et al. 1997). PgD_2 plays the role of a putative neuromodulator with strong somnogenic activity, mediated *via* specific PgD_2 receptors (Matsumura et al. 1994, Urade and Hayaishi 1999, Pinzar et al. 2000). Although PgD_2 biosynthesis in the brain has been demonstrated in numerous studies, regulation of this process is unclear. This particularly concerns the role of NMDA receptors and relation between the release of this prostanoid and NMDA receptor-mediated NO production.

It was demonstrated more than decade ago that the stimulation of NMDA receptors in cultured brain neurones results in Ca^{2+} - mediated release of arachidonic acid and its metabolite prostanoids (Łazarewicz et al. 1988, 1990, Dumuis et al. 1988, Shimazu and Wolfe 1990). More recently, the same effect has been demonstrated in the rabbit hippocampus *in vivo* (Łazarewicz and Salińska 1995). Apart from the known role of prostanoids in the regulation of vasoconstriction and platelet aggregation, these substances may play a key role in signal transduction, including NMDA receptor mediated regulation of gene expression (Lerea et al. 1995, 1997), a basic phenomenon for neuronal plasticity. Stimulation of NMDA receptors also leads to the release of another neuro- and vasoactive substance, nitric oxide (NO), synthesised by nitric oxide synthase (NOS) (Garthwaite et al. 1988, Wood et al. 1990, Yang and Chang 1998). A number of studies clearly demonstrated the role of NO in post-traslational regulation of COX activity in the control or pathological conditions including brain ischaemia (Salvemini et al. 1993, Landino et al. 1996, Maccarrone et al. 1997, Nogawa et al. 1998). In spite of these observations, *in vitro* experiments demonstrated that, in cultured rat dentate gyrus neurones, activation of NMDA receptors triggers the synthesis of various prostanoids but not PgD_2 (Lerea et al. 1997). It was unclear if this distinct regulation of PgD_2 biosynthesis could also be demonstrated in brain *in vivo*.

Microdialysis of selected brain regions proved useful in the *in vivo* study on release of neurotransmitter and other neuroactive substances (for review, see Benveniste 1989, Ungerstedt 1991). It has been utilised in our laboratory in studies of Ca^{2+} fluxes (Łazarewicz and Salińska 1993). Different prostanoids, products of arachidonate metabolism *via* the cyclooxygenase pathway, may be followed in the brain after ischaemia or injury by microdialysis combined with HPLC or radioimmunoassay (Yergey and Hayes 1990, Patel et al. 1992, Łazarewicz and Salińska 1995). The method of microdialysis combined with the determination of nitrite and nitrate, stable products of NO oxidation, or with detection of methemoglobine in oxyhemoglobine solution may be utilised for *in vivo* determination of NO levels in brain *in vivo* (Balcioglu and Maher 1993).

The aim of this work, utilising *in vivo* brain microdialysis, was to characterise in the rabbit hippocampus the NMDA receptor-dependence of NMDA--evoked release of PgD_2, in relation to changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) and NO levels.

METHODS

Albino rabbits (n = 84), weighing 3.5-4.0 kg were used in this study. Experimental groups consisted of 4-5 animals. The local Ethical Committee of the Medical Research Centre in Warsaw approved these experiments.

The house-made transhippocampal microdialysis probes were made of 10 mm long dialysis tubing (Cuprofan, Gambro, m.w. cut-off 10,000) glued between polyethylene tubes. Under pentobarbital anaesthesia, the probes were implanted stereotaxically into the dorsal part of the hippocampus, 4 mm behind and 18 mm lateral to the bregma, according to the method of Hamberger et al. (1982). During dialysis experiments performed 24 h after implantation, the probes were perfused at a rate of 2.5 µl/min with Krebs-Ringer bicarbonate (KRB) medium containing 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 25 mM NaHCO3, pH 7.4, with 1.2 mM $CaCl_2$. After at least 2 h equilibration, NMDA and other pharmacologically-active substances were added and samples of dialysate were collected for subsequent analysis.

For the content of PgD_2, the samples of dialysate, without extraction of prostanoids, were diluted in the assay buffer and analysed using a commercial RIA kit (Amersham). Radioactivity was counted in duplicate samples in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland) with 10 ml of Bray's scintillation mixture. According to the manufacturer, the PgD_2 antiserum used is highly specific for this prostanoid.

Therefore, the content of immunoreactive materials will be expressed as concentration of PgD_2 for simplification.

Changes in extracellular calcium concentrations ($[Ca^{2+}]_e$) were determined by measurements of $^{45}Ca^{2+}$ efflux from the pre-labelled hippocampus, according to a previously described method (Łazarewicz et al. 1986). The microdialysis probes were dialysed for 1 h against medium containing 25 μCi of $^{45}CaCl_2$, followed by 2.5 h equilibration - perfusion with non-radioactive KRB medium. The samples were collected every 5 min. After control steady-state efflux, the medium containing NMDA was introduced for 20 min, followed by a control KRB medium. $^{45}Ca^{2+}$ radioactivity was measured by liquid scintillation counting. The control values for each time point were extrapolated by a semilog regression analysis from the radioactivity of samples collected during microdialysis with control media and the results were expressed as a percentage of this control. In the rabbit hippocampus changes in the efflux of $^{45}Ca^{2+}$ exactly follow changes in Ca^{2+} concentration in dialysate, and thus reflect changes in $[Ca^{2+}]_e$ (Łazarewicz et al. 1986).

Determination of NO release in the dialysate was based on oxidation of oxyhemoglobin applied in the dialysis medium to methemoglobin by NO released in the hippocampus *in vivo* (Balcioglu and Maher 1993). The absorption of samples of dialysate at 401 μm (maximal absorbance of Met-Hb) and at 411 μm (isobestic point specific for the OxyHb/MetHb two component system) was measured with a Beckman DU-65 spectrophotometer. The calibration curve was constructed *in vitro*, using sodium nitroprusside standard.

The results are presented as mean values ± SD. Differences between means were tested for statistical significance by analysis of variance (ANOVA), followed by Dunnett's two-tailed test.

RESULTS

NMDA-induced prostaglandin D_2 release

A control, steady-state concentration of PgD_2 in the dialysate of the rabbit hippocampus was found at the

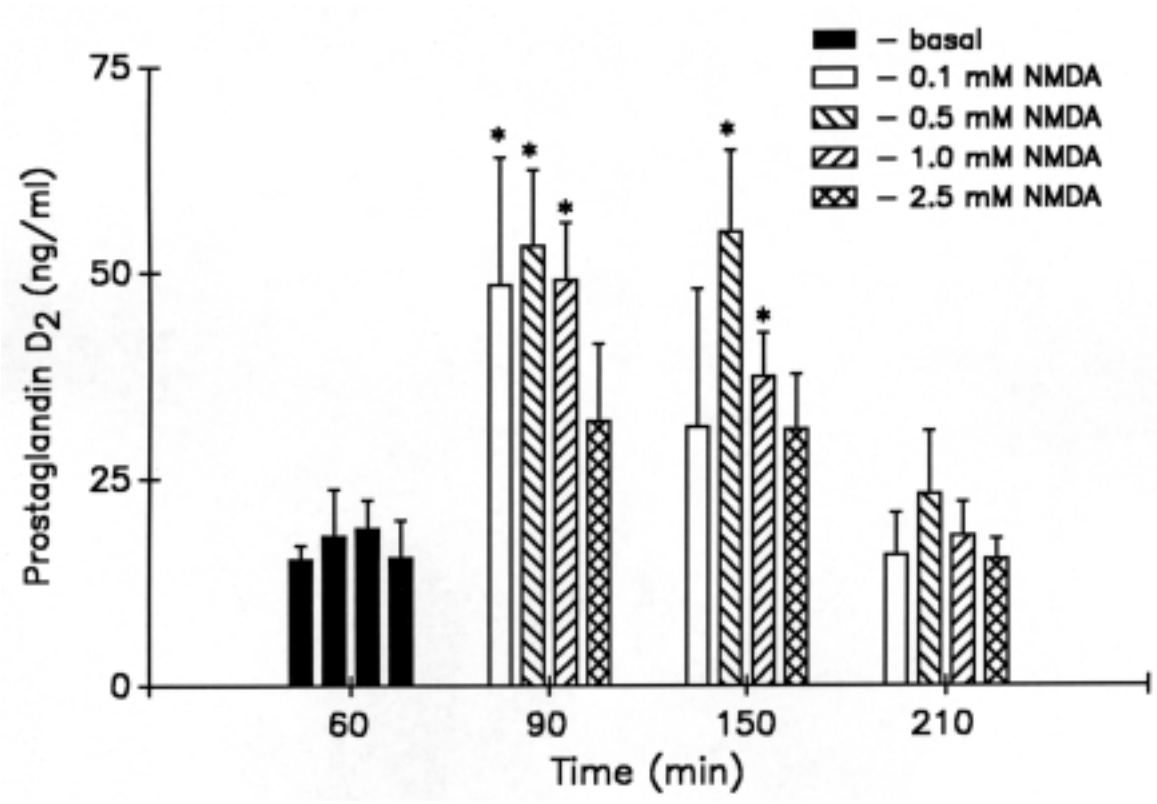

Fig. 1. NMDA - induced prostaglandin D_2 release in rabbit hippocampus *in vivo*: dose - effect relation. Data represent prostaglandin D_2 concentration in samples of dialysate collected after 2.5 h of washout perfusion. Samples were collected over 30-min or 60-min periods, as indicated in the graph. NMDA-containing dialysis medium was applied for 20 min after 1 h of basal perfusion. Values represent means ± SD (n=4-5). *Means significantly different from basal level (0-60 min), P<0.05.

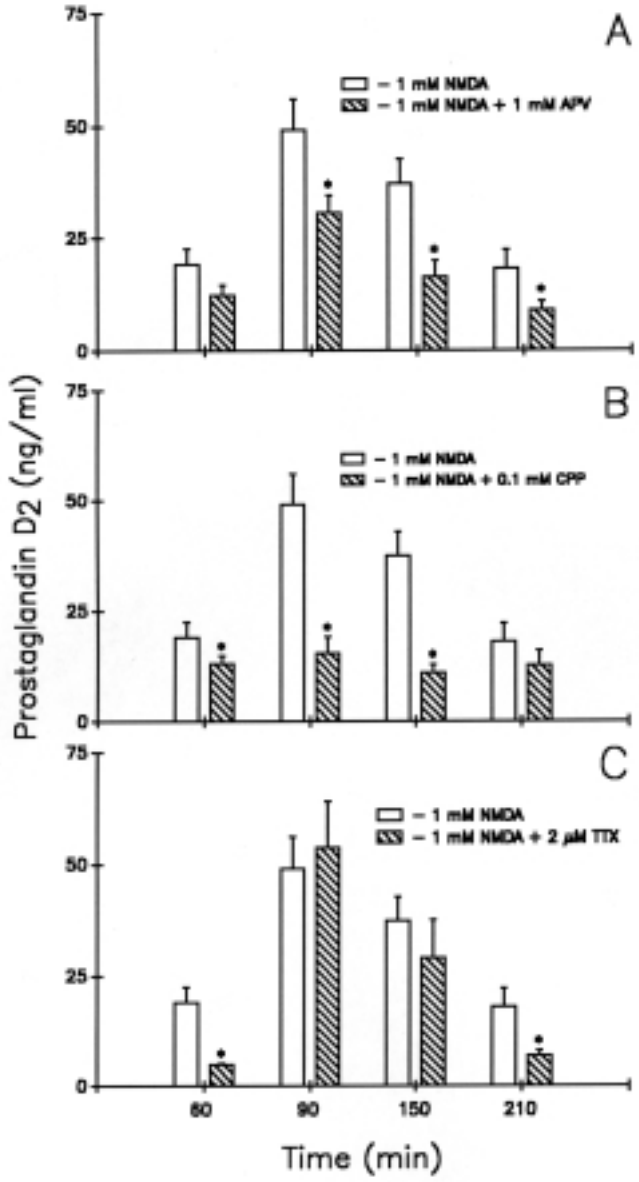

Fig. 2. NMDA - induced prostaglandin D_2 release in rabbit hippocampus *in vivo*: modulation by NMDA receptor and Na^+ channel antagonists. (A) Effect of 1 mM 2-amino-5-phosphonovaleric acid (APV). (B) Effect of 0.1 mM (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP). (C) Effect of 2 μM tetrodotoxin (TTX). Samples were collected over 30-min or 60-min periods, as indicated in the graph. NMDA-containing dialysis medium was applied for 20 min after 1 h of basal perfusion. Antagonists were present during the entire period of the experiment. Values represent means ± SD (n=4). *Means significantly different from the group treated with 1 mM NMDA, P<0.05.

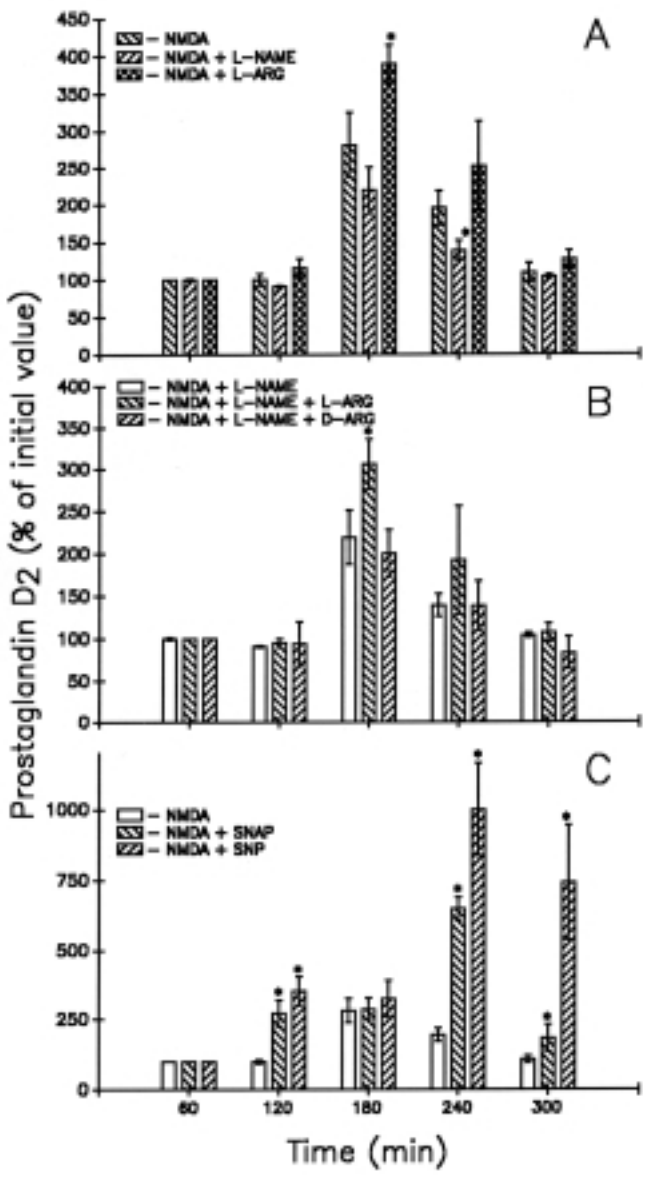

Fig. 3. Prostaglandin D_2 release in rabbit hippocampus *in vivo* induced by 1 mM NMDA: modulation by NO-related substances. (A) Effects of 1 mM N^G-nitro-L-arginine methyl ester (L-NAME) and 10 mM L-arginine (L-ARG). (B) Effects of 10 mM L-ARG and D-arginine (D-ARG) on NMDA-evoked effect in the presence of 1 mM L-NAME. (C) Effects of 25 mM sodium nitroprusside (SNP) and 25 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP), NO donors. Data represent relative concentration of prostaglandin D_2 in dialysate, in percent of initial value (0–60 min), collected after 2.5 h of washout perfusion. Samples were collected over 60-min periods, as indicated in the graph. NO-related substances were applied after 1 h of basal perfusion, NMDA-containing dialysis medium was applied for 20 min after 2 h of perfusion. Values represent means ± SD (n=4). *Means significantly different from the group treated with 1 mM NMDA, P<0.05.

level of 15-20 ng/ml (Figs. 1 and 2). During a 2.5 h microdialysis experiment the level of PgD_2 declined

constantly at a rate of < 5% per h (data not shown). As presented in Fig. 1, application of NMDA to dialysis medium induced a long-lasting stimulation of the release of PgD_2. The dose-response relation between NMDA and PgD_2 concentrations in the dialysate was complex. The NMDA-evoked release of PgD_2 to samples of dialysate collected during a 20-min period of NMDA application and 10 min after the pulse was already saturated at 0.1 mM NMDA and declined at 2.5 mM NMDA. In samples of dialysate collected during the next 60-min period, a bell-shaped dose-response relation was observed. A persistent PgD_2 release was highest at 0.5 mM NMDA. In samples collected 2.5 h after NMDA application, PgD_2 concentrations returned to the basal level.

As presented in Fig. 2A and B, competitive antagonists of the NMDA receptor, 1 mM 2-amino-5-phosphonovaleric acid (APV) and particularly 0.1 mM (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), decreased the basal level of PgD_2 and strongly inhibited its NMDA-induced release. Tetrodotoxin (TTX), an inhibitor of Na^+ channels strongly suppressing neuronal activity applied as a 2 μM solution, significantly decreased the basal level of PgD_2 in dialysate but had no effect on its NMDA-induced release (Fig. 2C).

In the presence of 1 mM N(G)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase (NOS), the basal level of PgD_2 in dialysate of the rabbit hippocampus was slightly lowered, whereas the prolonged release of PgD_2 after NMDA application was significantly reduced. In contrast, 10 mM L-arginine, an immediate precursor of NO, enhanced NMDA-evoked PgD_2 release (Fig. 3A). Inhibition of PgD_2 release by L-NAME was prevented by L-arginine but not by 10 mM D-arginine (Fig. 3B). Nitric oxide donors, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP), applied directly to dialysis medium in 25 mM concentrations significantly enhanced the basal level of PgD_2 in dialysate and potentiated the late phase of NMDA-evoked PgD_2 release, whereas the early phase of NMDA-induced release was unchanged (Fig. 3C).

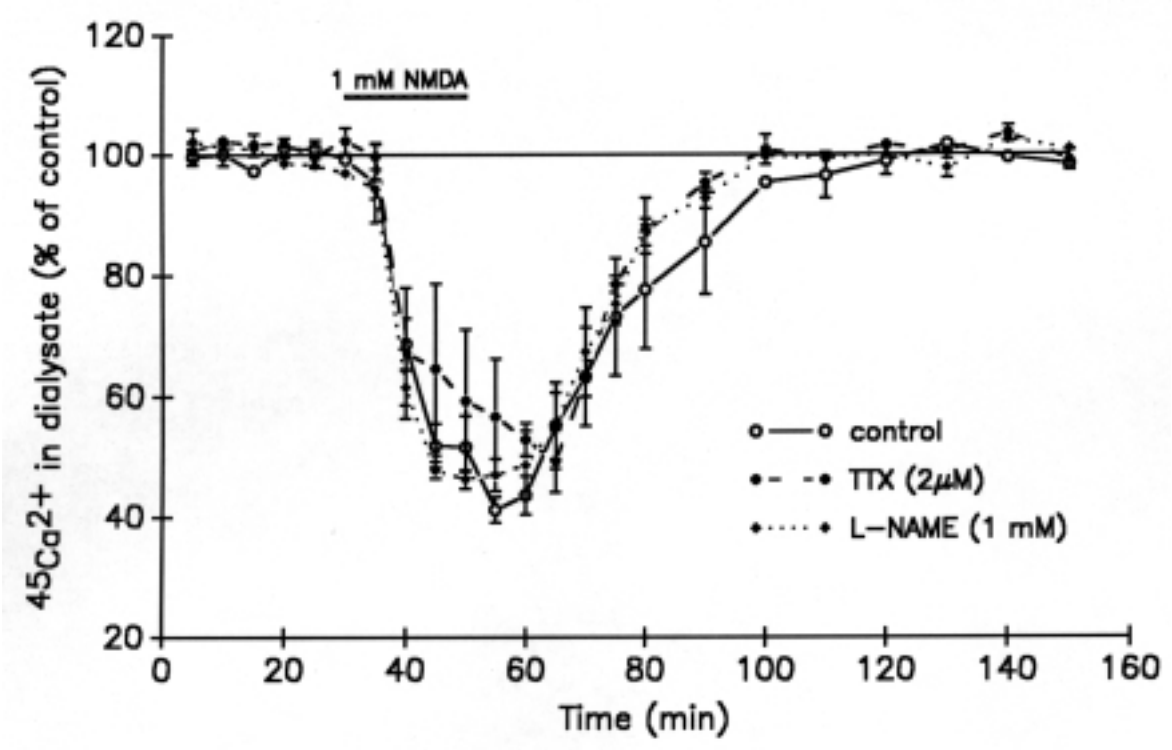

Fig. 4. NMDA - induced changes in extracellular Ca^{2+} concentration in the rabbit hippocampus *in vivo*: effect of 2 mM tetrodotoxin (TTX) and 1 mM N(G)-nitro-L-arginine methyl ester (L-NAME). Data represent relative changes in $^{45}Ca^{2+}$ level in dialysate, as a percent of control, indicative for extracellular calcium concentration (Łazarewicz et al. 1986). Values represent means ± SD (*n*=4).

NMDA-evoked decrease in extracellular Ca^{2+} level

As presented in Fig. 4, application of NMDA to dialysis medium induced a decrease in the efflux of $^{45}Ca^{2+}$ from the pre-labelled rabbit hippocampus to dialysate, which is known from our previous studies to indicate a decrease in $[Ca^{2+}]_e$. This long-lasting effect was insensitive to 1 mM L-NAME. In the presence of 2 μM TTX, an inhibitor of Na^+ channels, there was only an insignificant tendency for the reduction of a decrease in the efflux of $^{45}Ca^{2+}$ in the early phase of NMDA-evoked calcium imbalance.

NMDA-evoked NO release

NMDA-induced changes in the concentration of NO in dialysate of the rabbit hippocampus, measured by an indirect method utilising MetHb determinations, are presented in Fig. 5. In samples of dialysate collected only during the application of NMDA in dialysis medium, a significant increase in NO concentration was observed. This effect was completely cancelled in the presence of the NOS antagonist, 1 mM L-NAME.

DISCUSSION

The main finding of this *in vivo* microdialysis study is that NMDA receptors regulate production of PgD_2 in the rabbit hippocampus . These data are contradictory to the results of the *in vitro* study demonstrating the lack of an effect of NMDA receptors on PgD_2 biosynthesis in cultured rat dentate gyrus neurones (Larea et al. 1997). Hence, we will focus the discussion on possible explanations for this discrepancy between the results of *in vitro* and *in vivo* studies and on suggested mechanisms of NMDA receptor-mediated modulation of PgD_2 biosynthesis in the brain. Our data indicate that these regulatory mechanisms may encompass NO-induced activation of prostanoid biosynthesis.

Our results demonstrated that NMDA-evoked release of PgD_2 in the rabbit hippocampus was NMDA receptor-mediated, since the competitive NMDA receptor antagonists APV and particularly CPP significantly inhibited this process. The relation between NMDA dose and PgD_2 release was more complex. The release of PgD_2 was maximal at 0.1-0.5 mM NMDA and declined at higher concentrations of the ligand. In a previous microdialysis study (Łazarewicz and Salińska 1995) the

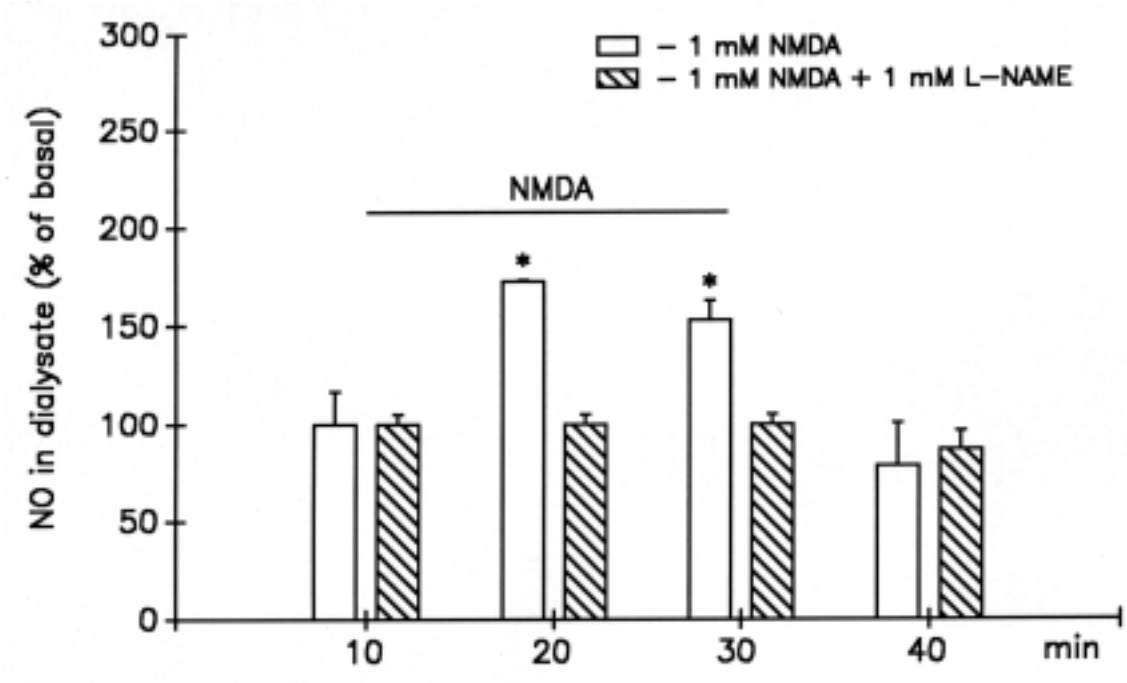

Fig. 5. NMDA - induced nitric oxide (NO) release in rabbit hippocampus *in vivo*: inhibition by 1 mM N(G)-nitro-L-arginine methyl ester (L-NAME). Data represent NO level in dialysate estimated by an indirect spectrophotometric method of MetHb measurement. Values represent means ± SD (n=4). *Means significantly different from the group treated with 1 mM NMDA, P<0.05.

release of 6-keto-$PgF_{1\alpha}$ and TxB_2 evoked by NMDA was saturated at 1 mM NMDA. On the other hand, a linear dose-effect relation of NMDA-evoked $[Ca^{2+}]_e$ decrease in the rabbit hippocampus *in vivo* was observed up to 5 mM NMDA (Łazarewicz and Salińska 1993). Most probably, lipolysis evoked by NMDA and mediated by the influx of Ca^{2+} to neurones may be saturated by much lower doses of NMDA than changes in $[Ca^{2+}]_e$. Application of the Na^+ channel blocker TTX resulted in a decrease of the basal PgD_2 level in dialysate whereas NMDA-evoked release was unaffected. A strong inhibitory effect of TTX on veratridine-evoked decrease in $[Ca^{2+}]_e$ was previously observed in our microdialysis experiments (Łazarewicz et al. 1986), whereas the present study demonstrated that TTX has no significant effect on NMDA-evoked changes in calcium homeostasis. Thus, ongoing trans-synaptic neuronal stimulation involving voltage-dependent Na^+ channels contributes to the basal prostanoid release, whereas NMDA receptors and Ca^{2+} influx to neurones *via* NMDA channels are specifically responsible for the NMDA - evoked PgD_2 release.

Our *in vivo* brain microdialysis experiments demonstrating NMDA-evoked stimulation of PgD_2 production utilised rabbits, whereas cultured rat brain neurones were used in the *in vitro* studies of Lerea et al. (1997) which failed to show such an effect. It seems that a discrepancy between these results cannot be attributed to species-related differences. It was found in an *in vivo* microdialysis study that PgD_2, the main prostanoid of the rodent brain, significantly increases in dialysate of the rat hippocampus after brain ischaemia and injury (Yergey and Hayes 1990, Huttemeier et al. 1993). Considering the postulated role of Ca^{2+} and excitatory amino acid receptors in the pathogenesis of brain ischaemia, this result indicates that *in vivo* PgD_2 biosynthesis may also be regulated by NMDA receptors in the rat hippocampus. A more probable explanation may be based on the predominant production of PgD_2 in brain in the extracellular fluid. It was shown that prostaglandin D synthase in the brain *in vivo* is secreted from its sites of biosynthesis in the leptomeninges and choroid plexus to the cerebrospinal fluid (Hoffmann et al. 1993). Thus, in pure cultures of dentate gyrus neurones the activity of prostaglandin D synthase may be very low if any, resulting in negligible PgD_2 production. Under such conditions its activation by NMDA may be also insignificant . Consequently, *in vitro* systems, and particularly neuronal cultures, are inadequate models for studies of brain PgD_2 biosynthesis and its regulation, whereas *in vivo* microdialysis experiments could provide relevant information.

The mechanism of NMDA receptor-mediated stimulation of PgD_2 release in brain *in vivo* is complex. The release of PgD_2 evoked by NMDA is accompanied by a decrease in $[Ca^{2+}]_e$, which reflects influx of extracellular calcium to stimulated neurones. As has been demonstrated in our previous study (Łazarewicz and Salińska 1993), this effect is NMDA dose - dependent and sensitive to NMDA receptor antagonism. A prolonged (3 h) perfusion of dialysis probe with Ca^{2+}-free medium, which greatly reduces $[Ca^{2+}]_e$ in the adjacent tissues (Łazarewicz et al. 1986), resulted in a significant inhibition of NMDA-evoked prostanoid release in the rabbit hippocampus *in vivo* (Łazarewicz and Salińska 1995). The influx of extracellular Ca^{2+} to neurones *via* NMDA channels, resulting in the increase of intracellular calcium concentration, triggers arachidonic acid release (Łazarewicz et al. 1988, 1990, Sanfeliu et al. 1990, Pellerin and Wolfe 1991). In NMDA-stimulated neurones, phospholipase A_2 plays a predominant role in Ca^{2+}-mediated arachidonic acid release (Łazarewicz et al. 1990). Thus, activation of NMDA receptors leads to an enhanced release of arachidonic acid, which is an initial substrate for prostanoid biosynthesis.

Subsequent steps of prostanoid biosynthesis include sequential oxidation of arachidonic acid by COX and hydroperoxidase to form prostaglandin H_2, a universal precursor of other prostanoids including PgD_2 (Smith and Marnett 1991, Maccarrone et al. 1997). As has been demonstrated previously in our microdialysis experiments in the rabbit hippocampus, application of NMDA induced a release of 6-keto-$PgF_{1\alpha}$ and TxB_2, and this effect was prevented by a COX inhibitor, indomethacin (Łazarewicz and Salińska 1995). Thus, COX may be considered as the other putative site of modulation of NMDA receptor-mediated rise in PgD_2 biosynthesis.

The results of our present experiments, namely suppression of NMDA-evoked PgD_2 release by NOS inhibitor L-NAME, prevention of this inhibition by L-arginine but not by D-arginine, and potentiation of PgD_2 production by NO donors, are indicative for the regulatory role of NO in NMDA receptor-mediated stimulation of PgD_2 production. When discussing these results the possible modulatory effects of NO on the activity of NMDA receptors should be considered. However, NO has been reported to inhibit NMDA receptors, possibly by their S-nitrosylation (Stamler et al. 1997), whereas our results show that NO enhances PgD_2 synthesis. Moreover, our

control experiments demonstrated the lack of effect of a NOS inhibitor, L-NAME, on NMDA-evoked changes in $[Ca^{2+}]_e$, pointing to interference of NO with sites located downstream of NMDA receptors. It has been demonstrated in several studies that COX activity is susceptible for stimulation by NO (Salvemini et al. 1993, Wu 1995, Landino et al. 1996, Maccarrone et al. 1997). Moreover, since NO has been shown to inhibit lipoxygenase activity (Maccarrone et al. 1996), NO could modulate the prostanoid metabolism in a dual way, by activating the COX-mediated pathway of the arachidonate cascade and inhibiting the lipoxygenase pathway (Maccarrone et al. 1997). According to these authors, inhibition of the latter may additionally enhance a stimulatory effect of NO on the COX-peroxidase complex constituting prostaglandin H synthase, since products of the lipoxygenase pathway inhibit this enzyme. Two different types of COX exist in brain cells, both subjects for NO stimulation. COX-1 is expressed constitutively, whereas COX-2 is inducible and up-regulated in injured neurones (Nogawa et al. 1998). It seems that both enzymes may be involved in NMDA-evoked PgD_2 release observed in this study and may be activated by NO, since injury evoked by the implantation of a microdialysis probe 24 h before the principal experiment could result in a delayed COX-2 up-regulation.

The results of this study indicate that NMDA receptors may stimulate *in vivo* PgD_2 synthesis in brain not only by enhanced production of its primary precursor, arachidonic acid, by calcium-mediated activation of phospholipase A_2 but also by the stimulation of synthesis of its immediate precursor prostaglandin H_2 throughout NO-mediated activation of COX. A functional role for this effect in the hippocampal region remains unclear. The only known defined physiological function of PgD_2 is the induction of sleep. However, the PgD_2-sensitive sleep promoting zone has been identified specifically in the ventral surface of the rostral basal forebrain (Matsumura et al. 1994). Thus, the role of PgD_2 in the hippocampus remains to be determined.

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