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Role of nitric oxide (NO) in ocular inflammation

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1 The actions of nitric oxide (NO) have been investigated in the rabbit eye, with particular emphasis on the relationship between NO and C-fibres and on those effects of NO that may be of importance in the inflammatory response to C-fibre stimulation.

2 The NO synthase inhibitor, N^G-nitro-L-arginine (L-NAME; 10–200 mg kg^{-1}), but not the inactive analogue D-NAME (200 mg kg^{-1}), was found to block the inflammatory response induced by infrared irradiation of the iris in a dose-dependent manner. The inhibitory effects of L-NAME (200 mg kg^{-1}) were partially reversed by L-arginine (500 mg kg^{-1}), but not by D-arginine (500 mg kg^{-1}).

3 L-NAME (200 mg kg^{-1}) virtually abolished the ocular effects of intravitreal injection of calcitonin gene-related peptide (CGRP) (0.3 nmol).

4 The concentration of CGRP in aqueous humour from untreated rabbit eyes was 0.1 ± 0.001 nmol l^{-1}. Irradiation of the iris raised the CGRP concentration to 8.9 ± 1.5 nmol l^{-1}. L-NAME (200 mg kg^{-1}) greatly suppressed the irradiation-evoked release of CGRP, the concentration in the aqueous humour being 1.2 ± 0.2 nmol l^{-1} ($P<0.001$). L-Arginine reversed the L-NAME-induced inhibition of release of CGRP, the concentration of CGRP in the aqueous humour being 9.7 ± 0.6 nmol l^{-1}.

5 In addition, a NO donor, sodium nitroprusside (0.9 μmol), was found to raise the concentration of CGRP in the aqueous humour (14.8 ± 0.8 nmol l^{-1}) and to induce symptoms of ocular inflammation. The elevation in concentration of CGRP induced by sodium nitroprusside was not affected by L-NAME (200 mg kg^{-1}) (14.5 ± 1.2 nmol l^{-1}). Ocular responses were not inhibited by L-NAME.

6 Our findings suggest that NO plays an important role in ocular inflammation by activating C-fibres (directly or indirectly) and by mediating CGRP-induced responses.

Keywords: Nitric oxide; C-fibres; transmitter release; calcitonin gene-related peptide; ocular inflammation; rabbit eye

Introduction

The inflammatory response in the eye consists of miosis, conjunctival hyperaemia and breakdown of the blood-aqueous barrier with subsequent leakage of protein into the aqueous humour. There is much evidence to suggest that C-fibre neurotransmitters, such as substance P and calcitonin gene-related peptide (CGRP), play a key role in the ocular response to injury (Stone *et al.*, 1987; Unger, 1990). As there is no barrier separating the iris and the ciliary body from the anterior chamber, any agent that is released locally will diffuse into the anterior chamber, making the eye an excellent model for studies of transmitter release.

NO is a short-lived molecule displaying numerous bioactivities (Moncada *et al.*, 1991). It is generated from L-arginine by the enzyme NO synthase (NOS), which is inhibited effectively, both *in vitro* and *in vivo*, by analogues of L-arginine, e.g. N^G-nitro-L-arginine (L-NAME) (Moncada *et al.*, 1991). Also, some nitrovasodilators, such as sodium nitroprusside, release NO spontaneously (Moncada *et al.*, 1991).

NOS has been demonstrated by immunostaining in the nerve fibres in the rat eye; the nerve fibres were moderate in number in the choroid and few in the anterior uvea (Yamamoto *et al.*, 1993). NOS activity has also been measured in the anterior uvea of the rabbit (Osborne *et al.*, 1993) where intravenous injection of L-NAME was found to reduce the regional blood flow (Seligsohn & Bill, 1993). These findings suggest that NO may be of physiological and/or pathophysiological significance in the control of ocular function. In the present study, we have investigated the actions of NO in the rabbit eye, with particular emphasis on the relationship between NO and C-fibres and on those effects of NO that may be of importance in the inflammatory response to C-fibre stimulation.

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Methods

Ocular inflammation induced by infrared irradiation of the iris

Inflammation in the eye was induced by infrared irradiation of the iris for 2 min, which results in a minor and reversible damage of the blood-aqueous barrier (Dyster-Aas & Krakau, 1964). The breakdown of the blood-aqueous barrier was determined by photoelectric measurement of the aqueous flare response (AFR) in the anterior chamber (Anjou & Krakau, 1961). This response is a Tyndall phenomenon in the anterior chamber reflecting protein leakage across the blood-aqueous barrier. Briefly, a narrow beam of light is passed through the anterior chamber. In the presence of large molecules (mainly proteins) in the aqueous humour, light scattering (aqueous flare) occurs. A correlation between the density of the AFR and the protein concentration has been established (Anjou & Krakau, 1961; Dyster-Aas & Krakau, 1964). Conjunctival hyperemia was assessed visually. The pupillary diameter was measured with a transparent ruler under constant and uniform illumination.

L-NAME (10–200 mg kg^{-1}, in 3 ml saline) was given by intravenous injection 30 min before the irradiation in order to establish a relationship between dose and the degree of inhibition of the AFR (Figure 1a). In most of the experiments, L-NAME was used at a dose of 200 mg kg^{-1} to obtain a near-maximal (Figure 1a) and relatively long-lasting inhibition of NOS-dependent responses. This choice of dose was supported by earlier reports by Persson *et al.* (1991) and Seligsohn & Bill (1993), showing that 100 mg kg^{-1} failed to induce maximal inhibition of NOS and that 30 mg kg^{-1} reduced the regional blood flow in rabbit uvea only for a short period of time (<20 min).

The rabbits were divided into five groups: group 1 was pretreated with L-NAME (200 mg kg^{-1}); group 2 was pretreated with D-NAME (200 mg kg^{-1}); group 3 was pretreated

with L-NAME + L-arginine (500 mg kg^{-1}); group 4 was pretreated with L-NAME + D-arginine (500 mg kg^{-1}); group 5 served as control (no injection before irradiation). Each group included six to eight rabbits.

Intravitreal injection of CGRP and sodium nitroprusside

CGRP (0.3 nmol) or sodium nitroprusside (0.9 μmol) was given by intravitreal injection (30 μl) into the corpus vitreum, 3–4 mm posteriorly to the limbus; the contralateral eye received vehicle (Holmdahl *et al.*, 1981).

In each study, the rabbits were divided into two groups: one group was pretreated with L-NAME (200 mg kg^{-1}, in 3 ml saline) given by intravenous injection 30 min before the intravitreal injection of either CGRP or sodium nitroprusside, the other group served as control (no injection of L-NAME before the intravitreal injection). Each group included six to eight rabbits.

Radioimmunoassay of CGRP in the aqueous humour

In another series of experiments, samples of aqueous humour were collected from the anterior chamber just after the AFR had reached maximum (1 h after the infrared irradiation and 4 h after the intravitreal injection of sodium nitroprusside). Samples of aqueous humour were also collected from untreated rabbits and from the rabbits injected with L-NAME only (1 h after the injection). The samples were frozen on dry ice and stored at −80°C until assayed for CGRP. Whenever aqueous humour was collected or intravitreal injections were given, the rabbits were anaesthetized by an injection of methohexitone sodium (5 mg kg^{-1}) into an ear vein. No anaesthesia was required during the rest of the experiments.

CGRP was measured by radioimmunoassay (RIA), using [^{125}I]-(Tyr°) CGRP (rat) as a tracer. The ID_{50} of the assay is 860 pmol l^{-1}. The detection limit is about 100 pmol l^{-1} and the interassay variation is 10%. CGRP variants (human CGRP I and II) cross-react with the antiserum better than 100% on a molar basis, but there is no cross-reaction with calcitonin, katacalcin, C-terminal adjacent peptide, tachykinins, neuropeptide Y and VIP (Grunditz *et al.*, 1986; Wahlestedt *et al.*, 1986).

Drugs

CGRP was from Peninsula (Merseyside, St. Helens, U.K.) and other drugs were from Sigma (St. Louis, MO, U.S.A.). They were dissolved in 0.9% saline. Since the sodium nitroprusside solution had a pH of 3.8, control eyes received saline in which the pH had been adjusted to this value by the addition of a small amount of 1 M hydrochloric acid.

Analysis of results

Data are expressed as mean ± s.e.mean. Student's *t* test was used for statistical analysis and the difference between unpaired groups was considered significant when $P<0.05$.

Results

Ocular responses to infrared irradiation of the iris

Irradiation induced miosis with pupil sizes of 1.8 ± 0.1 mm after 2 min compared with 5.9 ± 0.1 mm in the non-irradiated eye ($n=8$). The miosis subsided within 1.5 h; at that time the pupil sizes were 5.7 ± 0.2 and 5.9 ± 0.3 mm in irradiated and non-irradiated eyes, respectively. An AFR was noted about 15 min after the irradiation, reaching maximum after 1–2 (Figure 1). The conjunctiva displayed moderate hyperaemia (lasting for 2–3h). A transient and mild conjunctival hyperaemia (lasting for about 30 min) could be observed also in the non-irradiated eye but no miosis and no AFR. L-NAME inhibited the irradiation-evoked AFR in a dose-dependent manner (Figure 1a). The conjunctival hyperaemia was virtually abolished by L-NAME (200 mg kg^{-1}), while the miosis was unaffected, the pupil size being 1.8 ± 0.3 and 5.9 ± 0.2 mm, 2 min and 1.5 h after the irradiation, respectively. Irradition-evoked responses were not affected by D-NAME (not shown). The inhibitory effect of L-NAME on the conjunctival hyperaemia and the AFR could be partially reversed by L-arginine (Figure 1b) but not by D-arginine (not shown).

Ocular responses to CGRP and sodium nitroprusside

Intravitreal injection of CGRP induced severe conjunctival hyperaemia after 30–60 min. Also, the AFR started about 30 min after the injection and reached maximum after 2–3 h (Figure 2). No miosis was observed. In rabbits pretreated with L-NAME, CGRP failed to induce AFR and conjunctival hyperaemia (Figure 2).

Intravitreal injection of sodium nitroprusside also induced moderate conjunctival hyperaemia after 30–60 min. The AFR started about 30 min after the injection and reached maximum after about 4 h (Figure 3). No miosis was observed. In rabbits pretreated with L-NAME, sodium nitroprusside-induced AFR was only marginally suppressed; the significant differences between L-NAME-pretreated and control rabbits could only be observed 6–8 h after the injection of sodium nitroprusside (Figure 3).

Control eyes (injected with vehicle) displayed a minor and short-lasting (15–30 min) conjunctival hyperaemia but neither miosis nor AFR could observed. The hyperaemia was abolished by L-NAME (not shown).

Release of CGRP into the aqueous humour

The concentration of CGRP in the aqueous humour from the untreated rabbit eye was 0.1 ± 0.001 nmol l^{-1} ($n=12$) and was 0.14 ± 0.4 nmol l^{-1} after L-NAME injection ($n=6$) ($P>0.05$).

After irradiation, the concentration of CGRP was elevated greatly in the aqueous humour (Figure 4). In the L-NAME-pretreated rabbits, the concentration of CGRP was also elevated but much less than without L-NAME (Figure 4) ($P<0.001$). The inhibitory effect of L-NAME was reversed completely in the presence of L-arginine (Figure 4). The concentration of CGRP in the aqueous humour was elevated

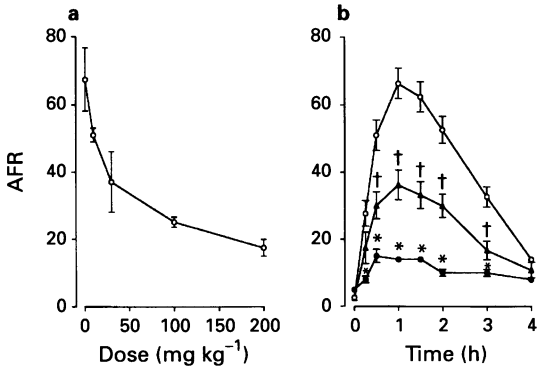

Figure 1 Infrared irradiation of the iris for 2 min results in a minor and reversible damage of the blood-aqueous barrier with subsequent leakage of protein into the aqueous humour, reflected in the so-called aqueous flare response (AFR). (a) The NOS inhibitor, L-NAME, inhibited the AFR in a dose-dependent manner. (b) L-NAME (200 mg kg^{-1}), inhibited the AFR almost completely (●). The inhibition could be partially reversed by L-arginine (500 mg kg^{-1}) (▲). Control rabbits did not receive L-NAME (○). Student's *t* test was used for statistical analysis at each time point. *Indicates significant difference ($P<0.05$ or 0.01) between L-NAME-pretreated group and control group. †: Indicates significant difference ($P<0.05$ or 0.01) between L-NAME + L-arginine pretreated group and L-NAME pretreated group and also between L-NAME + L-arginine pretreated group and control group. Means ± s.e. of mean. Six to eight rabbits in each group (a and b).

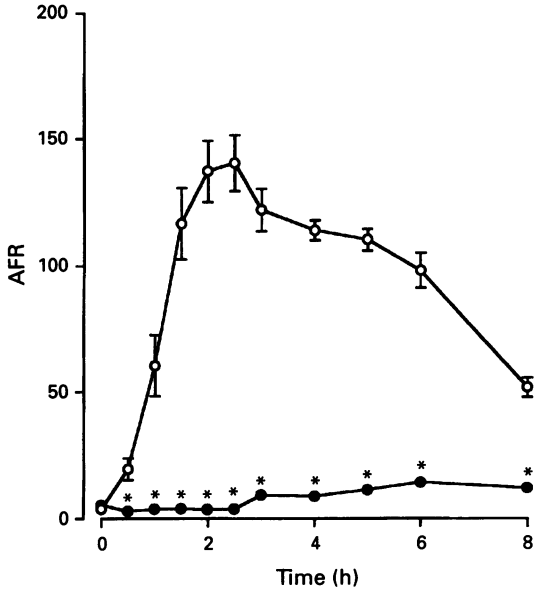

Figure 2 CGRP induces some of the symptoms of ocular inflammation. AFR induced by intravitreal injection of 0.3 nmol of CGRP was abolished by pretreatment with L-NAME (●) (200 mg kg^{-1}). Control rabbits did not receive L-NAME (○). Means ± s.e.mean. Six to eight rabbits in each group. Student's *t* test was used for statistical analysis at each time point. *Indicates significant difference ($P<0.01$ or 0.001) between L-NAME-pretreated group and control group.

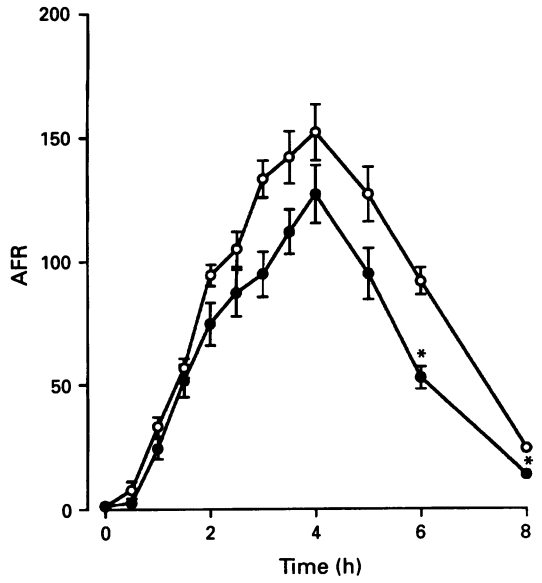

Figure 3 Sodium nitroprusside, which releases NO spontaneously, induces some of the symptoms of ocular inflammation, including AFR. L-NAME (200 mg kg^{-1}) only slightly inhibited the sodium nitroprusside-evoked AFR (●). Control rabbits did not receive L-NAME (○). Means ± s.e. of mean. Six to eight rabbits in each group. Student's *t* test was used for statistical analysis at each time point. *Indicates significant difference ($P<0.05$ or 0.01) between L-NAME-pretreated group and control group.

greatly after intravitreal injection of sodium nitroprusside (Figure 4); L-NAME did not affect this response (Figure 4) ($P>0.05$).

Discussion

The results show that L-NAME, but not D-NAME, induces specific blockade of ocular responses to infrared irradiation of the iris and to CGRP, probably because L-NAME inhibits

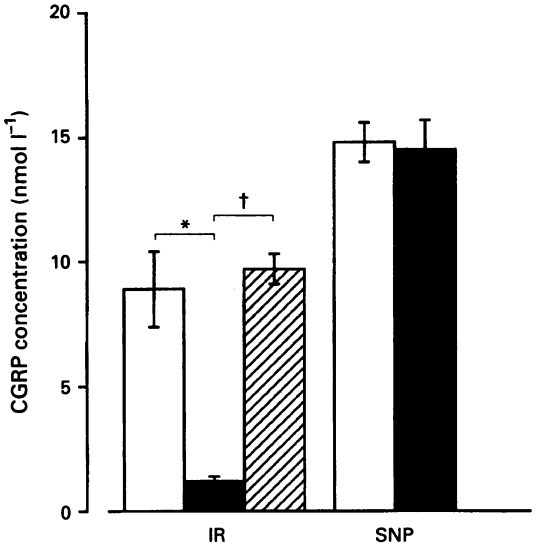

Figure 4 Noxious stimuli release neuropeptides from C-fibres into the aqueous humour. The concentrations of CGRP were elevated greatly in the aqueous humour of eyes exposed to infrared irradiation (IR) or injected with sodium nitroprusside (SNP). Open columns represent rabbits that did not receive the NOS inhibitor L-NAME. Solid columns represent rabbits pretreated with L-NAME (200 mg kg^{-1}). L-Arginine (500 mg kg^{-1}) reversed the L-NAME-induced inhibition of release of CGRP induced by IR (hatched column). Means ± s.e.mean. Six to eight rabbits in each group. Student's *t* test was used for statistical analysis. *Indicates significant difference ($P<0.001$) between L-NAME-pretreated group and control group. †Indicates significant difference ($P<0.001$) between L-NAME pretreated group and L-NAME + L-arginine pretreated group. There was no significant difference between L-NAME + L-arginine group and control group in response to IR and between L-NAME pretreated group and control group in response to SNP.

NOS. The L-NAME-induced inhibition was reversed by the L-form of arginine but not by D-arginine. The stereospecific effects of arginine and the arginine ester support the view that the effect of L-NAME on NO synthesis is a specific one.

CGRP, which is a well known C-fibre constitutent, is thought to play an important role in ocular inflammation (Wahlestedt *et al.*, 1986; Krootila *et al.*, 1988). The concentration of CGRP in the aqueous humour was greatly elevated in the inflamed eye, probably reflecting its release from excited C-fibres. Moreover, exogenously applied CGRP induces hyperaemia and breakdown of the blood-aqueous barrier (Wahlestedt *et al.*, 1986). The present study demonstrates that L-NAME inhibits the ocular responses to infrared irradiation, probably by inhibiting the release of transmitters/mediators from the C-fibres as well as by inhibiting CGRP-induced ocular responses. Moreover, sodium nitroprusside, which releases NO spontaneously (Moncada *et al.*, 1991), raised the concentration of CGRP in the aqueous humour greatly and caused breakdown of the blood-aqueous barrier. Hence, our results suggest that NO plays an important role in ocular inflammation and that in fact NO may have dual effects: first, NO is involved in the activation of C-fibres, causing release of transmitters such as CGRP; secondly, NO mediates the effects of CGRP (see also Andersson, 1992). Recently it was reported that L-NAME inhibited the capsaicin-induced (and C-fibre-mediated) increase in blood flow in rabbit skin, suggesting a role for NO in the activation of C-fibres (Hughes & Brain, 1994). Interestingly, NO has been found to accelerate the synthesis of prostaglandins which in turn may cooperate with NO in activating C-fibres (Sautebin *et al.*, 1995). Immunocytochemical studies, suggesting the existence of NOS in neurones and fibres of the dorsal root ganglia (Morris *et al.*, 1992), have raised the possibility that NO may have a signalling function within the sensory nervous system. Capsaicin releases transmitters from cultured dorsal root ganglion cells

by increasing intracellular cyclic GMP levels (Holzer, 1991). Conceivably, the stimulating effect of NO on sensory neurotransmitter release reflects its ability to increase cyclic GMP. Alternatively, NO has been shown to be released from non-adrenergic noncholinergic nerves in response to stimulation (for reviews, see Snyder & Bredt, 1991; Sanders & Ward, 1992). NO may enhance the transmitter release prejunctionally (Grider *et al.*, 1992).

The vascular actions of sensory transmitters concern vasodilatation as well as protein extravasation. Conceivably, vasodilatation occurs first followed by extravasation from the dilated blood vessels. In the course of the response of the rabbit eye to infrared irradiation, conjunctival hyperaemia (reflecting vasodilatation) appears promptly (within 2 min) while the AFR (reflecting protein extravasation) can be observed 15–30 min later. From the present results, it cannot be excluded that NO mediates CGRP-induced vasodilatation and that CGRP is responsible for the subsequent extravasation from dilated blood vessels. Alternatively, NO may induce vasodilatation and extravasation directly if a relatively large amount of NO is produced. Hence, NO and CGRP (possibly together with other C-fibre transmitters) may cooperate in controlling vascular functions although the precise mechanisms that underly such cooperation remain to be clarified.

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References

ANDERSSON, S.E. (1992). Glibenclamide and L-N^G-nitro-arginine methyl ester modulate the ocular and hypotensive effects of calcitonin gene-related peptide. *Eur. J. Pharmacol.*, **224,** 89–91.

ANJOU, C.I.N. & KRAKAU, C.E.T. (1961). Aqueous flare and protein content in the anterior chamber of normal rabbits' eyes. *Acta Ophthalmol.*, **39,** 95–101.

DYSTER-AAS, H.K. & KRAKAU, C.E.T. (1964). Aqueous flare determination in the rabbit by means of a minimal eye trauma. *Invest. Ophthalmol.*, **3,** 127–134.

GRIDER, J.R., MURTHY, K.S., JIN, J.-G. & MAKHLOUF, G.M. (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am. J. Physiol.*, **G774–778.**

GRUNDITZ, T., EKMAN, R., HÅKANSON, R., RERUP, C., SUNDLER, F. & UDDMAN, R. (1986). Calcitonin gene-related peptide in the thyroid nerve fibres and C cells: effects on thyroid hormone secretion and response to hypercalcemia. *Endocrinology*, **119,** 2313–2324.

HOLMDAHL, G., HÅKANSON, R., LEANDER, S., ROSELL, S., FOLKERS, K. & SUNDLER, F. (1981). A substance P antagonist, D-Pro^2, D-$Trp^{7,9}$-SP, inhibits inflammatory responses in the rabbit eye. *Science*, **214,** 1029–1031.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43,** 143–201.

HUGHES, S.R. & BRAIN, S.D. (1994). Nitric oxide-dependent release of vasodilator quantities of calcitonin gene-related peptide from capsaicin-sensitive nerves in rabbit skin. *Br. J. Pharmacol.*, **111,** 425–430.

KROOTILA, K., UUSITALO, H. & PALKAMA, A. (1988). Effect of neurogenic irritation and calcitonin gene-related peptide (CGRP) on ocular blood flow in the rabbit. *Curr. Eye Res.*, **7,** 695–703.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43,** 109–142.

MORRIS, R., SOUTHAM, E., BRAID, D.J. & GARTHWAITE, J. (1992). Nitric oxide may act as a messenger between dorsal root ganglion neurones and their satellite cells. *Neurosci. Lett.*, **137,** 29–32.

OSBORNE, N.N., BARNETT, N.L. & HERRERA, A.J. (1993). NADPH diaphorase localization and nitric oxide synthetase activity in the retina and anterior uvea of the rabbit eye. *Brain Res.*, **610,** 194–198.

PERSSON, M.G., WIKLUND, N.P. & GUSTAFSSON, L.E. (1991). Nitric oxide requirement for vasomotor nerve-induced vasodilatation and modulation of resting blood flow in muscle microcirculation. *Acta Physiol. Scand.*, **141,** 49–56.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262,** G379–392.

SAUTEBIN, L., IALENTI, A., IANARO, A. & ROSA, M.D. (1995). Modulation by nitric oxide of prostaglandin biosynthesis in the rat. *Br. J. Pharmacol.*, **114,** 323–328.

SELIGSOHN, E.E. & BILL, A. (1993). Effects of N^G-nitro-L-arginine methyl ester on the cardiovascular system of the anaesthetized rabbit and on the cardiovascular response to thyrotropin-releasing hormone. *Br. J. Pharmacol.*, **109,** 1219–1225.

SNYDER, S.H. & BREDT, D.S. (1991). Nitric oxide as a neuronal messenger. *Trends Pharmacol. Sci.*, **12,** 125–128.

STONE, R.A., KUWAYAMA, Y. & LATIES, A.M. (1987). Regulatory peptides in the eye. *Experientia*, **43,** 791–800.

UNGER, W.G. (1990). Mediation of the ocular response to injury. *J. Ocul. Pharmacol.*, **6,** 337–353.

WAHLESTEDT, C., BEDING, B., EKMAN, R., OKSALA, O., STJERNSCHANTZ, J. & HÅKANSON, R. (1986). Calcitonin gene-related peptide in the eye: release by sensory nerve stimulation and effects associated with neurogenic inflammation. *Regul. Pept.*, **16,** 107–115.

YAMAMOTO, R., BREDT, D.S., SNYDER, S.H. & STONE, R.A. (1993). The localization of nitric oxide synthase in the rat eye and related cranial ganglia. *Neurosci.*, **54,** 189–195.

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