British Journal of Pharmacology (1998) 125, 1–3
http://www.stockton-press.co.uk/bjp

SPECIAL REPORT

Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by 18-α glycyrrhetinic acid

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The gap junction inhibitor 18-α-glycyrrhetinic acid (α-GA, 100 μM) attenuated endothelium-dependent relaxations to acetylcholine and cyclopiazonic acid by ~20% in rings of pre-constricted rabbit iliac artery. The nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) inhibited relaxations to both agents by ~65% and these were further attenuated by α-GA to <10% of control. In endothelium-denuded preparations, relaxations to sodium nitroprusside were not affected by α-GA. Heterocellular gap junctional communication may therefore account for nitric oxide-independent relaxations evoked both by receptor-dependent and -independent mechanisms in rabbit iliac artery.

Keywords: Gap junctions; glycyrrhetinic acid; nitric oxide; endothelium-derived hyperpolarizing factor (EDHF)

Introduction Endothelium-dependent relaxations of blood vessels to agonists such as acetylcholine (ACh) are mediated by nitric oxide (NO), prostanoids and an endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Garland *et al*., 1995). The relative contribution of these factors may vary under different experimental conditions as all three may cause hyperpolarization of vascular smooth muscle (Parkington *et al*., 1993), release of EDHF is attenuated by NO (Bauersachs *et al*., 1996), and NO may stimulate the synthesis of prostanoids (Salvemini *et al*., 1993). The chemical nature of EDHF is unknown, candidate mediators including the endocannabinoid, anandamide (Randall & Kendall, 1998), and epoxyeicosatrienoic acids (EETs) which are cytochrome P_{450} mono-oxygenase derived metabolites of arachidonic acid (Bauersachs *et al*., 1994).

Ultrastructural studies have demonstrated the presence of gap junctional plaques in rabbit conduit arteries (Spagnoli *et al*., 1982), and functional studies have provided evidence that heterocellular gap junctional communication (GJC) contributes to NO- and prostanoid-independent relaxations mediated *via* the endothelium in rabbit aorta and mesenteric artery (Chaytor *et al*., 1998). It is possible that this mechanism of relaxation involves transfer of an EDHF from the endothelium to smooth muscle *via* gap junctions, which permit direct cell to cell communication through the diffusion of ions and small signalling molecules (for review see Evans, 1997). Constituent connexin proteins form a hexameric hemichannel or connexon containing a central pore, with docking of apposing connexons being effected by interactions between their extracellular loops. Chaytor *et al*. (1997) have shown that connexin 43 is the most prevalent subtype in endothelium-denuded rabbit superior mesenteric artery, and connexins 37, 40 and 43 have been identified in endothelial cells (Carter *et al*., 1996). Gap junction peptide 27, which possesses sequence homology with a region of the second extracellular loop of these connexins rapidly and reversibly attenuates endothelium-dependent relaxations of rabbit conduit arteries to agonists such as ACh and ATP without affecting NO-mediated relaxations or smooth muscle force development (Chaytor *et al*., 1998). This peptide also attenuates the NO-independent component of relaxation to cyclopiazonic acid (CPA), an inhibitor of Ca^{2+} sequestration by the endothelial endoplasmic reticulum, which stimulates depletion-activated Ca^{2+} influx and synthesis of both NO and EDHF in a receptor-independent fashion (Fukao *et al*., 1995; Chaytor *et al*., 1998).

Glycyrrhetinic acid (GA) is a lipophilic aglycone with a steroidal structure that can exist in α and β isoforms. Tracer studies have shown that α-GA and a variety of substituted derivatives reversibly interrupt GJC in cultured cells at micromolar concentrations; β-GA also inhibits GJC but shows non-specific effects at concentrations ~25 μM in human fibroblasts that are not seen with α-GA at concentrations up to 100 μM (Davidson *et al*., 1986, 1988; Guan *et al*., 1996; Goldberg *et al*., 1996). The effects of GA against GJC have not previously been evaluated in vascular tissues. In the present study we have therefore examined the efficacy of α-GA as an inhibitor of the gap junctional component of endothelium-dependent relaxations evoked by ACh and CPA in the rabbit isolated iliac artery.

Methods Male New Zealand White rabbits (2–2.5 kg) were killed by i.v. sodium pentobarbitone (120 mg kg^{-1}). Iliac arteries were removed and rings 2–3 mm in width cut and mounted in tissue baths containing Holmans buffer (composition in mM: 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 2.5 $NaHCO_3$, 11 glucose, 10 sucrose), aerated with 95% O_2, 5% CO_2 at 37°C. Tension was initially set at ~0.6 g and reset at intervals following stress relaxation during an equilibrium period of 1 h. The rings were contracted with 1 μM phenylephrine (PE) and cumulative concentration-response curves to ACh and CPA then constructed. Once maximum relaxation had been achieved the rings were washed, reconstricted, and further ACh concentration-response curves obtained in the presence of 100 μM α-GA, 300 μM N^G-nitro-L-arginine methyl ester (L-NAME) or their combination. Responses to sodium nitroprusside (SNP) were investigated in endothelium-denuded rings in the presence and absence of 100 μM α-GA. Removal of the endothelium was achieved by

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gentle abrasion of the intima with a roughened probe, denudation being confirmed by the absence of a relaxant response to 1 μM ACh at the beginning of each experiment. The effects of 100 μM β-GA were also investigated in endothelium-intact and -denuded rings. Data are given as mean $\pm$ s.e.mean. Fisher's test was used to compare concentration-response curves and the Student's t-test to compare maximal relaxations and EC_{50} values.

All reagents were obtained from Sigma. 18-α GA, 18-β GA and CPA were dissolved in DMSO; control experiments confirmed no significant effect on constrictor tone or ACh-induced relaxations at the final vehicle concentration employed. Other drugs were dissolved in buffer.

Results The tension developed by the iliac artery in response to 1 μM PE was 2.48 ± 0.07 g in endothelium-intact rings ($n=24$) and 2.62 ± 0.10 g in endothelium-denuded rings ($n=14$, no significant difference). Tension development in response to 1 μM PE in endothelium-intact rings was not significantly affected by incubation with α-GA (100 μM) for 1 h ($n=14$, 2.51 ± 0.14 g). By contrast, tension in pre-constricted preparations was reduced by $90 \pm 16\%$ following acute administration of β-GA (100 μM) in endothelium-intact rings, and by $75 \pm 18\%$ in endothelium-denuded rings ($n=4$, $P<0.01$ cf. control in each case).

Tension was maximally reduced by $88 \pm 2\%$ in response to ACh at a concentration of 3 μM ($n=13$, Figure 1a). In control experiments maximum relaxations remained at $93 \pm 6\%$ of their initial level after $\sim$6 h, confirming that there was no significant loss of response over time ($n=10$). In the presence of α-GA (100 μM) maximal reductions in tension to ACh occurred at 10 μM and were significantly decreased to $74 \pm 3\%$ (Figure 1a; $P<0.01$, $n=8$). EC_{50} values for ACh-induced relaxations were 0.22 ± 0.06 μM and 0.34 ± 0.10 μM in control and α-GA treated rings, respectively (no significant difference). Relaxation was inhibited by incubation with L-NAME (300 μM; 1 h) when ACh maximally reduced tension by $24 \pm 4\%$ ($P<0.01$, $n=5$), a value further reduced in the additional presence of 100 μM α-GA to $6 \pm 2\%$ (Figure 1a; $P<0.01$, $n=5$). The EC_{50} for ACh-induced relaxations in the presence of L-NAME was 1.79 ± 1.18 μM which was significantly larger than control ($P<0.05$, $n=8$).

In response to CPA, tension was maximally reduced by $92 \pm 3\%$ at a concentration of 60 μM ($n=11$, Figure 1b). α-GA (100 μM) significantly decreased this relaxation to $68 \pm 3\%$ ($P<0.01$, $n=6$). EC_{50} values for CPA-induced relaxation were 6.23 ± 0.83 μM and 5.50 ± 0.71 μM for control and α-GA treated tissues, respectively (no significant difference). Maximal reductions in tension were attenuated to $38 \pm 2\%$ in the presence of L-NAME (300 μM) ($P<0.01$, $n=5$), and to $14 \pm 7\%$ in the additional presence of α-GA (100 μM) (Figure 1b, $P<0.01$, $n=5$). Control EC_{50} values for CPA-induced relaxations were not affected by L-NAME (7.06 ± 0.60 μM). CPA exerted a small effect on tension in endothelium-denuded rings causing relaxations of $9 \pm 6\%$ at 100 μM (Figure 1b, $n=4$).

In experiments evaluating the effects of α-GA on nitrovasodilator-induced relaxation in endothelium-denuded rings, tension was maximally reduced by $89 \pm 6\%$ in response to 3 μM SNP. α-GA (100 μM) had no effect on maximum relaxation ($94 \pm 3\%$, $n=4$) or EC_{50} values (control: 0.43 ± 0.13 μM, α-GA treated tissues: 1.16 ± 0.57 μM). In experiments evaluating the possible role of prostanoids, ACh reduced tension in control endothelium-intact rings by $74 \pm 8\%$ with an EC_{50} of 0.26 ± 0.06 μM ($n=3$). Indomethacin (10 μM) had no effect on maximum relaxation ($72 \pm 10\%$, $n=3$) or EC_{50} value (0.39 ± 0.06 μM).

Discussion The present study has shown that the NO synthase inhibitor L-NAME, causes a $\sim$60–70% decrease in the relaxations induced by acetylcholine (ACh) and cyclopiazonic acid (CPA) in the rabbit iliac artery, thus confirming NO as the major mediator of endothelium-dependent relaxations in this vessel type. The residual L-NAME insensitive component of relaxations to these agents was attenuated by α-glycyrrhetinic acid (α-GA), an inhibitor of gap junctional

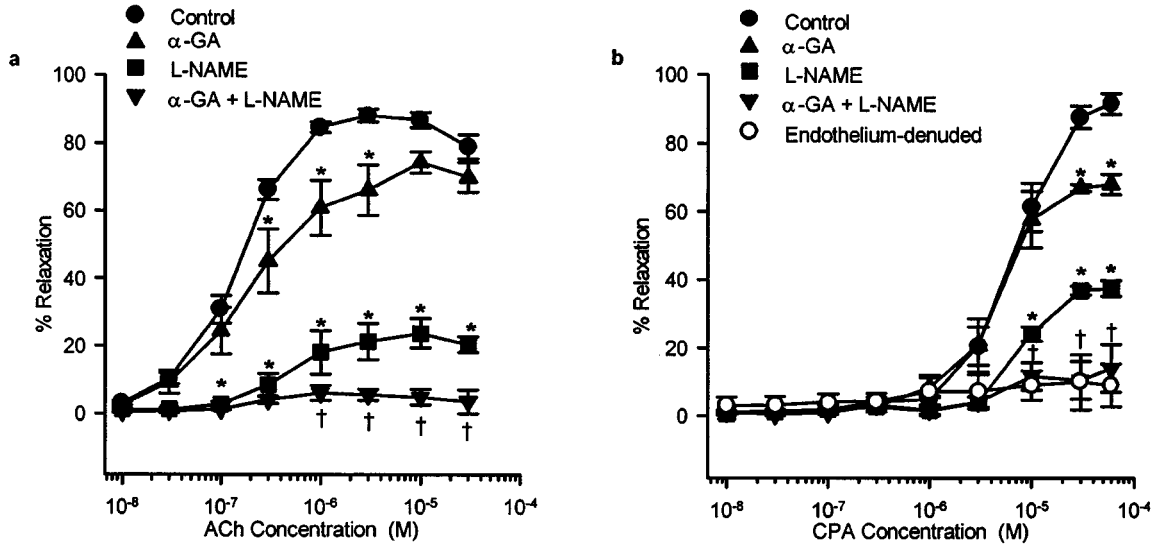

Figure 1 (a) Effect of L-NAME and α-GA on acetylcholine (ACh) induced relaxations. α-GA (100 μM, $n=8$) significantly inhibited relaxation compared to control ($n=13$). L-NAME (300 μM, $n=5$), inhibited relaxations to $\sim$20%, and these were almost abolished by α-GA ($n=5$). (b) Effects of L-NAME and α-GA on cyclopiazonic acid (CPA) induced relaxations. α-GA ($n=6$) significantly inhibited relaxations compared to control ($n=11$). L-NAME ($n=5$) inhibited relaxations to $\sim$40% and these were almost abolished by α-GA ($n=5$). CPA caused minimal relaxation of endothelium-denuded rings ($n=4$). $^{*}P<0.01$ cf. control, $\dagger P<0.01$ cf. L-NAME group.

communication (GJC). The possible contribution of prostanoids was excluded by experiments involving the cyclooxygenase inhibitor indomethacin, which did not affect responses to ACh. Relaxations to sodium nitroprusside (SNP), an exogenous donor of NO, were unaffected by α-GA confirming that it does not modulate the smooth muscle effects of NO. These findings accord with previous studies showing that a short connexin-mimetic peptide (Gap peptide 27) attenuates the NO- and prostanoid-independent component of the endothelium-dependent responses to ACh, ATP and CPA in other rabbit conduit arteries, without affecting relaxations to endogenous or exogenous sources of NO (Chaytor *et al*., 1998). 'Sandwich' experiments have shown that Gap peptide 27 may block the transfer of a diffusible factor from the endothelium to subjacent smooth muscle *via* gap junctions (Chaytor *et al*., 1998). The present findings therefore suggest that α-GA, which is known to inhibit GJC in non-vascular cell types, inhibits endothelium-dependent relaxation through a similar mechanism.

NO- and prostanoid-independent relaxations mediated *via* the endothelium, are generally attributed to a hyperpolarizing factor (EDHF), whose functional importance may differ between artery types. Thus, rabbit iliac and mesenteric arteries both relax by ~90% in response to ACh alone, but EDHF-type relaxations to ACh in the presence of L-NAME amount to ~20% and ~40% of developed tone respectively (this study, Chaytor *et al*., 1998). In the present study α-GA and L-NAME inhibited endothelium-dependent relaxation synergistically, almost abolishing the responses to ACh and CPA. This suggests that NO and GJC may together account for endothelium-dependent relaxation almost entirely, and that the putative EDHF normally does not diffuse freely through the extracellular space. Synergistic inhibition of relaxation has previously been noted with Gap peptide 27 and L-NAME in rabbit aorta and mesenteric artery (Chaytor *et al*., 1998).

The mechanism by which the α- and β-isoforms of glycyrrhetinic acid interrupt GJC are incompletely understood, but are not mediated by an action at mineralocorticoid/glucocorticoid receptors or changes in protein kinase C activity (Davidson, 1986). Rapid inhibition of gap-junctional dye coupling followed by reversible disaggregation of connexin 43 gap junction plaques by β-GA is associated with dephosphorylation (Guan *et al*., 1996). While the α-isoform also promotes gap junction plaque disassembly, this does not appear to involve connexin dephosphorylation (Goldberg *et al*., 1996). The present findings confirm that β-GA possesses actions not shared by α-GA as 100 μM α-GA had no significant effect on arterial tone, whereas 100 μM β-GA caused a gradual decrease in tension through a direct action on smooth muscle. The β-GA isoform is therefore a less suitable probe to assess the contribution of GJC to arterial relaxations.

In conclusion, α-glycyrrhetinic acid, an inhibitor of GJC, abolishes EDHF-type relaxations in rabbit iliac artery. The results therefore provide further evidence that EDHF mediates relaxation following diffusion *via* gap junctions.

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(Received May 29, 1998
Revised June 17, 1998
Accepted June 23, 1998)