Comparison of the effects of the novel vasodilator FK409 with those of nitroglycerin in isolated coronary artery of the dog

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1 The vasorelaxant effects of FK409, a new nitrovasodilator synthesized from a microbial product, were compared with those of nitroglycerin in isolated coronary artery rings of the dog contracted with U46619 (10^{-7} M).

2 FK409 (10^{-11} – 10^{-5} M) and nitroglycerin (10^{-9} – 10^{-4} M) each produced a concentration-dependent relaxation. Comparison of EC_{50} values showed that FK409 was about 25 times more potent than nitroglycerin.

3 Submaximum concentrations of nitroglycerin (10^{-6} M) and FK409 (3×10^{-8} M) elevated guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels, effects associated with vasorelaxation. Adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were unaffected.

4 The concentration-relaxation curves for nitroglycerin and FK409 were shifted to the right by methylene blue (3×10^{-6} – 3×10^{-5} M), an inhibitor of soluble guanylate cyclase, and to the left by M&B22,948 (3×10^{-6} – 3×10^{-5} M), an inhibitor of cyclic GMP phosphodiesterase.

5 After exposure of coronary arteries to the maximally-effective concentration of nitroglycerin (10^{-4} M), the mean EC_{50} value of FK409 did not change significantly, although that of nitroglycerin increased about 60 fold. After exposure to the maximally-effective concentration of FK409 (10^{-5} M), the mean EC_{50} value of FK409 increased about 6 fold and that of nitroglycerin about 11 fold.

6 These results suggest that the vasorelaxant effect of FK409, like that of nitroglycerin, is due to activation of soluble guanylate cyclase and a resultant increase in intracellular cyclic GMP. However, compared with nitroglycerin, there was less self-tolerance to the relaxant effects of FK409 and relatively little cross-tolerance between the two agents.

Keywords: FK409; nitroglycerin; dog coronary artery; self-tolerance; cross-tolerance; cyclic GMP

Introduction

(±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (FK409; FR900409) (Figure 1) is a structurally unique vasodilator discovered in the search for compounds with vasodilator and antiplatelet activities in fermentation products of *Streptomyces griseosporus* (Hino *et al.*, 1989). FK409 showed a potent relaxant activity on rat isolated aortae contracted with noradrenaline (Hino *et al.*, 1989). The drug also exerted a relaxant effect on dog isolated arterial strips contracted with KCl, and its relaxant potency was greater in coronary than in basilar, renal, mesenteric and saphenous arteries (Sato *et al.*, 1990). In anaesthetized dogs whose hearts underwent ischaemia by partial coronary occlusion, FK409 decreased left ventricular end-diastolic pressure and arterial blood pressure, and increased endomyocardial blood flow and increased the flow ratio of endomyocardium/epimyocardium (Sato *et al.*, 1990). These effects of FK409 are very similar to those observed with nitroglycerin in patients with ischaemic heart disease and in animal models of cardiac ischaemia (Becker & Pitt, 1971). In addition, FK409 inhibits platelet aggregation (Hino *et al.*, 1989), and nitrates also exert similar effects (Schafer *et al.*, 1980; Mellion *et al.*, 1981). Despite the close similarity between FK409 and nitroglycerin in vasodilator and anti-platelet aggregation activities, FK409 is not a nitrate. However, in view of its chemical structure it may produce

nitric oxide by biotransformation, although this has not yet been elucidated.

Tolerance to nitroglycerin is a significant clinical problem (Horowitz & Henry, 1987; Packer *et al.*, 1987; Katz, 1990). Unlike nitroglycerin, however, FK409 has been reported to develop neither self-tolerance nor cross-tolerance to nitroglycerin (Sato *et al.*, 1990). Thus, in the present study we initially compared the vasorelaxant effect of FK409 with that of nitroglycerin in dog isolated coronary arteries. Secondly, we investigated changes in cyclic nucleotide levels produced by the two vasodilators simultaneously with their relaxant effect. Thirdly, we determined whether the effects of FK409 were modified by methylene blue, an inhibitor of soluble guanylate cyclase (Ignarro *et al.*, 1981), or by M&B22,948, an inhibitor of cyclic GMP phosphodiesterase (Souness *et al.*, 1989). Finally, we studied the extent to which self-tolerance and cross-tolerance developed to the effects of FK409 and nitroglycerin.

Methods

Isolated coronary ring preparation

Mongrel dogs of either sex, weighing 7 to 12 kg were anaesthetized with pentobarbitone sodium (30 mg kg^{-1} , i.v.) and their hearts were removed. The proximal portion of the circumflex branch of the left coronary artery was dissected out from surrounding myocardial tissue and isolated coronary arterial rings (about 2 mm in length) were prepared by cutting with scissors. After the endothelium had been removed by rubbing, each ring was suspended in a 25 ml, thermostatically-controlled (37°C) organ bath containing Krebs-Henseleit solution bubbled with a mixture of 95% O_2 and 5% CO_2 . The composition of this solution was as follows (mM): NaCl 118, NaHCO_3 24.9, KCl 4.7, KH_2PO_4 1.2, CaCl_2 2.5, MgSO_4 1.2

Figure 1 Chemical structure of FK409.

and glucose 11.1. A resting tension of 2 g was applied throughout the 60 min equilibration period. Isometric changes in muscle tension were recorded on pen recorders (TOA, FBR252A) by means of isometric force transducers (NEC San-ei, 45196A).

Experimental protocol

Cumulative concentration-effects of FK409 and nitroglycerin were evaluated on preparations contracted with 10^{-7} M U46619 (an EC_{80}). Successive concentrations were applied when the vasorelaxant effect of the previous concentration had reached a nadir. Methylene blue (3×10^{-6} – 3×10^{-5} M) and M&B22,948 (3×10^{-6} – 3×10^{-5} M) were added to the Krebs-Henseleit solution 30 min before introduction of U46619. Tolerance was induced by means of the method of Henry *et al.* (1989). In brief, preparations were exposed for 60 min to the maximally-effective concentration of either nitroglycerin (10^{-4} M) or FK409 (10^{-5} M), and washed every 10 min for an additional 60 min before introduction of U46619.

Cyclic nucleotide assay

In experiments in which guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) contents were measured simultaneously with tension, coronary artery rings were removed from the baths at predetermined times after introduction of nitroglycerin or FK409, and immediately frozen in liquid nitrogen. The cyclic GMP and cyclic AMP contents of these frozen samples were measured with sensitive radioimmunoassay methods described elsewhere (Endoh & Taira, 1983).

Drugs

Nitroglycerin (0.05% aqueous solution in ampoule; Nippon Kayaku) FK409 (Fujisawa) and M&B22,948 (2-*o*-propoxyphenyl-8-azapurine-6-one; May & Baker) were generous gifts from each company. U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin $F_{2\alpha}$, Sigma), methylene blue (Wako Pure Chemical), and cyclic GMP-[125 I] and cyclic AMP-[125 I] assay kits (Amersham) were purchased. FK409 was dissolved in distilled water at a concentration of 10^{-2} M and methylene blue at a concentration of 10^{-1} M. M&B22,948 was dissolved in 10% triethanolamine at a concentration of 10^{-1} M. U46619 was dissolved in 99.5% ethanol at a concentration of 10^{-3} M. These stock solutions and nitroglycerin solution were diluted with distilled water to the desired concentrations when necessary.

Analysis of concentration-relaxation curves and statistics

In concentration-relaxation curves for nitroglycerin and FK409, relaxant effects were expressed as % inhibition of 10^{-7} M U46619-induced contraction, which we defined as 100%, and computer fitted to a logistic equation:

$$Y = M \times D^p / (K^p + D^p)$$

where M is the maximum effect of each drug, D is drug concentration, K is EC_{50} value of each drug and p is the slope parameter (Parker & Waud, 1971).

Experimental values are expressed in terms of mean $\pm$ s.e.mean. Comparisons of concentration-relaxation curves were made by analysis of variance. Statistical significance of differences between mean values was estimated by t test. A t test for the paired comparison was used when it was applicable. A P value smaller than 0.05 was considered to be significant.

Figure 2 Representative traces showing the relaxant effects of (a) nitroglycerin and (b) FK409 on dog coronary artery rings contracted with U46619 (10^{-7} M). The drugs were added at the points shown. Concentrations of the drugs are shown as $-\log [M]$.

Results

Vasorelaxant effects of FK409 and nitroglycerin

In isolated coronary artery rings contracted with U46619, cumulative administration of FK409 (10^{-11} – 10^{-5} M) or nitroglycerin (10^{-9} – 10^{-4} M) induced a concentration-dependent relaxation, and the maximum relaxation produced by each was a little more than 100%. Typical experiments are reproduced in Figure 2, and concentration-relaxation curves for both drugs are presented in Figure 3. The vasorelaxant effect of FK409 developed more slowly than that of nitroglycerin (Figure 2). The mean EC_{50} values of FK409 and nitroglycerin derived from 24 preparations for each were 4.2×10^{-9} M and 1.0×10^{-7} M, respectively. Thus, the vasorelaxant potency of FK409 was 24.4 times that of nitroglycerin. Slope parameters and maxima were not significantly different between nitroglycerin and FK409 (Figure 3).

Time course of relaxation and that of changes in cyclic nucleotide levels produced by nitroglycerin and FK409

The basal contents of cyclic GMP and cyclic AMP in isolated coronary rings contracted with U46619 were 29.1 ± 4.3 fmol mg^{-1} wet wt and 405.7 ± 90.3 fmol mg^{-1} wet wt, respectively. U46619 had no significant effect on either the cyclic GMP or the cyclic AMP content of the tissues. The time course of the relaxation and that of the changes in cyclic GMP and cyclic AMP levels in response to submaximum concentrations of nitroglycerin (10^{-6} M) and FK409 (3×10^{-8} M) are shown in Figures 4 and 5. After administration of nitroglycerin cyclic GMP levels increased rapidly

Figure 3 Mean concentration-relaxation curves for FK409 (○) and nitroglycerin (●) in U46619-contracted dog coronary artery rings. Each point represents the mean of 24 experiments; s.e.mean shown by vertical bars if larger than the point.

Figure 4 Time course of (a) the relaxation (○) and (b) of the changes in cyclic GMP (△) and cyclic AMP (▲) levels in response to nitroglycerin (10^{-6} M) in U46619-contracted dog coronary artery rings. Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

and reached a peak in 1 min, and then decreased, although the cyclic GMP content remained higher than basal values. At their peak, cyclic GMP levels increased 3.3 ± 0.9 fold ($n = 6$). However, the mechanical relaxation reached a nadir 2 min after the introduction of nitroglycerin with a maximum inhibition of $90.7 \pm 3.5\%$ ($n = 6$) (Figure 4). On exposure to FK409, cyclic GMP levels increased gradually and reached a maximum value after 5 min at which time cyclic GMP levels had increased 2.8 ± 0.5 fold ($n = 6$). The relaxation produced by FK409 reached a nadir after 10 min at which time the relaxation was $91.5 \pm 3.5\%$ ($n = 6$) (Figure 5). Both drugs, however, had no significant effects on cyclic AMP levels.

Figure 5 Time course of (a) the relaxation (○) and (b) of the changes in cyclic GMP (△) and cyclic AMP (▲) levels in response to FK409 (3×10^{-8} M) in U46619-contracted dog coronary artery rings. Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Figure 6 Mean concentration-relaxation curves for (a) nitroglycerin and (b) FK409 in U46619-contracted dog coronary artery rings in the presence of methylene blue added 30 min before introduction of U46619: (○) control; (●) 3×10^{-6} M; (□) 1×10^{-5} M and (■) 3×10^{-5} M. Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Modifications by methylene blue and M&B22,948 of the vasorelaxant effects of nitroglycerin and FK409

In the presence of methylene blue (3×10^{-6} – 3×10^{-5} M) the concentration relaxation curves for nitroglycerin and FK409 were shifted to the right (Figure 6). The mean EC_{50} values for nitroglycerin were significantly increased by 10^{-5} and 3×10^{-5} M methylene blue, and those of FK409 were also significantly increased by 3×10^{-6} , 10^{-5} and 3×10^{-5} M methylene blue ($n = 6$) (Table 1). The magnitudes of changes in the mean EC_{50} values produced by each concentration of methylene blue were similar for both nitroglycerin and FK409 (Table 1).

The concentration-relaxation curves for nitroglycerin and FK409 were shifted to the left in the presence of M&B22,948

Table 1 pEC_{50} values (negative logarithm of EC_{50} values) of nitroglycerin and FK409 in the absence (control) and presence of methylene blue and M&B22,948

	pEC_{50} value	
	Nitroglycerin	FK409
Methylene blue		
Control	7.01 ± 0.33	8.35 ± 0.14
3×10^{-6} M	6.45 ± 0.32	$7.74 \pm 0.08^*$
1×10^{-5} M	$6.05 \pm 0.25^*$	$7.42 \pm 0.09^*$
3×10^{-5} M	$6.01 \pm 0.21^*$	$7.01 \pm 0.33^*$
M&B22,948		
Control	6.93 ± 0.21	8.62 ± 0.10
3×10^{-6} M	7.30 ± 0.19	$9.23 \pm 0.12^*$
1×10^{-5} M	$7.59 \pm 0.17^*$	$9.47 \pm 0.24^*$
3×10^{-5} M	$7.68 \pm 0.19^*$	$9.89 \pm 0.26^*$

Values are means $\pm$ s.e.mean of six experiments. * $P < 0.05$ compared with control values.

Figure 7 Mean concentration-relaxation curves for (a) nitroglycerin and (b) FK409 in U46619-contracted dog coronary artery rings in the presence of M&B22,948 added 30 min before addition of U46619: (○) control; (●) 3×10^{-6} M; (□) 1×10^{-5} M and (■) 3×10^{-5} M. Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

(3×10^{-6} – 3×10^{-5} M) (Figure 7). The mean EC_{50} values for nitroglycerin were significantly decreased by 10^{-5} and 3×10^{-5} M M&B22,948, and those of FK409 by 3×10^{-6} , 10^{-5} and 3×10^{-5} M M&B22,948 ($n = 6$) (Table 1). The magnitudes of changes in the mean EC_{50} values produced by each concentration of M&B22,948 were similar for both nitroglycerin and FK409 (Table 1).

Contractions induced by U46619 were unaffected by methylene blue but were reduced by about 43% in the presence of the highest concentration (3×10^{-5} M) of M&B22,948 ($n = 6$).

Self-tolerance and cross-tolerance to the vasorelaxant effects of nitroglycerin and FK409

Isolated coronary artery rings of the dog pre-exposed for 60 min to either nitroglycerin (10^{-4} M) or FK409 (10^{-5} M) became significantly less responsive to the vasorelaxant actions of each drug than time-matched control rings (Figure 8). The mean EC_{50} value of nitroglycerin increased 61.5 fold ($n = 6$), but the increase in the mean EC_{50} value of FK409 was only 5.7 fold ($n = 6$) (Table 2).

Table 2 pEC_{50} values of nitroglycerin and FK409 in control and after exposure to nitroglycerin or FK409

	pEC_{50} value	
	Nitroglycerin	FK409
Control	7.29 ± 0.40	8.25 ± 0.10
Nitroglycerin (10^{-4} M)	$5.50 \pm 0.21^*$	8.10 ± 0.20
Control	7.07 ± 0.11	8.56 ± 0.15
FK409 (10^{-5} M)	$6.03 \pm 0.07^*$	$8.00 \pm 0.15^*$

Values are means $\pm$ s.e.mean of six experiments. * $P < 0.05$ compared with control values.

Figure 8 Mean concentration-relaxation curves for (a) nitroglycerin and (b) FK409 in U46619-contracted dog coronary artery rings in the tolerance study: (○) control; (●) after exposure to the maximum concentration of nitroglycerin (a, 1×10^{-4} M) or FK409 (b, 1×10^{-5} M). Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Rings pre-exposed to 10^{-5} M FK409 also became less responsive to the vasorelaxant action of nitroglycerin than time-matched control rings, with a 10.9 fold increase in the mean EC_{50} value of nitroglycerin ($n = 6$) (Figure 9). The concentration-relaxation curve for FK409 tended to shift to the right following pre-exposure to 10^{-4} M nitroglycerin, but the resulting mean EC_{50} value of FK409 was not significantly different from that of the time-matched control group ($n = 6$) (Table 2).

Pre-exposure to either nitroglycerin (10^{-4} M) or FK409 (10^{-5} M) for 60 min had no effect on contraction induced by U46619 (10^{-7} M).

Discussion

In the present experiments on dog isolated coronary artery rings, FK409 was a very potent vasorelaxant; comparing EC_{50} values, FK409 was about 25 times more potent than nitroglycerin, while the vasorelaxant effect of FK409 developed more slowly than that of nitroglycerin. There was, however, no significant difference between the slopes and maxima of concentration-relaxation curves to FK409 and nitroglycerin.

The vasorelaxant mechanism of nitroglycerin is currently understood to involve an increase in intracellular cyclic GMP levels (Kukovetz *et al.*, 1979). In the present experiments, nitroglycerin increased cyclic GMP levels and the peak increase coincided with its relaxant effects. FK409 at a sub-maximal concentration (3×10^{-8} M) also elevated cyclic GMP levels. This increase occurred more slowly than with nitroglycerin and remained elevated in the presence of FK409. Thus, as is the case with nitroglycerin, FK409 seems to produce vasorelaxation via an increase in intracellular cyclic

Figure 9 Mean concentration-relaxation curves for (a) nitroglycerin and (b) FK409 in U46619-contracted dog coronary artery rings in the cross-tolerance study: (○) control; (●) after exposure to the maximum concentration of FK409 (a, 1×10^{-5} M) or nitroglycerin (b, 1×10^{-4} M). Each point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

GMP. It is known that this effect of nitroglycerin is the result of activation of soluble guanylate cyclase (Ignarro *et al.*, 1981). Consequently, the vasorelaxant effect of nitroglycerin is inhibited by methylene blue, an inhibitor of soluble guanylate cyclase (Ignarro *et al.*, 1981; Martin *et al.*, 1984; Shibata *et al.*, 1986; Ohba *et al.*, 1987). In the present experiments, the vasorelaxant effect of FK409 was also inhibited by methylene blue as was that of nitroglycerin. M&B22,948 is a selective inhibitor of cyclic GMP phosphodiesterase (Souness *et al.*, 1989). If an increase in intracellular cyclic GMP plays a key role in the vasorelaxant effect of FK409, its effect should be potentiated by M&B22,948. Indeed, the present study showed that the vasorelaxant effect of FK409 was potentiated by M&B22,948

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essentially to the same extent as that of nitroglycerin. Thus, it is highly likely that FK409, although not a nitrate, activates soluble guanylate cyclase and produces vasorelaxation through accumulation of cyclic GMP as does nitroglycerin. The greater potency of FK409 than nitroglycerin may indicate that FK409 activates soluble guanylate cyclase more efficiently than nitroglycerin.

Recently nitroglycerin tolerance has become a significant clinical problem, because it limits the usefulness of nitroglycerin (Horowitz & Henry, 1987; Packer *et al.*, 1987). In the present experiments, after exposure to the maximally-effective concentration of nitroglycerin (10^{-4} M) the vasorelaxant potency of this agent was reduced to about 1/60. In contrast the potency of FK409 after exposure to its maximally-effective concentration (10^{-5} M) was reduced to only about 1/6. Thus, the degree of FK409 tolerance was about 1/10 that of nitroglycerin tolerance. Furthermore, after exposure to nitroglycerin there was no significant change in the vasorelaxant potency of FK409, although the potency of nitroglycerin was reduced to about 1/10 after exposure to FK409. The mechanism of tolerance to nitrovasodilators is probably closely associated with their metabolism within the tissue (Horowitz *et al.*, 1987; Fung *et al.*, 1989; Katz, 1990). Organic nitrates must first undergo reduction by organic nitrate reductase to form nitrite ions and consequently nitric oxide or S-nitrosothiols, and in these processes thiol donors are required (Ignarro *et al.*, 1981). Thus, the depletion of such donors results in the failure to form nitric oxide or S-nitrosothiol and thus eventually a reduced vasorelaxation. Nitric oxide and/or S-nitrosothiol are thought to be intermediates capable of activating soluble guanylate cyclase (Ignarro *et al.*, 1981). Based on this conceptual framework, the lower liability of FK409 for developing self-tolerance or cross-tolerance to nitroglycerin could be explained if the initial steps required for the metabolism of other organic nitrates are somehow bypassed by FK409 to allow the formation of nitric oxide or S-nitrosothiols more readily. Similar explanations have been put forward for other non-nitrate drugs such as molsidomine or its active metabolite SIN-1 (Fung *et al.*, 1989; Katz, 1990). Alternatively it is possible that FK409 directly stimulates guanylate cyclase. Whatever mechanism may be involved in the lower liability of FK409 to produce self-tolerance and cross-tolerance to nitroglycerin, this property should make this drug advantageous over tolerance-producing nitrates currently in clinical use.

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