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Study of the *in vivo* and *in vitro* cardiovascular effects of a hydralazine-like vasodilator agent (HPS-10) in normotensive rats

Francisco Orallo

Departamento de Farmacología, Facultad de Farmacia, Universidad de Santiago de Compostela, Campus Universitario Sur, E-15706 Santiago de Compostela (La Coruña), España

1 In this work, the cardiovascular effects of HPS-10, a new vasodilator agent, were studied in rats.

2 In conscious normotensive rats, oral administration of HPS-10 (4–9 mg kg^{-1}) produced a dose-related and long-lasting fall in systolic arterial blood pressure (ED_{30} of 5.32 mg kg^{-1}), accompanied by an increase in heart rate (ED_{30} of 8.43 mg kg^{-1}). This tachycardia was totally inhibited by pretreatment with (±)-propranolol (10 mg kg^{-1}, p.o.).

3 In anaesthetized normotensive rats, HPS-10 (0.3–0.6 mg kg^{-1}, i.v.) produced a gradual, dose-dependent and sustained decrease in systolic, diastolic and mean arterial pressure (MAP) (ED_{30} for MAP of 0.41 mg kg^{-1}, i.v.), accompanied by a significant bradycardia at high doses (>0.4 mg kg^{-1}; ED_{20} of 0.61 mg kg^{-1}, i.v.). HPS-10 (0.5 mg kg^{-1}, i.v.) did not modify the positive chronotropic effects induced by intravenous administration of noradrenaline (NA; 5 μg kg^{-1}), angiotensin II (AII; 0.2 μg kg^{-1}) and nicotine (200 μg kg^{-1}) but markedly inhibited the hypertensive response produced by these agents.

4 In rat isolated rubbed aorta, HPS-10 (0.1–1 mM) non-competitively and with almost equal effectiveness antagonized the contractions induced by NA, AII (in normal Krebs solution) and Ca^{2+} (in depolarizing Ca^{2+}-free high-K^+ 50 mM solution). In the experiments in Ca^{2+}-free medium, HPS-10 (1 mM) considerably inhibited the contractions induced by NA, AII and caffeine in rat aorta.

5 Furthermore, in the studies with radioactive Ca^{2+}, HPS-10 (1 mM) did not modify the basal uptake of $^{45}Ca^{2+}$ but strongly decreased the influx of $^{45}Ca^{2+}$ induced by NA, AII and K^+ in rat aortic rings.

6 In rat isolated atria, HPS-10 (1 mM) produced a positive inotropic/negative chronotropic effect.

7 HPS-10 (0.3 mM) significantly inhibited the sustained and transient Ba^{2+} inward current (I_{Ba}) recorded in whole-cell clamped rat aortic myocytes.

8 These results indicate that the non-selective vasorelaxant effects of HPS-10 in rat aortic rings can be attributed to transmembrane Ca^{2+}-antagonist activity and an intracellular action on smooth muscle cells. The direct vasodilator action of HPS-10 observed in rat isolated aorta may be responsible for the HPS-10 hypotensive activity in anaesthetized normotensive rats.

Keywords: Conscious and anaesthetized normotensive rats; rat aorta; HPS-10; rat atria; hydrazinopyridazines; vasodilator agent; hypotension; bradycardia; whole-cell clamped rat aortic myocytes

Introduction

Hydrazinophthalazines were first discovered by Gross *et al.* in 1950, the most important being hydralazine. Hydralazine is a peripheral vasodilator, which has been used for many years in the treatment of essential hypertension (Campos-Toimil & Orallo, 1996a). There is general agreement that the fall in blood pressure produced by hydralazine results from a direct action of the drug on vascular smooth muscle (Khayyal *et al.*, 1981; Orallo *et al.*, 1991; Moína *et al.*, 1994). Although the basis for its direct vasodilator effects has been studied extensively, the mechanism has not been clearly established. However, several hypotheses have been postulated (for a review see Gurney, 1994; Campos-Toimil & Orallo, 1996b). Recently, the therapeutic interest of the drug has considerably increased given that hydralazine may have potential cytotoxic activity because it notably decreases the blood flow to tumours (Hasegawa & Song, 1991; Horsman *et al.*, 1995).

In order to develop new drugs with greater activity and fewer side effects than hydralazine and in order to improve the pharmacological treatment of hypertension, we have been working, for several years, on the synthesis and pharmacological screening of related compounds (modified hydrazino pyridazines), most of which have proved to have considerable antihypertensive activity (Gil-Longo *et al.*, 1993).

The present study is the first time a detailed and comparative *in vivo* and *in vitro* investigation (in normotensive rats) on the cardiovascular effects of a novel hydrazino pyridazine derivative, 6-phenyl-5-(1-morpholinylmethyl)-3-hydrazine pyridazine (HPS-10) (Figure 1), has been described.

Methods

Animals

Male Wistar rats (Iffa-Credo) and male Swiss mice (Charles River CD-1), purchased from Criffa (Barcelona, Spain), were used throughout this study. They were housed (groups of five) to a macrolon cages (Panlab, Barcelona, Spain) on poplar shaving bedding (B&K Universal, G. Jordi, Barcelona, Spain) in a standard experimental animal room, illuminated from 08 h 00 min to 20 h 00 min (12 h light: 12 h dark cycle) and maintained at a temperature of 22–24°C. The animals had free access to food pellets (B&K Universal, G. Jordi, Barcelona, Spain), drinking fluid (tap water) and were allowed to acclimatize for one week before the experiments.

Conscious normotensive rats

A group of five normotensive male Wistar rats weighing 250–300 g, which had been fed standard food and water *ad libitum* and starved for 18 h before experiments, received deionized water (control), p.o., (10 ml kg^{-1}) and another similar group (treated) received HPS-10 solutions (4–9 mg 10 ml^{-1}, 10 ml kg^{-1}, p.o.). The systolic arterial pressure and heart rate were measured in the caudal artery by an indirect tail-cuff method before and 1, 3, 5, 7 and 24 h after treatment, by a programmed Narco Bio-Systems PE-300 sphygmomanometer equipped with a Narco pneumatic pulse transducer and a Scientific Instrument Center Mod. 2125 plotter. The rats were kept warm for 15 min at 37°C before measurements were taken.

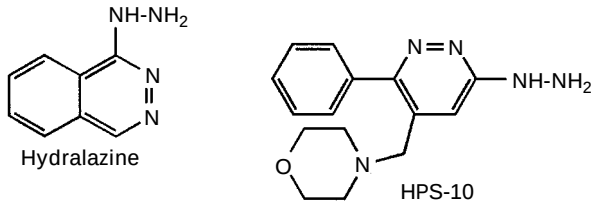

Figure 1 Chemical structures of HPS-10 and hydralazine.

In some experiments, rats received (±)-propranolol solution p.o. (10 mg (10 ml)$^{-1}$, 10 ml kg^{-1}). The systolic arterial pressure and heart rate were measured (as described above) before and 1, 2, 4, 6, 8 and 24 h after treatment.

When the effects of (±)-propranolol on HPS-10 were studied, the rats were treated with (±)-propranolol (10 mg kg^{-1}, p.o.) 1 h before administration of HPS-10 (10 mg kg^{-1}, p.o.). Simultaneously, control animals were treated with de-ionized water in place of (±)-propranolol and HPS-10.

Anaesthetized normotensive rats

Normotensive male Wistar rats weighing 250–300 g were anaesthetized by intraperitoneal injection of urethane (1.25 g (10 ml)$^{-1}$, 10 ml kg^{-1}) and the rectal temperature was kept at 36.5–37.5°C with an overhead lamp. The trachea was cannulated to facilitate spontaneous respiration and a patent airway. Polyethylene cannulae (PE-60 and PE-50), containing heparin-treated saline (120 iu ml^{-1}), were inserted into the left carotid artery (for arterial pressure and heart rate measurement) and in the left femoral vein (for i.v. bolus administration of drugs). Systolic and diastolic arterial pressures were monitored by means of a TRA 021 Letica pressure transducer on a Letica Unigraph 1000-506 device. The polygraph was coupled to a digital counter which was, in turn, connected to a programmed timer that entered the heart rate value, obtained from the arterial pulse wave, into a Panlab printer once a minute. Upon completion of the surgery, cardiovascular parameters were allowed to stabilize for 30 min.

In the first set of experiments, after blood pressure and heart rate had stabilized, saline (1 ml kg^{-1}, for control group) and HPS-10 solutions (0.2–0.6 mg ml^{-1}, 1 ml kg^{-1}, for treated groups) were injected intravenously via the left femoral vein, in order to observe the effects on blood pressure and heart rate.

In the second set of experiments, noradrenaline (NA), angiotensin II (AII) and nicotine were injected via the left femoral vein. After the cardiovascular responses produced by these drugs had been observed and the haemodynamic parameters (blood pressure and heart rate) had returned to basal values (10–15 min), a dose of saline or HPS-10 (0.5 mg kg^{-1}) was injected. After 15–20 min, the same dose of the pressor agent was administered to test (a) the reproducibility of the cardiovascular effects induced by successive administrations of NA, AII and nicotine in the control group or (b) whether HPS-10 could modify the cardiovascular responses induced by the pressor agents in the treated preparations.

Acute toxicity

For the study of acute toxicity, groups of 10 male Swiss mice (20–30 g in body weight) were used. Different solutions of HPS-10 in de-ionized water (200–325 mg (10 ml)$^{-1}$) were administered intraperitoneally (10 ml kg^{-1}). After drug administration, the behaviour of animals was carefully observed for three days. Lethal dose (LD_{50}) and s.e.mean values were determined according to the method of Miller & Tainter (1944).

Contraction studies in rat isolated thoracic aorta rings

Male Wistar rats weighing 250–300 g were killed by stunning and exsanguination. The thoracic aorta was rapidly removed, placed in a Petri dish with Krebs bicarbonate solution (oxygenated with carbogen (95% O_2 + 5% CO_2) at room temperature (20 ± 2°C)) of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$.2 H_2O 2.5, $MgSO_4$.7 H_2O 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA (disodium salt of ethylenediaminetetraacetic acid) 0.03, (+)-ascorbic acid 0.6 and glucose 11 (pH = 7.4), cleaned of fat and connective tissue, deprived of endothelium by rubbing the intimal surface with a cotton bud and cut into approximately 4 mm long cylindrical segments of 6–9 mg.

Aorta rings were immediately transferred to an organ bath containing 20 ml of the above solution, thermoregulated at 37°C and bubbled with carbogen. Two stainless steel pins were inserted through the lumen of each arterial segment: one pin was fixed to the organ bath and the other was connected to a CPOL force-displacement transducer to record isometric tension, with a computerized Celaster IOS 1 system. Before specific experimental protocols were initiated, aortae were equilibrated at a resting tension of 1 g for at least 1 h during which the physiological solution was replaced every 10 min. The absence of acetylcholine (1 μM) vasorelaxant action in aortic rings precontracted with NA (10 μM) and a simple haematoxylin-eosine staining technique were used to verify the removal of endothelial cells and the integrity of underlying smooth muscle (Orallo *et al*., 1995).

NA and AII concentration-response curves Cumulative concentration-response curves were obtained by the method of van Rossum (1963), in which progressively higher concentrations are applied when a steady state level had been reached for the preceding concentration. In control rings, four consecutive concentration-response curves were obtained at 60 min intervals, to allow washout and to minimize the possibility of receptor desensitization. Before the fourth curve was constructed, rings were preincubated for 20 min with de-ionized water, in order to test whether this vehicle could modify the NA and AII contractile response. The first curve differed from the last three, which were reproducible. In treated rings, after two similar curves with the agonist had been obtained, tissues were incubated with HPS-10 for 20 min (a sufficient exposure time to achieve equilibrium) and a fourth was constructed.

Calcium concentration-response curves After an equilibration period of 1 h in Krebs bicarbonate solution, tissues were incubated for 30 min in Ca^{2+}-free depolarizing Krebs bicarbonate solution (containing 50 mM KCl instead of the equivalent amount of NaCl, in order to maintain osmolarity). Calcium chloride (10 μM–10 mM) was then added to the bath in stepwise fashion. As above, after two reproducible control concentration-response curves had been obtained, tissues were incubated with HPS-10 for 20 min and a fourth curve was then constructed.

Contractile responses in calcium-free medium Aorta preparations were equilibrated for 60 min in normal Krebs solution and then washed 4 times over a 20 min period with a Ca^{2+}-free Krebs solution, containing 0.5 mM EGTA (ethylene glycol-*bis* (β-aminoethylether) N,N,N′,N′-tetraacetic acid); contraction was then elicited with the vasoconstrictor agents (NA 10 μM; AII 1 μM and caffeine 10 mM). In control rings, three consecutive and reproducible contractions were obtained at least at 80 min intervals following the above protocol. Before the three contractions were obtained, in order to test whether the deionized water affected the contractile response of the agonists, the preparations were washed in normal Krebs solution for 60 min (to fill the depleted Ca^{2+} stores). There followed a further 10 min preincubation in Ca^{2+}-free solution before deionized water was added, followed 10 min later by the corresponding dose of the vasoconstrictor agents. Likewise, aorta

rings (treated group) were simultaneously subjected to the same procedure but with addition of HPS-10 in place of de-ionized water.

In all the contraction studies, only one agonist and one concentration of HPS-10 were used in each experiment (per aortic ring).

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

Aorta rings were equilibrated for at least 60 min in Krebs bicarbonate solution (containing 0.2 mM instead of 2.5 mM $CaCl_2.2H_2O$) maintained at 37°C and bubbled with carbogen. Afterwards, the tissues were incubated for 5 min in a solution containing a single concentration (0.18 μM) of $^{45}Ca^{2+}$ (specific activity 28.60 mCi mg^{-1}) with or without NA (10 μM), AII (1 μM) or high K^+ (50 mM), instead of the equivalent amount of NaCl, to analyse the effect of these vasoconstrictor agents on basal $^{45}Ca^{2+}$ uptake. To investigate the actions of de-ionized water (for control) or HPS-10 (for treated group) on basal and induced $^{45}Ca^{2+}$ uptake, the arteries were exposed to them 20 min before and during the incubation period with $^{45}Ca^{2+}$. Thereafter, the preparations were washed for 30 min in 250 ml of an ice cold La^{3+} solution, in order to remove extracellular Ca^{2+} from the tissue (composition (mM): NaCl 119, KCl 4.7, tris(hydroxymethyl)aminomethane 5, $MgSO_4.7\ H_2O$ 1.2, $LaCl_3.7\ H_2O$ 50 and glucose 11; pH adjusted to 6.8 with HCl 1 N). Arteries were then blotted dry, weighed and digested in vials with 1 ml H_2O_2 (110 volumes) at 119°C for 90 min. After cooling, 5 ml of liquid scintillation counting cocktail (Ready-Safe Beckman) were added to each vial and the radioactivity of the samples measured in a liquid scintillation counter (Beckman LS 6000LL), following appropriate standard control procedures.

Rat isolated atria

Male Wistar rats (250–300 g) were killed by a blow to the head and exsanguinated. The chest was opened by midsternal incision, the heart rapidly taken out, transferred to a Petri dish with Krebs bicarbonate solution at room temperature (20±2°C) and the atria dissected free. Afterwards, these atria were set up in a 10 ml organ-bath under 1 g resting tension in Krebs solution, maintained at 37°C and bubbled with carbogen, which was changed every 10 min. The rate and force of contraction were measured with a force-displacement transducer (Letica TRI-110) and recorded on a Leti-Graph 1000-506 device. The polygraph was coupled to a digital counter which was, in turn, connected to a programmed timer that entered the atrial rate, obtained from the spontaneous atrial contraction, into a Panlab printer once a minute. After a 60 min stabilization time period, de-ionized water (for control group) or HPS-10 solution (for treated group) was added and measurements of force and rate of contraction registered each minute for a period of 15 min, in order to observe whether the inothropic and/or chronothropic properties were modified by the vehicle (de-ionized water) and the drug.

Culture of rat aortic smooth muscle cells

Primary cultures of smooth muscle cells were prepared from aortae of 10-week-old Wistar rats, essentially as we described elsewhere (von der Weid *et al*., 1993). Briefly, rats were stunned and exsanguinated. Aortae were rapidly dissected out under sterile conditions, cleaned of adhering fat and connective tissue and placed in a defined, serum-free culture medium MEM-Ham's F-12 (1 : 1 v/v; Gibco), supplemented with 2% synthetic serum substitutes Ultroser SF/Ultroser G (3 : 1 v/v; IBF), L-glutamine (1 mM) (+)-ascorbic acid (50 μM), L-proline (10 μM), penicillin G (100 u ml^{-1}), streptomicin (100 μg ml^{-1}) and amphotericin B (1 μg ml^{-1}) (obtained from Sigma). A first digestion was carried out in collagenase B (0.5 u ml^{-1}, Boehringer Mannheim) in Hanks' balanced salt solution (Gibco) for 0.5 h in a 37°C incubator Jouan EB 115, equipped with a three-dimensional platform suspension shaker (Heidolph Reax-3). Tissues were then replaced in culture medium and the adventitia and intima gently removed. The remaining media (cut in small pieces) was then incubated in a second digestion medium containing Hanks' balanced salt solution, collagenase B (2.5 u ml^{-1}) and elastase (40 u ml^{-1}; Boehringer Mannheim) for 60–90 min at 37°C. Single cells were dispersed by trituration with flame-polished Pasteur pipettes. The cell suspension was diluted with supplemented MEM-Ham's F12 and following centrifugation (1000 r.p.m. for 12 min), pelleted cells were resuspended in culture medium. An aliquot of sample (50 μl) was used to determine, in a haemacytometer, the cell viability (>80%) by dye exclusion, by use of a 0.1% trypan blue solution (Flow). The rest of the cell suspension was plated: (1) at 2000 cells $(cm^2)^{-1}$ onto glass coverslips in 60 mm Petri dishes (Corning). (2) At 10,000 cells $(cm^2)^{-1}$ in T-25 tissue culture flasks (Corning). After confluence, smooth muscle cells were treated with a Ca^{2+}-free/EDTA trypsin 0.25% solution (Sigma) and subcultured in 60 mm Petri dishes (glass coverslips), as described above.

After being plated, the cells were kept at 37°C in an incubator Heraeus B5061 EK gassed with 5% CO_2/95% air.

Electrophysiology: whole-cell voltage-clamp recordings

Single cells were studied usually 1–10 days after plating by standard patch-clamp techniques, as we previously described (Orallo & Takeda, 1992; von der Weid *et al*., 1993). In some cases, cells from first or second passages were also used. Briefly, glass coverslips containing rat aortic myocytes were transferred to 25 mm Petri dishes (Nunc; volume 5 ml) with Ba^{2+} external solution and placed for electrophysiological experiments in the recording chamber of a patch-clamp tower (List Electronic, Germany), equipped with an inverted light microscope (Axiovert 135 Zeiss).

The Ba^{2+} external bath solution contained (mM) NaCl 120, $MgCl_2.6H_2O$ 2, $BaCl_2.2H_2O$ 20, HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid], Sigma) 10, glucose 11; pH adjusted to 7.4 with NaOH.

Patch pipettes (thin wall 1.5 mm o.d./1.17 mm i.d. borosilicate glass capillaries, GC150TF-10, Clark Electromedical Instruments) were pulled and polished by a L/M-3P-A patch pipette puller and a L/M-CPZ-101 microforge (List Electronic). These pipettes had a resistance of 3–8 MΩ when filled with Cs/TEA internal solution (in mM): CsCl 140, EGTA 3, $MgCl_2.6H_2O$ 2, HEPES 20, magnesium adenosine trisphosphate (MgATP, Sigma) 5, lithium guanosine trisphosphate (LiGTP, Sigma) 0.2, tetraethylammonium chloride (TEA Cl, Sigma) 2, at pH 7.4 (adjusted with CsOH). Junction potentials between the intrapipette electrode (internal solution) and the reference electrode (bath solution) were subtracted by the offset of the patch clamp amplifier (L/M-EPC-7, List Electronic) before the gigaseals were obtained. To minimize associated capacitance, pipettes were coated with beeswax and dipped in Sigmacote (Sigma). Compensation for series resistance and capacitance was made by use of the inbuilt circuitry of the patch clamp amplifier.

Whole-cell currents through Ca^{2+} channels, with Ba^{2+} as the charge carrier instead of Ca^{2+} (I_{Ba}), were elicited by test pulses (500 or 800 ms in duration for transient and maintained currents, respectively) to depolarizations between −60 and +60 mV in 10 mV increments at 10–20 s intervals, from a holding potential (V_h) of −80 mV. The rundown of Ba^{2+} currents was usually between 15–25% and no greater than 40–50% over 15–20 min, after the access of the patch pipette to the interior of the cell. I_{Ba} (in pA) was measured as the difference between the maximal inward current amplitude and the steady current at the end of the pulse (zero current level). Experiments were made at room temperature (20–24°C). The data illustrated in results are non-leak subtracted.

HPS-10 (0.3 mM) was directly added to the bath 20 min before to study their effects on current-voltage (*I*-V) curves.

The control application of Ba^{2+} external bath solution alone had no effect on I_{Ba}.

For cell stimulation and for recording and analysis of data, a commercial software package (pCLAMP 6.0.3) with Digidata 1200 interface (Axon Instruments, U.S.A.), a personal computer (MC-486DX, 66 MHz) and a digital oscilloscope Nic-310 (Nicolet) were used.

Expression and statistical analysis of results

Unless otherwise specified, results shown in the text and figures are expressed as means ± s.e.mean. Significant differences between two means ($P<0.05$ or $P<0.01$) were estimated by Student's two-tailed t test for paired or unpaired data, where appropriate.

In conscious and anaesthetized normotensive rats, blood pressure and heart rate values are expressed in mmHg and beats min^{-1}, respectively.

Hypotensive activity and effects on heart rate in conscious rats are expressed as ED_{30}, the dosage of HPS-10 required to reduce normal systolic arterial pressure or to increase heart rate by 30%, which was calculated by the least squares linear regression, by use of the software of Tallarida and Murray (1987), of log dosage (in mg kg^{-1}) on maximum pharmacological response (% reduction in systolic arterial pressure or % increase in heart rate, achieved 1 h after treatment).

In anaesthetized normotensive rats, mean arterial pressure (MAP) was calculated according to the formula: (2 diastolic pressure + systolic pressure)/3. Unless otherwise specified, increases or decreases of mean blood pressure and heart rate values are expressed as absolute values with respect to basal values/values obtained in the presence of HPS-10. Hypotensive activity is expressed as ED_{30} for MAP and the effects on heart rate as ED_{20}, calculated as described above.

In mice toxicity studies, acute toxicity is expressed as LD_{50} (dose of HPS-10 that produces the death in 50% of the animal population), which was calculated as described in the corresponding section of Methods.

In functional postsynaptic studies in rat aorta, concentration-responses curves were analysed by use of a curve-fitting analysis programme (Origin 4.0). Contractile responses to vasoconstrictor agents (in the presence or absence of HPS-10) are expressed as a percentage of the maximal contraction ($E_{max}=100\%$) reached in the concentration-response curves obtained before incubation with HPS-10.

Vasoconstrictor agent pD_2 values (negative $\log_{10}$ of the molar concentration of agonist required to elicit 50% of maximal response) were obtained according to Van Rossum (1963). HPS-10 pD'_2 values (negative $\log_{10}$ of the molar concentration of antagonist required to cause a 50% depression of the maximal contractile effect of the corresponding agonist) were calculated (by use of the software of Tallarida & Murray, 1987), as the X-intercept of the regression of log (X − 1) on − log [antagonist], where X is the ratio of the maximal control response of the vasoconstrictor agent to the maximal effect of the agonist in the presence of the antagonist (HPS-10).

In contraction studies with a Ca^{2+}-free medium, contractile responses induced by the different vasoconstrictor agents before and after treatment with HPS-10 are expressed in absolute tension values (mg).

In the experiments with radioactive calcium, $^{45}Ca^{2+}$ vascular tissue (aortic rings) uptake was calculated from the formula: $^{45}Ca^{2+}$ uptake (nmol kg^{-1} wet tissue) = (c.p.m. in tissue kg^{-1} wet tissue wt.) × [(nmol $^{45}Ca^{2+}$ in 1 litre solution)/(c.p.m. l^{-1} solution)]. Note that the numerator of the second factor in this expression is the concentration of $^{45}Ca^{2+}$, not the total Ca^{2+} concentration.

In rat isolated atria, the rate and force of contraction values before and after incubation with HPS-10 are expressed in beats min^{-1} and mg, respectively.

In the whole-cell voltage-clamp experiments, I_{Ba} was measured as described above (in the corresponding section of electrophysiology).

Drugs, chemicals and radioisotopes

The drugs and radioisotopes used in the experiments were: HPS-10 hydrochloride (synthesized in the Department of Organic and Medicinal Chemistry, Faculty of Pharmacy, University of Santiago de Compostela), (±)-propranolol (Zeneca Farma, S.A.), heparin (Analema), (−)-NA bitartrate, (−)-nicotine di-(+)-tartrate, AII acetate, acetylcholine chloride, caffeine, tetraethylammonium chloride (TEA Cl) and urethane (purchased from Sigma Chemical Co), $^{45}Ca^{2+}$ (New England Nuclear).

The HPS-10 hydrochloride was dissolved in saline (for i.v. administration), in a Ba^{2+} external solution (for electrophysiological recordings) or in de-ionized water (for all the other experiments), immediately before use. Caffeine and (±)-propranolol solutions were prepared daily in Ca^{2+}-free medium and de-ionized water, respectively. Urethane and heparin were dissolved in saline to make several solutions of 25 g 100 ml^{-1} and 12,000 iu 100 ml^{-1}, kept at 4°C.

The appropriate dilutions of the following drugs were prepared daily from concentrated stock solutions kept at −20°C: (−)-NA bitartrate from 500 μg ml^{-1} or 100 mM stock solutions in saline (for i.v. administration) or de-ionized water (for all the other experiments), respectively (sodium bisulphite (0.2%) was added to the NA stock solutions to prevent oxidation); acetylcholine chloride (to test the absence of endothelium) from a stock solution (10 mM) in de-ionized water; AII acetate from 20 μg ml^{-1} and 10 mM stock solutions in saline (for intravenous administration) or de-ionized water (for the *in vitro* experiments), respectively and (−)-nicotine di-(+)-tartrate from a stock solution (2 mg ml^{-1}) in saline.

All chemicals and materials used for cell culture and electrophysiology were purchased from the suppliers indicated in the corresponding sections. All the other chemicals, including the reagents used in the preparation of physiological solutions, were of the best quality available commercially.

Results

Conscious normotensive rats

The mean basal values of systolic blood pressure and heart rate (measured in control and treated groups before administration) were 104 ± 4 mmHg and 383 ± 9 beats min^{-1} ($n=20$).

The mean values of systolic blood pressure and heart rate, measured 5 times in a 24 h period in control animals, after administration of saline, were not significantly different from the basal ones ($P>0.05$, $n=5$).

HPS-10 (4–9 mg kg^{-1}, p.o.) produced a pronounced, dose-dependent and long-lasting fall in systolic blood arterial pressure (Figure 2a). The peak effect was reached within 60 min. The fall in blood pressure was accompanied by a marked, dose-related and fairly well-correlated increase in heart rate that also peaked 1 h after administration (Figure 2b). ED_{30} for systolic arterial pressure was 5.32 ± 0.24 mg kg^{-1}, p.o., and for heart rate 8.43 ± 0.36 ($n=5$). Decrease in systolic arterial pressure and positive cronotropic effect fell to zero after 24 h (Figure 2a and b).

(±)-Propranolol (10 mg kg^{-1}, p.o.) produced a significant decrease in heart rate and had no effect on systolic arterial blood pressure in conscious normotensive rats (Figure 3a and b). Pretreatment with (±)-propranolol totally inhibited the increase in heart rate produced by HPS-10 (Figure 3b). (However, this pretreatment had no significant effect on the hypotension induced by HPS-10 (Figure 3a).

Anaesthetized normotensive rats

The mean basal values of MAP and heart rate in anaesthetized normotensive rats (measured in control and treated groups before treatment) were 91 ± 3 mmHg and 355 ± 6 beats min^{-1} ($n=40$). In control animals, these values made no significant

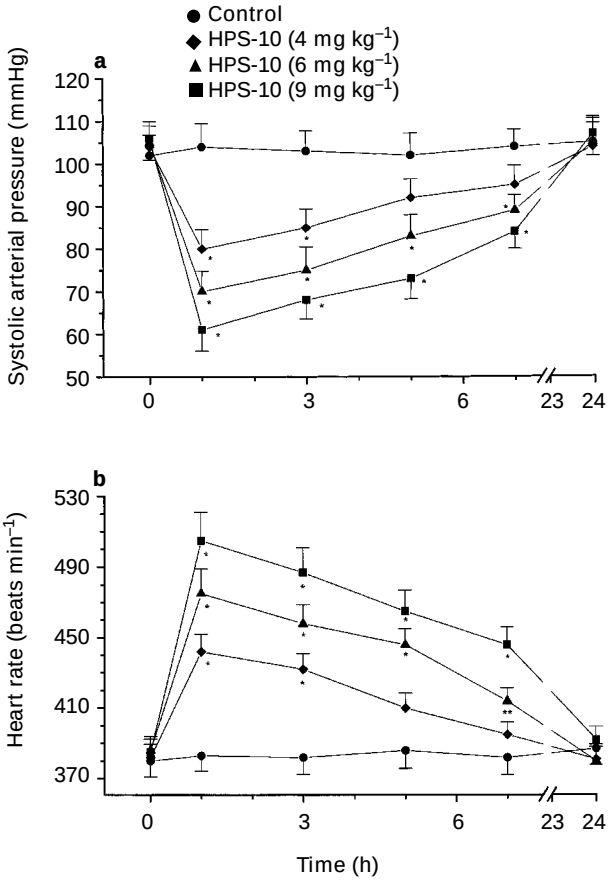

Figure 2 Effects of HPS-10 (4 mg kg^{-1} 6 mg kg^{-1} and 9 mg kg^{-1}, p.o.) on (a) systolic arterial pressure and (b) heart rate in conscious normotensive rats. Each point is the mean of five experiments and vertical lines show s.e.mean. Level of statistical significance with respect to control values: $*P<0.01$ or $**P<0.05$.

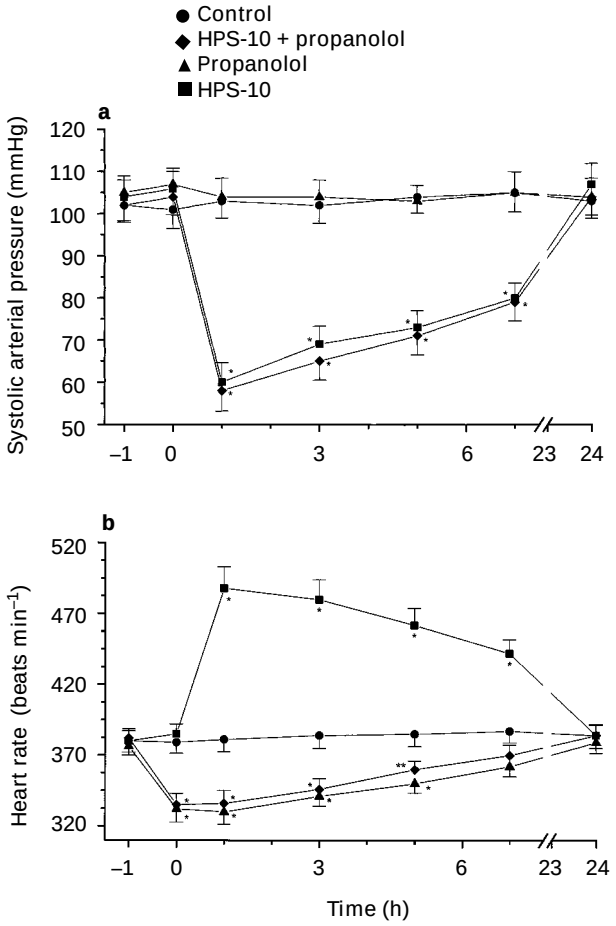

Figure 3 Effects of (±)-propranolol (10 mg kg^{-1}, p.o.) and HPS-10 (9 mg kg^{-1}, p.o. 1 h after de-ionized water or 9 mg kg^{-1}, p.o. 1 h after treatment with (±)-propranolol 10 mg kg^{-1}, p.o.) on systolic arterial pressure (a) and heart rate (b) in conscious normotensive rats. Each point represents the mean, and vertical lines show s.e.mean, from 5 experiments. Level of statistical significance with respect to control values: $*P<0.01$ or $**P<0.05$.

difference throughout the period in which the measurements were carried out ($P>0.05$, $n=5$).

Administration of HPS-10 (0.3–0.6 mg kg^{-1}, i.v.) caused a gradual and dose-dependent fall in systolic, diastolic and MAP. The maximal effect of the drug was reached in the 5–10 min after treatment and persisted for over 30 min. The ED_{30} for MAP was 0.41 ± 0.05 mg kg^{-1}, i.v. This hypotensive effect was accompanied by irregular and no significant changes in heart rate at low doses (<0.4 mg kg^{-1}, i.v.). However, at high doses, a significant decrease in heart rate was observed. The ED_{20} was 0.61 ± 0.07 mg kg^{-1}, i.v.

At the dosage of 5 mg kg^{-1}, i.v., the minimum MAP and heart rate values were 52 ± 5 mmHg and 318 ± 9 beats min^{-1} (decreased values in mmHg and beats min^{-1} of 39 ± 4 and 37 ± 8, respectively; $P<0.01$ with respect to basal and/or control values, $n=5$) and persisted for over 30 min.

Two successive injections of a single dose of vasoconstrictor agents (NA, AII and nicotine) induced reproducible cardiovascular effects (see below) in anaesthetized normotensive rats. Administration of saline (1 ml kg^{-1}, i.v., for control group) did not significantly modify these effects ($P>0.05$, $n=5$).

NA (5 μg kg^{-1}, i.v.) produced a maximum value of MAP and heart rate of 148 ± 7 mmHg (increase value in mmHg: 57 ± 5) and 419 ± 12 beats min^{-1} (increase value in beats min^{-1}: 64 ± 9) ($P<0.01$ with respect to basal and/or control values, $n=5$). HPS-10 (0.5 mg kg^{-1}, i.v.) was injected and the values of blood pressure and heart rate, after 15–20 min, were 56 ± 5 and 320 ± 10 ($n=5$). Hence, the same dosage of NA produced a maximal increase of these two haemodynamic parameters, whose values were 74 ± 5 mmHg and 379 ± 10 beats min^{-1}. The increment in beats min^{-1} (59 ± 9) was not different from that obtained in the absence of HPS-10 ($P>0.05$, $n=5$). However, HPS-10 significantly inhibited the hypertensive response induced by NA ($P<0.01$, $n=5$) because the increment in mmHg (18 ± 4) was significantly less than that obtained without HPS-10 ($P<0.01$, $n=5$).

AII (0.2 μg kg^{-1}, i.v.) significantly increased MAP and heart rate, the maximum values were 139 ± 6 mmHg and 395 ± 12 beats min^{-1} (variation in mmHg and beats min^{-1}: 48 ± 4 and 40 ± 8, respectively; $P<0.01$ with respect to basal and/or control values, $n=5$). The values of MAP and heart rate were 53 ± 6 mmHg and 317 ± 9 beats min^{-1} ($n=5$) 15–20 min after administration of HPS-10 (0.5 mg kg^{-1}, i.v.). Again, a new dose of AII was administered and, as above, a characteristic pressor response accompanied by tachycardia was produced, the maximum values being: 68 ± 5 mmHg and 354 ± 12 (increased values in mmHg and beats min^{-1} of: 15 ± 4 and 37 ± 9, respectively). There were no statistically significant differences between the values of the increments of heart rate induced by AII before and after administration of HPS-10 ($P>0.05$). However, HPS-10 strongly inhibited the pressor response induced by AII ($P<0.01$, $n=5$).

Nicotine (200 μg kg^{-1}, i.v.) increased MAP and heart rate to a maximum value of 145 ± 7 mmHg (variation in mmHg: 54 ± 6) and 401 ± 9 beats min^{-1} (variation in beats min^{-1}: 46 ± 8) ($P<0.01$ with respect to basal values, $n=5$). HPS-10 was administered and, after 15–20 min, MAP and heart rate values were 55 ± 6 mmHg and 322 ± 11 beats min^{-1} ($n=5$).

Afterwards, the same dosage of the pressor agent produced a maximal increase in MAP and heart rate, the values being 73 ± 4 mmHg and 367 ± 12 beats min^{-1}. Also, in this case, only the values of the increments in MAP (18 ± 4), but not the values of the increments in heart rate (45 ± 9) were significantly less than those obtained without the drug ($P < 0.01$, $n = 5$).

Acute toxicity

The LD_{50} value of HPS-10 was 264 ± 8 mg kg^{-1}, i.p. In most cases, the death of animals, probably due to severe convulsions, occurred within 30–60 min after intraperitoneal administration. The other main toxic symptoms detected in mice were respiratory stimulation and an increase in heart rate. The surviving animals exhibited almost normal behaviour 24 h after treatment.

Contraction studies in rat isolated thoracic aortic rings

This preparation lacked spontaneous activity. Resting tone was unaffected by de-ionized water and/or HPS-10 (0.1–1 mM) ($n = 5$).

NA induced dose-related contractions in endothelium-denuded rat aorta rings. The pD_2 and the maximal tension (mg) were 7.98 ± 0.14 and 6430 ± 220, respectively ($n = 20$). HPS-10 (0.1–1 mM) shifted the concentration-response curve for NA to the right with depression of the maximal response (Figure 4a). The pD'_2 value was 3.49 ± 0.16.

AII elicited dose-related contractions in rubbed aortic rings. The pD_2 was 7.93 ± 0.12 and the maximal tension 2032 ± 80 mg ($n = 20$). HPS-10 (0.1–1 mM) significantly inhibited AII-induced contractions in the aorta rings and caused a shift to the right of the concentration-response curves for this vasoconstrictor agent, with depression of the maximal response (Figure 4b). The PD'_2 was value 3.68 ± 0.18 ($n = 5$).

In depolarizing Ca^{2+}-free high-K^+ (50 mM) medium, addition of Ca^{2+} to the bath induced a gradual increase in tension. The maximal effect reached was 5112 ± 216 mg and the EC_{50} value (50% effective concentration) was 316 ± 11 μM ($n = 20$). HPS-10 (0.1–1 mM) antagonized non-competitively the Ca^{2+}-induced contractions with depression of the maximal effect (Figure 4c). The pD'_2 value was 3.7 ± 0.2 ($n = 5$).

There were no statistically significant differences between the pD'_2 values of HPS-10 against the above vasoconstrictor agents.

In Ca^{2+}-free medium, NA (10 μM) and AII (1 μM) produced a characteristic contraction with two distinct components: an initial transient contraction (fast component) that relaxed to a sustained tension (slow component), (tension values: 2129 ± 145 and 1204 ± 62 mg for NA and 642 ± 32 and 375 ± 24 mg for AII; $n = 10$). Caffeine (10 mM) produced only an initial transient contraction of 535 ± 20 mg ($n = 10$). De-ionized water had no significant effect on NA-, AII- and caffeine-induced contractions.

HPS-10 (1 mM) strongly inhibited the contractile effect induced by NA (transient contraction: 629 ± 85 mg and sustained contraction: 318 ± 38 mg), AII (transient contraction: 152 ± 31 mg and sustained contraction: 78 ± 14 mg) and caffeine (transient contraction: 269 ± 28 mg) ($P < 0.01$, $n = 5$).

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

Basal uptake of $^{45}Ca^{2+}$ by the segments of rat aorta was 22.4 ± 1.9 nmol kg^{-1} ($n = 5$). De-ionized water and/or HPS-10 (1 mM) did not significantly modify this value ($^{45}Ca^{2+}$ tissue content: 21.6 ± 1.8 nmol kg^{-1} and 23.2 ± 2.1 nmol kg^{-1}, $P > 0.05$, $n = 5$).

NA (10 μM), AII (1 μM) and high K^+ (50 mM) significantly increased $^{45}Ca^{2+}$ uptake (induced uptake) ($^{45}Ca^{2+}$ tissue content: 38.1 ± 2.2 nmol kg^{-1} (NA), $P < 0.01$ with respect to basal value, $n = 5$; 32.5 ± 1.6 nmol kg^{-1} (AII), $P < 0.01$ with respect to basal value, $n = 5$; 45.2 ± 2.4 nmol kg^{-1} (K^+), $P < 0.01$ with respect to basal value, $n = 5$). De-ionized water had no effect on NA-, AII- and K^+-induced $^{45}Ca^{2+}$ uptake. However, HPS-10 (1 mM) significantly reduced these values ($^{45}Ca^{2+}$ tissue content in presence of HPS-10: 30.2 ± 1.3 nmol kg^{-1} (NA), $P < 0.01$ with respect to evoked value, $n = 5$; 25.4 ± 1.2 nmol kg^{-1} (AII), $P < 0.01$ with respect to induced value, $n = 5$; 33.6 ± 1.9 nmol kg^{-1} (K^+), $P < 0.01$ with respect to evoked value, $n = 5$).

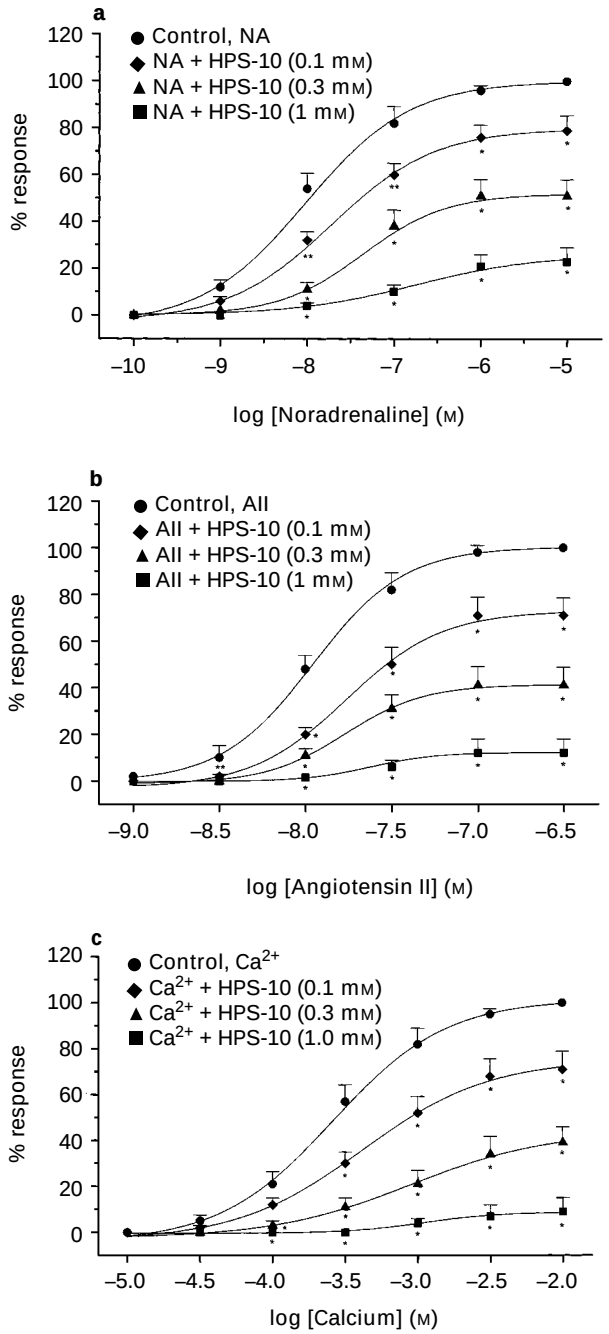

Figure 4 Endothelium-denuded rat aortic rings: cumulative concentration-response curves for (a) NA in the absence and presence of HPS-10 (0.1 mM, 0.3 mM and 1 mM); (b) AII in the absence and presence of HPS-10 (0.1 mM, 0.3 mM and 1 mM); (c) Ca^{2+} in depolarizing Ca^{2+}-free high-K^+ 50 mM solution in the absence and presence of HPS-10 (0.1 mM, 0.3 mM and 1 mM). Each point represents the mean value ± s.e.mean (indicated by vertical lines) from 5 experiments. Level of statistical significance with respect to control curves: $^*P < 0.01$ or $^{**}P < 0.05$.

Rat isolated atria

The mean basal values of force and rate of contraction, measured in control and treated groups before administration, were 495 ± 12 mg and 297 ± 7 beats min^{-1} ($n = 10$). In the control

preparations, the mean values obtained in the 15 min period were not significantly different from the basal ones ($P>0.05$, $n=5$).

HPS-10 (1 mM) significantly modified the mean atrial force and rate values, for a period of 15 min, compared against basal and control values ($P<0.01$, $n=5$) (Figure 5a and b). In fact, 1 min after the addition of HPS-10 (1 mM), the heart rate fell from 298 ± 10 to 222 ± 8 beats min^{-1} ($n=5$, $P<0.01$) and thereafter increased again until values of about 242 ± 11 (Figure 5a). On the other hand, within 5 min of its addition, HPS-10 increased the force of the contraction from 498 ± 17 mg to a peak of 622 ± 21 mg ($n=5$, $P<0.01$), after which the contractile force gradually decreased (Figure 5b).

Electrophysiology: whole-cell voltage-clamp experiments

When K^+ currents were blocked with Cs^+/TEA internal and 20 Ba^{2+} external solutions, depolarization-activated whole-cell I_{Ba}, having a fast onset and a rapid time course of inactivation, were recorded in whole-cell clamped rat aortic myocytes (Figure 6a). In most cells, following the decay of the initial transient I_{Ba}, no perceptible net sustained I_{Ba} was present. From the *I*-V relationships, the threshold for activation was about -50 mV with peak I_{Ba} occurring at -20 mV (Figure 6b). Preincubation for 20 min with HPS-10 (0.3 mM) strongly decreased I_{Ba} (Figure 6a and b).

In some cells, a sustained I_{Ba} was observed in addition to the initial transient current (Figure 7a). From the *I*-V curves, the threshold for activation was about -30 mV with peak I_{Ba} occurring at 10 mV. This maintained current was considerably inhibited by preincubation with HPS-10 (0.3 mM) for 20 min (Figure 7a and b).

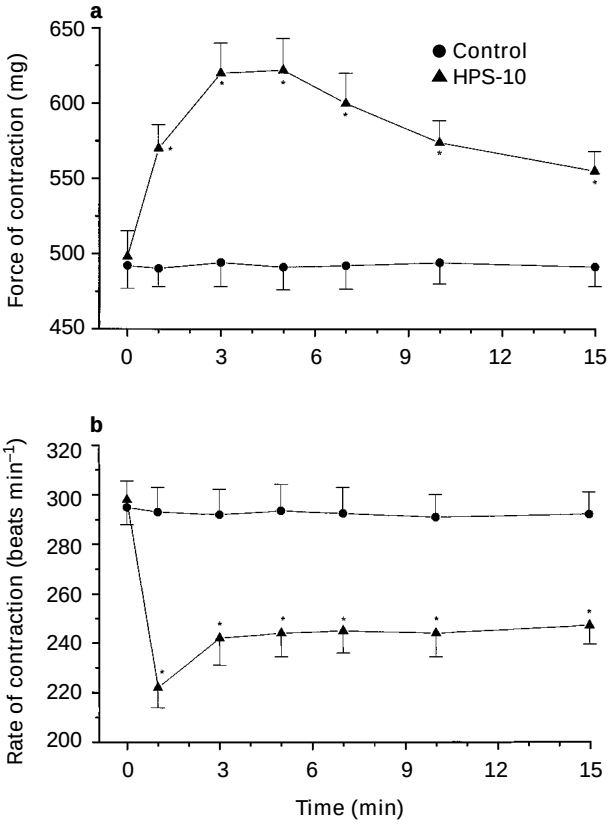

Figure 5 Effects of HPS-10 (1 mM) on (a) force of contraction and (b) rate of contraction in rat isolated atria. Each point represents the mean value ± s.e.mean (indicated by vertical lines) from 5 experiments. Level of statistical significance with respect to control values: $*P<0.01$.

Discussion

In this work, the potential activity on the rat cardiovascular system of a novel vasodilator compound (HPS-10) was studied for the first time. In conscious normotensive rats, HPS-10 exhibited a marked, prolonged and dose-related hypotensive activity, which was greater than that observed for hydralazine, in the same experimental conditions (see, for example, Orallo, 1984; Raviña *et al.*, 1991). This hypotensive activity was, in all doses, accompanied by a significant and fairly well correlated tachycardia. Since this tachycardia was totally inhibited by (±)-propranolol, it may be due to a reflex sympathetic response similar to that previously described for hydralazine (Orallo *et al.*, 1992).

In anaesthetized normotensive rats, HPS-10 was also more effective than hydralazine in reducing MAP (ED_{30} for hydralazine of about 0.48 mg kg^{-1}, i.v., in the same experimental conditions; see Orallo, 1984). However, the HPS-10 hypotensive effect was not accompanied by prolonged tachycardia, as in conscious normotensive rats. This may be due to the fact that urethane inhibits the cardiovascular reflexes responsible for HPS-10-induced tachycardia in conscious normotensive rats, as previously described for hydralazine by Orallo *et al.* (1992).

In order to elucidate the precise mechanism of the action of HPS-10 *in vivo*, a series of experiments in anaesthetized

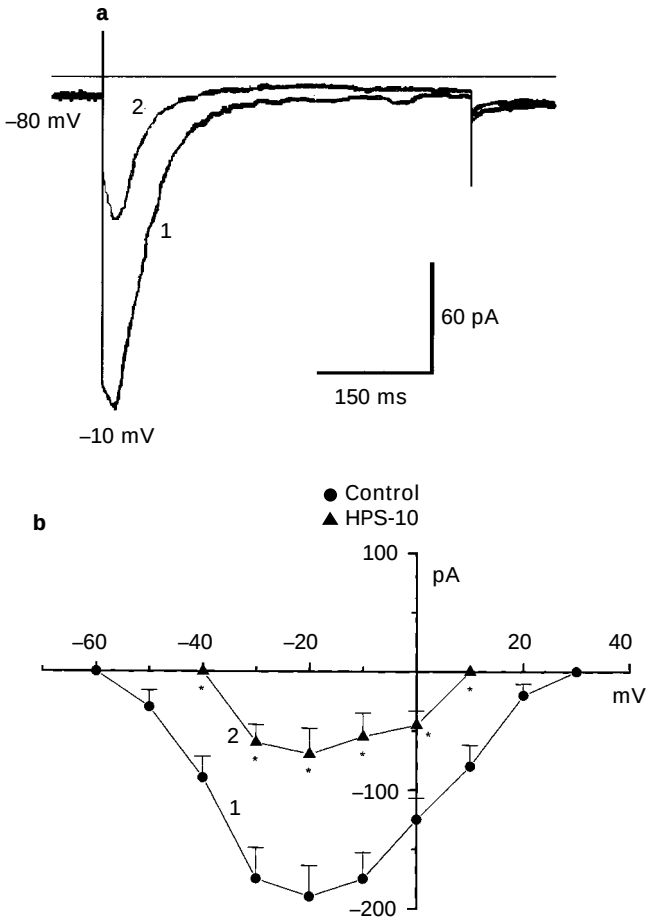

Figure 6 Effects of HPS-10 (0.3 mM) on transient I_{Ba} in rat cultured aortic myocytes. (a) During depolarizations to -10 mV from a V_h of -80 mV, I_{Ba} was measured in the presence (trace 2) and absence (trace 1) of HPS-10. (b) Peak *I*-V relations for I_{Ba} measured in the presence (trace 2) and absence (trace 1) of HPS-10 (0.3 mM) from a V_h of -80 mV to depolarizations by test pulses (500 ms duration) between -60 mV and $+60$ mV in 10 mV increments at 10–20 s intervals. Each point represents the mean value ± s.e.mean (indicated by vertical lines) from 5 experiments. Level of statistical significance with respect to control I-V curves: $*P<0.01$.

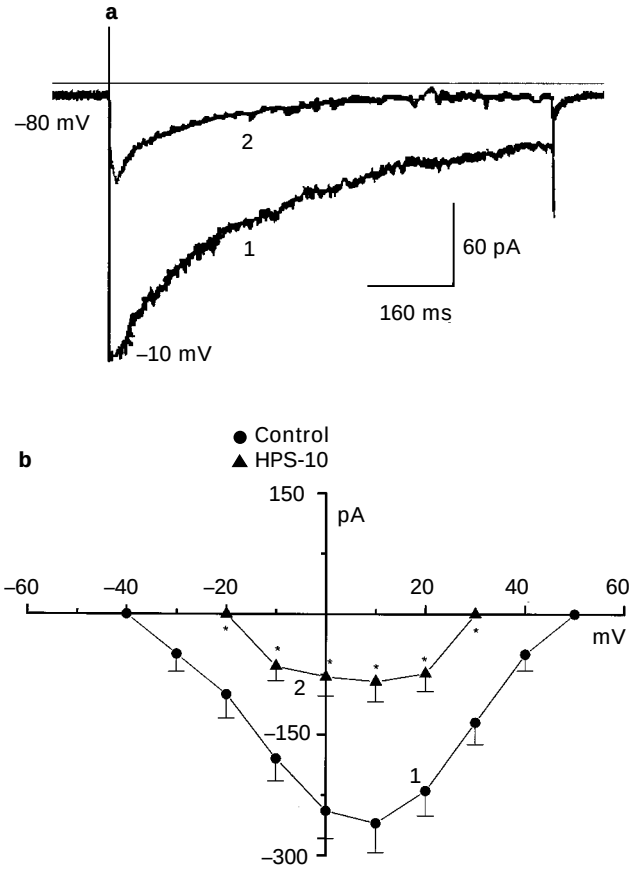

Figure 7 Effects of HPS-10 (0.3 mM) on sustained I_{Ba} in rat cultured aortic myocytes. (a) During depolarizations to −10 mV from a V_h of −80 mV, I_{Ba} was measured in presence (trace 2) and absence (trace 1) of HPS-10. (b) Peak *I*-V relations for I_{Ba} measured in presence (trace 2) and absence (trace 1) of HPS-10 (0.3 mM) from a V_h of −80 mV to depolarizations by test pulses (800 ms duration) between −60 mV and +60 mV in 10 mV increments at 10–20 s intervals. Each point represents the mean value ±s.e.mean (indicated by vertical lines) from 5 experiments. Level of statistical significance with respect to control *I*-V curves: $*P<0.01$.

normotensive rats were designed. In this preparation, the dosage of HPS-10 chosen for these experiments was 0.5 mg kg^{-1}, i.v., because this is considered to be the most effective therapeutic dosage, according to the values of ED_{30}, for studying the effects of HPS-10 on the cardiovascular response induced by the different vasoconstrictor agents used (NA, AII and nicotine).

HPS-10 inhibited, in a non-selective manner, the pressor response induced (via different mechanisms) by the above vasoconstrictor drugs and did not modify their positive chronotropic effects, which suggests that: (1) The hypotension induced by HPS-10 is not due to selective blockade of nicotinic receptors, α-adrenoceptor subtypes and/or AII postsynaptic vascular receptors (mainly AT_1 receptors) but probably a non-specific and direct vasodilator action on vascular smooth muscle cells (see below). (2) The bradycardia produced by HPS-10 in anaesthetized normotensive rat at doses higher than 0.4 mg kg^{-1}, i.v., cannot be attributed to a blocking action of cardiac β_1-adrenoceptors, AII receptors (possibly AT_1) and/or a ganglionic blocking effect. In this context, it is interesting to note that this bradycardia may be due to a putative direct action of the drug on the heart since HPS-10 notably decreased the basal values of the rate of contraction in rat isolated atria (see above).

The vasorelaxant effect of HPS-10 was examined in rat aorta without endothelium by evaluating its action on the contractile responses induced by NA, AII and Ca^{2+}, in order to ascertain whether it correlated well with the hypotensive activity observed *in vivo* and to investigate whether the *in vitro* action of this drug is selective.

In the present work, HPS-10, at concentrations within the same range in which hydralazine is active in rat isolated aorta (see, for example, Orallo *et al*., 1991), antagonized in a non-specific/non-competitive way and with almost equal effectiveness contractions induced by NA, AII and Ca^{2+} in rat rubbed aortic rings. This suggests that the vasorelaxant effects of HPS-10 are due to a non-selective direct vasodilator action, possibly related to either (a) intracellular activity, as shown previously for hydralazine in vascular smooth muscle (for a review see Campos-Toimil & Orallo, 1996b) and rat vas deferens (Campos-Toimil *et al*., 1994a), or (b) a non-selective effect on the cell membrane, as previously described for hydralazine (for a review see Campos-Toimil & Orallo, 1996b; see also below).

However, this vasorelaxant activity of HPS-10 in rat endothelium-denuded rat aorta rings was more potent than the vasodilator action previously described for hydralazine in rat isolated rubbed aorta (Orallo, 1984; Moína *et al*., 1994). Furthermore, there is a good correlation between the non-selective effect of HPS-10 on the vasoconstrictor responses induced by NA and AII *in vivo* (anaesthetized normotensive rat) and *in vitro* (rat isolated aorta), which suggests that the direct vasodilator action of HPS-10 observed *in vitro* at high concentrations may be responsible for its hypotensive activity noted *in vivo*, as previously shown for hydralazine (see Introduction).

The hypothesis proposed above that the vasorelaxant action of HPS-10 may be due, at least in part, to an intracellular effect on rat aorta smooth muscle cells, is supported by the experiments carried out in a Ca^{2+}-free medium. Under these conditions, NA, AII and caffeine induced a contractile response in the vascular smooth muscle, which was due only to the release of intracellular Ca^{2+} from the internal stores sensitive to the agonists. The mechanism whereby NA, AII and caffeine cause Ca^{2+}-release is not entirely known, but it has been suggested that the two first vasoconstrictor agents (via activation of postsynaptic α_1-adrenoceptor subtypes and AT_1 receptors, respectively) induce a fast contractile response (attributed to inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} by interacting with a specific receptor of intracellular stores (Chiu *et al*., 1987; Bosnjak, 1993)) and a subsequent sustained contraction (which is thought to involve the breakdown of phosphoinositide to diacylglycerol, followed by the activation of protein kinase C and induction of contraction in the presence of a low concentration of Ca^{2+} (Nishizuka, 1984; Koch *et al*., 1990)). However, caffeine induces Ca^{2+}-release by a different mechanism (Sato *et al*., 1988; Noguera & D'Ocon, 1992), no type of membrane receptor being involved, but probably by interaction with ryanodine specific receptors located in a sensitive intracellular store, which could partially overlap with the sensitive pool of NA and 5-HT (for review see Kuriyama *et al*., 1995 and Orallo, 1996).

In Ca^{2+}-free medium, HPS-10 strongly inhibited NA-, AII- and caffeine-induced contractile responses, which suggests that it may act intracellularly. Similar results have been obtained previously for hydralazine (Gurney & Allam, 1995; Campos-Toimil & Orallo, 1996b). This HPS-10 intracellular action may be direct and/or indirect (mediated, for example, by cyclic nucleotides, mainly guanosine 3′:5′-cyclic monophosphate (cyclic GMP); for discussion of hydralazine effects on intracellular cyclic GMP levels in vascular smooth muscle see Campos-Toimil & Orallo, 1996b).

The fact that pD'_2 values for HPS-10 were almost identical for contractions induced by NA, AII and Ca^{2+} would also tend to suggest the existence of a non-selective mechanism of action on the cell membrane, involving (among other mechanisms), the opening of K^+ channels and/or the blockage of Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels (if they exist).

The effect of HPS-10 on the smooth muscle cell membrane of rat aorta does not seem to be due to the opening of K^+ channels, since cromakalim and other K^+ channel agonists do

not inhibit contractions induced by Ca^{2+} (in depolarizing Ca^{2+}-free high-K^+ solution) at concentrations greater than about 30 mM of KCl in vascular smooth muscle. This last effect is due to the fact that, at high extracellular KCl concentrations, the cell membrane is depolarized to a level far from the K^+ equilibrium potential and, under these conditions, the K^+ channel openers do not hyperpolarize the smooth muscle cells. As a consequence, the vasorelaxant effects of these openers are negligible (for review see Quast, 1992).

The effect of HPS-10 on transmembrane Ca^{2+} channels is illustrated by the experiments with radiolabelled calcium. Basal $^{45}Ca^{2+}$ uptake, i.e. the amount of Ca^{2+} entering by means of leak channels (Orallo, 1996) was unchanged by the addition of HPS-10 (1 mM), whereas the same concentration of the drug strongly inhibited the uptake of $^{45}Ca^{2+}$ induced by K^+, NA and 5-HT in rat rubbed aortic rings. These results suggest that HPS-10 may inhibit, at least in part, the contractions induced by Ca^{2+} (in depolarizing Ca^{2+}-free high-K^+ medium), NA and AII by blocking transmembrane Ca^{2+} influx through voltage- and receptor-dependent Ca^{2+} channels. In this context, contradictory effects have been obtained previously for hydralazine (Campos-Toimil *et al*., 1994b; Campos-Toimil & Orallo, 1996b). The effect of HPS-10 on transmembrane calcium channels may be direct and/or indirect, mediated, for example, by an increase in cytosolic cyclic nucleotide concentrations (mainly cyclic GMP), which involves the inhibition of Ca^{2+} influx, possibly as a result of depohosphorylation of calcium channels, due in turn to phosphorylation of a protein phosphatase 2A (for a review see Orallo, 1996).

In the electrophysiological experiments carried out in the present work, a transient low-voltage-activated T-type Ca^{2+} channel current was largely predominant in rat aortic myocytes cultured in a defined, serum-free medium with the sustained L-type current being evident in some cells, as previously described (see Serebryakov & Takeda, 1992; Neveu *et al*., 1993).

In whole-cell clamped rat aortic myocytes, HPS-10 con siderably decreased depolarization-activated transient and sustained I_{Ba}, which confirms its inhibitory action on the calcium influx through transmembrane voltage-dependent L and T Ca^{2+} channels described above (Ca^{2+} antagonist activity). Similar results have been described for hydralazine by Orallo & Takeda (1992).

On the other hand, in the present work, it was not possible to study the potential effects of HPS-10 on receptor-operated Ca^{2+} channels, because, up to date, they have not been clearly identified by means of standard patch-clamp techniques (see above). In addition, NA and other vasoconstrictor agents produce variable effects on I_{Ba} in vascular smooth muscle and it seems quite probable that the so-called receptor-operated calcium channels are in reality receptor-operated non-specific cation channels with some degree of selectivity for divalent species, as has been recently reviewed (Orallo, 1996).

Finally, it should be pointed out that the calcium antagonist activity shown by HPS-10 in rat aortic smooth muscle cells could explain, at least in part, the decrease in the automaticity of sinus node pacemaker cells (negative chronotropic effects) produced *in vitro* (rat isolated atria) by high concentrations of the drug (1 mM) and previously obtained by a number of authors for hydralazine in several isolated cardiac tissues (for a review see Campos-Toimil & Orallo, 1996b). However, despite these negative chronotropic effects on sinus node pacemaker cells, HPS-10 paradoxically increased the force of contraction of the rat isolated atria. Similar results have been obtained for hydralazine and the mechanism responsible for this effect on the atrial myocardial cells remains, at present, unknown (Campos-Toimil & Orallo, 1996a).

To summarize, the novel vasodilator HPS-10 has been characterized as an agent with pronounced and long-lasting hypotensive activity in the conscious and anaesthetized normotensive rat and to have clear non-specific vasorelaxant effects on rat aorta (probably due to a transmembrane Ca^{2+}-antagonist properties and an intracellular action on smooth muscle cells).

There is a good correlation between the results obtained *in vivo* (anaesthetized normotensive rats) and *in vitro* (isolated rat aorta and rat aortic myocytes), which suggests that, if HPS-10 exhibits a similar behaviour in resistance blood vessels, its hypotensive activity in anaesthetized normotensive rats (5 mg kg^{-1}) may be due, at least in part, to a decrease in peripheral vascular resistance as a result of a direct vasodilator effect on vascular smooth muscle cells.

The new compound shows a pharmacological profile similar to the profile exhibited by hydralazine, a greater hypotensive activity and a lower toxicity (LD_{50} for hydralazine of about 124 mg kg^{-1}, i.p., in mice, in the same experimental conditions; see Orallo, 1984). Therefore, and bearing in mind that the ratio DL_{50} (mg kg^{-1}, i.p., in mice)/DE_{30} (mg kg^{-1}, p.o., in rat) for HPS-10 is quite favourable, compared with the ratio for hydralazine (see Orallo, 1984), it is concluded that HPS-10 may have therapeutic potential as a model for the development of new, less toxic and more efficient vasodilator drugs.

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