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PF9404C, a new slow NO donor with beta receptor blocking properties

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1 PF9404C is the S-S diesteroisomer of a novel blocker of beta adrenergic receptors with vasodilatory properties. It causes a concentration-dependent relaxation of rat aorta helical strips pre-contracted with 10^{-6} M noradrenaline (NA; IC_{50} 33 nM). It was equipotent to nitroglycerin (NTG; IC_{50} 49 nM), but much more potent than isosorbide dinitrate (ISD; IC_{50} 15,000 nM).

2 Oxyhaemoglobin (10 μM) shifted to the right the concentration-response curve for the relaxation induced by PF9404C (IC_{50} 530 nM) or NTG (IC_{50} 61 nM).

3 Either methylene blue (MB) or ODQ (1 μM each) largely prevented the vasorelaxing responses to increasing concentrations of PF9404C or NTG.

4 In rat aorta smooth muscle cells, PF9404C increased the formation of cyclic GMP from 3 pmol mg^{-1} protein in basal conditions, to 53 pmol mg^{-1} protein in 10 μM PF9404C. Neither metoprolol nor carvedilol enhanced cyclic GMP.

5 In the electrically driven guinea-pig left atrium, PF9404C blocked the inotropic effects of isoprenaline in a concentration-dependent manner. Its IC_{50} (30 nM) was similar to that for S-propranolol (22.4 nM) and lower than the IC_{50}s for metoprolol (120 nM) and atenolol (192 nM). The beta-adrenergic ligand (−)-[^{3}H]-CGP12177 (0.2 nM) was displaced from its binding to rat brain membranes with K_i of 7 nM, 17 nM, 170 nM and 1.2 μM respectively for PF9404C, S-(−)propranolol, metoprolol, and atenolol.

6 The data are consistent with the idea that the S-S diesteroisomer PF9404C, is a potent vasorelaxing agent, as well as a blocker of cardiac beta adrenergic receptors. The mechanism of its vasorelaxing effects involves the slow generation of NO. This molecule can, therefore, exhibit antihypertensive and cardioprotective actions through a double mechanism, NO donation and beta blockade.

Keywords: PF9404C; NO donors; beta blockers

Abbreviations: ACE, angiotensin-converting enzyme; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Hb, haemoglobin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IBMX, 3-isobutyl-1-methylxanthine; ISD, isosorbide dinitrate; ISO, isoprenaline; MB, methylene blue; NA, noradrenaline; NO, nitric oxide; NOR1, (±)-(E)-methyl-2-[(E)-hydroxyminol]-5-nitro-6-methoxy-3-hexenamide]; NOR2, [(±)-(E)-Methyl-2-[(E)-hydroxyminol]-5-nitro-3-hexenamide]; NOR3, [(±)-(E)-ethyl-2-[(E)-hidroxyminol-5-nitro-3-hexenamide]; NOR4, [3-((±)-(E)-ethyl-2′-[(e)-hydroxyminol]-5-nitro-3-hexenecarbamoyl)-pyridine]; NTG, nitroglycerin; ODQ, (1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one); SNAP, S-nitroso-N-acetyl-penicillamine; SNP, sodium nitropruside

Introduction

For over two decades, blockers of beta adrenergic receptors, calcium antagonists, diuretics, vasodilators, and inhibitors of angiotensin-converting enzyme (ACE) are being used in the treatment of hypertensive patients. Recent meta-analytic (Furberg *et al*., 1995) and observational studies (Psaty *et al*., 1995) have shown that upon long-term treatments, only the beta-blockers show a clear-cut cardioprotective effect in hypertensive patients. The intriguing questions are through which mechanism(s) do these drugs afford cardioprotection and whether vasodilation added to beta blockade offers additional advantages. Considering the results of recent clinical trials with carvedilol, a combined alpha-beta blocker (McTavish *et al*., 1993), the answer seems to be affirmative. Carvedilol improves rest cardiac function and lessens symptoms in patients with heart failure (Olsen *et al*., 1995). Vasodilation (alpha-blockade) and protection against the arrhythmogenic effects of excessive circulating catecholamines (beta-blockade) could be the two mechanisms involved in its therapeutic effects, in addition to its ability to scavenge free-radicals and to prevent cell death (Yue *et al*., 1993).

In a search for antihypertensive compounds endowed with this profile, we attempted to incorporate into the molecule of classical beta-blockers, functional groups capable of generating nitric oxide (NO) when in contact with tissues and cells. We came across with PF9104, a derivative of metoprolol having two functional NO_2 groups in the aliphatic chain. This molecule kept its beta-blocking properties, and provided a powerful vasodilating action both *in vitro* and *in vivo*. As a result, the molecule produced bradicardia and a fall in blood pressure in both the anaesthetized or awake spontaneously hypertensive rat, either after oral or i.v. administration (Antón *et al*., 1996). Since PF9104 has two assymmetric carbon

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centres, one in each alkylic side chain; it was therefore important to resolve the four different enantiomers (Bull *et al*., 1995; Antón *et al*., 1996). The S-S enantiomer, (2′S),(2S)-3-isopropylamine,1-[4-(2,3-dinitroxy)propoxymethyl]-phenoxy-2′-propranol, exhibited the highest potency as beta-blocker and vasodilator, was named PF9404C, and selected for further development (see formula in Figure 1). In this report we present its vasodilatory properties in rat thoracic aorta strips, as well as data clarifying the mechanism mediating such vasodilation. This action is most likely related to its ability to liberate NO, as suggested by pharmacological and biochemical experiments. These properties were compared with those of various classical vasodilators (nitroglycerin, NTG; sodium nitroprusside, SNP; isosorbide dinitrate, ISD) and other fast and slow donors of NO (NOR1, NOR2, NOR3, NOR4, and S-nitroso-N-acetyl-penicillamine, SNAP). In addition, the beta-blocking properties of PF9404C, compared to those of metoprolol, S-propranolol and atenolol are also presented.

Methods

Contractions of rat thoracic aorta

Male Sprague-Dawley rats weighing 225–250 g were used in all the experiments. The animals were killed by a blow on the head. The segment of thoracic aorta as close as possible to the heart was quickly removed and placed in a Petri dish containing Krebs-bicarbonate solution (in mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KPO_4H_2 1.2, $CaCl_2$ 1.5, $NaHCO_3$ 25, glucose 11, and the excess fat and connective tissue were removed. The aorta was cut spirally into a strip approximately 2–3 mm wide. In each experiment, segments of 1.5–2 cm long were used; the strips were kept in Krebs solution gassed with 95% oxygen and 5% CO_2 throughout the experiment.

The segments of aorta were mounted in an organ bath (muscle chamber with a capacity of 40 ml, kept at 37°C) so that one end was fixed to an isometric transducer connected to an amplifier and recorder. In some experiments, 3-mm-wide rings from the aorta were also used; they were mounted in the bath through two wire hooks carefully introduced into the vessel's lumen. In all experiments the muscles were loaded with 1 g of tension. The endothelium was removed by pressing the intimal surface of the tissue on a wet filter paper.

Preparation of oxyhaemoglobin

Commercial haemoglobin contains a mixture of oxyhaemoglobin and the oxidized derivative, methemoglobin. Pure haemoglobin (oxyhaemoglobin) was prepared by adding to a 1 mM solution of commercial haemoglobin in distilled water, a 10 fold molar excess of sodium dithionite ($Na_2S_20_4$). Sodium dithionite was then removed by dialysing the samples successively in 3 Erlenmeyer with 5 liters of a solution of ethylenediaminetetraacetic acid (EDTA) 0.001% in distilled water each, and gassed continuously with N_2 during 4 h.

The purity of the oxyhaemoglobin solution was determined spectrophotometrically and the solutions were frozen in aliquots at −20°C and stored for up to 14 days.

Culture of smooth muscle cells

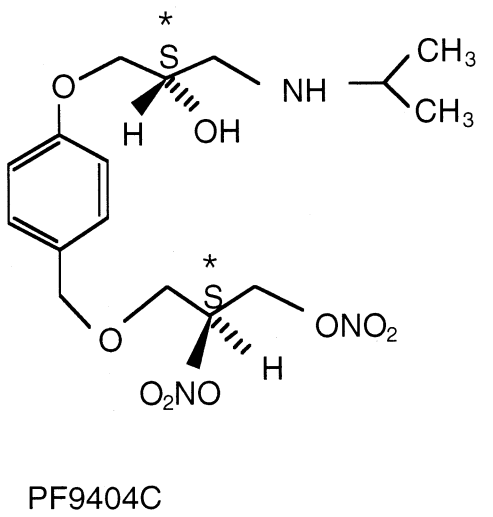
Rat aortic smooth muscle cells were isolated from male Sprague-Dawley rats weighing 225–250 g. The animals were killed by a blow on the head and the thoracic aorta was quickly dissected out, placed in a Petri dish containing Locke's solution (in mM): NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, glucose 5.6, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES 10 mM) supplemented with 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, and opened longitudinally.

The intimal layer was scraped off, and the medial layer was mechanically stripped from the adventitia. The medial layer was then cut into pieces and dispersed into single cells by incubation with a solution of 4 mg ml^{-1} collagenase type II in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% bovine serum albumin, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin for 90 min in an incubator at 37°C in a water-saturated, 5% CO_2 atmosphere with agitation.

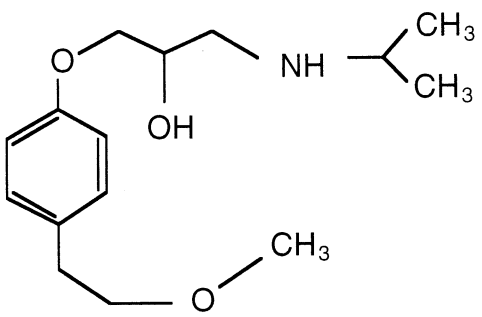
After two centrifugations in fresh DMEM, the cells were resuspended in the same medium supplemented with 10% foetal calf serum, and after differential plating in a Petri dish during 20 min to separate endothelial cells, the smooth muscle cells were seeded in 24-multiwell Costar plates. The medium was changed every 2–3 days.

Cyclic GMP measurement in cultured rat aortic smooth muscle cells

Rat aortic smooth muscle cells were grown to confluence in 24-multiwell Costar plates. Before the experiment, cells were



PF9404C



METOPROLOL

Figure 1 Molecular structure of compound PF9404C and metoprolol. Asterisks indicate the two assymetric carbons, one in each lateral chain. The diesteroisomer used in this study was the S-S.

washed twice with 0.5 ml Krebs-HEPES solution of the following composition (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1, Glucose 11, HEPES 10, at pH 7.2 and 37°C.

Cells were incubated for 1, 5, 15, 30 or 60 min with 1 μM of the different drugs, for the time-dependence experiments, or with 0.3, 1, 3, 10, 30 or 100 μM of the drugs for 5 min, in the concentration-dependence experiments. In all cases, drugs were dissolved in Krebs-HEPES containing 1 mM Ca^{2+}, in the presence of 0.3 mM 3-isobutyl-1-methylxanthine (IBMX) to prevent the degradation of the cyclic GMP formed.

After the incubation period, the medium was aspirated from the wells and 300 μl of ice-cold 65% (v v^{-1}) ethanol was added to each well. After 20 min, the extract was transferred to Ependorf tubes. Another 300 μl of ice-cold 65% (v v^{-1}) ethanol was added and 5 min later, this second fraction was collected and added to the same Ependorf tubes. The extracts were then centrifuged at 11,000 r.p.m. for 2 min and the supernatant was transferred to fresh tubes that were introduced open in an oven at 60°C until the ethanol was completely evaporated.

The content of cyclic GMP in the dry extract (expressed as a function of the protein content in each well) was determined using the kit for enzyme immunoassay RPN 226 (acetylation assay) from Biotrak (Amersham).

To determine the protein content of the wells, 500 μl of 0.5 N NaOH was added and after a few minutes, the bottom of the wells was scraped with a plastic pipette tip. The extract was then added to the sediment that remained in the Ependorf tubes used for the centrifugation at the beginning of the experiment; after mixing, the protein content was determined by the method of Bradford using the Bio-Rad Protein Assay reactive.

Isolated guinea-pig left atrium

Male guinea-pigs weighing 300–400 g were killed by a blow on the head. The heart was quickly removed and placed in oxygenated Krebs-bicarbonate solution. The left atrium was carefully dissected and placed between bipolar platinum electrodes. The base of the atrium was tied to one of the electrodes and the tip to an isometric transducer connected to an amplifier and recorder (Cibertec, Madrid, Spain), with 1 g baseline tension. The preparation was placed in a 10-ml glass bath at 32°C, in Krebs-bicarbonate solution of the following composition (in mM): NaCl 119, KCl 4.7, $MgS0_4$ 1.2, $KP0_4H_2$ 1.2, $CaCl_2$ 1.8, $NaHC0_3$ 25, glucose 11, pH 7.4.

An initial 30-min period of electrical drive (4 V, 1 ms, 1 Hz) allowed the stabilization of the basal contraction. Thereafter, 3×10^{-8} M isoprenaline (ISO) was added to the bath to enhance the contraction and increasing concentrations of each drug were added in order to study their blocking effects.

Displacement by PF9404C of the binding to membranes of CGP-1277

Male Wistar rats weighing 250–300 g were sacrificed and their brains were extracted and homogenized in 10 volumes per mg of tissue in Tris-HCl 50 mM, pH 7.7 by means of a glass-glass Potter homogenizer. The homogenate was first centrifuged at 16,000 r.p.m. during 10 min and the pellet was resuspended in Tris-HCl 50 mM and centrifuged at $35,000 \times g$ during 20 min; this last procedure was repeated once more. The final pellet was resuspended in Tris-HCl 50 mM, pH 7.7 and kept in aliquots of 1 ml at −70°C. Quantitation of proteins was performed with the method of Bradford using the Bio-Rad Protein Assay reactive.

For equilibrium binding experiments, cerebral membranes (300 μg of proteins) were incubated in Tris-HCl with increasing concentrations of (−)-[^{3}H]-CGP12177 in a final volume of 2 ml. Each concentration of the ligand was assayed in duplicate; nonspecific binding was determined in the presence of 5 μM metoprolol. The samples were incubated in a bath with agitation for 60 min at 37°C. Incubation was stopped by filtration through glass fibre Watman GF/C filters under reduced pressure. Filters were washed three times with 5 ml of ice cold Tris-HCl 50 mM, pH 7.7 and dried; they were introduced in vials with 4 ml of scintillation liquid and radioactivity counted in a scintillation counter Packard (model L1500). Specific binding was estimated by subtracting nonspecific from total binding and accounted for 65–75% of total binding.

For displacement experiments, the experimental conditions were essentially the same as above. Cerebral membranes (300 μg protein) were incubated with 0.2 nM (−)-[^{3}H]-CGP12177 and increasing concentrations of metoprolol, atenolol, (S)-propranolol and PF9404C in a final volume of 2 ml, completed with Tris-HCl 50 mM, pH 7.7. IC_{50} values were transformed into K_i using the equation of Cheng & Prussoff (1973).

Materials and solutions

The following materials were used: Collagenase type II, bovine serum albumin fraction V, EGTA, sodium nitroprusside (SNP), isosorbide dinitrate (ISD), methylene blue, bovine haemoglobin, acetylcholine chloride, metoprolol, atenolol, and S-(−)propranolol (Sigma); foetal calf serum, penicillin and streptomycin (GIBCO); S-nitroso-N-acetylpenicillamine (SNAP) (RBI); nitroglycerin solution 1% (Merck); 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one, ODQ) (Tocris Cookson, U.S.A.); (±)-(E)-methyl-2-[(E)-hydroxyminol]-5-nitro-6-methoxy-3-hexenamide] (NOR1), [(±)-(E)-Methyl-2-[(E)-hydroxyminol]-5-nitro-3-hexenamide] (NOR2), [(±)-(E)-ethyl-2-[(E)-hidroxyminol-5-nitro-3-hexenamide] (NOR3) and [3-((±)-(E)-ethyl-2′-[(e)-hydroxyminol]-5-nitro-3-hexenecarbamoyl)-pyridine] (NOR4) (Alexis Corporation, Switzerland); PF9404C was from Almirall Prodesfarma (Barcelona, Spain); [^{3}H]-CGP12177 (Amersham). All other chemicals used were reagent grade.

NOR1, NOR2, NOR3, NOR4, SNAP and ODQ were dissolved in dimethylsulphoxide (DMSO, Merck) at 10^{-1} or 10^{-2} M, and diluted in saline solutions to the desired concentration. The highest concentrations of DMSO used (no more than 0.1%) had no effects on any of the parameters studied. PF9404C was dissolved in distilled water at 10^{-2} M, and diluted in the saline solutions to be used.

Statistical analysis of the results

Data are expressed as means ± s.e.mean. IC_{50}s for each drug were estimated through non-linear regression anaylsis using ISI software for a PC computer. Differences between non-paired groups were compared by Student's *t*-test or ANOVA test with the statistical programme Statworks TM; a value of P equal or smaller than 0.05 was taken as the limit of statistical significance.

Results

Vasorelaxation caused by PF9404C and known NO donors

In every individual experiment, each aorta strip was initially equilibrated for 2 h in oxygenated Krebs-bicarbonate solution

at 37°C. Then the strip was contracted with a submaximal concentration of noradrenaline (10^{-6} M); the amine was washed out and after full relaxation, the vessel was contracted again with the same concentration of noradrenaline. Usually, after 2–3 additions and washouts of noradrenaline, a stable contraction was obtained which exhibited little decay for about 2 h. Unstable preparations tending to relax spontaneously were discarded. At 10^{-6} M, noradrenaline caused a stable contraction of 8.2±0.53 mN (average of 13 strips from six different animals and experiments).

Figure 2a shows an example of a stable vessel contraction caused by noradrenaline. In this vessel the endothelium was removed and hence, acetylcholine (10^{-6} M) did not cause its relaxation (Furchgott & Zawadsky, 1980). After washout, the artery relaxed and was contracted back with noradrenaline. Then, PF9404C added in cumulative concentrations (10^{-9}–10^{-6} M) caused a step-wise relaxation of the artery (Figure 2b). Similar results were obtained in the presence of endothelium

Table 1 IC_{50} and time constants (τ) for the relaxation of aorta strips induced by PF9404C and various NO donors

Compound	*IC_{50}* (nM)	n	τ *for relaxation* (min)
SNP	1.9±1	8	—
NOR3	5.8±0.5	3	2.32
PF9404C	33±12	9	3.23
NTG	49±22	14	1.70
NOR4	58±24	3	5.88
NOR2	110±17	3	1.92
SNAP	190±80	6	—
NOR1	240±71	3	1.21
ISD	15000±5900	11	—

Mean IC_{50} were calculated by non-linear regression analysis for the individual concentration-response curves which form the averaged curves in Figure 2c,d. τ were calculated from the decay rate of the relaxation curves. *n*, number of strips (from at least three different animals). SNP, sodium nitroprusside; NTG, nitroglycerine; ISD, isosorbide dinitrate.

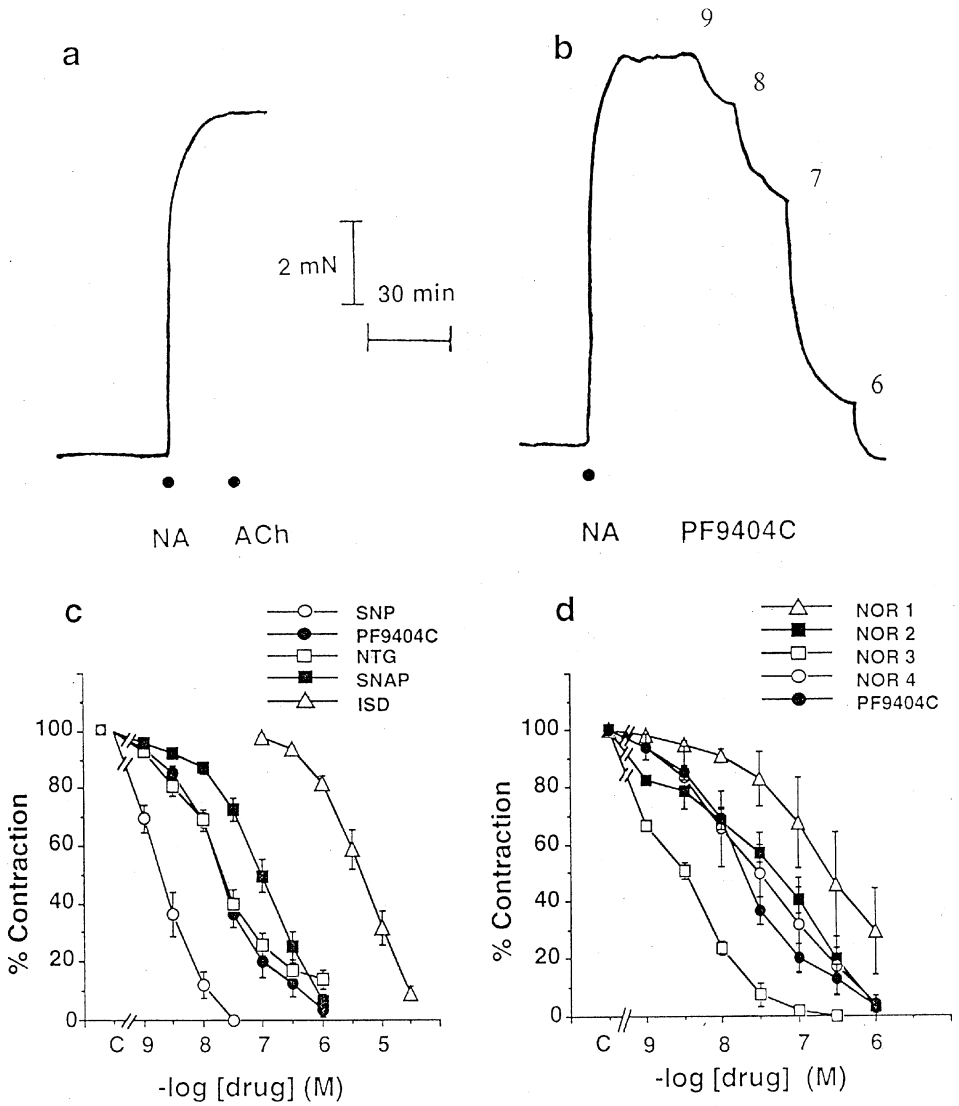


Figure 2 (a) Shows a typical sustained contraction of a rat thoracic aorta strip induced by 10^{-6} M noradrenaline (NA, dot at bottom); this concentration corresponds to the third of the three noradrenaline additions performed initially in each experiment, made at 3-min intervals. Acetylcholine (ACh, 10^{-6} M) was added as shown on the second dot at the bottom. (b) shows the vasorelaxing effects of cumulative concentrations of PF9404C in an endothelium-denuded artery strip (numbers on the trace represent the negative logarithm of each concentration). (c and d) Show the concentration-response curves for various NO donors causing relaxation of rat aorta strips pre-contracted with NA. The protocol used with the different compounds was similar to that followed for PF9404C in (b). (c) Shows the curves for SNP, NTG, PF9404C, SNAP, and ISD, while panel d shows data-sets for NO donors NOR1, NOR2, NOR3, NOR4, and again PF9404C. Data are means±s.e.mean of 3–7 strips from at least three different animals. See the calculated IC_{50}s in Table 1.

(not shown). Unless otherwise specified, the subsequent experiments were performed in endothelium-deprived tissues.

Using this protocol, the vasorelaxant effects of various known NO donors were compared with those of PF9404C (Figure 2c,d and Table 1). The most potent vasorelaxing agent was SNP (IC_{50} of only 1.9 nM), and the least potent was ISD (IC_{50} of 15,000 nM). NOR3 (5.8 nM), NTG (49 nM), PF9404C (33 nM) and NOR4 (58 nM) were in the nM range. Finally SNAP, NOR2 and NOR1 had IC_{50}s in the submicromolar range (around 0.2 μM).

Rate and duration of relaxation

The hypothesis underlying the mechanism for the relaxation of the aorta implies NO liberation through a tissue-dependent mechanism. Several of the reference compounds used here are known to release NO at different rates. It was therefore plausible that the rate of relaxation by these compounds, and the duration of such relaxing effects could depend on such rate of release of NO. To study the functional consequences of these variations, the following experimental protocol was designed.

After the standard initial equilibration period, the aorta strip was contracted with noradrenaline. Once this tonic contraction stabilised, a concentration of 30 nM of PF9404C was added and left in contact with the tissue for about 2 h. Figure 3a shows that this concentration of PF9404C relaxed the aorta tending to reach slowly the baseline tonus. The preparation remained relaxed as long as the compound was kept in the bath.

A different behaviour was seen with 1 μM NOR1, that caused a faster relaxation, that however did not reach the baseline (Figure 3b). Contrary to PF9404C (30 nM), NOR1 (1 μM) could not maintain the relaxation and after about 10 min, the vessel commenced to contract back to near its

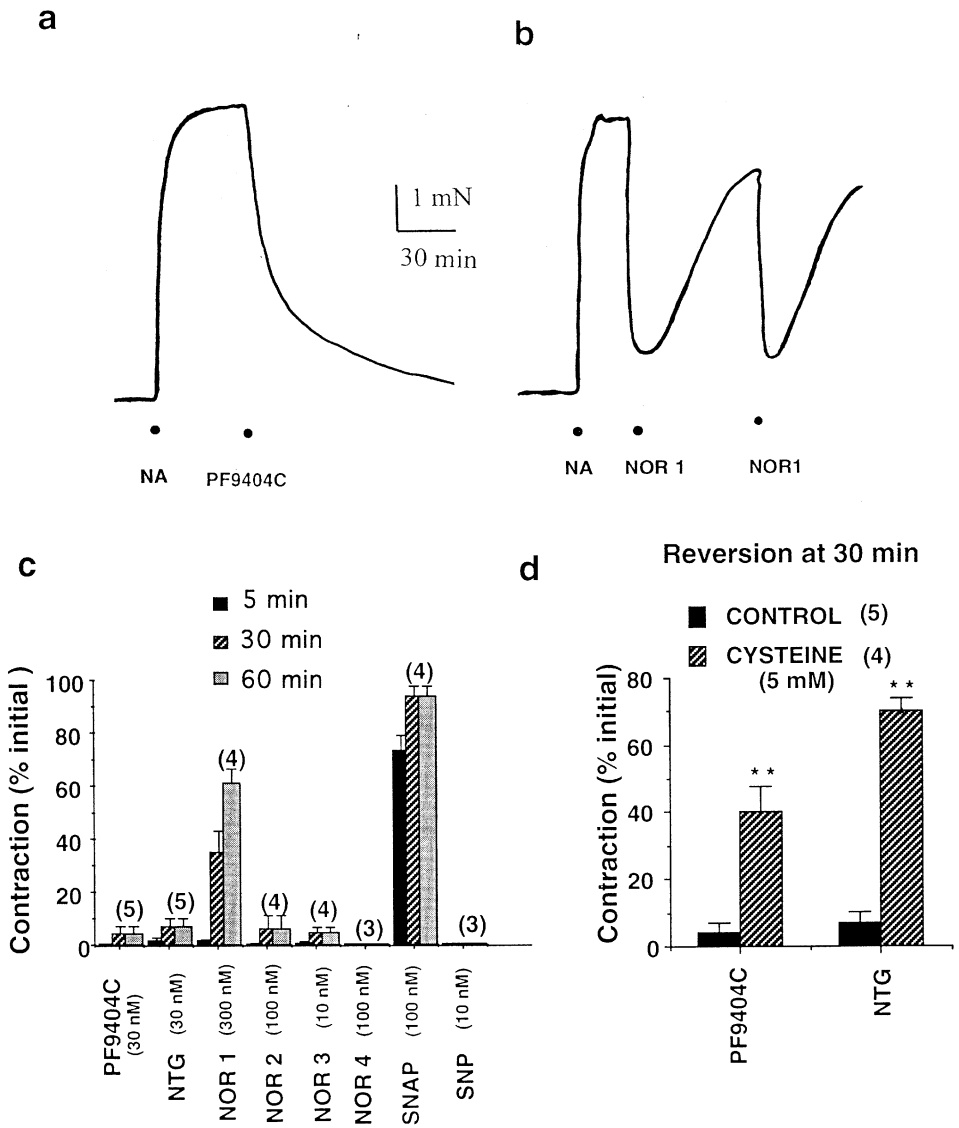


Figure 3 Rate and duration of the relaxation induced by PF9404C, NTG and NOR compounds. Aortic strips pre-contracted tonically with noradrenaline (NA, 1 μM) were exposed to 30 nM PF9404C (a) or 1 μM NOR1 (b). These concentrations were added to the organ bath as shown by the dots at the bottom of each trace, and were left in contact with the tissue during the rest of the experiment. When the tonic contraction recovered, a second dose of NOR1 (1 μM) was added; a new fast relaxation, similar to the first one, was produced. (c and d) Show the reversal of the aortic strip relaxation induced by NO donors, and the effects of cysteine. (c) Shows the recovery of contraction after 5, 30 or 60 min of incubation with each compound, at concentrations causing full relaxation. Note that during these recovery time periods, the tissues were continuously exposed to the compounds. (d) Shows the reversal of the relaxation induced by 1 μM PF9404C or NTG, in the absence and the presence of 5 mM cysteine, after 30 min incubation. Data are means$\pm$s.e.mean of the number of strips shown in parentheses. $^{**}P<0.01$, $^{***}P<0.001$ with respect to controls.

previous level. A new addition of NOR1 (1 μM) produced again a fast and reversible relaxation, suggesting that this was not due to development of tolerance but rather, to the likely consumption of the compound, which is known to be the fastest NO donor of all NOR compounds.

The rate of relaxation of six different compounds, at concentrations causing full relaxation of pre-contracted arteries, 30 nM for PF9404C and NTG; 10 nM for NOR3; 100 nM for NOR2 and NOR4; and 300 nM for NOR1 were calculated. Relaxation curves were best fitted to a single exponentials; the τ for NOR1 was 1.21 min, followed by NOR2 (1.92 min), NOR3 (2.32 min) and NOR4 (5.88 min). NTG and PF9404C had τ of 1.7 and 3.23 min, respectively.

Figure 3c shows the results of averaged experiments performed to study whether the relaxation induced by PF9404C and the other reference compounds was maintained along the time period of tissue exposure to the molecules. Once the relaxation reached its maximum at each drug concentration, the degree of contraction of the preparation was measured at 5, 30 and 60 min and its size normalized and expressed as per cent of the initial contraction before addition of each compound. In tissues exposed to NOR4 and SNP, no recovery of contraction was seen. The vessels incubated with PF9404C (30 nM), NTG (30 nM), NOR2 (100 nM) and NOR3 (10 nM) recovered only 10% of the initial contraction after 1 h. The tissues exposed to NOR1 recovered 35% of their initial contraction after 30 min, and 60% after 60 min. The vessels exposed to SNAP recovered their initial contraction by 80% in only 5 min; full recovery was achieved after 30 min.

Maintenance of the relaxation in the presence of PF9404C and of other reference compounds, could be due to slow and continued release of NO from the molecules, through a tissue-dependent mechanism. If so cysteine, known to accelerate such NO generation (Ignarro & Gruetter, 1980; Feelisch & Noack, 1987) should increase the rate of 'consumption' of the drugs as NO donors, thereby limiting the duration of their relaxing effects. Figure 3d shows that this was so. Thirty minutes after adding PF9404C or NTG (30 nM each), the relaxation was reversed little. However, in the presence of cysteine (5 mM), the preparations exposed to

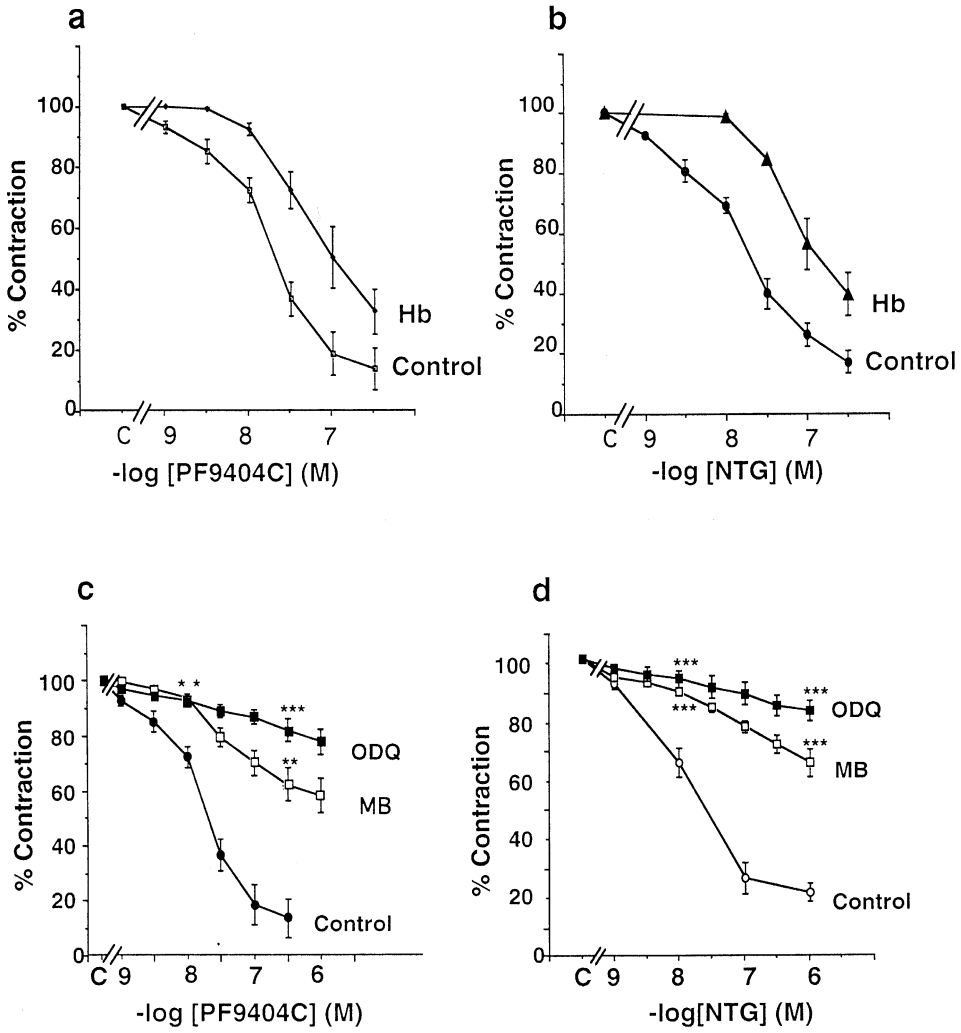


Figure 4 (a and b) Show that oxyhaemoglobin (Hb, 10 μM) displaces to the right the concentration-response curves for the vasorelaxing effects of PF9404C (a) or NTG (b). These curves were obtained using protocols similar to that shown in Figure 2b. Data are means$\pm$s.e.mean of six strips from three different animals. The statistical significant differences were determined by ANOVA test with an appropiate multiple comparison (Fisher PLSD). *$P<0.05$ with respect to controls. (c and d) Show that soluble guanylate cyclase inhibitors methylene blue (MB) and ODQ strongly counteracted the vascular relaxing effects of PF9404C and NTG. A single concentration-response curve for PF9404C or NTG was performed in each individual aorta strip pre-contracted with noradrenaline (10^{-6} M), either in the absence (control) or in the presence of 1 μM MB or ODQ. Data are means$\pm$s.e.mean of 4–6 strips from different animals. **$P<0.01$, ***$P<0.001$ when compared to controls at the same concentration of each compound.

PF9404C recovered 40% of their initial contraction, and those incubated with NTG recovered 70% of their initial tonic contraction.

Effects of oxyhaemoglobin, methylene blue (MB) and ODQ on the relaxation of aorta strips induced by PF9404C and NTG

Haemoglobin has the ability to bind avidly NO (Ignarro, 1989; Moncada *et al*., 1991), thereby preventing the stimulation of guanylate cyclase activity (Miki *et al*., 1977; Murad *et al*., 1978), and its tissue effects. A NO-sequestering compound such as oxyhaemoglobin delays or prevents the relaxation induced by NO release. Therefore it was reasonable to test the effects of oxyhaemoglobin on the vasorelaxing actions of PF9404C and NTG.

Figure 4a shows the concentration-response curves for the relaxation of aorta strips (previously pre-contracted with 1 μM noradrenaline), for PF9404C in the absence (control) and in the presence of 10 μM oxyhaemoglobin, added 10 min before initiating the cumulative concentration-response curve. The presence of oxyhaemoglobin decreased the vasorelaxing potency of PF9404C; thus the IC_{50} of 19 nM obtained in control conditions went up to 53 nM. A similar shift was observed for NTG, showing an IC_{50} of 14 nM before oxyhaemoglobin, and of 61 nM in its presence. In both cases, the effects of oxyhaemoglobin were significant over the total concentration range of PF9404C and NTG.

The previous data suggest that PF9404C might cause vascular relaxation through a NO-releasing mechanism. NO relaxes the vessels by activating a soluble guanylate cyclase to form cyclic GMP in smooth muscle cells. Therefore it was interesting to study the effects of two well known inhibitors of the enzyme, MB (Gruetter *et al*., 1981) and ODQ (Garthwaite *et al*., 1995; Moro *et al*., 1996), on the relaxation induced by PF9404C and NTG.

Figure 4c,d show concentration-response curves for the relaxation of rat aorta strips tonically contracted with 10^{-6} M noradrenaline. In control strips, PF9404C and NTG caused 80–90% relaxation of the tissues at submicromolar concentrations. In the presence of MB (1 μM) PF9404C caused only about 40% relaxation at 1 μM. ODQ (1 μM) was even more potent in preventing the relaxing effects of PF9404C, since at 1 μM it caused only 20% relaxation. At 1 μM, NTG relaxed the aorta by 90% in control conditions; the relaxation decreased to 30% in the presence of MB and to 15% in the presence of ODQ.

Generation of cyclic GMP induced by PF9404C and NTG, in rat aorta smooth muscle cells

After knowing the ability of PF9404C to generate NO in rat aorta smooth muscle cells, the obvious question was whether a parallel production of cyclic GMP was observed. Figure 5 indicates that the answer to this question is positive.

Rat aorta smooth muscle cells grown to confluence were incubated at 37°C for 5 min, in the presence of increasing concentrations of PF9404C or NTG. Formation of cyclic GMP above basal levels (around 3 pmol mg protein^{-1}) was already observed at submicromolar concentrations of the two compounds. The formation of cyclic GMP increased in a concentration-dependent manner. In the case of PF9404C it saturated at 10 μM (53 pmol mg protein^{-1}), while with NTG the formation of cyclic GMP continued increasing up to 30 μM (78 pmol mg protein^{-1}). At the concentrations of 1 and 30 μM, metoprolol did not enhance the basal cell levels

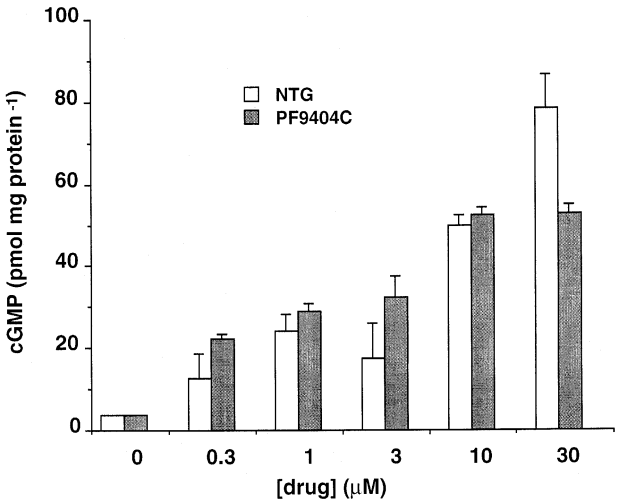


Figure 5 Generation of cyclic GMP induced by PF9404C and NTG, in rat aorta smooth muscle cells. Cells were incubated for 5 min with increasing concentrations (abscissa) of PF9404C (black columns) or NTG (white columns). After this incubation period, the cell contents of cyclic GMP were estimated as described in Methods. They are expressed as pmol mg cell protein^{-1} (ordinate). Data are means $\pm$s.e.mean of four wells from the same cell culture.

of cyclic GMP. Carvedilol was not effective at 1 μM; at 30 μM it produced 12 pmol mg protein^{-1} of cyclic GMP.

Effects of PF9404C on the inotropic response to isoprenaline of the guinea-pig left atrium

The idea behind PF9404C was to get a hybrid molecule having vasodilating and beta adrenergic receptor blocking effects. To test this last property we studied its actions on the inotropic effects of the beta receptor agonist isoprenaline. After an initial equilibration period, isoprenaline addition (3×10^{-8}) produced a marked enhancement of atrial contractions; this concentration was selected because it produced a submaximal and measurable inotropic effect, that was reproducible after 7–10 additions of isoprenaline, given at 30-min intervals. In ten atria, the net increase in the force of contraction induced by isoprenaline amounted to 11 ± 1.3 mN.

Concentration-response curves for the inhibition of isoprenaline-induced inotropism were obtained by intercalating increasing concentrations of each compound between two isoprenaline additions. Each individual preparation served to perform a full concentration-response curve for a given compound (Figure 6a).

At 10^{-7} M PF9404C blocked the isoprenaline inotropic response almost fully (Figure 6a). The concentration-response curve for this compound (Figure 6b) allowed the calculation of an IC_{50} of 30 nM (Table 2). It was, therefore, more potent than metoprolol (IC_{50}, 120 nM), and atenolol (IC_{50}, 192 nM) and similar to S-propranolol (IC_{50}, 22.4 nM).

Binding of (−)-[^{3}H]-CGP12177 to rat cerebral membranes

(−)-[^{3}H]-CGP12177 binding to rat cerebral membranes was linear in the concentration range 50–500 μg of protein, at 37°C, using 1 nM (−)-[^{3}H]-CGP12177 and 5 μM metoprolol for nonpecific binding (data not shown). The amount of membranes selected for saturation and displacement studies was 300 μg protein, since the radioactive signal was good and nonspecific binding amounted to 30–40%.

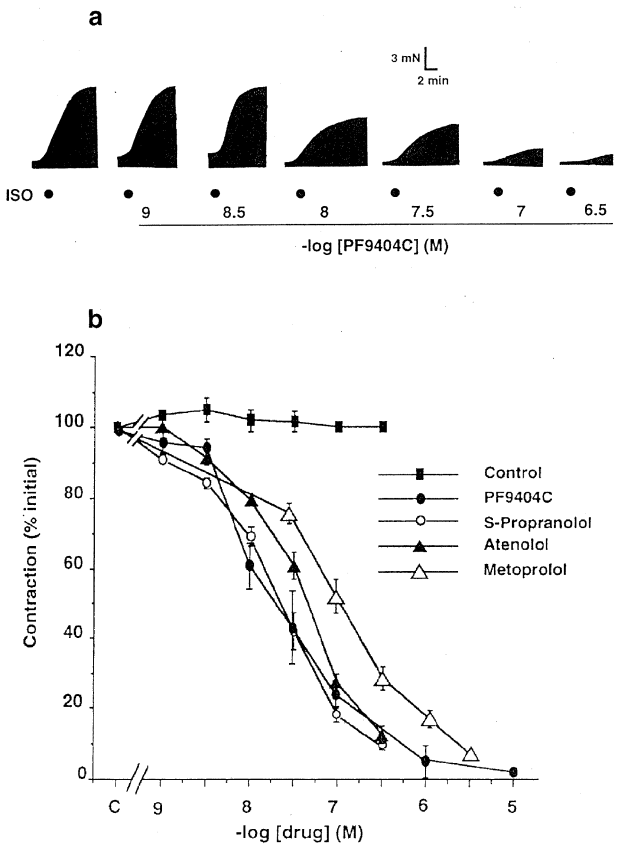


Figure 6 Concentration-response curves for the inhibition by PF9404C and other beta-blockers, of the inotropic effects of isoprenaline in the guinea-pig left atrium. The atrium, driven at 1 Hz, was incubated with a submaximal concentration of isoprenaline (3×10^{-8} M) for 5 min, to get a stable inotropic effect. This addition of isoprenaline was repeated at 30 min intervals (dots at the bottom of each trace in (a). The increase in force of contraction above basal amounted to 11 ± 1.3 mN (mean of ten atria) and was reproducible for 7–10 additions of isoprenaline. The compounds were added at increasing concentrations (abscissa), and were present since 10 min before and during isoprenaline incubation. Data in (b) are means $\pm$s.e.mean of 5–8 experiments performed with tissues from different animals, following the protocol shown in (a).

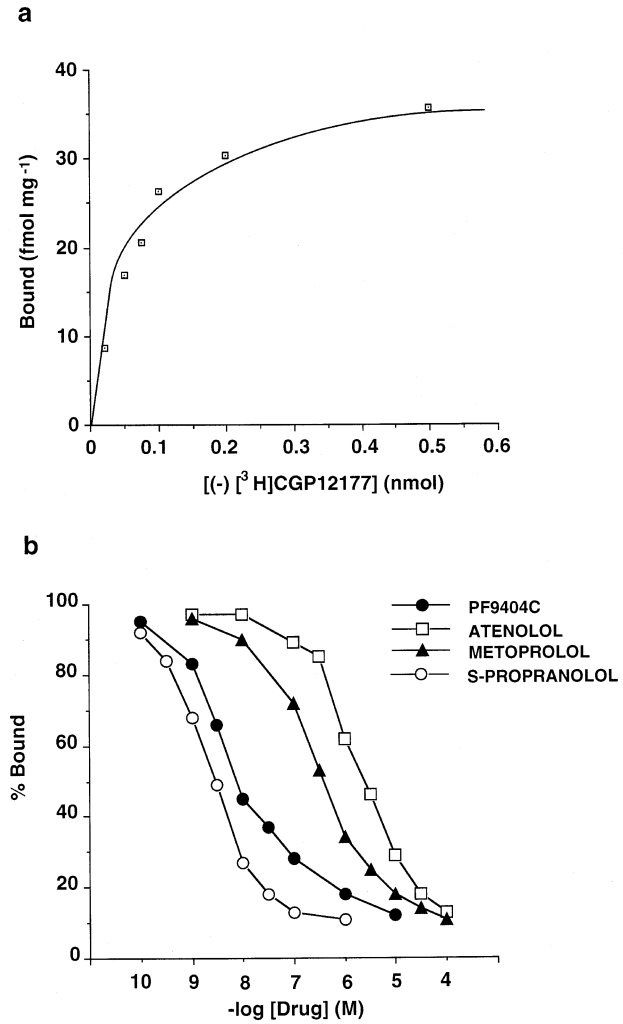


Figure 7 (a) Typical example of saturation binding of (−)-[^{3}H]-CGP12177 to rat cerebral membranes. Net binding (total binding minus non-specific binding) is shown. (b) Displacement of (−)-[^{3}H]-CGP12177 binding by PF9404C and other beta-blockers. Data are means $\pm$ s.e.mean of four experiments, performed in duplicate from four different batches of membranes.

The saturation curve of (−)-[^{3}H]-CGP12177 obtained in the presence of 5 μM metoprolol is shown in Figure 7a. The K_D was 0.1 nM, and the B_{max} was 42.7 fmol mg protein^{-1} ($n=4$ experiments in duplicate, performed in four different batches of membranes).

Figure 7b shows the displacement curves of (−)-[^{3}H]-CGP12177 binding by the beta-antagonists metoprolol, atenolol, S(−)-propranolol and PF9404C. All drugs tested displaced (−)-[^{3}H]-CGP12177 binding (used at 0.2 nM) in a concentration-dependent manner. The most potent was PF9404C (K_i, 0.007 ± 0.001 μM) followed by S-(−)propranolol (K_i 0.0017 ± 0.001 μM), metoprolol (K_i 0.17 ± 0.04 μM), and atenolol (K_i, 1.2 ± 0.5 μM). The IC_{50} values are shown in Table 2, where they are compared with the blockade of the isoprenaline inotropic effects in the guinea-pig atrium; a good correlation between both parameters is evident.

Table 2 Effects of PF9404C and other beta-blockers, on the inotropic effects of isoprenaline in the guinea-pig left atria and [3H]CGP12177 binding to brain membranes

Compound	n	*Inotropic effect of isoprenaline* (IC_{50s} nM)	*Displacement of [^{3}H]-CGP12177 binding* (IC_{50s} nM)
PF9404C	8	30 ± 9.9	23 ± 4
S-Propranolol	5	22.4 ± 3.8	53 ± 4
Metoprolol	5	120 ± 20.6	530 ± 110
Atenolol	5	192 ± 53	$3{,}700 \pm 1{,}600$

Mean IC_{50} were calculated by non-linear regression analysis for the individual concentration-response curves which form the averaged curves in Figure 6b (atrial contraction) and Figure 7b (displacement of [^{3}H]-CGP12177 binding).

Discussion

For a long time, the combination of beta-blockers with nitrovasodilators has been, and still is an efficient therapeutic approach in coronary heart diseases. Therefore, we believed that nitration of beta-blockers could produce a NO donor while keeping its beta adrenergic receptor blocking effects. We came across with the S-S enantiomer of a metoprolol derivative named PF9404C that, as this study demonstrates, has both properties, vasodilation and beta blockade.

Vasodilation shows up at submicromolar concentrations; hence PF9404C behaves as a potent relaxing agent in rat aortic strips, previously contracted with noradrenaline, the physiological neurotransmitter at vascular sympathetic neuroeffector junctions. Noradrenaline has β adrenoceptor agonist action, that could contribute to the vasorelaxant effects of PF9404C. However, the compound relaxed equally well the contractions of rabbit aorta strips precontracted with phenylephrine, a pure α adrenoceptor agonist (data not shown). The mechanism underlying such vasorelaxant effects seems to be mediated by the release of NO from PF9404C, as judged by two types of experiments (Furchgott & Zawadszky, 1980; Moncada *et al*., 1991): (a) Pharmacological analysis of vasorelaxation and (b) induction of cyclic GMP formation.

As a candidate NO donor compound, PF9404C was compared with various known NO donors concerning their ability to cause relaxation of rat aortic strips precontracted with noradrenaline. PF9404C was between the most potent nitrovasodilators (SNP, NOR3, NTG and NOR4), far away from the less potent vasodilators (SNAP, NOR2, NOR1 and ISD). Though by itself this fact does not prove that the mechanism of action of PF9404C involves NO release, several other pharmacological experiments suggest that this is so. For instance, oxyhaemoglobin (a NO sequestering agent) displaced to the right the concentration-dependent vasorelaxing responses of PF9404C (Figure 4). And cysteine, that is known to accelerate NO formation (Ignarro *et al*., 1980; Feelisch & Noack, 1987), accelerated the reversion of the vasorelaxant effects of the compound (Figure 3d).

A final argument rests on the fact that the vasodilatory effects of NO seem to be mediated by the activation of a soluble guanylate cyclase and the subsequent formation of cyclic GMP (Ignarro & Kadowiz, 1985). So, two inhibitors of this enzyme, methylene blue and ODQ, prevented almost completely the vasorelaxant actions of PF9404C (Figure 4c). In addition, direct measurements of cyclic GMP formation in rat aorta smooth muscle cells, indicate that PF9404C is efficient and potent in generating this intracellular messenger.

All these pharmacological and biochemical experiments support beyond doubt the idea that when in contact with living cells, PF9404C can generate substantial amounts of NO, leading to cyclic GMP formation and to vasorelaxation. The question now arises to whether PF9404C differs from the NO donors taken as reference compounds in this study. Rapid NO donors such as NOR1 or SNAP produced a transient vasorelaxation of only few minutes in duration. This was likely due to rapid NO formation and consumption of the compound. This was not the case for PF9404C, that produced a slowly developing and sustained relaxation of the vessel (Figure 3). The prototype of nitrates having still a wide range of clinical indications is NTG. This was the reason of the thorough comparison between NTG and PF9404C. The results obtained proved that PF9404C followed very closely the behaviour of NTG.

Concerning beta blockade, two types of experiments, pharmacological and biochemical, were also performed. The antagonism of the inotropic actions of isoprenaline in the guinea-pig left atrium proof that PF9404C causes a blockade of cardiac beta adrenergic receptors. Its beta blocking potency was close to that of S(-)propranolol, 4 fold higher than metoprolol, and 6 fold higher than atenolol. This was corroborated with radioligand binding experiments in rat brain membranes, where the binding of (−)-[^{3}H]-CGP12177 was displaced with the following order of potencies: PF9404C > S-(−) propranolol > metoprolol > atenolol.

With this pharmacological profile we envision a potential cardiovascular use of PF9404C in the following clinical framework. The rationale for the combination of a beta-receptor antagonist with a vasodilator in the same molecule is clearly established (Strein & Sponer, 1990). When betablockers alone are administered to hypertensive patients, the peripheral vascular resistance rises initially and the cardiac output decreases. Consequently, at the start of treatment the arterial pressure remains to a large extent unaltered, it decreases gradually during chronic treatment, whereas the cardiac output is consistently reduced. However, even during chronic treatment the total peripheral resistance remains higher than in normotensives. Thus, pure beta-blockers are normally not capable of correcting the haemodynamic disturbance, i.e. elevated total peripheral resistance underlying the arterial hypertension. The peripheral vascular resistance generally remains raised even during long-term therapy, and the cardiac output is reduced (Lund-Johansen, 1984). The administration of a vasodilator alone leads to a fall of peripheral resistance and of the arterial blood pressure. However, heart rate and plasma catecholamine levels increase and the renin-angiotensin-aldosterone system is activated. These counter-regulatory responses can be prevented by simultaneous beta-blockade. In addition to these properties, that are currently being investigated *in vivo*, as a NO donor PF9404C may exert protective effects on cardiovascular tissues through the following mechanism. It is known that NO exerts potent vasodilating actions (Palmer *et al*., 1987), inhibits leukocyte-endothelial cell interaction (Moilanen *et al*., 1992; Ma & Lefer, 1993), as well as platelet adherence and aggregation (Graaf *et al*., 1992) and vascular smooth muscle cell proliferation (Garg & Hassid, 1989). These actions constitute important protective mechanisms against atherogenesis and myocardial ischemia-reperfusion injury. Hence, the reduction in NO activity observed in hypercholesterolemia may promote atherogenesis and aggravate reperfusion injury. Conversely, the preservation of NO activity through a slow NO donor such as PF9404C may exert beneficial effects in hypercholesterolemia and ischaemic heart injury. We are currently investigating all these possibilities both *in vitro* and *in vivo*.

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