Effect of nitric oxide synthase inhibition on long-term potentiation at associational-commissural and mossy fibre synapses on CA3 pyramidal neurones

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1 The sensitivity of long-term potentiation (LTP) to nitric oxide synthase (NOS) inhibition was determined for two synaptic input systems onto CA3 pyramidal neurones the LTP of which display differential sensitivity to N-methyl-D-aspartate (NMDA) receptor antagonists: the fimbrial input which activates the associational-commissural synapses on the distal apical dendrites and the mossy fibre input which synapses on the proximal apical dendrites of CA3 pyramidal neurones.

2 Following high-frequency stimulation (HFS) of the fimbrial input, average e.p.s.p. amplitude increased by $92.4 \pm 22.0\%$ (mean $\pm$ s.e.mean; $n = 6$ cells) when compared to the pre-HFS average. In the presence of $100 \mu\text{M}$ N^ω-nitro-L-arginine methyl ester (L-NAME), the enhancement was reduced significantly to $32.2 \pm 11.6\%$ ($n = 5$ cells; $P < 0.05$). In the presence of $300 \mu\text{M}$ L-NAME, the inhibition was more complete, with post-HFS e.p.s.p. amplitude increasing an average $6.2 \pm 9.3\%$ ($n = 7$ cells, $P < 0.05$).

3 Following high frequency stimulation of the mossy fibre input, average e.p.s.p. amplitude increased by $57.9 \pm 13.0\%$ ($n = 6$ cells) when compared to the pre-HFS average. The presence of $100 \mu\text{M}$ L-NAME had no significant effect on the enhancement, averaging $63.6 \pm 5.9\%$ ($n = 4$ cells; $P > 0.05$). Similarly, increasing the concentration of L-NAME to $300 \mu\text{M}$ had no significant effect on the potentiation, with the post-HFS amplitude increasing by an average $55.6 \pm 9.5\%$ ($n = 5$ cells, $P > 0.05$).

4 These results suggest that LTP at associational-commissural synapses (fimbrial input) is significantly depressed in the presence of the NOS inhibitor L-NAME, while mossy fibre LTP is unchanged.

Keywords: Long-term potentiation; CA3 pyramids; nitric oxide; mossy fibres; fimbria

Introduction

The induction of long-term potentiation (LTP) by high-frequency stimulation of the mossy fibre tract to a CA3 pyramidal neurone is insensitive to blockers of N-methyl-D-aspartate (NMDA) receptors (Harris & Cotman, 1986; Jaffe & Johnston, 1990; Zalutsky & Nicoll, 1990; Katsuki *et al.*, 1991). This contrasts with the LTP described at other excitatory synapses in the hippocampus (including the perforant path to granule cells, associational-commissural to CA3 pyramidal neurones and the Schaeffer collateral to CA1 pyramidal neurones), which have all been shown to be blocked by such treatment (Harris & Cotman, 1986; Errington *et al.*, 1987; Zalutsky & Nicoll, 1990; Katsuki *et al.*, 1991). The high NMDA receptor density in the distal regions of the CA3 apical dendrites, where the fimbrial (associational-commissural) inputs terminate, but low receptor density in the more proximal regions of the dendrites, where the mossy fibres synapse, is consistent with these observations (Monaghan & Cotman, 1985). A number of postsynaptic pharmacological alterations (e.g. postsynaptic injection of calcium chelators, G-protein inhibitors) have also been shown to inhibit selectively the NMDA receptor-dependent form of LTP while having no effect on the non-NMDA receptor mediated mossy fibre LTP (Zalutsky & Nicoll, 1990; Katsuki *et al.*, 1991; 1992; but see Jaffe & Johnston, 1990; Johnston *et al.*, 1992).

NMDA receptor activation can elevate levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in rat hippocampi by activating the conversion of L-arginine to nitric oxide (NO) via nitric oxide synthase (NOS; East & Garthwaite, 1991). The pharmacological inhibition of NOS by the L-arginine analogue, N^ω-nitro-L-arginine-methyl ester (L-NAME) inhibits the induction of the NMDA receptor-mediated LTP

at the Schaeffer-collateral to CA1 synapse (Bon *et al.*, 1992; Haley *et al.*, 1992). These results suggest that NO may be a retrograde messenger the postsynaptic productions of which is stimulated by the activation of NMDA receptors (Gally *et al.*, 1990; Böhme *et al.*, 1991; O'Dell *et al.*, 1991; Schuman & Madison, 1991; Bon *et al.*, 1992; Haley *et al.*, 1992; but see Bliss & Collingridge, 1993).

Fimbria-evoked LTP in CA3 pyramidal neurones exhibits all the characteristics commonly used to describe a Hebbian synapse (associativity, cooperativity and specificity; Katsuki *et al.*, 1991; Zalutsky & Nicoll, 1990; 1992; but see Jaffe & Johnston, 1990) while mossy fibre LTP only displays specificity (Zalutsky & Nicoll, 1992). The fimbria-evoked LTP engages the associational-commissural fibres of the CA3 neurones and so involves transmission at synapses with NMDA receptors (Katsuki *et al.*, 1991). The question arises as to whether these synapses on the distal apical dendrites of CA3 pyramidal neurones utilize NO as a retrograde messenger in the generation of LTP and whether the mossy fibre synapses on the proximal apical dendrites do not. The present work shows that this may be the case.

Methods

Hippocampal slices (400 μm thick) were prepared from 3–5 week old male Wistar rats by conventional techniques (Dingledine *et al.*, 1980). The slices were maintained in a submersion chamber at room temperature, approximately 21°C and continuously perfused with artificial cerebro-spinal fluid (ACSF) containing the GABA_A antagonist, picrotoxinin (20 μM) at a rate of 2–3 ml min⁻¹. The ACSF had the following composition (mM): NaCl 123.8, NaHCO₃ 26.2, KCl 2.0, MgSO₄ 1.0, KH₂PO₄ 1.3, CaCl₂ 2 and glucose 10.0. This solution was constantly bubbled with 5% CO₂ and 95% O₂.

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in order to maintain a pH of approximately 7.3. Microelectrodes of resistances between 80 M Ω and 120 M Ω (filled with 4 M potassium acetate) were positioned in the CA3 region of the hippocampal slices and CA3 pyramidal neurone impalements obtained by making short punctate movements into *stratum pyramidale*. The quality of impalement was determined by the presence of stable resting membrane potentials more negative than -65 mV, stable input resistance and an action potential greater than 80 mV in amplitude. Rectangular stimulating pulses (40–100 μ s in duration) were delivered at 0.5 Hz through a bipolar tungsten electrode (tip spacing $\approx$ 75 μ m) positioned either on the mossy fibre tract well inside the dentate gyrus (to activate mossy fibre synapses) or straddled across the fimbria (to activate associational-commissural synapses). The intensity of the stimulation was set to evoke excitatory postsynaptic potentials (e.p.s.ps) which were sufficiently below the action potential threshold (usually <5 mV) as to prevent non-linear summation of enhanced responses following tetanic stimulation. Evoked e.p.s.ps from these neurones were recorded with an Axoclamp 2A amplifier (Axon Instruments), digitally stored (on line) and later analysed with pCLAMP software (Axon Instruments) on an IBM compatible computer.

The high frequency stimulation (HFS) paradigm delivered to the mossy fibre and fimbrial pathways differed. HFS delivered to the mossy fibre tract consisted of a single 100 Hz train lasting for 1 s (100 impulses) at an intensity just below the action potential threshold. The paradigm for fimbrial stimulation consisted of 5 trains of 10 impulses at 100 Hz, separated by 1 s (a total of 50 impulses) at an intensity just below the action potential threshold. E.p.s.p. amplitude was measured as the difference (mV) between the baseline and peak of a response. The mean amplitude of between 60 and 150 consecutive evoked (0.5 Hz) e.p.s.ps recorded before and 15 to 20 min after the tetanic stimulation were used to assess the degree of potentiation for a given cell. The increase in efficacy of synaptic transmission following tetanic stimulation was calculated as the percentage increase in mean e.p.s.p. amplitude 15 to 20 min after high frequency stimulation.

Both the GABA_A antagonist, picrotoxinin and the nitric oxide synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma.

Data are expressed as mean percentage increase ($\pm$ standard error of the mean, s.e.mean) in e.p.s.p. amplitude 15 to 20 min after HFS. The significance of the difference between drug treatments and bath controls was calculated by Student's two-tailed unpaired *t* test, with *P* values of less than 0.05 considered to represent a significant difference.

Results

High frequency stimulation (HFS) of the mossy fibre or fimbrial pathways resulted in the sustained enhancement of excitatory postsynaptic responses. Changes in the amplitude of the e.p.s.p. determined between 15 and 20 min after HFS were used to measure the extent of mossy fibre and fimbrial LTP. The mean amplitudes of the e.p.s.ps were significantly increased for mossy fibre LTP ($57.9 \pm 13.0\%$; $n = 6$ cells; $P < 0.05$; Figure 1b) and fimbrial LTP ($92.4 \pm 22.0\%$; $n = 6$ cells; $P < 0.05$; Figure 2b) using a single sample Student's *t* test. This potentiation of synaptic efficacy was observed for the duration of the impalements following HFS. To test whether nitric oxide synthase participated in the formation of mossy fibre and fimbrial LTP, hippocampal slices were perfused with normal ACSF containing 100 μ M or 300 μ M L-NAME, and then subjected to the appropriate tetanic stimulation. The mean e.p.s.p. amplitude after HFS of the mossy fibres rose by an average $63.6 \pm 5.9\%$ ($n = 4$ cells) in the presence of 100 μ M L-NAME. This enhancement was not significantly different from the control rise in e.p.s.p. amplitude ($P > 0.05$; Figure 1b), thereby suggesting that

Figure 1 (a) Effect of high frequency stimulation (HFS) of the mossy fibre tract on the amplitude of evoked e.p.s.ps in the presence of 300 μ M N^ω-nitro-L-arginine methyl ester (L-NAME). (b) The percentage increase in the mean mossy fibre evoked e.p.s.p. amplitude measured following a high frequency stimulation ($n = 6$ cells) was unaffected by the presence of 100 μ M ($n = 4$ cells; $P > 0.05$) or 300 μ M L-NAME ($n = 5$; $P > 0.05$).

mossy fibre LTP was not sensitive to the inhibition of NOS with L-NAME. A further increase in the L-NAME concentration to 300 μ M did not produce any significant change in the magnitude of LTP ($55.6 \pm 21.3\%$; $n = 5$ cells; $P > 0.05$; Figure 1a and b).

In the presence of 100 μ M L-NAME, the mean e.p.s.p. amplitude rose by an average of $32.2 \pm 11.6\%$ ($n = 5$ cells) following HFS of the fimbrial pathway. This was significantly different when compared to the bath control ($P < 0.05$; Figure 2b). In the presence of 300 μ M L-NAME, mean e.p.s.p. amplitude rose by an average $6.2 \pm 9.3\%$ ($n = 7$ cells) following HFS (Figure 2a). This higher concentration of L-NAME had a significant effect on the magnitude of LTP when compared to the control rise in e.p.s.p. amplitude in the bath control ($P < 0.05$; Figure 2b). These results suggest that fimbrial LTP, but not mossy fibre LTP, can be modulated by the inhibition of NOS.

Discussion

The induction of NMDA-receptor dependent LTP at the associational-commissural synapses with CA3 pyramidal neurones appears to involve the synthesis of NO in much the same way as the induction of NMDA-receptor dependent LTP at the Schaeffer collateral to CA1 synapses (Gally *et al.*, 1990; Böhme *et al.*, 1991; O'Dell *et al.*, 1991; Schuman & Madison, 1991; Bon *et al.*, 1992; Haley *et al.*, 1992; but see Gribkoff & Lum-Ragan, 1992; Field *et al.*, 1992). The sug-

Figure 2 (a) Effect of high frequency stimulation (HFS) of the fimbria on the amplitude of evoked e.p.s.ps in the presence of 300 μM N^o-nitro-L-arginine methyl ester (L-NAME). (b) The percentage increase in the mean fimbrially evoked e.p.s.p. amplitude measured following a high frequency stimulation ($n = 6$ cells) is significantly decreased in the presence of 100 μM L-NAME ($n = 5$ cells; $P < 0.05$) and 300 μM L-NAME ($n = 7$ cells; $P < 0.05$).

gested role for NO in LTP has been that of a putative retrograde messenger, released from a postsynaptic neurone following coincident pre- and postsynaptic activity (Gally *et al.*, 1990). The failure of NOS inhibition to block LTP at the mossy fibre to CA3 pyramidal neurone synapses, at which

neither associative nor NMDA-receptor dependent LTP occur, is consistent with the putative link between NMDA receptors, NOS and associative LTP (Gally *et al.*, 1990; East & Garthwaite, 1991).

NOS has been found in hippocampal neurones by use of both the NADPH diaphorase reaction as well as antibodies to NOS-I obtained from the cerebellum. The most intense staining is present in the *stratum radiatum* and subgranular zone of the dentate gyrus, outside the pyramidal cell layer in the *corpus ammonis* as well as in some of the pyramidal cells and their apical dendrites in the CA3 and CA1 regions (Valtschanoff *et al.*, 1992; Vincent & Hope, 1992; Vincent & Kimura, 1992; Schmidt *et al.*, 1992). The nitric oxide-sensitive soluble guanylate cyclase is found in all the CA3 and CA1 pyramidal neurones (Schmidt *et al.*, 1992). Neurones located outside the pyramidal cell layer in the *corpus ammonis* stain both for NOS as well as GABA and are thought to be inhibitory interneurones.

It seems likely that NOS is present in only a proportion of the CA1 and CA3 pyramidal neurones. The presence of guanylate cyclase in all pyramidal neurones suggests that NO released from pyramidal neurones that do contain NOS may act to produce cyclic GMP in other pyramidal neurones that do not contain NOS. The presence of NOS within inhibitory interneurones may indicate that NO could be acting as a paracrine factor, being released from these interneurones to act on neighbouring pyramidal neurones during intense synaptic activity, thereby modulating synaptic transmission in these neurones (Valtschanoff *et al.*, 1992; Vincent & Hope, 1992; Vincent & Kimura, 1992).

The question arises as to how a rapidly diffusing molecule like NO, with a radius of action of around 100 μm (Garthwaite, 1991) may selectively influence the fimbrial but not mossy fibre terminals; if NOS is localized only in the more distal postsynaptic spines of CA3 pyramidal neurones where fimbrial inputs terminate, it may only act on synapses local (within 100 μm) to these sites. Another possibility is that the nitric oxide-sensitive soluble guanylate cyclase occurs only in the associational-commissural but not mossy fibre terminals and NOS is located non-specifically along the CA3 apical dendrite or in some extrasynaptic site. We are at present using antibodies to NOS-I and guanylate cyclase to determine the ultrastructural localization of this enzyme in the apical dendrites of CA3 pyramidal neurones.

Previous work has shown a number of differences in the subcellular mechanisms responsible for the expression of a LTP at the mossy fibre and associational-commissural synapses. In addition, this study suggests the differential presence of a NOS-dependent LTP at these two synaptic pathways which share the same postsynaptic neurone.

References

- BLISS, T.V.P. & COLLINGRIDGE, G.L. (1993). A synaptic model of memory – long-term potentiation in the hippocampus. *Nature*, **361**, 31–39.
- BÖHME, G.A., BON, C., STUTZMANN, J.-M., DOBLE, A. & BLANCHARD, J.-C. (1991). Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.*, **199**, 379–381.
- BON, C., BÖHME, G.A., DOBLE, A., STUTZMANN, J.-M. & BLANCHARD, J.-C. (1992). A role for nitric oxide in long-term potentiation. *Eur. J. Neurosci.*, **4**, 420–424.
- DINGLELINE, R.Y., DODD, Y. & KELLY, J.S. (1980). The *in vitro* brain slice technique as a useful neurophysiological preparation for intracellular recording. *J. Neurosci. Methods*, **2**, 323–362.
- EAST, S.J. & GARTHWAITE, J. (1991). NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. *Neurosci. Lett.*, **123**, 17–19.
- ERRINGTON, M.L., LYNCH, M.A. & BLISS, T.V.P. (1987). Long-term potentiation in the dentate gyrus: induction and increased glutamate release blocked by D(-)aminophosphonovalerate. *Neuroscience*, **20**, 279–284.
- FIELD, A.C., REDMAN, S.J. & STRICKER, C. (1992). NMDA receptor activation at synapses on CA1 neurons is not necessary for the induction of LTP. *Soc. Neurosci. Abstr.*, **18**, 1347.
- GALLY, J.A., READ-MONTAGUE, P., REEKE Jr., G.N. & EDELMAN, G.M. (1990). The NO hypothesis: possible effects of a short lived, rapidly diffusible signal in the development and function of the nervous system. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 3547–3551.
- GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, **14**, 60–67.
- GRIBKOFF, V.K. & LUM-RAGAN, J.T. (1992). Evidence for nitric oxide synthase inhibitor-sensitive and insensitive hippocampal synaptic potentiation. *J. Neurophysiol.*, **68**, 639–642.
- HALEY, J.E., WILCOX, G.L. & CHAPMAN, P.F. (1992). The role of nitric oxide in hippocampal long-term potentiation. *Neuron*, **8**, 211–216.
- HARRIS, E.W. & COTMAN, C.W. (1986). Long-term potentiation of guinea pig mossy fibre responses is not blocked by N-methyl D-aspartate antagonists. *Neurosci. Lett.*, **70**, 132–137.

- JAFFE, D. & JOHNSTON, D. (1990). Induction of long-term potentiation at hippocampal mossy-fiber synapses follows a hebbian rule. *J. Neurophysiol.*, **64**, 948–960.
- JOHNSTON, D., WILLIAMS, S., JAFFE, D. & GRAY, R. (1992). NMDA-receptor-independent long-term potentiation. *Annu. Rev. Physiol.*, **54**, 489–505.
- KATSUKI, H., KANEKO, S. & SATOH, M. (1992). Involvement of postsynaptic G proteins in hippocampal long-term potentiation. *Brain Res.*, **581**, 108–114.
- KATSUKI, H., KANEKO, S., TAJIMA, A. & SATOH, M. (1991). Separate mechanisms of long-term potentiation in two input systems to CA3 pyramidal neurons of rat hippocampal slices as revealed by the whole-cell patch-clamp technique. *Neurosci. Res.*, **12**, 393–402.
- MONAGHAN, D.T. & COTMAN, C.W. (1985). Distribution of N-methyl-D-aspartate sensitive L-[3H]glutamate-binding sites in the rat brain. *J. Neurosci.*, **5**, 2909–2919.
- O'DELL, T.J., HAWKINS, R.D., KANDEL, E.R. & ARANCIO, O. (1991). Tests of the roles of 2 diffusible substances in long-term potentiation – evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11285–11289.
- SCHMIDT, H.H.H.W., GAGNE, G.D., NAKANE, M., POLLOCK, J.S., MILLER, M.F. & MURAD, F. (1992). Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J. Histochem. Cytochem.*, **40**, 1439–1456.
- SCHUMAN, E.M. & MADISON, D.V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science*, **254**, 1503–1506.
- VALTSCHANOFF, J.G., KHARAZIA, V.N. & WEINBERG, R.J. (1992). Neurons containing nitric oxide synthase in rat hippocampus. *Soc. Neurosci. Abstr.*, **18**, 639.
- VINCENT, S.R. & HOPE, B.T. (1992). Neurons that say NO. *Trends Neurosci.*, **15**, 108–113.
- VINCENT, S.R. & KIMURA, H. (1992). Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience*, **46**, 755–784.
- ZALUTSKY, R.A. & NICOLL, R.A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurones. *Science*, **248**, 1619–1624.
- ZALUTSKY, R.A. & NICOLL, R.A. (1992). Mossy fiber long-term potentiation shows specificity but no apparent cooperativity. *Neurosci. Lett.*, **138**, 193–197.

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