British Journal of Pharmacology (1996) 118, 1639-1644

Action of probucol in arteries from normal and hypercholesterolaemic rabbits

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1 The effect of probucol on the vascular reactivity of different arteries isolated from rabbits was studied as well as its effects on the development of atherosclerosis in a cholesterol-fed rabbit model.

2 Probucol $10^{-6}-5\times10^{-4}$ M produced a concentration-dependent inhibition of the contractile responses induced by KCl (80 mM), the sequence for the IC_{50} was: mesenteric artery (5th branch, $4.8\pm2.6\times10^{-5}$ M) >aorta ($8.2\pm2.3\times10^{-5}$ M) >femoral artery ($>5\times10^{-4}$ M). The response to noradrenaline was: mesenteric artery (5th branch, $4.2\pm1.3\times10^{-5}$ M) >aorta ($3.2\pm3.0\times10^{-4}$ M) >femoral ($>5\times10^{-4}$ M).

3 In the aorta, probucol ($10^{-5}-10^{-4}$ M) shifted the concentration-response curves to Ca^{2+} downward and to the right.

4 Probucol at 5×10^{-5} M and 5×10^{-4} M showed a reduction in the $^{45}Ca^{2+}$ uptake in resting, non-stimulated aortic rings as well as the uptake induced by both noradrenaline 10^{-6} M and KCl 80 mM.

5 In experiments *in vivo*, probucol did not affect lipid profiles; however, drug-treatment significantly decreased the cholesterol content of aortic tissue and the extent of intimal surface covered with atherosclerotic lesions.

6 The vascular reactivity was recovered in femoral arteries from rabbits on the atherogenic diet plus probucol.

7 It is concluded that the effect of probucol in vascular smooth muscle can be attributed to an inhibition of Ca^{2+} entry through both potential- and receptor-operated pathways. Moreover our findings suggest that the effects of probucol on movement of calcium in vascular smooth muscle may play an important role in the mechanism of antiatherogenic properties of this drug.

Keywords: Probucol; rabbit arteries; atherosclerosis

Introduction

Two of the major mechanisms implicated in atherogenesis are the oxidation of LDL (Henry, 1990) and the overload of calcium in smooth muscle cells (Fleckenstein *et al.*, 1990). Both oxidized LDL and calcium are cytotoxic to smooth muscle cells, endothelial cells and other cells implicated in atherogenesis (van Hinsbergh, 1984). Several investigations suggest that peroxidative modification of intimal lipoprotein (VLDL, LDL), could play a pivotal role in mediating the cellular responses of early atherosclerotic lesions (Steinberg *et al.*, 1989; Steinberg & Witztum, 1990). It has been observed that both LDL and calcium overload may be implicated in structural alterations associated with the atherogenic process such as intimal thickening (Stary, 1990; Atkinson, 1992). Moreover, they were related to functional changes occurring in atherosclerosis, since impairment of vascular smooth muscle and endothelial responses have been observed in vitamin D-nicotine-treated animals (Atkinson, 1992) and in endothelium-dependent relaxation in rabbit aorta (Kugiyama *et al.*, 1990).

Several studies in cholesterol-fed animal models (Ginsburg *et al.*, 1983; Blumlein *et al.*, 1984; Watanabe *et al.*, 1987) have indicated that calcium antagonists can reduce the extent of lesion formation and the number of new lesions in patients (Lichtlen *et al.*, 1990). In addition, it has been demonstrated that some antioxidant phenol derivates (probucol, khellin, di-BHA, etc.) inhibit, in a concentration-dependent manner, the $BaCl_2$-induced contraction in rat non-vascular smooth muscle. In this experiment probucol was found to be the most potent compound studied (Sgaragli *et al.*, 1993). Furthermore, khellin has been shown to depress both contractility and $^{45}Ca^{2+}$ uptake in vascular smooth muscle (Ubeda *et al.*, 1991).

In this way, molecules which may combine both free radical scavenging and calcium antagonist properties would be of particular value in protecting against atherosclerotic damage.

Probucol reduces the extent of aortic atherosclerosis produced by diet-induced hypercholesterolaemia in rabbits. This reduction occurs in the absence of any significant change in the characteristics of plasma lipoproteins (Daugherty *et al.*, 1989). The latter suggests that probucol may act by other mechanisms in addition to reducing oxidation of LDL (Parthasanathy *et al.*, 1986).

The mechanism of the hypolipidaemic effect in man has been attributed to a variety of mechanisms: decreased *de novo* cholesterol biosynthesis, increased activity of hepatic LDL receptors, increased secretion of bile acids (Steinberg, 1986) and induction of a 'selective' uptake of HDL cholesterol esters to the liver using cultured Hep G2 human hepatoma cells (Pfueffer *et al.*, 1992).

In order to elucidate these mechanisms, we have studied the effects of probucol on the vascular reactivity of different arteries isolated from rabbits. In this study, we have also investigated the effects of probucol on the development of atherosclerosis in a cholesterol-fed rabbit model; the biochemical and morphological changes as well as the vascular reactivity alterations that occur in atherosclerosis.

Methods

Experiment 1: In vitro *effects of probucol on arteries from normal rabbits*

General procedure Male New Zealand white rabbits weighing 2.5-3 kg were obtained from Biocentre S.A. (Barcelona, Spain). The animals were anaesthetized with ethyl ether and

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killed by exsanguination from the common carotid artery. The thoracic aorta, femoral and mesenteric (5th branch) arteries were rapidly removed. Thoracic aorta and femoral arteries were placed in Godfraind solution of the following composition (mM): NaCl 122, KCl 5.9, $NaHCO_3$ 15.5, glucose 11, $CaCl_2$ 1.25 and $MgCl_2$ 1.22. Adherent fat and surrounding tissue were cleaned off and the arteries were cut into rings approximately 2–3 mm wide. The rings were then suspended between two stainless steel hooks in organ baths containing 10 ml Godfraind solution. The solution was kept at 36±0.5°C and gassed continuously with a 95% O_2: 5% CO_2 gas mixture. The aorta and femoral arteries were mounted under 2 and 1.5 g tension respectively. Each preparation was allowed to equilibrate for 90–120 min. Contractile responses were measured isometrically by means of force-displacement transducers (Grass FT 03) and were recorded on a Grass polygraph as previously described (Tejerina *et al.*, 1988).

Mesenteric resistance vessels were mounted on a myograph. Two 40 μm tungsten wires were passed through the lumen of an isolated cylindrical segment (2 mm long) of the fifth branch (approximately 175 μm inside diameter) of the superior mesenteric arteries. One wire was fastened with screws to a fixed tissue mounter and the other was pulled taut by parallel hooks which were attached to a string-gauge force transducer (U-gauge, Shinko Co., Ltd.); the positions were adjusted with a micromanipulator. Mesenteric resistance vessels were equilibrated in physiological saline solution (PSS) of the following composition (mM): NaCl 140, KCl 4.6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10 and HEPES 5. The solution was kept at 36±0.5°C, gassed continuously with O_2, and maintained at an optimal resting tension of 40 mg (Cauvin *et al.*, 1982; Tejerina *et al.*, 1992).

After equilibration the following experiments were carried out: (1) each aorta or femoral ring was exposed to single submaximal concentrations of KCl (80 mM) and noradrenaline (NA 10^{-6} M). An initial 10–25 min control contraction was obtained in each experiment with the appropriate stimulating agent. The rings were then washed and rested for a minimum of 45–60 min. Control contractile responses for each agonist were obtained at the beginning of the experiment when two successive responses were of almost identical height. The rings were then exposed to probucol (10^{-6} M – 5×10^{-4} M) for 45 min before the addition of KCl or NA. The method used for assessing inhibitory effects of probucol on mesenteric resistance vessels was to contract the vessels with a submaximal concentration of KCl (80 mM) or NA (10^{-4} M), then wash the activating agent out and repeat the stimulus after 15 min of preincubation of the vessel with PSS containing probucol (Cauvin *et al.*, 1987). Only one agonist was used in each experiment.

(2) To determine if the inhibitory effects of probucol were dependent on the calcium concentration, aortic rings were incubated in Ca^{2+}-free Godfraind for 120 min then in Ca^{2+}-free high K^+ (80 mM) depolarizing Godfraind solution for 10 min. Cumulative concentration-response curves to Ca^{2+} were then obtained by increasing the Ca^{2+} concentration in the bath (1–5 mM) stepwise over the next 45 min. Ca^{2+} was then washed out and the rings were re-incubated in Ca^{2+}-free Godfraind solution for 60 min (Barrigon *et al.*, 1984). The high K^+ depolarizing procedure was repeated, but probucol was added to the bath 45 min before the first addition of Ca^{2+}. The results are expressed as a percentage of the maximal contractile response induced by 5 mM $CaCl_2$.

$^{45}Ca^{2+}$ fluxes

$^{45}Ca^{2+}$ uptake was determined as previously described (Meisheri *et al.*, 1981; Leijten & van Breeman, 1984). Aortic rings were equilibrated for 120 min in Godfraind solution. After equilibration, experimental rings were treated with probucol for 45 min and control rings with an equivalent amount of solvent (i.e. Godfraind solution). The rings were then exposed for 90 s to $^{45}Ca^{2+}$ solution (specific activity 4 μCi ml^{-1}). In some experiments NA (10^{-6} M) or KCl (80 mM) was added simultaneously with $^{45}Ca^{2+}$. At the end of 90 s exposure to the stimulatory agent, the tissues were washed in ice-cold EGTA-Godfraind solution for 45 min to remove extracellular calcium. The rings were then removed, blotted, weighed, placed in scintillation vials and 0.5 ml of Soluene-350 (Packard) was added.

Experiment 2 In vivo *effects of probucol on arteries from hypercholesterolaemic rabbits*

General procedure Three groups ($n=9$) of New Zealand White male rabbits, weighing 1.8–2.2 kg (3 months old) at the beginning of the study were used. The control group was maintained on a standard diet (normal control). Group II was maintained on a diet containing 1% cholesterol (wt/wt) (U.A.R., Paris, France) (high-cholesterol control). Group III also received the diet containing 1% cholesterol but supplemented with probucol 1% (wt/wt) (U.A.R., Paris, France). The experiment lasted 16 weeks. All the animals were initially fed with a standard laboratory diet (Panlab S.L., Barcelona, Spain) for at least 7 days after delivery to our laboratory. Diet and tap water were available *ad libitum*. Food intake was monitored daily for the two control groups and the probucol-treated group. The body weight of each rabbit was determined before starting the treatment and weekly thereafter. At the end of the 16th week, the animals were anaesthetized with ethyl ether and killed by exsanguination from the common carotid arteries. The aorta, mesenteric (5th branch) and femoral arteries were then rapidly removed and cleaned of grossly adherent adventitial tissues. The mesenteric and femoral arteries were cut into rings and mounted as described above. After equilibration the following experiments were carried out: (1) Mesenteric rings from each group (GI, GII and GIII) were exposed to single submaximal concentrations of KCl (80 mM) and NA (10^{-4} M). (2) Femoral rings from the same groups were exposed to cumulative increasing concentrations of KCl (20–120 mM) and noradrenaline (10^{-8} M – 10^{-4} M).

Morphometric and biochemical procedure

After the aortic arteries were removed and cleaned, the samples were cut into 2 longitudinal halves. One of the halves was further divided into aortic arch (from the ascending arch to the exit of the left subclavian artery) and thoracic aorta (the remaining thoracic aorta). Both parts were fixed in buffered 10% formalin and stained with osmium tetroxide (Saso *et al.*, 1992), and were then laid out flat with the intimal side up. The intimal aortic side was macroscopically photographed. The whole area corresponding to both the aortic arch and thoracic aorta as well as the surface of intimal area covered by atherosclerotic plaques was measured. The measurements were performed from the negative prints by delineating the perimeter of the aorta, or the perimeter of the lesions, using an interactive eletronic pen and plaque of a CUE2 morphometry system. Measurements were analysed by a morphometric subroutine of CUE2 software. The other half was used for determination of cholesterol content. The cholesterol was extracted from the aorta with isopropyl alcohol/heptane (4:1 v/v) as described previously (Dole, 1956) and determined with a commercially available enzyme kit (Menarini Diagnostics, Firenze, Italy).

Plasma concentrations of total cholesterol, triglycerides and phospholipids were also determined with commercially available enzyme kits (bioMerieux, Marcy, France). All protocols concerning animals were approved by the University Complutense of Madrid (EEC official registration 28079-15ABC).

Drugs

The following drugs were used: probucol (Merrell Dow S.A.); noradrenaline bitartrate (Sigma), potassium chloride and calcium chloride (Merck) and $^{45}Ca^{2+}$ (specific activity

2 mCi ml^{-1}) (Amersham). Stock solutions of probucol (10^{-2} M) were prepared by dissolving probucol powder in a solution containing Tween 80 (10% v/v) in water; working solutions were made in Godfraind solution or PSS, since control experiments had demonstrated that the highest Tween 80 level used had no effect on contractile responses to KCl or noradrenaline. The concentration for each chemical or drug is expressed as final concentration in the bath in terms of the salt. Ascorbic acid was added to each daily prepared solution of noradrenaline.

Statistical analyses

In vitro results are expressed throughout as mean ± s.e.mean. Concentration-response curves were used to determine the concentration of probucol producing 50% inhibition of the maximal contractile response (IC_{50}), using linear regression analysis over the response range of 20–80% of the maximal inhibition.

All *in vivo* values used in analyses represent mean ± s.e.mean of 7–9 rabbits in each group. Comparisons between the different groups were performed by analysis of variance (ANOVA), and Fisher's test. A level of probability $P<0.05$ was accepted as statistically significant.

Results

Experiment 1: In vitro *effects of probucol on arteries from normal rabbits*

Effects on contractions induced by KCl and noradrenaline The inhibitory effects of probucol on the contractile responses induced by high K^+ (80 mM) and NA (10^{-6} or 10^{-4} M) in rabbit aorta, femoral and mesenteric (fifth branch) arteries were studied. As shown in Figures 1–2, preincubation with probucol at concentrations ranging from 10^{-6} M to 5×10^{-4} M produced a concentration-dependent inhibition of the contractile response induced by the stimulating agents (K^+ or NA) in mesenteric and aortic arteries. However, probucol 5×10^{-4} M suppressed the contractile response induced by K^+ in the mesenteric artery (fifth branch) (Figure 1) while this concentration left unaltered around 20% and 45% of the contraction induced in the aorta and femoral artery, respectively (Figure 1). On the other hand, probucol (5×10^{-4} M) inhibited by 60% the response elicited by NA both in the mesenteric artery and in the aorta but only by 10% in the femoral artery (Figure 2).

The concentrations at which probucol inhibited 50% of the maximal contractile response (IC_{50}) induced by high K^+ and noradrenaline are shown in Table 1.

Effects on Ca^{2+}-induced contractions

In the aortic artery previously depolarized by high K^+, probucol produced a concentration-dependent decrease of the contraction induced by Ca^{2+} and shifted the concentration-response curve downwards and to the right. Probucol, 10^{-5} M, reduced the maximal response of the aorta to 5 mM Ca^{2+} to 58.5 ± 6.5 ($n=13$) and at 10^{-4} M to 31.0 ± 6.8 ($n=13$) (Figure 3).

Effects on $^{45}Ca^{2+}$ influx

$^{45}Ca^{2+}$ influx was studied in resting, non-stimulated aortic rings as well as in rings stimulated by high-K^+ depolarization and agonist activation (noradrenaline) (Figure 4). In resting rings, probucol at 5×10^{-4} M reduced $^{45}Ca^{2+}$ content below control values (42.3 ± 22.6 versus 11.7 ± 2.2 μmol kg^{-1} wet tissue; $n=6$, $P<0.05$). Addition of noradrenaline (10^{-6} M) or high-K^+ (80 mM KCl) increased the $^{45}Ca^{2+}$ influx to 235.0 ± 76.4 and to 221.0 ± 38.0 μmol kg^{-1} wet tissue, respec-

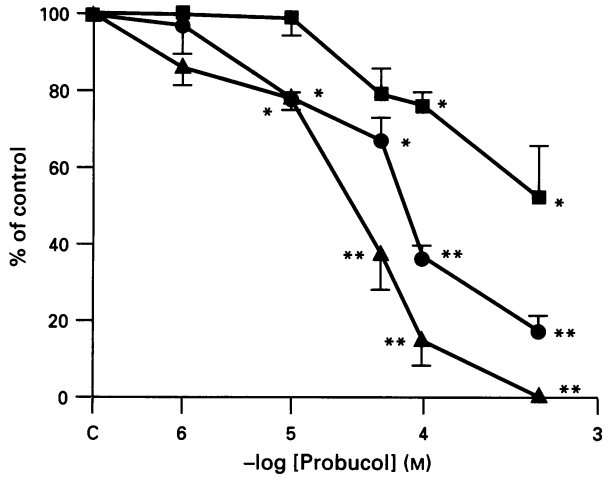

Figure 1 Inhibitory effect of probucol (10^{-5}–5×10^{-4} M) on the contractile responses induced by high K^+ (80 mM) in (●) rabbit aorta, (■) femoral, and (▲) mesenteric arteries. Each point represents the means of 6–8 experiments ± s.e.mean. *$P<0.05$, **$P<0.01$.

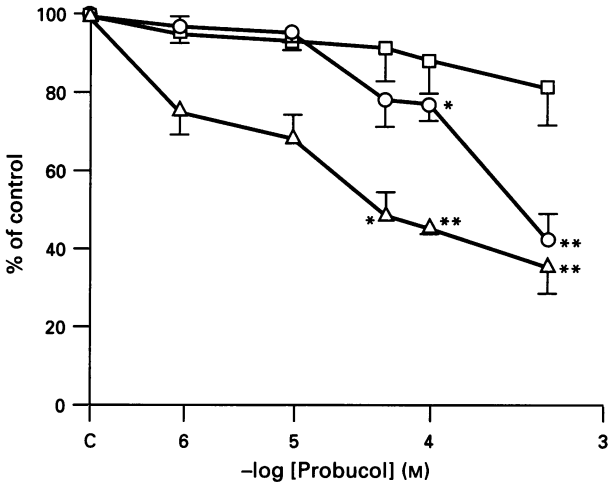

Figure 2 Inhibitory effect of probucol (10^{-5}–5×10^{-4} M) on the contractile responses induced by noradrenaline (10^{-6} or 10^{-4} M) in (○) rabbit aorta, (□) femoral, and (△) mesenteric arteries. Each point represents the means of 6–8 experiments ± s.e.mean. *$P<0.05$, **$P<0.01$.

Table 1 Concentrations at which probucol (M) inhibits 50% of the maximal contractile response (IC_{50}) induced by different agents

Artery	*KCl* (80 mM)	*NA* (10^{-6} or 10^{-4} M)
Aorta	$8.2\pm2.3\times10^{-5}$ M	$3.2\pm3.0\times10^{-4}$ M
Femoral	$>5\times10^{-4}$ M	$>5\times10^{-4}$ M
Mesenteric (5th branch)	$4.8\pm2.6\times10^{-5}$ M	$4.2\pm1.3\times10^{-5}$ M

tively ($n=8-10$). Probucol at 5×10^{-5} M reduced the uptake induced by NA from 235.0 ± 7.0 to 192.54 ± 42.0 ($n=8-10$), and to 80.9 ± 10.2 μmol kg^{-1} wet tissue at 5×10^{-4} M ($n=9$). When the $^{45}Ca^{2+}$ influx was induced by high-K^+, the reduction was from 221.8 ± 38.0 to 191.4 ± 3.0 ($n=8-10$) and to 46.7 ± 3.7 μmol kg^{-1} wet tissue ($n=7$) respectively for probucol 5×10^{-5} M and 5×10^{-4} M.

Experiment 2: In vivo *effects of probucol on arteries from hypercholesterolaemic rabbits*

General findings The rabbits in experiment 2 tolerated the diets well throughout the 16-week study period. Body weight increased gradually and rabbits gained an average of 0.7–0.9 kg in all groups by the end of the study.

Effect of probucol on development of atherosclerosis in hypercholesterolaemic rabbits We investigated biochemical and morphological alterations present in atherosclerosis. Probucol treatment did not significantly affect plasma concentration of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and phospholipids (data not shown). In spite of the lack of effect of probucol on the above mentioned parameters, the administration of the drug decreased the cholesterol content of aortic tissue (Figure 5a) and the extent of intimal aortic surface covered with discernible atherosclerotic lesions (Figure 5b).

The effects of *in vivo* treatment with probucol on the contractile responses induced with cumulative concentrations of NA (10^{-7} M to 10^{-4} M) and of KCl (20–120 mM) were studied in hypercholesterolaemic fermoral arteries. As shown in Figure 6a and b, the vascular reactivity recovered in femoral arteries of rabbits on the atherogenic diet containing probucol. Concentration-response curves to NA as well as to high-K were shifted upwards and to the left in the probucol-treated group compared to the control-hypercholesterolaemic group. The responsiveness of small resistance vessels (mesenteric, 5th branch) appeared not to be affected by hypercholesterolaemia or probucol-treatment (data not shown).

Discussion

The results of the first part of this study, experiments *in vitro*, demonstrate that probucol prevents contractile responses in different arteries isolated from normal rabbits. Probucol in-

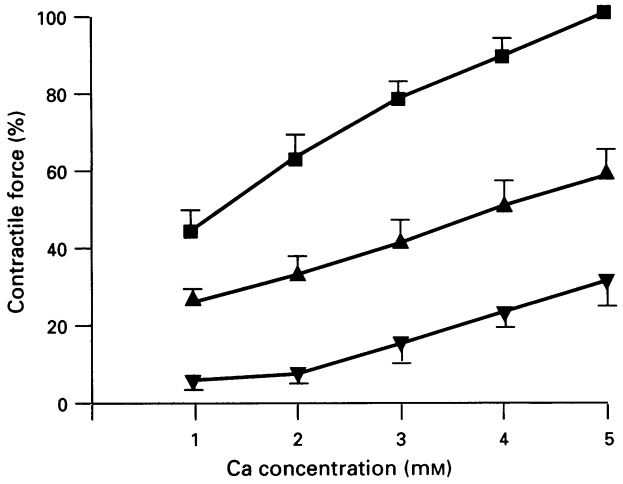

Figure 3 Effect of probucol on restoration of the isometric contraction of aortic rings by addition of calcium (1–5 mM) to Ca-free high K^+ (80 mM) medium. Ordinate scale: percentage of the maximum control contractions obtained with the highest concentration of calcium in each experiment. Each point represents the mean ± s.e.mean of 13 experiments: (■) control, (▲) after probucol 10^{-5} M and (▼) 10^{-4} M.

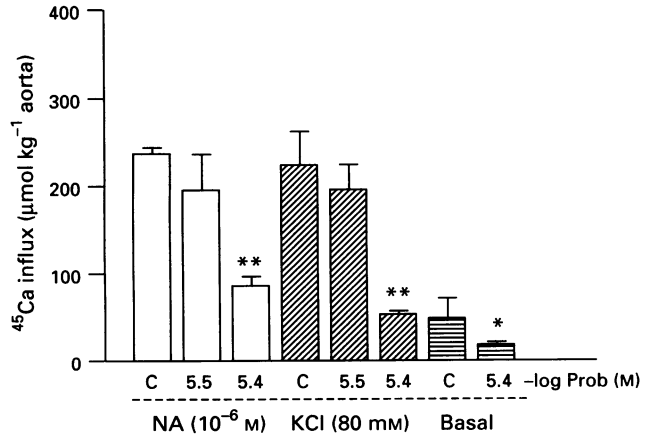

Figure 4 Effects of probucol on $^{45}Ca^{2+}$ influx in resting aortic rings and in rings stimulated with 80 mM KCl or 10^{-6} M noradrenaline (NA). The values are the mean with s.e.mean of 7–10 experiments.

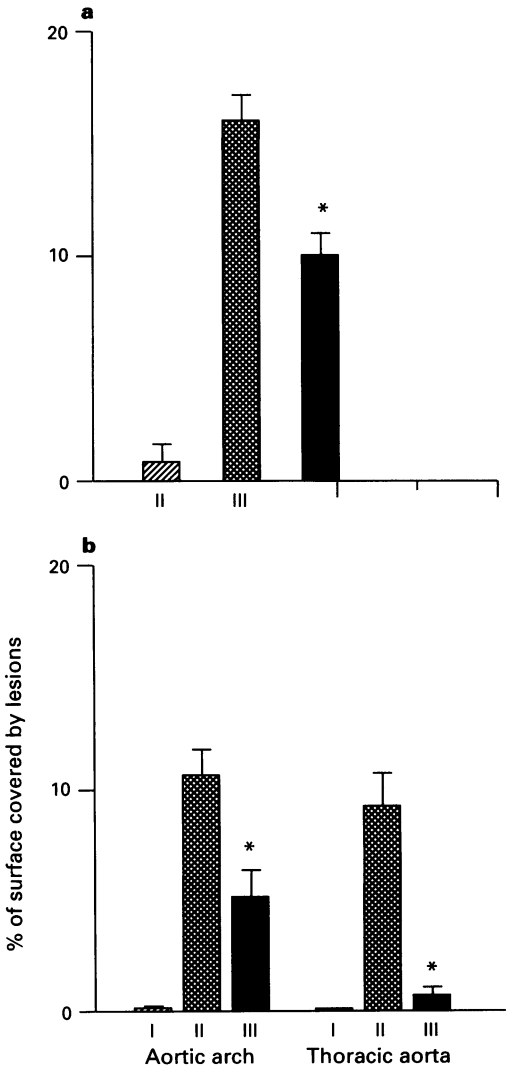

Figure 5 (a) Total cholesterol content of aortic tissue. (b) Percentage of intimal surface area covered by discernible atherosclerotic lesions. I: Control group (standard diet); II: hypercholesterolaemic group (1% cholesterol); III, 1% cholesterol plus probucol. Each column represents the mean with s.e.mean of 7–10 rabbits in each group. *$P<0.05$.

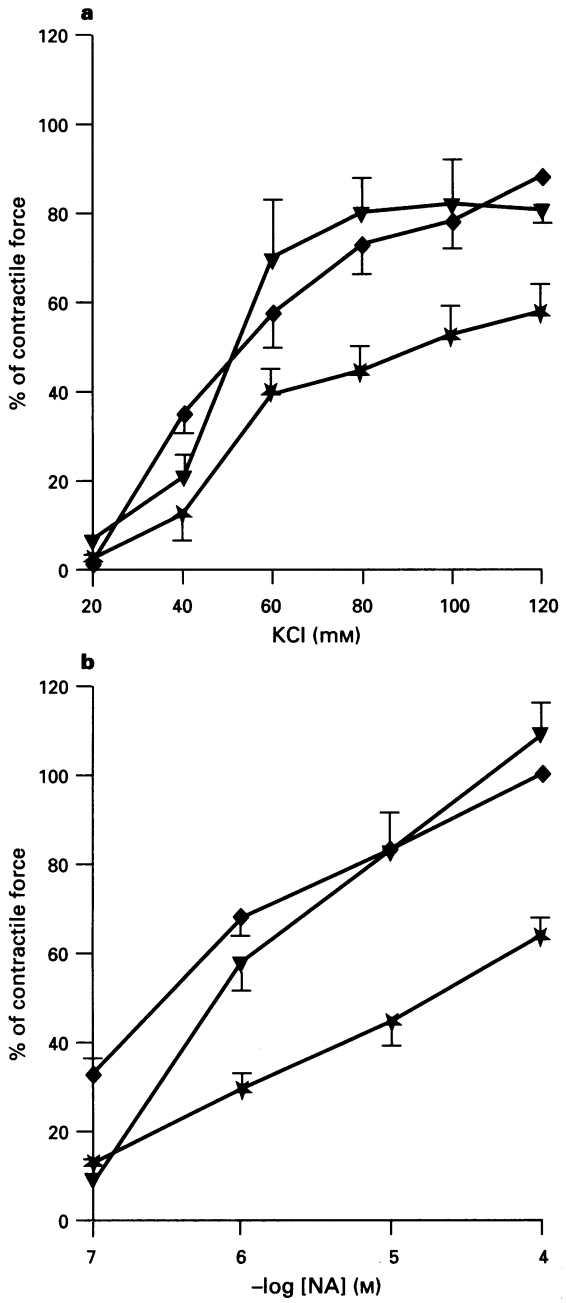

Figure 6 Effects of probucol *in vivo* on the concentration-response curves to KCl (a) and NA (b) in femoral arteries from (◆) normal, (★) hypercholesterolaemic and (▼) probucol (1%)-treated rabbits. Each point represents the mean ± s.e.mean of 8–10 experiments.

hibited the contractile responses induced by high-K^+ depolarization in all arteries studied (mesenteric > aorta > femoral) and the Ca^{2+}-induced contractions in K^+-depolarized aortic rings. These responses have been attributed to Ca^{2+} entry through voltage-operated channels (VOCs) (Hudgins & Weiss, 1968; Sigurdsson *et al.*, 1975; Bolton, 1979; Cauvin *et al.*, 1983). Moreover, probucol also exerted an inhibitory effect on total contractile responses to noradrenaline (mesenteric > aorta > femoral), suggesting that it may also block Ca^{2+} in response to this agonist. It has been proposed that vasoconstrictor agents increase Ca^{2+} entry by opening receptor-operated channels (ROCs) (Godfraind & Kaba, 1969; Bolton, 1979; Cauvin *et al.*, 1983; Khalil *et al.*, 1987). Thus, these findings suggest that probucol may inhibit Ca^{2+} uptake through voltage- and receptor-operated channels. To test the possibility that probucol inhibits Ca^{2+} entry through VOCs and ROCs, its effect on Ca^{2+} uptake was studied on aortic rings stimulated by high K^+ and NA. Probucol inhibited Ca^{2+} entry in aortic rings previously stimulated by high K^+ and by NA but also reduced Ca^{2+} entry in non-stimulated rings. The latter indicates that probucol behaves as a non-specific inhibitor of Ca^{2+} influx since it reduces the Ca^{2+} uptake through VOCs and ROCs and also the Ca^{2+} entry through passive leak channels. In accordance with our results, Sgaragli *et al.* (1993) have shown that probucol and other antioxidant compounds with phenol or phenol derivative structures have calcium antagonist properties in rat ileal longitudinal muscle (Sgaragli *et al.*, 1993). Moreover, Bay K8644, a well-known calcium channel agonist, was found to completely reverse the inhibition caused by these antioxidants. In addition, other authors have suggested that some other phenols, such as khellin (Ubeda *et al.*, 1991) or quercetin (Abdalla *et al.*, 1989) possess calcium antagonist properties on vascular smooth muscle contraction. Nordihydroguaiaretic acid (NDGA), a drug structurally related to probucol, was shown to be able to produce a reversible concentration-dependent inhibition of Ca^{2+} channel currents on pituitary cell lines (Korn & Horn, 1990). Thus, it seems possible that probucol acts on calcium channels in vascular smooth muscle.

During the past years, it has become evident that vascular reactivity is altered during the progression of atherosclerotic plaques. However, there is some controversy around this fact. In several studies a decrease in the contractile responses induced by phenylephrine in aortic rings isolated from Watanabe hyperlipidaemic heritable rabbits has been described (Kolodgie *et al.*, 1990), whereas in others, an increase in total resistance induced by NA in hyperlipidaemic monkeys before the development of atherosclerosis has been reported (Heistad *et al.*, 1984).

In order to investigate the role of probucol on vascular disorders associated with the development of atherosclerosis, we have studied the effects of the drug on the vascular reactivity of diet-induced hypercholesterolaemic rabbits.

Despite there being no difference in cholesterol (total, LDL and HDL) plasma level between the hypercholesterolaemic group (Group II) and the probucol-treated group (Group III), the drug could reverse the hyporeactivity found in femoral arteries isolated from hypercholesterolaemic rabbits. Drugs such as nifedipine, diltiazem and verapamil that block calcium entry in vascular smooth muscle have been shown to have a beneficial effect on atherosclerosis in animal models (Ginsburg *et al.*, 1983; Blumlein *et al.*, 1984; Watanabe *et al.*, 1987). Probucol, a drug in clinical use for treatment of hypercholesterolaemia, is a potent inhibitor of *in vitro* oxidation of LDL (Parthasarathy *et al.*, 1986) and recently it has been reported that dietary probucol and other antioxidants prevent the inhibition of endothelium-dependent relaxation in aorta from cholesterol-fed rabbits (Simon *et al.*, 1993; Keaney *et al.*, 1995) as well as in human subjects (Plane *et al.*, 1993). Our findings suggest that the effect of probucol on vascular reactivity might contribute to the mechanism of the antiatherogenic properties of this drug.

This work was supported by a FISS grant (93/0291). We are indebted to Ruben Vara for his excellent technical assistance. T.C. and M.D.R. were supported by FISS fellowships.

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(Received January 22, 1996
Revised March 26, 1996
Accepted April 10, 1996)