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Inhibition of nitrergic relaxations by a selective inhibitor of the soluble guanylate cyclase

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1 The actions of 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), a specific inhibitor of the soluble guanylate cyclase (SGC), were investigated in the rabbit anococcygeus muscle.

2 ODQ (1 nM-1 μM) inhibited in a concentration-dependent manner the relaxations induced by electrical field stimulation (EFS; 50 V, 0.3 ms duration, 1 Hz, for 5 s, every 120 s).

3 ODQ (1 μM) also inhibited the relaxations elicited by EFS (50 V, 0.3 ms duration, 1, 2.5, 5, 10 Hz, for 5 s) and sodium nitroprusside (SNP; 1 μM) without affecting those induced by isoprenaline (1 μM), atrial natriuretic peptide (ANP; 100 nM) or an analogue of cyclic GMP (8-pCPT-cyclic GMP; 500 μM).

4 ODQ (1 μM) inhibited the elevations in the concentration of cyclic GMP induced by SNP or EFS, but not by ANP. ODQ did not affect the concentrations of cyclic AMP.

5 Nitrergic relaxation in this tissue appears, therefore, to be mediated via activation of SGC.

Keywords: Nitrergic neurotransmission; soluble guanylate cyclase; anococcygeus (rabbit); cyclic GMP; ODQ

Introduction

The L-arginine: nitric oxide (NO) pathway (Moncada *et al.*, 1991) is now known to be responsible for neurotransmission in some nerves previously described as non-adrenergic and non-cholinergic (Rand, 1992). Pharmacological, physiological and immunohistochemical evidence confirms that this 'nitrergic transmission' (Rand & Li, 1990) operates in a variety of autonomically innervated organs from many species including human (Brookes, 1993; Rand & Li, 1995). Nitrergic transmission in the rabbit anococcygeus muscle has been characterized (Graham & Sneddon, 1993; Kasakov *et al.*, 1995). Circumstantial evidence suggests that nitrergic transmission operates via stimulation of the soluble guanylate cyclase (SGC), leading to increases in cyclic GMP (Bowman & Drummond, 1984; Mirzazadeh *et al.*, 1991) although this has not been fully confirmed due to the absence of a specific inhibitor of SGC.

1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) is a novel compound which has been shown to inhibit NO-induced increases in cyclic GMP concentrations in the brain (Garthwaite *et al.*, 1995). Furthermore, ODQ selectively inhibits SGC in vascular tissue and platelets without any affect on NO (Moro *et al.*, 1996), unlike other SGC inhibitors such as methylene blue (Mayer *et al.*, 1993) or LY83583 (Mulsch *et al.*, 1988) which have other biological actions. We have therefore investigated the effect of ODQ on nitrergic neurotransmission in the rabbit anococcygeus muscle to determine the involvement of SGC in this process.

Methods

Preparation of tissues

Male New Zealand rabbits (2.8-3.5 kg) were killed by an overdose of pentobarbitone and the bilateral anococcygeus muscles were excised as described previously (Kasakov *et al.*, 1995). The isolated preparations (3 x 15 mm) were placed horizontally between two ring electrodes in 1 ml plastic double-jacketed tissue chambers (37°C) perfused at a constant flow of 0.6 ml min^{-1} with a medium of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, glucose 11.5, and gassed with 5% CO_2 in O_2 (pH 7.4-7.6). One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Linearcorder WR 3101 (Graphtec) for registration of isometric changes in tension. The preparations were stretched until they reached approximately the *in situ* length (2 to 5 mN) and were allowed to equilibrate for 90 min. Electrical field stimulation (EFS; 50 V, 0.3 ms pulse duration, 0.2-40 Hz, for 5-60 s) was delivered by Grass S88 stimulators. Drugs were applied either into the medium reservoir or directly into the chamber at a rate of 50 μl in 30 s. The concentration-response studies with ODQ were performed by cumulative addition of the drug into the medium reservoir and the tissues were allowed to incubate with each concentration for at least 30 min.

Measurement of cyclic GMP and cyclic AMP concentrations in the tissue

After inhibition of adrenergic and cholinergic components with guanethidine (10 μM) and scopolamine (10 μM), the tone of the tissue was raised with histamine (0.5 μM). The tissue was then allowed to equilibrate for at least 20 min without any EFS or drug application. After EFS (50 V, 0.3 ms duration, 1 Hz, for 1 min) or 1 min after application of the drugs (sodium nitroprusside (SNP), isoprenaline (Iso), atrial natriuretic peptide (ANP)) in the absence or presence of ODQ (1 μM), the tissue was freeze-clamped and stored at -70°C. The frozen tissues were homogenized in a stainless steel pestle and mortar and the homogenate was then incubated in 1 ml of ice cold perchloric acid (0.5 M) at 4°C for 1 h. After centrifugation at 12,000 *g* for 5 min at 4°C and neutralisation with K_3PO_4 (1 M), cyclic GMP and cyclic AMP concentrations in the supernatant were measured separately (as pmol per mg soluble protein) using specific enzyme immunoassay kits (Amersham, U.K.). The soluble protein concentration of each sample was measured in the supernatant with BioRad reagent (BioRad, Germany).

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Chemicals used

The chemicals used were: atrial natriuretic peptide (ANP), guanethidine monosulphate, histamine hydrochloride, isoprenaline hydrochloride (Iso), K_3PO_4, sodium nitroprusside (SNP), scopolamine hydrochloride, all from Sigma, U.K.; 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (8-pCPT-cyclic GMP) from Biolog, U.K.; perchloric acid from Analar, U.K.; and 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; code NNC07-9008) from Novo Nordisk, Denmark.

Statistical analysis

Quantitative data are expressed as means ± s.e.mean and the differences between two means were evaluated by Student's two-tailed *t* test for unpaired observations. A probability of less than 0.05 was considered statistically significant; *n* denotes the number of preparations.

Results

Mechanical responses

EFS (50 V, 0.3 ms duration, 1–10 Hz, for 5 s, every 120 s) of histamine-contracted tissues treated with scopolamine and guanethidine elicited reproducible relaxations. The magnitude of EFS-induced relaxations was frequency-dependent (not shown). Infusion of SNP (1 μM), Iso (1 μM), ANP (100 nM) and 8-pCPT-cyclic GMP (500 μM) caused relaxation of the tissue of similar magnitude to that induced by EFS at 1 Hz (Figure 1a).

ODQ elevated the basal tone of the tissue by ~10% (not shown). EFS-induced relaxations were inhibited in a concentration-dependent manner by ODQ (Figure 2). ODQ (1 μM) completely abolished the relaxations elicited by EFS at 1 Hz, significantly inhibited the relaxations at 2.5–10 Hz and those elicited by SNP, but did not affect the relaxations induced by infusion of Iso, ANP or 8-pCPT-cyclic GMP (Figure 1b and Table 1). After washing the tissue with perfusion solution for 1 h, the relaxations induced by EFS and SNP were similar to those obtained before treatment with ODQ.

Cyclic GMP concentrations

The basal cyclic GMP concentration in rabbit anococcygeus muscle was 43.3 ± 3.9 pmol mg^{-1} protein ($n=4$). Application of EFS (50 V, 0.3 ms duration, 1 Hz, for 1 min), SNP (1 μM) or ANP (100 nM) increased the cyclic GMP concentrations significantly ($P<0.001$ for EFS and ANP; $P<0.01$ for SNP; $n=4$; Figure 3a). Iso (1 μM) caused a slight decrease in cyclic GMP concentrations ($P=0.0434$; $n=4$; Figure 3a).

In the presence of ODQ (1 μM), basal cyclic GMP concentrations were reduced significantly ($P<0.001$; $n=4$; Figure 3a). The elevation of cyclic GMP concentrations elicited by EFS or SNP was significantly inhibited by ODQ ($P<0.0001$ for EFS and $P<0.005$ for SNP; $n=4$; Figure 3a) but that elicited by ANP was not affected ($P>0.05$; $n=4$; Figure 3a). ODQ did not affect the slight reduction in cyclic GMP concentrations elicited by Iso ($P>0.05$; $n=4$; Figure 3a).

Cyclic AMP concentrations

The basal cyclic AMP concentration was 385.5 ± 3.6 pmol mg^{-1} protein and was not affected by EFS,

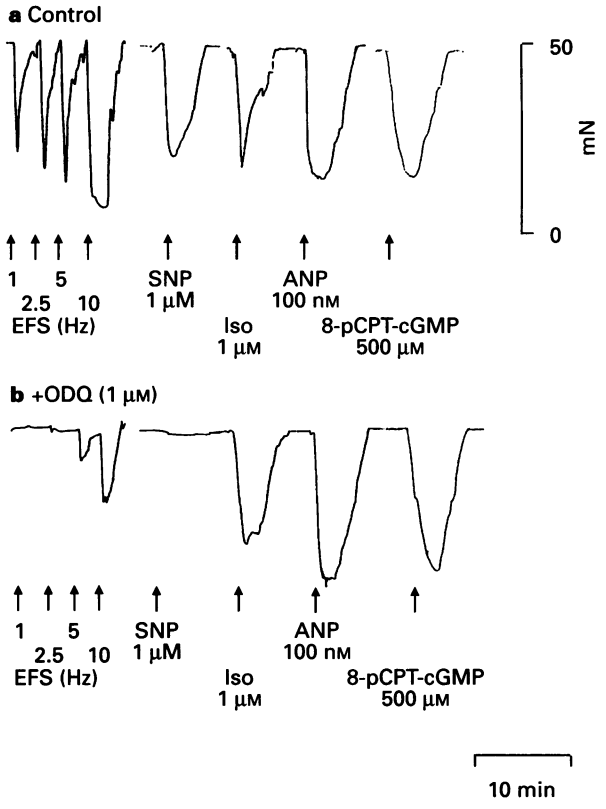

Figure 1 Relaxation responses elicited by electrical field stimulation (EFS, 50 V, 0.3 ms duration, 1, 2.5, 5, 10 Hz, for 5 s) and by infusion of sodium nitroprusside (SNP, 1 μM), isoprenaline (Iso, 1 μM), atrial natriuretic peptide (ANP, 100 nM), cyclic GMP analogue (8-pCPT-cGMP, 500 μM) in the rabbit anococcygeus muscle in the absence (a) and presence (b) of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM). Trace is representative of six experiments.

Table 1 Relaxations of rabbit anococcygeus in the absence and presence of ODQ

	Control		*+ODQ* (1 μM)	
	AUC (mm^2)	*Magnitude* (mN)	*AUC* (mm^2)	*Magnitude* (mN)
ANP	435.8 ± 65.7	33.0 ± 1.0	400.7 ± 32.6	32.0 ± 2.0
Iso	194.5 ± 4.0	29.0 ± 1.0	201.4 ± 12.4	31.0 ± 3.0
SNP	177.2 ± 12.3	27.0 ± 2.0	12.3 ± 6.7*	1.0 ± 1.0*
8-pCPT-cyclic GMP	311.7 ± 43.0	32.0 ± 2.0	320.4 ± 24.8	30.0 ± 2.0
EFS, 1 Hz	52.8 ± 1.8	24.0 ± 2.0	0 ± 0*	0 ± 0*
EFS, 2.5 Hz	71.5 ± 0.4	31.0 ± 1.0	6.5 ± 2.0*	2.8 ± 0.5*
EFS, 5 Hz	100.8 ± 1.3	36.0 ± 1.0	19.0 ± 1.2*	7.0 ± 1.0*
EFS, 10 Hz	202.5 ± 2.0	45.0 ± 1.0	39.5 ± 7.3*	23.0 ± 2.0*

Relaxation responses induced by atrial natriuretic peptide (ANP, 100 nM), isoprenaline (Iso, 1 μM), sodium nitroprusside (SNP, 1 μM), cyclic GMP analogue (8-pCPT-cyclic GMP, 500 μM) or electrical field stimulation (EFS, 50 V, 0.3 ms duration, 1–10 Hz, for 5 s) in the absence or in the presence of ODQ (1 μM) expressed as area under the curve of relaxation (AUC, mm^2) and magnitude of relaxation in mN ($n=6$). *Significantly different from control value ($P<0.0001$).

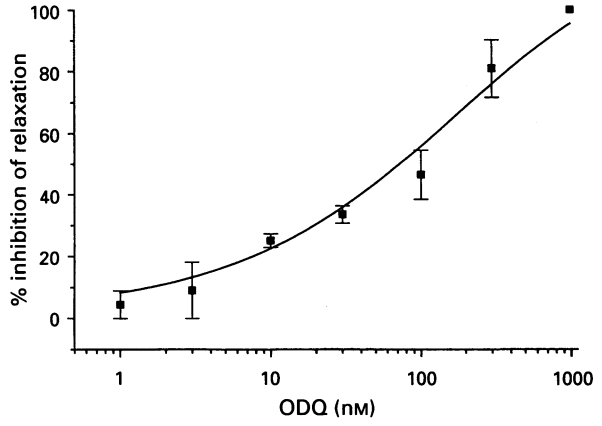

Figure 2 Relaxation responses elicited by electrical field stimulation (50 V, 0.3 ms duration, 1 Hz, for 5 s, every 120 s) were inhibited concentration-dependently by ODQ (1 nM–1 μM). Inhibition of relaxation is expressed as percentage decrease in the amplitude of relaxation ($n=4$).

SNP or ANP ($P>0.05$; $n=4$; Figure 3b). Administration of Iso however, elicited a significant increase in cyclic AMP concentrations ($P<0.005$; $n=4$; Figure 3b). ODQ did not affect the basal concentration of cyclic AMP or its concentration in the presence of EFS, SNP or ANP ($P>0.05$; $n=4$; Figure 3b), nor did it affect the elevated concentration of cyclic AMP elicited by Iso ($P>0.05$; $n=4$; Figure 3b).

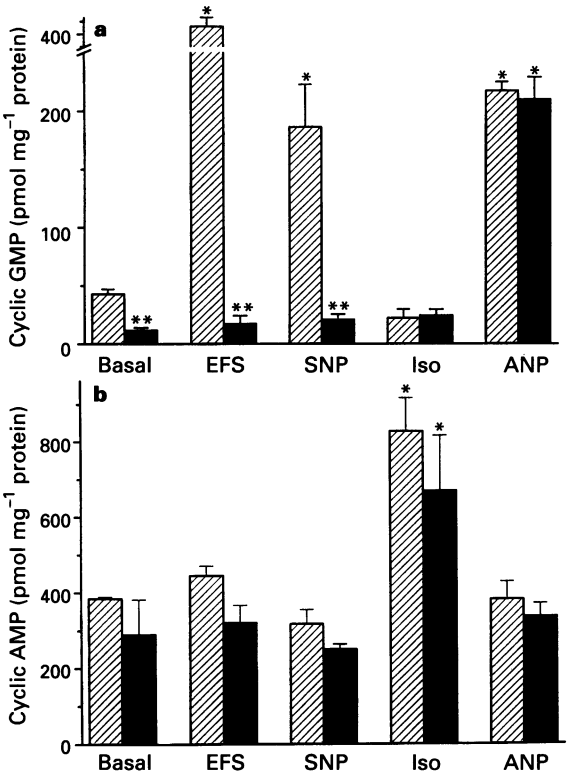

Figure 3 (a) Cyclic GMP concentrations in basal conditions or with electrical field stimulation (EFS, 50 V, 0.3 ms duration, 1 Hz, for 1 min), sodium nitroprusside (SNP, 1 μM), isoprenaline (Iso, 1 μM) or atrial natriuretic peptide (ANP, 100 nM) in the absence (control; hatched columns) or presence of ODQ (1 μM; filled columns). $n=4$; *Significantly different from basal concentrations ($P<0.05$); **Significantly different from control concentrations in the absence of ODQ ($P<0.05$). (b) Cyclic AMP concentrations in basal conditions or with EFS (50 V, 0.3 ms duration, 1 Hz, for 1 min), SNP (1 μM), Iso (1 μM) or ANP (100 nM) in the absence (control; hatched columns) or presence of ODQ (1 μM; filled columns). $n=4$; *Significantly different from basal concentrations ($P<0.05$).

Discussion

ODQ has been shown to inhibit both N-methyl-D-aspartate-induced increases in cyclic GMP concentrations in rat cerebellar slices and NO-stimulated activity of the purified SGC (Garthwaite *et al.*, 1995). Furthermore, ODQ abolishes the NO-dependent increases in cyclic GMP in platelets and in vascular tissue in a concentration-dependent and non-competitive manner and inhibits SGC activity in the crude extract of rat aortic smooth muscle (Moro *et al.*, 1996). Thus this compound, which does not inactivate NO or inhibit its release, acts as an inhibitor of SGC in the brain, platelets and vascular tissue.

The relaxation response elicited by EFS in the rabbit anococcygeus muscle is neuronal in origin and nitrergic in nature (Graham & Sneddon, 1993; Kasakov *et al.*, 1995). We have now shown that in this tissue ODQ inhibits nitrergic relaxations elicited by EFS in a manner which is concentration-dependent and slowly reversible. In addition, it abolishes the relaxation response induced by SNP and inhibits the increases in cyclic GMP concentrations induced by SNP and EFS. The inhibitory effect of ODQ is specific for SGC since it did not affect the relaxation or increase in cyclic GMP elicited by ANP, which activates particulate GC. Furthermore, ODQ did not affect the increase in cyclic AMP elicited by Iso, indicating that its action is confined to GC. In addition, ODQ did not inhibit the relaxation to a cyclic GMP analogue, 8-pCPT-cyclic GMP, suggesting that its inhibitory site is on the SGC and not at a further step in the transduction mechanism of this enzyme.

The reduction in basal cyclic GMP concentrations and elevation of the basal tone by ODQ support the earlier demonstration of the existence of a basal release of NO from nitrergic nerves (Ward *et al.*, 1992; Wiklund *et al.*, 1993; Kasakov *et al.*, 1995) akin to that from vascular endothelium (Rees *et al.*, 1989) and from perivascular nitrergic nerves (Toda & Okamura, 1992).

Our finding that ODQ inhibits nitrergic relaxation and relaxation induced by an NO-donor in the rabbit anococcygeus muscle, as well as inhibiting basal and nitrergic/NO stimulated production of cyclic GMP, further indicates the potential usefulness of this compound in elucidating the role of cyclic GMP in the biological actions of NO.

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