The effect of nitric oxide on the efficacy of synaptic transmission through the chick ciliary ganglion

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1 The effect of nitric oxide on the efficacy of synaptic transmission in the chick ciliary ganglion of post-hatched birds has been determined by use of the size of the postganglionic compound action potential resulting from chemical transmission through the ganglion as a measure of synaptic efficacy.

2 Sodium nitroprusside (100 μM) increased the synaptic efficacy by an average 26%. This is likely to be due to its ability to release nitric oxide, as potassium ferricyanide (100 μM) did not cause a potentiation. Sodium azide (100 μM), shown in sympathetic ganglia to stimulate production of cyclic GMP, did not modulate synaptic efficacy significantly.

3 8-Br-cyclic-GMP (100 μM) increased synaptic efficacy by an average 61%. The addition of 8-Br-cyclic-AMP (100 μM) had less effect, increasing transmission by on average 46%.

4 The nitric oxide synthase blocker, N^G -nitro-L-arginine methyl ester (L-NAME, 100 μM) was added prior to the tetanic stimulation of the preganglionic nerves at 30 Hz for 20 s, a procedure known to produce both post-tetanic potentiation and long-term potentiation of synaptic transmission through the ganglion. L-NAME reduced the long-term potentiation by an average of 47% but did not significantly change the post-tetanic potentiation.

5 Following the brief application of 8-Br-cyclic AMP, 8-Br-cyclic GMP and sodium nitroprusside there was an enhancement of the efficacy of synaptic transmission that persisted after the withdrawal of the drugs. The maximum increase in synaptic efficacy following the brief addition of 8-Br-cyclic GMP was 116%, sodium nitroprusside was 110% and 8-Br-cyclic AMP was 126%.

6 These results suggest that nitric oxide modulates synaptic transmission through the ganglion by acting on an endogenous guanylate cyclase that produces cyclic GMP.

Keywords: Nitric oxide; ciliary ganglia; cyclic GMP

Introduction

Nitric oxide has been implicated in the maintenance of long-term potentiation (LTP) in the hippocampus (see for example, Haley *et al.*, 1992). As a form of LTP occurs in the avian ciliary ganglion (Scott & Bennett, 1993), it is possible that a component of the maintenance of this LTP is also due to nitric oxide. In avian ciliary neurones, nitric oxide synthase is exclusively found in the giant nerve terminal calyx in the avian ciliary ganglion (Chan, 1992), so that it is unlikely that nitric oxide acts as a retrograde messenger in the way that has been suggested in CA1 pyramidal neurones in the hippocampus (for a review see, Bredt & Snyder, 1992). Nevertheless, nitric oxide is known to decrease calcium-activated potassium currents in the ganglion by a mechanism that is independent of any effect on the calcium influx through calcium channels (Cetiner & Bennett, 1992). This result supports the hypothesis that nitric oxide might act to increase transmitter secretion by increasing the duration of the terminal action potential following blockade of the fast calcium-dependent potassium current, I_{K} . Such a mechanism has been suggested to underlie the potentiation of transmitter secretion from nerve terminals in *Aplysia* neurones (Kandel & Hawkins, 1992). One problem with this idea is that nitric oxide also decreases the transient and sustained whole-cell calcium currents in the ganglion (Khurana & Bennett, 1993). Thus on the one hand calcium influx across the nerve terminal membrane may be increased by nitric oxide to increase the duration of the action potential and therefore the open time of voltage-sensitive calcium channels. On the other hand however, these channels themselves may be depressed, thus decreasing the calcium influx. In the present work it is shown that an endogenous nitric oxide synthase in the nerve terminal of the ganglion does play a role in the maintenance of

LTP and the method by which this might occur is investigated.

Methods

One to five day post-hatched white leghorn chicks (*Gallus gallus*) were used in all experiments. Animals were decapitated and a craniectomy performed to expose the right ciliary ganglion. The ciliary ganglion with the attached right eye and oculomotor nerves were removed. The right eye was dissected to free the ciliary nerve from within the eye (see Pilar & Tuttle, 1982 for a further description of the dissection). The ciliary ganglion with its attached oculomotor and ciliary nerves were then placed in a tissue bathing chamber. The bath was perfused at a rate of approximately 2 ml min⁻¹ with Tyrode solution of the following composition (in mM): Na⁺ 140, K⁺ 5, Mg²⁺ 1, Ca²⁺ 3, Cl⁻ 153, glucose 10, HEPES 10, equilibrated by bubbling with 95% O₂, 5% CO₂ and pH adjusted to 7.2–7.4 with NaOH. Stimulation was applied to the oculomotor nerve through a glass suction electrode. The application of the stimulating pulse (a square wave of 14 V and duration 0.09 ms) was through a radio-frequency isolation unit. Experiments were conducted at room temperature (17 to 20°C).

Recordings of compound action potentials from the postganglionic ciliary nerve were made by using a fine suction electrode. Signals obtained from this nerve were amplified by an Axoclamp 2A amplifier (Axon Instruments). These signals were digitized using a Labmaster A-D board and recorded and analysed on an IBM-compatible computer using the pCLAMP (Fetchex, Fetchan) software package (Axon Instruments).

Drugs used here were hexamethonium chloride, 8-

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bromoadenosine 3':5'-cyclic monophosphate (8-Br-cyclic AMP), 8-bromoguanosine 3':5'-cyclic monophosphate (8-Br-cyclic GMP), sodium azide (NaN_3), potassium ferricyanide (FCN), sodium nitroprusside (SNP) and N^G -nitro-L-arginine methyl ester (L-NAME). These were purchased from Sigma Chemical Company.

Hexamethonium ($300\text{ }\mu\text{M}$), a nicotinic cholinergic antagonist, was routinely added to the bathing solution in order to reduce the size of the chemical component of the biphasic compound action potential. Supramaximal stimulation was applied to the preganglionic oculomotor nerve. The reduction in the compound action potential by hexamethonium permitted measurement of an increase in synaptic efficacy. An increase in the synaptic efficacy, such as that which might follow tetanic stimulation during post-tetanic potentiation (PTP) and LTP, or following the addition of a potentiating drug, was reflected by a significant increase in the number of the postsynaptic population reaching action potential firing threshold. This was indicated by an increase in the amplitude of the chemical component of the compound action potential.

Protocols for the measurement of LTP and PTP were followed routinely. Upon the attachment of the suction electrodes to the pre- and postganglionic nerves, the preganglionic oculomotor nerve was stimulated at a frequency of 0.033 Hz . Only preparations with evoked compound action potentials larger than 1 mV were used. Hexamethonium ($300\text{ }\mu\text{M}$) was then added to the bathing solution. This was allowed to perfuse the ganglion for at least 60 min so that the chemical phase of the compound action potential could be reduced (by at least 60% of its original value) and a control measurement for its amplitude established. The preparation was stimulated at 0.033 Hz during this period.

Activity-dependent potentiation was produced by tetanic stimulation of 30 Hz for 20 s and applied following the establishment of a control level of synaptic efficacy. It has been shown that the frequency of this tetanic stimulation occurs within the normal operating range of ciliary ganglia (Fujii, 1992). Following tetanic stimulation of the ciliary ganglion, recordings of the PTP and LTP time courses were made by measuring the amplitude of the chemical phase of the evoked compound action potential. Addition of drugs to the bathing solution was carried out at least 60 min after the previous tetanic stimulation. Any drug was allowed to perfuse the ganglion for at least 60 min before tetanic stimulation was reapplied.

For the brief exposure of the ganglia to NO, 8-Br-cyclic AMP and 8-Br-cyclic GMP (as defined by the horizontal bars in Figure 3), the drug was applied directly to the bath and then quickly washed from the bath. A test was made to ensure that any change in synaptic efficacy in these experiments was due to the drug action, and not to another factor to do with the exchange and washing. This involved the addition of normal bath solution directly to the bath for 3 min followed by the same washing procedure as used with drug application. No change in the synaptic efficacy was observed over the time course of an hour indicating that the effects of the brief drug addition were the result of the drugs action.

The ordinates of the figures are expressed in terms of percentage of control. A control value (V_{ctl}) was obtained by averaging the amplitude of the chemical component of the compound action potential for the 15 min prior to drug addition or tetanic stimulation (measured by evoking compound action potentials at a frequency of 0.033 Hz). Following this, compound action potentials were again evoked at a frequency of 0.033 Hz . The amplitudes of these post-tetanic compound action potentials (V) were measured. The percentage increase was calculated as:

$$\% \text{ of control} = 100 \times \frac{V}{V_{\text{ctl}}}$$

Data are expressed as mean $\pm$ standard error of the mean

(s.e.mean) and n gives the number of experiments. The significance of the difference between n pairs of observations made before and after application of a drug was calculated by Student's paired t test. P values of 0.05 or less were considered to represent significant differences.

Results

The efficacy of transmission through the ciliary ganglion was determined as described previously (Scott & Bennett, 1993). The size of the compound action potential recorded in the postganglionic ciliary nerve due to chemical transmission

Figure 1 The effects of 8-Bromo-cyclic GMP (8-Br-cGMP), 8-bromo-cyclic AMP (8-Br-cAMP) and sodium nitroprusside (SNP) on transmission through the ciliary ganglion. The ordinate scale gives the percentage of the amplitude of the chemical component of the compound action potential with respect to its amplitude during the control period recorded from the postganglionic ciliary nerve. Measurement is made by stimulating at 30 s intervals and the control amplitude is established by averaging these recordings for 15 min before application of the drug or tetanic stimulation. (a) Shows the effect of addition of $100\text{ }\mu\text{M}$ 8-Br-cyclic GMP at time zero; (b) shows the effect of the addition of $100\text{ }\mu\text{M}$ 8-Br-cyclic AMP at time zero; (c) shows the effect of the addition of $100\text{ }\mu\text{M}$ SNP at time zero. The horizontal bar indicates the time of application of the respective drugs.

through the ganglion in the presence of $300\text{ }\mu\text{M}$ of hexamethonium was used as a measure of synaptic efficacy. The oculomotor nerve was stimulated every 30 s and the size of the chemical component of the action potential noted. Application of $100\text{ }\mu\text{M}$ 8-Br-cyclic GMP increased the synaptic efficacy in all 5 ganglia studied, with an average increase of $61 \pm 31\%$ (Figure 1a). Application of $100\text{ }\mu\text{M}$ of 8-Br-cyclic AMP also enhanced transmission through the ganglion, by an average of $46 \pm 20\%$ in 5 experiments (Figure 1b).

In order to determine if an endogenous nitric oxide-sensitive, soluble guanylate cyclase was likely to be present in the ganglion for the production of cyclic GMP, sodium nitroprusside ($100\text{ }\mu\text{M}$) was applied to the preparation. This increased the size of the compound action potential by an average of $26 \pm 12\%$ in 12 ganglia (Figure 1c). Sodium azide ($100\text{ }\mu\text{M}$), an agent shown to stimulate the formation of cyclic GMP in sympathetic ganglia (Quenzer *et al.*, 1980a,b; Ariano *et al.*, 1982; Ando *et al.*, 1983; Volle & Quenzer, 1983), was applied to the ganglion in five experiments. Surprisingly, this showed no significant effect on the efficacy of synaptic transmission. The action of sodium azide in stimulating cyclic

GMP formation is known to be much less effective in autonomic ganglia than is the action of sodium nitroprusside (Quenzer *et al.*, 1980a). In order to check that the effects of sodium nitroprusside ($\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$) were due to its production of nitric oxide, five ganglia were bathed in $100\text{ }\mu\text{M}$ potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$). There was no change in the compound action potential.

The existence of a nitric oxide-sensitive system in the ganglion, presumably operating through cyclic GMP, raises the question of whether an endogenous nitric oxide modulates transmission. In order to test this, the nitric oxide synthase blocker L-NAME ($100\text{ }\mu\text{M}$) was added to the preparation,

Figure 2 The effect of blocking nitric oxide synthase with N^G -nitro-L-arginine methyl ester (L-NAME) on post-tetanic potentiation and long-term potentiation of chemical transmission in the ganglion. The ordinate scale gives the percentage of control of the chemical component of the compound action potential as in Figure 1. Each point represents the mean $\pm$ s.e. mean for five experiments. (a) Tetanic stimulation of the ganglion at 30 Hz for 20 s produces the usual post-tetanic and long-term potentiation time courses; (b) the same ganglia were perfused for at least 60 min with $100\text{ }\mu\text{M}$ L-NAME and tetanic stimulation was reapplied to the presynaptic oculomotor nerve.

Figure 3 The result of a brief application of 8-bromo-cyclic AMP (8-Br-cAMP), 8-bromo-cyclic GMP (8-Br-cGMP), and sodium nitroprusside: (a) exposure of the ganglion to 1 mM 8-Br-cyclic AMP for 3 min at time zero increased the efficacy of synaptic transmission which was maximal at time 40 min and measured as 134% of the control in this case. (b) Exposure of the ganglion to 1 mM 8-Br-cyclic GMP for 3 min at time zero increased the efficacy of synaptic transmission which was maximal at time 10 min and measured as 125% of the control in this case. (c) Exposure of the ganglion to 1 mM sodium nitroprusside (SNP) for 3 min at time zero increased the efficacy of synaptic transmission which was maximal at time 10 min and measured as 114% of the control in this case.

Figure 4 The dose-response curve of the maximal increase in the efficacy of synaptic transmission following drug exposure at the indicated concentration for 3 min. The maximal responses for cyclic AMP (●), cyclic GMP (○) and sodium nitroprusside (▲) exposure were taken at time 40 min, 10 min and 10 min respectively after initial drug exposure.

and the effect of this on transmission and potentiation monitored. L-NAME did not affect transmission through the ganglia but it did significantly decrease LTP by an average of $48\% \pm 13\%$ in 5 preparations ($P < 0.05$; Figure 2). There was no significant effect on PTP ($P > 0.25$). In one preparation the LTP doubled in the presence of L-NAME; we have no explanation for this.

Given that NO, cyclic GMP and cyclic AMP are likely to be involved in the enhancement of synaptic efficacy, ganglia were exposed for 3 min to each of these drugs at a concentration of 1 mM to see whether the resultant potentiation would be maintained following the drug removal (Figure 3). Application of 1 mM 8-Br-cyclic AMP produced a slowly increasing potentiation that reached a maximum of $126 \pm 4\%$ of control ($n = 3$) at time 40 min (Figure 3a). The exposure of the ganglion to 8-Br-cyclic GMP produced a potentiation that was maximal at 10 min after the drug application to a value of $116 \pm 7\%$ of control ($n = 3$; Figure 3b). The potentiation produced by SNP reached a maximal level 10 min after stimulation ($110 \pm 2\%$; $n = 3$; Figure 3c). Both the 8-Br-cyclic AMP and SNP evoked effects returned to control levels approximately 25 min after drug application. Each of the potentiations resulting from the actions of these drugs was proportional to the drug concentration over the range $10 \mu\text{M}$ to 1 mM (Figure 4).

Discussion

Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (Dawson *et al.*, 1991; Hope *et al.*, 1991) can be used as a marker for neurones that contain NO synthase. In the autonomic nervous system such neurones are found in the intermedio-lateral cell column of the spinal cord giving rise to the preganglionic sympathetic nerves (Valtschanoff *et al.*, 1992; Blottner & Baumgarten, 1992) and in pelvic ganglia, as well as in the intramural plexuses of the gastrointestinal tract and lower urinary tract (Grozdanovic *et al.*, 1992). Antibodies to nitric oxide synthase have confirmed these findings with NADPH-diaphorase histochemistry (Bredt *et al.*, 1990) as well as allowing for its ultrastructural localization. This has shown that the synthase is not associated with any subcellular organelle or with the plasma membrane

(Llewellyn-Smith *et al.*, 1992), but is patchily distributed in both the neurone cell bodies as well as in nerve terminals. NADPH-positive stained neurones are found throughout the ciliary ganglion of avian embryos but are restricted to the superficial layers of the ganglion after hatching (Scott *et al.*, 1992). Ultrastructural localisation of antibodies to the synthase show that it is exclusively found in the giant calyciform preganglionic terminal on the ciliary neurones, with no antibody in the neurone somas (Chan, 1992). Therefore, it appears very likely that nitric oxide may act as a cotransmitter or modulator of transmission in the ciliary ganglion rather than a retrograde messenger as has been proposed for neurones in the hippocampus (see for example, Böhme *et al.*, 1991).

There is now considerable evidence suggesting that nitric oxide acts as a transmitter in the peripheral nervous system. The inhibitory neurones of the gastrointestinal tract that use neither noradrenaline nor acetylcholine as transmitters (Bennett *et al.*, 1963), the so called NANC neurones, appear in most cases to use nitric oxide as their transmitter (Gillespie *et al.*, 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Bult *et al.*, 1990; Desai *et al.*, 1991; Boeckxstaens *et al.*, 1991; Christinck *et al.*, 1991). There is also evidence that nitric oxide can act as an autoexcitatory agent to potentiate the release of transmitters. For example, the secretion of noradrenaline in the regulation of haemodynamics is facilitated by the action of endogenous nitric oxide (Halbrugge *et al.*, 1991).

Endogenous nitric oxide is generated and released from the ciliary ganglion during LTP, as this was reduced by about 40% in the presence of L-NAME without any affect on PTP. Cyclic GMP may be involved in the action of nitric oxide, as addition of 8-Br-cyclic GMP increased the efficacy of transmission through the ganglion by about 40%, that is to about the same extent as did sodium nitroprusside, a result similar to that observed for sympathetic ganglia (Briggs, 1992). The classical pathway for the action of nitric oxide, namely through the haeme group of guanylate cyclase capturing nitric oxide, with a subsequent conformational change in the enzyme leading to the production of cyclic GMP and phosphorylation of a substrate through cyclic GMP-dependent kinase (Bredt & Snyder, 1992), is one mode of action observed in sympathetic ganglia (Dun *et al.*, 1978). In these ganglia the increase in cyclic GMP accompanying nerve stimulation (Weight *et al.*, 1974) is probably confined to the nerve terminals as depolarization of the ganglion with high potassium concentrations does not lead to an increase in cyclic GMP if the ganglion has been denervated (Quenzer *et al.*, 1980a,b; Ando *et al.*, 1983). This increase in the terminal cyclic GMP may be due to the action of secreted acetylcholine acting on terminal muscarinic receptors to inhibit secretion (Morita *et al.*, 1982), as acetylcholine increases the cyclic GMP content of the ganglion through an atropine-sensitive mechanism (Kebabian *et al.*, 1975). Whether acetylcholine secreted at the ciliary nerve terminals also acts to stimulate the production of cyclic GMP in these terminals in order to increase transmitter secretion has not been determined.

In the present case it has been shown that 8-Br-cyclic AMP can increase the efficacy of synaptic transmission through the ganglion by about 40% as is the case for sympathetic ganglia (Briggs, 1992). As this is about the same increase in efficacy as that produced by sodium nitroprusside, it is possible that nitric oxide also acts through the cyclic AMP pathway. In sympathetic ganglia, analogues of cyclic AMP give a long-lasting increase in the evoked secretion of acetylcholine through a mechanism that does not involve extracellular calcium (Briggs *et al.*, 1985; 1988). The question immediately arises as to how nitric oxide could increase cyclic AMP and also elevate transmitter secretion. One way involves the ability of cyclic GMP to inhibit cyclic AMP phosphodiesterase (Maurice & Haslam, 1990a,b): this mechanism ensures that if cyclic GMP levels in the nerve terminal are elevated by nitric oxide then so will those of cyclic AMP. It

is interesting in this regard that recent biochemical studies have shown that there is a large increase in the phosphorylation of a protein kinase A substrate in the ciliary ganglion after its exposure to sodium nitroprusside (Lengyel *et al.*, 1993). In the hippocampus cyclic AMP is elevated in an NMDA-receptor dependent way during LTP indicating an involvement of cyclic AMP-dependent protein kinase (Chetkovitch *et al.*, 1991). The effect of blocking cyclic AMP production on LTP in the ciliary ganglion has not yet been investigated.

The effects of cyclic AMP in elevating transmission through the ciliary ganglion were more sustained than those due to cyclic GMP (see Figures 1 and 3). One possible explanation is that cyclic GMP has two actions in the ganglion: one already mentioned involves the elevation of cyclic AMP through the ability of cyclic GMP to inhibit cyclic AMP phosphodiesterase; the other could involve the elevation of a protein phosphatase through protein kinase G. It has recently been shown that calcium-activated potassium channels in rat pituitary tumour cells are stimulated by cyclic GMP acting through a protein phosphatase (White *et al.*, 1993). If cyclic AMP stimulation through cyclic GMP inhibits these channels in the ciliary ganglion but cyclic GMP-dependent protein phosphatase stimulates them, then the action of exogenous cyclic GMP may be transient compared with that of exogenous cyclic AMP. It remains to be seen whether this hypothesis survives experimental testing.

The present results suggest the following model for the action of nitric oxide in the maintenance phase of LTP in the ciliary ganglion. High-frequency stimulation of the calyciform nerve terminal leads to the influx of sufficient calcium into the terminal to activate nitric oxide synthase

(Chan, 1992; Garthwaite, 1991); the nitric oxide so produced acts on guanylate cyclase located in the terminal resulting in the increase in transmitter secretion in autonomic ganglia but no change in the sensitivity of postsynaptic receptors (Kuba & Kumamoto, 1990; Scott & Bennett, 1993). The principal effect of the cyclic GMP produced is to phosphorylate cyclic AMP phosphodiesterase, so that this can no longer metabolize cyclic AMP (Maurice & Haslam, 1990a). The subsequent increase in cyclic AMP in the terminal leads to the phosphorylation of a protein kinase A substrate (Lengyel *et al.*, 1993), which may be identified as the calcium-activated potassium channels in the terminal. This results in a decrease in the opening of these channels (Cetiner & Bennett, 1992), with a consequent increase in the duration of the terminal action potential and a very large increase in the transient calcium concentration required during vesicle exocytosis at the active zones of the terminal. Whether there is an auto-phosphorylation form of protein kinase A in the terminal which will allow for a sustained effect of this pathway on transmitter secretion is not known. Nor is it known whether the decrease in calcium channel opening produced by nitric oxide is such as to compensate for the enhancement expected from the increase in duration of the action potential. The present work suggests however that the processes described in the above model may occur: blocking nitric oxide synthase with L-NAME decreases LTP whereas adding nitric oxide exogenously increases the efficacy of transmission through the ganglion as does the addition of either cyclic GMP or cyclic AMP. Whether there is a pathway from cyclic GMP to calcium-calmodulin kinase II remains to be investigated, as blocking this kinase prevents LTP (Scott & Bennett, 1993), indicating that it is important in the expression of LTP.

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