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Inhibition by 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) of Responses to Nitric Oxide-donors in Rat Pulmonary Artery: Influence of the Mechanism of Nitric Oxide Generation

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Abstract

ODQ, (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, an inhibitor of soluble guanylate cyclase) inhibits vasorelaxant responses to nitric oxide (NO)-donor drugs, but the extent of the inhibition varies depending on the NO donor studied. The purpose of this study was to test the hypothesis that these variations in the effects of ODQ reflect differences in the mechanisms whereby each NO donor generates NO.

On pulmonary artery preparations pre-contracted submaximally with phenylephrine, ODQ (3 μM) almost abolished the relaxant responses to glyceryl trinitrate, isosorbide dinitrate and nitroprusside; each of these drugs requires activation in the tissue (by enzymes or reducing agents) to generate NO. In contrast, ODQ (3 μM) caused a parallel shift in the concentration–relaxation curves to linsidomine (SIN-1), FK409, MAHMA NONOate and spermine NONOate (1·63 to 2·54 log units) with no depression in maximum response; each of these NO donors generates NO in the physiological bathing solution without requiring tissue activation. For the four drugs in this group, the effects of 10 μM ODQ were not significantly greater than the effects of 3 μM ODQ; thus there was an ODQ-resistant component to the response suggesting that part of the response involved a mechanism that was independent of soluble guanylate cyclase.

NO donors that require tissue activation probably generate NO within the smooth-muscle cell, whereas those that do not require tissue activation generate NO outside the cell. Hence it is concluded that the site of NO generation (intra- or extracellular) might determine whether or not there is an ODQ-resistant component in the relaxation response.

The novel drug, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), is a selective inhibitor of soluble guanylate cyclase (Garthwaite et al 1995) and is therefore a potentially useful tool for characterizing drugs that act via this enzyme. Nitric oxide (NO)-donors are considered to act, at least in part, via soluble guanylate cyclase, and this leads to relaxation of vascular smooth muscle. Examination of the literature shows that the inhibition of vasorelaxant responses by ODQ is not necessarily the same for all NO donors. Because these drugs generate NO in several different ways (Feelisch & Stamler 1996), it is possible that the disparate effects of ODQ reflect the different mechanisms of NO generation. The purpose of this study was to test this hypothesis by examining the effects of ODQ on responses to seven different NO donors in a single type of blood vessel (rat pulmonary artery). The NO donors studied included three drugs (glyceryl trinitrate, isosorbide dinitrate and nitroprusside) that, when studied on isolated tissues, require activation in the tissue to generate NO and four drugs (FK409 ((±)-*E*-4-ethyl-2-[*E*-hydroximino]-5-nitro-3-hexenamide), linsidomine (SIN-1; 3-morpholinosydnonimine), MAHMA NONOate (*Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl) amino]}diazen-1-ium-1,2-dioloate) and spermine NONOate (*Z*-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}diazen-1-ium-1,2-dioloate)) that generate NO independently of the tissue (Feelisch & Stamler 1996).

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A preliminary account of some of this work was presented at the XIIIth International Congress of Pharmacology, Munich, July, 1998 (Wanstall & Homer 1998).

Materials and Methods

Drugs

FK409 was a gift from Fujisawa, Japan. Glyceryl trinitrate (ampoules) was from Pohl; MAHMA NONOate and spermine NONOate from Cayman; linsidomine (SIN-1) from Biomol; ODQ from Tocris Cookson; and acetylcholine chloride, atrial natriuretic peptide, isosorbide dinitrate, nitroprusside, phenylephrine hydrochloride and U46619 from Sigma. MAHMA NONOate and spermine NONOate were dissolved and diluted in 0·01 M NaOH; FK409 and linsidomine were dissolved and diluted in 0·1 mM HCl. Solutions of acetylcholine and nitroprusside were prepared in deionized water; atrial natriuretic peptide in physiological salt solution (PSS); glyceryl trinitrate, isosorbide dinitrate and U46619 in ethanol; phenylephrine in 0·01 M HCl; and ODQ in dimethylsulphoxide. Dilutions of these drugs were prepared in PSS except for isosorbide dinitrate, which was diluted with deionized water. All solutions were kept ice-cold throughout the experiment.

Blood vessel preparations and experimental protocols

Ring preparations (endothelium intact) of the main pulmonary artery (length 3 mm) and an intralobar pulmonary artery ($1{\cdot}54 \pm 0{\cdot}11$ mm; $n = 6$) from male Wistar rats, 200–300 g, were mounted in vertical organ baths or small vessel myographs (Mulvany-Halpern type; Model 400A; AJP Trading, Aarhus, Denmark), respectively, as described in detail by Wanstall et al (1997). The preparations were set up in physiological salt solution (composition (mM): NaCl 118; KCl 5·9; $CaCl_2$ 1·5; $MgSO_4$ 0·72; $NaHCO_3$ 25; glucose 11·7; Na_2EDTA 0·025) at 37°C and gassed with 95% O_2–5% CO_2. The resting forces were 10 mN for the main pulmonary artery and $1{\cdot}56 \pm 0{\cdot}33$ mN for the intralobar pulmonary artery ($n = 6$, preparations individually normalized). Changes in force in the circular muscle were measured isometrically.

The presence of a functional endothelium was confirmed by obtaining a relaxant response to acetylcholine (1 μM) after precontracting the preparations submaximally with phenylephrine (0·1 μM; main pulmonary arteries) or U46619 (thromboxane-mimetic; 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; 0·3 μM; intralobar pulmonary arteries). Contraction to potassium-depolarizing PSS (80 mM NaCl replaced with 80 mM KCl) was then obtained to stabilize the preparation. The tissues were washed and two or three consecutive cumulative concentration–response curves to one of the vaso-relaxant drugs were obtained on preparations contracted submaximally with phenylephrine (0·1 μM; main pulmonary artery) or U46619 (0·3 μM; intralobar pulmonary artery). The first curve was a control curve; the second curve was in the presence of ODQ (3 or 10 μM, 30 min incubation); and the third curve, when obtained, was in the presence of ODQ (10 μM, 30 min incubation). In separate experiments it was shown that repeated concentration–response curves in the absence of ODQ were reproducible.

Data analysis

Relaxant responses to the vasorelaxant drugs were expressed as percentage reversal of the contraction induced by phenylephrine or U46619. For those NO donors for which ODQ caused a parallel shift in the concentration–response curves, the magnitude of the shift was expressed, in log units, as the difference between the negative log EC50 value in the absence (control curve) and presence of ODQ (where EC50 is the concentration giving 50% of the maximum response to the particular NO donor).

Data are expressed as means ± standard error of the mean (s.e.m.). The statistical significance of any differences between mean values of log unit shift was assessed by use of Student's *t*-test or by analysis of variance followed by the Tukey-Kramer post hoc test, as indicated in the Results section.

Results

ODQ (3 μM) almost abolished the relaxant responses induced in the main pulmonary artery by glyceryl trinitrate, isosorbide dinitrate and nitroprusside (Figure 1). The small residual response to nitroprusside was not inhibited by 10 μM ODQ (Figure 1). ODQ (3 μM or 10 μM) did not abolish responses to linsidomine, FK409, MAHMA NONOate or spermine NONOate but, instead, caused a parallel shift in the concentration–response curves to a higher concentration range, with no depression of the maximum response. For three of these drugs, FK409, MAHMA NONOate and spermine NONOate, 10 μM ODQ caused a slightly greater (2-fold) shift

than 3 μM ODQ, but this difference was not statistically significant (Table 1). The shifts of the curves for MAHMA NONOate and spermine NONOate were significantly less than the shifts for FK409 or linsidomine, but there was no difference between the two NONOates or between FK409 and linsidomine (Table 1). ODQ (3 μM) had no effect on responses to atrial natriuretic peptide (Figure 1). This confirmed the selectivity of ODQ for soluble, as opposed to particulate, guanylate cyclase.

The effects of two of the drugs, isosorbide dinitrate and FK409, on intralobar pulmonary artery were also examined. The data obtained agreed with the findings for the effects of these drugs on main pulmonary artery, i.e. ODQ (3 μM) abolished responses to isosorbide dinitrate (n = 3) but caused a parallel shift in the concentration–response curve to FK409 to a higher concentration range, with no change in the maximum response. The shift (2·64 ± 0·21 log units; n = 3) was not significantly different from the corresponding value for the main pulmonary artery (Table 1; $P > 0{\cdot}05$, Student's *t*-test).

Discussion

The seven different NO donors examined in this study of rat pulmonary artery could be clearly divided into two groups on the basis of the inhibitory effects of ODQ on vasorelaxant responses to the various drugs. In one group, ODQ (3 μM) caused a marked depression in the maximum relaxation, to the point that responses were almost abolished; this group comprised the three drugs—glyceryl trinitrate, isosorbide dinitrate and nitroprusside—that require tissue activation (either by enzymes or by reducing agents) to generate NO. In the other group, the same concentration of ODQ (and also a higher concentration, 10 μM) caused only partial inhibition of the responses, seen as a parallel shift in the concentration–response curves with no depression in maximum relaxation; this group comprised the four drugs—FK409, linsidomine, MAHMA NONOate and spermine NONOate—that do not require tissue activation to generate NO.

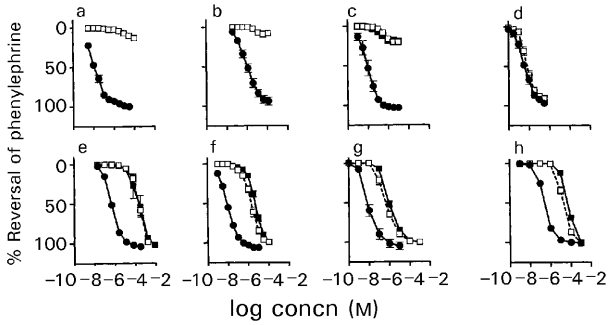

Figure 1. Mean concentration–relaxation curves to glyceryl trinitrate (a), isosorbide dinitrate (b), nitroprusside (c), atrial natriuretic peptide (d), linsidomine (e), FK409 (f), MAHMA NONOate (g) and spermine NONOate (h) on rat main pulmonary artery. Data in the absence (●) or presence of 3 μM (□) or 10 μM (■) ODQ are shown. Note that the concentration axes on the upper graphs are the same as those on the lower graphs. Points are mean values, and the standard errors of the means, when larger than the symbols, are shown by the vertical bars. n = 4, with the exception of linsidomine in the absence of ODQ (n = 9) and in the presence of ODQ 3 μM (n = 6).

Table 1. Effects of ODQ on concentration–relaxation curves to nitric oxide (NO) donors.

NO-donor drug	Log unit shift*	
	ODQ (3 μM)	ODQ (10 μM)
Linsidomine	2·54 ± 0·05 (6)	2·60 ± 0·20 (4)
FK409	2·35 ± 0·13 (4)	2·72 ± 0·05 (4)
MAHMA NONOate	1·63 ± 0·04 (4)†	1·96 ± 0·02 (4)†
Spermine NONOate	1·86 ± 0·07 (4)†	2·22 ± 0·03 (4)‡

Values are means ± standard errors of the mean with numbers of preparations in parentheses. *Log unit shift = negative log EC50 (ODQ absent) − negative log EC50 (ODQ present), where EC50 is the concentration giving 50% of the maximum response to the particular NO donor. †$P < 0{\cdot}05$, value significantly different from the corresponding value for linsidomine and FK409; ‡$P < 0{\cdot}05$, value significantly different from the corresponding value for FK409; for each NO donor the value for 3 μM ODQ was not different from the value for 10 μM ODQ (analysis of variance and Tukey-Kramer post hoc test).

Inhibition of soluble guanylate cyclase by ODQ is reported to be either non-competitive (Garthwaite et al 1995) or competitive but irreversible (Schrammel et al 1996); hence the nature of the inhibition of the three NO donors that require tissue activation, i.e. non-parallel shift in the curves and depression of the maximum, was the predicted result. This pattern of inhibition is comparable with the effect of ODQ on responses to NO donors when activation of guanylate cyclase is being measured, rather than relaxation of smooth muscle (Brunner et al 1995; Schrammel et al 1996). The contrasting pattern of inhibition by ODQ of relaxant responses to the four drugs that do not require tissue activation, i.e. parallel shift in the curves and no depression of the maximum, was not the predicted result for an irreversible or non-competitive inhibitor. This, together with the finding that responses to this second group of drugs were not completely inhibited even when the concentration of ODQ was increased, indicated that part of the response was ODQ-resistant and, by implication, independent of soluble guanylate cyclase. This suggestion is compatible with recent reports that, in some situations, at least part of the response to NO does not involve guanylate cyclase and cyclic GMP (Brun-

ner et al 1995; Hussain et al 1997; Onoue & Katusic 1998).

On the basis of existing literature we considered that the most likely candidate for any non-cyclic GMP component was activation of potassium channels in the cell membrane (Bolotina et al 1994; Plane et al 1996; Zhao et al 1997; Onoue & Katusic 1998). Surprisingly, preliminary experiments with spermine NONOate and FK409 have indicated that potassium-channel activation cannot account for the ODQ-resistant component of the responses of rat pulmonary artery (Homer & Wanstall, unpublished data). However, the possibility remains that other type(s) of membrane ion-channels might be involved. Another possible explanation of the data is that the ODQ-insensitive component of the responses involves a pool of guanylate cyclase that is not inhibited by ODQ; this has been suggested in relation to results on rat aorta with the NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine (van der Zypp & Majewski 1998). In this context the possible involvement of particulate guanylate cyclase, which is located in the cell membrane, cannot be excluded (Murad 1994). Particulate guanylate cyclase is not inhibited by ODQ (Garthwaite et al 1995) and our data with atrial natriuretic peptide, which acts via particulate guanylate cyclase, confirmed this. A detailed study is now in progress to determine the mechanism(s) involved in the ODQ-resistant component of relaxation to these NO donors in rat pulmonary artery, including an investigation of various different membrane ion-channels.

The reason why some NO donors have an ODQ-resistant component but others do not is presently speculative. In a recent study with rabbit carotid artery it was suggested that differences in the inhibitory effects of ODQ on responses to NO derived from the endothelium, compared with NO derived from a NO donor drug, might reflect differences in the rate at which NO is generated (Plane et al 1998). This concept cannot explain the current data if one considers the findings with MAHMA NONOate and spermine NONOate. Under experimental conditions comparable with those in the current study these two NONOates generate NO at very different rates, i.e. the half-lives for the decomposition of these compounds in the generation of NO are only 1·3 min for MAHMA NONOate but 73 min for spermine NONOate (Homer & Wanstall 1998). Despite these markedly different rates of NO generation, the inhibition of responses by ODQ was virtually identical for both drugs.

An alternative speculation, compatible with the data from the current study, is that the site where NO is generated is important. NO generated in the smooth-muscle cell (as occurs with glyceryl trinitrate, isosorbide dinitrate and probably nitroprusside) could arguably have ready access to soluble guanylate cyclase in the cytosol. In contrast, NO that reaches the smooth muscle from outside the cell, as occurs with FK409, linsidomine and the NONOates, might be more likely to exert part of its action via a mechanism that involves the cell membrane.

This is the first study to examine the effect of ODQ on responses to as many as seven NO donor drugs in a single type of blood vessel, but there are several reports on individual drugs, in a variety of blood vessels, that are in agreement with the current findings. For instance *S*-nitrosoglutathione (Brunner et al 1995; Moro et al 1995), *S*-nitroso-*N*-acetyl-D,L-penicillamine (van der Zypp & Majewski 1998), DEA-NONOate (Onoue & Katusic 1998), and linsidomine (SIN-1; Plane et al 1996) all release NO without tissue activation, and relaxant responses to all of these NO donors have an ODQ-resistant component. Glyceryl trinitrate (Brunner et al 1995; Hussain et al 1997; van der Zypp & Majewski 1998) and nitroprusside (Brunner et al 1995; van der Zypp & Majewski 1998) require tissue activation and relaxant responses are almost abolished by ODQ. A preliminary report (Schmidt et al 1998) of a study on rabbit aorta which included several, but not all, of the NO donors examined in the current study described results comparable with our findings. Thus the concept that NO donors which do not require tissue activation have an ODQ-resistant component to their action is well supported in the literature. However, there is at least one exception to this generalization because responses of the rabbit carotid artery to linsidomine were completely blocked by ODQ (Plane et al 1998). Hence the pattern of inhibition by ODQ might, in most instances, depend on the mechanism of NO generation, as suggested by the present study on rat pulmonary artery, but the particular type of blood vessel being studied might also have an influence.

Although we have yet to establish the mechanism of the ODQ-resistant component to the response to linsidomine, FK409 and the two NONOates in rat pulmonary artery, the findings of this study are important for two reasons. Firstly, ODQ is being used increasingly as a pharmacological tool in functional studies on NO donors. It is therefore important to characterize the effects of the drug on responses to a wide range of NO donors in a single study. Secondly, and more importantly, NO donors are frequently used as a convenient source of NO in pharmacological studies. Our data emphasize that it is invalid to assume that all NO donors have the

same profile of action with respect to the cellular mechanism involved in smooth muscle relaxation, even though they all generate NO. This should be borne in mind when selecting an NO donor as a source of NO for a particular experimental purpose or in a particular clinical setting.

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