Cross talk between receptors mediating contraction and relaxation in the arterioles but not the dilator muscle of the rat iris

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1 Sympathetic nerve stimulation causes contraction of the dilator muscle and the large arterioles of the iris via the activation of α_{1B} -adrenoceptors. We have investigated whether increases in adenosine 3': 5'-cyclic monophosphate (cyclic AMP) and the activation of receptors in these tissues can modulate these nerve-mediated contractions.

2 Increasing intracellular cyclic AMP with dibutyryl cyclic AMP (1 mM), forskolin (50 μ M) or isobutylmethylxanthine (100 μ M) produced relaxation of both the dilator and the arterioles, abolished the nerve-mediated constriction of the arterioles, but potentiated the nerve-mediated contraction of the iris dilator.

3 Pretreatment of the preparations with cholera toxin, to activate G_s permanently, caused a dilatation of the arterioles and abolished the nerve-mediated constriction but had no effect on the dilator muscle.

4 The β -adrenoceptor agonist, isoprenaline (1 μ M), the adenosine- A_1 - A_2 agonist, N-ethylcarboxamidoadenosine NECA (100 nM), in the presence of the A_1 -selective antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX, 10 nM), and calcitonin gene-related peptide (CGRP, 10 nM) all separately caused a dilatation of the arterioles and abolished the nerve-mediated constriction, while only isoprenaline (1 μ M) produced an effect on the dilator, i.e. a relaxation but a potentiation of the nerve-mediated contraction. These results suggest the presence of at least 3 types of receptor linked to G_s and an increase in cyclic AMP in the arterioles, i.e. β -adrenoceptor, adenosine- A_2 and CGRP, but only 1 G_s -linked receptor, i.e. β -adrenoceptors, on the dilator muscle cells.

5 Neither the A_1 -selective agonist, cyclohexyladenosine (CHA, 10 nM) nor the A_1 -selective antagonist, DPCPX (10 nM) had any significant effect on the nerve-mediated constriction of the arterioles, suggesting that presynaptic A_1 -receptors do not play a role in modulating the sympathetic nerve-mediated constriction.

6 Forskolin (50 μ M), in the presence of capsaicin (10 μ M) to inactivate unmyelinated CGRP-containing sensory nerves, still caused a dilatation of the arterioles and abolished nerve-mediated constriction suggesting that the effects on the blood vessels were due to increases in cyclic AMP in the arteriolar cells and not in the sensory nerves.

7 Using reverse transcription polymerase chain reaction, we have demonstrated expression of the α_{1B} and α_{1C} molecular subtypes in the tissues of the iris. These molecular subtypes most likely correspond to the α_{1B} and α_{1A} pharmacological subtypes, respectively.

8 Preincubation in N^G -nitro-L-arginine methyl ester (L-NAME, 10 μ M), but not D-NAME (10 μ M), reduced the efficacy of forskolin in inhibiting the sympathetic nerve-mediated vasoconstriction. These results suggest that the inhibition of sympathetic nerve-mediated constriction by cyclic AMP in the arterioles is indirect via the production of nitric oxide.

9 Our results demonstrate that there is 'cross talk' between receptors linked to increases in cyclic AMP and α_{1B} -adrenoceptors in the arterioles of the rat iris, but that no such interaction occurs between cyclic AMP and α_{1B} -adrenoceptors in the dilator muscle. The interaction in the arterioles occurs as a result of cyclic AMP-mediated stimulation of nitric oxide synthesis, presumably by the arteriolar endothelial cells.

Keywords: Cyclic AMP; α_{1B} -adrenoceptor; arteriole; iris dilator muscle; sympathetic nerve; nitric oxide; cross talk

Introduction

A small proportion of neurones in the superior cervical ganglion give rise to the adrenergic innervation of the iris (Olson *et al.*, 1988). Studies that use the formaldehyde-induced fluorescence technique for the detection of catecholamines have shown that these sympathetic axons form a rich network of terminals covering the dilator muscle and the largest of the arterial blood vessels which are found within the stromal layer of the rat iris. Stimulation of these nerves causes the release of noradrenaline and a subsequent contraction of the iris dilator and a constriction of the arterioles (Takayanagi *et al.*, 1992; Hill *et al.*, 1993; Gould & Hill, 1994). In both these tissues, nerve-mediated contractile responses were 100 fold more sensitive to the α_1 -adrenoceptor antagonist, prazosin, than the α_2 -

adrenoceptor antagonist, yohimbine. Responses were also relatively insensitive to the α_1 -antagonist, WB4101, but were abolished following exposure to chloroethylclonidine (CEC) (Hill *et al.*, 1993; Gould & Hill, 1994). While CEC has been reported to inactivate α_{1B} -adrenoceptors, it has also been shown to have α_2 -adrenoceptor agonist actions (Bultmann & Starke, 1993) which, in the case of sympathetic nerves, could lead to a decrease in neurotransmitter release and a concomitant decrease in the postsynaptic response. In the arterioles of the rat iris, the action of CEC is postsynaptic, as it abolishes the effect of exogenous noradrenaline as well as the nerve-mediated contractile response (Gould & Hill, 1994). In view of these results, the receptors mediating nerve-induced contractile responses in both the iris dilator and arterioles have been identified pharmacologically as α_{1B} -adrenoceptors (Takayanagi *et al.*, 1992; Hill *et al.*, 1993; Gould & Hill, 1994).

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Activation of G-protein-coupled α_{1B} -adrenoceptors has been reported to lead to an increase in inositol phospholipid hydrolysis and release of intracellular calcium (Han *et al.*, 1987). Consistent with the involvement of this pathway, the nerve-mediated contractions of the dilator muscle and the arterioles are not dependent on the activation of voltage-sensitive calcium channels, which permit the movement of extracellular calcium into the muscle cells (Takayanagi *et al.*, 1992; Gould & Hill, 1994). Intracellular interactions between receptor-activated second messenger pathways which modulate the level of cyclic AMP, have been shown to alter the activity of the pathway involving inositol trisphosphate (IP_3) hydrolysis in smooth muscle (Abdel-Latif, 1989; 1991). In bovine sphincter there is a reciprocal effect between muscarinic receptor activation of IP_3 formation and cyclic AMP formation. For example, elevation of cyclic AMP with isoprenaline or forskolin inhibits carbachol-induced IP_3 formation and sphincter contraction (Tachado *et al.*, 1989; 1992). Thus it has been suggested that interaction between these two second messenger pathways may be one mechanism for regulating contraction and relaxation of smooth muscle (Abdel-Latif, 1991). Similar results have been found in the rat aorta, where increases in cyclic AMP have an inhibitory effect on noradrenaline-stimulated contraction due to α_1 -adrenoceptor activation (Lincoln & Fisher-Simpson, 1984; Manolopoulos *et al.*, 1991). This was reported to be due to a reduction in intracellular calcium and is perhaps accompanied by a reduction in the sensitivity of the contractile elements to calcium. The site at which cyclic AMP interacts with the IP_3 pathway was thought to occur distal to the membrane receptor coupling to phospholipase C. Both cyclic AMP and cyclic GMP have recently been shown to inhibit IP_3 -dependent intracellular calcium release and contraction due to agonist stimulation (Murthy *et al.*, 1993).

The present study investigates whether α_{1B} -adrenoceptor-mediated contractions of the dilator muscle and of the arterioles of the iris can be modulated by increases in intracellular cyclic AMP. We further investigate the possible existence, on the arterioles and on the dilator muscle, of specific receptor subtypes which, when activated, could be responsible for elevated cyclic AMP levels *in vivo*.

Methods

In vitro experiments

Wistar rats of either sex (4–6 weeks postnatal) were killed with an overdose of either anaesthetic and the irides removed. The irides were cut in half, the sphincter removed and the dilator pinned out in a 1 ml bath which was perfused with Krebs solution of the following composition (mM): NaCl 119.8, KCl 15.0, $CaCl_2$ 2.5, $MgCl_2$ 2.0, NaH_2PO_4 1.0, $NaHCO_3$ 24.4 and glucose 22.0, and gassed with a mixture of 95% O_2 plus 5% CO_2 . Preparations were equilibrated for 30 min before commencing transmural nerve stimulation, via platinum electrodes, at 10 Hz for 1 s (0.1 ms pulse width, 45 V) every 3 min. This stimulation produced consistent contractile responses of the dilator and blood vessels (mean resting diameter of $31.7 \pm 1.0 \mu m$). Hyoscine (1 μM) was added to the Krebs solution to prevent the effect of cholinergic nerves. Contractions were measured using video microscopy and the computer programme, DIAMTRAK (Nield, 1989), which uses averaging techniques to locate the edges of the arterioles and display their difference as arteriolar diameter on a chart recorder. At least 3 consistent control contractions were recorded before adding any drug. The magnitude of the vasoconstrictor responses to nerve stimulation in both control and drug solutions were expressed as a percentage of the resting vessel diameter. This was done in order to standardize nerve-mediated responses in vessels of different resting diameter. Dilator muscle contractile responses were measured after cutting off the sphincter muscle and locating the cut edge of the dilator with the programme DIAMTRAK. Drug effects were expressed as a percentage of

the average of 3–4 control responses prior to the addition of drug. Experimental values are given as the mean $\pm$ s.e. mean of results from at least 3 preparations. Vehicle controls were found to have no effect on nerve-mediated contractions. The adenosine- A_2 antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), was present 30 min before the addition of the adenosine- A_1 - A_2 agonist, N-ethylcarboxamidoadenosine (NECA). In experiments to test the effect of forskolin in the presence of capsaicin, N^G -nitro-L-arginine methyl ester (L-NAME) or D-NAME, the capsaicin, L-NAME or D-NAME was present in the Krebs solution for the entire experiment.

The significance of drug effects was tested by Analysis of Variance with 95% confidence limits, followed by unpaired *t* tests with Bonferroni correction for multiple groups.

Drugs and solutions

The following drugs were used: rat calcitonin gene-related peptide 1-37 (CGRP, Auspep, Australia), capsaicin (Fluka Chemie, Switzerland), N^G -nitro-L-arginine methyl ester (L-NAME) and N^G -nitro-D-arginine methyl ester (D-NAME, Sapphire Bioscience Pty. Ltd., Australia). Cholera toxin, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), ($\pm$)-isoprenaline hydrochloride, N^6 -cyclohexyladenosine (CHA), $N^6,2'$ -O-dibutyryl-adenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP), N-ethylcarboxamidoadenosine (NECA) and (–)-hyoscine (scopolamine) hydrochloride were all from Sigma, U.S.A.

All drugs were made up at the start of each experiment as 1000 $\times$ stocks, except for dibutyryl cyclic AMP which was dissolved directly in Krebs solution. Capsaicin, CHA, DPCPX, forskolin and IBMX stocks were dissolved in absolute ethanol, NECA stock was dissolved in 0.1 M HCl. Forskolin was also dissolved in 0.1% dimethyl sulphoxide. Stocks for all other drugs were dissolved in H_2O . Dilutions of all stocks were made in Krebs for final concentrations.

Expression of mRNA for α_1 -adrenoceptor subtypes in the iris

RNA isolation Total cellular RNA was isolated from freshly dissected iris tissue, from adult Wistar rats killed with an overdose of anaesthetic, using a single-step protocol for RNA isolation ('RNAzol B', Cinna/Biotech). Both the sphincter muscle and the ciliary processes were dissected from the iris. The iris tissue thus included the dilator muscle and the arterioles found within the stromal layer of the iris. Separation of these latter two tissues was not physically possible as the stroma overlies the dilator. The manufacturers recommended protocol for isolation of RNA was followed. Briefly, tissue was collected into RNAzol solution, homogenized at room temperature and the homogenate separated into two phases. The RNA was precipitated from the aqueous phase with isopropanol and the RNA pellet dissolved in water. Optical density readings were done to estimate the amount of total RNA before it was used in reverse transcription-polymerase chain reaction (RT-PCR).

Reverse transcription-polymerase chain reaction Oligodeoxynucleotide primers for use in RT-PCR were synthesized using an Applied Biosystems Model 380B Nucleic Acid Synthesiser and solid phase synthesis. Oligonucleotides were designed from published sequences of rat α_{1A} cDNA. Forward primer 5'-AGTGGGTGTCTTCTCTGGCGGCCCTT-3', and reverse primer 5'-GATCACTGCCATGGGTAGGTAGAA-3' (Lomasney *et al.*, 1991b) were used to generate a 517 base pair (bp) fragment; α_{1B} cDNA forward primer 5'-AGGGCCATCTCTGTGGGCCTGGTG-3', and reverse primer 5'-GATGGAGATGACCGTGGACAAGAC-3', (Voigt *et al.*, 1990) were used to generate a 405 bp fragment; the α_{1C} specific oligonucleotide forward primer was 5'-TGGCCATCATTC-TGGTTATGT-3' and reverse primer 5'-GCAACCCACCA

CGATGCCAG-3' (Alonso-Llamazares *et al.*, 1993) and these were employed to generate a 251 bp fragment.

Prior to RT-PCR, total RNA was treated with DNase I to avoid false positives being generated from the DNA present in RNA preparation. The DNase-pretreated RNA (3.9 μg) was reverse transcribed with oligo dT (300 ng) and 50 u of reverse transcriptase (StrataScript RNase H⁻). The reaction was carried out in a volume of 50 μl , and 2 μl was used in each PCR reaction.

PCR was performed in a capillary tube thermal cycler (Corbett Research, Sydney, Australia) using 20 μl aliquots under the following conditions: 10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.1% Triton X-100, deoxyribonucleoside triphosphates (dNTPs) - 200 μM each of dATP, dCTP, dGTP, dTTP, 24 pmol of each primer and 0.2 u of SuperTaq DNA polymerase (P.H. Stehelin and Cie AG, Basel). Samples were heat sealed and amplifications were performed for 30 cycles. The amplification products were separated in 2% agarose gel impregnated with ethidium bromide.

Results

Effects of increasing intracellular cyclic AMP

Three agents were used to test the effect of increasing intracellular cyclic AMP on the nerve-mediated contractile responses of the blood vessel and the dilator muscle: dibutyryl cyclic AMP (1 mM), IBMX (100 μM), a phosphodiesterase inhibitor, and forskolin (50 μM), a direct activator of adenylate cyclase. Increasing cyclic AMP with any of these three agents abolished the nerve-mediated constriction of the arterioles but potentiated the contractile response to nerve stimulation in the dilator muscle (Figures 1, 2). In addition, increasing cyclic

Figure 1 Effect of forskolin (50 μM) on the nerve-mediated contractions of the arterioles (a) and the dilator muscle (b). Arrows indicate nerve stimulation (10 Hz, 1 s). Dotted line represents previous resting diameters of the arteriole in control solution.

AMP caused a dilatation of the arterioles ($38.5 \pm 1.1 \mu\text{m}$, $n = 3$, in forskolin, 50 μM , compared to $33.3 \pm 1.2 \mu\text{m}$, $n = 3$, in control, $P > 0.05$) and a relaxation of the dilator muscle (Figure 1). Thus, an increase in cyclic AMP affected the nerve-mediated contractile responses differently in the two tissues.

Involvement of the GTP-binding protein, G_s

To investigate the physiological relevance of the effect of cyclic AMP on sympathetic nerve-mediated responses, the possible presence of the GTP-binding protein, G_s , was examined using cholera toxin. Cholera toxin ($2 \mu\text{g} \cdot \text{ml}^{-1}$) treatment for 1.5 h caused a dilatation of the arterioles and prevented the nerve-

Figure 2 Effect of (left to right) dibutyryl cyclic AMP (dbcAMP, 1 mM), forskolin (50 μM) and isobutylmethylxanthine (IBMX, 100 μM) on the nerve-mediated contractile responses of the arterioles (a) and on the nerve-mediated contractile responses of the dilator muscle (b). Columns represent the mean of 3 preparations. Vertical bars represent the s.e.mean.

Figure 3 Effect of treatment with cholera toxin ($2 \mu\text{g} \cdot \text{ml}^{-1}$) on the nerve-mediated contractions of (a) the arteriole and (b) the dilator muscle. Arrows indicate nerve stimulation (10 Hz, 1 s). Dotted line represents previous resting diameter of the arteriole in control solution.

mediated vasoconstriction, but had no effect on the nerve-mediated contraction of the dilator muscle (Figure 3).

Identity of receptors coupled to G_s in the dilator and arterioles

β -Adrenoceptors The β -agonist, isoprenaline (1 μ M), significantly reduced the sympathetic vasoconstriction (Figure 4). Isoprenaline also produced a relaxation of the dilator ($n=5$) and a concomitant potentiation of the nerve-mediated contraction of the dilator muscle.

Adenosine receptors Presynaptic adenosine- A_1 -receptor activation causes a decrease in neurotransmitter release from sympathetic nerves (Starke *et al.*, 1989; von-Kugelgen *et al.*, 1992), and thus could decrease the response to nerve stimulation. The presence of adenosine receptors was therefore examined. The A_1 -selective agonist, CHA (10 nM) did not significantly reduce the sympathetic vasoconstriction (Figure 4).

The A_1 -selective antagonist, DPCPX (10 nM), had no significant effect on nerve-mediated vasoconstriction, confirming the absence of a role of presynaptic A_1 -receptors (Figure 4). In the presence of DPCPX (10 nM), the adenosine- A_1 - A_2 agonist, NECA (10 and 100 nM), concentration-dependently reduced the sympathetic vasoconstriction (Figure 4) but did not relax the dilator.

CGRP receptors CGRP (100 nM–100 pM) caused a vasodilatation and a concentration-dependent reduction of sympathetic vasoconstriction (Figure 5) but had no effect on the dilator. The inhibitory action of CGRP on nerve-mediated vasoconstriction rapidly desensitized (within 3 min for the higher concentrations). In the presence of CGRP, while the

Figure 4 Effect of (left to right) isoprenaline (Iso 1 μ M, $n=5$), CHA (10 nM, $n=4$), DPCPX (10 nM, $n=6$), DPCPX (10 nM) and NECA (10 nM, $n=3$), DPCPX (10 nM) and NECA (100 nM, $n=3$) on the nerve-mediated constriction of the arterioles (Control, $n=15$). Columns represent the mean $\pm$ s.e. mean of the number of preparations in parentheses. *Differs significantly from control ($P<0.05$); ** differs significantly from DPCPX alone ($P<0.05$). For abbreviations, see text.

responses to nerve stimulation had desensitized, the arteriole continued to relax.

Does the effect of increased cyclic AMP in the arterioles occur in the arteriolar cells or in the sensory nerves?

After inhibiting sensory nerve transmission by treatment with capsaicin (10 μ M) for 15 min, forskolin (50 μ M) abolished sympathetic nerve-mediated vasoconstriction (Figure 6).

Expression of mRNA for α_1 -adrenoceptor subtypes

In order to confirm the molecular identity of the α_1 -adrenoceptor subtypes identified pharmacologically as α_{1B} in the dilator and the arterioles, we used RT-PCR with subtype-specific primers. The results from RT-PCR on the rat iris show two PCR products. Both α_{1B} (405 bp) and α_{1C} (251 bp) primers have amplified products of appropriate size from the same pool of cDNA that was synthesized with oligo dT primers (Figure 7, lanes 4, 6). Under similar conditions and using

Figure 5 Concentration-response curve for the effect of rat calcitonin gene-related peptide (CGRP) on the nerve-mediated constriction of the arterioles. Points represent the means $\pm$ s.e. mean of data from 5 preparations.

Figure 6 Effect of capsaicin treatment on the response to forskolin. (a) Shows nerve-mediated constriction of the arterioles after perfusing with capsaicin (10 μ M) for 15 min; (b) shows the effect of subsequent addition of forskolin (50 μ M) on the nerve-mediated constriction of the arteriole in the continued presence of capsaicin (10 μ M). Traces are representative of 3 preparations. Arrows indicate nerve stimulation (10 Hz, 1 s). Dotted line indicates previous resting diameter of the arteriole in capsaicin solution.

Figure 7 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA from dilator muscle cells. RNA was isolated with RNazol and treated with DNaseI, then reverse transcribed with oligo dT (lanes 2,4,6) or with oligo dT but no reverse transcriptase (lanes 3, 5, 7). PCR was carried out in the capillary tube thermal cycler using oligonucleotide primers specific for α_{1A} (lanes 2,3), α_{1B} (lanes 4,5), and α_{1C} -adrenoceptors (lanes 6,7) for 30 cycles. Control PCR reaction without RNA is shown in lane 8 and DNA size markers (ϕ X174/HaeIII) in lane 1.

Figure 8 Effect of forskolin on nerve-mediated vasoconstriction in the presence of N^G -nitro-D-arginine methyl ester (D-NAME, 10 μ M, open columns) and L-NAME (10 μ M, stippled columns). Columns represent means and s.e. means of 3-4 preparations. *Differs significantly from equivalent forskolin concentration in the presence of D-NAME ($P \leq 0.05$).

cDNA from the same reverse transcription pool, no PCR fragments were detected in samples which used oligonucleotide specific primers for α_{1A} -receptors (Figure 7, lane 2).

Is the inhibition of vasoconstriction by increased cyclic AMP indirect via the production of nitric oxide?

In vessels such as the rat aorta, vascular relaxation by elevated cyclic AMP may be produced indirectly via the production of nitric oxide (Gray & Marshall, 1992a,b). Forskolin caused a concentration-dependent inhibition of nerve-mediated vasoconstriction in the presence of the inactive, D-NAME (10 μ M). This inhibition was significantly reduced ($P < 0.05$) in the presence of the nitric oxide synthase inhibitor, L-NAME (10 μ M, Figure 8). There was no significant difference between the size of the nerve-mediated constriction, expressed as a percentage of the resting vessel diameter, recorded in the presence of L-NAME ($28 \pm 3.1\%$, $n = 10$) or D-NAME ($25.5 \pm 2.2\%$, $n = 10$) alone.

Discussion

Previous pharmacological studies have shown that, in the rat, both the nerve-mediated constriction of the larger irideal arterioles and the nerve-mediated contraction of the dilator muscle of the iris result from the activation of α_{1B} -adrenoceptors (Hill *et al.*, 1993; Gould & Hill, 1994). In the present study, we have examined the effect of elevated cyclic AMP formation on the nerve-mediated contractions of these two tissues. Intracellular cyclic AMP production was increased by one of three different agents: (1) dibutyl cyclic AMP, (2) forskolin, which activates adenylate cyclase to elevate cyclic AMP production or (3) IBMX, a non-selective phosphodiesterase inhibitor, to prevent the breakdown of cyclic AMP. Elevation of cyclic AMP by any of these means produced a relaxation of the dilator and of the arterioles. On the other hand, an increase in cyclic AMP abolished nerve-mediated constriction of the arterioles but potentiated the nerve-mediated contraction of the dilator. Thus cyclic AMP had opposite effects on these two nerve-mediated responses, both of which result from the activation of α_{1B} -adrenoceptors.

The physiological relevance of the above results was tested by examining the possible presence of the GTP binding protein, G_s , in the dilator and arterioles using cholera toxin which causes ADP ribosylation and permanent activation of G_s and adenylate cyclase (Cassel & Selinger, 1977; Milligan, 1988). In the present study, cholera toxin caused a vasodilatation and prevented the nerve-mediated constriction of the arterioles, but had no effect on the nerve-mediated dilator contraction. These results suggested that G_s is present in the cells of the arterioles but not in the cells of the dilator muscle. We addressed the possibility that receptors coupled to G_s are present on the arterioles, but not the dilator, by testing the effect of agonists from receptors which are known to be coupled to G_s , on nerve-mediated responses. Such receptors could be activated by transmitters released from sympathetic or sensory nerves. Responses to the activation of parasympathetic cholinergic nerves were prevented by the presence of hyoscine in the Krebs solution.

The sympathetic neurotransmitter noradrenaline may activate both α - and β -adrenoceptors. Activation of presynaptic β -adrenoceptors has been reported to have a facilitatory action on the release of noradrenaline (Medgett, 1983) and would potentiate the response to noradrenaline and not reduce it. Activation of postsynaptic β -adrenoceptors, on the other hand, would lead to an increase in cyclic AMP and an inhibition of nerve-mediated responses. The β -agonist isoprenaline relaxed the dilator, suggesting that β -adrenoceptors on the surface of the dilator can mediate relaxation. Isoprenaline reduced the nerve-mediated vasoconstriction, also suggesting the presence of β -adrenoceptors on the arterioles.

ATP is a cotransmitter with noradrenaline in the sympathetic nervous system (Burnstock, 1990a,b; Starke *et al.*, 1991). ATP is rapidly hydrolysed to adenosine, which is a potent vasodilator. Adenosine- A_1 and - A_2 receptors are negatively and positively coupled to adenylate cyclase and cyclic AMP formation, respectively. Previous studies have shown an inhibitory purinergic prejunctional modulation of noradrenaline release in the rat iris via activation of A_1 -receptors (Fuder & Muth, 1993). Activation of A_1 -receptors by the selective agonist, CHA, had no effect on the dilator or arteriolar constriction. The antagonist DPCPX, which is selective for A_1 -receptors (Collis *et al.*, 1987; Vials & Burnstock, 1993), also had no significant effect on sympathetic vasoconstriction. These results suggest that presynaptic A_1 -receptors do not play a role in regulating neurotransmitter release from sympathetic nerves innervating the dilator or the arterioles. In the presence of DPCPX, the adenosine- A_1 - A_2 agonist, NECA, abolished the sympathetic vasoconstriction but had no effect on the dilator muscle. This indicates that adenosine- A_2 -receptor activation may modulate the sympathetic nerve-stimulated contraction of the arterioles but that no adenosine- A_2 response exists in the dilator.

The dilator muscle of the rat iris is supplied with nerves immunoreactive for the sensory neurotransmitters substance P and CGRP (Olson *et al.*, 1988). While both substance P and CGRP are potent vasodilators, only CGRP stimulates cyclic AMP production following activation of its receptors (Kubota *et al.*, 1985; Hirata *et al.*, 1988; Pernow, 1989; Maynard *et al.*, 1990). CGRP has also been identified as the neurotransmitter which mediates the inhibitory effect of sensory nerves on sympathetic nerve-mediated constriction in rat mesenteric arteries and rabbit ear arteries (Han *et al.*, 1990; Maynard *et al.*, 1990). Transmural nerve stimulation utilized in the present study will activate all the nerves present in the iris and so CGRP may have been released from sensory nerves. Exogenous CGRP caused vasodilatation and reduced nerve-mediated constriction of the arterioles but it neither caused relaxation of the dilator nor affected nerve-mediated contraction. These results suggest the presence of receptors for CGRP on the arterioles but not the dilator. The inhibitory effect of CGRP on nerve-mediated vasoconstriction showed a rapid desensitization compared to the longer lasting vasodilatation produced by the peptide. The different time-courses of the two effects of CGRP on the arterioles suggest that they may be produced by different mechanisms.

These experiments indicate that at least three types of receptor coupled to G_s activation are present on the arterioles and can have an inhibitory effect on nerve-mediated vasoconstriction. Only one of the three receptor types examined is present on the dilator muscle. Elevated cyclic AMP caused a relaxation of the dilator muscle, but this relaxation was not observed in the dilator after treatment with cholera toxin to activate G_s . One explanation for this apparent discrepancy may be that the concentration of cholera toxin used in this study may not have activated all the G_s present in the iris.

Increases in cyclic AMP in sensory axons or activation of presynaptic receptors leading to an increase in cyclic AMP leads to an increase in neurotransmitter release from sensory nerves (Rhoden & Barnes, 1990). For example, in the rabbit iris sphincter, activation of adenosine- A_2 -receptors on the sensory nerves enhances sensory nerve transmission (Gustafsson & Wiklund, 1986). It is possible, then, that the A_2 -agonists, and elevated cyclic AMP, may be acting on the sensory nerves to produce an increased release of other neuropeptides, such as substance P. This increased release of neuropeptides may be responsible for the inhibition of sympathetic vasoconstriction via a postsynaptic mechanism other than increased cyclic AMP. We tested this hypothesis by examining the effect of forskolin on nerve-mediated responses after treatment with capsaicin, to inhibit sensory nerve transmission (Holzer, 1991); forskolin still inhibited sympathetic vasoconstriction as well as producing a relaxation of the arterioles and the dilator. These results thus show that the differential effect of cyclic AMP in inhibiting arteriolar constriction but not dilator contraction is not due to an effect on sensory nerves.

The results of this study show that an increase in cyclic AMP leads to the abolition of nerve-mediated constriction of arterioles in the iris, but has no effect on the nerve-mediated contraction of the dilator in spite of apparently identical receptor subtypes mediating these two responses. The ability of the β -agonist, isoprenaline, to relax the dilator suggests that this difference is not due to the absence of receptors coupled to G_s and increases in cyclic AMP. The differential effect of cyclic AMP is also independent of sensory nerve stimulation, suggesting that the difference in responses is occurring postsynaptically. The sympathetic nerves innervating the arterioles and the dilator muscle both contain neuropeptide Y (NPY), in

addition to noradrenaline (Olson *et al.*, 1988), and so the difference between the two tissues is also unlikely to result from differences in the content of co-released substances from sympathetic nerves.

One explanation for the difference in responsiveness to cyclic AMP may be that there is more than one subtype of α_1 -adrenoceptor present in the iris. Unfortunately, the arterioles lie in the stroma anterior to the single dilator cell layer and separation of the two tissue types by dissection is not possible. Using RT-PCR, we have determined that both the α_{1B} - and the α_{1C} -adrenoceptor subtypes are expressed in the tissues of the iris. Recent reviews have highlighted the confusion in the nomenclature of α_1 -adrenoceptors based on the results of pharmacological and molecular biological studies (Ford *et al.*, 1994). According to this latter classification, the two receptor subtypes detected in the iris correspond to the pharmacological α_{1B} - and α_{1A} -adrenoceptor subtypes respectively. It seems unlikely that the receptors in either the arterioles or the dilator could be mistakenly identified as α_{1B} instead of α_{1A} as these two subtypes differ in both their sensitivity to WB4101 and to CEC (Lomasney *et al.*, 1991a). These results then lend support to the pharmacological characterization of the subtype of synaptic receptors in both tissues as α_{1B} . The location of the α_{1A} -adrenoceptors is unknown although it is tempting to speculate that they may be located on melanophores in the irideal stroma (Hill *et al.*, 1993).

In blood vessels, such as the rat aorta and porcine palmar lateral vein, elevated cyclic AMP levels produce an increase in cyclic GMP suggesting that an interaction occurs between these two second messengers (Gray & Marshall, 1992a,b; Wright *et al.*, 1994). In the rat aorta, effects of increased cyclic AMP following activation of either β -adrenoceptors or CGRP receptors are mediated indirectly via the production of nitric oxide by the endothelium (Gray & Marshall, 1992a,b). The differences in the responses to cyclic AMP of the α_{1B} -adrenoceptors in the irideal arterioles compared to those in the dilator muscle may therefore simply be due to the release of nitric oxide from the vascular endothelial cells. In the rat irideal arterioles, the nitric oxide synthase inhibitor, L-NAME, prevented the inhibition of sympathetic nerve-mediated vasoconstriction produced by forskolin. This suggests that in the arterioles, elevation of cyclic AMP may inhibit nerve-mediated vasoconstriction indirectly via the production of nitric oxide. The activity of nitric oxide synthase have been reported to be affected by phosphorylation by a number of cellular kinases (Bredt *et al.*, 1992). A phosphorylation site for the cyclic AMP-dependent protein kinase A on nitric oxide synthase has been identified (Bredt *et al.*, 1992) and this may provide a mechanism for the above response in the arterioles.

The present results provide evidence, in living arterioles, for cross talk between α_{1B} -adrenoceptors, coupled to IP_3 -dependent intracellular calcium release, and receptors coupled to increases in cyclic AMP. This interaction, however, occurs only as a result of the cyclic AMP-dependent activation of nitric oxide, presumably in the endothelial cells. In the dilator muscle, we have no evidence for an interaction between the α_{1B} -adrenoceptors and receptors coupled to increases in cyclic AMP. The potentiation of the α_{1B} -adrenoceptor response in the dilator muscle may simply reflect the change in resting tone of the preparation due to cyclic AMP. These results suggest that there is no nitric oxide synthase present in the dilator muscle cells or in the underlying epithelium.

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