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Inhibition of NO-mediated responses by 7-ethoxyresorufin, a substrate and competitive inhibitor of cytochrome P_{450}

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1 The effects of 7-ethoxyresorufin (7-ER), which is a substrate for and competitive inhibitor of cytochrome P_{450}, were studied on responses to nitric oxide (NO), the NO donors sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), acetylcholine-induced endothelium-dependent relaxations of rat and rabbit aortic rings and nitrergic nerve stimulation-induced relaxations of rat anococcygeus muscles.

2 In rat and rabbit aortic rings, 7-ER (2 μM) inhibited the relaxations to acetylcholine in endothelium-intact preparations and the relaxant action of NO in endothelium-denuded preparations. Relaxant responses to SNP and GTN were inhibited by 7-ER in the rat but not rabbit aortic rings. However, the relaxant actions of papaverine and 8-bromo-cyclic GMP were not affected by 7-ER.

3 In rat anococcygeus muscles, 7-ER (2 μM) inhibited the relaxant action of NO, but relaxations elicited by nitrergic nerve stimulation were only partly inhibited by a higher concentration of 7-ER (10 μM).

4 After inhibition by 7-ER, superoxide dismutase (100 u ml^{-1}) restored NO-induced relaxations of the rat aortic rings, but not acetylcholine-, SNP or GTN-induced relaxations, and restored NO- and nitrergic nerve stimulation-induced relaxations of anococcygeus muscles.

5 Another cytochrome P_{450} inhibitor, troleandomycin (10-30 μM), had no effect on NO- or acetylcholine-induced relaxations of rat aortic rings and NO- or nitrergic nerve stimulation-induced relaxations of anococcygeus muscles. However, resorufin, an analogue of 7-ER, inhibited responses to acetylcholine, NO and GTN in rat aortic rings.

6 The results suggest that 7-ER inhibited responses to NO and nitrergic nerve stimulation through generation of superoxide radicals. However, an additional mechanism may be involved in the reduction in acetylcholine-induced responses in aortic rings.

7 A 7-ER sensitive P_{450} system may be involved in the bioactivation of GTN and SNP in rat aortic rings, but not in rabbit aorta or rat anococcygeus muscles.

Keywords: Anococcygeus muscle; cytochrome P_{450}; endothelium-derived relaxing factor (EDRF); 7-ethoxyresorufin; nitrergic nerves; nitric oxide; nitric oxide synthase

Introduction

There is a structural resemblance between NO synthase (NOS) and cytochrome P_{450} reductase (Bredt *et al.*, 1991) and the formation of nitric oxide (NO) by NOS may involve similar mechanisms to those in reactions catalysed by P_{450} enzymes. Findings in support of this view are: (1) NOS is a P_{450} type haemoprotein; (2) a specific P_{450} isoenzyme has the ability to catalyse the oxidation of L-OH-arginine to NO; and (3) NO can inhibit both NOS and P_{450} enzymes (White & Marletta, 1992; Klatt 1992; Boucher *et al.*, 1992; Renaud *et al.*, 1993; Wink *et al.*, 1993; Stadler *et al.*, 1994). Therefore, it is possible that P_{450} inhibitors may also be NOS inhibitors; in fact, phencyclidine and clotrimazole, which are both P_{450} inhibitors, inhibited NOS activity in the brain (Osawa & Davila, 1993; Wolff *et al.*, 1993) and ebselen, which breaks a cysteine thiolate/Fe^{3+} bond of some P_{450} enzymes, inhibited endothelial NOS (Zembowicz *et al.*, 1993).

7-Ethoxyresorufin (7-ER) is generally regarded as a substrate and selective inhibitor of the 1A1 isoform of the P_{450} family of enzymes (Tassaneeyakul *et al.*, 1993; Nelson *et al.*, 1993). 7-ER has been frequently used as a P_{450} inhibitor to study the metabolism of arachidonic acid (Fulton *et al.*, 1992; Imig *et al.*, 1994; Oyekan *et al.*, 1994). In addition, it has been suggested that a 7-ER-sensitive cytochrome P_{450} system may be involved in the bioactivation of glyceryl trinitrate (GTN) (Bennett *et al.*, 1992). Evidence that it might inhibit NOS is suggested by the findings that it inhibited acetylcholine-induced relaxations of rat aortic rings (Bennett 1992) and bradykinin-induced relaxations in the rat kidney (Oyekan *et al.*, 1991; Fulton *et al.*, 1992). More recently, 7-ER was found to inhibit NOS activity in homogenates of rat cerebellum and human placenta by a mechanism that differs from that of L-arginine analogues (Li & Rand, 1993; Di Iulio *et al.*, 1993). The NOS activity of the human placenta appears to be of the endothelial type (Gude 1994; Garvey *et al.*, 1994).

In the light of the above findings, we set out to investigate the effects of 7-ER on acetylcholine-induced relaxant responses of rat and rabbit aortic rings that are mediated by endothelium-derived nitric oxide (EDNO), and on nitrergic nerve stimulation-induced relaxations of rat anococcygeus muscles that appear to be mediated by NO or a NO-donating compound (Rand & Li, 1995a,b). The effects of 7-ER on responses to exogenous NO and the NO donors, sodium nitroprusside (SNP) and GTN, were also studied. Additionally, the effects of some compounds related to 7-ER were also tested.

Preliminary results of the findings reported in this paper have been communicated to the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists (Li & Rand, 1993; Rand & Li, 1993a) and to the British Pharmacological Society (Rand & Li, 1995c).

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Methods

Male Sprague-Dawley rats (250–400 g) and New Zealand White rabbits (2.5–5 kg) were killed by cervical dislocation and the proximal thoracic aorta and anococcygeus muscles (rat only) were removed.

Rings of aorta about 6 mm in length were prepared and set up for measurement of isometric tension as described previously (Rand & Li, 1993b) and sustained contractions were produced by phenylephrine (1 μM) so that relaxant responses could be elicited. The endothelium was present when acetylcholine was used, and endothelium-denuded preparations were used for the study of NO and NO donors.

Anococcygeus muscles were set up as described previously

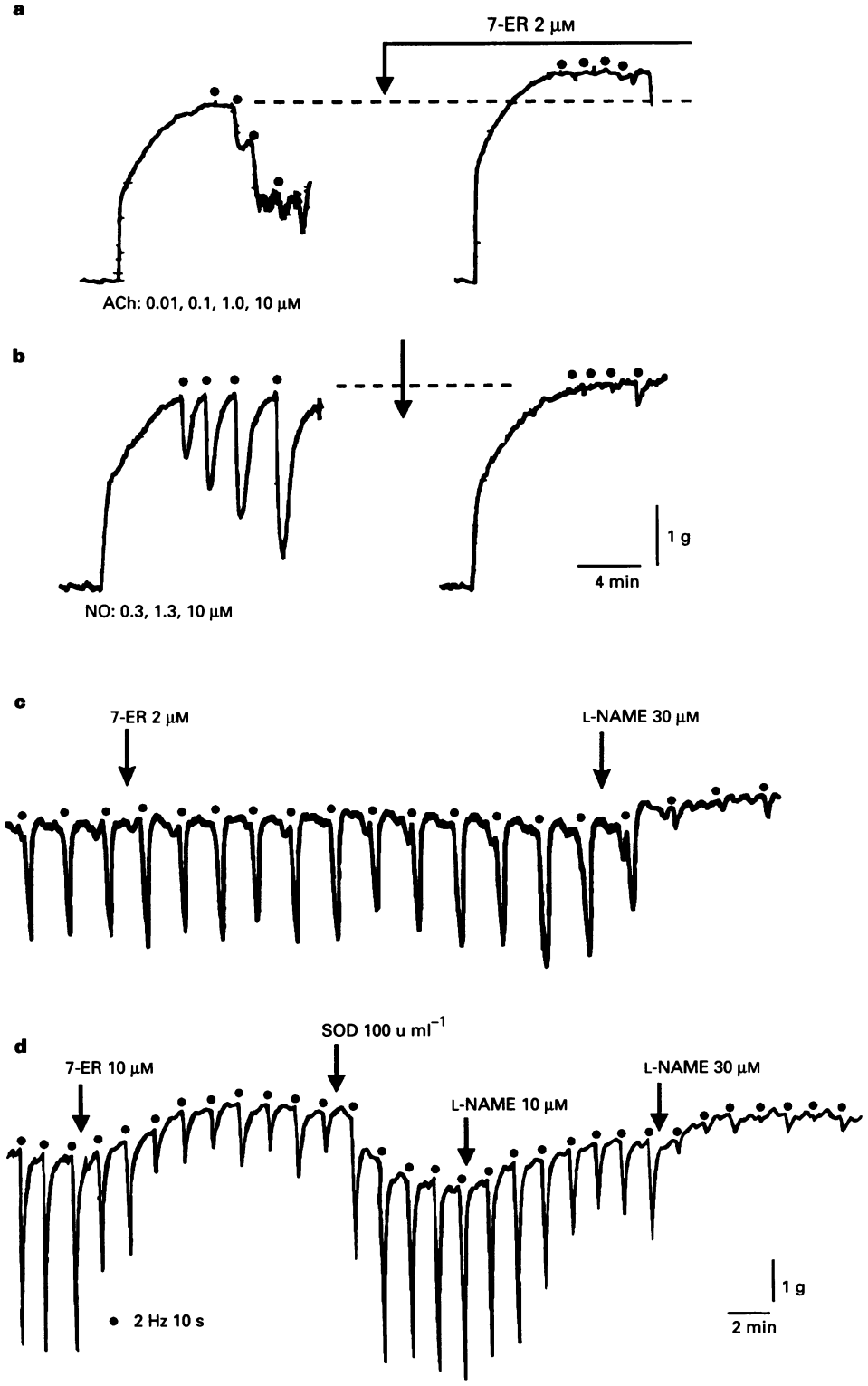

Figure 1 Tracings illustrating some effects of 7-ER. (a) Enhancement of phenylephrine-induced contraction and inhibition of acetylcholine-induced relaxations in an endothelium-intact rat aortic ring. (b) Inhibition of NO-induced relaxations in an endothelium-denuded rat aortic ring. (c) Lack of appreciable effect of 2 μM 7-ER on nitrergic nerve stimulation (2 Hz 10 s)-induced relaxations in an anococcygeus muscle. (d) With 10 μM 7-ER, there was a reduction of nitrergic nerve stimulation-induced relaxations and an increase in tone, and these effects were abolished by SOD (100 u ml^{-1}).

(Gillespie, 1972; Li & Rand, 1989). The tone was raised and nitrergic relaxations in response to field stimulation (2 Hz for 10 s) were revealed by guanethidine (10–30 μM).

The effects of 7-ER and other P_{450} inhibitors were studied after initial control responses had been obtained and parallel experiments without addition of 7-ER or related compounds were elicited in tissues from the same donor animal to serve as time controls.

The composition of the physiological salt solution (PSS) was as follows (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067, ascorbic acid 0.14.

The following drugs were used: acetylcholine perchlorate (British Drug Houses Ltd, U.K.); L-arginine, 8-bromo-cyclic GMP (8-bromo-cGMP), catalase, 7-ethoxyresorufin (7-ER), papaverine, phenylephrine hydrochloride, resorufin, sodium nitroprusside (SNP), superoxide dismutase (SOD), and troleandomycin (Sigma Chemical Co., U.S.A.); N^G-nitro L-arginine methyl ester (L-NAME; Wellcome Research Laboratory, U.K.); guanethidine sulphate (Ciba, Australia); glyceryl trinitrate (GTN; David Bull, Melbourne, Vic, Australia); nitric oxide (compressed gas, CIG, Melbourne). Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanayagam *et al.*, 1992). Stock solutions of resorufin, 7-ER and troleandomycin were made in dimethylsulphoxide (DMSO, ICN, U.S.A.); DMSO alone, in concentrations equivalent to those when it was used as a vehicle (up to 0.1%) had no detectable effects in the test systems used.

Data are expressed as mean values and s.e.means. Pairs of means were compared with Student's *t* test and groups of data were compared by analysis of variance (ANOVA). Values of $P<0.05$ were considered significant.

Results

Effects of 7-ER on relaxations elicited by acetylcholine, NO, SNP, GTN, papaverine and 8-bromo-cyclic GMP in rat aortic rings

In preliminary experiments it was established that 7-ER (0.3–2 μM), added 10–15 min previously, reduced the relaxant actions of acetylcholine (0.01–10 μM) in endothelium-intact rings and of NO (0.3–10 μM) in endothelium-denuded rings in concentration-dependent manners. These relaxations were completely abolished by 10 μM 7-ER. The following experiments were then carried out with a single concentration of 7-ER (2 μM) to characterize the mechanisms of actions of 7-ER.

In endothelium-intact preparations, contractions elicited by phenylephrine were significantly enhanced, being 121.5±3.8% ($n=6$) of the control responses ($P<0.01$, *t* test), and the relaxant action of acetylcholine was markedly inhibited, as illustrated in Figure 1a and mean data are shown Figure 2a.

In endothelium-denuded preparations, 7-ER did not affect the contractile action of phenylephrine but markedly inhibited NO-induced relaxations, as illustrated in Figure 1b and mean data are shown in Figure 2b. The relaxant actions of GTN and SNP were also inhibited by 7-ER (Figure 3a), but relaxant responses to papaverine or 8-bromo-cyclic GMP in endothelium-denuded preparations were not affected (Figure 4).

The inhibitory effect of 7-ER on NO-induced relaxations was not significantly changed when the incubation time for 7-ER was increased to 30 min, but it was slightly reduced (<15%, $n=4$) when the incubation time was decreased to 5 min.

Effects of 7-ER on relaxations elicited by acetylcholine, NO, SNP and GTN in rabbit aortic rings

7-ER (2 μM) inhibited relaxations of rabbit aortic rings to acetylcholine in endothelium-intact preparations (Figure 2c) and to exogenous NO in endothelium-denuded preparations (Figure 2d), but it did not affect relaxations elicited by GTN or SNP in endothelium-denuded preparations (Figure 3b).

Effects of 7-ER on relaxations elicited by nitrergic nerve stimulation, NO, SNP and GTN in rat anococcygeus muscles

At a concentration of 2 μM, 7-ER had no significant effect on relaxations induced by nitrergic nerve stimulation (Figure 1c and 5a), although they were subsequently reduced as usual by L-NAME; however, 7-ER greatly reduced NO-induced relaxations (Figure 5b). A higher concentration of 7-ER (10 μM) reduced NO-induced relaxations to a greater extent (result not shown) and also reduced relaxations elicited by nitrergic nerve stimulation (Figures 1d and 5a) and increased the tone, as illustrated in Figure 1d.

The relaxant actions of SNP and GTN were not affected by 2 μM 7-ER (Figure 3c).

Effects of SOD, catalase and L-arginine on inhibition of relaxant responses by 7-ER

In rat aortic rings, SOD (100 u ml^{-1}) did not significantly affect the relaxant action of acetylcholine and had no effect on relaxations that had been reduced by 7-ER (Figure 2a). However, the relaxant action of NO was slightly but significantly enhanced, and NO-induced relaxations that had been reduced by 7-ER were largely restored (Figure 2b). When SOD was added before 7-ER, it prevented the inhibitory effect of 7-ER on responses to NO. Catalase (100 and 200 u ml^{-1}) did not significantly affect relaxations to NO or the inhibitory effect of 7-ER on NO-induced relaxations.

The inhibition by 7-ER of relaxations of rat aortic rings induced by GTN or SNP was not affected by SOD (100 u ml^{-1}) (results not shown).

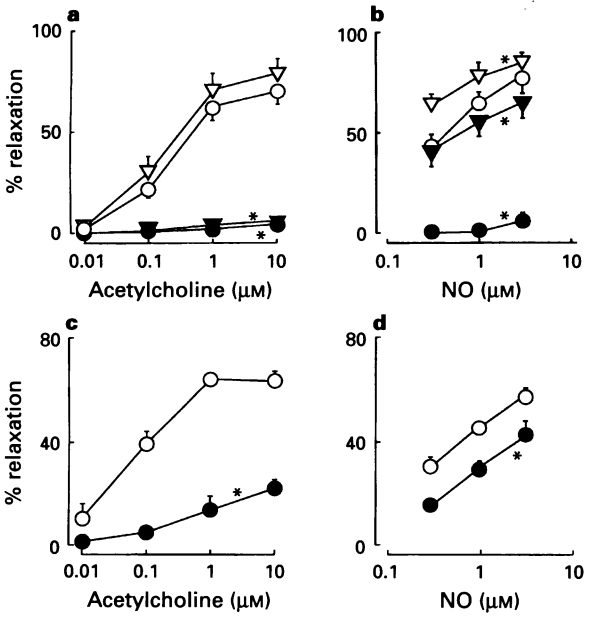

Figure 2 Effects of 7-ER (2 μM) and SOD (100 u ml^{-1}) alone and in combination. (a) and (c): Relaxations of endothelium-intact aortic rings elicited by acetylcholine. (b) and (d): Relaxations of endothelium-denuded rings by nitric oxide. (a) and (b): Relaxations of rat aortic rings. (c) and (d): Relaxations of rabbit aortic rings. Symbols are as follows: (○) time control observations; (▽) in the presence of SOD alone; (●) in the presence of 7-ER alone; (▼) SOD plus 7-ER. Symbols represent means with s.e.mean (which in some cases were smaller than the size of the symbol) of 5–6 experiments. *Concentration-response curve differs significantly ($P<0.05$, two-way ANOVA) from the corresponding control curve.

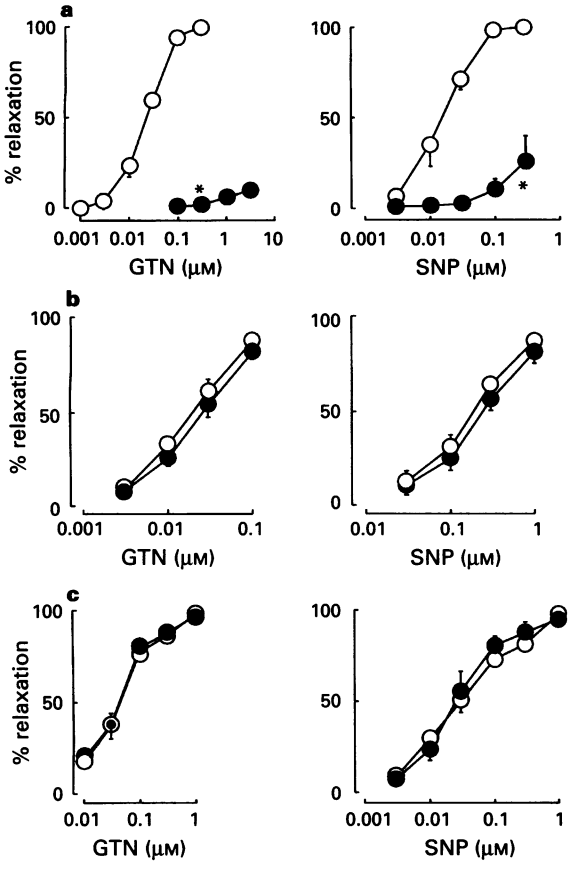

Figure 3 Effects of 7-ER (2 μM) on relaxations elicited by GTN (left panels) and SNP (right panels) in rat aortic rings (a), rabbit aortic rings (b) and rat anococcygeus muscles (c). Symbols indicate mean control observations (○) and mean observations in the presence of 7-ER (●). Values are means ± s.e.means (which in some cases were smaller than the size of the symbol) of 5 experiments. *Significant difference from the control curve ($P<0.05$, two-way ANOVA).

In anococcygeus muscles, SOD (100 u ml^{-1}) had no effect on relaxations produced by nitrergic nerve stimulation (Figure 5a), but restored stimulation-induced relaxations that had been inhibited by 7-ER and reduced the tone back to the initial control level (Figures 1d and 5a). The inhibitory effect of L-NAME was not affected by the presence of SOD. NO-induced relaxations that had been reduced by 7-ER were restored to their control levels by SOD (Figure 5b), and when SOD was added first it prevented the inhibitory effect of 7-ER.

Preincubation of aortic rings or anococcygeus muscles with L-arginine (1 mM) had no effect on the inhibition by 7-ER (2 or 10 μM) of acetylcholine-, NO- or nitrergic nerve stimulation-induced relaxations (results not shown).

Effects of agents related to 7-ER on relaxant responses

Resorufin, an analogue of 7-ER, also inhibited relaxant responses to acetylcholine, NO and GTN in rat aortic rings, although it was less effective than 7-ER (Figure 6; cf. Figures 2a, 2b and 5a).

The P_{450} inhibitor, troleandomycin (10–100 μM) had no effect on acetylcholine-induced relaxations of rat aortic rings and NO- or nitrergic nerve stimulation-induced relaxations of anococcygeus muscles (results not shown).

Discussion

7-ER strongly inhibited EDNO-mediated relaxations elicited by acetylcholine and endothelium-independent relaxations

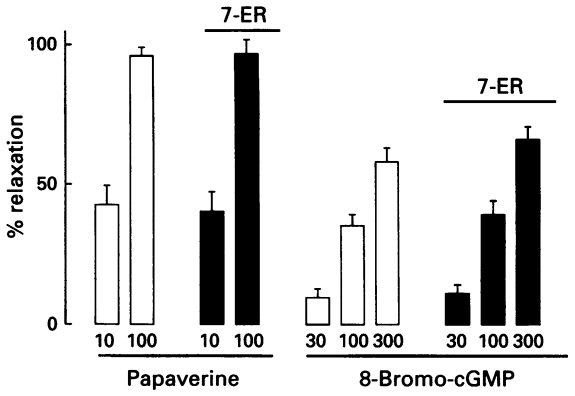

Figure 4 Effects of 7-ER (2 μM) on relaxations elicited by papaverine (10 and 100 μM) and 8-bromo-cyclic GMP (30–300 μM) in rat aortic rings. Columns represent means with s.e.mean of 5 experiments. Open columns are control observations and solid columns are in the presence of 7-ER.

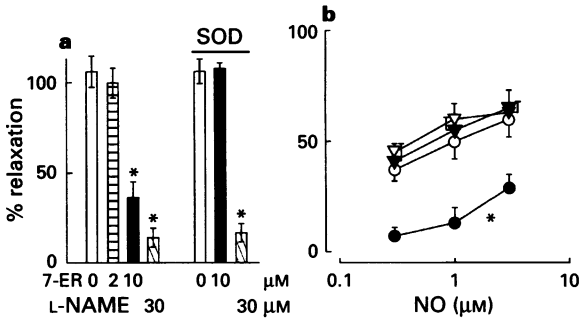

Figure 5 Effects of 7-ER on relaxations induced by nitrergic nerve stimulation (2 Hz for 10 s) and nitric oxide (0.3–3 μM) in rat anococcygeus muscles. (a) Nitrergic nerve stimulation-induced relaxations were reduced by 10 μM 7-ER, but were restored to control levels by SOD (100 u ml^{-1}). The inhibitory effect of L-NAME (30 μM) was not affected by the presence of SOD. Columns represent means with s.e.means of 4–6 experiments. *Significant difference from control (*t* test). (b) NO-induced relaxations: (○) control; (▽) SOD (100 u ml^{-1}) alone; (●) 7-ER (2 μM); (▼) SOD + 7-ER. Symbols indicate means with s.e.means. *Curve differs significantly from the control curve ($P<0.05$, two-way ANOVA).

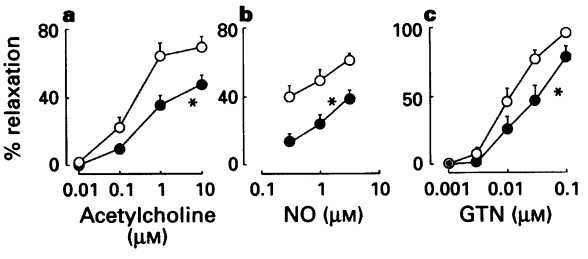

Figure 6 Effects of resorufin (2 μM) on relaxations of rat aortic rings elicited by acetylcholine (0.01–10 μM) in endothelium-intact rings (a) and by nitric oxide (0.3–3 μM, b) and GTN (0.001–0.1 μM, c) in endothelium-denuded rings. Symbols represent means with s.e.means for control observations (○) and observations in the presence of resorufin (●). $n=4-6$ experiments. *Curve differs significantly ($P<0.05$, two-way ANOVA) from the control.

elicited by NO but did not affect NO-independent relaxations elicited by papaverine or 8-bromo-cyclic GMP. These results are consistent with the findings that 7-ER inhibited acetylcholine-induced relaxations and accumulation of cyclic GMP in the rat or rabbit aorta (Bennett *et al.*, 1992; Oyekan *et al.*,

1994), but it did not affect relaxations elicited by the β-adrenoceptor agonist, isoprenaline or the K^+ channel activator, diazoxide (Oyekan *et al.*, 1994).

The mechanisms underlying the inhibition of 7-ER of NO-mediated relaxations have not previously been elucidated. The finding that SOD but not catalase overcame the blockade by 7-ER of responses to exogenous NO suggests that superoxide anions are involved, since they rapidly inactivate free NO (Moncada *et al.*, 1991). The source of the superoxide generated by 7-ER could be redox recycling of 7-ER to a semiquinoneimine compound through a NADPH cytochrome reductase, which results in the conversion of oxygen to superoxide anions (Dutton *et al.*, 1989). According to Dutton *et al.* (1989) the reactions could proceed in both directions and superoxide anions are capable of reoxidizing reduced resorufin compounds. This view is supported by the finding that resorufin, an analogue of 7-ER, inhibited responses to EDNO and NO. However, it has yet to be confirmed that the rat aorta contains such a reductase and that the proposed reactions take place under the present experimental conditions. Alternatively, other unknown mechanisms may be involved in the 7-ER-induced generation of superoxide radicals. In fact, it is not known whether NOS itself may have the ability to metabolize 7-ER, generating superoxide in the process.

However, the inhibition by 7-ER of acetylcholine-induced relaxations of rat aortic rings was not affected by SOD, indicating that this effect may not be due to inactivation of endothelium-derived nitric oxide (EDNO) by superoxide. It remains to be determined, however, whether this was due to the poor access of SOD to intracellular superoxide radicals generated by 7-ER or to the involvement of additional mechanisms. One such mechanism is that 7-ER may directly inhibit endothelial nitric oxide synthase (eNOS), since 7-ER has been found to inhibit e-type NOS activity in homogenates of human placenta (Li & Rand, 1993; Di Iulio *et al.*, 1993). However, it has also been reported that 7-ER failed to inhibit the conversion of L-arginine to L-citrulline by endothelial cells (Oyekan *et al.*, 1994). On the other hand, inhibition of nitrergic nerve stimulation-induced relaxations of anococcygeus muscles by 7-ER was overcome by SOD, indicating that this effect can be attributed to generation of superoxide, despite the fact that neuronal NOS in homogenates of rat cerebullum was inhibited by 7-ER (Li & Rand, 1993; Di Iulio *et al.*, 1993). Thus, it appears that the NOS of nitrergic nerves in the anococcygeus muscle was not inhibited in the intact tissue.

It is generally thought that enzymatic bioactivation of organic nitrates such as GTN to an active species (possibly NO) is needed for their biological actions. Enzymes suggested for this role include glutathione S-transferases and cytochrome P_{450} (Yeates *et al.*, 1989; Schröder, 1992; McDonald & Bennett, 1993). Since 7-ER reduced relaxant responses to GTN in rat but not rabbit aortic rings, it appears that the bioactivating mechanism of GTN is 7-ER-sensitive in the rat aorta, as suggested by Bennet *et al.* (1992), whereas in the rabbit aorta or rat anococcygeus muscle it is not. This may be related to the findings that the bioactivation of GTN in the rabbit aorta was not inhibited either by carbon monoxide or by other P_{450} inhibitors, but was inhibited by the glutathione-*S*-transferase inhibitor ethacrynic acid (Liu *et al.*, 1992; Kenkare & Benet, 1993). Therefore, based on evidence from a number of studies, it seems likely that the bioactivation of GTN may involve enzymes that differ between tissues and species.

The bioactivation mechanism for SNP differs from that for GTN (Bates *et al.*, 1991; Kowaluk *et al.*, 1992), and since 7-ER reduced SNP-induced relaxations in rat but not rabbit aortic rings or rat anococcygeus muscles, it again appears the mechanisms differ between tissues and species.

The relaxation of the rat anococcygeus muscle elicited by stimulation of nitrergic nerves is mediated by a transmitter that does not behave like the free radical form of NO but could be an NO-adduct (for reviews see Rand & Li, 1995a,b). The finding that a low concentration (2 μM) of 7-ER blocked responses to NO but a higher concentration was required to reduce responses to nitrergic nerve stimulation suggests that the nitrergic transmitter of the rat anococcygeus muscle may be in a form (such as a NO-containing compound) which is more resistant to inactivation by superoxide anions than is free NO. The finding is consistent with those for other superoxide generators, including hydroquinone and LY-83583 (Hobbs *et al.*, 1991; Barbier & Lefebvre, 1992). However, Boeckxstaens *et al.* (1994) suggested that the difference in the sensitivity of NO- and nitrergic nerve stimulation-induced responses to superoxide generators may be due to failure of these agents to gain access to neuroeffector junctions. On the other hand, Martin *et al.* (1994) found that the SOD inhibitor, diethyldithiocarbamate, revealed the inhibition of nitrergic relaxations by superoxide generators in bovine retractor penis muscles, indicating that these agents can gain access to the nitrergic junction, and that the nitrergic transmitter is protected from inactivation by SOD, at least in the tissue used. Additional work is needed to elucidate the exact mechanism of actions of 7-ER in the rat anococcygeus muscle and in particular the effect of SOD inhibitors on the inhibition by 7-ER of nitrergic nerve stimulation-induced responses.

In conclusion, these results suggest that the inhibition by 7-ER of exogenous NO- and nitrergic nerve stimulation-induced relaxations involves the generation of superoxide radicals, whereas inhibition of EDNO-mediated relaxations may involve additional mechanisms. In addition, these results indicate that a 7-ER-sensitive system is required for the bioactivation of GTN and SNP to NO in the rat but not in the rabbit aorta or in the rat anococcygeus muscle. A final consideration arising from this study is that although a biochemical mechanism may explain certain effects of a drug, it would be rash to predict a functional consequence of a biochemical action.

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