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Vasorelaxant effects induced by the antiangiogenic drug linomide in aortic and saphenous vein preparations of the rabbit

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1 Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxoquinoline-3-carboxamide) inhibits vascular proliferation and has been proposed as an antiangiogenic drug. We have investigated the vascular effect of linomide in rabbit aortic and saphenous vein ring preparations and in rat cultured vascular smooth muscle cells (VSMCs).

2 Linomide (25–300 μg ml^{-1}) did not alter the basal tone of the preparations. The drug induced a concentration-dependent relaxant effect in aortic rings with endothelium, preconstricted by noradrenaline (NA), 5-hydroxytryptamine (5-HT) and by the thromboxane mimetic U46619.

3 The degree of relaxation induced by linomide was significantly reduced by exposure to the cyclo-oxygenase inhibitors indomethacin (3 μM) and acetylsalicylic acid (500 μM), and was not influenced by pretreatment with the nitric oxide synthase inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) (100 μM) in aortic rings with endothelium, preconstricted with NA.

4 Endothelium removal significantly reduced the relaxant response to linomide in aortic ring preparations.

5 A concentration-dependent relaxant response was observed also in rabbit saphenous vein preparations deprived of endothelium and preconstricted either by NA or U46619. The degree of relaxation obtained in a high potassium solution was consistently smaller than that observed in NA-pretreated venous preparations.

6 The vasorelaxant effect of linomide was consistently blunted by the adenylate cyclase inhibitor SQ 22536 (50 μM), both in intact aortic rings and in those deprived of endothelium.

7 In rat cultured vascular smooth muscle cells, linomide (100–200 μg ml^{-1}) induced a significant increase in cyclic AMP levels, which was blocked by exposure to 50 μM SQ 22536.

8 In endothelium-deprived aortic ring preparations, the linomide-induced relaxant effect was greatly reduced in high potassium medium (KCl = 25 mM). Pretreatment with the ATP potassium channel inhibitor glibenclamide (3 μM) significantly reduced the linomide-induced relaxation.

9 The results show that linomide possesses a vasorelaxant effect which is attributable to both endothelium-dependent and -independent properties. While the former component of the drug's activity is apparently due to the release of a prostanoid from endothelial cells, the endothelium-independent mechanism involved in linomide relaxation is linked to cyclic AMP accumulation and to ATP-sensitive potassium channel activation in VSMCs.

Keywords: Linomide; antiangiogenic drugs; vascular relaxation; cyclic AMP; K^+ channels; vascular smooth muscle cells

Introduction

Angiogenesis, i.e. the development of new blood vessels, is necessary for the continuous growth of solid tumours and is involved in their metastatic spread; it is currently proposed that pharmacological treatments able to inhibit angiogenesis may represent a promising rational approach to the therapy of malignancies (Folkman, 1995). Antiangiogenic drugs could be used to potentiate conventional chemotherapy and radiotherapy (Teicher *et al*., 1994). Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxoquinoline-3-carboxamide) has been shown to inhibit the growth rate and development of metastases of murine melanoma cells and rat prostatic cancer *in vivo* (Kalland, 1986; Ichikawa *et al*., 1992). This drug possesses relevant antiangiogenic properties both in non-tumour assay models of angiogenesis (Vukanovic *et al*., 1993; Ziche *et al*., 1997) and in rat prostatic carcinoma *in vivo* (Vukanovic *et al*., 1995; Vukanovic & Isaacs, 1995; Joseph *et al*., 1996), where blood flow and microvascular density appear to be reduced following drug administration (Vukanovic *et al*., 1993; Vukanovic & Isaacs, 1995). The mechanism of linomide antiangiogenic activity involves its ability to reduce the infiltration of macrophages and to inhibit specifically angiogenic factor-induced growth and migration of microvascular endothelial cells *in vitro* (Vukanovic & Isaacs, 1995; Parenti *et al*., 1996).

Drugs with antiangiogenic properties act on the highly proliferative endothelium and do not exhibit cytotoxic effects (Fan *et al*., 1995). Despite the fact that the target of antiangiogenic drugs is the vasculature, the pharmacological profile of their vascular effects has never been investigated. The aim of the present study was to assess the vascular effect of the antiangiogenic drug linomide, by investigating its functional and biochemical effects on rabbit aortic and saphenous ring preparations and on rat cultured vascular smooth muscle cells (VSMCs).

Methods

Aortic and saphenous ring preparations

The thoracic segment of the aorta and the saphenous vein were obtained from male New Zealand rabbits (2.5–3 kg) and cut into rings of 3–4 mm width. The preparations were suspended between stainless steel hooks and mounted in a 10 ml organ bath filled with warmed (37°C) and oxygenated (95% O_2, 5% CO_2) Krebs solution. The incubation solution had the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7,

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KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10. When a high potassium solution (25 mM) was used, an equimolar reduction in NaCl was performed. A tension of 1–2 g was applied and isometric contraction was recorded by a transducer on a polygraph chart (Battaglia Rangoni). After 60–90 min of equilibration, various concentration-response curves for noradrenaline (NA), U46619 and 5-hydroxytryptamine (5-HT) were performed and a concentration of each agonist able to induce an increase in tension of approximately the same degree was chosen. The relaxant effect of linomide was tested in preconstricted preparations by obtaining cumulative response-curves for the drug at concentrations ranging from 25 to 300 μg ml^{-1} (70–860 μM). These concentrations were chosen since they were similar to that previously shown able to inhibit the angiogenic factor-induced growth and migration of endothelial cells *in vitro* (Vukanovic & Isaacs, 1995; Parenti *et al*., 1996). The increase in tonic tension induced by different vasoconstrictor agents was taken as 100% value, and the vasodilating effect induced by linomide was referred to as percentage of change from this value. Since the effect of linomide was scarcely reversible, it was not possible to test the interference by other drugs on linomide action by obtaining a second curve in the same preparation. Thus, the effects of different pharmacological tools were evaluated by comparing the relaxant response to linomide in control preparations to that observed in preparations treated with the interfering drugs. Rabbit aortic rings with and without endothelium, and endothelium-deprived saphenous vein preparations were used for the study. The presence of functional endothelium was assessed by testing the relaxant response to 1 μM acetylcholine (ACh); the preparations in which this concentration of ACh relaxed the preparations by less than 50% were discarded. Endothelium-deprived preparations were obtained by carefully removing the intimal surface by polyethylene tubing. The lack of any relaxant response to ACh and the presence of full relaxation induced by the nitric oxide-donor drug sodium nitroprusside (10 μM) and/or by the adenylate cyclase activator forskolin (1 μM) were tested in these preparations, in order to confirm that the procedure was successful and that no damage was caused to smooth muscle tissue.

Cell cultures

VSMCs were isolated from thoracic aorta, obtained from male Wistar rats, weighing 200-250 g. The vessel was aseptically excised and placed in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). Adhering fat and connective tissue were removed by blunt dissection. The aorta was then opened longitudinally and preincubated in DMEM containing 0.1% collagenase for 30 min at 37°C in 95% air and 5% CO_2. The adventitia was carefully removed as well as the luminal surface, scraped with forceps to remove endothelial cells, and then minced into 1 mm pieces and incubated again with collagenase for 20 min. After centrifugation the pellet was resuspended in medium containing 20% calf serum (CS). Cells were cultured in DMEM supplemented with 10% CS, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, and kept in a humidified incubator at 37°C in 5% CO_2; they were split twice a week 1:2 with trypsin-EDTA solution. Cells used for this study were from 5–6 passages and were characterized by immunohistochemical assay with a monoclonal antibody against α-actin (Sigma); 90% of the cells were positive to α-actin.

Cyclic AMP and cyclic GMP determination

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in smooth muscle cells were measured as described by Masini *et al*. (1991). Briefly, the cells were plated in 100 mm diameter dishes and allowed to grow to 90% confluence (about 1.5–2×10^6 final cell number). Stimulation was carried out in phosphate buffered saline (PBS). Cell monolayers were pretreated for 15 min with 50 μM isobutyl-1-methylxanthine (IBMX) to block phosphodiesterase activity. The cells were stimulated with the test substances for 10 min. At the end of incubation the cells were washed and scraped off in 500 μl ice-cold trichloroacetic acid (TCA) and centrifuged at 13000 $g \times 10$ min at 4°C. After centrifugation the supernatant was extracted with tri-n-octylamine (0.5 M) in 1,1,2-trichlorotrifluoroethane. The levels of cyclic AMP and cyclic GMP in the aqueous phase were measured by commercially available radioimmunoassays in duplicate with iodinated kits from Amersham. The cyclic AMP and cyclic GMP contents of each dish were expressed as pmol mg^{-1} protein; the drug-induced changes in this value were expressed as a percentage baseline.

Statistical analysis

All results were expressed as mean values ± s.e.mean. Statistical analysis was performed by analysis of variance followed by the LDS test in order to evaluate the differences between groups. Student's *t* test for unpaired data was used in the experiments in which cyclic AMP levels were assayed. A P value <0.05 was considered significant.

Drugs and chemicals

The drugs and chemicals used were: linomide(N-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxoquinoline-3-carboxamide) (kindly supplied by Dr Beryl Hartley-Asp of Kabi Pharmacia Therapeutics); acetylcholine hydrochloride (ACh), (−)-noradrenaline hydrochloride (NA), forskolin, tri-n-octylamine, acetylsalicylic acid, collagenase, sodium nitroprusside, indomethacin, glibenclamide, 5-hydroxytryptamine (5-HT), 3-isobutyl-1-methylxanthine (IBMX), tetraethylammonium chloride (TEA), DMEM, trypsin-EDTA solution, streptomycin and penicillin were purchased from Sigma Chemical Co.; N^G-nitro-L-arginine methyl ester (L-NAME) was from NovaBiochem; 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22356) and U46619 (9,11-dideoxymethano-epoxy-9α,11α-prostaglandin $F_{2\alpha}$) from Research Biochemicals International. Cell culture reagents were purchased from Boehringer-Mannheim. Calf serum (CS) was purchased from Gibco. Tissue culture plastic was from Costar Ltd.

Stock solutions of indomethacin were dissolved in ethanol and those of IBMX in dimethyl sulphoxide; all the other substances were dissolved in water and further dilutions were made in Krebs solution.

Results

Relaxant effect of linomide in preconstricted aortic ring preparations with endothelium

Exposure for 7 min to concentrations of linomide ranging from 25 to 300 μg ml^{-1} did not modify the resting tone of the preparations. An increase in tension to 1396 ± 39 mg ($n = 36$) was induced by NA (0.3–1 μM) pretreatment of the preparations. In NA-preconstricted aortic rings, a concentration-dependent relaxing effect was induced by linomide (25–300 μg ml^{-1}), as shown in Figure 1. The maximum effect observed with 300 μg ml^{-1} of the drug consisted of a relaxation of 64.4 ± 3.6%, a degree similar to that induced by 1 μM ACh (about 70%) in the same preparations. Administration of 5-HT (1–3 μM) and U46619 (0.3 μM) increased the resting tension very similarly to that obtained with NA (1300 ± 80 and 1396 ± 43 mg, respectively, $n = 5$). The vasorelaxant effect induced by linomide in preparations treated with these two agonists was superimposable on that observed in NA-treated preparations (Figure 1). Since the relaxant response to linomide was not influenced by the kind of vasoconstrictor agent employed, the following experiments were performed in NA-preconstricted preparations.

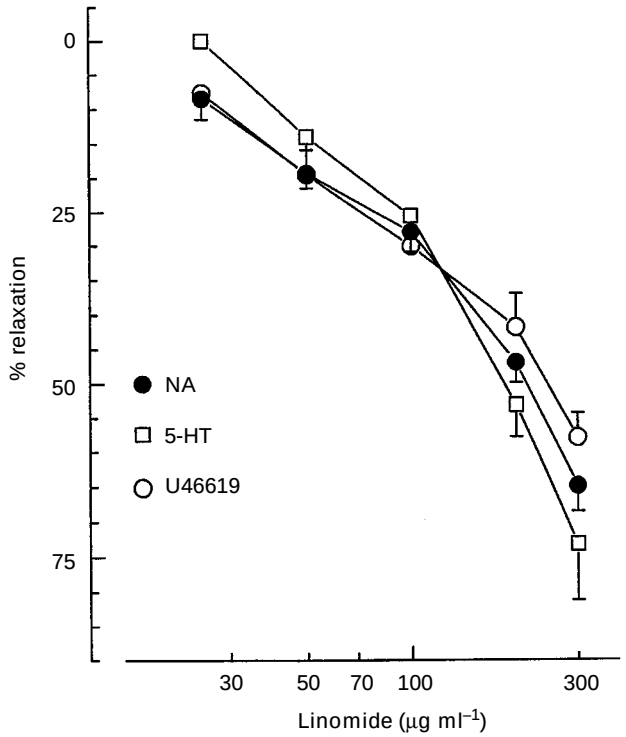

Figure 1 Relaxant effect of linomide in aortic ring preparations with endothelium and preconstricted by noradrenaline (NA), U46619 and 5-hydroxytryptamine (5-HT). Each point represents the mean of at least 7 experiments; vertical lines show s.e.mean. $*P<0.05$.

The treatment for 30 min with L-NAME (100 μM) did not modify the degree of contractile response to NA, and was able to block fully the ACh-induced relaxation. However the linomide-induced relaxation was not reduced by previous exposure to the NO-synthase inhibitor (Figure 2a). Exposure of the preconstricted aortic rings for 60 min to a concentration of indomethacin (3 μM) able to block cyclo-oxygenase activity (Flower, 1984) did not alter the contractile response to NA, but significantly reduced the relaxant effect of the higher concentrations of linomide tested (Figure 2b). Similar results were obtained by using a different cyclo-oxygenase inhibitor, acetylsalicylic acid, at the concentration of 500 μM (Figure 2b).

Relaxant effect of linomide in endothelium-deprived aortic ring preparations

The size of the contractile response to NA was not significantly changed by previous endothelium deprivation. However, the relaxant effect of linomide was reduced in endothelium-deprived preparations (Figure 3); the differences between the effects observed in the intact and endothelium-deprived preparations reached significance at the two higher concentrations of linomide tested (200 and 300 μg ml^{-1}). The concentration-relaxant effect curve to linomide obtained in endothelium-deprived preparations was almost completely superimposable on those observed in the presence of indomethacin and acetylsalicyclic acid.

Relaxant effect of linomide in endothelium-deprived saphenous vein ring preparations

In the rabbit saphenous vein ring preparations deprived of endothelium, NA (0.1–3 μM) induced an increase in tonic tension which amounted to 650 ± 10 mg ($n=7$). In such preparations linomide (25–300 μg ml^{-1}) induced a concentration-dependent relaxant response the extent of which was very similar to that observed in endothelium-deprived aortic ring preparations (Figure 4). Results similar to those described

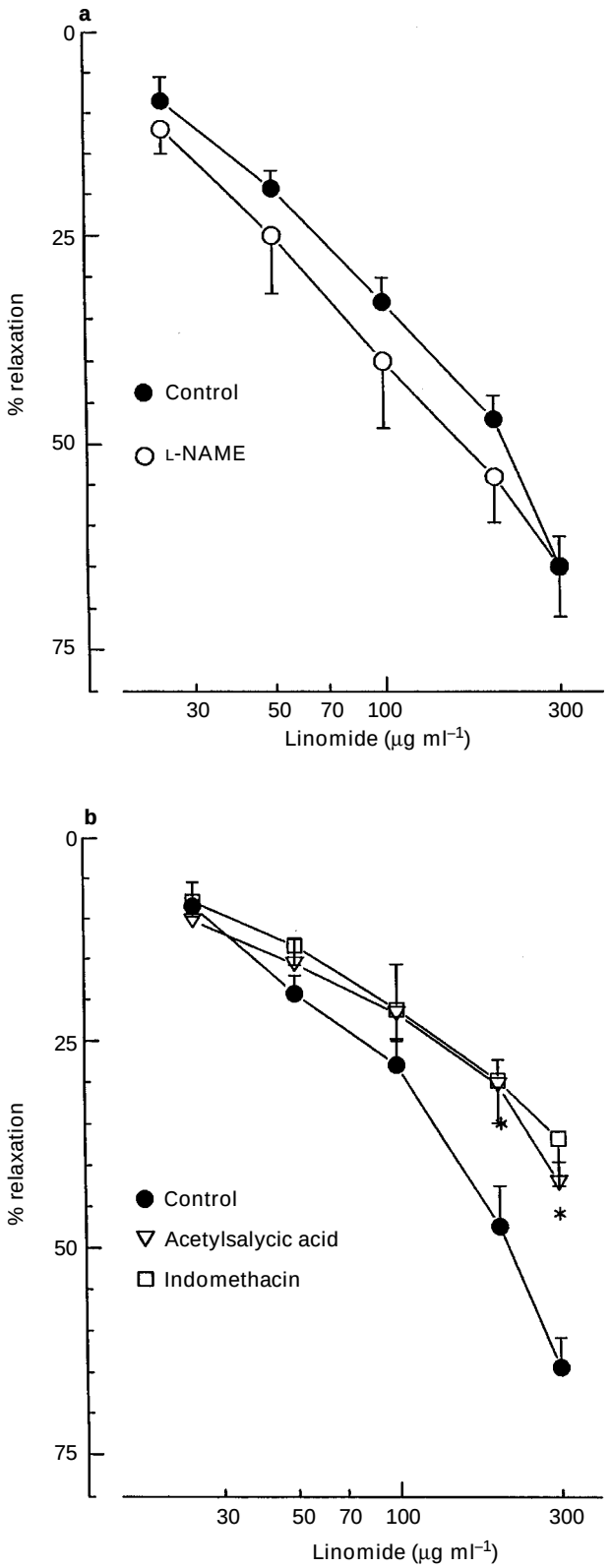

Figure 2 Effect of 100 μM L-NAME (a), 3 μM indomethacin and 500 μM acetylsalicylic acid (b) on linomide-induced relaxation in NA-preconstricted aortic ring preparations with endothelium. Each point represents the mean of at least 6 experiments; vertical lines show s.e.mean. $*P<0.05$.

above were obtained in preparations preconstricted by U46619 (data not shown, $n=3$).

The size of the contractile response to KCl (25 mM) was not significantly different from that induced by NA. However, the vasorelaxant response to linomide in high K^+ medium was

significantly smaller than that observed in the presence of NA (Figure 4).

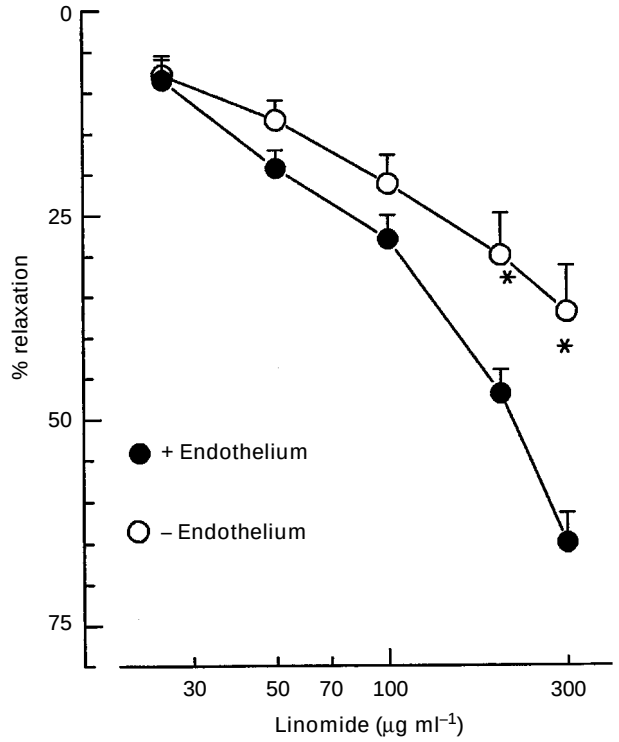

Figure 3 Relaxant effect of linomide in aortic ring preparations with (+) and without (−) endothelium preconstricted by NA. Each point represents the mean of at least 7 experiments; vertical lines show s.e.mean. $*P<0.05$.

Effect of linomide on cyclic AMP levels and cyclic-AMP-dependent relaxant mechanisms in aortic ring preparations and VSMCs

Since an increase in cyclic AMP levels could be involved in the direct relaxant response to linomide in preconstricted preparations, the effect of SQ 22536, an inhibitor of the adenylate-cyclase, was investigated. The relaxant effect of linomide was reduced by pretreatment for 30 min with 50 μM SQ 22536 (Figure 5) both in preparations with and without endothelium. However, this reduction was greater in intact preparations than in those deprived of endothelium (Figure 5).

The direct effect of linomide on cellular cyclic AMP levels was also assessed in cultured VSMCs. The basal levels of cyclic AMP in the cells amounted to 6 ± 0.1 pmol mg^{-1} protein. The 10 min treatment with linomide 100 and 200 μg ml^{-1} induced a significant rise in cyclic AMP levels, which increased by $31.6\pm2.5\%$ and $58.3\pm13\%$, respectively (Figure 6). The levels of the cyclic nucleotide in the cells exposed to 50 μM SQ 22536 for 30 min were slightly reduced (5.23 ± 0.4 pmol mg^{-1} protein). The increases in the cyclic AMP levels induced by the two linomide concentrations were completely blocked in the VSMCs pretreated with the adenylate cyclase inhibitor (Figure 6), at a concentration which was able to reduce by 50% the rise in cyclic AMP levels induced by a maximal effective concentration (3 μM) of the direct adenylate cyclase activator forskolin (data not shown). Conversely, in the same experimental conditions, cyclic GMP levels were unaffected by exposure to linomide (data not shown).

Effect of potassium channel inhibitors on linomide-induced relaxation in aortic endothelium-deprived preparations

In aortic ring preparations deprived of endothelium and precontracted by incubation in a medium containing a high K^+ concentration ($[K^+]=25$ mM) plus prazosin (1 μM), the

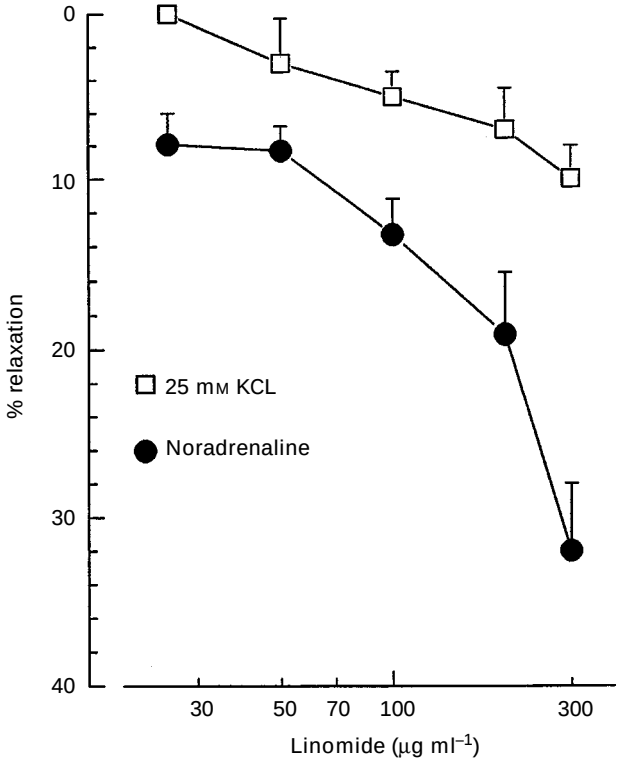

Figure 4 Relaxant effect induced by linomide in saphenous endothelium-deprived preparations preconstricted by NA and by exposure to a high potassium medium (25 mM KCl). Each point represents the mean of at least 6 experiments; vertical lines show s.e.mean. $*P<0.05$.

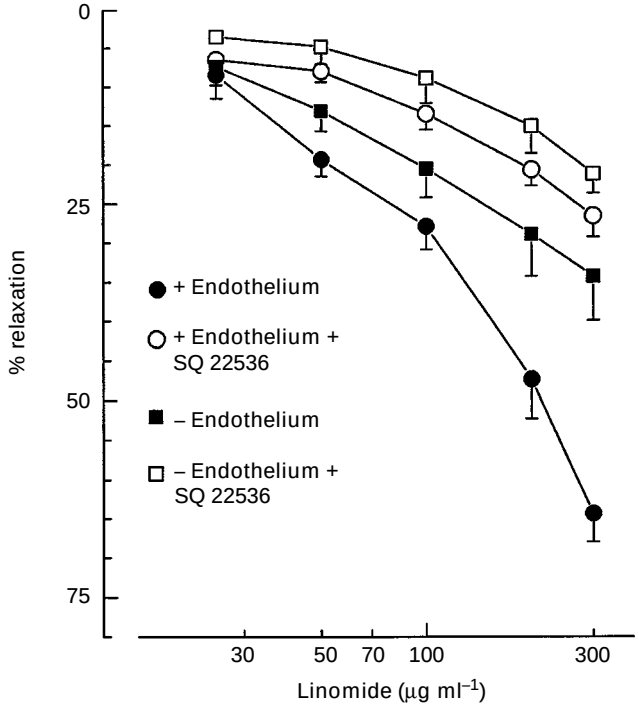

Figure 5 Influence of the adenylate-cyclase inhibitor SQ 22536 (50 μM) on the linomide-induced relaxant effect in aortic ring preparations with (+) and without (−) endothelium. Each point represents the mean of at least 6 experiments; vertical lines show s.e.mean. All the points are significantly different ($P<0.05$) from their control at concentrations of 50 μg ml^{-1} and above.

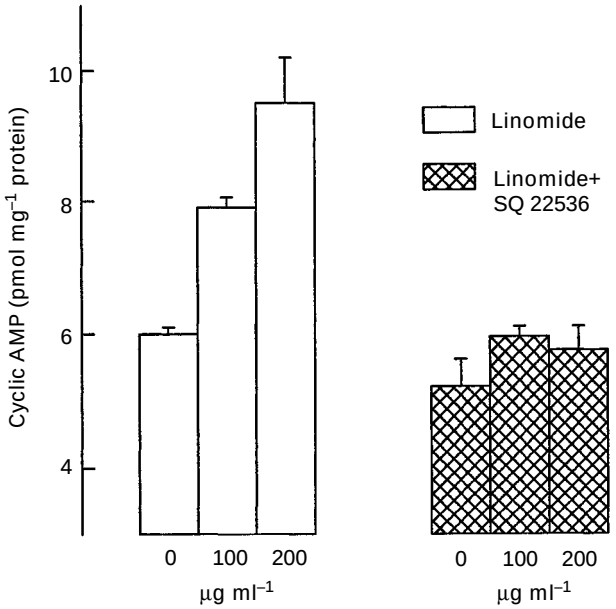

Figure 6 Effect of two different concentrations of linomide on cyclic AMP levels in untreated smooth muscle cells, and in cells previously exposed to 50 μM SQ 22536. Each column represents the mean ± s.e.mean of at least 3 experiments in duplicate.

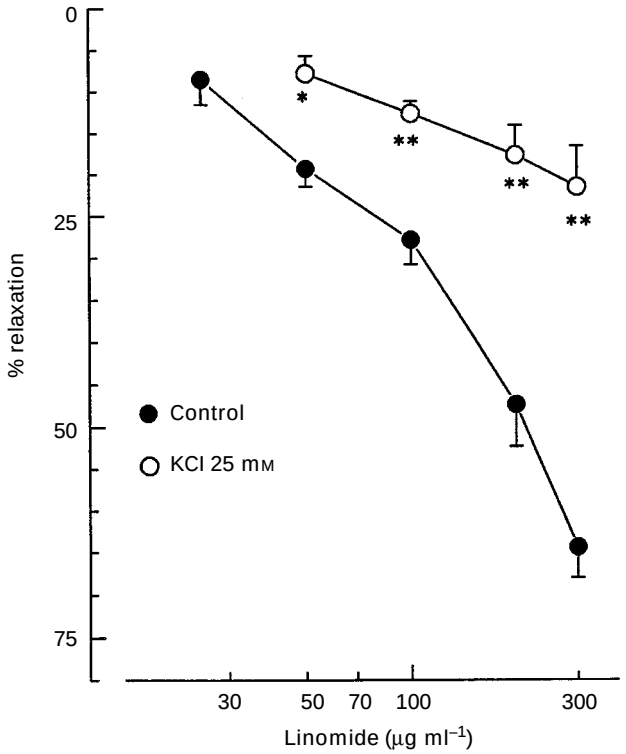

Figure 7 Comparison between the relaxant effect of linomide in NA-preconstricted aortic ring preparations and that in preparations exposed to a high potassium (KCl = 25 mM) medium. Each point represents the mean of at least 8 experiments; vertical lines show s.e.mean. $*P<0.05$, $**P<0.01$.

relaxant activity of linomide was strongly reduced (Figure 7). The tension, which increased to 1530 ± 152 mg in the presence of the high-K^+ medium, was reduced by only about 20% by the higher concentration of linomide tested (300 μg ml^{-1}).

In preparations preconstricted by NA, 30 min exposure to glibenclamide (3 μM), an inhibitor of ATP-sensitive K^+ channels, consistently reduced the relaxant effect of linomide. The degree of relaxation (36.8 ± 5.7%) observed with the higher concentration of linomide in endothelium-deprived preparations was reduced to 50% (13.4 ± 2.7%) by glibenclamide (Figure 8). In contrast, the Ca^{2+}-activated K^+ channel blocker tetraethylammonium (TEA), at a concentration of 7.5 mM, was not able to modify the relaxant effect of linomide (data not shown, $n=4$).

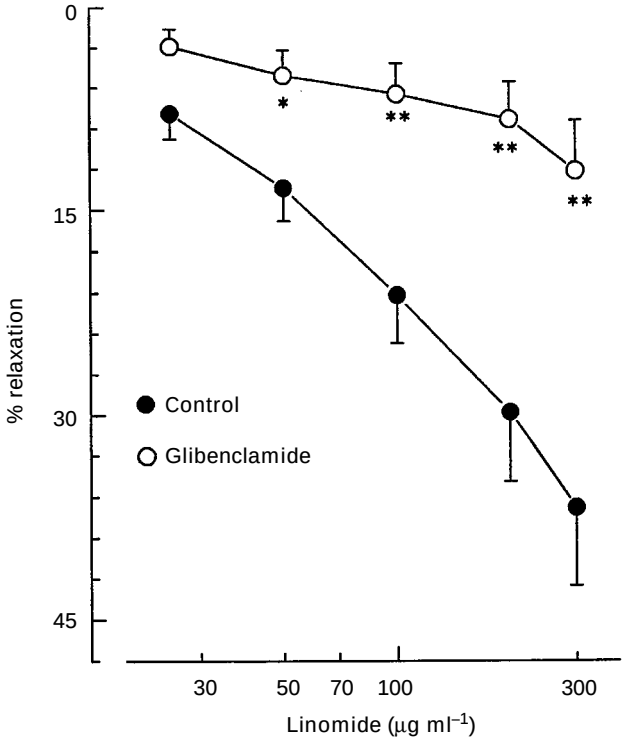

Figure 8 Effect of 3 μM glibenclamide on linomide-induced relaxation in endothelium-deprived aortic ring preparations preconstricted by NA. Each point represents the mean of at least 6 experiments; vertical lines show s.e.mean. $*P<0.05$, $**P<0.01$.

Discussion

Continuous and long-term therapy with antiangiogenic drugs has been proposed in association with conventional chemotherapy to reduce the spread of metastasis. An important issue in this type of therapy is to limit tumour diffusion by eliminating access to the vasculature of tumour cells while delivering cytotoxic drugs to the tumour. The goal of an antiangiogenic drug is to inhibit proliferation of capillary endothelia undergoing rapid mitosis in response to growth factors. By doing so, antiangiogenic drugs could also reduce drug diffusion to the tumour. Thus the pharmacological profile of the vascular effects of an antiangiogenic drug is of interest.

The present study, which represents the first attempt to describe the vascular effects of the antiangiogenic drug linomide, demonstrates that the drug has concentration-dependent vasorelaxant activity both in arterial and venous preparations. The observation that the response to linomide was detectable not only in NA-precontracted preparations, but also in those exposed to other vasoconstrictor agents such as 5-HT and U46619, indicates that the vasorelaxant properties of the drug are not merely attributable to α-adrenoceptor antagonist activity.

In arterial preparations preconstricted by NA, the relaxant effect of linomide is attributable to both an endothelium-dependent and -independent mechanism of action. This conclusion is based chiefly upon results showing that the relaxant effect of linomide was significantly reduced by removing endothelium from the preparations.

The endothelium-dependent component of the effect of linomide seems to be completely attributable to the production of a prostanoid by the endothelial cells. In fact, linomide-induced relaxation was inhibited by concentrations of indomethacin and acetylsalicylic acid able to block cyclo-oxygenase activity (Flower, 1984). The degree of relaxation induced by linomide in the presence of the two cyclo-oxygenase inhibitors was almost completely superimposable on that observed in endothelium-deprived aortic preparations. Furthermore, L-NAME did not influence the linomide relaxing effect in intact preparations, thus indicating that the NO-pathway is not implicated in the endothelium-dependent component of the drug's effect. However, the observation that a consistent part of the linomide relaxant effect was still present in aortic endothelium-deprived preparations and in those treated with cyclo-oxygenase inhibitors, suggests that linomide has a direct effect on vascular smooth muscle cells. This finding prompted us to study the response to linomide of endothelium-deprived preparations exposed to molecules able to interfere with smooth muscle relaxation. Both in aortic and saphenous vein preparations the relaxant activity of linomide is strongly reduced in a high K^+ medium. Since the increase in tonic tension obtained in this experimental condition is due to the opening of calcium channels (Spedding & Cavero, 1984), the reduced effectiveness of linomide in relaxing vascular smooth muscle argues against the hypothesis that the drug exhibits calcium entry blocking activity. On the other hand, linomide is able to increase the cyclic AMP level through stimulation of adenylate cyclase. In fact, cyclic AMP levels were significantly and concentration-dependently increased in cultured smooth muscle cells; this effect was antagonized by SQ 22536, an adenosine analogue with a specific inhibitory effect on the adenylate-cyclase activity both in isolated cells and in intact preparations (Haslam *et al*., 1978; Lippe & Ardizzone, 1991).

The present results also suggest that K^+ channels are involved in the linomide mechanism of action, as shown in endothelium-deprived aortic preparations, where the relaxant effect of linomide was unaffected by the unspecific K^+ channel blocker TEA, but was effectively antagonized by the ATP-sensitive K^+ channel inhibitor glibenclamide (Edwards & Weston, 1993). The functional presence in vascular smooth muscle cells of K^+ channels inhibited by intracellular ATP, the opening of which causes relaxation, is now firmly established (Standen *et al*., 1989). Such channels are recognized as targets of various vasodilators acting as ATP-sensitive channel openers, such as cromakalim and pinacidil, the electrophysiological and relaxant effects of which are effectively antagonized by glibenclamide (Standen *et al*., 1989; Cavero *et al*, 1989; Wickenden *et al*., 1991; Edwards & Weston, 1993). This kind of channel is also involved (Nelson *et al*., 1990; Quayle *et al*., 1994) in the potent vascular relaxant effect induced by calcitonin gene-related peptide (CGRP). Thus the results obtained in endothelium-deprived preparations point to adenylate-cyclase stimulation and ATP-sensitive K^+ channels as the mechanisms involved in the direct effect exerted by linomide on vascular smooth muscle. Our findings are not sufficient to explain whether or not a link could exist between these two mechanisms. What can be stated is that such a correlation has indeed been suggested in the case of CGRP, which has a potent relaxant effect in vascular (Nelson *et al*., 1990; Amerini *et al*., 1994; Quayle *et al*., 1994) and non vascular smooth muscle (Santicioli *et al*., 1995) that has been attributed to cellular mechanisms identical to those demonstrated in the present study as responsible for the direct component of linomide's activity. In particular, it has been proposed that, following CGRP-induced stimulation of adenylate-cyclase, a protein kinase A-dependent phosphorylation step is responsible for ATP-sensitive K^+ channel opening (Quayle *et al*., 1994).

In conclusion, linomide is used as an angiogenesis inhibitor in clinical trials in patients with advanced cancer (Vukanovic *et al*., 1993). Our data demonstrate that linomide is active on the vascular wall leading to arterial and venous relaxation. We have previously shown that linomide specifically inhibits endothelial cell sprouting and proliferation, *in vitro*, induced by vascular endothelial growth factor and does not block the spontaneous replication of normally quiescent cells (Parenti *et al*., 1996). In the present study, we demonstrated that linomide has vasorelaxant properties due to both endothelium-dependent and -independent mechanisms. Therefore, together with its efficacy in inhibiting capillary growth in tumours, linomide appears to possess vascular effects that might favour drug diffusion to the tumour, thus possibly contributing to improved anticancer therapy.

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